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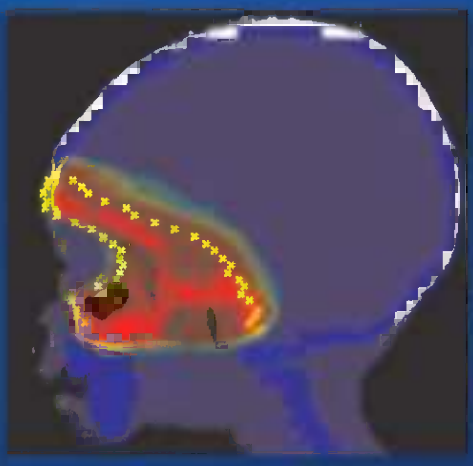
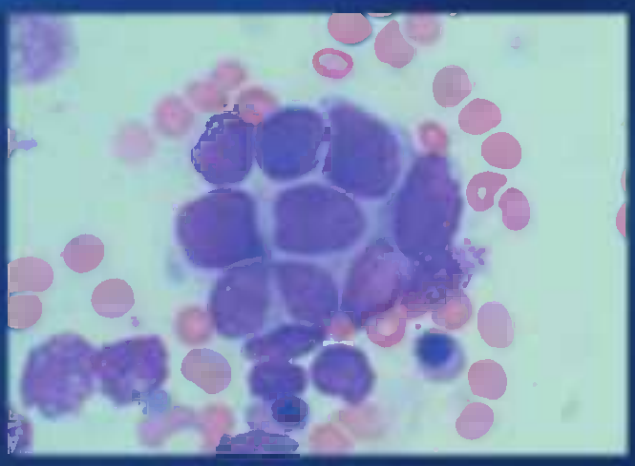
PEDIATRIC ONCOLOGY

Neuroblastoma





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Preface

The rapid advances in our understanding of the biology and treatment of neuroblastoma make it difficult to keep up to date. The clinical facets of neuroblastoma are endlessly fascinating. Its "natural history" overtly displays the difference between cancer and a truly extraordinary non-malignant proliferative disease. An interesting and potentially promising research emphasis is to unravel the difference between the "good" and "bad" forms of the disease. Our interest in neuroblastoma was kindled by clinical observations going back many decades. For example, is it likely that neuroblastoma "metastasizes" from one adrenal to the other and to the posterior mediastinum, or that malignant secondary deposits in these three unlikely sites will disappear spontaneously? Our early observations of this phenomenon were made in the days when there were no effective treatments for neuroblastoma so it was easier willy-nilly to observe the natural history.

We have seen disease wax and wane over time, such as skin lesions which became increasingly mature with each new "crop"; thus, the last one seen at 36 months was diagnosed as a neurofibroma. The results coming from the screening programs underline these concepts. They have shown that many more infants actually harbor occult neuroblastoma than are diagnosed clinically (in the nonscreened cohort population). This establishes that most such foci would have regressed spontaneously had they not been detected through screening. Observations such as these suggest that 4S neuroblastoma could teach us more about what clonal growth implies than clonal growth teaches us about neuroblastoma.

Obviously neuroblastoma can be a relentless, malignant disease, and these children need far better therapies than we now can muster. But the future may not lie so much in new classes of compounds or even drug adjuvants. It lies, instead, in the final understanding of what makes neuroblastoma mature into ganglioneuroma or, even more importantly, what prompts it to disappear spontaneously. Success will be measured when widespread disease in children with high-risk neuroblastoma is made to vanish through molecular genetic manipulations. Then cure will have achieved its true and very special meaning: disappearance of a life-threatening malignant disease without incurring the side effects of currently available avenues of treatment.

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Epidemiology

Andrew F. Olshan

Contents

This chapter reviews the epidemiology of neuroblastoma including the descriptive epidemiology and the evidence for an association with environmental exposures such as parental occupation, medication use during pregnancy, parental smoking and alcohol consumption, pregnancy history, and other exposures.

1.1 Descriptive Epidemiology

In the United States neuroblastoma accounts for 7.2% of all cancers among children younger than 15 years of age (SEER 2003). It is the most common extracranial solid tumor of childhood. Approximately 650 children are diagnosed with neuroblastoma in the United States each year (Goodman et al. 1999).

Based upon 1424 incident cases identified by the Surveillance, Epidemiology, and End Results Program of the U.S. National Cancer Institute (NCI) for 1975–2000, the total incidence of neuroblastoma was 10.2 per million children under age 15 years (age-adjusted to the 2000 U.S. standard million population; SEER 2003). The rates were 10.3 per million for males and 10.1 for females. Rates by race and ethnicity were 10.8 for whites, 8.4 for black children, and 7.5 for children of other racial/ethnic groups. The incidence rates by age category were 19.6 per million for ages 1–4 years, 2.9 for ages 5–9 years, and 0.7 for 10–14 years. Neuroblastoma is the most common malignancy among infants (61.3 per million). The incidence rate among infants was slightly higher among males (62.8) than females (59.8).

Based upon international registry data, the incidence of neuroblastoma is highest among Caucasians

Table 1.1. Neuroblastoma survival by gender, race, age, and stage

	5-year relative survival rate (%)[a]
Male	64
Female	65
White	65
Black	60
<1 year old at diagnosis	86
1–4 years at diagnosis	54
5–9 years at diagnosis	44
10–14 years at diagnosis	61
Local and regional stages (all ages)	85
Local and regional stages (<1 year old)	95
Local and regional stages (≥1 year old)	80
Distant metastatic stage (all ages)	48
Distant metastatic stage (<1 year old)	77
Distant metastatic stage (≥1 year old)	34

[a] Based on SEER (www.seer.cancer.gov) registry data 1985–2000

from North American, Europe, Australia, and Israeli Jews (Stiller and Parkin 1992). Lower rates were found for registries in southern and eastern Asia, including India and China, and in Latin America. Overall, the incidence appeared to be higher for regions or ethnic groups with a higher standard of living (Stiller and Parkin 1992). A previous study by SEER data found no total increase in incidence over time but reported a 3.4% average annual percentage (APC) increase for infants diagnosed between 1973 and 1992 (Gurney et al. 1996). The average annual increase was twice as high for infant boys as for infant girls. Other studies in the United States and elsewhere have noted increases in the incidence of neuroblastoma (Olshan and Bunin 2000). Improvements in diagnostic procedures, prenatal diagnosis, and possibly screening in some countries contributed to some of the increase during the 1970s through the early 1990s; however, analysis of the most recent SEER data (1973–2000) showed no significant increase in incidence overall (annual percentage change=0.3%) or among infants (APC=0.7%). There is some variability in 5-year relative survival rates based on age and stage (Table 1.1). Based upon SEER data for the years 1985–2000 the 5-year relative survival rate for neuroblastoma was 65%. No overall differences were found by race or gender. Survival was highest among infants and those with local or regional disease. Poorer survival was found for older children and those with distant metastases disease.

1.2 Risk Factors

The odds ratio provides an estimate of the relative risk, the risk among those with the exposure relative to the risk among those without the exposure. The odds ratio is estimated using exposure data collected in a case-control study, an efficient study design for a rare disease such as neuroblastoma. Odds ratios above the null value of 1.0 (indicating no case-control differences in the prevalence of a given exposure or factor) suggest a positive association, whereas odds ratios below 1.0 suggest that the factor may be associated with decreased risk. The assessment of the statistical associations should also include consideration of study design and analysis issues, such as the role of chance, confounding variables, and selection and exposure misclassification bias. Except where specifically indicated, the majority of epidemiologic studies have not examined any potential heterogeneity in risk among neuroblastoma subgroups defined by stage, age, or molecular markers.

1.2.1 Pregnancy and Childhood Factors

Several epidemiologic studies have investigated the role of reproductive history and birth characteristics in the etiology of neuroblastoma (See Review by Olshan and Bunin 2000). Conflicting results have been found for risk of neuroblastoma and maternal history of prior miscarriage, history of one or more induced abortions (Hamrick et al. 2001; Buck et al. 2001), repeat Cesarean birth and history of vaginal infection during pregnancy and sexually transmitted infection (Michalek et al. 1996; Hamrick et al. 2001). Studies also conflict with regard to the relationship

between preterm birth (<37 weeks gestation) and low birth weight (<2500 g). One study reported that neuroblastoma patients were less likely than controls to have been born preterm (Johnson and Spitz 1985); however, a trend toward increasing risk with lower birth weight was observed among term births. Two recent studies have reported positive associations: one with very preterm birth (<33 weeks odds ratio=1.9; Hamrick et al. 2001); and preterm birth among patients with stage-3 or stage-4 neuroblastoma (odds ratio=3.4; Schuz et al. 2001). Low birth weight and very low birth weight (<1500 g) have been found to increase risk in two studies (Johnson and Spitz 1985; Hamrick et al. 2001). Two studies have reported no differences in birth weight or gestational age, and no associations with birth order, maternal age, or parity have been reported (Hamrick et al. 2001).

A German study reported that a history of tonsillectomy and/or appendectomy increased the risk threefold for stage-3 or stage-4 neuroblastoma patients (Schuz et al. 2001). Breastfeeding for more than 6 months was shown to decrease the risk of neuroblastoma by 40% in one study (Daniels et al. 2002).

A recent review indicated that some studies have found a significant excess of birth defects among children with neuroblastoma compared directly with controls or using expected rates (Foulkes et al. 1997). Associations with defects, including neurofibromatosis type 1, Beckwith-Wiedemann syndrome, Hirschsprung's disease, musculoskeletal and cardiovascular malformations, Turner's syndrome, and neurodevelopmental abnormalities, have been reported. Some of these associations were not consistent across studies, and over- or under-ascertainment could bias the comparisons. Molecular studies of familial neuroblastoma cases have not provided evidence of linkage with the genes thought to be responsible for Hirschsprung's disease or neurofibromatosis (Maris et al. 1997).

1.2.2 Medication Use

Case series reports from Israel, Australia, and Japan identified a possible relationship with maternal use of hormones for bleeding, history of miscarriage, and ovulation induction (see review by Olshan and Bunin 2000). Four case-control studies have examined hormone use before or during pregnancy. Positive associations (odds ratio>2.0) have been reported with maternal use of sex hormones 3 months prior to or during pregnancy and among women with a history of miscarriage or stillbirth. Indications for hormone usage included contraception, vaginal bleeding, and previous miscarriage. The largest case-control study (504 cases) found no overall association with infertility, infertility treatment, and other hormone use, although an elevated risk (odds ratio=4.4) was found for Clomid use among male offspring, consistent with a previous finding (Olshan et al. 1999a). Other maternal medications used during pregnancy that have been found in some studies to increase the risk of neuroblastoma include a group termed "neurally active" drugs (amphetamines, antidepressants, antipsychotics, muscle relaxants, prescription pain medications, and tranquilizers), antinauseants, diuretics, analgesics, and antibiotics (odds ratio=2.8; Kramer et al. 1987).

A recent report suggested that maternal multivitamin use during pregnancy was associated with a 30–40% reduction in the risk of neuroblastoma (Olshan et al. 2002). The analysis was unable to isolate any specific vitamin that might be responsible for the association. The finding requires replication in epidemiologic studies and possible investigation in laboratory experiments.

1.2.3 Lifestyle Exposures

Although a possible increased risk exists for mother's smoking during pregnancy, other studies have failed to confirm this finding (Olshan and Bunin 2000; Yang et al. 2000). No association with paternal smoking has been found. A possible association between fetal alcohol syndrome and neuroblastoma has been reported (Kinney et al. 1980). Case-control studies have not reported a difference in the proportion of case and control mothers who reported alcohol consumption during pregnancy; however, daily drinking or drinking three or more drinks on one occasion was associated with a nine- and sixfold elevated risk, respectively (Kramer et al. 1987). Other studies have not found an association with the amount or frequency of alcohol consumption.

1.2.4 Parental Occupation and Environmental Exposures

Parental occupation, specifically paternal occupation, has been found to increase the risk of neuroblastoma in offspring in several studies (Olshan and Bunin 2000; Olshan et al. 1999b; DeRoos et al. 2001a–c). Two of the studies found an association with fathers employed in electronics-related occupations including electricians, electric and electronics workers, electrical equipment assembly, linemen, utility employees, welders, and electric equipment salesmen and repairmen. The risk estimates were relatively large (>2.0) but imprecise. Other paternal occupations and industries that had an increased risk included food product packers and warehouse men, farmers and agricultural workers, rubber processing, painting, chemistry occupations, tire manufacturing, rubber/plastics/synthetics industry, service occupations, packaging and materials handling, and processing occupations.

Bunin et al. (1990) reported an increased risk (odds ratio=2.2) for maternal occupations including stock girls, textile and food product packers, plastic product packers, electrical products assembly, and metal product fabrication workers. Parental occupational exposures reported to be associated with risk included dusts, aromatic and aliphatic hydrocarbons, electromagnetic fields, metal fumes and dusts, benzene, asbestos, and pesticides/herbicides. The studies did not include direct measurement of occupational exposures but indirectly inferred the potential exposures using job title and industry.

All the parental occupation studies thus far have a number of limitations including the lack of a complete occupational history in studies using birth certificates, broad occupational groupings, crude exposure assessment, and small sample sizes. Nonetheless, the current epidemiologic evidence suggests that the risk of several occupations (e.g., electronics-related jobs) warrants further evaluation.

Two studies have reported associations with self-reported use of home and garden pesticides (Daniels et al. 2001; Schuz et al. 2001). One study reported a twofold increased risk for garden pesticides among children with stage-3 or stage-4 disease (Schuz et al. 2001). Another study did not find any differences in risk among subgroups defined by MYCN tumor status or stage (Daniels et al. 2001). There have been reports of "clusters" of neuroblastoma cases in communities in the United States. Environmental factors, such as proximity to hazardous waste sites, have been suggested as possibly related to the etiology of these clusters, but firm evidence to confirm the causality of these speculative associations has been consistently lacking.

1.3 Conclusions

Several epidemiologic studies have been conducted to evaluate potential risk factors for neuroblastoma. No causal factor(s) has been isolated. Few of the reported associations have been replicated in multiple studies. Moreover, the studies have suffered from methodologic limitations such as small sample size (most studies had fewer than 200 cases), incomplete exposure data collection, and inadequate control groups.

Despite these limitations, the previous studies have provided leads that warrant evaluation in future studies. Certain pregnancy and birth factors, parental occupation, and medications deserve more careful investigation. Besides addressing the methodologic concerns outlined above, future studies should take advantage of the developments in molecular epidemiology and identification of specific genetic variation in the human genome. The sharp distinction between favorable (local reginal-4S) and high-risk (stage 4 and MCYN-amplified stage 3) groups, as well as apparent biologic heterogeneity of neuroblastoma, should be incorporated directly in future epidemiologic studies. Biologic subgroups that represent different etiologic pathways for neuroblastoma can refine our ability to detect risk factors operating in those pathways. Finally, the incorporation of genetic susceptibility factors, such as common polymorphisms for genes involved in carcinogen metabolism and DNA repair, may help elucidate gene-environment interactions that have otherwise gone undetected with only the exposure data.

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Screening for Neuroblastoma

William G. Woods

Contents

2.1 Introduction

Pediatricians are by training the most prevention oriented of all the primary care physicians. Immunizations for various potentially life-threatening infectious diseases and early screening for inborn errors of metabolism are two shining examples of childhood disease prevention. Prior to 1970, no one had attempted to reduce the morbidity, or more importantly, the mortality, of any childhood cancer through preclinical detection, specifically by mass screening for this disease. Over the past 30 years there has been much effort put into better understanding the role of preclinical detection of neuroblastoma, and potentially lowering mortality from this most challenging of childhood solid tumors. This chapter addresses various aspects of screening for neuroblastoma in children.

2.2 The Rationale for Neuroblastoma Screening

Neuroblastoma has an incidence of about 10 per million children 0–14 per year throughout the developed world (Young et al. 1986; Bernstein et al. 1992). In North America neuroblastoma will develop in approximately one in 7000 children before the age of 5 years, and over 700 cases are expected to be diagnosed annually. The incidence of neuroblastoma is about twice that of phenylketonuria, almost tenfold higher than that of galactosemia, and slightly less common than neonatal hypothyroidism (Woods and Tuchman 1987), all diseases which are mandated by most neonatal screening programs throughout the U.S.

Neuroblastoma is a fascinating neoplasm because of several clinical and biologic characteristics. The

tumor is unique biochemically because it possesses metabolic pathways for catecholamine synthesis and metabolism. Homovanillic acid (HVA), the main metabolite of dopamine, and vanillylmandelic acid (VMA), the main metabolite of adrenalin and noradrenalin, are sensitive and convenient markers of neuroblastoma since they are excreted in excessive amounts in a patient's urine (Hinterberger and Bartholomew 1969). Homovanillic acid and VMA have routinely been measured in patients with neuroblastoma for the past 30 years, and have been found to be invaluable aids in both neuroblastoma diagnosis and follow-up.

The treatment and outcome of neuroblastoma are highly age- and stage dependent. Children who are diagnosed with early-stage localized disease or under 1 year of age, irrespective of stage, can often be treated with limited therapy and have excellent survival (Bernstein et al. 1992; Matthay et al. 1989). In contrast, children over the age of 1 year who present with advanced-stage disease have a very poor survival despite aggressive chemotherapeutic treatment regimens, including bone marrow transplantation (Bernstein et al. 1992; Matthay et al. 1989). While some authors have hypothesized that neuroblastoma presents as at least two discreet clinical pathologic entities (Woods et al. 1992; Brodeur and Nakagawara 1992), others believe that malignant progression is a natural transition from benign-acting neuroblastoma in an infant to advanced-stage disease in a child. We presently know that favorable prognosis is strongly associated with specific tumor cellular characteristics (see Chaps. 4, 5, and 8) But in the 1980s, in the absence of this molecular genetic information, it was hypothesized that this natural transition may be interrupted by early detection to eradicate preclinical neuroblastomas.

2.3 Early Pioneering Studies Investigating Neuroblastoma Screening in Japan

The identification of elevated urinary catecholamines in infants with neuroblastoma was first made in 1957 (Mason et al. 1957). Over the next 15 years, methods for measuring the main urinary metabolic byproducts of dopamine and epinephrine, HVA, and VMA were refined. Twenty-four-hour collections were the rule, and elevated urinary catecholamines became extremely important in aiding in the diagnosis of children with "small round cell tumors" and subsequent follow-up of catecholamine-secreting neuroblastomas (Tuchman et al. 1987). A urinary VMA "spot test" based on the reaction of phenolic acids with diazotized p-nitroaniline became commonplace in pediatric oncology practice (LaBrosse 1968). In the early 1970s, Sawada and colleagues from the Kyoto Prefectural University of Medicine began pilot studies which led to implementing a mass screening program for 6-month-old children in eight cities and prefectures in Japan using the VMA spot test on random urine samples (Sawada et al. 1984). The annual incidence of neuroblastoma in Japan, 8 per million children, was similar to that reported in the U.S. at the time. Originally, 282,000 infants were screened by Sawada et al., representing 50–75% of all births in the areas studied (Sawada et al. 1984). Because of a positive test or logistic problems with the initial sample, almost 11,000 infants (3.8%) were retested. Among 264 infants (1 in 1000) who required clinical evaluation for neuroblastoma at a medical center because they had three consecutive positive urinary tests, 16 cases of neuroblastoma were subsequently identified, giving an incidence by screening of 1 in 17,600 infants. As opposed to the high expected incidence of metastatic disease at diagnosis, 5 patients were found with Evans stage-I tumor, 4 with stage II, 2 with stage III, 5 with stage IV-S, and none with stage IV. The 16 patients were treated with surgery and limited chemotherapy, 15 of whom were alive more than 5 years after diagnosis. The only death occurred 1 month after surgery in a patient with stage-II disease. Of the original screened cohort, an additional 6 children were found to have neuroblastoma 14–29 months after their urinary spot tests gave negative results. Hence, the false-negative rate of the Kyoto screening program was 6 of 22, or 27%, similar to what one would have expected using a VMA spot test (Sawada et al. 1984).

This encouraging early trial was reconfirmed by Sawada in longer-term follow-up of the screened population (Sawada 1986). Subsequently, many other screening trials were initiated in Japan, increasingly

using quantitative assays for measuring both VMA and HVA. In an important trial from Sapporo City, Nishi, Takeda and colleagues demonstrated markedly improved survival in children in that city offered screening compared with neighboring rural areas in Hokkaido Prefecture, in which no screening was available (Nishi et al. 1987). Several childhood cancer experts throughout Europe and North America called for the institution of neuroblastoma screening programs on their continents. Many other Japanese investigators began trials in their own prefectures, and by 1986, screening for neuroblastoma was mandated by law in Japan.

A more careful analysis of the Japanese neuroblastoma screening studies revealed many methodologic limitations (Tuchman et al. 1990). Firstly, there was no utilization of a population-based cohort of infants: studies were generally performed in prefectures which did not have the ability to guarantee ascertainment of all neuroblastoma cases occurring in that region, either detected by screening, or missed and subsequently clinically found. Secondly, the data were all based on survival rather than mortality. To the untrained observer, one would surmise that mortality is the reverse of survival (or "one minus survival"). In fact, mortality represents the number of deaths in a given population, and is not affected by the incidence of a disease in that given population. This difference from "survival" becomes most important in evaluating neuroblastoma screening trials. For example, as the Japanese increasingly used more sensitive and specific quantitative assays for measuring VMA and HVA in their trials, there were increasing data suggesting a rise in neuroblastoma incidence (Yamamoto et al. 2002). As can be seen graphically in Fig. 2.1, if one has an incidence in a disease of 1X, with a survival of 50%, of 100 children, 50% will die. If one artificially raises the incidence to 2X, or 200 individuals in this case, and one maintains the same mortality (50 deaths), there is an artificial increase in the survival to 75% (150 of 200). Hence, looking at survival only, when the actual relevant end point is death rate, can greatly mislead an investigator. In addition, the early Japanese studies utilized no control groups other than historical controls; therefore, potential declines in neuroblastoma mortality could have been attributed to improvements in therapy, rather than preclinical detection. Finally, without the utilization of a population-based cohort trial, several other classic methodologic issues, such as lead time or length bias, could produce falsely optimistic results.

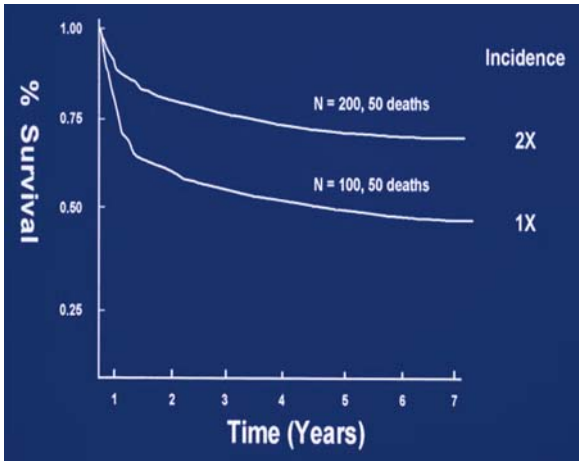

Figure 2.1
Effect of incidence on survival

2.4 Initial North American and European Neuroblastoma Screening Trials

In the context of the potentially exciting results coming out of Japan mixed with the realities of those studies' limitations, several groups throughout Europe and North America began early pilot studies looking at the potential effectiveness of neuroblastoma screening. Small exploratory studies were initiated in Quebec (Scriver et al. 1987), Minnesota (Tuchman et al. 1989), northern England (Craft et al. 1989), Germany (Schilling et al. 1991), France (Mathieu et al. 1996), Austria (Kerbl et al. 1997), and elsewhere (Bergeron et al. 1998). Newcastle hosted the first International Symposium on Neuroblastoma Screening in 1988, where investigators had the opportunity to share logistical challenges and early results. Several important methodologic aspects of

Table 2.1. Principles to be considered for a cancer screening program (Adapted from Prorok and Connor 1986)

1. The disease should be a "common" serious health problem, with substantial morbidity and mortality
2. The target population should be clearly defined and have a reasonable disease prevalence
3. The target population should be accessible, with reasonable compliance to screening expected
4. The screening test should be acceptable in its performance (sensitivity, specificity) and acceptable to those screened
5. Effective treatment should exist for the disease to be detected by screening
6. There should be a reasonable expectation that patients with positive screening will comply with recommended work-up, diagnosis, therapy, and follow-up
7. Sufficient resources should be available to perform the screening
8. Develop policies for early recall of patients testing positive and follow-up of those testing negative
9. Quality control procedures to maintain sensitivity and specificity of the screening test should be in place0

neuroblastoma were revealed. These aspects deserve some comment, given that they represent challenges of any population-based screening approaches for any diseases:

- Sample collection. In a series of important studies, Tuchman and colleagues from Minnesota demonstrated that measuring spot urines to determine HVA and VMA were as valid as 24-hour sample collections, thus obviating long collections for children in whom neuroblastoma was suspected clinically (Tuchman et al. 1985). In Japan, urine was squeezed out of diapers into plastic soy sauce bottles which held 5–10 ml of urine. North American and European investigators began collecting urine on diapers blotted against a 10×10 cm piece of filter paper which, when dried, could be mailed by regular mail to the screening laboratory for accurate determination of VMA and HVA, with urinary creatinine as the internal standard.
- Assays. Multiple laboratory assays for measuring catecholamines were debated, from the totally qualitative VMA spot test and the semi-quantitative thin layer chromatographic approach, through high performance liquid chromatography (HPLC), ELISA immuno-assays, and ultimately gas chromatography/mass spectroscopy (GC-MS) as the gold standard.
- Compliance. No adequate population-based screening trial can be done without a high compliance rate among the participants. Early studies in Minnesota (Tuchman et al. 1989), Texas (Ater et al. 1998), and Austria (Kerbl et al. 1997) found compliance rates of returning filter papers by parents of 6 month olds to be as low as 9 percent, pointing out the need for a massive public health infrastructure to support adequate compliance, even for something as simple as collecting urine from a diaper. Because of such issues, investigators in Minnesota joined forces with those in Quebec, combining clinical trials expertise (Bernstein et al. 1992), an infrastructure already in place for collecting urine in a large majority of 3-week olds, as part of a urinary metabolic screening program for various inborn errors of metabolism (Scriver et al. 1987); and a rapid GC-MS assay for VMA and HVA determination (Tuchman et al. 1983).
- Sample sizes. It became rapidly clear that to adequately study neuroblastoma screening, one might need a trial studying up to a million children or more to get meaningful results (Esteve et al. 1995). This sobering reality led to major modifications in many trials, some of which were abandoned due to the cost, and others that waited years for adequate funding before they proceeded.
- Case and control ascertainment. In-place state and country-wide tumor registries collecting incidence and mortality data with greater than 90–95 % ascertainment are an important requirement for an adequate prevention study.

Table 2.1, from Prorok and Connor (1986), lists principles to be considered for a cancer screening program. One could argue that any childhood disease is not a "common" serious health problem with substantial morbidity and mortality, to warrant the expense of a screening program; however, based on past precedent and the fact that children represent the future of the world, should screening of any childhood disease lower mortality, implementation would be seriously considered.

2.5 Follow-up Studies from Japan and Europe

Since 1986, when screening for neuroblastoma in Japan was mandated by law, compliance with the Japanese screening program has been much greater than 80% nationwide (Sawada and Takeda 2000). Although highly successful in recruiting parents to participate in this program, such widespread mass screening also led to less ability to measure the efficacy of this approach, for example, by comparing mortality from neuroblastoma in a population offered screening versus that not offered screening; however, subsequent attempts to document screening efficacy were performed in Japan on relatively small populations. Investigators in general found no diminution in the incidence of late-stage disease, an early marker of potential screening success, in the incidence of the disease with unfavorable biologic features, or in mortality (Yamamoto et al. 2002; Bessho et al. 1991; Yamamoto et la. 1995; Kaneko et al. 1990; Suita et al. 1998).

During the 1990s, as noted above, several smaller studies were also performed in Europe, usually without controls, with preliminary results suggesting that screening increased the incidence of the disease (Mathieu et al. 1996; Bergeron et al. 1998). Ultimately, only two prospective population-based controlled trials examining the role of neuroblastoma screening in reducing mortality from this disease were implemented that had adequate funding to guarantee a high screening compliance rate; uniform neuroblastoma evaluation, staging, treatment, and follow-up; and optimum ascertainment procedures for determining incidence and mortality. These were the Quebec Neuroblastoma Screening Project (Woods et al. 1996, 2002) and the German Project on Neuroblastoma Screening (Schilling et al. 1998, 2002). Both of these studies deserve special mention, noting similarities and differences.

2.6 Definitive Controlled Trials from Quebec and Germany

2.6.1 Studies, Designs, and Logistics

The greatest strength of both the Quebec and German trials was that they were prospective, population-based controlled studies in which neuroblastoma mortality was the definitive end point, rather than survival (vide supra). Both studies had considered a randomized trial approach, but "randomized controlled trials in population-based intervention studies are not always feasible" (Woods et al. 1999) as pointed out by the Quebec researchers. To clarify, the North American group had to decide what they were actually studying by introducing a new screening procedure in an infant population. Were they going to evaluate the screening test itself (urine sampling of 6-month-old babies by parents at home), or were they going to study the entire public health intervention which included introducing a new screening test? They decided that the latter question was much more relevant to improving scientific knowledge, and that to achieve a reasonable compliance rate multiple population-based education methods would be necessary, as noted below. If these measures led to a "halo effect," with an increased incidence in the non-screened population, the study results would have been viewed with skepticism. There were also practical matters including the fact that there were no other infant urinary screening programs in place in North America with a high compliance rate. Hence, control populations were picked throughout North America where no public health interventions were performed, and where had such been attempted they would have required millions of dollars in resources to be successful. These areas included the states of Minnesota and Florida, the Greater Delaware Valley, and the Province of Ontario (Woods et al. 1996). German investigators faced a similar problem. They im-

plemented screening in six German states selected on the basis of the "feasibility of implementing the screening program" (Schilling et al. 2002).

The Quebec Neuroblastoma Screening Project was a joint collaboration of 31 investigators throughout North America. The Quebec trial was designed specifically to answer the question of whether screening infants at or before 6 months of age (and the public health interventions associated with it) would lower mortality from this disease. After appropriate sample-size estimates were performed, geared at lowering overall mortality by 40%, it was decided to offer screening to a 5-year birth cohort in the Province beginning 1 May 1989, once NIH funding was secured. The only screening data available around the world at that time was for infants screened at 6 months of age in Japan. Investigators hence decided that they would screen at the same age, to be able to confirm or refute results from Japan. It was furthermore decided that infants would be screened at two ages: once at 3 weeks to take advantage of the urinary screening infrastructure which had been in place for well over 10 years (Scriver et al. 1987), and again at 6 months of age with a new public health intervention. Parents were given a "screening kit" at the birth of their child. The kit included filter paper collection instructions and a bilingual consent form with a "passive" informed consent process specifically explained, approved by an NIH-certified review board. Parents knew that if they did not want to screen their infants for either inborn errors or neuroblastoma, they did not need to return the filter paper. On the other hand, if they wanted their infants screened for the already-in-place program for metabolic abnormalities but not for neuroblastoma, they simply needed to check a box indicating refusal to participate in the "cancer test," mailing the consent form with the filter paper. Greater than 90% compliance was expected with the 3-week test, as compliance had consistently been above that level for several years for the metabolic screen (Scriver et al. 1987). Because the 6-month screen represented a new public health measure, multiple mechanisms were put in place to achieve compliance of about 75%. Some of these mechanisms included radio/television appearances and public service announcements, newspaper and magazine articles, posters in physicians' office and health clinics, information given to parents at birth, notices included with the Provincial "subsistence checks" which generally were mailed to all parents of infants, and even reminder inserts in diaper boxes.

Initial analyses of filters from both time periods were done in Sherbrooke utilizing thin-layer chromatography. The assays were geared towards the highest sensitivity and accepted a lower specificity: all positive filters, representing between 5 and 10% of infants screened, were then sent to Minneapolis where definitive, highly specific GC-MS assays were performed on the same sample. If the results were positive, parents were contacted and a second sample was requested, which was again studied by GC-MS. All children with a second positive sample were referred to one of the four Quebec pediatric cancer centers for uniform neuroblastoma evaluation (Table 2.2).

In the German Project on Neuroblastoma Screening, initial pilot studies examined the feasibility of performing a screening study in infants at 6 months of age (Schilling et al. 1991). Subsequently, pilot studies in Japan were instituted looking at screening at a later age; and preliminary data were emerging from Quebec suggesting a greatly increased incidence of the disease by screening at or earlier than 6 months, with no evidence of lowering the incidence of advanced-stage disease (Woods et al. 1996). Investigators worldwide believed that any reduction of mortality from a screening approach would be potentially heralded by a lower incidence of children "destined" to do poorly. Stuttgart and Hamburg researchers hypothesized that if neuroblastoma screening at 6 months of age was not going to lower mortality, perhaps screening at 1 year would be more successful, as well as potentially lower the incidence of disease by not detecting cases which would have spontaneously regressed before that age. After securing funding from the German government, screening was offered to all children at 1 year of age born in six German states, between 1 July 1994 and 31 October 1999.

German investigators hoped to achieve a compliance of over 70% to insure accurate sample-size estimates geared at lowering mortality. Unfortunately,

Table 2.2. Comparison of trials. *NA* not applicable

Characteristic	Quebec trial	German trial
Screening birth cohort period	1 May 1989 to 30 April 1994	1 July 1994 to 31 October 1999
Location	All of Quebec	Six German states
Number in cohort offered screening	476,654	2,581,188
Age at screening	3 weeks and 6 months	1 year
Screening compliance	89% at 3 weeks 73% at 6 months } 92% overall	61%
Concurrent control cohorts[a]	I. Rest of Canada II. 4 Specific control groups: Ontario Minnesota Florida Greater Delaware Valley	Remaining ten German states
Number in control cohorts	Rest of Canada, 1,509,000 Specific control groups, 2,718,000	2,117,000
Ascertainment procedures for screened and control cohorts	Two independent procedures, both complete with high correlation	One collaborative procedure, complete
Screening assays	Thin layer chromatography → Gas chromatography/mass spectroscopy	High-performance liquid chromatography
Number of (+) assays required before referral for neuroblastoma evaluation	2	2
Type of analysis	Entire cohort (8% not screened)	Screened sub-cohort only (excludes 39% not screened)
Screen (+) requiring neuroblastoma evaluation	82 (1/5300 screened)	1754 (1 of 840 screened)
False (+)	39	1605
True (+)	43	149
Positive predictive value	52%	8%
Missed by screening (never screened)	66 (3) (Excludes 20 patients diagnosed clinically prior to 3 weeks of age)	55 (NA) (Excludes unknown number of cases diagnosed prior to screening)
Total cases	132	204
Standardized incidence ratio (SIR) for neuroblastoma, comparing the study to control groups	2.0	1.9
SIR of advanced stage-3 and stage-4 disease ≥1 year	1.5	1.2
Uniform neuroblastoma staging, treatment, and follow-up	Yes	Yes
Deaths:		
Total	22	17
Screen detected	0	3
Diagnosed prior to 3 weeks of age	3	NA
Missed by screening	18	14
Not screened	1	NA

Table 2.2. Continued

Characteristic	Quebec trial	German trial
Cumulative mortality in study (per 100,000 children)	4.8 (0–8 years)	1.3 (1–5 years)
Cumulative mortality control groups (per 100,000 children)	3.3–5.3 (0–9 years)[b]	1.2 (1–5 years)
Standardized mortality Ratio for neuroblastoma comparing study to control groups	1.4[c]	1.1

[a] Two separate ascertainment procedures in Quebec
[b] Four specific areas in North America
[c] Versus rest of Canada

despite major efforts, early compliance in their trial was low, less than 50%, demonstrating how difficult obtaining good compliance is. Although the ultimate compliance rates approached 65%, overall compliance was 61%. The study was approved by a state ethics committee of the German Medical Association. They agreed that parents gave informed consent by mailing in urine-saturated filter papers for testing. The parents of each child in the screening area were offered screening once, at the time of the general checkup when the child was about 1 year of age (Schilling et al. 2002). Urine collected was analyzed for catecholamines by high-performance liquid chromatography. Similar to the Quebec trial, if a child had a positive assay, a second sample was requested. If that sample was positive, parents were contacted and asked to bring their child to a center for neuroblastoma evaluation. The assay was purposely geared to be as sensitive as possible, knowing that such an approach might lead to lower specificity, thus generating a much larger number of false-positive cases than in the North American trial; hence, there were some very interesting and important differences between this and the Quebec trial (Table 2.2).

Compliance in the Quebec trial closely approximated that used to calculate sample size estimates, and further analyses of the Quebec cohort were done using the entire birth cohorts, i.e., children were included whether screened (overall 92%) or not. Because of the lower than expected compliance rate in the German trial, many analyses in their definitive paper were based on results in those individuals only screened (Schilling et al. 2002), as noted in Table 2.2. Almost 2.6 million children were born in the six states during the 5 years of the trial, with 1.5 million actually undergoing screening. On the other hand, both trials successfully utilized concurrent control groups with millions of infants born in those areas. Quebec investigators used two completely independent ascertainment procedures for identifying cases, and more importantly, neuroblastoma deaths. One procedure utilized resources set up by the pediatric oncologists in the various study and control areas noted above, with major input from the North American cooperative groups, the Pediatric Oncology Group and Children's Cancer Group. Collectively, these groups treated 95% of all young children diagnosed with cancer in North America (Ross et al. 1996). The second ascertainment approach was performed independently by investigators at the Laboratory Center for Disease Control and Statistics Canada, part of Health and Welfare Canada, utilizing the whole of Canada without Quebec as the control. A remarkable congruence was found between the two procedures. In Germany, 10 of its 16 states in whom infants were not offered screening were used as the controls and included populations in the former East Germany. Fortunately, there were excellent childhood cancer registries in both East and West Germany before unification. Investigators were highly confident that these registries would be able to help them ascertain and follow patients (Schilling et al. 2002). Cas-

es were identified by the German Childhood Cancer Registry, "which receives information from all cases of childhood cancer in Germany, including all neuroblastomas. Follow-up of all cases was conducted in cooperation with the neuroblastoma treatment trial of the German Society Pediatric Hematology–Oncology" (Schilling et al. 2002). That the German ascertainment procedure was near complete was demonstrated by the fact that only two children diagnosed with neuroblastoma in Germany over a 5-year period were lost to follow-up within 5 years.

There were considerable differences in the sample sizes of the two studies. The much smaller Quebec trial was able to perform its study with fewer expected cases and deaths, in large part because it was investigating mortality from birth rather than from 1 year of age, as in the German study: about one-fifth of all neuroblastoma deaths occur in the first year of life. However, their study may have been underpowered (Esteve et al. 1995) had results been less conclusive. The much larger German trial still relied on sample-size estimates that required a 50 % reduction in mortality to see "significant benefit for the study population" (Schilling et al. 2002). Finally, both studies nicely utilized uniform neuroblastoma evaluation, treatment, and follow-up in all study cases and in many of the control areas.

2.6.2 Studies' Results

Despite some substantial and interesting differences between the two trials, overall results were strikingly similar, and hence very revealing vis-à-vis neuroblastoma behavior. Firstly, the Quebec trial nicely confirmed reports from smaller studies in Japan that highly sensitive assays measuring catecholamine metabolites would markedly raise the incidence of neuroblastoma in infants screened at 6 months of age or younger (Woods et al. 1996). The incidence of neuroblastoma almost doubled over controls in the Quebec birth cohort. The potential for neuroblastomas to regress had been documented for over 30 years (d'Angio et al. 1971), but the magnitude of such regressing cases was never appreciated. The Quebec data suggest that in countries in which there is a very strong medical surveillance of infants, neuroblastoma incidence may rise, as previously noted in studies from Denmark (Carlsen 1986). The data also suggest that as newer perinatal technologies become widespread, such as intrauterine ultrasonography, neuroblastoma incidence will also rise. Furthermore, initial studies examining the incidence of neuroblastoma in children born in Quebec during the 5 years immediately after screening was discontinued, 1 May 1994 to 30 April 1999, document that there has been a reduction in cases compared with the screened population, although not to baseline (WGW: personal observation).

Although many investigators may have predicted the marked rise in neuroblastoma incidence in Quebec, the German results vis-à-vis incidence were almost "shocking," they, too, found a doubling of the neuroblastoma incidence (Table 2.2), all over the age of 1 year. These data strongly suggest that tumors destined to regress may be present and excrete catecholamines for a much longer time than neuroblastoma researchers previously would have hypothesized.

Both studies documented significant neuroblastoma deaths in the study population. In the Quebec trial, there were 22 deaths, with none in the screened detected cases (Woods et al. 2002); however, three infants diagnosed prior to screening at 3 weeks of age, all with extremely high catecholamine levels that would have been detected by screening, and all with stage 4-S disease, died. Two of these infants had classic stage 4-S disease with rapidly expanding liver masses leading to respiratory compromise, despite heroic surgical attempts at relief. The third infant, despite a clinical stage of 4-S, had unfavorable biologic features, including amplified MYCN gene. The patient responded initially to chemotherapy but ultimately relapsed and died; otherwise, only one child in the Quebec population who died was not screened. In the German trial, investigating only those individuals who were screened, there were 17 deaths, 14 in children missed by screening, and 3 who died after preclinical detection. Of these three, "two children died from complications from surgery (one with stage 2-B disease, and the other with stage 3 disease), and one died from complications of chemotherapy (for stage 2-B disease)" (Schilling et

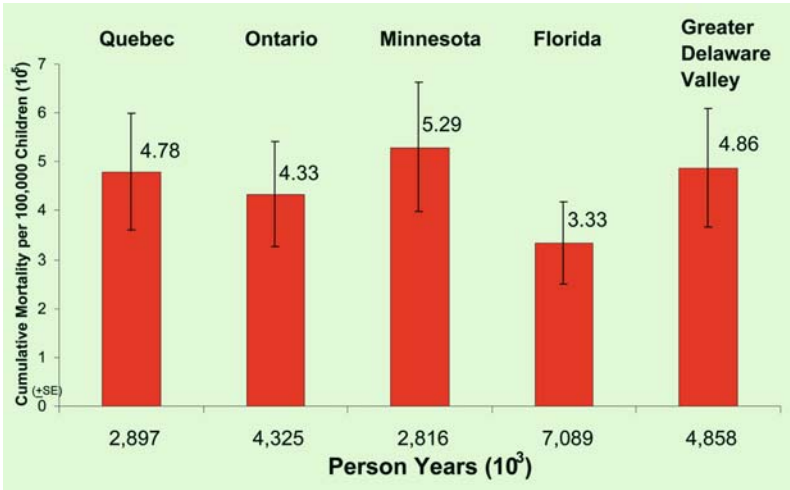

Figure 2.2
Cumulative mortality due to neuroblastoma among children younger than 8 years of age

al. 2002). Despite no deaths in the screened detected children in the Quebec trial, in 1 child with stage 2-B neuroblastoma that was detected by screening at 6 months and who was treated with doxorubicin and cyclophosphamide, a secondary leukemia with an abnormality in chromosome 11q23 subsequently developed. That child underwent bone marrow transplantation and is alive but has severe graft-vs-host disease. An additional child whose disease was detected by screening is in a persistent vegetative state as a result of complications of surgery for severe gastrointestinal obstruction and necrosis. The gastrointestinal problems were attributed to adhesions that resulted from the surgery to remove the neuroblastoma 7 years previously (Woods et al. 2002).

As an intermediate end point, both studies examined the incidence of advanced-stage neuroblastoma (INSS 3–4) in children over 1 year of age. Both showed, if anything, an increase in that incidence in the screened groups (Table 2.2). These results suggested that the screening procedure or the public health interventions instituted as part of the screening projects actually raised the incidence of advanced-stage disease in older infants, perhaps through clinical detection of cases that may have spontaneously regressed, the "halo effect." In the Quebec trial, there was a significant increased incidence in neuroblastoma over 1 year of age (Woods et al. 1996). In the German trial, even the incidence of advanced-stage disease over the age of 2 years was not lowered by the screening procedure (Schilling et al. 2002).

Finally, and most importantly, cumulative mortality in the study populations in both the Quebec and German trials was not reduced compared with appropriate controls. Mortality was higher in the Quebec screened cohort than in the German, but included a 9-year analysis (Woods et al. 2002). Only preliminary mortality results were presented for the German trial, examining cumulative figures between 1 and 5 years of age (Schilling et al. 2002); however, in examining standardized mortality ratios (SMR) of neuroblastoma comparing study versus control groups, neither showed any reduction, with an SMR of 1.4 in Quebec and 1.1 in Germany (Table 2.2). In fact, in the Quebec trial, examining the rate of death due to neuroblastoma compared with the rest of Canada, as compiled by Statistics Canada, the overall SMR for Quebec was 1.39, with 95 % confidence intervals of 0.85–2.30. Figure 2.2 displays cumulative deaths in the Quebec population versus the four control populations whose deaths were ascertained by study investigators.

2.7 Biologic, Psychologic, Economic, and Clinical Aspects of Neuroblastoma Screening

2.7.1 Biologic Aspects

Had neuroblastoma screening actually been associated with a reduction in mortality, one should have seen children with unfavorable biology detected preclinically with subsequent good outcomes. In general, this was not the case. Even in early Japanese trials, virtually all children with neuroblastomas detected by screening demonstrated favorable biologic features, including histology, triploid DNA content, and lack of MYCN amplification (Kaneko et al. 1990). In only one international trial, that conducted in Austria, were any substantial number of patients found through screening with unfavorable biology (Kerbl et al. 1997): results from this study are a bit controversial because of various methodologic issues. Preliminary results from the Quebec trial documented that, similar to the Japanese uncontrolled studies, virtually all children detected clinically had favorable biologic features (Brodeur et al. 1998, 2001); however, the vast majority of children who died after being missed by screening had unfavorable biologic features; for example, amplified MYCN oncogene identified in 11 of 19 patients studied (Woods et al. 2002). The German project is expected to publish biologic results in the future.

In summary, the current data overwhelmingly suggest that patients with favorable biology neuroblastoma are able to be successfully detected preclinically; however, those with poor biologic characteristics are missed by screening at 3 weeks, 6 months, and 1 year of age. This suggests that such tumors are either in general not present at these ages, or small enough not to be excreting catecholamines in excess of normal urinary amounts, with subsequent other cellular events leading to a great expansion of the cancer, often with metastatic spread, and clinical detection at an advanced stage.

2.7.2 Psychologic Aspects

Unfortunately, very few studies have examined the potential psychological implications of screening infants for neuroblastoma (Bell et al. 1994). Investigators in the Quebec trial tried unsuccessfully to obtain funding for what they believed to be an important secondary aim of their trial. Austrian investigators fortunately were able to conduct interviews on parents of children who underwent neuroblastoma screening with negative results (Dobrovoljski et al. 2003). They found that a large portion of parents of infants who were referred to cancer centers because of elevated catecholamines and were found not to have neuroblastomas remained very concerned about their children, even years later. Hence, the screening procedure was felt to be very psychologically stressing with long-term consequences. The Quebec screening trial was geared toward a very high specificity, and in the end, less than 1 in 10,000 normal children were evaluated at medical centers for neuroblastoma and found not to have the cancer. The number and percent of such children who falsely tested positive was a log higher in the German study, and remains high in Japan today.

2.7.3 Economic Aspects

Very little has been written on the potential cost-effectiveness of neuroblastoma screening. Because screening has been found to be ineffective, one could argue that such studies would by necessity be negative. The Quebec investigators, however, did prospectively examine cost-effectiveness and the data have yet to be published; however, preliminary results lead to a very important and provocative conclusion: over $8 million USD were spent on the Quebec trial in funds provided through the peer-review grant mechanism of the National Cancer Institute. In addition, significant resources were provided by the Quebec Institute of Genetic Medicine for neuroblastoma screening, including costs associated with setting up the infrastructure for metabolic screening that enabled this study to be done as economically as possible. The German Trial cost more than $20 million USD; hence, at first glance these were highly expen-

sive "negative" studies. But over 4 million children are born in the U.S. every year, compared with 100,000 in Quebec. To put in place an effective infrastructure to screen a large portion of American newborns would have cost easily hundreds of millions of dollars. As importantly, such an infrastructure would have cost tens of millions of dollars to maintain on an annual basis. As noted above, major pediatric voices clamored for institution of neuroblastoma screening in the U.S. before definitive trials proving or disproving its efficacy were performed. Not only did the Quebec and German trials show that neuroblastoma screening was ineffective, but ultimately they saved the American, Canadian, German, and other health care system billions of dollars over a generation. The economic value of well-done research cannot be overestimated, even if results obtained are negative.

2.7.4 Clinical Implications

With the determination that a substantial number of preclinically detected neuroblastomas undergo spontaneous regression, it is highly likely that a substantial amount of favorable-biology neuroblastoma detected clinically would also spontaneously regress; hence, the results of the neuroblastoma screening studies may have practical implications for the care of infants with clinically detected disease. Yamamoto and colleagues have now defined criteria for observing patients with neuroblastomas detected by screening without incurring any untoward risk. The criteria include the identification of small masses on radiographic examinations but no invasion of the intraspinal canal or infiltration around the great vessels; relatively moderate catecholamines secretions; and parental consent (Yamamoto et al. 1998). Their initial results reveal that a substantial proportion of observed tumors regress, and even those infants that need subsequent treatment do well. It is therefore likely that a similar proportion of infants in whom neuroblastoma is detected clinically at less than 6 months of age can also be observed for potential regression of the tumor, rather than undergo major surgery.

2.8 Conclusions

The idea that one could detect childhood cancer preclinically by screening has been and remains an appealing prospect. In well-performed trials in the only childhood cancer in which proper studies could be performed at the end of the twentieth century, neuroblastoma screening for elevated urinary catecholamines led to a marked increase in the incidence of the disease with no reduction in its mortality; hence, in 2004 using the markers studied, neuroblastoma screening has been and should be abandoned throughout the world: in Japan, screening was finally halted in March of 2004 (Tsubono et al. 2004). In the future, there may be better opportunities as more selective markers for poor-biology neuroblastoma are discovered that can be utilized as screening tools. In the meantime, one needs to remember that even collecting urine from a wet diaper may have horrendous long-term consequences, as evidenced by the outcome of some infants screened in the Quebec, German, and Japanese trials. Physicians should always practice the "golden rule" of medicine: *primum non nocere*.

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Genetics

John M. Maris, Garrett M. Brodeur

Contents

3.1 Introduction

Multiple somatically acquired genetic alterations have been described in neuroblastoma, but the genetic events that initiate tumorigenesis remain largely unknown. Like most other human cancers, a small subset of neuroblastoma cases have an apparent heritable genetic etiology. Familial neuroblastoma was first described in 1945 (Dodge and Brenner 1945), and multiple pedigrees have been reported in the literature since that time (Knudson and Strong 1972; Kushner et al. 1986; Maris and Brodeur 2001). In addition, there have been several patients with neuroblastoma and associated constitutional abnormalities and/or other conditions reported, suggesting the underlying genetic defect predisposed to the development of neuroblastoma.

This chapter reviews the genetics of neuroblastoma, emphasizing germline aberrations that predispose to the development of this neoplasm. Somatic genetic events associated with neuroblastoma pathogenesis and with clinical phenotype are reviewed in Chaps. 4 and 5.

3.2 Associated Genetic Conditions

It is likely that any neuroblastoma predisposition gene will have an essential role in the normal development of neural crest-derived tissues. Interestingly, the coincident occurrence of neuroblastoma in patients with global disorders of neural crest-derived cells (i.e., "neurocristopathies"), such as Hirschsprung disease (HD) and/or congenital central hypoventilation syndrome (CCHS, Ondine's curse),

Table 3.1. Candidate regions for neuroblastoma predisposition gene

Regions with evidence for genetic linkage	Regions of allelic deletion	Regions of chromosomal gain	Regions containing genes mutated in HD, CCHS, and/or NF1
4p16	1p36.2-.3	1q21–32	1p36.1 (*ECE1*)
16p12–13	3p21-pter	2p24 (*MYCN*)	2q22 (*ZFHX1B*)
	4p16	7q	4p12 (PHOX2B)
	9p21–24	17q23–25	5p12 (*GDNF*)
	11q14–23		10q11 (*RET*)
	14q32		11p13 (*BDNF*)
	16p12–13		13q22 (*EDNRB*)
	18q21		17q11.2 (NF1)
	19q13		20q13 (EDN3)

has been described (Verloes et al. 1993). In addition, there have been reports of the coexistence of neuroblastoma and neurofibromatosis type 1, including the coincidence of these disorders in familial neuroblastoma (Maris et al. 1997, 2002). Indeed, homozygous inactivation of the NF1 gene in primary neuroblastomas has also been described (Origone et al. 2003; Martinsson et al. 1997). These data suggest that the genes implicated in the genesis of Hirschsprung disease (*RET, EDNRB, EDN3, GDNF, ECE1*, and *ZFHX1B*), central hypoventilation (*RET, GDNF, EDN3, BDNF*, and *PHOX2B*) and/or NF1 may be causally involved in the initiation or progression of human neuroblastoma, especially in the context of a neurocristopathy (Table 3.1). A recent study reported a germline mutation in *PHOX2B* in a patient with neuroblastoma (Amiel et al. 2003), although previous reports have found no evidence for linkage at the 4p12 *PHOX2B* locus (Maris et al. 2002). GDNF and related molecules, neurturin (*NRTN*), artemin (*ARTN*), and persephin (*PSPN*), signal through a unique multicomponent receptor system consisting of RET tyrosine kinase and glycosyl-phosphatidylinositol-anchored coreceptor (*GFRalpha1–4*) (Sariola and Saarma 2003; Takahashi 2001); however, other than *RET* and *GDNF*, mutations in the genes encoding these ligands and coreceptors have not yet been implicated in the pathogenesis of HD or CCHS.

3.3 Constitutional Chromosomal Abnormalities

Discovery of cancer predisposition genes has been facilitated by the identification of rare patients with constitutional genomic DNA aberrations. Although neuroblastoma patients with de novo karyotypic abnormalities are rare, detailed analyses of these cases have been informative. Satge and colleagues recently reviewed 51 cases of constitutional karyotypic aberrations in neuroblastoma patients and confirmed recurrent constitutional deletions at chromosomal regions 1p36, 2p23, 3q, 11q14–23, and 15q (Satge et al. 2003). High-resolution genetic mapping of some of these deletions has aided in determining the location of putative neuroblastoma suppressor genes at chromosomes 1p36 and 11q14–23 (White et al. 2001; Mosse et al. 2003). The three children with constitutional 1p36 interstitial deletions all had profound neurocognitive deficits and were diagnosed with neuroblastoma during infancy. The constitutional deletions overlap the location of a putative 1p36 tumor suppressor gene (see Chaps. 4 and 5), suggesting that germline absence of a gene within this region may predispose to the development of neuroblastoma. Constitutional balanced translocations have been identified rarely in neuroblastoma patients, and no common region is apparent. Whole chromosome gains or losses are also rare in neuroblastoma pa-

tients. Interestingly, there appears to be an excess incidence of neuroblastoma in patients with 45,X (Turner) syndrome and perhaps trisomy 13, whereas trisomy 21 (Down syndrome) appears to be associated with a decreased risk for developing neuroblastoma (Satge et al. 2003; Blatt et al. 1997; Satge et al. 1998).

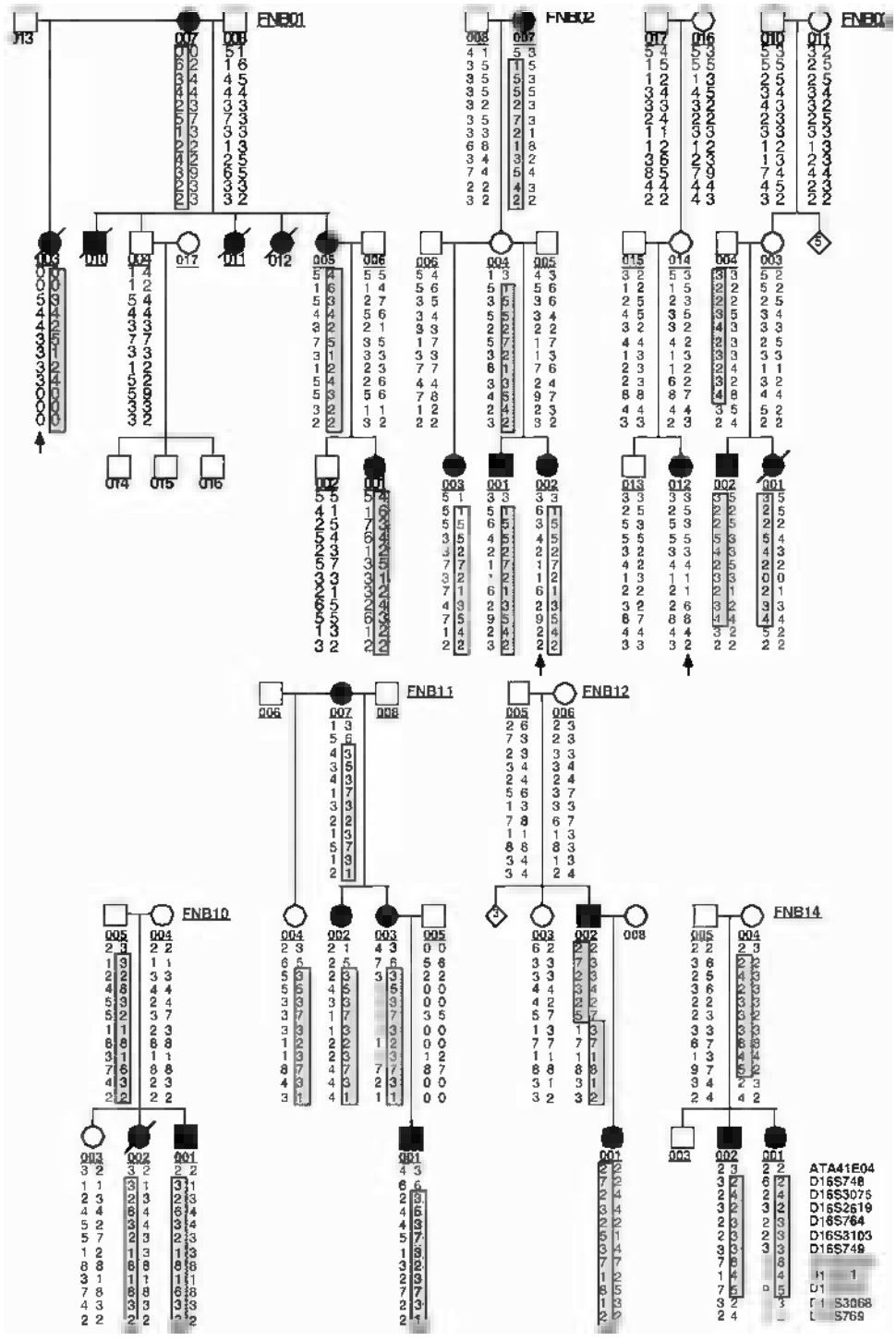
3.4 Hereditary Neuroblastoma

Neuroblastoma, like retinoblastoma and Wilms' tumor, is an embryonal malignancy that is notable for both a sporadic and hereditary form of the disease. Knudson and Strong provided the first genetic hypothesis of neuroblastoma tumorigenesis in 1972. In a comparison of 29 cases of hereditary neuroblastoma (from 13 families) to 504 unselected cases, they showed that 56% of familial cases were diagnosed at less than 1 year of age compared with 26% of the nonfamilial cases. In addition, 23% of the familial cases had multiple primary tumors documented, compared with 5% of the nonfamilial cases. Analysis of the pedigree structures was consistent with an autosomal-dominant mode of inheritance with incomplete penetrance that they calculated to be 0.63 (63% chance carriers will be affected). These data strongly suggested that the genetics of neuroblastoma initiation are similar to retinoblastoma, and subsequent studies indicated that, like *RB1*, a hereditary neuroblastoma predisposition gene should be a tumor suppressor. Nevertheless, for many of the reasons listed below, the genetic etiology of neuroblastoma has remained elusive over three decades since Knudson and Strong's original observations.

Despite the unequivocal data supporting a genetic hypothesis for the initiation of neuroblastoma tumorigenesis, it is relatively uncommon to obtain a positive family history of the disease for an individual neuroblastoma patient. Shojaei-Brosseau and colleagues recently used an epidemiological approach to show that only 5 of 426 consecutive neuroblastoma patients (1.2%) at a single institution had documentation of at least one first- or second-degree relative with neuroblastoma (Shojaei-Brosseau et al. 2004). This translates to a relatively high standardized incidence ratio (SIR) of 11.4 (95% confidence interval of 3.7–26.5) for the development of neuroblastoma among index-case relatives, but the risk to siblings was estimated at only 0.2%. Patients who present with multiple primary tumors or congenital neuroblastoma are more likely to harbor a germline mutation in a predisposition gene, but in many cases these may be de novo mutations. Taken together, these data suggest that heritable neuroblastoma is a rare phenomenon, and the pediatric oncologist should reassure parents of any newly diagnosed patient that the risk to siblings (particularly in the absence of high-risk features such as multifocal primary tumors) is very low.

The vast majority of reported neuroblastoma pedigrees are small, and large, multiplex, or three-generation families have been identified only rarely. Analyses of the published pedigrees in the past three decades strongly support the original conclusion of an autosomal-dominant mode of inheritance with incomplete penetrance. Although some families show multiple-affected individuals with few unaffected individuals between generations (obligate carriers), other families show multiple-affected individuals in the same generation (i.e., cousins) with no disease detected in intervening relatives (Maris et al. 2002; Perri et al. 2002; Lemire et al. 1998). Therefore, it is very difficult to determine precisely the penetrance of a mutant hereditary neuroblastoma gene segregating within a family, and this interfamilial heterogeneity may suggest that there is more than one heritable predisposition gene with different likelihoods of initiating neuroblastoma tumorigenesis.

Similar to patients with sporadic neuroblastoma, the clinical course in familial cases is also extremely variable (intrafamilial heterogeneity), with often striking contrast in the ages at presentation, disease stage, biological features of the tumor, and disease outcome. In addition, there are several reports of asymptomatic obligate carriers with elevated urinary catecholamines or in whom clinically occult tumors have been detected (Maris et al. 1997); therefore, reduced penetrance secondary to clinically occult or spontaneously regressing tumors, on the one hand, and the lethality of the condition prior to reproductive age, on the other, may both contribute to the


FNB01
FNB02
FNB03
FNB10
FNB11
FNB12
FNB14
ATA41E04
D16S748
D16S3075
D16S2619
D16S764
D16S3103
D16S749

◀ **Figure 3.1**

The pedigrees from seven neuroblastoma families with evidence for linkage to chromosome bands 16p12–13. *Filled symbols* indicate individual affected with neuroblastoma, ganglioneuroblastoma, or ganglioneuroma. Genotyping data are arranged into probable haplotypes based on minimization of recombination events for 16p polymorphic markers listed at *bottom right* and are displayed for each individual with an available DNA sample. *Gray box* indicates common haplotype segregating with disease in each family and shows genetic homogeneity at 16p. *Arrowheads* indicate haplotype lost when LOH was detected in corresponding tumor specimen

rarity of familial neuroblastoma. These facts have also contributed to the difficulty in approaching this disease with classic genetic approaches in order to isolate genes that predispose to the development of neuroblastoma when mutated in the germline.

3.5 Genetic Studies of Familial Neuroblastoma

There are two published studies that used classic genetic linkage methods to localize hereditary neuroblastoma predisposition genes. In a genome-wide search for linkage in seven pedigrees with at least two first-degree relatives affected with neuroblastoma, convincing evidence was discovered that a hereditary neuroblastoma predisposition gene (*HNB1*) is located on the distal short arm of chromosome 16 (16p12–13; Fig. 3.1) (Maris et al. 2002). Subsequent identification of a three-generation family with seven individuals affected with neuroblastoma appeared to confirm linkage to 16p with a cumulative LOD score of 3.7 (Maris et al. 2003). Loss of heterozygosity has been observed in 13% of sporadic neuroblastomas, suggesting that somatic inactivation of a 16p tumor suppressor gene might contribute to neuroblastoma initiation or progression in at least a subset of non-familial cases (Furuta et al. 2000). The genomic region likely to harbor *HNB1* remains relatively large and the positional cloning of this gene is ongoing.

Perri and colleagues studied two families in which ≥ third-degree relatives (cousins) were affected with neuroblastoma. They showed no evidence for linkage to 16p (Perri et al. 2002), in agreement with the original 16p linkage report in which two families consisting of cousins with neuroblastoma also showed no evidence of linkage to 16p (Maris et al. 2002). However, using a candidate-locus approach, they did show evidence for linkage to the distal short arm of chromosome 4p that overlapped a common region of hemizygous deletion observed in some primary neuroblastomas (Perri et al. 2002). Of note, the seven families linked to 16p showed strong evidence refuting linkage to 4p (J.M. Maris, unpublished data). Taken together, these observations support the hypothesis that at least two hereditary neuroblastoma predisposition genes exist, and that the penetrance of the two predisposition genes is different. The literature also strongly suggests that each of the major candidate loci and/or genes listed in Table 3.1 have been excluded as harboring a hereditary neuroblastoma predisposition gene through candidate-locus and/or genome-wide analyses (Maris et al. 1996, 2002; Tonini et al. 2001).

3.6 Conclusions

The rare neuroblastoma patients with a family history of the disease, associated genetic disorder, and/or constitutional chromosomal abnormality offer unique insights into the molecular pathogenesis of this enigmatic tumor. The identification of at least two putative familial neuroblastoma predisposition loci supports the assumption that neuroblastoma is a complex disease genetically, with multiple pathways to tumor initiation. Although identification of hereditary neuroblastoma predisposition genes would be of immediate benefit to those rare families that show evidence for predisposition to the disease, it is likely that the larger impact will be drawn from the insights these discoveries will provide for neuroblastoma tumorigenesis in general.

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Molecular Cytogenetics

Manfred Schwab

Contents

4.1 Introduction

It a major tenet in cancer research that alterations in cellular genes lead to the malignant transformation of normal cells. Two major classes of cancer-related genes have been identified: (a) oncogenes, which contribute to cancer "dominantly" by positive modulation of cellular growth; and (b) tumor suppressor genes, which are thought to control normal cellular growth and differentiation and act in a "recessive" negative way, contributing to cancer through functional inactivation. Both sporadic and familial genetic factors contribute to the pathogenesis of most types of cancer and, as reviewed in Chap. 3, a small subset of neuroblastoma cases have an apparent heritable genetic etiology; however, the vast majority of patients appear to develop neuroblastoma through spontaneously acquired somatic events rather than germline aberrations.

This chapter reviews our current understanding of the somatic genetic events that are associated with neuroblastoma pathogenesis and with clinical phenotype.

4.2 Classical Cytogenetics

In 1965, minute chromatin bodies, now referred to as double minutes (DMs; Fig. 4.1a), were first discovered in neuroblastoma cells (Cox et al. 1965). Subsequently, another novel chromosome abnormality, homogenously staining chromosomal region (HSR; Fig. 4.1b), was identified in human neuroblastoma cells as well as in antifolate-resistant hamster cells (Biedler and Spengler 1976); however, the biological

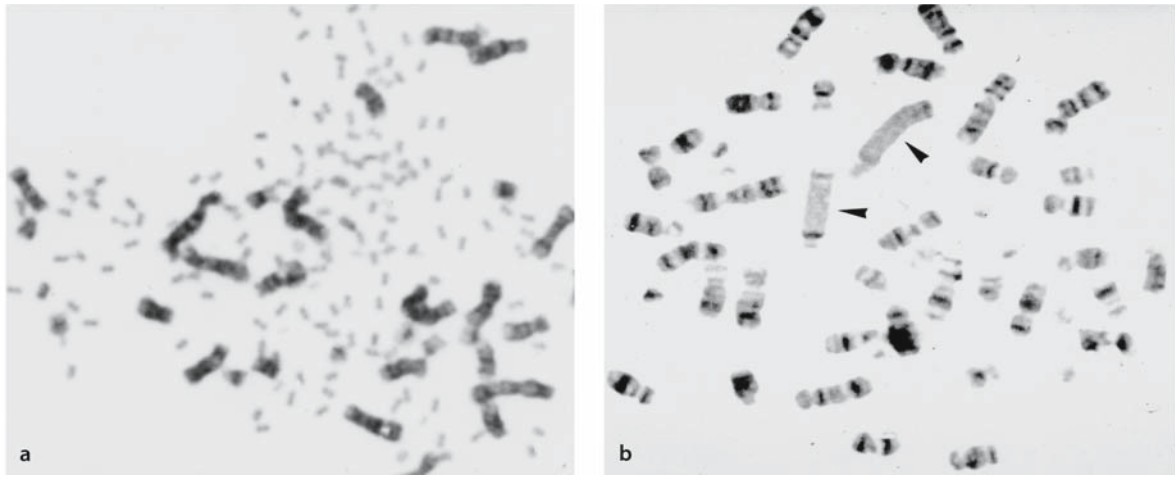


Figure 4.1 a,b

Cytogenetic manifestations of amplified DNA in human neuroblastoma cells. **a** Double minutes (DMs). **b** Homogeneously staining chromosomal region (HSRs; *arrowheads*)

significance of these cytogenetic aberrations remained unclear for many years. Some investigators speculated that DMs and HSRs were chromosomal manifestations of multiplicated drug-resistance genes; others hypothesized that DMs may inhibit neoplas-tic growth (Sandberg et al. 1972), or that loss of DMs through fragmentation from an HSR might be associated with the loss of the malignant phenotype of that cell (Balaban-Malenbaum and Gilbert 1977).

The advent of chromosome banding techniques in 1968 led to the unequivocal identification of all human chromosomes (Caspersson et al. 1968). The first systematic search for neuroblastoma-associated chromosomal alterations dates back to 1977, when Brodeur and co-workers noted the presence of chromosome 1p deletion in a conspicuous number of neuroblastoma cell lines and primary tumors (Brodeur et al. 1977). The high incidence of 1p deletions was confirmed in larger studies (Brodeur et al. 1981; Gilbert et al. 1982), and the authors speculated that this deletion represented the first "hit" in the two-step genetic sequence of tumor development proposed by Knudson (Knudsson 1971; Brodeur et al. 1977, 1981; Gilbert et al. 1982).

4.3 Oncogene Expression Profiling

The discovery of retroviral oncogenes (*v-onc*) and their cellular homologues (*c-onc*) in the early 1980s (Bishop 1982; Varmus 1982) quickly led to the identification of mutated *c-oncs* in human cancer cells (Der et al. 1982; Parada et al. 1982; Santos et al. 1982). In many types of cancer, these genes were found to be altered in structure (Groffen et al. 1984; Heisterkamp et al. 1983) or expression (Dalla-Favera et al. 1982; Neel et al. 1982) as a result of non-random chromosomal translocation. The first mRNA expression array (Onco-Array) used *v-onc* cDNAs spotted on filter membranes (Schwab et al. 1983b) to which a complex reverse-transcribed, radioactively labeled cDNA from neuroblastoma cells was hybridized. This principle technology was the forerunner of more recent large-scale expression array platforms (see Chap. 8). Profiling of neuroblastoma cell lines quickly established the strong expression of a *c-onc*, seemingly the *MYC* gene, the cellular homologue of the chicken retroviral gene (Fig. 4.2).

Subsequent DNA analyses quickly established an increased DNA copy number of a gene that was not

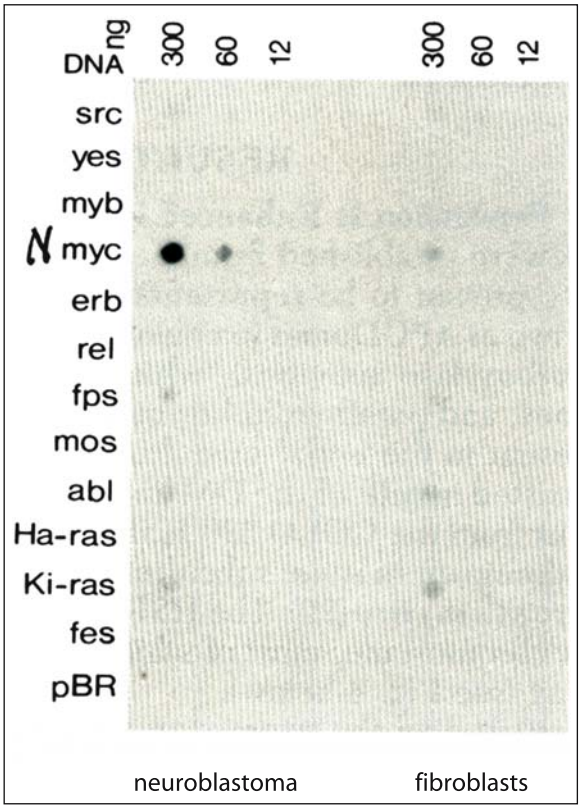


Figure 4.2

Expression profiling of oncogenes in neuroblastoma cell line Kelly to detect oncogene overexpression. Oncogene specific DNAs, many as cDNA of retroviral oncogenes, were spotted on a nitrocellulose filter, which subsequently was probed with radioactively labeled cDNA generated by reverse transcription of total polyadenylated RNA extracted from the tumor cells. Under conditions of reduced-stringency hybridization, a strong signal was seen for *MYC* which, by DNA analysis, turned out to result from the enhanced expression, consequent to DNA amplification, of a *MYC*-relative, the *MYCN* gene

the authentic *MYC* gene, but rather a close relative, initially referred to as *N-myc* (Schwab et al. 1983a; *MYCN is the correct human gene nomenclature*). Molecular cytogenetic analyses identified DMs and HSRs as the site of amplified *MYCN* (Schwab et al. 1984). Enhanced expression of the *MYCN* gene also contributed to tumorigenic cellular growth (Schwab et al. 1985). The activities of the *MYCN* protein, and the clinical significance of the amplified *MYCN* gene, have been the subject of previous reviews (Schwab 1998; Schwab et al. 2003). Amplified *MYCN* has been referred to as the "clinical debut of oncogenes," and because of the strong association between *MYCN* amplification and poor outcome, determining *MYCN* status in neuroblastoma tumors prior to initiating therapy is now considered an international clinical standard (Schwab et al. 2003). Array technology can now probe thousands of genes. While this technology is still evolving, large-scale expression profiling of neuroblastoma tumors has already begun (see Chap. 8; Alaminos et al. 2003; Berwanger et al. 2002; Fan et al. 2004; Khan et al. 2001; Mora et al. 2003; Sotiriou et al. 2002).

4.4 "Neuroblastoma Suppressor Genes" and Loss of Heterozygosity

The concept of tumor suppressor genes evolved from seminal observations made while studying retinoblastoma (Knudson 1971). The identification of the molecular pathway of retinoblastoma development by successive inactivation of the two *RB1* alleles at a gene locus was seen as a paradigm for tumor suppressor gene inactivation in other human cancers. Statistical analyses indicated that a two-hit genetic pathway, similar to the one identified in retinoblastoma, would lead to the development of neuroblastoma (Knudson 1971). Furthermore, the consistent 1p deletion detected in neuroblastoma tumors suggested that loss of a putative "neuroblastoma suppressor gene" (NSG) on chromosome 1p may represent the first "hit." The second hit was presumed to be a point mutation – or other subtle alterations – of the NSG on the other allele (although the sequence of "hits" can be either way). To identify the candidate NSG, LOH studies on a large number of tumors have been performed to define smallest region of overlapping deletions (SRO).

4.4.1 Chromosome 1p Deletion

Overall, up to 35% of neuroblastomas have LOH of chromosome 1p (Fong et al. 1989; Maris et al. 2000;

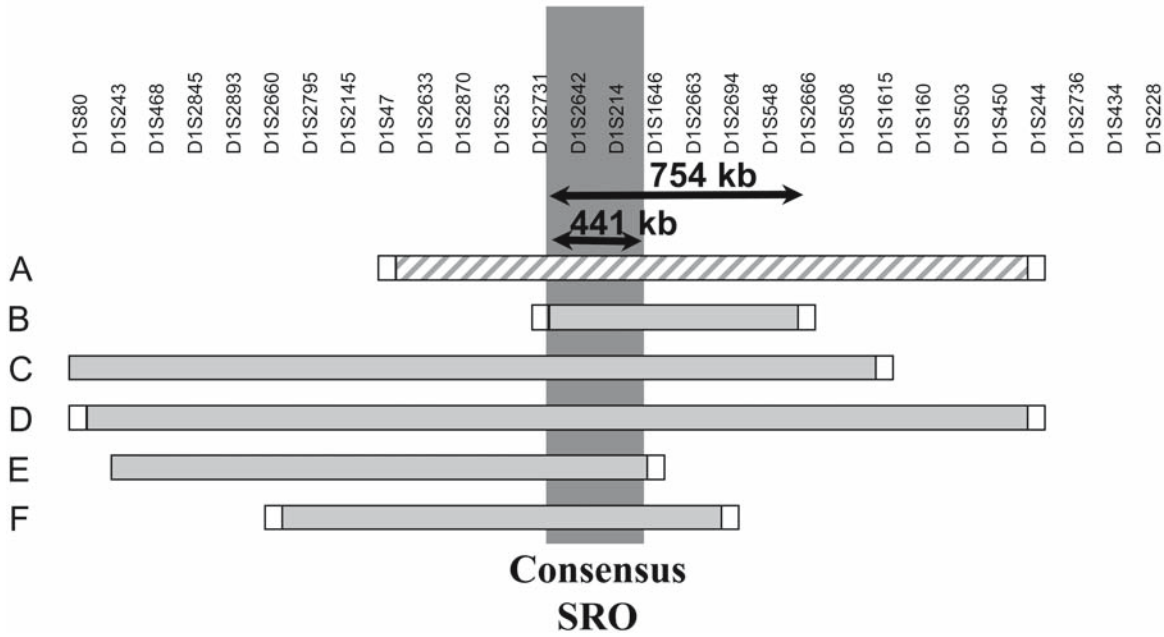


Figure 4.3

Comparison of 1p smallest region of overlapping deletions (SROs) identified by LOH studies in neuroblastoma. *A* Schwab et al. (1996); *B* Bauer et al. (2001); *C* Caron et al. (2001); *D* Martinsson et al. (1997), Ejeskar et al. (2001); *E* Maris et al. (2001b); *F* Hogarty et al. (2000), White et al. (2001). *Open boxes at the end of bars* represent the first non-deleted marker. *Arrows* give the distances between markers defining the SRO B (Bauer et al. 2001) and between markers bounding the consensus region of deletion. Order of markers is from Bauer et al. (2001) and the UCSC genome browser (http://genome.ucsc.edu) and was confirmed using the NCBI resource UniSTS (http://www.ncbi.nlm.nih.gov)

Takayama et al. 1992; Takita et al. 1995). A large number of molecular analyses in primary tumors has refined the SRO, mainly detecting LOH with polymorphic markers mapped to 1p (Caron et al. 2001; Ejeskar et al. 2001; Fong et al. 1989, 1992; Hogarty et al. 2000; Maris et al. 2001a; Martinsson et al. 1995; Schwab et al. 1996; Weith et al. 1989; White et al. 1995, 2001). These efforts resulted in an SRO within 1p36 defined proximally by *D1S244* and distally by *D1S80* (Fig. 4.3) The low incidence of small interstitial deletions within 1p36 has made it difficult to further narrow the SRO, a prerequisite for positional cloning. Furthermore, although several 1p36 rearrangements have been identified in neuroblastoma cell lines, along with a constitutional translocation t(1;17)(p36.31–36.13;q1 1.2–12) in a patient with multifocal neuroblastoma (Laureys et al. 1990), these chromosomal breakpoints are dispersed throughout a large genomic region.

Recently, an SRO was refined to a 1 Mb region within 1p36.3 defined by LOH in a primary tumor that extends distally from *D1S214*, and by a constitutional deletion between *D1S468* and *D1S2826* in a patient with neuroblastoma (White et al. 2001). Independently, a smaller candidate region of approximately 1 Mb (between *D1S2731* and *D1S2666)* was mapped to 1p36.3 (Bauer et al. 2001). Both regions appear to overlap in the vicinity of marker *D1S214*. Neuroblastoma cell line NGP has a translocation t(1;15)(36.2;q24), including a 2-Mb DNA duplication at 1p36.2 (Amler et al. 1995). Although the proximal breakpoint defined by the duplication appears to

map outside the 1p36.3 SRO, the distal breakpoint, which maps to between *D1S160* and *D1S214*, probably lies within the 1-Mb SRO. Several new genes mapping near this breakpoint region were identified recently that are currently being further characterized (Amler et al. 2000; K.O. Henrich et al., submitted). A homozygous deletion spanning approximately 500 kb at *D1S244* has been reported in two neuroblastoma cell lines (Ohira et al. 2000); however, this homozygous deletion is localized proximal to the refined 1 Mb SRO, which would make a single tumor suppressor gene within 1p36.3 unlikely. Also, it has not been established that the two cell lines have been derived from different patients.

A terminal 1p36 deletion syndrome has been described which is associated with mental retardation and craniofacial features (Shaffer and Heilstedt 2001; Shapira et al. 1997). The prevalence of this deletion (1p36.3) is estimated to be 1 in 5000, making it the most common terminal deletion (Shaffer and Heilstedt 2001). The deletion is distal to *D1S228*, and in some cases the large deletions include the 1-Mb SRO within 1p36.3 (Wu et al. 1999). To date, 2 patients with terminal 1p36.3 deletion syndrome have developed neuroblastoma (Biegel et al. 1993; White et al. 2001); however, neuroblastoma has not been detected in any of the originally published cases (Wu et al. 1999), suggesting that neuroblastoma is not a common feature of this syndrome. It remains unclear whether some rare patients with 1p36.3 deletion syndrome may have a predisposition to neuroblastoma depending on their specific deleted regions, or whether the two published cases were simply due to coincidence.

4.4.1.1 One or More "Tumor Suppressor Gene" Loci in 1p

Several observations suggest that more than one 1p locus may be affected in neuroblastoma. Outcome has reported to be poorer in patients with tumors that have large 1p deletions than patients with short or interstitial deletions (Takeda et al. 1994). Furthermore, while tumors with large 1p deletions were associated with adverse prognostic factors, such as diploidy or tetraploidy, and amplified *MYCN*, tumors with small interstitial deletions had DNA content in the triploid range and a high proportion of tumors were detected by mass screening. The existence of two distinct deleted regions was also suggested by LOH at polymorphic loci in clinically identified neuroblastomas (Caron et al. 1995; Schleiermacher et al. 1994). Additional studies have demonstrated that tumors with and without *MYCN* amplification show different types of SRO (Cheng et al. 1995; Gehring et al. 1995; Caron et al. 1993; Fong et al. 1989). In *MYCN*-amplified tumors, 1p deletions are very common and are large, always at least including a region from 1p35–1p36 to telomere. In contrast, 1p deletions occur in only 15–20 % of tumors that lack *MYCN* amplification, and the deletions are consistently smaller and commonly map to 1p36.3; thus, a second tumor suppressor locus inactivated by the 1p deletions in *MYCN*-non-amplified neuroblastomas has been postulated (Caron et al. 1995; Schleiermacher et al. 1996). This TSG was suggested to be localized at 1p35–36.1, just distal to the deletion border of the smallest 1p deletion found in *MYCN*-amplified cases (Caron et al. 1995; Spieker et al. 2001). The smallest SRO of the *MYCN* single-copy tumors is included into the larger SRO of *MYCN*-amplified tumors, implying that a distal suppressor locus in 1p36.2–3 must also be deleted in *MYCN*-amplified tumors.

The genomic complexity of the 1p region and the large size of its deletions have made it difficult to identify a neuroblastoma TSG. Although several candidate genes have been proposed, none has been shown to contain tumor-specific mutations, indicating that alternate mechanisms of TSG inactivation, such as epigenetic silencing or haploinsufficiency, may have to be considered. In addition, structural alterations of chromosome 1 have to be evaluated together with coincident genetic changes in other genomic regions, such as amplified *MYCN*, 17q gain, and diploidy/triploidy.

4.4.2 Deletion of 11q

Cytogenetic analyses have demonstrated the presence of 11q deletions in about 15 % of neuroblastoma tumors (Mertens et al. 1997). In LOH studies, 11q loss has been detected in 5–32 % of the tumors (Takeda

et al. 1996). Loss of the whole chromosome 11 appears to be strongly associated with low stage tumors, whereas unbalanced deletion of 11q is predominantly observed in high-stage tumors without amplified *MYCN* (Guo et al. 1999, 2000; Maris et al. 2001b). Deletion events affecting 11q are predominantly large and terminal. A single region of 2.1 cM within 11q23.3, flanked by markers *D11S1340* and *D11S1299*, was deleted in all tumors with 11q LOH (Guo et al. 1999). Constitutional rearrangements of 11q have been observed in some neuroblastoma patients, including a deletion of 11q23-qter, balanced translocations involving 11q21 and 11q22, and an inversion of 11q21-q23 (Bown et al. 1993; Hecht et al. 1982; Koiffmann et al. 1995). The role of these constitutional changes is not clear, but it has been speculated that disruption of one or more 11q genes may predispose to the development of neuroblastoma.

4.4.2.1 Chromosome 11 Deletion and 17q Gain

Fluorescence in situ hybridization (FISH) analyses have demonstrated that, after 1p, chromosome arm 11q is the second most common partner for 17q translocations (van Roy et al. 1994). Such translocations, resulting in concurrent loss of distal 11q and gain of 17q, account for approximately half of the 11q deletion cases (Vandesompele et al. 2001); thus, LOH studies assessing the prognostic value of chromosome losses must take into account the 17q status of each individual tumor.

4.4.3 LOH of Additional Chromosomes

Genome-wide surveys at randomly selected loci have revealed several chromosomal regions with LOH including. 9p21 (Marshall et al. 1997), 14q32 (Hoshi et al. 2000; Thompson et al. 2001), and others (Westermann and Schwab 2002). Although numerous investigators have speculated that TSGs may reside in these sites, to date, in spite of laborious efforts, not one neuroblastoma TSG has been identified.

4.4.4 LOH and Tumor Suppressor Genes: an Evasive Connection or Flawed Hypothesis?

There are several possible explanations for the failure to identify a neuroblastoma TSG. Firstly, the two-hit model, in its original form, may not be applicable to neuroblastoma. In addition, the current logic of utilizing LOH studies to determine the SRO and then surveying the chromosomally intact homologue for genes in this region and for mutations may be flawed. One possibility is that the loss of a single allele by deletion may be sufficient to produce a biological effect. Evidence for haploinsufficiency is accumulating (Goss et al. 2002; Gruber et al. 2002; Kucherlapati et al. 2002; Spring et al. 2002; Venkatachalam et al. 1998) for a number of genes, including *BLM*, *Fen1*, *TP53*, *ATM*, and others (Table 4.1). It is also possible that deletion of a single allele, such as in 1p, alone or in combination with deletion at another genetic locus, may contribute to tumorigenesis simply by dosage effect, without any mutational or epigenetic change of the remaining allele. Evidence is also emerging that slight gene dosage changes, like segmental duplications, can contribute to human malignant and non-malignant disorders (Corvi et al. 1995; Gratacos et al. 2001; Savelyeva et al. 2001). Genetic imbalance for 1p36 (at least 2 copies of chromosome 1 present with additional 1p36-deleted chromosome 1 copies) may also be associated with poor prognosis, similar to that seen in patients with tumors with 1p deletion or amplified *MYCN* (Spitz et al. 2002).

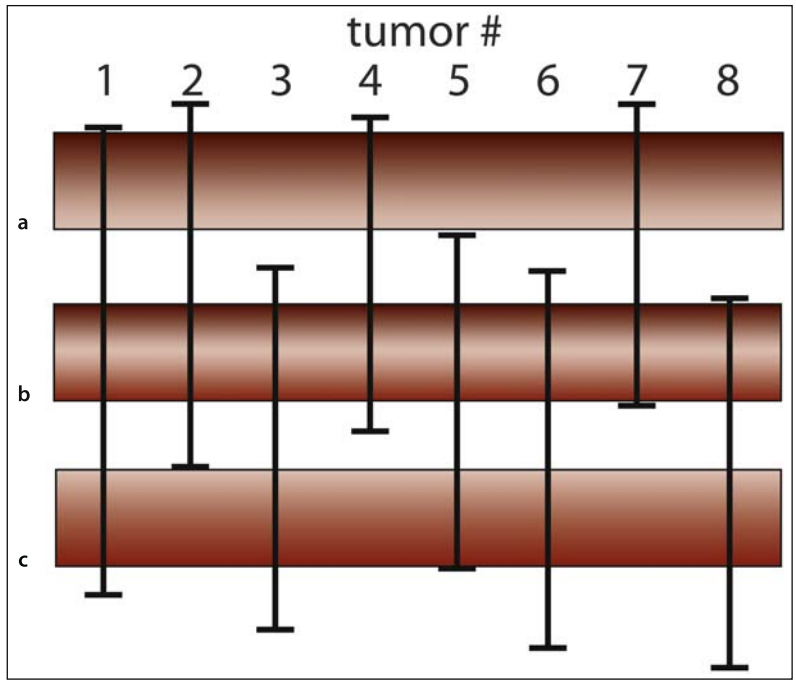
Another problem could be genetic heterogeneity for particular LOH regions among neuroblastoma subtypes, and thus, the strategy of analyzing the genes of a consensus region deduced from a larger number of tumors is flawed. One scenario, hypothetical but in principle suggested earlier (Takeda et al. 1994), could be that one biological or clinical group (group 1) of tumors results from the inactivation of one gene, while another clinical group of neuroblastomas (group 2) depends on the inactivation of another gene. Both genes may be in 1p36, but the group-2 gene may be several megabases away from the group-I gene. When LOH data are combined from these two groups, the SRO will be extremely unlikely to harbor the damaged second allele (Fig. 4.4).

Table 4.1. Genes and haploinsufficiency in tumorigenesis

Gene	Function	Reference
AML1/CBFA2	Transcription factor	Barton and Nucifora (2000); Song et al. (1999)
Cdh1	Cell-cell adhesion	Smits et al. (2000)
Dmpl	Cell-cycle control	Inoue et al. (2001)
Lkb1	Kinase with unknown target	Miyoshi et al. (2002)
NF1	Signal transduction	Zhu et al. (2002)
P27^{Kip1}	Cell-cycle control	Fero et al. (1998)
Ptch	Signal transduction	Wetmore et al. (2000)
Pten	Signal transduction	Kwabi-Addo et al. (2001)
Atm	DNA damage response	Spring et al. (2002)
Blm	DNA repair	Goss et al. (2002)
Fenl	DNA repair	Kucherlapati et al. (2002)
p53	Cellular stress response	Ide et al. (2003); Venkatachalam et al. (1998)
H2AX	DNA repair	Celeste et al. (2003)
Nbn	DNA repair	Dumon-Jones et al. (2003)
Anx7	DNA repair	Srivastava et al. (2003)

Figure 4.4

Genetic heterogeneity of LOH regions (depicted by *black capped bars*) may explain the failure to find the "neuroblastoma suppressor gene." In case of genetic heterogeneity, there could actually be at least two (*B*, *C*), if not more genes involved, and these might be separated many megabases from each other. Combining these LOH regions into a single consensus region (*A*) would inevitably initiate a gene search in a region that is unlikely to harbor the long-sought NSG



According to this hypothesis, there would actually be two SROs, each harboring a different gene that is damaged only in tumors of the corresponding group. As mentioned above, previous studies have indicated that two, or even more, "neuroblastoma genes" may reside in chromosome 1p (Caron et al. 2001). The existence of two separate 1p regions with relevance to neuroblastoma is also supported by an independent study that indicated two regions of loss, at 1p36 and 1p22 (Mora et al. 2000). Another study concluded that there were three regions of loss at 1p36.1–2, 1p36.3, and 1p32–34, and each was associated with different neuroblastoma groups (Hiyama et al. 2001).

4.5 Comparative Genomic Hybridization

In CGH, differentially labeled tumor DNA and normal DNA are competitively hybridized to normal human metaphase chromosomes (Kallioniemi et al. 1992). This methodology detects quantitative chromosomal changes, such as deletions, duplications or amplifications on the basis of the ratio of the hybridization of the two differently labeled DNAs. The advantage of this approach is that the complete set of quantitative genomic changes can be determined in a single experiment. Limitations are the low level of resolution (several Mb) and the fact that absolute quantitation of the changes is not precise. More recently, array CGH has been performed in an effort to increase the resolution of this technique. This strategy utilizes an array of DNA targets, and both cDNA and BAC arrays have been used (Beheshti et al. 2003; Cowel and Nowak 2003).

Both CGH and array CGH (Cunsolo et al. 2000; Plantaz et al. 2001; Schleiermacher et al. 2003; Stallings et al. 2003; Vettenranta et al. 2001) have largely confirmed previous cytogenetic and LOH studies revealing a high-frequency of 1p loss, 11p loss, 2p gain, and 17q gain (Schleiermacher et al. 2004; Brinkschmidt et al. 1997; Lastowska et al. 1997b, 2002; Plantaz et al. 1997; Vandesompele et al. 1998). The CGH studies have also revealed that about 50% of neuroblastomas have an additional segment of 17q, indicating that gain of 17q is the most frequent genetic alteration in neuroblastoma. Gain of 17q appears more common in advanced-stage tumors, in tumors from children aged over 1 year, and in tumors showing 1p loss, amplified *MYCN*, and diploidy or tetraploidy. In contrast, triploidy with whole chromosome 17 gain is associated more often with neuroblastomas showing favorable clinical and genetic features (Bown et al. 1999). Amplified *MYCN* rarely, if ever, occurs without either 1p deletion or 17q gain or both, implying that *MYCN* amplification is a later event in the sequence of genetic aberrations underlying neuroblastoma progression (Bown et al. 1999). Although several studies appear to suggest 17q gain as a powerful prognostic factor (Abel et al. 1999; Bown et al. 1999, 2001; Caron 1995; Caron et al. 1996; Lastowska et al. 1997a), a recent study could not confirm this association (Spitz et al. 2003).

4.6 Tumor Cell Ploidy

Many neuroblastomas have higher than normal DNA content or hyperploidy. Kaneko and Knudson have suggested that in neuroblastoma, aneuploidy may be a consequence of tetraploidization with subsequent bipolar, tripolar, or tetrapolar divisions (Kaneko and Knudson 2000). Supernumerary centrosomes leading to multipolar divisions have been implicated in both chromosome missegregation and the generation of aneuploid cells in various cancer types, including neuroblastoma (Brinkley 2001). A defect of spindle formation may cause incomplete segregation during mitosis; thus, such a defect in a tetraploid cell undergoing a tripolar division could lead to one near-triploid and one near-pentaploid cell. In fact, in neuroblastoma tumors with more than one tumor cell clone, near-pentaploid tumor cells are often observed together with near-triploid tumor cells.

Recently, Kaneko and Knudson have developed an attractive hypothesis explaining the association between ploidy and neuroblastoma phenotype (Kaneko and Knudson 2000). This hypothesis is based on the assumption that both clinically "favorable" triploid tumors and clinically "unfavorable" diploid tumors arise through the same genetic event, as suggested from observations in familial cases (Knudson and Strong 1972; Kushner et al. 1986). The initiating

tumorigenic event may be a mutation in a classical tumor suppressor gene with recessive effect at cellular level (Comings 1973; Knudson and Strong 1972). Tetraploidization and subsequent multipolar division of a diploid cell heterozygous for a mutation in such a gene would give rise to diploid and tetraploid daughter cells with no normal allele and highly malignant phenotype, or triploid daughter cells with at least one normal allele and less malignant phenotype.

4.7 Conclusion

Despite many advances in understanding the genetics and developmental molecular pathways, they have not yet translated into more effective therapy for high-risk neuroblastoma Nevertheless, the fascinating multiplicity of its clinical and biological phenotypes has attracted a growing number of clinical and basic scientists. Their combined efforts will inevitably resolve the intricate pathways that govern both progression and spontaneous regression of this disease. This knowledge should provide the platform for the development of new diagnostic tools and novel therapeutic strategies. Until then, we should be careful and avoid offering simplified suggestions for a rapid clinical translation.

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Molecular and Developmental Biology of Neuroblastoma

Akira Nakagawara

Contents

5.1 Neural Crest Development and Neuroblastoma

Cancer has its own face reflecting the characteristics of the tissue from which it is derived. This can be demonstrated by histopathologic examination, by immunohistochemistry, and/or by in situ hybridization. Recent advances in molecular biology and genetics have also revealed that these morphological distinctions among cancers are associated with differences in gene expression profiles within tumor cell and stromal cell components. Furthermore, the patterns of gene expression unique for each cancer are dictated by genetic abnormalities which have occurred in progenitors of the specific developmental lineage. Neuroblastoma originates from the sympathoadrenal lineage, and its biology is closely related to that of normal sympathetic neurons. In this chapter, the molecular and cellular bases for the genesis and biology of neuroblastoma are summarized.

5.1.1 Genes of Neural Development and Molecular Targets of Neuroblastoma

During neural development, neural crest cells migrate and differentiate into several cell lineages, e.g., melanocytes, sensory neurons, enteric ganglion cells, and sympathetic neurons (Fig. 5.1). The first signaling molecules which trigger crest cells to differentiate or migrate are bone morphogenetic proteins (BMPs) and their receptors (Huber et al. 2002). The commitment to differentiate into sympathetic neurons is associated with the transient expression of (a) basic helix-loop-helix transcription factors, e.g., *MASH1* (a proneural gene homologous to *drosophila achaete-*

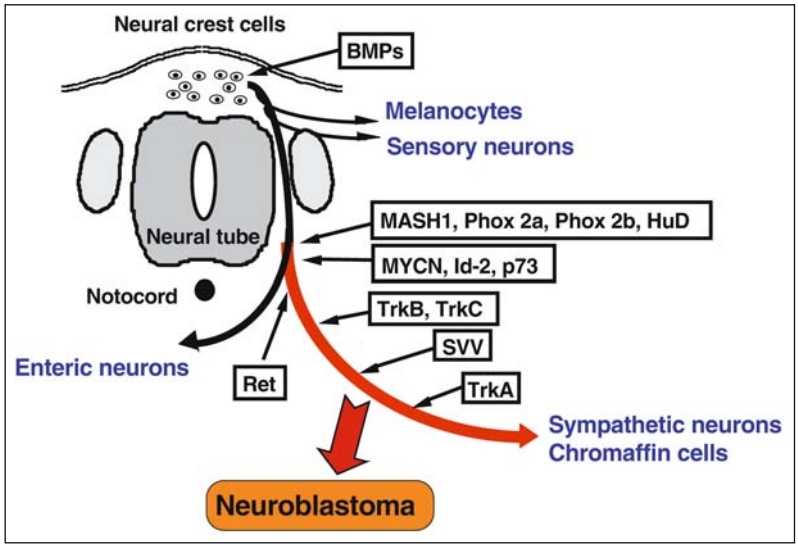


Figure 5.1

Neuroblastoma originates from the sympathoadrenal lineage of neural crest. The bone morphogenetic protein (BMP) signals may be important at the early stage of differentiation of neural crest cells. *MASH1* (*hASH1*) may function as one of the key transcription factors which define the direction of differentiation to sympathetic neurons. The other important nuclear factors, e.g., Phox2a, Phox2b, HuD, *MYCN*, Id2, and p73, may also be involved in the cell-fate determination. Some of those genes are often upregulated or amplified in aggressive neuroblastomas (Nakagawara 2004). At the stage of terminal differentiation of sympathetic neurons followed by programmed cell death, the signals through neuronal tyrosine kinase receptors, e.g., Ret, TrkB, TrkC, and TrkA, are necessary sequentially and/or in a form of crosstalk. The many genes involved in regulation of neuronal terminal differentiation or programmed cell death are often expressed at high levels in favorable neuroblastomas

scute), *HES1*, *MYCN*, HIF1α and HuD, (b) homeobox genes, e.g., Phox2a and Phox2b, and (c) p73 (a family member of the tumor suppressor gene p53; Nakagawara 2004). Several lines of investigation support the importance of these genes. *MASH1* null mice lack sympathetic ganglion cells (Guillemot et al. 1993). Notch signaling, through its intracellular domain translocation into the nucleus, stimulates the transcriptional activation of the *HES1* and *HES5* genes whose products in turn inhibit transcription of the *MASH1* gene (Radtke and Raj 2003). *MYCN* is indispensable for the normal neural development. It induces Id2 which is a negative regulator of *HES1* and pRb, a retinoblastoma suppressor (Lasorella et al. 2000). p73 knockout mice also show abnormalities in cell survival in both the nervous and immune systems (Yang et al. 2000). Gene targeting of HIF2α disturbs the catecholamine metabolism in sympathetic neurons (Tian et al. 1998). All these genes regulate each other in an orchestrated manner to drive the correct differentiation of neural crest cells into sympathetic neurons.

Further downstream, terminal differentiation to mature sympathetic cells is strongly regulated by the signaling of neurotrophin family members and their receptors (Nakagawara 2001, 2004). In addition, other genetic aberrations associated with neuroblastoma have been mapped to specific genomic regions or genes well known to be important in regulating the normal development of neurons (Nakagawara 2001, 2004). It seems obvious that a relationship should exist between the genetic or biological targets of neuroblastoma and the key molecules involved in the normal development of neural crest cells.

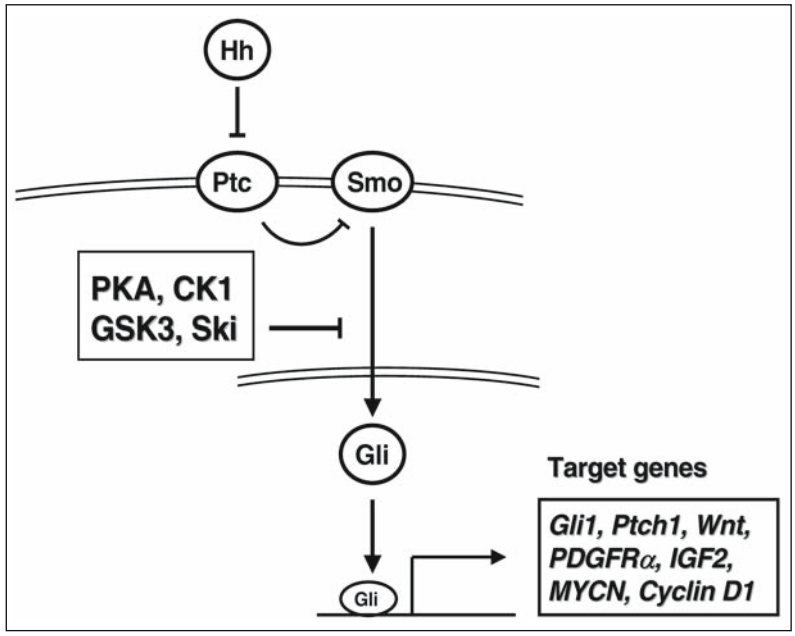


Figure 5.2

Hedgehog-Gli signaling in neural development and tumorigenesis. Sonic hedgehog (Hh) signaling activates Gli transcription factors which then induce the target genes important for regulating neural differentiation as well as neuronal tumorigenesis. They include *MYCN*, *cyclin D1*, *IGF2*, and *PDGFRα*, all of which are known to be players characterizing neuroblastoma biology. *T bars* show inhibitory interactions. *Arrows* show positive interactions

5.1.1.1 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, may be the first signal that defines the early phase of differentiation and migration of neural crest cells during development (Oppenheim 1991). The ligand-dependent activation of BMP receptors transduces its signal into the nucleus through the sequential activation of Smad signaling molecules by phosphorylation. Although the role of BMPs in neuroblastoma has long been elusive, Nakamura et al. (2003) have recently reported that SH-SY5Y and RTBM1 neuroblastoma cell lines are responsive to BMP2 leading to growth arrest and differentiation. Of interest, BMP treatment also induces the downregulation of p53 family members including p53 and p73, as well as their target gene, $p21^{WAF1}$. In contrast, a similar cyclin-dependent kinase inhibitor, $p27^{KIP1}$, is markedly induced at the protein level by downregulation of Skp2, a component of its E3 ubiquitin ligase complex. BMP is also a direct transcriptional target of retinoic acid which induces neuroblastoma differentiation (see Chap. 15; Rodriguez-Leon et al. 1999). The DAN family members are inhibitors of BMP, and are also expressed in neuroblastomas (Enomoto et al. 1994). The DAN gene itself, which is mapped to chromosome 1p36, is a transcriptional target of BMP (Nakamura et al. 2003; Shinbo et al. 2002), suggesting that the BMP signaling network may be important in the differentiation and survival of neuroblastoma (Nakamura et al. 2003). The role of other important signals which function during neuronal development, including Sonic Hedgehog (Shh) and Wnt, is less well known in neuroblastoma. Interestingly, the Shh downstream signaling molecule, Gli, can transactivate *MYCN* and cyclin D1 (Altaba et al. 2004) (Fig. 5.2).

5.1.1.2 *MASH1/hASH1*

Achaete-Scute homolog-1 (*MASH1* in rodents and *hASH1* in humans) is a basic helix-loop-helix transcription factor which plays an important role in the early development of neural and neuroendocrine progenitor cells (Ball 2004). Helix-loop-helix proteins include achaete-scute homologs, E proteins, *MYCN*, *Math*, *NeuroD*, *neurogenin*, *Id*, and *HES*. Targeted disruption of *MASH1* in mice has led to the absence of

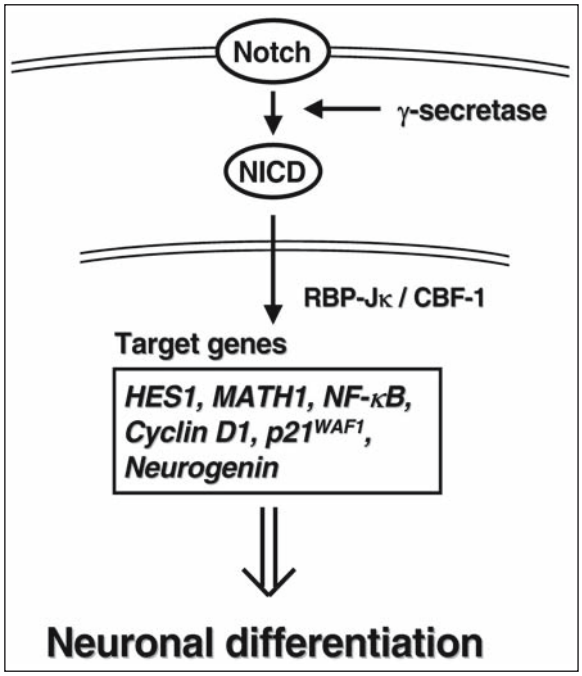


Figure 5.3

Notch signaling transactivates gene expression to induce neuronal differentiation. Binding of the ligand delta to its receptor notch triggers intramembrane proteolytic cleavage by γ-secretase. This results in the release of the notch intracellular domain (NICD), which then translocates to the nucleus where it associates with the CSL family of DNA binding proteins and transactivates gene expression. The target genes include *HES1, MATH1, NF-kB, cyclin D1, p21,* and *neurogenin. HES1* then inhibits transactivation of *MASH1* (*hASH1*)

sympathetic neurons, suggesting the important role of *MASH1* in sympathetic differentiation (Guillemot et al. 1993). *MASH1* is transiently induced during neural development to promote neuronal cell differentiation; however, high *hASH1* expression persists in neuroblastoma tumors and cell lines (Soderholm et al. 1999; Ichimiya et al. 2001). Retinoic acid treatment decreases the expression of *hASH1* and induces neurite extension (Ichimiya et al. 2001). *hASH1* also directly represses the expression of PACE4, a mammalian subtilin-like proprotein convertase that activates TGF-β-related proteins (e.g., BMPs) in neuroblastoma cell lines (Yoshida et al. 2001). The Notch signaling pathway also plays a key role during neuronal development (Axelson 2004). One of the important regulators of *hASH1* is a basic HLH protein, *HES1* (Fig. 5.3). *HES1* is regulated, at least in part, by Notch signaling and is induced at the transcription level. *HES1* directly binds to the promoter of *hASH1* and inhibits its transcriptional activation. A constitutively active form of Notch could block neurite extension during the induced differentiation of human neuroblastoma cells, possibly by inhibiting *hASH1* through the induction of *HES1* (Radtke and Raj 2003).

5.1.1.3 Phox2a and Phox2b

Phox2a and Phox2b are paired-like homeodomain transcription factors with complete conservation in their homeodomain. They are specifically expressed in noradrenergic neurons and activate the tyrosine hydroxylase and dopamine-β-hydroxylase genes (Schneider et al. 1999; Stanke et al. 1999; Ernberger 2000). While the expression of Phox2a is regulated by *MASH1*, Phox2b is not (Lo et al. 1999) (Fig. 5.4). The genetic disruption of either Phox2a or Phox2b gene de-monstrated that both genes are essential for the development of autonomic neural crest derivatives (Morin et al. 1997; Pattyn et al. 1999). Interestingly, Trochet et al. (2004) reported that the Phox2b gene was mutated in a family case of neuroblastoma and in a neuroblastoma patient with Hirschsprung's disease.

5.1.1.4 Id

Id proteins generally function as inhibitors of differentiation and as positive regulators of proliferation in neuronal development (Lavarone and Lasorella 2004). Id is a protein with the helix-loop-helix domain without a basic region and forms heterodimers with bHLH proteins, e.g., *MASH1* and *HES1* to inhibit their transactivation function (Massari and Murre 2000). In pediatric cancers, *MYC* oncoproteins and EWS-Ets fusion proteins are targeted to induce Id2 which in turn inhibits Rb and other target proteins including bHLH proteins, Ets and Pax. In neuroblastoma, *MYCN* has been shown to induce Id2 which stimulates cell proliferation by inhibiting Rb function (Lasorella et al. 2000).

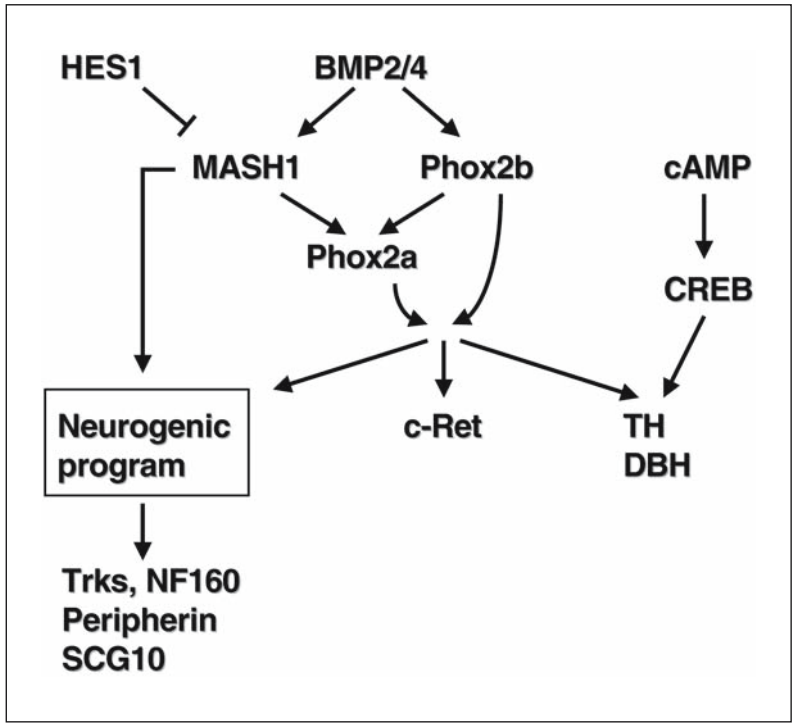


Figure 5.4

Regulatory network controlling sympathetic neuron development. BMP2 and BMP4 are required for the expression of *MASH1* and *Phox2b*. *HES1* induced by notch signaling inhibits expression of *MASH1*. *MASH1* and *Phox2b* are genetically upstream of *Phox2a*, and *Phox2b* is genetically upstream of *Gata3*. Expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) depends on *MASH1*, Phox2b, and Gata3. Cyclic AMP also controls expression of TH and DBH. Phox2a and Phox2b may affect induction or maintenance of *MASH1* expression. *MASH1*, Phox2a, and Phox2b regulate the downstream neurogenic program, leading to terminal differentiation of sympathetic neurons by inducing the genes, e.g., Trks, NF160, peripherin, and SCG10

5.1.1.5 *MYCN*

MYCN is a member of the group of *MYC*-box genes, and its product is a bHLH protein (Schwab et al. 2003). *MYCN* is transiently expressed during normal neural development and defines the direction of neuronal differentiation. *MYCN* is frequently amplified in advanced-stage neuroblastoma (Schwab et al. 1983, 1984; Brodeur et al. 1984; Seeger et al. 1985), and the biology of high-risk neuroblastoma is influenced by the subsequent overexpression of *MYCN* oncoprotein and its targets including telomerase and those functioning in ribosome biogenesis and protein synthesis (Mac et al. 2000; Boon et al. 2001).

5.2 Molecular Bases of Differentiation and Programmed Cell Death

5.2.1 Molecular Aspect of Spontaneous Regression

It is well known that some subsets of neuroblastoma can regress spontaneously. One of the most important hints to understand the mechanism of spontaneous regression is age of the patient at the onset of neuroblastoma. Regression rarely occurs when the tumor is found in patients over 1 year of age. The dramatic regression of the stage 4s tumor after its rapid growth usually occurs within 6 months after birth; therefore, it is plausible that epigenetic regulations, timed with the development of sympathetic neurons, might also control neuroblastoma regression. It is well known that massive death of sympathetic neurons is induced during the perinatal period – a process called developmentally regulated neuronal programmed cell death following deprivation of tar-

get tissue-derived neurotrophins (Oppenheim 1991). This same death mechanism appears to be conserved in primary neuroblastomas found in infants, leading to the induction of their spontaneous regression (Nakagawara 1998b).

5.2.2 Neurotrophic Factors and Their Receptors

5.2.2.1 Neurotrophins and Their Receptors in Neuroblastoma

The neurotrophin family of growth factors consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5; Huang and Reichardt 2003). The corresponding high-affinity neurotrophin receptors with tyrosine kinase activity have been identified as TrkA, TrkB, and TrkC (Snider 1994) (Fig. 5.5 a, b). TrkA is a preferred receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower-affinity neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor (TNFR)/Fas family (Snider 1994). The targeted disruption of neurotrophins and their receptors has demonstrated that NGF/TrkA signaling supports the survival and differentiation of sympathetic and sensory neurons responsive to temperature and pain, while BDNF/TrkB, NT-4/TrkB, and NT-3/TrkC signaling supports those of sensory neurons responsive to tactile stimuli and motor and sensory neurons responsive to limb movement and position, respectively (Klein 1994). These results suggest that neural development and maintenance of the neural network are spatiotemporally controlled by neurotrophin signaling with or without some redundancy in both peripheral and central nervous systems.

In neuroblastoma, high levels of TrkA are expressed in subsets of tumors with good prognosis, often showing spontaneous regression (Nakagawara et al. 1992, 1993; Suzuki et al. 1993; Kogner et al. 1993). Such tumors usually occur in patients under 1 year of age, and their DNA ploidy is aneuploid. A very limited amount of NGF may be supplied from stromal cells, e.g., Schwannian cells and fibroblasts, which at least partly regulate the differentiation and programmed cell death of neuroblastoma cells (Nakagawara 1998a). On the other hand, TrkA expression is strongly downregulated in tumors with aggressive behavior that usually possess amplification of the *MYCN* oncogene and allelic loss of chromosome 1p36 (Nakagawara et al. 1992, 1993). TrkB is preferentially expressed in aggressive neuroblastomas together with its preferred ligands BDNF and NT-4/5 which stimulate in an autocrine/paracrine manner, conferring an enhanced malignant phenotype to the tumor cells (Nakagawara et al. 1994; Matsumoto et al. 1995). TrkC is expressed in favorable neuroblastomas at variable levels (Yamashiro et al. 1996), but its preferred ligand, NT-3, is nearly undetectable by RT-PCR in primary neuroblastomas (Nakagawara 1998a); thus, in regressing neuroblastomas, tumor cells expressing the TrkA receptor may be dependent on a limited amount of NGF supplied from stromal cell. In the presence of NGF the cells mature, whereas they will die in the absence of this ligand (Nakagawara 1998a,b); however, in clinically aggressive neuroblastomas, the TrkA is downregulated and the downstream signaling cascades are disturbed, and these cells utilize the BDNF or NT-4/TrkB autocrine system for efficient growth. Neurotrophin signaling may also regulate tumor metastasis (Matsumoto et al. 1995), proliferation (Matsumoto et al. 1995), and angiogenesis (Canete et al. 2000). The role of $p75^{NTR}$ in neuroblastoma is unclear. The $p75^{NTR}$ receptor is expressed in both neuroblastoma cell lines (Azar et al. 1990) and primary neuroblastomas (Nakagawara et al. 1993). Interestingly, the expression levels of $p75^{NTR}$ mRNA are significantly higher in favorable neuroblastomas (stages 1, 2 and 4s) as compared with the advanced stage tumors, especially those with *MYCN* amplification (Nakagawara et al. 1993).

5.2.2.2 Neurotrophin Signaling in Neuroblastoma

In a rat pheochromocytoma cell line PC12, differentiation signals by NGF may be mediated through the tyrosine phosphorylation of the Trk receptor and through the subsequent activation of Shc/Grb2/SOS, Ras, Raf, MEK, and ERKs, while survival signals in the same cells may be transduced through the direct

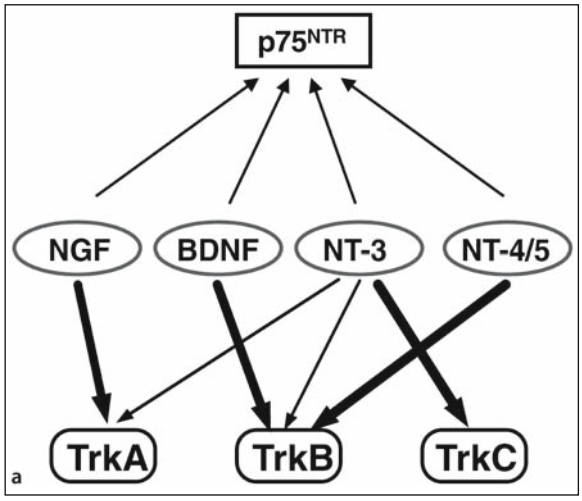
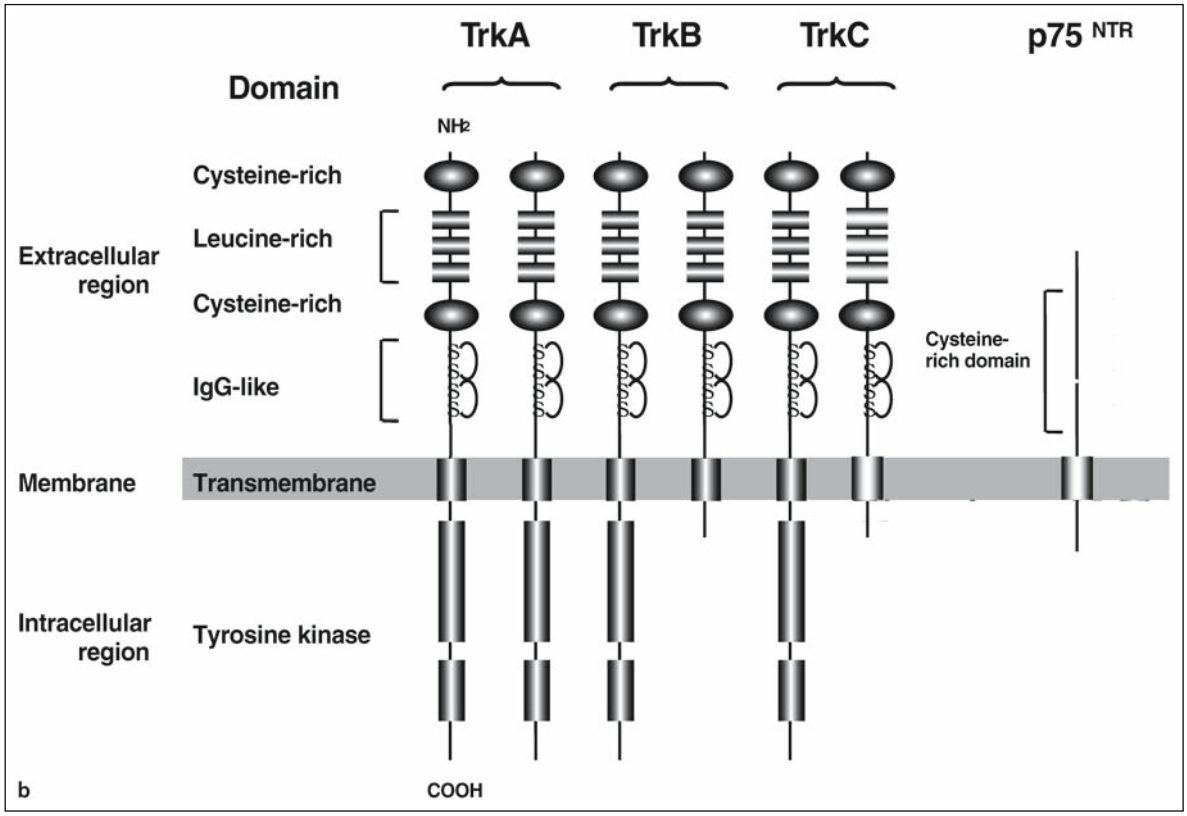


Figure 5.5 a, b

Neurotrophins and their receptors. **a** TrkA is a preferred high-affinity receptor for NGF, TrkB for BDNF, and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower affinity neurotrophin receptor $p75^{NTR}$. **b** The structures of neurotrophin family receptors. The extracellular domains of TrkA, TrkB, and TrkC have high structural similarity. The intracellular domain of Trks possesses tyrosine kinase activity. TrkB and TrkC receptors have truncated forms which lack the tyrosine kinase domain. The low-affinity receptor, $p75^{NTR}$, has a short intracellular region containing the death domain, and belongs to the Fas/TNFR family of the receptors



activation of PI3-kinase which in turn activates downstream molecules, e.g., Akt and Bad (Klesse and Parada 1999). On the other hand, in normal sympathetic neurons, the activation of PI3-kinase is mediated not by the tyrosine phosphorylation of the receptor but by the Ras activation which promotes neuronal survival, suggesting that the Trk intracellular signaling pathway might be deregulated in cancer cells. This is also the case in neuroblastoma. In the neuroblastoma cell lines with a single copy of *MYCN*, NGF can induce differentiation when exogenous TrkA is overexpressed (Eggert et al. 2000). In the cell lines with *MYCN* amplification, however, the NGF-stimulated TrkA receptors which were overexpressed cannot normally activate downstream signaling molecules, resulting in unresponsiveness to the ligand. Furthermore, it is surprising that BDNF/TrkB signaling appears to be functioning in the same cells by promoting survival (Nakagawara et al. 1994; Hishiki et al. 1998), although the signaling pathway might be different from that of sympathetic neurons (Klesse and Parada 1999).

5.2.2.3 GDNF Family Receptors

Neurotrophic factors of the glial cell line-derived neurotrophic factor (GDNF) family, which include GDNF, artemin and neurturin, are secreted by neuroblastoma cells as well as stromal cells and activate their receptor complex composed of Ret tyrosine kinase and the GFRα co-receptors expressed in neuroblastoma cells (Hishiki et al. 1998; Ichikawa et al. 2004). In contrast to NGF/TrkA and BDNF/TrkB, however, the GDNF/Ret/GFRα autocrine system is functioning in both favorable and unfavorable neuroblastomas to enhance the survival and differentiation of tumor cells (Hishiki et al. 1998).

5.2.2.4 Other Factors and Receptors

Neuroblastoma cells express other growth factors and receptors. Both pleiotrophin (PTN) and midkine (MK) are factors in the same family with neurotrophic function (Kadomatsu et al. 1990; Li et al. 1990; Kadomatsu and Muramatsu 2004). PTN is expressed significantly at high levels in favorable neuroblastomas, while MK is highly expressed in almost all neuroblastomas with a tendency to be expressed at high levels in tumors in advanced stages (Nakagawara et al. 1995). Neuroblastoma also expresses many other receptors, e.g., fibroblast growth factor receptor (FGFR; Schweigerer et al. 1991), insulin-like growth factor (IGFR; El-Badry et al. 1991), DCC (deleted in colon cancer) (Reale et al. 1996), and neuronal leucine-rich repeat receptors (NLRRs; Hamano et al. 2004), as well as a novel plasma membrane enzyme ECEL1, which is significantly highly expressed in favorable neuroblastomas (Kawamoto et al. 2003). The biological significance of these factors and receptors in neuroblastoma are not currently known.

5.2.3 Functional Role of p53 Family Genes

Recent lines of evidence suggest that both the p53 tumor suppressor protein and its related protein p73 are involved in the induction of programmed cell death and growth arrest in neuronal cells (Pozniak et al. 2000). p73 is a recently identified candidate tumor suppressor gene mapped to chromosome 1p36.2, a frequently deleted region in many human cancers including neuroblastoma and oligodendroglioma (Ichimiya et al. 1999; Billon et al. 2004). In cultured neonatal sympathetic neurons, p53 protein levels are increased in response to NGF withdrawal as well as $p75^{NTR}$ activation, and it functions downstream of c-Jun NH_2-terminal kinase (JNK) and upstream of Bax to induce apoptosis (Aloyz et al. 1998) (Fig. 5.6). Indeed, in $p53^{-/-}$ mice, naturally occurring sympathetic neuron death is inhibited. Pozniak et al. (2000) have also reported that p73 is primarily present in developing neurons as ΔNp73, an NH_2-terminally truncated isoform, whose level is decreased when sympathetic neurons undergo apoptosis after NGF withdrawal, and that p53 becomes activated to be pro-apoptotic. In contrast to the truncated form of p73, full-length p73 has induced neuronal differentiation in a mouse neuroblastoma cell line N1E115 (Laurenzi et al. 2000). These data suggest that the neuronal apoptosis induced by NGF withdrawal is at least partly regulated by a reciprocal balance between levels of pro-apoptotic p53 and anti-apoptotic ΔNp73.

Figure 5.6

A model of signaling pathway for survival and death in sympathetic neurons regulated by NGF. NGF depletion may induce activation of JNK/p53 pathway which could be modified by p73/ΔNp73 regulatory system. $p75^{NTR}$ activation, which sends signals of both survival and death, may also regulate downstream p53/p73/ΔNp73 pathway

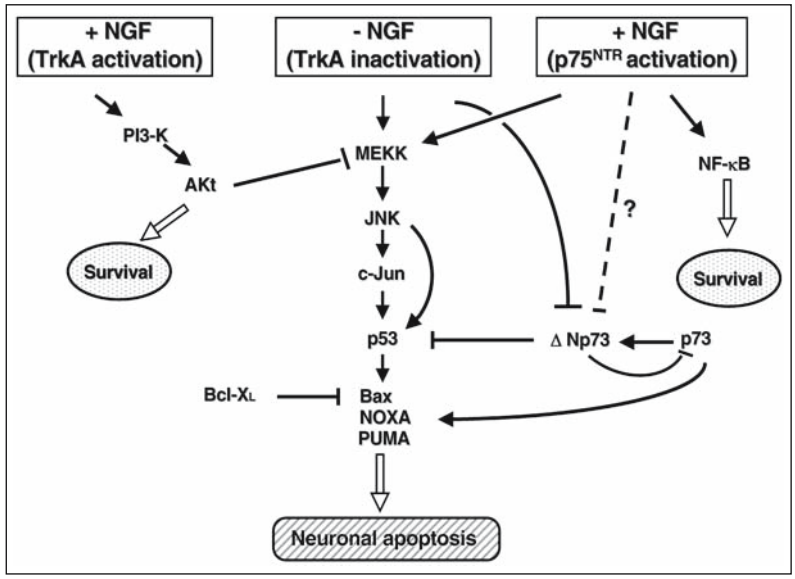
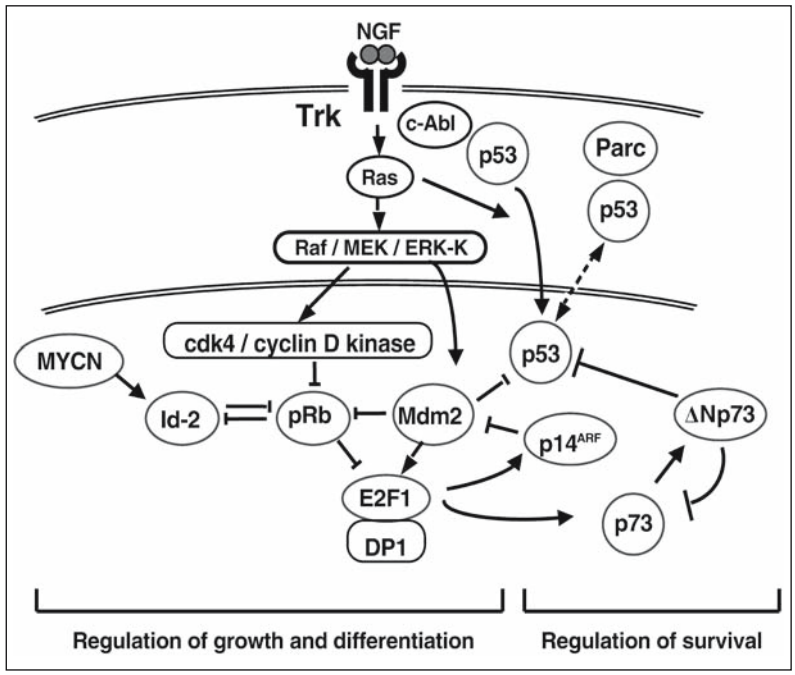


Figure 5.7

A possible signaling pathway regulating growth, differentiation and survival in neuroblastoma cells or sympathetic neurons. The NGF-triggered autophosphorylation of TrkA tyrosine kinase receptor induces activation of Ras/MAPK pathway, which in turn regulates nuclear pRB and Mdm2. In some poor-outcome neuroblastomas, p53, which is shuttling between cytosol and nucleus, is trapped in the cytosol by Parc, an anchoring protein of p53. *MYCN* induces expression of *Id-2* whose protein product in turn inhibits pRB. E2F1 negatively regulated by pRB directly induces expression of p73. p73 is regulated by ΔNp73 in a negative autoregulatory manner (Nakagawa et al. 2002), and ΔNp73 also inhibits p53



The importance of p53 and p73 has also been emphasized by the important observation that, in cultured neuroblastoma and other cancer cells, p73 directly transactivates the ΔNp73 gene by binding to its promoter after treating the cells with genotoxic reagents, e.g., cisplatin (Nakagawa et al. 2002). The induced ΔNp73 protein in turn interacts with either wild-type p53 or TAp73 and inhibits their proapoptotic function; thus, ΔNp73 can act as an oncogene and as an inhibitor of wild-type p53 and TAp73. The presence of this autoinhibitory feedback loop among p53, TAp73, and ΔNp73 may at least in part explain why there is no mutation of the p73 gene in cancers.

p53 is associated with TrkA via the proto-oncogene product c-Abl as an adaptor or bridging molecule, suggesting that it may also play a role in Trk signaling (Yano et al. 2000) (Fig. 5.7). The activation of Ras by NGF stimulation of the TrkA receptor induces p53 nuclear translocation and growth arrest in PC12 cells (Hughes et al. 2000). The c-Ha-Ras gene could be a target of p53, and protein products induce a positive feedback loop by activating $p14^{ARF}$ which counteracts the negative feedback loop mediated by mdm2 (Deguin-Chambon et al. 2000). These observations strongly suggest that p53 and p73 tumor suppressors function in neurotrophin signaling and modulate the growth, differentiation, and apoptosis of neurons.

In neuroblastoma and some other human cancers, wild type p53 is often localized in the cytoplasm (Moll et al. 1995). Although the regulatory mechanism of cellular localization of p53 and p73 is still unknown, activated Ras in NGF/TrkA signaling stimulates the nuclear translocation of p53 and leads to growth arrest by the induction of $p21^{WAF1}$ in PC12 cells (Hughes et al. 2000). Furthermore, some fractions of recurrent neuroblastomas and neuroblastoma cell lines acquire mutation of the p53 gene (Tweddle et al. 2001).

5.2.4 Apoptotic Signals in Neuroblastoma

To date, the spontaneous regression of neuroblastoma, has occurred only *in vivo*. Although this makes the analysis difficult, there are some important reports. An anti-apoptotic protein, Bcl-2, is expressed in primary neuroblastomas and neuroblastoma cell lines. The expression levels of Bcl-2 and Bcl-X_L are high in aggressive tumor cells but are low in regressing cells (Ikeda et al. 1995; Ikegaki et al. 1995). Caspase-1 and caspase-3 are expressed at significantly higher levels in favorable neuroblastomas (Nakagawara et al. 1997), and caspase-8 is silenced in aggressive neuroblastomas by the methylation of its promoter as one of mechanisms (Teitz et al. 2000). Silencing of caspase-8 is observed in 25–35 % of primary neuroblastomas with a high frequency in more aggressive tumors (Teitz et al. 2000; Eggert et al. 2001; van Noesel et al. 2003). Survivin, a member of the inhibitors of apoptosis protein (IAP), is mapped to the long arm of chromosome 17. In neuroblastoma, survivin is highly expressed in high-risk tumors, and its overexpression inhibits cellular apoptosis (Islam et al. 2000). Kitanaka et al. (2002) have recently reported an interesting observation that "autophagy" may be involved in the regression of neuroblastoma cells.

5.3 Conclusions

Development of neuroblastoma may be triggered by a genetic event(s) that leads to chromosome and/or the genomic DNA abnormalities such as amplification of the *MYCN* gene and deletions or gains in chromosomal regions including 1p, 11q, and 17q. Together with other epigenetic mechanisms of gene activation or gene silencing, they affect gene and protein expression which in turn deregulate cellular signaling. In neuroblastoma the normal biology of developing neuronal cells and cancer biology appear to overlap. A further understanding of the mechanisms involved in the transformation of progenitors or the stem cells into neuroblastoma with significant cellular heterogeneity may provide clues for the development of novel therapeutic strategies for this often aggressive lethal disease.

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Cellular Heterogeneity

Robert A. Ross

Contents

6.1 Introduction

Cellular heterogeneity, a hallmark of cancer, probably accounts for the variability of its clinical presentation and non-uniform response to treatment. For neuroblastoma (NB), this heterogeneity results from the plasticity of the embryonic neural crest, from which this tumor originates (Biedler et al. 1997; Brodeur 2003). This chapter briefly reviews the lineages manifested by the developing neural crest and the biology of the distinct cell types in human NB.

6.2 Neural Crest Differentiation

The neural crest is a transient embryonic cell structure generated from the neuroectodermal plate upon closure of the neural tube (Le Douarin and Ziller 1993). Migrating neural crest cells from the trunk region of the embryo generate neuronal and glial cells of the peripheral nervous system, neuroendocrine and sensory ganglion cells, as well as non-neural pigment and smooth muscle-like cells. An important aspect of neural crest development germane to NB is that cell division continues along with the progressive restriction of differentiation potential and is even present in adrenal medullary cells postnatally (Mascorro and Yates 1989). Thus, two seemingly divergent cellular programs are operating simultaneously: proliferation and differentiation.

Excellent studies have highlighted the amazing pluripotent nature of the neural crest anlage. Detailed studies using a chick/quail chimera showed that the local tissue microenvironment plays a pivotal role in effecting the differentiation lineages (Le Douarin and

Ziller 1993). More recently, restrictive signaling factors that promote commitment to particular cell fates in migratory and postmigratory neural crest precursor cells have been delineated (Lo et al. 2002; Hemmati et al. 2003; Luo et al 2003). For example, achaete–scute complex (e.g., HASH1) and atonal (ato) homologs (e.g., neurogenin) are required in vivo for development of autonomic and sensory neurons, respectively. By contrast, melanocytes are generated by Wnt signaling, while TGFβ promotes smooth muscle cell development. Finally, Notch and neuregulin promote satellite glial and Schwann cell differentiation.

6.3 Neuroblastoma Cellular Heterogeneity

Many of the cell phenotypes characteristic of the developing neural crest – neuroblasts, non-neuronal (Schwann, perineurial, or satellite) cells, and even melanocytes –are evident in the same NB (Shimada et al. 1999). Moreover, cellular heterogeneity and extent of maturation (e.g., stroma-rich and stroma-poor tumors or high- and low-risk tumors based on histological grade) correlate with clinical behavior and are useful for prognostication of the disease (see Chap. 7; Shimada et al. 1999).

This same cellular heterogeneity is seen in NB cell lines. Three distinct cellular phenotypic variants have been described (Rettig et al. 1987; Biedler et al. 1988, 1997): sympathoadrenal (N-type) neuroblasts; large flattened, substrate-adherent (S-type) cells; and morphologically intermediate (I-type) cells (Fig. 6.1). Studies over the past 25 years have shown that each phenotype represents a particular lineage within the neural crest. The availability of cell lines of the three cell types has led to an increased understanding of the differentiation and malignant potentials of each.

6.4 N-type Neuroblastic Cells

In vitro, the predominant neuroblastic (N) cells resemble sympathoadrenoblasts – immature neural/neuroendocrine precursors, with small rounded cell bodies and neuritic processes that vary widely in number and length. Cells adhere poorly to the underlying substrate but adhere well to each other to form cell clumps (pseudoganglia), achieving high saturation densities in culture (Rettig et al. 1987; Biedler et al. 1997; Spengler et al. 1997). Biochemically, they express proteins for synthesis, binding, and degradation of norepinephrine and acetylcholine (the two major neurotransmitters of the peripheral nervous system), as well as opioid and cholinergic receptors. They express the neuroectodermal stem cell intermediate filament nestin, as well as all three neurofilament proteins and chromogranin A (CgA) and secretogranin II (SgII), depending on their degree of differentiation (Biedler et al. 1997; Ross et al. 2002; Thomas 2003). In addition, they express dHAND and HASH-1, transcription factors that are markers of the early stages of neural crest development (Jögi et al. 2002).

Another transcription factor associated with NB and early neuronal development is *MYCN* (Chap. 4). Expression of the oncoprotein is associated with increased mitosis and a dedifferentiated state in neuroectodermal cells of the CNS. High-level expression requires a neuroblastic phenotype, as non-neuronal variants do not express the protein even in the cell lines with transcriptionally active, amplified *MYCN* genes (Spengler et al. 1997).

N-type cells are tumorigenic. They form colonies in soft agar and tumors in mice, with variable degrees of malignancy (Spengler et al. 1997); however, too few *MYCN*-nonamplified N-type cell lines have been tested to discern a relation between *MYCN* amplification status and malignant potential.

Experimental protocols can induce N-type cells to differentiate along either a neuronal or a neuroendocrine pathway or de-differentiate to an immature neural crest-like phenotype. Neuronal differentiation following addition of retinoids or cyclic AMP-elevating agents is characterized by decreases in cell division and amounts of CgA and *MYCN* protein and increases in SgII and neurofilament proteins and in the number and length of neurites (Ross et al. 2002). Neuroendocrine differentiation induced by synthetic glucocorticoids results in cell flattening, increases in CgA and *MYCN* levels, and decreases in neurite formation, SgII, and neurofilaments (Ross et al. 2002). Hypoxia has also been shown to affect neuroblastic

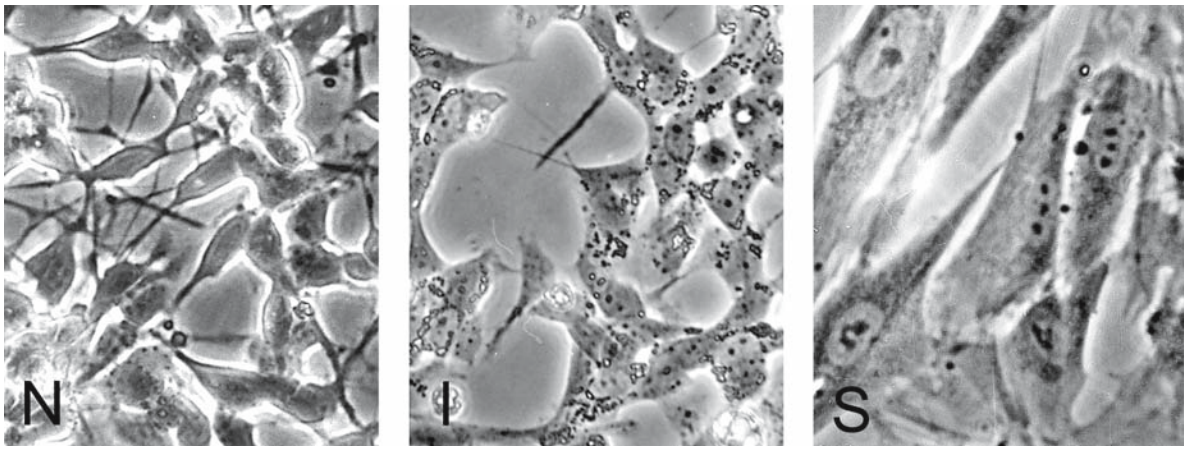

Figure 6.1

Phase-contrast photomicrographs of phenotypic cell variants derived from the LA-N-1 (*N*), SK-N-BE(2) (*I*), and SMS-KCN (*S*) neuroblastoma cell lines (magnification ×500)

differentiation. Growth of N-type cells under hypoxic conditions causes decreased expression of neuronal/ neuroendocrine-specific genes (e.g., CgA and neuropeptide Y) and increased expression of genes present in early neural crest development (c-kit, Notch-1, and HES-1) – indicators of de-differentiation (Jögi et al. 2002).

6.5 S-type Non-Neural Cells

In addition to neuroblasts, a second, clearly non-neuronal cell type is frequently observed in NB cell lines. Termed S, for "substrate adherent", it exhibits contact inhibition of growth, extensive migration on a substrate, and a limited lifespan in culture. Unlike the clearly defined neuronal lineage of N cells, the biochemical signature of S-type cells is more variable. Studies have identified melanocytic properties (tyrosinase, melanosomal glycoproteins, and melanosomes), Schwann or glial cell markers (chondroitin sulfate proteoglycans and large amounts of laminins and fibronectin), and/or smooth muscle cell features (alpha-smooth muscle actin and calponin) (Rettig et al. 1987; Tsokos et al. 1987; Jessen and Mirsky 1999; Sugimoto et al. 2000). All of these lineages are consistent with a neural crest origin for the S cell, as developing crest cells of the trunk give rise to non-neuronal Schwann, glial, melanocytic, and smooth muscle cell components in vivo. The presence of nestin in these cells is consistent with the S-cell phenotype as a neuroectodermal precursor of the non-neuronal lineages of the neural crest (Thomas 2003).

S cells differ from N cells in two other aspects. Firstly, S cells display markers for HLA class-I antigens and β2-microglobulin, which are absent on N-type cells (Rettig et al. 1987). Secondly, unlike N cells, S-type cells will not grow in soft agar or form tumors in nude mice (Biedler et al. 1988; Spengler et al. 1997).

The NB tumors with abundant stroma (stroma-rich) generally have a better prognosis than stroma-poor tumors (Ambros and Ambros 1995; Shimada et al. 1999; Brodeur 2003). The discovery that, in vitro, N and S cells arise from a common precursor suggested that, in vivo, stromal cells could be of tumor origin. One study, using paraffin nonisotopic in situ hybridization, concluded that stromal cells are of nontumor origin, presumably recruited by the neuroblasts in the tumor (Ambros and Ambros 1995). Subsequent studies, using short-term culture of

bone marrow tumor cells or laser-capture microdissection with bicolor fluorescence in situ hybridization, showed that both neuroblasts and Schwann cells had identical genetic markers – strong evidence that they arise from a neoplastic precursor (Valent et al. 1999; Mora et al. 2001). This topic is still under debate.

Equally important is the extent of the interaction between Schwann (or S-type) cells and neuroblastic (or N-type) cells in the survival/proliferation/differentiation of each phenotype. In early experiments, N cells co-cultured with S cells were much more differentiated and grew more slowly (B.A. Spengler and J.L. Biedler, personal communication). Such results are consistent with studies of developing neurons and Schwann cells which show that reciprocal contact determines survival and differentiation (Jessen and Mirsky 1999). Also, conditioned medium from normal Schwann cells in culture increases NB cell survival and differentiation (Kwiatkowski et al. 1998) and contains a potent inhibitor of angiogenesis, thus providing a mechanistic basis for the benign behavior of stroma-rich tumors (Huang et al. 2000).

6.6 I-type Stem Cells

The I-type cell was initially identified in cultured cell lines because it appeared "intermediate" in morphology between N and S cells. It exhibits morphological features of both N-type cells (short neurite-like cell processes and growth to high saturation densities) and S-type cells (strong adhesion to and extensive migration over the substrate) (Biedler et al. 1997). These cells also express proteins of both differentiation pathways – noradrenergic biosynthetic enzymes, granins (CgA and SgII), and neurofilament proteins of neuroblasts as well as S cell proteins vimentin, EGF receptor, and CD44. Examples of I-type cells include the cell lines GOTO, NUB-7, BE(2)-C, SH-IN, and LA-N-2 (Biedler et al. 1988, 1997; Ross et al. 1995, 2002).

Continuing research indicates that this cell represents a unique cell type within the NB repertoire. Its ability to generate daughter cells with the same phenotype (self-renewal) and to differentiate bidirectionally along either neuroblastic or Schwann/glial pathways suggests that it is a neural crest cancer stem cell.

First demonstrated for BE(2)-C cell clones and subsequently for other I-type cell lines, I-type cells become neuroblastic when treated for 7–14 days with retinoic acid (RA), but differentiate into S-type cells following treatment with BUdR (Ross et al. 1995). Unlike N (or S) cells, I cells retain the ability to convert to two distinctly different cell lineages.

The most provocative finding regarding the NB I-type stem cell is its malignant potential. As a group, these stem cells are more malignant than neuroblastic variants; they have four- to fivefold higher colony-forming efficiencies in soft agar than N cells and have an over sixfold greater capacity to form tumors in nude mice (Ross et al. 2003; Spengler et al. 1986; Walton et al. 2004). Moreover, phenotype rather than *MYCN* amplification/overexpression determines malignancy; e.g., NB I-type stem cells lacking *MYCN* amplification are more tumorigenic than N-type cells which contain >150-fold amplified genes. Thus, research to date on cell lines suggests that the I-like stem cell could be the truly tumorigenic cell component of NB tumors.

Malignant stem cells in tumors could exert a significant negative effect on prognosis and long-term survival; however, distinguishing putative stem cells from those with a neuroblastic phenotype in tumor sections by routine hematoxylin–eosin analysis is difficult, if not impossible. To specifically search for I-like cells, tumor sections immunostained conjointly with antibodies specific for N or S cells were examined for the presence and frequency of double-labeled cells (Ross et al. 2003). In preliminary analyses, doubly labeled I-like cells were present in all tumors (Fig. 6.2). When the tumors were grouped as either good risk (typically local regional or stage 4s) or poor risk (stages 3 or 4), the frequency of I-like cells was significantly higher (~ fivefold) in the latter group (B.A. Spengler, personal communication). The characterization of this previously unnoticed NB cell type in cell lines and its potential role in refractory high-risk tumors may have identified an important new target for experimental therapeutics.

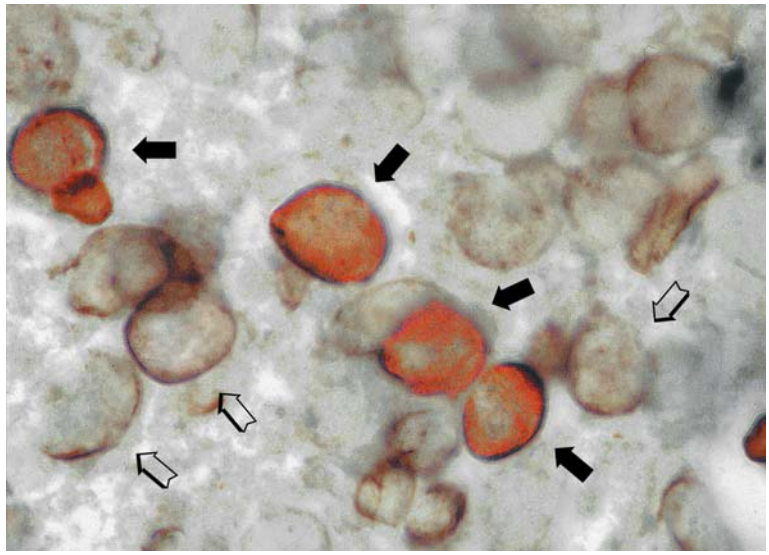

Figure 6.2

Neuroblastoma tumor section stained conjointly for expression of S100A6 (an S cell marker; *red*) and neurofilament 160 (an N-cell marker; *gray*). Examples of I-like cells expressing both proteins are indicated by *filled arrows*, whereas N cells expressing only neurofilament protein are denoted by *open arrows*

6.7 Transdifferentiation

Transdifferentiation is the process whereby cells change from one unique phenotype into another unique phenotype without going through a developmentally less mature stage (Liu and Rao 2003). This process has been reported for cells from all three germ layers and, in particular, for cells of the hematopoietic and neural systems. However, distinguishing de-differentiation/re-differentiation (a two-stage process involving reversion to a more immature stage before expression of the novel phenotype) from transdifferentiation is not easy, especially in heterogeneous populations of cells or those where the intermediate cell type is not readily identifiable (Liu and Rao 2003). In studies with SH-SY5Y (N-type) and SH-EP (S-type), clones of the SK-N-SH cell line, cells with morphological and biochemical features of the other phenotype arose spontaneously and were subcloned (Ross et al. 1995). Of importance, transdifferentiated subclones each have a marker chromosome unique to the clone of origin; therefore, these lines did not arise by clonal selection of pre-existing variants, as has been suggested (Cohen et al. 2003), but represent the conversion to a new cell phenotype. Similar phenotypic conversions have been seen for the LA-N-1 and SK-N-BE(2) cell lines: N-type LA1–55n arose spontaneously and was cloned from the S-type LA1–5s as were S-type LA1–19Bs cells from the N-type LA1–19n clonal cell line. Likewise, the twice-cloned BE(2)-M17 cell line gave rise to the BE(2)-M17F S-type clone. In all cases, the interconversion/transdifferentiation process occurred spontaneously and morphological, biochemical, and cytogenetic criteria were used to confirm the phenotype and cell of origin. Transdifferentiation is very rare and it is the ability to select for the different cell types in culture that has permitted its documentation. Whether the phenomenon observed in NB represents true transdifferentiation or a more complex process involving de-differentiation followed by differentiation along a second neural crest pathway has not been resolved. Nevertheless, the interconversion of N- and S-type cells in culture would suggest that it may occur in vivo. The evolution of quiescent S-type cells into highly proliferative N or I cells mimics the clinical picture of a rapidly recurrent neuroblastoma following a period of clinical remission.

6.8 Conclusions

Cellular heterogeneity is a common feature of human NB tumors and cell lines. Moreover, the different phenotypes identified in fresh tumors are similar, if not identical, to those seen in cell lines; thus, cell lines may serve as useful surrogates in the investigation of the biochemical, differentiable, and tumorigenic heterogeneity of human NBs. It is clear that the cell variants differ markedly in growth potential, both in vitro and in vivo. A small amount of experimental data would also suggest that variants differ in the intrinsic sensitivities to commonly used chemotherapeutic agents. It is also clear that "cross-talk" may occur between cell variants within tumors, further influencing tumor viability, tumorigenicity, or response to therapy. The identification of putative stem cells within tumors and characterization in cell culture may offer new opportunities for developing strategies for more effective control of NB.

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Clinical Presentation

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Contents

7.1 Introduction

For almost 140 years physicians have been aware of the enigmatic tumor in young children called "neuroblastoma." In 1864 Virchow was the first to describe a child with an abdominal tumor which he designated as "glioma." For more than a century the gliomatous aspect of neuroblastoma has been neglected, but it is now vehemently debated whether the stromal part is neoplastic (Mora et al. 2001) or reactive (Ambros et al. 1996) in nature. Marchand disclosed in 1891 the common features of tumors from the sympathetic nervous system and the adrenal medulla. In 1901 Pepper described the prenatal metastases of an adrenal sarcoma to the liver with ascites, scrotal edema, anemia, and recurrent fever (Pepper type; Pepper 1901) which is presently known as stage-4S neuroblastoma, the prototype of a spontaneously regressing tumor. Hutchinson observed in 1907 the characteristic bilateral periocular hematomas with proptosis of the eyes as symptoms of orbital and skeletal metastases of an adrenal tumor (Hutchinson type; Hutchinson 1907), the prototype of a progressing, still widely treatment-resistant neuroblastoma.

The term neuroblastoma was introduced in 1910 by Wright when he demonstrated that the tumor originated from embryonal neuroblasts of the sympathetic peripheral nervous system (Wright 1910). The ability of neuroblastoma for spontaneous maturation into ganglioneuroma was first described in 1927 by Cushing and Wolbach. The detection of catecholamine metabolites as tumor markers was first reported by Mason et al. (1957) in a patient with hypertension, a quite rare feature in this disease. Two years

later, vanillylmandelic acid (VMA) was identified as one of the main metabolites and tumor markers (Greenberg 1957; Stickler et al. 1959). In 1971 Evans et al. proposed the first internationally accepted staging system (Evans et al. 1971). Schwab and coworkers (1983) detected MYCN amplification as an important molecular feature of cell lines and primary tumors which proved to be a reliable marker for indicating rapid tumor progression (Seeger et al. 1985) and is now considered an essential parameter for risk estimation.

This chapter describes the clinical presentation of children with neuroblastoma and a comprehensive international perspective of the current criteria that are used for diagnosis, staging, and risk classification. The data regarding clinical presentation are derived from the German experience during the past 24 years. Greater than 95% of all German neuroblastoma patients known to the central children's tumor registry were enrolled in national trials during this time period, and only those with the wrong diagnosis were excluded from analysis; thus, these clinical data can be considered representative of neuroblastoma in a Western country.

7.2 Diagnosis

According to international agreement (Brodeur et al. 1993), the diagnosis of neuroblastoma is established if (a) unequivocal pathologic diagnosis is made from tumor tissue by light microscopy (with or without immunohistology, electron microscopy, increased urine, or serum catecholamine metabolites), or (b) bone marrow aspirate or trephine biopsy contain unequivocal tumor cells (e.g., syncytia or immunocytologically positive clumps of cells) *and* increased urine or serum catecholamine metabolites.

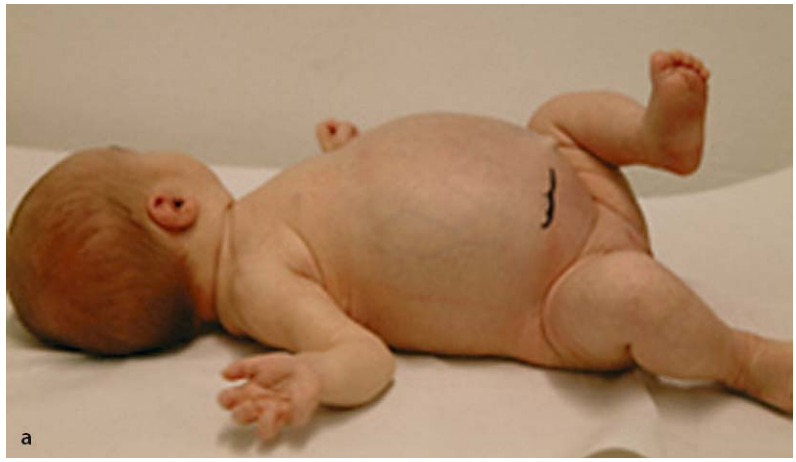
A "suspected clinical diagnosis" of neuroblastoma may be established in emergency situations based on its radiographic features together with distinctly elevated catecholamine metabolites and MIBG avidity. This diagnosis must be considered preliminary (because these tests do not rule out mature ganglioneuroma or pheochromocytoma) and incomplete; thus, tissue histology should always follow.

In addition to tumor histology and urinary catecholamines, the investigation of tumor tissue for genetic abnormalities can aid in the diagnosis of neuroblastoma and provide clinically relevant prognostic information. While certain genetic aberrations are characteristic of neuroblastoma (deletion of 1p36; *MYCN* amplification), specific tumor karyotypic abnormalities [e.g. (t11; 22) for Ewing's sarcoma, (t2;5), (t8;14) for non-Hodgkin's lymphoma, t(2;13) for rhabdomyosarcoma] exclude a diagnosis of neuroblastoma.

7.2.1 Diagnostic Tumor Tissue

Due to the considerable heterogeneity that can be present in neuroblastoma tumors, a single biopsy may not be representative of the tumor's histology or biology. Recently, specific guidelines for pathology and biology studies have been published (Ambros and Ambros 2001). It is recommended that at least two samples, each of 1×1×1 cm size from morphologically different appearing areas, be examined. For unresectable tumors, open biopsies are preferred and, if possible, two different areas should be biopsied by the surgeon. If only Tru-Cut biopsies are feasible, due to the poor general condition of the child, four samples from different areas are recommended (at least 1 cm long; 0.1 cm thick; needle size 18 G). Fine-needle aspiration cytology (22-G needle size; at least 10^5–10^6 cells) with or without ultrasound guidance do not provide an assessment of the tissue architecture and are not recommended but may be sufficient in some cases for cytological and molecular diagnosis.

To ensure the correct sampling and quick handling of the tumor tissue, the presence of an experienced pediatric oncologist in the operation room is highly recommended. Together with the pathologist, and optimally within 20 min from resection, touch preparations for cytology and fluorescence in situ hybridization (FISH) investigations are made before formalin fixation for the pathological examination. Pieces are snap frozen for molecular studies (e.g., Southern blot, PCR) and put in tissue culture medium for classic cytogenetic investigations and tissue culture studies. In the case of small specimens, histology with touch preparations and snap freezing should be priorities.



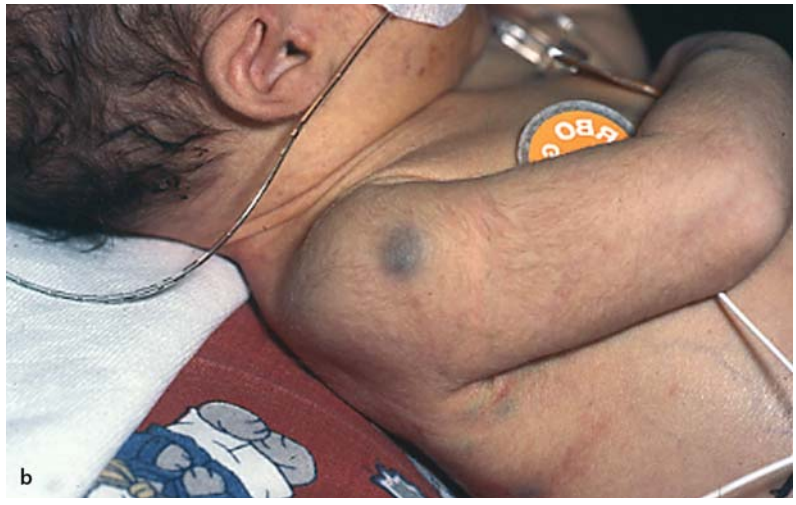


Figure 7.1 a,b

a A 6-week old girl with abdominal distension by massive liver enlargement (lower rim is marked) and bilateral adrenal primaries. Minimal bone marrow involvement (<1 %), good general condition (stage 4S). **b** Blueberry appearance of subcutaneous metastases in a newborn with stage-4S disease

7.3 Clinical Presentation

7.3.1 Symptoms

7.3.1.1 Frequent Symptoms

Presenting signs and symptoms of children with neuroblastoma reflect both the location of the primary tumor and the extent of disease. The typical patient with neuroblastoma is an infant or a toddler. Pain from abdominal distension or metastases to bone is common (30 %). Many patients fail to gain weight or have weight loss (11 %). The characteristic bilateral periorbital ecchymosis, which is a sign of metastatic disease, is typically caused by intraorbital masses.

In contrast, infants with stage 4S commonly present with abdominal distension resulting from massive liver infiltration (Fig. 7.1 a) and subcutaneous nodules (most of them better palpable than visible, sometimes blueberry appearance; Fig. 7.1b). The massive hepatomegaly can lead to respiratory distress, and kidney or bowel function can be impaired due to obstruction by the tumor. Their medical condition can rapidly deteriorate within hours or days (Berthold et al. 1990).

Table 7.1. Rare but characteristic symptoms of neuroblastoma (*n*=1878, trials NB90/97)

Symptom	Pathogenesis	Occurrence (%)	5-year survival (%)[a]
Transverse myelopathy	Dumbbell tumor with intraspinal, extradural extension	5.4	84±4
Treatment-resistant diarrhea	Vasointestinal peptide secretion by the tumor	3.9	55±7
Horner's syndrome	Cervical tumor with involvement of the cervical ganglion	2.4	79±7
Opsomyoclonus–ataxia syndrome	Unknown ("paraneoplastic")	1.3	87±8
Hypertension	Secretion of pressure active catecholamine metabolites by the tumor or compression of the renal artery	1.3	74±11

[a] The overall survival ratios reflect the data of the designated group. They have not been balanced according to risk factors such as age, stage, *MYCN* amplification, etc. The 5-year overall survival of the entire group was 71±1%

Patients with localized disease are often asymptomatic, and in many cases the diagnosis of neuroblastoma is made following a radiograph or ultrasound performed for unrelated reasons. The rate of incidental diagnosis has varied considerably between countries, even before screening programs were introduced. In Great Britain 7.7% of the neuroblastoma patients were diagnosed at visits for routine health examination or following an investigation of an unrelated condition, compared with 27.1% in Austria and 33.9% in Germany (Powell et al. 1998).

7.3.1.2 Rare but Characteristic Symptoms

Rare but characteristic symptoms of neuroblastoma are shown in Table 7.1.

Transverse Myelopathy

Transverse myelopathy can result from growth of a cervical, intrathoracic, or intraabdominal neuroblastoma through neural foramina into the spinal canal. Approximately half of the patients with dumbbell lesions initially present with neurological symptoms (Katzenstein et al. 2001), but myelopathy may develop soon afterwards, e.g., during surgery; thus, the degree of intraspinal tumor extension should be evaluated by MRI before surgery in order to avoid decompensation of a labile steady state. The neurological abnormalities associated with these tumors include motor deficit (>95%), radicular or back pain (54%), sphincter abnormalities, (34%), and sensory (12%) deficits (de Bernardi et al. 2001). The frequency of complete neurological recovery appears to be inversely correlated with the severity of the presenting neurological deficits (Katzenstein et al. 2001). Forty to 50% of the severely affected surviving children experience long-term neurological sequelae (Katzenstein et al. 2001; de Bernardi et al. 2001). There is a high likelihood of permanent neurological dysfunction in patients who experience neurological symptoms for more than 1 week prior to the initiation of treatment. Chemotherapy, radiotherapy, and surgical decompression with laminectomy have been shown to result in similar rates of neurological recovery, but chemotherapy may be associated with fewer long-term sequelae (Katzenstein et al. 2001; de Bernardi et al. 2001).

Opsomyoclonus–ataxia syndrome

The opsomyoclonus–ataxia syndrome (Kinsbourne syndrome) is characterized by rapid, irregular movements of the eyes ("dancing eyes"; may continue during sleep) and/or by myoclonus and ataxia of the limbs ("dancing feet"), the trunk, and the eyelids.

Many patients experience developmental delays including cognitive and motor delays, language deficits, and behavioral abnormalities (Russo et al. 1997; Rudnick et al. 2001). The pathogenesis is still unclear, although extensive lymphocytic infiltration of the tumor tissue (compared with neuroblastoma patients without opsomyoclonus) (Cooper et al. 2001) and the presence of anti-neuronal antibodies (Rudnick et al. 2001) suggest that the disorder is immunologically mediated. Removal of the primary tumor may not necessarily cure the neurological manifestation. The pharmacological treatment of the neurological symptoms includes glucocorticoids (prednisone or ACTH), high-dose immunoglobulins, and cytotoxic drugs. Anecdotal reports indicate that 60–80% of the patients respond to treatment, but long-term neurodevelopmental results are still poor (60–70% permanent handicaps). The survival rate of children with opsomyoclonus–ataxia syndrome is generally favorable, because the majority of them have localized tumors and present at a young age (Rudnick et al. 2001) (see Chaps. 11 and 13; Table 7.1).

Horner's Syndrome

Horner's syndrome (ptosis, miosis, enophthalmos) and heterochromia (difference in color between the two irises) is caused by disturbances of the cervical sympathetic ganglia which are responsible for normal eye color and development. An association has been found with neuroblastoma only and not with other tumors of the same area (Jaffe et al. 1984) indicating an intimate cooperation between the ganglia and the neuroblastoma development.

Treatment-Resistant Diarrhea

The association of treatment-resistant diarrhea, hypokalemia, and dehydration with neuroblastoma is observed in approximately 4% of patients and is thought to result from overproduction of the vasointestinal peptide (VIP) by maturing neuroblastomas (El Shafie et al. 1983) or ganglioneuromas. It usually resolves after surgical removal of the primary tumor. The use of chemically designed VIP antagonists is still in the preclinical phase (Lilling et al. 1994).

Hypertension

Hypertension is usually caused by tumor pressure on the renal artery with consequent stimulation of the renin–angiotensin system, rather than by tumor secretion of vasoactive catecholamine metabolites (dopamine, epinephrine, norepinephrine). With chemotherapy hypertension can worsen before it gets better. If α- and β-blockers or angiotensin-converting-enzyme inhibitors fail to control the blood pressure and the tumor remains unresectable, one may consider surgically freeing the renal artery without attempting tumor resection.

7.3.2 Tumor Markers

Neuroblastoma is one of the few pediatric tumors in which tumor markers have been shown to have a role in the diagnosis, prognosis, and disease monitoring. Generally, the frequency of abnormal levels increases with tumor burden (stage) and cell turnover (Table 7.2). The reference ranges are strongly age-dependent, with considerably higher values the newborn period and infancy.

Table 7.2. Stage dependence of abnormal tumor markers in neuroblastoma. *HVA* homovanillic acid, *VMA* vanillylmandelic acid, *INSS* International Neuroblastoma Staging System

Tumor marker	*N*	INSS stage			
		1–3 %	4 %	4S %	All %
HVA and/or VMA in urine and/or serum	1280	82	96	93	89
Neuron-specific enolase	1572	57	97	80	75
Lactate dehydrogenase	1809	38	88	45	58
Ferritin	1476	16	53	25	32

7.3.2.1 Catecholamine Metabolites

Catecholamine metabolites represent the most sensitive and specific tumor markers. While the determination of vanillylmandelic acid (VMA) and homovanillic acid (HVA) in a clean void urine sample is considered essential, the additional value of dopamine is less clear. The simultaneous measurement of urinary creatinine permits reliable VMA and HVA estimates in spot urine samples avoiding the uncomfortable 24-h urine collection. The determination of VMA, HVA, and dopamine in serum samples may be useful in some instances, but is 10–15% less sensitive. The usefulness of catecholamine metabolites as early markers of recurrence may be limited. In one study only 54% of patients demonstrated abnormal values at the time of recurrence (Simon et al. 2003). Using HPLC or mass spectrometry, the number of false-positive values is substantially reduced. False positives (predominantly HVA levels) were observed during a large screening program after massive apple juice intake, with active neurodermatitis, and with some congenital neurodevelopmental disorders. The ratio VMA/HVA as an indicator for prognosis has been diminished by the advent of new molecular markers, while the clinical utility of the dynamical response of tumor markers to treatment is just emerging (Hero et al. 2001).

7.3.2.2 Neuron-Specific Enolase

Neuron-specific enolase (NSE) is synthesized by neuroblastoma cells and used as an immunohistochemical marker. Elevated serum levels have been reported in other neuroectodermal tumors such as Ewing's sarcoma, small cell lung cancer, and pheochromocytoma, as well as in acute lymphoblastic leukemia and non-Hodgkin's lymphoma (Hann and Bombardieri 2000). High levels at diagnosis were associated with poor outcome in several studies (cutoff levels 30–100 ng/ml) when corrected for stage (Zeltzer et al. 1983). Neuron-specific enolase is less specific for neuroblastoma than the catecholamine metabolites, but is more prognostic, and similarly valuable for monitoring recurrent disease (Simon et al. 2003).

7.3.2.3 Ferritin

Neuroblastoma cell lines and tumors produce and secrete ferritin which is biochemically different (glycosylated, electrophoretic characteristics) from that secreted by normal cells (Hann and Bombardieri 2000). Elevated serum ferritin levels have been observed not only in neuroblastoma but also in Hodgkin's disease, leukemia, and breast cancer. While tumor cells from infants with stage-4 and stage-4S tumors contained equivalent amounts of ferritin (Hann et al. 1981), the highest serum levels were only observed in children with stage-4 disease with poor prognosis (Table 7.2; Hann et al. 1981). Although ferritin is a robust prognostic marker at diagnosis (Hann et al. 1985; Berthold et al. 1994), it is unsuitable for monitoring the disease, because it becomes elevated from frequent blood transfusions during chemotherapy; thus, ferritin appears helpful for estimating the prognosis, but not for diagnosis and monitoring.

7.3.2.4 Lactate Dehydrogenase

Several multivariate analyses demonstrated that elevated serum lactate dehydrogenase (LDH) levels provide additional prognostic information that is independent of stage, age, and other factors (Berthold et al. 1992a, 1994; Shuster et al. 1992; Lau 2002). The majority of children with localized neuroblastoma have normal LDH levels at diagnosis, whereas LDH is elevated in most children with stage-4 disease. Since LDH is not tumor specific, it is not useful for differential diagnosis. Nevertheless, since high levels reflect fast cell turnover and tumor load (irrespective of primary tumor size), it can be a useful marker for monitoring high-risk disease. In the absence of modern molecular markers (e.g., lack of tissue), the LDH level may provide the best prognostic estimation within the various stage and age categories (Berthold et al. 1992a, 1994).

7.3.2.5 Other Tumor Markers

Chromogranin A is an acidic protein which is co-stored and co-released with catecholamines from storage vesicles. Mean serum chromogranin-A levels correlated with disease stage and prognosis in chil-

Table 7.3. Primary sites in 1967 patients with neuroblastoma by stage

Primary site	INSS stage			
	1–3 %	4 %	4S %	All %
Cervical	3.9	0.7	2.6	2.6
Thoracic	19.5	8.4	12.2	14.7
Adrenal	41.4	62.3	63.8	51.3
Abdominal (non-adrenal)	33.2	23.0	17.8	27.9
Combined sites	1.8	2.9	1.0	2.1
Other	0.2	0.1	0	0.2
Undetected	0	2.5	2.6	1.2
Total	100	100	100	100

dren over 1 year of age with stage-3 or stage-4 neuroblastoma (Hsiao et al. 1990). Its value as a measure of the degree of neuroendocrine differentiation has not yet been thoroughly investigated.

Neuropeptide Y is a 36 amino acid peptide which is co-localized with catecholamines and may act as neuromodulator of cardiovascular and neuroendocrine function, e.g., of noradrenalin release and enhancing its effect. High serum levels were detected in neuroblastomas and pheochromocytomas depending on stage (higher in disseminated disease) and differentiation (lower in differentiated tumors; Kogner et al. 1993). The additional clinical use of neuropeptide-Y determination in patients with neuroblastoma remains to be established.

7.3.3 Primary Tumors

7.3.3.1 Sites of the Primary Tumor

Primary tumors may originate in all sites of sympathetic ganglia or paraganglia; in particular along the sympathetic paravertebral chain, in the adrenal medulla, in the organ of Zuckerkandl, and in the ganglion stellatum at the seventh cervical vertebral transverse process. As shown in Table 7.3, adrenal origin predominates and cervical sites are exceptional in stage 4S and stage 4. With improved imaging techniques the number of undetected primaries decreased from 5% in the 1980s to 2% in 2000 – 2003. In localized disease, the incidence of thoracic primaries is usually higher than that of adrenal origin.

It is still unclear whether the site of the primary tumor is associated with the biological properties of the disease. Conspicuously, cervical neuroblastomas are associated only rarely with distant metastases. In contrast, in nearly 90% of patients with metastatic disease the primary site is located in the abdomen.

7.3.4 Metastases

7.3.4.1 Metastatic Sites

Metastases are non-randomly distributed and the pattern differs distinctly between the mainly progressive stage 4 and the mainly regressive stage 4S (Table 7.4). Major metastatic sites in stage-4 disease include bone marrow, bone, and lymph nodes, while liver, skin, and bone marrow are common metastatic sites in stage-4S infants. By definition, infants with stage-4S disease do not have bone involvement. The preference of the bony metastases to facial bones, including the orbits, may be related to their neural crest origin (de la Monte et al. 1983).

The site of recurrence may depend on the type of the preceding treatment. For example, with the infu-

Table 7.4. Localization of metastases in patients with neuroblastoma stage 4 and stage 4S at diagnosis and at first recurrence

Disease localization	Stage 4 (%)		Stage 4S (%)	
	Initial[a]	First recurrence[b]	Initial[c]	First recurrence[d]
Bone marrow	87.3	35.2	61.5	19.2
Bone	66.1	46.6	0.0	15.1
Lymph nodes	18.6	8.9	0.0	7.7
Liver	17.4	7.5	76.0	38.5
Skin	2.8	0	12.5	7.7
Intracranial/cerebral	9.1	19.0	0.0	15.4
Lung/pleura	4.7	3.1	0.0	0
Paratesticular	1.0	0	2.6	11.5
Ovary	0.3	0	0.0	0
Isolated local recurrence		17.0		26.9
Isolated metastatic recurrence		58.1		30.7
Combined local and metastatic recurrence		24.9		42.4

[a] Evaluable patients: 725
[b] Evaluable patients: 358
[c] Evaluable patients: 192
[d] Evaluable patients: 26

sion of autologous stem cells, lung metastases are now observed. Moreover, with the use of more intensive induction chemotherapy and the monoclonal anti-GD2 antibody ch14.18 for consolidation, the number of patients with recurrences in the bone marrow has decreased (Simon et al. 2004). Thus, the distribution pattern of metastases after first-line therapy may vary depending on the therapeutic modalities used.

7.3.4.2 Bone Marrow Assessment

Because of the uneven distribution of metastases to the bone marrow, at least two marrow aspirates and two biopsies (trephines) from the iliac crests are recommended (Brodeur et al. 1993). Alternatively, four aspirates from four different sites of the iliac crest or in infants from the proximal tibial bone are sufficient to rule out gross marrow involvement. For an adequate biopsy at least 1 cm of marrow (not cartilage, not bone) is necessary (Brodeur et al. 1993) which may not be feasible in young infants. The bone marrow aspiration consists of three sampling steps per site:

- First aspiration (0.1–0.4 ml) for bone marrow smears
- Second aspiration (2–5 ml, anticoagulated with heparin) for immunocytology
- Third aspiration (2–3 ml, anticoagulated with EDTA or directly into extraction medium) for PCR investigations (Ambros and Ambros 2001).

Figure 7.2 demonstrates characteristic syncytia and immunocytologically positive clumps of cells as requested for the bone marrow diagnosis of neuroblastoma. Although consensus on the specific antibodies for marrow immunocytology has not yet been reached, commercially available anti-GD2 antibodies are widely accepted (Ambros and Ambros 2001) since very few neuroblastomas are GD2 negative. Complementary markers include the neural cell adhesion molecule (NCAM, CD56), NSE, chromogranin A, and

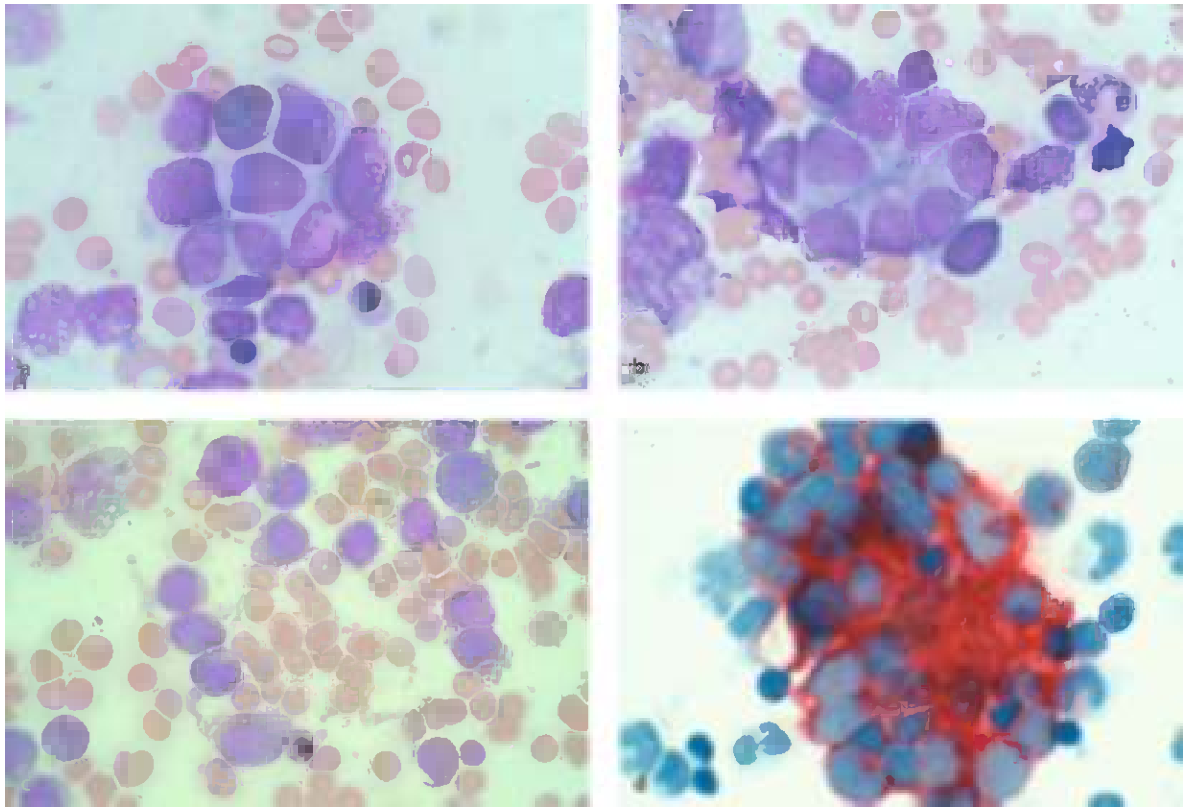


Figure 7.2 a–d

Neuroblastoma cells in bone marrow. **a** Characteristic clumps of cells (three to several hundred cells) closely adhering to each other. The cell size approximates two to three red blood cell diameters and appears a little bit larger and more polymorphic than hematopoietic blasts. High nucleus/cytoplasm ratio. The round-to-moderately oval nucleus contains fine grainy chromatin characteristically with small areas of higher density ("pepper-and-salt structure"). Nucleoli are not often visible. The basophilic cytoplasm is purely confined and the cell margins even invisible in cell clumps. Vacuoles are rarely seen, and granulation is never seen. Some neuroblastoma cells may demonstrate phagocytosis phenomena (not shown). **b** Homer-Wright rosette: typical neuroblastoma cells form a ring of cells around cytoplasmic material (neuropil) in the center. **c** Atypical "ALL like" neuroblastoma cells in bone marrow with many single cells of only one 1–2 red blood cell diameter size, but typical structure of the nucleus and ill-defined, fringed cytoplasm, no or only small clumps. **d** Small tumor clump consisting of cells with distinct membrane staining for GD2 (antibody 14G2a). Due to unspecific GD2 staining to other cell elements (e.g., some megakaryocytes, histocytes phagocytosing neuroblastoma cells), the morphology of the stained cell must be compatible with a neuroblastoma cell to be regarded as such. (Courtesy of R. Schumacher-Kuckelkorn, Cologne)

tyrosine hydroxylase. It is agreed that patients who meet the International Neuroblastoma Staging System (INSS) criteria for stages 1 – 3 should not be upstaged to stage 4 or stage 4S because of the detection of a few immunocytologically positive cells in the bone marrow in the absence of morphological evidence of disease by light microscopy (Brodeur et al. 1993).

The amount of marrow involvement in most infants with stage-4S disease is minimal (≤1% tumor

cells per nucleated marrow cells). An upper limit of 10% has been defined. If this is exceeded, an upstaging from stage 4S to stage 4 is recommended (Brodeur et al. 1993).

7.3.4.3 Definition of Cortical Bone Metastases

Bone metastases discriminate between stage 4S and stage 4 and are considered more difficult to treat than other metastatic sites (Ladenstein et al. 1998); therefore, independent methods for defining cortical bone disease are desirable.

In the case of an MIBG-avid neuroblastoma without any focal distant lesion, no further investigation is generally necessary. If the primary tumor is MIBG negative, a technetium bone scan is recommended (in two-thirds of the cases the primary itself is taking up the technetium tracer) in order to detect MIBG negative distant bone metastases.

In lesions with focal MIBG uptake, controversy exists about the accuracy of MIBG in detecting cortical bone metastases (Suc et al. 1996). Some investigators have suggested evaluation of bone lesions by technetium bone scan (highly sensitive), while others rely on osteosclerotic or osteolytic changes in the plain X-ray radiographs (less sensitive).

7.4 Differential Diagnosis

7.4.1 Small Blue Round Cell Tumors

Neuronal differentiating features are present in most neuroblastomas; therefore, the histological diagnosis of neuroblastoma is unequivocal in the vast majority of the patients. In the case of a completely undifferentiated histological pattern, other small blue round cell tumors need to be ruled out; those include Ewing's sarcoma and other peripheral neuroectodermal tumors (PNET), rhabdomyosarcoma (RMS), desmoplastic small round cell tumor (DSRCT) and malignant non-Hodgkin's lymphoma (NHL). These tumors do not excrete elevated catecholamine metabolites and are not MIBG avid (Ewing's/ PNET tumors may demonstrate faint MIBG uptake). The locations of tumors may help to discriminate further [bone involvement in Ewing's sarcoma, ribs and thoracic wall in PNETs (Askin tumors), main soft tissue mass in rhabdomyosarcoma, peritoneal implants in DSRCT, and frequent lymphoblastic bone marrow infiltration in NHL]; however, one has to be reminded that dumbbell tumors with an intrathoracic/intraabdominal spindle mass extending into the intraspinal space has been described for (extraskeletal) Ewing's sarcoma as well as for NHL and for soft tissue sarcomas. Immunohistochemistry using a panel of antibodies is necessary to make the correct differential diagnosis in those cases. The histological diagnosis of neuroblastoma in an adult patient presenting with catecholamine-negative, MIBG-negative primary tumor and metastases only to the lung should raise suspicion. In the near future, the characteristic differences of gene expression between the small blue round cell tumors may help further distinguish these tumor types (Khan et al. 2001). Before small round blue cell tumor gene chips become readily available, already established tumor-specific translocations, such as *EWS-FLI1* (t11;22) (q24;q12) in the Ewing sarcoma family of tumors, *PAX3-FKHR* (t2;13) (q35;q14) in alveolar rhabdomyosarcoma, the *WT1-EWS* (t11;22) (p13;q12) in DSRCT, and the *cmyc* gene involving translocation (t8;14) (q24;q32) in Burkitt's lymphoma, may be helpful for differential diagnosis.

7.4.2 Adrenal Hemorrhage in the Newborn

The differentiation of necrotic/hemorrhagic neuroblastoma from bleeding residues of the adrenal during the newborn period may be difficult. The determination of urinary VMA/HVA levels rarely resolves the differential diagnosis because of the tumor is generally too small to produce abnormal marker levels. Since most patients are not in critical condition and the prognosis is favorable, histological investigation can be delayed for 1–3 months with close follow-up of tumor size and structure using ultrasonography. The majority of infants can be spared invasive diagnostic procedures using this wait-and-see approach. In a series of 53 infants with postnatal suprarenal masses, 58% were localized neuroblastomas with favorable outcome. All other cases showed spontaneous regression of the lesion

(hemorrhage) or had a benign lesion (Sauvat et al. 2002).

7.4.3 Nephroblastoma

The European Wilms' tumor study has a long and successful tradition of preoperative chemotherapy where the initial diagnosis is based on radiological criteria only. Approximately 2 % of 1603 histologically proven neuroblastomas have been pretreated wrongly according to the Wilms' tumor protocol (Hero et al. 2002). The poor outcome of these patients was related more to their unfavorable biological features than to the preoperative Wilms' chemotherapy. If the radiological criteria (Wilms' tumor: intrarenal, destruction of the renal pelvis; neuroblastoma: suprarenal, tumor calcifications) are unable to discriminate, careful observation of the tumor response to preoperative Wilms' tumor chemotherapy is important and, if inadequate, a strong indicator for misdiagnosis.

7.4.4 Esthesioneuroblastoma (Olfactory Neuroblastoma)

Esthesioneuroblastoma, a rare neoplasm of the superior nasal cavity, is believed to arise from basal progenitors of the olfactory epithelium. It is neither a neuroblastoma, as the name implies, nor a PNET, since in situ hybridization and PCR studies have not confirmed the 11;22 translocation for the majority of cases (Dulguerov et al. 2001); thus, esthesioneuroblastoma is considered a distinct clinical and molecular entity not to be confused with neuroblastoma.

7.4.5 Ganglioneuroma, Pheochromocytoma, Paraganglioma, Chemodectoma

These incomplete or complete ganglionic (ganglioneuroma) or chromaffin (adrenal pheochromocytoma, extraadrenal paraganglioma) differentiated tumors may secrete VMA and HVA or different vasoactive catecholamines [norepinephrine, dopamine by pheochromocytoma and paraganglioma, although most do not (ganglioneuroma) (Geoerger et al. 2001)]. The clinical presentation of the ganglioneuroma is similar to neuroblastoma, and many investigators believe that these tumors represent a mature variant of neuroblastoma. Pheochromocytoma and extraadrenal paraganglioma are very rare in childhood and present typically with the triad of episodic headache, sweating, and palpitation as a result of the release of stored catecholamines from the tumor. Non-chromaffin paragangliomas (e.g., paraganglioma carotis=chemodectoma) arise from parasympathetic ganglia, predominantly in the head and neck, and present with local mass effects such as cranial nerve palsies and tinnitus (Dluhy 2002). The potential differential diagnostic problem is usually solved by the equivocal histology.

7.5 Clinical and Laboratory Evaluation

7.5.1 Staging

Disease stage is a powerful prognostic factor in neuroblastoma (Table 7.5). In 1988 an international staging proposal was developed, which was further refined in 1993 (Brodeur et al. 1993). The INSS has been readily accepted worldwide; therefore, other staging systems that were used to determine extent of disease in the past, such as the Evans' staging system, the Pediatric Oncology Group staging system, and the TMN system, are not discussed in this chapter (Evans et al. 1971; Castleberry et al. 1994; Ng and Kingston 1993). The INSS is a surgical-based staging system (Table 7.6), although modern imaging tools together with the estimation of an experienced pediatric surgeon is likely to achieve the same stage category in almost all cases. The criterion "crossing the midline" for discriminating between stage 2 and stage 3 is described as the infiltrative contiguous extension beyond the opposite side of the vertebral bodies. Characteristically, these tumors encompass large vessels and other vital structures. A tumor just overhanging the midline (e.g., large adrenal tumors) would not be sufficient for stage-3 categorization.

Although it was anticipated that the introduction of new sensitive diagnostic techniques (e.g., of MIBG scintigraphy and immunocytology or PCR for

Table 7.5. International Neuroblastoma Staging System (INSS) (Brodeur et al. 1993)

Stage	Definition
Stage 1	Localized tumor confined to the area of origin. Complete gross resection with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph node negative for tumor. Adherent lymph nodes in direct continuity with and removed with the tumor may be positive for the tumor. A *grossly resected* midline tumor without ipsilateral (with: stage 2A) or contralateral (with: stage 2B) lymph node involvement is considered stage 1
Stage 2A	Unilateral with incomplete gross resection; identifiable ipsilateral and contralateral lymph node negative for tumor
Stage 2B	Unilateral with complete or incomplete gross resection; with ipsilateral lymph node positive for tumor; identifiable contralateral lymph node negative for tumor
Stage 3	Tumor infiltrating across midline with or without regional lymph node involvement; or unilateral tumor with contralateral lymph node involvement or midline tumor with bilateral lymph node involvement
Stage 4	Dissemination of tumor to distant lymph nodes, bone marrow, liver, or other organs except as defined in stage 4S
Stage 4S	Localized primary tumor as defined for stage 1 or 2 with dissemination limited to liver, skin, and bone marrow (<10% of nucleated marrow cells are tumor cells)

Table 7.6. Variation of the relative stage incidence under and over 1 year of age in Japan (*J*; Ikeda et al. 2002), Germany (*G*) and United Kingdom (*UK*; Pearson and Philipp 2000), and North America (*NA*)

INSS stage	All ages				<1 year				⩾1 year			
No. of patients	J 644 %	G 670 %	UK 1266 %	NA 1253 %	J 485 %	G 271 %	UK 332 %	NA 450 %	J 159 %	G 399 %	UK 934 %	NA 803 %
Stage 1	38	18	5	21	45	26	9	23	16	13	4	19
Stage 2	19	10	12	15	22	13	23	18	7	9	8	13
Stage 3	14	20	17	17	15	19	21	17	9	20	15	17
Stage 4S	6	11	6	6	8	27	22	18	–	–	–	–
Stage 4	23	41	60	41	9	15	25	24	67	58	73	51

Germany: 1 January 1990 to 31 March 1995; with INSS system and before the nationwide screening
UK is based on ENSG data
NA is based on COG data from study ANBL00B1, 1 April 2001 to 30 August 2003 (S.L. Cohn et al., unpublished results)

bone marrow evaluation) would enhance the ability to detect metastatic disease, the absolute number of stage-4 neuroblastoma per children <14 years of age in Germany has been remarkably constant over the past 15 years (Poisson regression for linear yearly trend of the stage-4 case incidence; per population, years 1988 – 2002; p=0.92; data courtesy of C. Spix, Mainz, Germany). Instead, an increase in the incidence of lower stages has been observed. One reason for this increase is the change of the staging system from Evans (more stage-3 patients) to INSS (more stage-1 patients). A second reason is the screening program that was introduced in Germany from 1995–2000.

Table 7.7. Biological types of neuroblastoma

Type	Presentation	Adverse mole cular marker	Course of disease	Treatment approach	Current outcome
Regressive	Multilocular (stage 4S)	Absent	Progression(may be fast!) regression	Minimal therapy: inhibition of rapid tumor growth by "mild" chemotherapy; biopsy (resection)+observation only	80–85 % survival
	Unilocular (stages 1–3)	Absent			
Progressive	Metastatic (stage 4)	Present or absent	Progression	Maximum therapy: polychemotherapy; megatherapy with stem cell support; surgery; radiotherapy; immunotherapy	20–30 % survival
	Unilocular (stage 1–3)	Present			
Maturative	Unilocular (stage 1–3)	Absent	Maturation	No approach because this subtype can be identified only retrospectively	100 % (?)

From Berthold F, Hero B (2000) Neuroblastoma: current drug therapy recommendations as part of the total treatment approach. Drugs 59:1261–1277

The INSS classification utilizes surgical resectability as one of the criteria for stage determination; thus, depending on the skills of an individual surgeon, the radiologically same midline neuroblastoma may be classified as stage 3 if incompletely resected or stage 1 if completely resected. Discussions of the participating institutions with the trial office in Germany demonstrated that the discrimination between stage 2 and stage 3 may also be difficult in selected cases. Another controversy relates to the stage-4S classification. While age over 1 year and the presence of bone metastasis is uniformly accepted to qualify for stage 4, the size of the primary tumor and the presence of metastases to sites other than bone marrow (<10 %), liver, and skin are not. Some investigators believe that large stage-3 tumors and non-regional lymph node metastases may well be compatible with the biological characteristics of stage-4S neuroblastoma.

7.5.2 Biological Types of Neuroblastoma

The biological behavior of histologically identical neuroblastoma is extremely variable. At least three biological subtypes are known (Table 7.7).

7.5.2.1 Maturative Subtype

The maturative subtype can be identified only retrospectively after maturation to ganglioneuroma, and represents probably less than 5 % of all peripheral neuroblastic tumors. A model of maturation suggests that neuroblastoma cells with spontaneous maturation capacity (not induced by cytotoxic agents) attract and recruit extratumoral Schwann cells that themselves inhibit neuroblastic proliferation and promote maturation (Ambros et al. 1996). So far, parameters that indicate an ongoing or future maturation process are lacking, and it is also unclear whether all gan-

glioneuromas evolve from neuroblastoma. Although some researchers believe that alkylators induce maturation, the majority of investigators feel that chemotherapy does not play a role in the development of ganglioneuroma.

7.5.2.2 Regressive Subtype

Four observations suggest the potential of spontaneous regression in neuroblastoma:

1. The slow, but continuous disappearance of all tumor lesions in most patients with stage-4S neuroblastoma (Pepper type; Pepper 1901; Berthold et al. 1990; Nickerson et al. 2000)
2. The >90 % event-free and overall survival rates in patients with stage 1 that are treated with surgery only in spite of residual microscopic tumor (Brodeur et al. 1993; Berthold et al. 1994; Kushner et al. 1996)
3. The two- to threefold "overdiagnosis" of patients with neuroblastoma in areas where screening programs are performed compared with areas without neuroblastoma screening (Schilling et al. 2002; Woods et al. 2002) (see Chap. 2).
4. The observation of partial or complete disappearance of stage-2 and stage-3 neuroblastoma without cytotoxic therapy in particular during, but not limited to, infancy (Yamamoto et al. 1998; Berthold et al. 1998). The time span from diagnosis to the beginning of regression is considerable (1.5–18 months, *n*=24; Hero et al. 2000) and could be preceded by a period of progression before regression.

Expression of unfavorable molecular markers, such as *MYCN* amplification, 1p and 11q deletion, DNA diploidy are typically associated with advanced-stage disease, while the absence of the unfavorable factors and the presence of triploidy are typically found in infants with the regressive subtype of neuroblastoma (Lastowska et al. 2001; Brodeur et al. 1997; Mathew et al. 2001; Spitz et al. 2002, 2003a,b; Maris et al. 2001; White et al. 1995; Hallstensson et al. 1997; Vandesompele et al. 1998; Guo et al. 1999; Plantaz et al. 2001; Bown et al. 1999; Abel et al. 1999; Look et al. 1991; Ladenstein et al. 2001; Kramer et al. 1997; Combaret et al. 1996, 1997; Terpe et al. 1994; Norris et al. 1996); however, the molecular mechanisms underlying the phenomenon of spontaneous regression remain

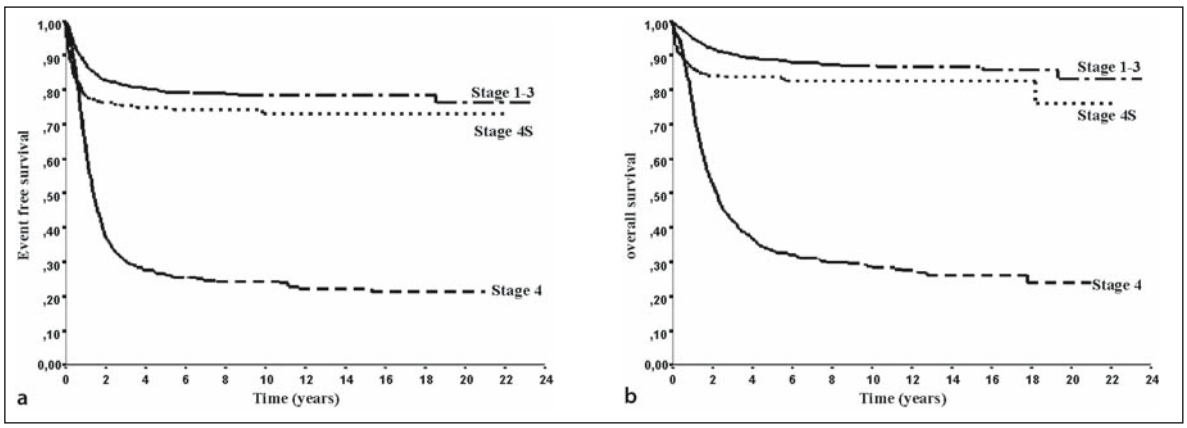


Figure 7.3 a,b

Event free survival (EFS) and overall survival (S) in 2779 consecutive patients with neuroblastoma stratified by stage*. **a** EFS: Stage 1–3 n=1428, 5-year-EFS 79.5 ± 1.1 %, Stage 4 n=1077, 5-year-EFS 26.4 ± 1.4%, Stage 4S n=274, 5-year-EFS 74.8 ± 2.7%. **b** S: Stage 1–3 n=1428, 5-year-OS 88.7 ± 0.9%, Stage 4 n=1077, 5-year-OS 33.3 ± 1.6%, Stage 4S n=274, 5-year-OS 83.7 ± 2.3%.

* 1979–1990 by the Evans'; 1990–2003 by the INSS staging system. The stages I–III (Evans') and 1–3 (INSS), Stages IVS and 4S, IV and 4 have been combined for this analysis

unclear and objective and reproducible criteria that discriminate between the regressive and the progressive subtypes have not been identified. To date, the frequency and profile of regressing stage-2 and stage-3 tumors in the different age groups remain incompletely defined. This uncertainty may result in the over-treatment of patients whose tumor would have regressed without any medical intervention (for details see Chap. 11).

7.5.2.3 Progressive Subtype

The vast majority of stage-4 tumors and a minor fraction of stages 1, 2, 3 and 4S neuroblastomas belong to the progressive subtype. Those patients are currently treated with all available therapeutic modalities (polychemotherapy including megatherapy with autologous stem cell transplantation, surgery, radiotherapy, immunotherapy, differentiation therapy) to improve the still poor event-free and overall survival (Figure 7.4; also see Chap. 11). The time to progression and the sites of progression depend on the biological profile of individual tumors and the type of therapy the patient received. A more precise definition of the clinical extremes (pure regressive/pure progressive subtype) is probably necessary before the molecular basis for the slowly progressing neuroblastoma groups is understood.

7.5.3 Prognostic Risk Groups

Recently, a number of risk stratification systems have been developed for treatment stratification purposes. Of the vast number of markers that have been investigated for prognostic impact, only a few are clinically useful to determine risk and treatment strategies. Virtually all classification systems utilize INSS stage, age at the time of diagnosis, and the status of the *MYCN* gene to determine risk. The Children's Oncology Group (COG) Risk Group Classification System also includes tumor histology and DNA ploidy. In Germany the presence of clinically threatening symptoms and the degree of "resectability" of the primary tumor are also stratifying parameters. The various factors that are used to classify patients as low-, intermediate-, or high risk in different cooperative groups are shown in Table 7.8. Until a uniform classification system is established, it will remain difficult to compare results

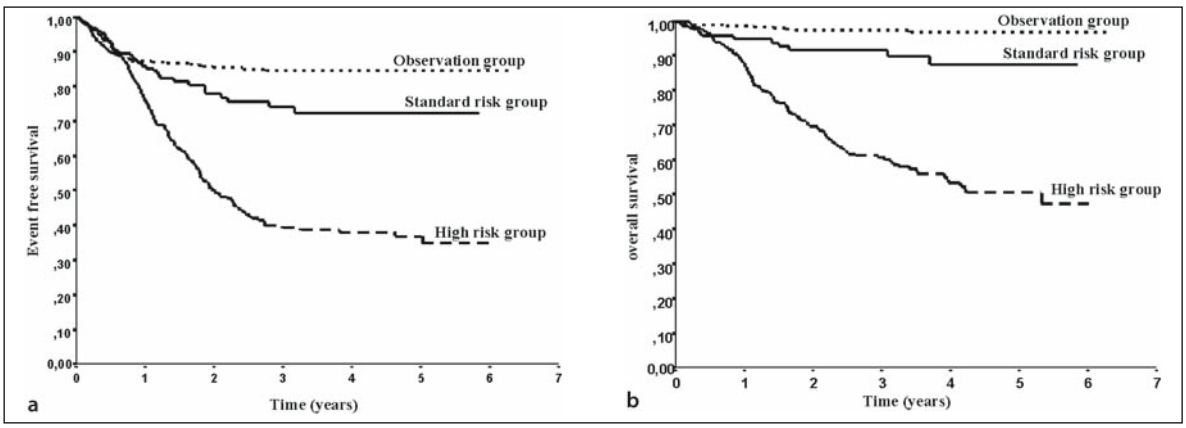


Figure 7.4 a,b

Event-free survival (EFS) and overall survival (OS) in 951 patients according to risk categories. **a** EFS: Observation group n=433, 3-year-EFS 84.5±1.9%, Standard-risk group n=140, 3-year-EFS 74.6±4.3%, High-risk group n=378, 3-year-EFS 39.4±3.0%. **b** OS: Observation group n=433, 3-year-OS 97.3±0.8%, Standard-risk group n=140, 3-year-OS 91.7±2.6%, High-risk group n=378, 3-year-OS 60.5±3.0%

Table 7.8. Synopsis of neuroblastoma risk definitions by major study groups

Study group	Risk factor INSS stage	Age (years)	*MYCN*	Other
Low-risk neuroblastoma				
COG[a]	1	0–21	Any	
	2	<1	Any	
		1–21	=	
		1–21	↑	+ Shimada histology favorable
	4S	<1	=	+ Shimada histology favorable, DNA index hyperdiploid
GPOH	1	Any	=	
	2–3, 4S	<1	=	No threatening symptoms
	2 (r)	>1	=	Resectable primary tumor, no threatening symptoms
E-SIOP[b]	1	Any	Any	
	2, 3 (r)	Any	=	Resectable primary tumor
	4S	<1	=	
	4 modified	<1	=	metastases not to bone, CNS, lung
Japan[c]	1, 2, 3, 4S	<1	=	
	1, 2	>1	=	
Intermediate-risk neuroblastoma				
COG[a]	3	<1	=	
		1–21	=	+ Shimada histology favorable
	4	<1	=	
	4S	<1	=	+ DNA index diploid or Shimada histology unfavorable
GPOH	2, 3, 4S	<1	=	+ Threatening symptoms
	2 (ur)	>1	=	+ Unresectable primary tumor
	3	>1	=	
E-SIOP[b]	2, 3 (ur)	Any	=	Unresectable primary tumor
	4	<1	=	+ metastases to bone, CNS, lung
Japan[c]	4	<1	=	
	3	>1	=	
High-risk neuroblastoma				
COG[a]	2	1–21	↑	+ Shimada histology unfavorable
	3	Any	↑	
		1–21	=	+ Shimada histology unfavorable
	4S	<1	↑	
	4	<1	↑	
		1–21	Any	
GPOH	1, 2, 3, 4S	Any	↑	
	4	Any	Any	
E-SIOP[b]	2, 3, 4S	Any	↑	
	4	<1	↑	
	4	>1	Any	
Japan[c]	Any	Any	↑	
	4	>1	Any	

[a] Courtesy of K. Matthay, San Francisco, Calif.
[b] Courtesy of B. de Bernardi, Genoa, Italy
[c] Courtesy of M. Kaneko, Tsukuba, Japan

Table 7.9. Incidence of selected potential molecular risk factors in neuroblastoma by stage. *FISH* fluorescence in situ hybridization, *CGH* comparative genomic hybridization

Risk factor	Frequency of unfavorable expression				No. of patients	Method	Reference
	Stage 1–3 %	4S %	4 %	All %			
MYCN amplification	(Stages 1+2:)		(Stages 3+4:)				
	4	8	31	22	3000	Various	Brodeur et al. (1997)
				16	642	FISH	Mathew et al. (2001)
	8	11	33	19	179	FISH	Spitz et al. (2003a)
Deletion 1p36	23	21	45	32	288	LOH	Maris et al. (2001)
				26	122	LOH	White et al. (1995)
	12	7	46	26	196	FISH	Spitz et al. (2002)
Deletion 3p26	(Stages 1–3, 4S:)						
	14		17	16	58	LOH	Hallstensson et al. (1997)
				25	36	CGH	Vandesompele et al. (1998)
	9	0	36	19	182	FISH	Spitz et al. (2003a)
Deletion 11q23	43	29	48	44	295	LOH	Guo et al. (1999)
				28	36	CGH	Vandesompele et al. (1998)
			43		83	CGH	Plantaz et al. (2001)
	13	19	48	29	182	FISH	Spitz et al. (2003a)
Gain 17q21	(Stages 1–3, 4S:)						
	28		85	54	313	CGH, FISH	Bown et al. (1999)
	52	50 (1/2)	71	60	48	FISH	Abel et al. (1999)
	55	31	72	61	193	FISH	Spitz et al. (2003b)
DNA diploidy	18	29	47	34	298	Flow cytometry	Look et al. (1991)
	(Stages 1–3, 4S:)						
	18		70	37	179	Flow cytometry	Ladenstein et al. (2001)
Lack of trkA expression	(Stages 1–3, 4S:)						
	8		66	35	113	Immuno-histology	Kramer et al. (1997)
	12	0	61	31	108	Immuno-histology	Combaret et al. (1997)
Lack of CD44 expression	2	0	45	16	121	Immuno-histology	Combaret et al. (1996)
	14	0	17	16	129	Immuno-histology	Terpe et al. (1994)
MRP overexpression	(Stages 1, 2, 4S:)		(Stages 3, 4:)				
	21		56	42	60	PCR	Norris et al. (1996)

of risk-based trials conducted in different regions of the world. While stage, age, and *MYCN* are generally accepted as key prognostic variables, a more refined system will have to wait for a better understanding of the biology underlying the diverse clinical types [regression/maturation/progression (fast/slowly)] underlying. It is also noted that risk factors are relevant only in the context of their respective treatments, since the latter can dramatically impact survival and may abolish even *MYCN* amplification as a risk factor (Kaneko et al. 2002).

In addition to stage and age, other clinical prognostic parameters include tumor markers (VMA/HVA; Laug et al. 1978; Berthold et al. 1992b), NSE (Zeltzer et al. 1983; Garaventa et al. 2002), Ferritin (Hann et al. 1981; Garaventa et al. 2002), LDH (Berthold et al. 1992a, 1994; Shuster et al. 1992; Lau 2002; Garaventa et al. 2002), chromogranin A (Hsiao et al. 1990), neuropeptide Y (Kogner et al. 1993), primary site and volume (Cotterill et al. 2000), and metastatic site (DuBois et al. 1999). An interdependence of the tumor markers has been demonstrated (Berthold et al. 1992a, 1994; Garaventa et al. 2002). A second set of clinical prognostic markers consists of response parameters like MIBG normalization as a consequence of chemotherapy (Hero et al. 2001; Ladenstein et al. 1998), clearing of tumor cell contaminated bone marrow (Chap. 11), response of tumor markers (Hero et al. 2001), and resectability of the primary tumor (Berthold et al. 1992a, 1994; Garaventa et al. 2002; von Schweinitz et al. 2002). Furthermore, the histological pattern of the tumor as defined by the mitosis–karyorrhexis index, the amount of Schwann cells, the neuroblastic differentiation and age has been used as prognostic parameter in particular in American trials (Shimada et al. 1984, 1999; Shimada and Roald 2000) (Table 7.8). In other series, the proliferation rate of neuroblastoma cells alone was of prognostic impact (Krams et al. 2002).

During the past 20 years a number of specific genetic changes have been found to be associated with biologic features, e.g., *MYCN* amplification and 1p36 deletion with the progressive type of neuroblastoma (Figure 7.5). Genetic investigation has become standard in most clinical trials, not for diagnostic purposes (Table 7.5) but rather for prognostic information (Brodeur et al. 1997; Mathew et al. 2001; Spitz et al. 2002, 2003a,b; Maris et al. 2001; White et al. 1995; Hallstensson et al. 1997; Vandesompele et al. 1998; Guo et al. 1999; Plantaz et al. 2001; Bown et al. 1999; Abel et al. 1999; Look et al. 1991; Ladenstein et al. 2001; Kramer et al. 1997; Combaret et al. 1996, 1997; Terpe et al. 1994; Norris et al. 1996) (Table 7.8). Genetic characteristics promise to reflect the natural course of the disease more directly than derived clinical parameters and present with a yes or no answer. Table 7.9 summarizes the factors that are believed to contribute to prognosis estimation. The incidence rates given for the unfavorable expression of the risk factors demonstrate that they describe only a fraction of the patients with unfavorable prognosis. Some factors are closely associated with each other, e.g., *MYCN* amplification and 1p36 alteration, deletion of 11q23 and 3p26, and loss of expression of trkA and CD44. Utilizing, for example, *MYCN*, del 1p, del 11q and del 3p together, 83 % of stage-4 and 30 % of stages 1 – 3 and stage-4S patients showed one or more of the chromosomal changes (Spitz et al. 2003a); however, the prediction of events of tumor progression in low-risk patients was possible roughly only in one-half using all the markers and their combinations listed in Table 7.9, while a substantial fraction of patients with chromosomal and/or immunohistological abnormalities in their tumors did not experience tumor progression (unpublished observation). Few of the listed molecular factors have been found unanimously to be of prognostic value. Contradictory results were obtained, for example, for *MYCN* amplification [lack of correlation with prognosis for localized (Cohn et al. 1995) and metastatic (Laug et al. 1978) neuroblastoma] and for 17q gain (Spitz et al. 2003b) which is likely to result from different treatment of the patients and different investigation methods in the lab. The suggested proposal for a standardized tumor tissue processing and analysis (Ambros and Ambros 2001) has been mentioned above. Apart from standardization issues, modern technologies that screen myriads of gene expression levels in small tumor biopsies are likely to define in the near future not only characteristic gene expression profiles (Sauvat et al. 2002), but also genetic key characteristics that allow the description of molecular risk categories. Using the Ewing's paradigm of seed growing in a soil,

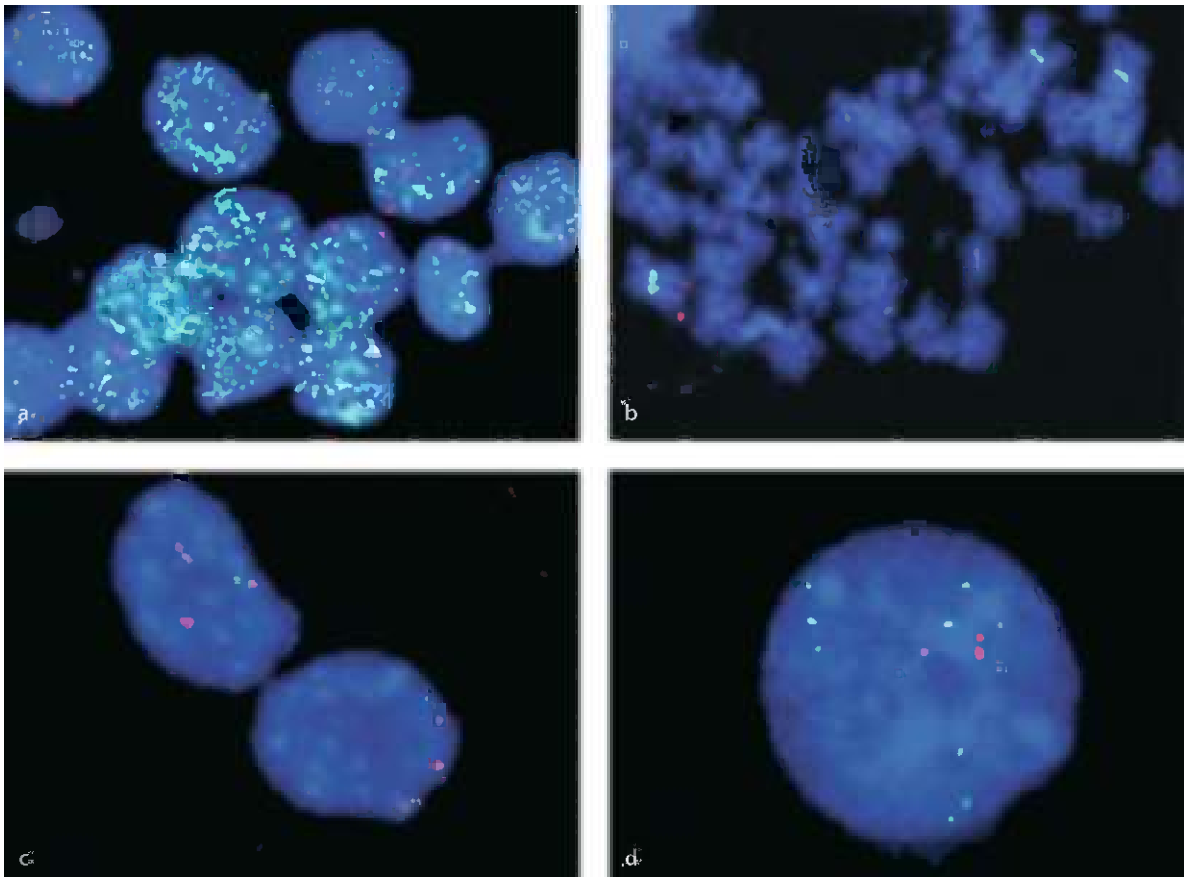


Figure 7.5 a–d

Interphase fluorescence in situ hybridization (FISH) studies of chromosomal loci frequently involved in neuroblastoma. **a** *MYCN* amplification (interphase FISH, tumor touch preparation): two red signals demonstrate centromeric regions of the two chromosome-2 copies. The dispersed green signals indicate multiple copies of the *MYCN* gene in 2p24. **b** Deletion in chromosome 1p (chromosomes of cell line IMR5): three chromosomes 1 demonstrate green centromeric signals (D1Z1), but only one derivative copy shows a red distal (1p36) signal. **c** Imbalance 11q: the nuclei show three red markings of chromosome 11 centromere, but only two green signals of the MLL-locus in 11q23. One of the three chromosome 11 alleles lost its distal long arm. **d** Gain 17q: the nucleus has four red dots of the chromosome 17, but ten green distal chromosome 17 signals (17q21). The distal part is overrepresented. (Courtesy of R. Spitz, Cologne)

understanding the genetic characteristics of the tumor (seed) in the context of the host (soil) should help define the natural history of neuroblastoma more directly than derived clinical parameters. Figure 7.4 shows an example of the relationship between clinical + molecular risk factors and survival. The observation group (surgery only) comprises 46 % of all patients, the standard-risk group (four blocs of chemotherapy) 15 %, and the high-risk group (all modalities) 40 %.

Table 7.10. International neuroblastoma response criteria (INRG) (Brodeur et al. 1993)

Response	Primary tumor	Metastatic sites
CR	No tumor	No tumor; catecholamines normal
VGPR	Decreased by 90 – 99%	No tumor; catecholamines normal; residual ^{99}Tc bone changes allowed
PR	Decreased by >50%	All measurable sites decreased by >50%. Bones and bone marrow: no. of positive bone sites decreased by >50%, no more than one positive bone marrow site allowed
MR	No new lesions; >50% reduction of any measurable lesion (primary or metastases) with <50% reduction in any other; <25% increase in any existing lesion	
NR	No new lesions; <50% reduction, and <25% increase in any existing lesion	
PD	Any new lesion; increase of any measurable lesion by >25%; previous negative marrow positive for tumor	

7.5.4 Response Criteria

The evaluation of the tumor response to the treatment is mandatory; however, the correlation between initial response and long-term outcome is still weaker in neuroblastoma than prednisone response in acute lymphoblastic leukemia. The criteria for defining response (Brodeur et al. 1993) are shown in Table 7.10. All test results that were abnormal at diagnosis need to be reinvestigated after 3–4 months, after completion of each major therapeutic step (e.g., at the end of chemotherapy, after surgery). Most protocols require additional evaluations for disease response during the treatment period and following the completion of therapy (Therassa et al. 2000).

Currently, the response status is assessed using standard radiographic measurements to determine tumor size and evidence of metastases in addition to histological evaluation of the bone marrow. Many efforts of refinement are being undertaken such as PCR-based detection of minimal residual disease in the bone marrow (Chap. 11), *MYCN*-DNA levels in serum (Combaret et al. 2002), and antibody-based scintigraphy (Chap. 14). These studies need confirmation by other independent groups before they are adopted for standard response evaluation reporting.

7.6 Conclusions

Children with neuroblastoma have a wide range of presenting signs and symptoms. This cancer must be considered in every child with hepatomegaly, skin nodules, periorbital ecchymosis, bone pain, Horner's syndrome, opsoclonus–myoclonus, and/or transverse myelitis. Neuroblastoma is characterized by a diversity of clinical behavior, but to a large extent, outcome can be predicted by the stage of disease, the age at diagnosis, and the presence or absence of *MYCN* amplification. Additional factors, such as tumor pathology, DNA content, genetic abnormalities, and the presence of clinically threatening symptoms, have also been found to have prognostic value. Most countries have developed treatment strategies that are tailored according to patient risk; however, currently uniform criteria for risk-group stratification do not exist. An International Neuroblastoma Risk Classification System is needed so that treatment protocols performed in different regions of the world can be compared and optimal therapeutic strategies for patients with low-, intermediate-, and high-risk disease can be identified.

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Pathology of Peripheral Neuroblastic Tumors

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Contents

8.1 Introduction

Peripheral neuroblastic tumors (pNTs), which include neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, are common pediatric tumors (Ross et al. 1996). These tumors are derived from immature sympathetic neuroblasts during embryonic, fetal, or early postnatal development, and their morphological features appear to recapitulate developmental stages of sympathetic ganglia. Their primary sites are anatomically related to the embryological distribution of neural crest cells, and include adrenal gland and structures of the sympathetic nervous system.

For many years pNTs were characterized as "enigmatic" because of their unexpected clinical behaviors, such as involution/spontaneous regression, maturation, or aggressive progression. Because of recent advances in clinical and basic research, pNTs now are considered to be biologically heterogeneous, and their individual molecular properties like account for their unique clinical behaviors (Brodeur and Maris 2002). Based primarily on their clinical biology, the International Neuroblastoma Pathology Classification (Shimada et al. 1999a,b) was established by adopting the concept of the original Shimada system (age-linked evaluation of the morphological features in this disease). In this chapter, histopathology of the pNTs is illustrated according to the Classification along with its biological relevance.

8.2 Historical Overview

Since the beginning of the twentieth century, attempts have been made to deduce prognostic information from the histological appearance of the individual tumors (Beckwith and Martin 1968; Hughes et al. 1974; Landau 1911; Mäkinen 1972; Wahl 1914). In 1914 Wahl suggested a sequence of maturation of the pNTs (Wahl 1914), and in 1968, Beckwith and Martin proposed a grading system based on the semi-quantitative assessment of neuroblastic cytodifferentiation (Beckwith and Martin 1968). In 1974 Hughes and co-workers proposed their grading system, but at this time based on non-quantitative assessment of neuroblastic/ganglionic cytodifferentiation (Hughes et al. 1974). As summarized in a review article by Dehner in 1988 (Dehner 1988), however, those attempts could not successfully satisfy the oncologists dealing with this "enigmatic" disease.

Interestingly, in the first half of the twentieth century, it was believed that older patients had a better prognosis (Landau 1911; Wahl 1914), which was probably due to the inability to distinguish local-regional from stage-4 metastatic disease. In the second half of the twentieth century, however, it was clearly recognized that younger patients (especially diagnosed before 1 year of age) had a significantly better prognosis than older patients (Gross et al. 1959). Furthermore, the majority of tumors in infants with clinically favorable outcome showed no or very limited morphological evidence of cytodifferentiation. Beckwith and Martin concluded that "differences in degree of maturation probably did not account for the more favorable outcome of the neuroblastomas in infancy" (Beckwith and Martin 1968).

In 1984 Shimada and colleagues proposed a classification system based on a unique concept of age-linked evaluation of morphological indicators (Shimada et al. 1984). First they made an age-appropriate framework of the maturational sequence of the pNTs. The maturational sequence was defined by two morphological indicators, grade of neuroblastic differentiation, and degree of Schwannian stromal development. Prior to their study, Schwannian stromal component, which is one of the major elements in the normal ganglionic structure of the sympathetic nervous system, had never been a subject of serious investigation in pNTs. According to this classification system, clinically favorable tumors can be less differentiated when diagnosed in younger patients, and should have morphological features of more advanced maturation in older children (for detailed explanation see Chap. 4). They also found increased numbers of karyorrhectic cells in highly aggressive tumors, and introduced a concept of mitosis–karyorrhexis index.

In 1992 Joshi and co-workers proposed histological grading by using mitotic rate (MR: low ≤10/10 high-power fields, high >10/10 high-power fields) and calcification (presence or absence; Joshi et al. 1992). In their report they also proposed a risk grouping by combining the histological grade and age of the patient at diagnosis (low risk: patients in all age groups with tumor having low MR and calcification, and patients ≤1 year of age with either low MR or calcification; high risk: patients >1 year of age with either low MR or calcification, and patients in all age group with high MR and no calcification; Joshi et al. 1992). They later published a modified histological grading by replacement of mitotic rate with MKI (Joshi et al. 1996).

In 1994 the International Neuroblastoma Pathology Committee was formed to establish a prognostically significant and biologically relevant classification for international use. The Committee first defined terminology and morphological criteria of pNTs, and then analyzed and tested mainly those two classifications proposed by Shimada et al. (1984) and Joshi et al. (1992, 1996). In 1999, after 5 years of collaborative work, the Committee developed the International Classification based on the original Shimada classification with minor modifications (Shimada et al. 1999a,b).

8.3 Basic Morphology

As proposed by Shimada et al. in their original classification (Shimada et al. 1984), pNTs are classified into four basic morphological categories (Shimada et al. 1999a): neuroblastoma (Schwannian stroma-poor); ganglioneuroblastoma, intermixed (Schwannian stro-

ma-rich); ganglioneuroma (Schwannian stroma-dominant); and ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor). Within each category one or more subtypes are recognized (see below). The first three categories and their subtypes are based on morphological changes according to the maturational sequence. The Shimada system distinguishes biologically favorable pNTs with a potential of age-appropriate maturation and biologically unfavorable pNTs without such a potential (see Chap. 4). In the last category, the tumor is composed of clearly distinct multiple clones, representing different states of maturation or maturational arrest. Among these categories, ganglioneuroma is not considered a separate entity, but rather as a fully mature form of tumor constituting the end of the biological continuum for all the pNTs, a model which postulates that ganglioneuromas are neuroblastomas at a later time in their development.

To date, there is no clear distinction in molecular characteristics between pNTs with a potential of regression and pNTs with a potential of maturation. In fact, during the maturational sequence of pNTs, the vast majority of neuroblastic cells probably die before or after reaching a certain degree of maturation (cellular death during the process of tumor maturation, comparable to cellular death seen in the process of normal organogenesis). By contrast, Schwannian stroma, once developed and established, are believed by many to constitute a persistent and dominant component in pNTs.

8.3.1 Neuroblastoma (Schwannian Stroma-poor)

Tumors in this category are composed of neuroblastic cells forming lobules separated by thin fibrovascular septa where Schwann cells (or their precursors) can (or may) be detected as slender S-100 positive cells (Shimada et al. 1985). Three subtypes, i.e., undifferentiated, poorly differentiated, and differentiating, are distinguished based on different grades of neuroblastic differentiation. It is noteworthy that in the original Shimada Classification, there were two subtypes, undifferentiated (including undifferentiated and poorly differentiated subtype of the International Classification) and differentiating (same as differentiating subtype), in this category. On gross examination, tumors are generally soft in consistency. Cut surfaces of those in the undifferentiated and poorly differentiated subtype are often hemorrhagic, while tumors in the differentiating subtype are usually tan-yellow, without hemorrhage.

Neuroblastoma, Undifferentiated Subtype (Fig. 8.1 a): In this rare subtype, tumor tissue is composed of undifferentiated neuroblastic cells without identifiable neuropil or rosettes. In order to establish the diagnosis, supplementary tests, such as immunohistochemistry, electron microscopy, and/or molecular/cytogenetic analysis, are usually required.

Neuroblastoma, Poorly Differentiated Subtype (Fig. 8.1 b): Diagnosis for tumor in this subtype is relatively easy because of the presence of varying amount of neuropil and/or rosettes of the Homer-Wright type. Most of the tumor cells are undifferentiated: less than 5 % of the population has morphological evidence of differentiation (see below).

Neuroblastoma, Differentiating Subtype (Fig. 8.1 c): Tumor of this subtype usually has abundant neuropil. Five percent or more of the tumor cells are differentiating neuroblasts: they are characterized by synchronous differentiation of the nucleus (enlarged, eccentrically located with vesicular chromatin pattern, and a single prominent nucleolus), and of the cytoplasm (eosinophilic/amphophilic with a diameter two or more times larger than the nucleus).

Mitosis–karyorrhexis index (MKI): One of three MKI classes is assigned to the given neuroblastoma tumor. Those classes are low MKI (<2 % or <100 of 5000 mitotic and karyorrhectic cells), intermediate MKI (2–4 % or 100–200 of 5000 mitotic and karyorrhectic cells), and high MKI (>4 % or >200 of 5000 mitotic and karyorrhectic cells). The MKI is defined by counting the number of tumor cells in mitosis and in the process of karyorrhexis (Fig. 8.1 d), and should reflect an average for all tumor sections available. Karyorrhectic cells show condensed and fragmented

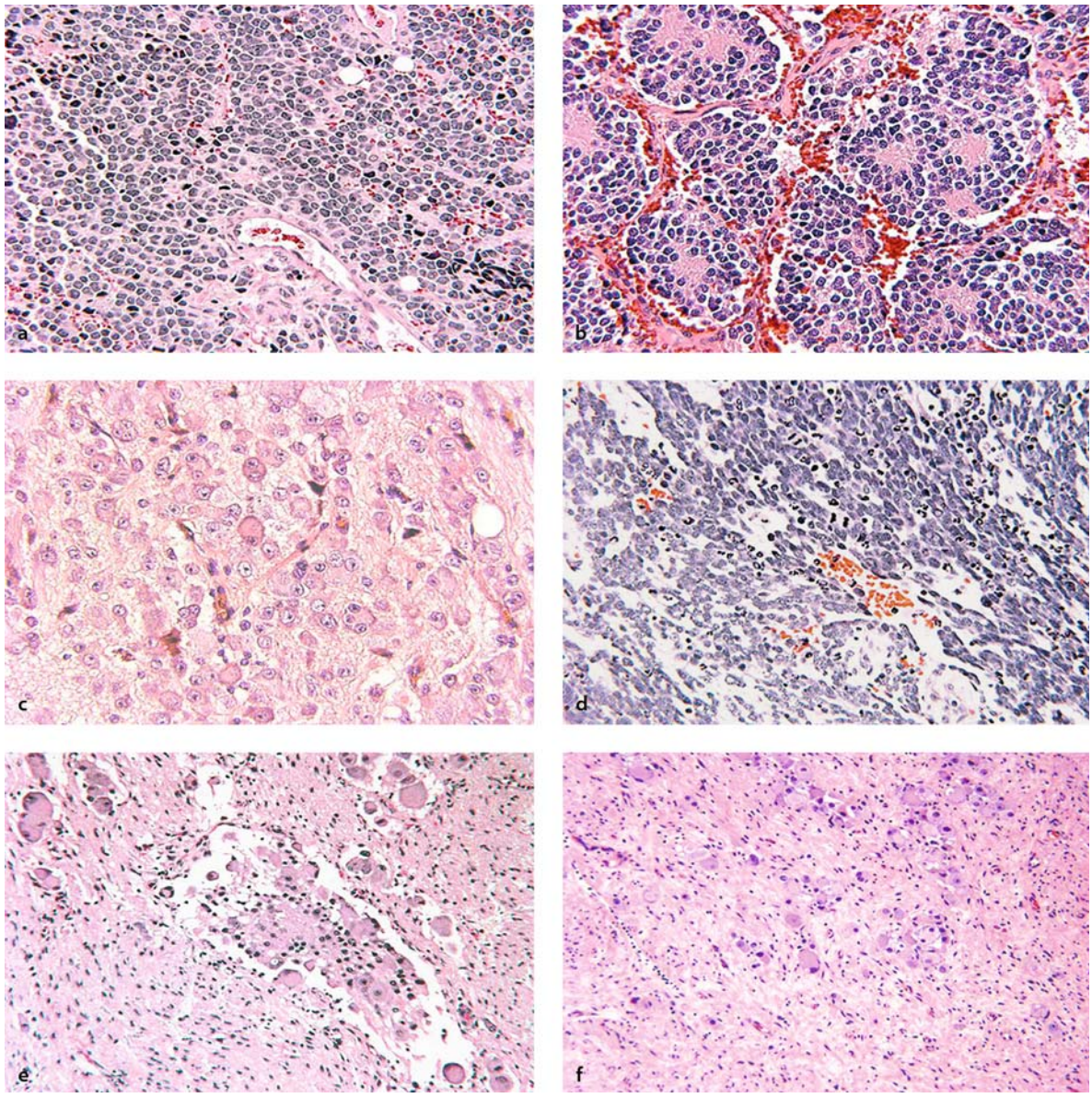

Figure 8.1 a–f

Histology of peripheral neuroblastic tumors. **a** Neuroblastoma (Schwannian stroma-poor), undifferentiated subtype. **b** Neuroblastoma (Schwannian stroma-poor), poorly differentiated subtype. **c** Neuroblastoma (Schwannian stroma-poor), differentiating subtype. **d** Neuroblastoma (Schwannian stroma-poor) with a high mitosis–karyorrhexis index. **e** Ganglioneuroblastoma, intermixed (Schwannian stroma-rich). **f** Ganglioneuroma (Schwannian stroma-dominant), maturing subtype.

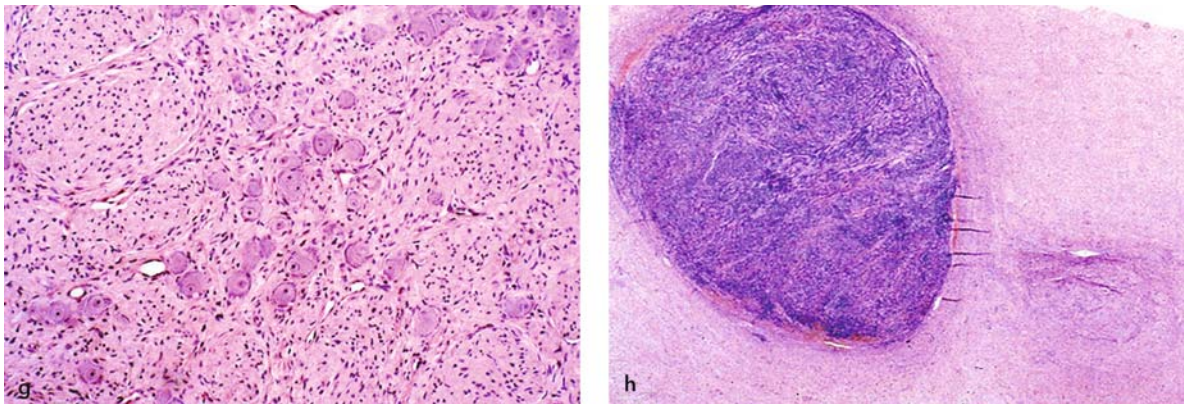

Figure 8.1 g–h

g Ganglioneuroma (Schwannian stroma-dominant), mature subtype. **h** Ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor)

nuclear material, usually accompanied by condensed eosinophilic cytoplasm. Simple hyperchromatic nuclei without chromatin fragmentation are not included in MKI counting.

8.3.2 Ganglioneuroblastoma, Intermixed (Schwannian Stroma-rich)

The international classification has stipulated that tumors having prominent Schwannian stromal development occupying more than 50% of the tumor tissue are upgraded to this category. Tumor histology is consistent with a transition to the full differentiation/maturation of ganglioneuroma (see below), but the process is not complete, as evidenced by scattered "residual" microscopic foci where neuroblastic cells in various stages of differentiation as well as varying numbers of maturing ganglion cells are found in the background of neuropil (Fig. 8.1 e).

8.3.3 Ganglioneuroma (Schwannian Stroma-dominant)

Tumors are predominantly composed of Schwannian stroma with individually distributed maturing/mature ganglion cells. Two subtypes, ganglioneuroma, maturing, and mature, are included in this category.

Ganglioneuroma, Maturing Subtype (Fig. 8.1 f): Tumor of this subtype was previously named "ganglioneuroblastoma, well differentiated" in the original Shimada classification. Some of the neuroblastic components appear to be on their way to fully mature ganglion cells and have appearances of differentiating neuroblasts and/or maturing ganglion cells.

Ganglioneuroma, Mature Subtype (Fig. 8.1 g): Tumor in this subtype is composed of fully developed Schwannian stroma and mature ganglion cells. Those mature ganglion cells are surrounded by satellite cells. Mature non-myelinating Schwann cells, the dominating component of tumor, characteristically form multiple fascicles covered with perineurial cells.

Tumors categorized as either ganglioneuroblastoma, intermixed, or ganglioneuroma have an elastic consistency, and their cut surfaces are always tan-yellow and homogenous with or without fibrous bands.

8.3.4 Ganglioneuroblastoma, Nodular (Composite, Schwannian Stroma-rich/ Stroma-dominant and Stroma-poor)

Tumor in this category is characterized by the presence of one or more macroscopic, usually hemorrhagic neuroblastomatous nodule(s) (stroma-poor

component) coexisting with ganglioneuroblastoma, intermixed (stroma-rich component) or with gang-lioneuroma (stroma-dominant component; Fig. 8.1h). On microscopic examination, there is typically abrupt demarcation (pushing border or even fibrous pseudo-capsular formation) between the neuroblastomatous nodule(s) and the stroma-rich or stroma-dominant tumor tissue. Some nodules, however, are not clearly demarcated but rather have a zone of neuroblastic infiltration into the adjacent Schwannian stromal tissue. In rare cases the neuroblastomatous nodule becomes so large, dominating the tumor tissue, that one can recognize stroma-rich/stroma-dominant area only by light microscopic examination.

Nodular formation is usually considered to be a feature of the primary tumor, but it may be overlooked on gross examination; thus, those cases with ganglioneuroblastoma, intermixed or ganglioneuroma at the primary site and neuroblastoma at the metastatic site should be classified into this category.

8.4 Prognostic Classification

The International Neuroblastoma Pathology Classification (the Shimada system) distinguishes favorable and unfavorable histology groups (Shimada et al. 1999b). Tumors in the favorable histology group fall within a conceptual framework of age-linked maturational sequence from poorly differentiated subtype (<1.5 years of age at diagnosis) to differentiating subtype (<5 years of age) of neuroblastoma (Schwannian stroma-poor) to ganglioneuroblastoma, intermixed (Schwannian stroma-rich) to ganglioneuroma (Schwannian stroma-dominant). The neuroblastoma tumors in this group should have a low (for those patients <5 years of age) or an intermediate (for those <1.5 years of age) MKI. By contrast, tumors in the unfavorable histology group have immature histologies for patient's age and include undifferentiated subtype (at any age), poorly differentiated subtype (≥1.5 years of age), and all subtypes (≥5 years of age) of the neuroblastoma. Among the neuroblastoma tumors, those with a high MKI (at any age) or an intermediate MKI (≥1.5 years of age) also qualify as unfavorable histology. Ganglioneuroblastoma, intermixed and ganglioneuroma are classified into a favorable histology group regardless of the patients' age, although these tumors are usually diagnosed in older children. Ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor) can be divided into two subsets, favorable and unfavorable, by applying the same criteria of age-linked histopathological evaluation to the nodular (neuroblastomatous) component (Peuchmaur et al. 2003; Umehara et al. 2000).

While arriving at the proposed Classification, the International Neuroblastoma Pathology Committee tested other morphological indicators (calcification, mitotic rate) and classifications (original risk grouping by combination of mitotic rate, calcification, and age (Joshi et al. 1992); modified risk grouping by combination of MKI, calcification, and age (Joshi et al. 1996), and analyzed their prognostic effects (Shimada et al. 1999b). Although these indicators and classifications all had prognostic effects by univariate analysis, calcification and mitotic rate did not add any significant prognostic information to the International Neuroblastoma Pathology Classification in multivariate analysis. Furthermore, the Classification could provide significantly better prognostic information than those risk groupings. The Committee also examined the age factor of the Shimada system, and confirmed that the two cutoff points, i.e., 1.5 and 5 years of age at diagnosis, used in the Classification distinguished prognostic groups most significantly (Shimada et al. 1999b).

8.5 Biological Relevance

In this section, the biological relevance of International Neuroblastoma Pathology Classification (the Shimada system) is summarized.

8.5.1 Schwannian Development in Neuroblastic Tumors

Peripheral neuroblastic tumors consist of two main cell populations: neuroblastic/ganglionic cells and Schwann cells. As described above, the International Neuroblastoma Pathology Classification uses morphological features of both neuroblastic differentia-

tion and Schwannian stromal development for defining maturational sequence of the pNTs. Based on embryological interactions between normal neuroblasts and Schwann cells (Reynolds and Woolf 1993), some postulate that neoplastic neuroblasts produce Schwann cell mitogens important for their proliferation and development (Ambros 2001). Schwann cells, in return, can secrete anti-proliferative and differentiation-inducing factors crucial to neuronal differentiation. This mutual interaction between neuroblastic cells and Schwannian stromal cells may explain the maturational processes of biologically favorable pNTs. In biologically unfavorable pNTs, there is generally less Schwannian component and limited tumor maturation. The origin of the tumor Schwann cells remains controversial. One study indicates that both cell types, i.e., neuroblastic/ganglionic cells and Schwann cells, arise from the same neoplastic neuroblastic clone or precursor cell (Mora et al. 2001); however, other studies present evidence to support that the Schwann cells in pNTs are reactive in nature and probably recruited from surrounding non-neoplastic tissue by tumor neuroblastic cells (Ambros et al. 1996).

8.5.2 Correlation of Histopathology with *MYCN* Amplification and trkA Expression

There is a reproducible correlation between the molecular event of *MYCN* amplification and the morphological manifestations in pNTs (Shimada et al. 1995; Goto et al. 2001). Those tumors with amplified *MYCN* typically are of the undifferentiated or poorly differentiated subtype of neuroblastoma (Schwannian stroma-poor) with markedly increased mitotic (proliferating) and karyorrhectic (apoptotic) activities (Shimada et al. 1995; Goto et al. 2001), an unfavorable histology group according to the International Neuroblastoma Pathology Classification. The presence of prominent nucleoli in neuroblastic cells of undifferentiated or poorly differentiated neuroblastoma, often associated with unfavorable prognosis (Ambros et al. 2002), can be an additional hallmark of *MYCN* amplification (own unpublished observations).

The balance appears to favor cellular proliferation (mitosis) more than cellular death (karyorrhexis) in a *MYCN* amplified tumor, which is well known to have a highly aggressive and rapidly progressive clinical behavior. In light microscopic sections from *MYCN* amplified tumors, however, the number of karyorrhectic cells always exceeds that of mitotic cells. This may be explained by the fact that the histologically visible stage of mitosis is much shorter than that of karyorrhexis (Bursch at al. 1991). Our preliminary data show that neuroblastoma tumors with favorable histology express significantly higher levels of trkA than those with unfavorable histology (Shimada et al. 2004). Favorable histology neuroblastoma tumors include both poorly differentiated and differentiating subtypes: although there is no difference in the level of trkA expression between these two histological subtypes, tumors of differentiating subtype are diagnosed in significantly older children (usually between 1 and 5 years of age) than those of poorly differentiated subtype (newborn to 1.5 years of age). This may suggest an in vivo latent period required for morphological evidence of neuroblastic differentiation among the neuroblastoma tumors in the favorable-histology group, and supports the concept of an age-linked Pathology Classification.

8.5.3 Composite Tumor

The term "composite" implies that the tumor is composed of histologically and, probably biologically, different clonal populations (Schmidt et al. 1993), a description possibly applicable to almost 10% of pNTs. In the International Neuroblastoma Pathology Classification, this composite form is designated as ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor). In this model the neuroblastic nodule(s) represents the evolution of one or multiple clones, either because of newly acquired genetic aberrations in late stage of tumor progression or the persistence of genetically and biologically distinct variants evolving early in tumor formation. Clinically, two-thirds of these composite tumors are aggressive (Peuchmaur et al. 2003; Umehara et al. 2000).

8.6 Conclusion

Neuroblastic tumors are known to be heterogeneous and their clinical behaviors are driven by complex molecular/genetic properties. The International Neuroblastoma Pathology Classification exploits a system of age-linked evaluation of morphological indicators, to distinguish among tumors with near-identical histological features but vastly different clinical behaviors. This classification offers a unique forum for finding the morphological link between clinical behavior and tumor genetics of the enigmatic cancer of childhood.

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Molecular Pathology of Neuroblastic Tumors Based on Genome-wide Expression Analysis

William L. Gerald

Contents

9.1 Introduction

Neuroblastomas (NBs) have heterogeneous biologic, genetic, and morphologic features and are characterized by diverse clinical behavior. Although the biological basis for this diversity is poorly understood, many molecular features, such as DNA index, oncogene amplification, and tumor suppressor gene loss, have been identified that correlate with clinically relevant aspects of the disease. Because of this strong relationship between biology and clinical phenotype, molecular classification is playing an increasingly important role in stratifying therapy for patients with NB. The identification of tumor-specific molecular alterations and the characterization of critical pathways regulating tumor growth are likely to further refine our ability to diagnose and classify NB, and may lead to the identification of therapeutic targets. Completion of a draft sequence of the entire human genome and the development of miniaturized high throughput technology for comprehensive genetic analysis now permit the monitoring of every gene in a single experiment and provide parallel analysis of the complex coordinated pathways that contribute to the clinical phenotype of cancers (Ramaswamy and Golub 2002). This chapter provides an overview to comprehensive gene expression profiling of NB as a means to define the molecular pathology of this disease.

9.2 Clinical Issues

Neuroblastomas are embryonal neoplasms that develop during fetal or early postnatal life from neural crest-derived cells that are still immature but differentiation restricted (Brodeur and Maris 2002). The clinical spectrum of NB is fascinating with at least three distinctly disparate presentations. One subset of tumors is associated with spontaneous regression of clinically apparent NB (most stage-4S patients; d'Angio et al. 1971). It is yet to be determined whether regressing NBs are persistent embryonic rests, represent hyperplasia, or are true malignancies. To date, the underlying mechanism(s) that lead to spontaneous tumor regression are not known. Recent studies indicate that there are genetic prerequisites for the process of regression to proceed, supporting the premise that stage-4S NB represents a unique biological entity and that individual genes and pathways will be identified that may define this unique subset of tumors (Ambros et al. 1995; Grosfeld et al. 1993; Mora et al. 2000).

A second category of NB is associated with differentiation and lack of distant metastasis [stages 1 – 3, herein referred to as local regional (LR) NB]. Interestingly, some LR NB can involve regional lymph nodes. A subset of these tumors have ganglionic differentiation and Schwannian stroma. Most stage-1 and stage-2 LR tumors can be successfully treated with surgery alone (Alvarado et al. 2000; Perez et al. 2000) (see Chap. 11). In addition, outcome is favorable for patients with stage-3 LR tumors that lack high-risk features following treatment with chemotherapy and surgery (Matthay et al. 1998) (see Chap. 11), and favorable outcome has been reported for stage-3 patients following surgery alone (Kushner et al. 1996). Nevertheless, some LR tumors are locally aggressive resulting in repeated recurrences and poor outcome.

Finally, the major clinical subcategory of NB (~60 % of patients) does not undergo spontaneous regression or maturation, but presents as advanced-stage tumors (stage 4). The hallmark of this disease is destructive bone metastasis. Although most patients older than 1 year with stage-4 tumors initially respond to chemotherapy, these patients frequently recur and become progressively resistant to medical treatment. It is very rare for stage-4S or LR NB to progress to stage-4 disease, implying that they are biologically distinct diseases despite histological and clinical similarities. In addition, infants with stage-4 NB constitute yet another distinct entity by virtue of their high curability. Irrespective of these clinical subgroups, there is a strong association between clinically aggressive NB and specific genetic alterations, i.e., *MYCN* amplification, deletions of 1p, gains of 17q, and a di/tetraploid DNA content (Mora et al. 2000; Look et al. 1991; Brodeur et al. 1984). These observations suggest that specific genetic alterations are likely to contribute to the clinical behavior of NBs, and that the clinical subtypes will have disparate molecular profiles. Molecular components that correlate with outcome may also be potential therapeutic targets.

9.3 Technical Aspects of Gene Expression Analysis

Given the limitations in our present understanding of the basic biology of human cancer, many investigators have turned to the use of high-throughput gene expression studies to provide a more complete characterization of this disease. The technology underlying comprehensive gene expression analysis is the culmination of several amazing accomplishments including sequencing of the entire human genome, identification of most human protein-encoding sequences, development of techniques for the efficient production, purification and attachment of tens of thousands of nucleic acid probes to a solid support in a miniaturized format, and the development of sensitive detection techniques coupled with sophisticated analytical software. With this degree of complexity it is not surprising that comprehensive gene expression studies have many inherent technical and analytical challenges; however, initial efforts are providing reason for optimism (Ladanyi and Gerald 2003).

9.3.1 Transcript Profiling Methods

Several methods have been used for high-throughput gene expression analysis; they include sequence analysis of cloned transcripts [differential display, subtrac-

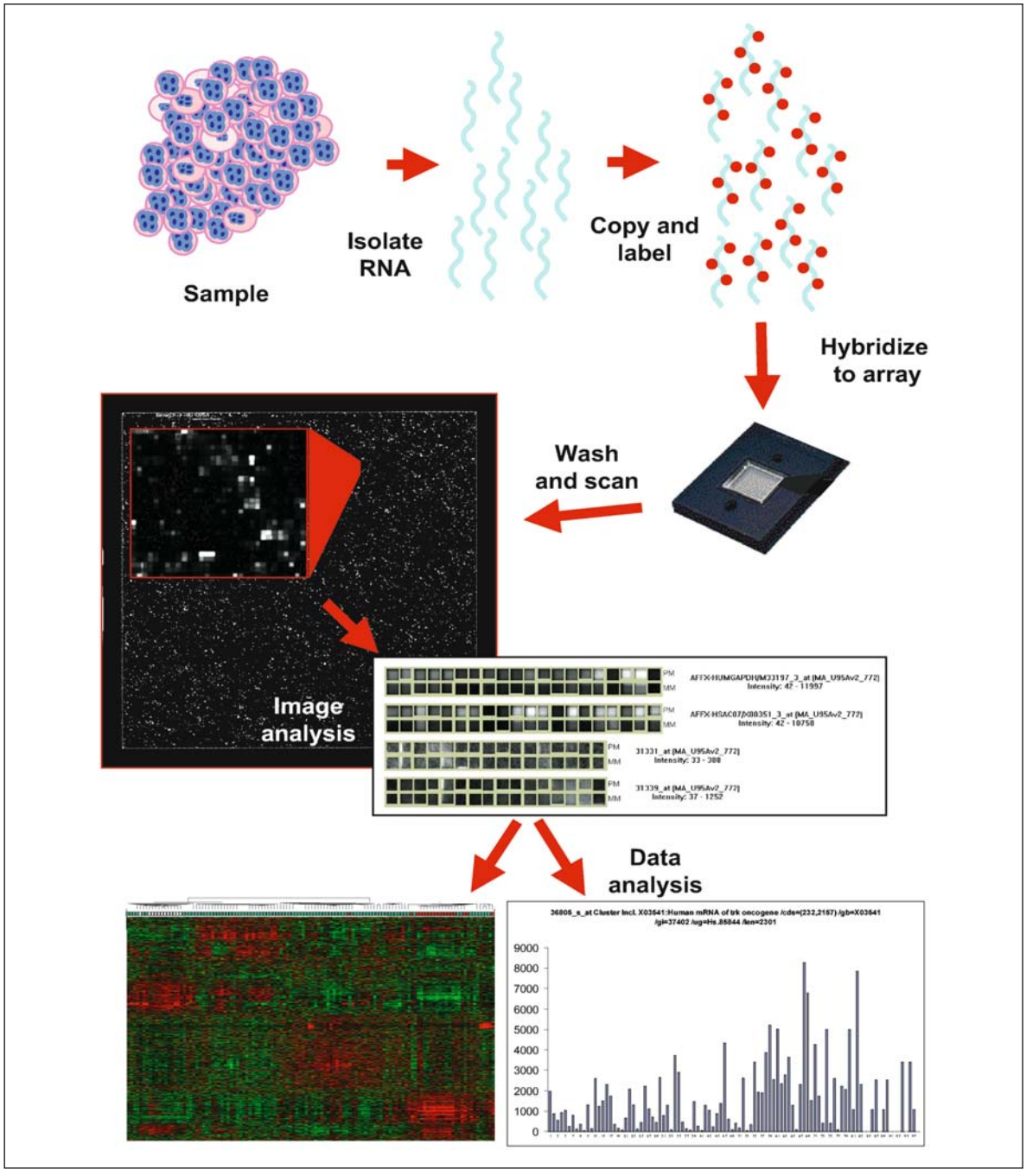

Figure 9.1

Steps involved in microarray-based gene expression analysis

tive hybridization, serial analysis of gene expression (SAGE) and sequencing of expressed sequence tags (ESTs)], and the use of microarrayed sequence probes for quantitative hybridization studies. Sequencing-based techniques can be very quantitative and precise but are dependent on the depth of sequencing, accurate sequencing, and authentic mapping of sequence to gene. They are also labor intensive. Microarray-based hybridization studies involve labeling of transcript representations from cells or tissues and hybridization of the labeled target to nucleic acid probes attached to a solid support in an arrayed pattern (Fig. 9.1). The bound label is proportional to the quantity of specific transcripts in the original RNA mixture. The probe arrays are miniaturized such that thousands of individual probes can be attached to a very small substrate providing high-throughput and experimental efficiency. This technique allows for the efficient analysis of virtually unlimited numbers of genes.

9.3.2 Data Analysis

The analysis of expression data is of primary importance but beyond the scope of this chapter; however, a few critical issues are highlighted here (Quackenbush 2001). Most studies are designed to identify important genes that participate in a critical process or to classify samples into previously unrecognized biologically or clinically important subsets. This can be accomplished by reducing the expansive data sets using statistical or metric thresholds to identify genes whose expression varies significantly between samples of interest. A number of methods can then be used to identify samples or genes with the desired properties. Unsupervised algorithms search the data with few user imposed restrictions in an effort to recognize molecular substructure and identify previously unrecognized classes of genes or samples. Supervised methods apply prior knowledge, such as histology, phenotype, stage or outcome, and identify genes with statistically significant expression differences between groups; however, because of the large volume of gene expression data and relatively small number of samples, some associations are likely due to chance. For this reason it is imperative that the significance of correlations be established by testing independent sample sets.

9.3.3 The Impact of Tissue Heterogeneity on Gene Expression Analysis

Neuroblastomas can demonstrate marked intra and intertumoral heterogeneity primarily due to the spectrum of differentiation that occurs (see Chap. 8). This may result in tumor samples with a predominant primitive neuroblast component and others with a predominant differentiated ganglionic or Schwannian cell component. The interpretation of observed gene expression is therefore dependent on understanding the complexity of the cellular content. In heterogeneous samples, gene expression corresponding to very small but biologically significant components of tumors may not be evident using current methods and inter-sample comparisons can be limited by their lack of consistency in tissue content. Few reports have addressed these issues, but it is obvious that cell content is a major factor in NB as shown in the analyses below.

9.4 Gene Expression Analysis of NB

9.4.1 The NB Transcriptome and Its Relationship to Neural Crest Development

The occurrence of NB in a wide anatomic distribution that parallels the sympathetic nervous system, and the spectrum of tumor phenotypes, including various degrees of differentiation, regression, and proliferation, are strong evidence that the origin of this tumor is closely linked to the development of neural crest-derived sympathogonia. These cells migrate from the neural tube to generate the primordia of the sympathetic chain along the abdominal aorta. A subpopulation migrates to the adrenal anlage to form the chromaffin cells of the medulla. There are many opportunities during this process for abnormalities to contribute to tumorigenesis including loss of controls on cell proliferation, differentiation, or apoptosis. A look at the transcriptome specific to NB reflects this developmental arrest and relationship to neural crest-derived tissues. In preliminary gene expression studies of a spectrum of NB, we have identified genes that are highly expressed in tumors rela-

Table 9.1. Genes involved in neurogenesis that are specifically expressed at increased levels in high-risk neuroblastoma relative to normal tissues

Gene title	Gene symbol	Chromosomal location	Gene ontology biological process description (Harris et al. 2004)
Embryonic lethal, abnormal vision, Drosophila-like 3 (Hu antigen C)	ELAVL3	19p13.2	Cell differentiation, neurogenesis
Midkine (neurite growth-promoting factor 2)	MDK	11p11.2	Cell differentiation, regulation of cell cycle, cell proliferation, cell–cell signaling, signal transduction, neurogenesis
Tyrosine hydroxylase	TH	11p15.5	Embryogenesis and morphogenesis, neurotransmitter biosynthesis, aromatic amino acid family metabolism, catecholamine biosynthesis, synaptic transmission
Tubulin, alpha 3	TUBA3	12q12–12q14.3	Glia cell differentiation, cell-shape and cell-size control, neurogenesis
Kallikrein 8 (neuropsin/ovasin)	KLK8	19q13.3-q13.4	Neurogenesis
Fetal Alzheimer antigen	FALZ	17q24.3	Neurogenesis
SMA3	SMA3	5q13	Neurogenesis, carbohydrate metabolism, skeletal development
Platelet-activating factor acetyl-hydrolase, isoform Ib, gamma subunit 29 kDa	PAFAH1B3	19q13.1	Neurogenesis, lipid catabolism
Paired-like homeobox 2b	PHOX2B	4p12	Regulation of transcription, DNA dependent, neurogenesis, development

tive to non-neoplastic tissues (including fetal and mature brain tissue, spinal cord, bone marrow, liver, lung, and kidney). Many of the functionally annotated genes that are highly over-expressed in high-risk NB are believed to play a role in cell growth, development, differentiation, and histogenesis (unpublished data). A significant number are specifically annotated as playing a role in neurogenesis (Table 9.1). It is also interesting that a high proportion (about 38% of the most strongly differentially expressed genes) of these NB-specific genes are expected to function in some aspect of transcription and a significant number (about 19%) are believed to be part of signaling pathways. Many of these genes are highly specific for NB and may enhance our ability for accurate diagnosis and identification of therapeutic targets.

From an unsupervised analysis (average linkage hierarchical clustering) of gene expression in NB, it is clear that differentiation is a key factor reflected in the profiles (Fig. 9.2). Cell lines composed of relatively pure populations of poorly differentiated neuroblastic cells are relatively distinct and aligned with a subset of stroma-poor NB that are similarly composed of poorly differentiated neuroblasts. Likewise, ganglioneuromas and NB containing a large component of Schwannian cells (stroma-rich) have expression profiles that are distinct from stroma-poor tumors. This is a reflection of the very different expression patterns of Schwann cells and neuroblasts. Stroma-poor (neuroblast-rich) tumors are characterized by a greater level of expression for genes associated with DNA replication and cell division, and molecules regulating early

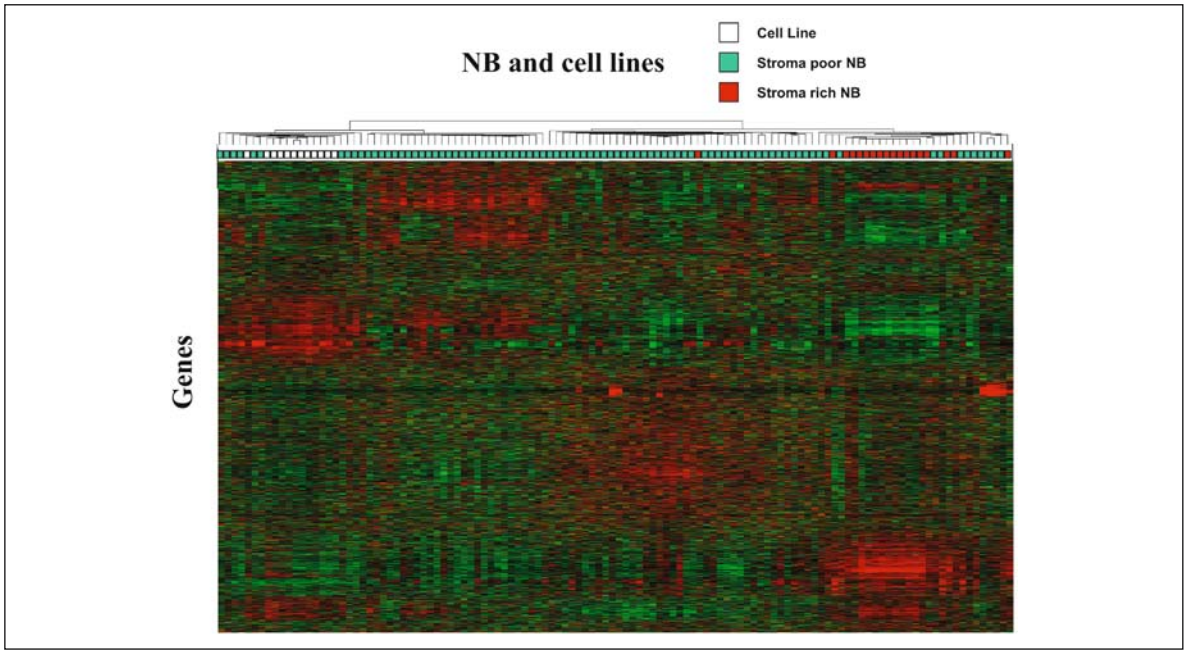

Figure 9.2

Graphic display of a hierarchical cluster analysis of NB gene expression. The dendrogram represents relationship between samples. Branch length is based on 1 – Pearson's correlation coefficient between the samples of that branch. *Columns* are individual samples and *rows* represent an individual gene. Sample types are indicated at the top of each column just below the *dendrogram*. Expression levels are *pseudocolored red* to indicate transcript levels above the median for that gene across all samples and *green* below the median. Color saturation is proportional to the magnitude of expression

Table 9.2. General biological function most commonly assigned to genes correlating with differentiation in neuroblastoma

General biological function	Differentially expressed genes over-expressed in stroma-poor NB (%)[a]	Differentially expressed genes over-expressed in stroma-rich NB (%)[a]
Cell cycle, DNA replication and repair	33	13
Neurogenesis or neural function	27	7
Development or histogenesis	27	11
Immune or inflammatory response	2	21
Cell adhesion	2	15
Apoptosis	2	8

[a] Genes may have more than one biological function

stages of neurogenesis (Table 9.2), while Schwannian stroma-rich tumors displayed over-expression of many genes associated with the immune response and cell adhesion. It is unclear at this time which cells in stroma-rich tumors contribute to the expression differences, but it is likely that many are intrinsic to the Schwannian component. This data also emphasizes the need to control for tissue content and differentiation in analysis of NB for correlations with clinical phenotype.

9.4.2 Gene Expression Associated with Clinically Relevant Subtypes of NB

Supervised approaches to data analysis allow the identification of genes that are differentially expressed in the biologically and clinically distinct subsets of NB tumors. For example, comparison of stroma-poor stage-4 NB with known bone metastasis to stroma-poor LR NB that were cured with surgery alone demonstrated a number of differentially expressed genes that are known to participate in cell cycle regulation, DNA replication, mitosis, and cell division. These genes reflect the striking differences in cell replication known to exist between these two groups and most are over-expressed in stage-4 tumors. Other differentially expressed genes encode proteins with a wide variety of proposed biological functions; however, it is of interest that some of the genes over-expressed in LR NB included several with anti-apoptotic activity (OPTN, TIAF1, PRKCZ) and some that play a role in neurogenesis (PAFAH1B1, PMP22; Li et al. 1998; Chang et al. 1998; Rust et al. 2000; Sweeney et al. 2000; Wulf et al. 1999).

Other studies investigating the gene expression patterns of individual NB risk groups have recently been published. Sequence analysis of cDNA libraries from NB defined as favorable (single-copy *MYCN*, high NTRK1 expression) or unfavorable (amplified *MYCN* and low NTRK1) revealed a large number of genes that were relatively over-expressed in the favorable tumors (Ohira et al. 2003), including genes that are believed to play a role in cell signaling, transcription, protein synthesis, and cell homeostasis. Few genes were identified that were over-expressed in unfavorable NB.

Telomerase is a ribonucleoprotein enzyme essential for the replication of chromosome termini in most eukaryotes. Activation of telomerase has been implicated in cell immortalization and cancer cell pathogenesis and is associated with outcome in NB (Choi et al. 2000). Comparison of NB with high or low telomerase activity, using a cDNA array corresponding to genes expressed in the human fetal brain, identified 63 genes over-expressed in tumors with high telomerase activity and 46 with low activity (Hiyama et al. 2003). The over-expressed genes in tumors with high telomerase activity included those involved in cell cycle, apoptosis escape, protein synthesis, and transcription. Those over-expressed in tumors with low-level telomerase included neural transmitters and several receptors associated with neural or neuroendocrine function. These genes again reflect the biology of distinct NB risk groups with active cell replication in high-risk groups and increased degrees of neural differentiation in low-risk tumors.

These preliminary results suggest that clinically relevant NB subgroups have distinct molecular profiles and that characterization of individual molecules will lead to a better understanding of NB biology and provide the means for molecular classification that may be used in conjunction with traditional clinical features for improved patient care. It is intriguing to speculate that the clinical behavior of a NB may one day be more accurately predicted by expression analysis than by the clinical and biological features that are currently utilized for patient risk stratification and treatment.

9.4.3 Molecular Pathology of *MYCN* Amplification

MYCN amplification occurs in about 20% of NB and is a well-established clinical marker of aggressive disease used for patient risk stratification. Amplification leads to high levels of *MYCN* expression in most cases and is believed to directly contribute to tumor biology. Although *MYCN* is a transcriptional regulator, few specific in vivo targets have been identified and the mechanisms by which *MYCN* contributes to aggressive tumor biology are not known (Seeger et al.

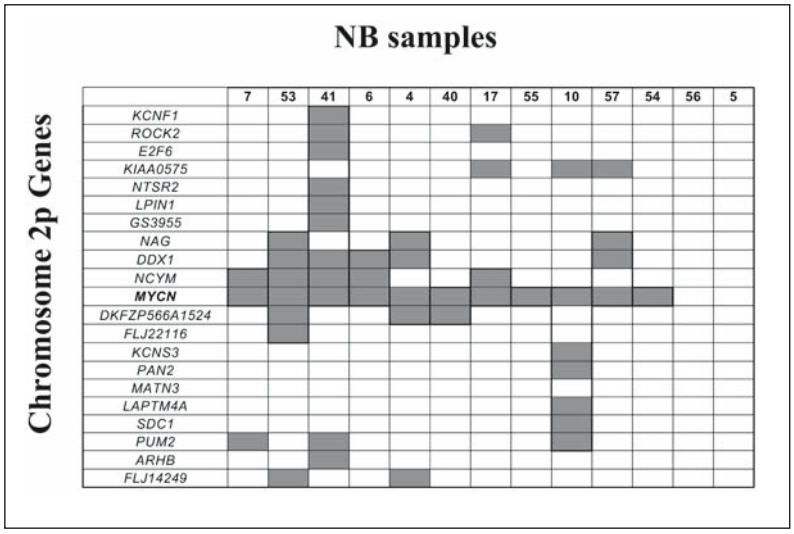

Figure 9.3

Expression of genes near *MYCN* in samples showing amplification or over-expression of this gene. Map is derived from the UCSC genome database (http://genome.ucsc.edu/cgi-bin/hgGateway). *Gray boxes* indicate expression at least one standard deviation above the mean for that gene in tumor samples

1985; Ma et al. 1993). Oligonucleotide arrays have been used to monitor the effects of *MYCN* on gene expression in NB through analysis of human tumors and cell lines (Alaminos et al. 2003). An interesting finding that has been noted in previous studies (Nisen et al. 1988; Slavc et al. 1990) is that *MYCN* mRNA expression levels did not always coincide with *MYCN* gene copy number. This implies that over-expression of *MYCN* occurs in some cases of NB without gene amplification and is probably due to alterations in transcriptional regulation. The clinical significance of over-expression in the absence of amplification is uncertain; however, in array studies, tumors with high levels of *MYCN* expression in the absence of amplification tend to cluster with amplified tumors with increased expression, demonstrating a correlation between level of *MYCN* and overall gene expression profile (Alaminos et al. 2003).

Alaminos and co-workers compared the expression profiles of tumors with and without high levels of *MYCN* mRNA using relatively stringent criteria and found that 222 of 62,839 probe sets identified genes with significant differential expression (Alaminos et al. 2003). Seventy-four probe sets detected genes that were up regulated and 148 that were down regulated in tumors with high levels of *MYCN* RNA. Some of these were believed to be direct targets of this oncogene based on altered expression in cell lines with induced expression of *MYCN*. The functional aspects of some of these genes included transcriptional regulators (*HTATIP*, *HTATIP2*, *DDX1*, *MI-ER1* and *NCYM*), oncogenes (*NCYM*, *RAB20*), cell proliferation (*CDCA7, CENPE, CDC2L2, PC-TAIRE2BP)*, and neural differentiation (*HOXC10, PTN*, *FMNL*, *DNER*, *CLU*, *GDA*, *NRCAM*, *ECEL1* and *SNPH*), and correlate well with the lack of differentiation and high mitotic–karyorrhectic index which is common for *MYCN* amplified tumors. An expression map of the region corresponding to NB with high levels of *MYCN* expression (Fig. 9.3) demonstrated that *MYCN* is the only gene consistently expressed in all NB with 2p amplification and agrees with the findings of others (George et al. 1996; Hiemstra et al. 1994). The significance of over-expression of co-amplified genes is unknown.

A separate study investigating the regulation of gene expression by *MYCN* in a NB cell line using SAGE analysis had a very different result (Boon et al. 2001). In that study the majority of genes that were upregulated by *MYCN* were associated with ribosome assembly and activity. Potential reasons for the differences between the findings in these studies include the over-representation of highly expressed genes in SAGE analysis, the different experimental systems (cell lines vs tumor samples), and the analytical methods used to identify differentially expressed

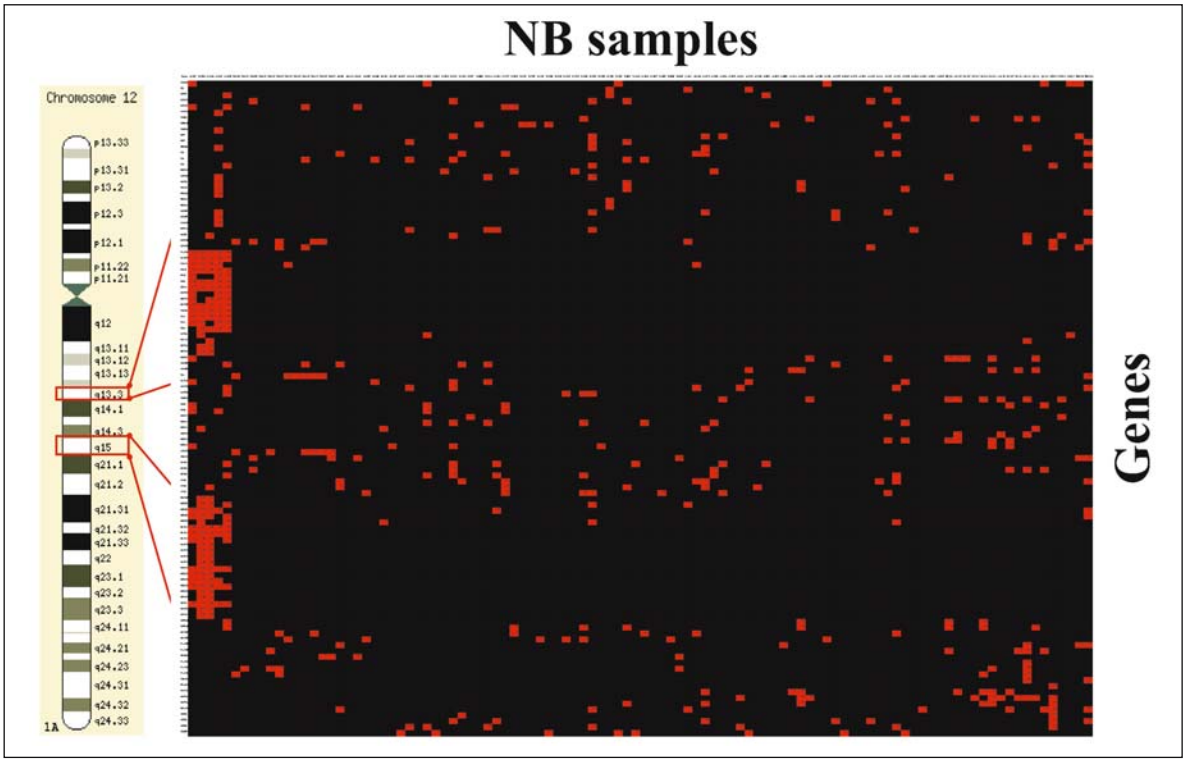

Figure 9.4

A positional gene expression map of 12q13–15 from 56416679 bp to 76539024 bp. Map is derived from the UCSC genome database (http://genome.ucsc. edu/cgi-bin/hgGateway). Each column is a single NB sample and each row is expression of an individual gene. *Red highlight* indicates expression level greater than two standard deviations above the mean. The five cases with obvious over-expression of this region are in the *first five columns* on the left. Note that the genes separate into two distinct regions, 12q13.3 and 12q15

genes. These disparate results emphasize the importance of technical and analytical factors in high-throughput molecular studies.

9.4.4 Distinct Molecular Features of NB Discovered Through Gene Expression Analysis

Gene expression profiling is a powerful technique to discover previously unrecognized tumor subtypes and distinct molecular phenotypes. For example, a search of SAGE libraries for genes related to development identified DLK1 as dramatically over-expressed in some NB cell lines (van Limpt et al. 2000). Further study suggested that DLK1 over-expression was not due to amplification or mutation but was associated with chromaffin differentiation (van Limpt et al. 2003). Data from our own studies suggest that high levels of DLK1 expression is more common in a subset of high-risk NB (unpublished data).

Analysis of genome-wide expression data for NB samples based on oligonucleotide arrays revealed a subset with over-expression of several contiguous genes located at 12q13–15 (W. Su et al., 2004). Regional over-expression suggests a chromosomal amplification event and consistently expressed genes

represent candidate oncogenes. About 5% of NB tumors demonstrate 12q gene over-expression. Positional expression mapping identified the narrowest region of overlap containing 21 genes, with 11 genes over-expressed by all cases. In cases with high levels of expression for genes at 12q, three- to more that tenfold increase in 12q gene copy number was detected by fluorescent in situ hybridization. Amplification of 12q has been identified in a large variety of other cancer types. The 12q expressed genes in NB mapped to a site similar to the complex amplicon reported in sarcomas and gliomas and identify critical genes and pathways affected by 12q gene amplification (Fig. 9.4). This use of positional gene expression mapping provides a means to efficiently filter and select genes within altered chromosomal regions that are prime candidates to contribute to neoplastic development. Importantly, the data described here suggest that gene expression analysis can identify molecular markers that segregate with biological phenotype and molecular classification of NB.

9.5 Conclusion

It is clear that comprehensive molecular studies will have a significant effect on research in cancer biology and oncology. It is also clear that these techniques should be used with discretion and common sense to avoid over interpretation of the large volumes of data, much of which has yet to be validated. Nonetheless, analysis of human tissue samples with a carefully controlled experimental design and rigorous data analysis could lead to new discoveries in gene-function relationships that will complement traditional focused experiments in model systems. Advanced molecular techniques may provide the tools to better understand the clinically heterogeneity and complex biology of NB. Although there are many challenges in moving complex assays from research laboratories to practical utility in diagnostic medicine, the significant potential for identifying therapeutic targets and improving treatment for children with high-risk disease make this a task worth pursuing.

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Anatomic and Functional Imaging

Sara J. Abramson, Barry L. Shulkin

Contents

10.1 Introduction

Primary neuroblastoma (NB) tumors may develop anywhere along the sympathetic chain or in the adrenal medulla. Although the majority of tumors are found in the retroperitoneum, primary sites in the posterior mediastinum, pelvis, and neck also occur but are less common (Abramson 1997). Neuroblastomas are often locally invasive and may encase surrounding vascular structures and extend into local lymph nodes. More than 50% of children with NB present with distant disease. Common sites of metastases include bone, bone marrow, liver, skin, and lymph nodes. Occasionally, patients may also present with lung and central nervous system metastases. Treatment strategies are based, in part, on the results of imaging studies that are used to evaluate the stage of disease at the time of diagnosis as well as disease response following therapy. This chapter reviews the imaging modalities that have been shown to be useful for clinical staging, assessing potential resectability, and examining response to therapy.

10.2 Imaging Modalities

10.2.1 Ultrasonography

Ultrasonography is the most commonly performed screening examination for abdominal and pelvic problems in pediatric patients. Unlike many other imaging studies, sedation is not needed for ultrasonography and there is no exposure to ionizing radiation. The location of the primary tumor, the presence of vascular encasement, and metastasis to the liver may be detected by ultrasonography. Calcifications are demon-

strated as focal areas of increased echogenicity, sometimes associated with acoustical shadowing. The aorta and inferior vena cava are usually anteriorly displaced by the retroperitoneal mass. Tumor invasion of the liver or kidney can be assessed by visualization of planes between the mass and the liver or kidney. Doppler may be used to demonstrate flow in vessels compressed by tumor; however, evaluation of the full extent of the disease is not possible with ultrasonography.

10.2.2 Computerized Axial Tomography

Computerized axial tomography (CT; Tables 10.1, 10.2) scans can be performed relatively quickly and tumor calcifications are readily detected (Cohen 1992). CT of the head and orbits should be performed in any patient with suspected cranial bone involvement, and chest CT scans are necessary for evaluating parenchymal lung disease. The presence of bony changes on CT at diagnosis is diagnostic of metastatic stage-4 NB, To obtain optimal CT scans of the abdomen, adequate contrast filling of bowel as well as bolus intravenous contrast enhancement are required. CT cannot be used

Table 10.1. Technique for computerized axial tomography radiation dose

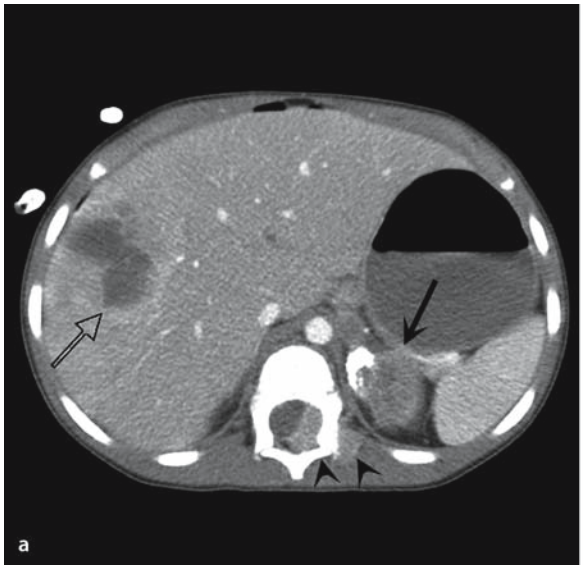
Age (years)	Effective mA	Effective kV
<1	60	80
1–5	60	100
5–10	80	100
10–17	100	100
17–25	120	120

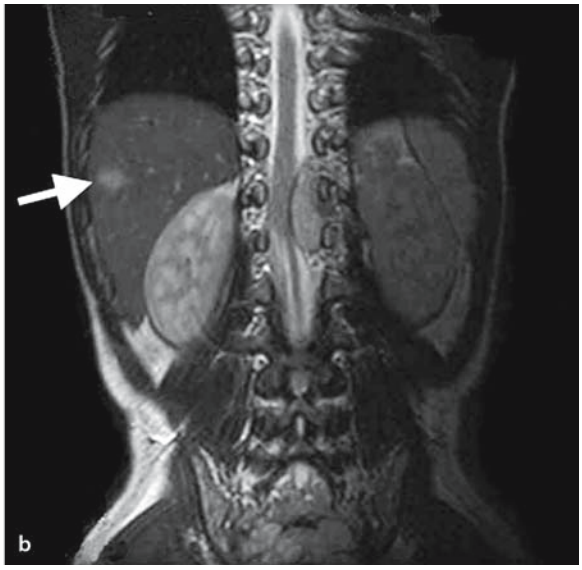
May need more mA if large patient

A CT examination in children should be performed using as little radiation as possible. The use of multi-detector scanners is an advantage in decreasing radiation dose and decreasing the duration of the examination.

Table 10.2. Preparation for CT scanning: oral contrast; intravenous contrast; and sedation

Oral contrast	
0–4 years	4 ml of Gastrografin mixed well with 8 oz of clear fluid
5–12 years	6 ml of Gastrografin mixed well with 12 oz of clear fluid
12–18 years	8 ml of Gastrografin mixed well with 16 oz of clear fluid
> 18 years	Adult dose
Note: The clear fluid may be juice or soda of the patient's choice.	
When receiving general anesthesia the oral contrast is given more than 2 h before the scan so that the patient will be NPO for 2 h before being anesthetized	
Intravenous contrast	
All patients receive nonionic contrast	
Dose 2 ml/kg for chest, abdomen, and pelvis not to exceed 150 ml	
Dose for neck, chest, abdomen, and pelvis total TOTAL 3 ml/kg; split dose 1/3 for neck, 2/3 for chest, abdomen and pelvis not to exceed 60 ml for neck and 120 ml for chest, abdomen, and pelvis	
Injection rate 1 ml/s	
0.8 ml/s for Mediports or Broviacs	
Hand injection for small peripheral lines	
PICC lines cannot be used for injection	
Sedation	
General anesthesia is usually necessary for patients under 3 years of age. Exception is made if fast (5–10 min) scanners are available, where the child may be fed a bottle containing oral contrast material mixed with clear fluid 15–20 min before being papoose-immobilized on the scanner table	





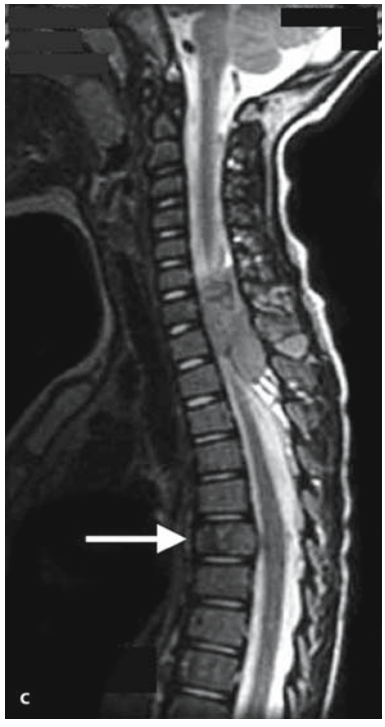


Figure 10.1 a–c

Retroperitoneal neuroblastoma with extension of disease. **a** Contrast-enhanced CT demonstrates the top of a partially calcified left adrenal mass (*arrow*). Note left para-aortic node as well as liver metastasis (*open arrow*). Note mass in the soft tissues of the left back with extension into the neural canal (*arrowheads*). The effect of the epidural component cannot be fully evaluated on CT. **b** Coronal T2-weighted MR image demonstrates the T10/11 – T12 left epidural mass compressing the spinal cord with extension into the adjacent left neural foramen. Note enhancing liver metastasis (*arrow*). **c** Sagittal T2-weighted MR image in the same patient demonstrates thoracic epidural disease with spinal cord compression. Note marrow involvement in mid-thoracic vertebral body (*arrow*)

to evaluate bone marrow, and this imaging study is inadequate for the evaluation of epidural disease (Fig. 10.1). In young children the lack of intraperitoneal fat also makes extent of disease evaluation by CT suboptimal. Younger children may require general anesthesia for CT studies, and in these cases distinguishing true lung involvement from atelectasis is not always possible. In addition, CT cannot reliably be used to evaluate the response of bone disease, as bony abnormalities may persist for months to years; however, active bone disease can be accurately diagnosed by CT if periosteal reaction and soft tissue extension are seen. Unlike ultrasound, there is radiation exposure from CT.

Table 10.3. Magnetic resonance imaging evaluation of patients with neuroblastoma. Suggested techniques, contrast, scan sequences, and anatomical sites

Machine specifications
Magnet: 1.5-T unit
Coils: Body coils or phased-array coils are used for most pediatric patients. Head coils are used for neonates and infants
Slice thickness: ranges from 5-mm slice thickness with 0- to 1-mm intersection gaps to 8-mm slice thickness with 0- to 2-mm intersection gaps depending on the size of the patient
Contrast
Gadolinium: 0.1 mm/kg
Scan sequences
T1-weighted spin echo or T1-weighted gradient echo (in phase)
T2-weighted fat-suppressed fast spin echo
T1-weighted fat-suppressed spin echo or T1-weighted fat suppressed gradient echo (in phase) after IV injection of gadolinium
Evaluation of anatomical sites
Pelvis and femurs to mid-femoral shafts for evaluation of marrow disease and pelvic involvement
Coronal T1-weighted spin-echo or gradient-echo (in phase) sequences
Transverse T2-weighted fat-suppressed fast spin-echo sequences
Chest and abdomen: to evaluate extent of disease
Coronal T1-weighted spin-echo or gradient-echo (in phase) sequence to include the neck for evaluation of possible neck nodes as a site for biopsy
Transverse T2-weighted fat-suppressed fast spin-echo sequence
Transverse T1-weighted fat-suppressed spin-echo or gradient-echo (in phase) sequence after injection of gadolinium
Coronal T1-weighted fat-suppressed spin-echo or gradient-echo (in phase) sequence after injection of gadolinium
Lumbar spine: to evaluate marrow disease
Sagittal T1-weighted spin-echo sequence
Sagittal T2-weighted fat-suppressed fast spin-echo sequence

10.2.3 Magnetic Resonance Imaging

Many investigators consider magnetic resonance imaging (MR; Table 10.3) the imaging modality of choice in the initial evaluation of NB (Siegel et al. 2002; Sofka et al. 1999). Magnetic resonance has distinct advantages over CT including (a) the absence of ionizing radiation, (b) the ability to evaluate neural foraminal and epidural involvement, and (c) the ability to detect bone marrow disease. In addition, MR is ideal for defining the precise extent of skeletal involvement, separating cortical bone from marrow involvement. Furthermore, invasion of the liver, kidney, and abdominal organs can readily be detected by MR imaging. This is particularly important in large, right-sided masses where separation of the mass from liver or kidney may be difficult by CT. By imaging in multiple planes, MR can usually demonstrate the relationship of the mass to adjacent organs. Gadolinium-enhanced scans can provide additional information in these cases (Kornreich et al. 1991). On MR neuroblastoma characteristically has prolonged

Table 10.4. Technetium-99m bone scintigraphy

Bone scanning principles	Scan technique
Image entire body with particular attention to patient position: knees and elbows should be as flat as obtainable to assess peri-articular uptake	Intravenous injection of 25 mCi Tc-99m-MDP/1.73 m^2, imaging begun 2–3 h after injection
Bladder should be empty for adequate visualization of the pelvis. In small children this may require bladder catheterization (if it can be safely done) or delayed images after voiding.	Multi-headed gamma camera with low-energy collimator preferred
	Computer acquisition
	Single photon emission CT as needed

T1 and T2 relaxation times, demonstrating heterogeneous low signal on T1 and high signal on T2. Bright signal in the mass on T1-weighted imaging sequences represents hemorrhage. Although calcifications are not as readily identified on MR as on CT, they are visualized as areas of signal void. Nevertheless, non-visualization of calcifications should not be a significant deterrent to using MR for the initial evaluation of NB.

10.2.4 Bone Scan

The bone scan (Table 10.4) has had a long-standing role for evaluating the entire skeleton for metastatic NB (Shulkin et al. 1992). In addition, many primary NBs also accumulate Tc-99m MDP because of their calcifications. The depiction of osseous lesions depends on the reaction of bone to the presence of tumor within bone. The bone scan provides a major diagnostic advantage over skeletal surveys in the assessment of bony NB lesions. Considerable attention to scan technique and positioning is critical to distinguish normal metaphyseal uptake from NB involvement, particularly when these lesions are symmetrical. Since NB frequently localizes in the metaphyseal region adjacent to the epiphyseal plates, sites of normally increased uptake of bone seeking tracers, metastatic involvement may be difficult to appreciate in these areas of normal high physiological accumulation.

10.2.5 MIBG Scintigraphy

Most NB tumors effectively concentrate tracers designed to image the sympathetic nervous system and other tracers with avidity for somatostatin receptors. Meta-iodobenzylguanidine (MIBG) scintigraphy was developed at University of Michigan in the late 1970s to image the adrenal medulla (Wieland et al. 1980), and was later successfully applied to functional imaging of pheochromocytoma (Sisson et al. 1981) and NB (Treuner et al. 1984; Geatti et al. 1985). MIBG is a tracer for the type-1 amine uptake and granular storage pathways (APUD: amine precursor uptake and decarboxylation). Once transported into the NB cell the majority of MIBG remains within the cytoplasm (Smets et al. 1989, 1990). In contrast to pheochromocytoma cells where MIBG is actively transported into catecholamine storage granules by a reserpine-sensitive pump, accumulation of MIBG in NBs depends on continued reuptake of the effluxed radiotracer. MIBG is not metabolized by the enzymes which metabolize catecholamines. Drugs that interfere with uptake of catecholamines and related compounds may impair visualization of NBs; these include over-the-counter non-prescription cough and cold preparations which contain pseudoephedrine or phenylpropanolamine, and labetolol, a beta-adrenergic antagonist. Before injection of the MIBG tracer, parents should be asked specifically about recent administration of all prescribed and over the counter drugs.

Table 10.5. MIBG imaging

Principles	Scan technique
Imaging of entire body	SSKI or other iodine solutions to block uptake of free radioiodine into the thyroid. Begin the day prior to injection and at minimum, 30 min prior to injection
I-123 MIBG produces images of much higher count density than I-131 MIBG. I-123 has less radiation hazard and requires less stringent thyroid protection	Intravenous injection of 10 mCi I-123-MIBG/1.73 m^2
Bladder should be empty for adequate visualization of the pelvis. In small children this may require bladder catheterization (if it can be safely done) or delayed images after voiding.	Whole-body imaging 18–24 h later, low-energy collimator
	Computer acquisition
	Single photon emission CT as needed

Review of 13 publications from eight countries on MIBG scintigraphy performed on 330 patients (Feine et al. 1987; Geatti et al. 1985; Heyman et al. 1988; Lumbroso et al. 1988; Schmiegelow et al. 1989; Shulkin and Shapiro 1990; Troncone et al. 1990) indicates that the sensitivity of disease detection is ~87% and the specificity is ~94%. The positive predictive value is ~98%, while the negative predictive value is ~70%. The majority of patients in the reported studies were evaluated with I-131 MIBG. Both I-131 and I-123 MIBG are excellent agents for imaging NB. In the United States both the I-131 and the I-123 labeled forms are commercially available, although I-123 MIBG has gained wider use in the past few years because of its superior scintigraphic properties and radiation safety considerations. The images obtained from I-123 MIBG studies have higher count densities and greater quality, and the sites of normal uptake are more readily recognized. In direct comparisons of the two compounds, one study showed that the same number of lesions were identified by both techniques, while another study suggested that more lesions were identified with I-123 MIBG scintigraphy (Gelfand 1996; Simon et al. 1992). High-quality single-photon-emission CT (SPECT) images can be obtained using I-123 MIBG. One study indicated that a greater number of abnormal sites of uptake can be detected by SPECT than by planar scintigraphy, with better anatomic localization of the lesions (Ruffini et al. 1996); however, other investigators did not find more lesions by SPECT, although an increase in the certainty that suspected abnormalities on planar imaging were indeed abnormal was reported (Gelfand et al. 1994).

MIBG is highly specific for NB in the usual pediatric context (Leung et al. 1997). The results of MIBG scintigraphy in 100 children with a variety of childhood tumors other than NB studied in five referral centers in three countries showed a specificity of >95%. In these studies, solid tumors of childhood, such as Wilms tumor and soft tissue sarcoma, failed to concentrate MIBG (0 of 14 and 0 of 15, respectively). The remainder of the patients also had negative scans except for 1 of 2 with infantile myofibromatosis, 1 of 2 with neuroendocrine carcinomas, 1 of 2 pancreatoblastomas, and 1 of 10 primitive neuroectodermal tumors; thus, MIBG is only rarely concentrated by non-neural crest tumors.

MIBG scans may also have prognostic value. A study by Suc and colleagues (1996) suggested that children older than 1 year with more than four deposits of MIBG avid NB at diagnosis were seven times less likely to achieve a complete remission after four courses of chemotherapy. Similar results have been reported by Perel and co-workers (1999). In addition, a recent study by Matthay et al. (2003) suggests that the number of MIBG-positive lesions identified after four cycles of induction therapy may be predictive of outcome.

10.2.6 Octreotide Scanning

Many neuroendocrine tumors express somatostatin receptors, and the currently available In-111DTPA-pentetreotide (Octreoscan) detects the majority of NBs and pheochromocytomas; however, this compound appears to be inferior to MIBG for imaging these tumor types (Lauriero et al. 1995; Manil et al. 1996; Sautter-Bihl et al. 1994). Visualization of NBs in the abdomen and pelvis can be impaired by the marked normal hepatic, splenic, renal, and bowel uptake of this agent. In contrast to specificity of MIBG, Octreoscan images many types of neuroendocrine tumors, including carcinoids, islet cell tumors, pituitary adenomas, and medullary carcinoma of the thyroid, and non-neuroendocrine tumors, such as lymphomas and small cell carcinoma of the lung. Inflammatory infiltrates may also show uptake.

10.2.7 Positron Emission Tomography Scanning

F-18 fluorodeoxyglucose (FDG) is concentrated by most NBs and pheochromocytomas (Fig. 10.2; Shulkin et al. 1996; Kushner et al. 2001). Unlike tracers specific for tissues of the adrenergic nervous system, FDG uptake does not depend on type-1 catecholamine uptake. Most NBs concentrate FDG and uptake prior to therapy is often intense, decreasing with treatment. FDG imaging in NBs is particularly valuable for monitoring those tumors which do not concentrate MIBG.

10.2.8 Other Tracers

Gallium-67 is rarely used for imaging of NB. Although nearly 80% sensitivity for detection of the primary tumor has been reported, gallium imaging did not visualize skeletal involvement (Garty et al. 1989). Tl-201 has been useful for imaging a variety of soft tissue tumors, but this tracer localizes poorly in NB (Howman-Giles et al. 1995). A number of ligands similar to MIBG have also been used to study sympathetic innervation of the heart as well as to image neuroendocrine tumors. These include I-123 amino iodobenzylguanidine (AIBG), F-18 fluorodopamine, F-18 fluronorepinephrine, F-18 fluorometaraminol, C-11 hydroxyephedrine (HED), C-11 epinephrine (EPI), C-11 phenylephrine, and fluoro-metaiodobenzylguanidine (FIBG); only three have been investigated in patients with neuroendocrine tumors (Shulkin et al. 1986, 1996, 1999). FIBG has been shown to be concentrated by NB cells in culture (Vaidynathan et al. 1994b). Benzylguanidine labeled with the alpha particle emitter Astatine 211 has been proposed as a potential radiotherapeutic agent (Vaidyanathan et al. 1994a). These derivatives of MIBG rely on the type-1 uptake pathway for entry into the cell.

The I-131 labeled monoclonal antibody 3F8 specific for ganglioside GD2 has been used for radioimmunoscintigraphy and radioimmunotherapy of NB. Because of the abundance of this antigen on neuroblastoma ($5–10\times10^{-6}$ molecules per cell), high levels of radiolabel can be deposited selectively to tumor instead of normal tissues (Miraldi et al. 1986; Yeh et al. 1991). The I-124 labeled form has also been used to

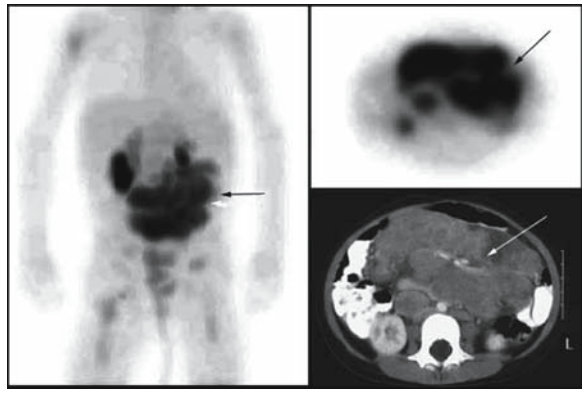


Figure 10.2

A 4-year-old with extensively treated neuroblastoma under consideration for bone marrow transplant. I-123 MIBG scan (not shown) showed no abnormal uptake. *Left panel:* Coronal image from PET-FDG scan shows large area of markedly increased uptake (*arrow*). Transverse image from PET-FDG scan (*right upper panel*) shows markedly increased uptake (*arrow*) occupying most of the abdomen. A CT scan of same area (*right lower panel*) shows the large abdominal mass (*arrow*) with mixed attenuation characteristics. The patient soon after died from progressive disease

improve tumor dosimetry (Larson et al. 1992). Several other monoclonal antibodies that may prove to be useful in imaging NB are under investigation (see Chap. 14 for a detailed discussion of antibody-based radioimmunotherapy).

10.3 Disease Evaluation

10.3.1 Primary Site

Primary retroperitoneal NB tumors frequently infiltrate behind the aorta and IVC (inferior vena cava) displacing these vessels anteriorly. In addition, tumors commonly encase the aorta, IVC, renal arteries, renal veins, celiac axis, and superior mesenteric artery. When compared with CT, MR appears superior in evaluating vascular displacement and encasement (Tanabe et al. 1993a, b). Measurement of tumor volume as a quantitative response to therapy has become important study endpoints, for prognosis and follow-up. In some therapeutic protocols, the percentage of tumor shrinkage as determined by CT scan or MR imaging is critical to the continuation or abandonment of specific therapies (Wheatley et al. 1995).

10.3.2 Local Invasion

Extension of disease to lymph nodes adjacent to primary abdominal NB tumors in the renal hila, porta, and retroperitoneum is commonly seen. Separation of the primary tumor from the adjacent involved nodes is often not possible, regardless of the imaging modality utilized. Distant nodal disease, particularly in the neck where they are readily accessible to biopsy, should be identified on CT scans or on coronal MR scans in patients with large unresectable primary tumors, possibly sparing the patient an open biopsy (Abramson et al. 1996). Mediastinal, hilar, paratracheal, subcarinal, and azygoesophageal adenopathy can be due to direct extension of large thoracoabdominal primary tumors or may represent metastatic disease.

Direct invasion of abdominal organs, including liver and kidney, can occasionally be seen. Invasion of the psoas muscle is not uncommon. Pleural effusions are uncommon and are typically observed in the presence of tumor masses that are pleural based or rib lesions. Epidural involvement may be partially identified on CT. Magnetic resonance is necessary for full evaluation of foraminal and epidural tumor extension and possible cord compression (Sofka et al. 1999; Siegel et al. 1986) (Fig. 10.1).

10.3.3 Distant Metastases

10.3.3.1 Bone Metastases

Bone metastases may be detected by bone scintigraphy, MIBG scan, and PET scan. Symmetrical skeletal involvement is easier to interpret by MIBG than by bone scan. Furthermore, bone scan can remain abnormal for months even after the successful treatment of the tumor, whereas MIBG will not be taken up by healing bone; however, since bone and bone marrow are two distinct compartments with disparate prognostic importance (e.g., stage 4 vs stage 4S in infants), caution should be exercised in assigning bony involvement solely on MIBG. False-negative I-123 MIBG scans for skeletal involvement have been reported, prompting some investigators to recommend both I-123 MIBG plus bone scans for the evaluation of NB (Gordon et al. 1990). In stage-4 patients, where bony and bone marrow involvement usually go hand in hand, concordance between MIBG scanning and bone scanning is generally the rule (Shulkin et al. 1992); however, some studies have indicated that more skeletal lesions may be evident on MIBG scan compared with bone scan (Hadj-Djilani et al. 1995). Bone lesions may also be identified on CT; however, these lesions persist as abnormalities on CT for years, even in the face of bone and MIBG scans that have reverted to normal.

10.3.3.2 Bone-Based, Dural-Based, Leptomeningeal, and Brain Metastases

Computed tomography scan or MR imaging of skull metastases shows bony erosion or abnormal bone signal and soft tissue masses, which may extend into the soft tissues of the scalp or push through the inner table of the skull (Egelhoff and Zalles 1996). Dural

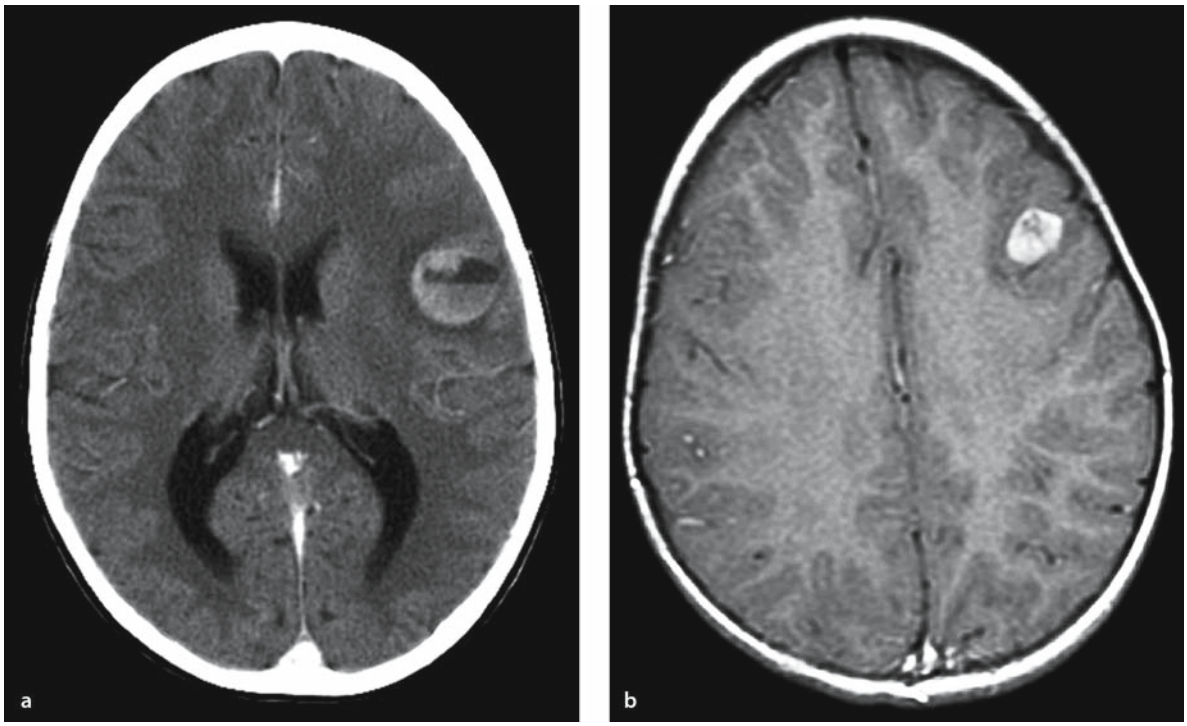


Figure 10.3 a,b

Intracerebral metastases. **a** Patient 1. Axial post-contrast CT of the brain reveals a hemorrhagic left posterior frontal brain metastasis. **b** Patient 2. Post-contrast axial T1-weighted image of the brain demonstrates a left frontal brain metastasis

disease, which is common, may be well demonstrated with either contrast-enhanced CT scan or MR imaging. Once through the dura, these tumors can impinge on the brain parenchyma. Sphenoid bone involvement may extend into the orbits as a soft tissue mass causing proptosis. Diffuse leptomeningeal disease may be visualized by CT (Sener 1993) or MRI. Although MR with gadolinium contrast has high sensitivity in detecting meningeal metastasis, occasionally false-positive results can be seen following general anesthesia and lumbar puncture. Intracerebral metastatic lesions are usually solid (Kramer et al. 2001) and occasionally hemorrhagic (Aronson et al. 1995) (Fig. 10.3) or cystic (Kenny et al. 1995). These cystic lesions may display contrast-enhancing rims and be confused with infection or inflammation.

10.3.3.3 Marrow Metastasis

Histological examination is the gold standard for the diagnosis of marrow metastasis. MIBG is the most efficient test to estimate the distribution and severity of bone marrow involvement (Fig. 10.4; Osmanagaoglu et al. 1993). Spotty marrow involvement can be missed by tissue biopsy. In addition, MIBG and MRI may show bone marrow involvement in areas not accessible to biopsy.

MRI may show more bone marrow lesions than MIBG (Corbett et al. 1991).

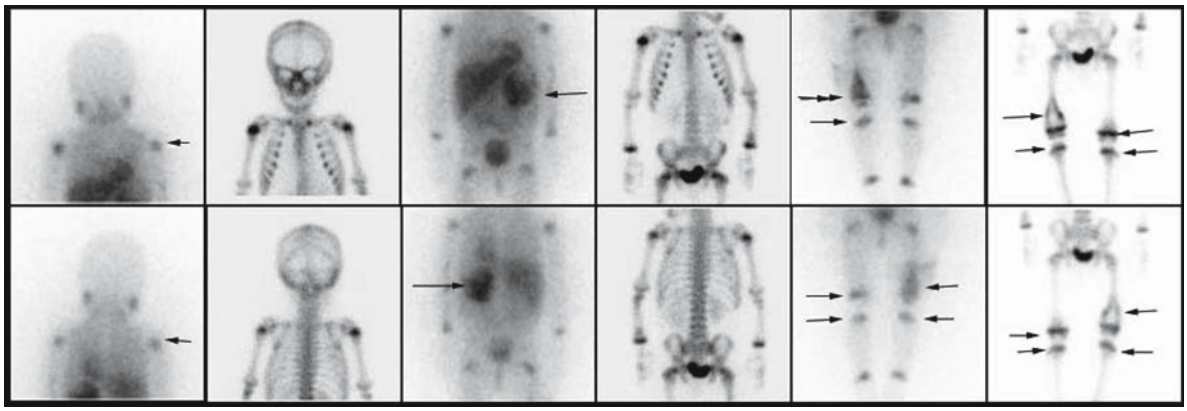


Figure 10.4

A 3-year-old with widespread neuroblastoma at presentation. Alternating panels of MIBG scan and bone scan. Foci of disease are much better delineated on the MIBG scan. *Top row* are anterior views, *bottom row* are posterior views. The MIBG scan shows extensive skeletal involvement: there is abnormal uptake in both shoulders (*arrows*, *panels 1 and 2*), both wrists (*panels 3 and 4*), the proximal femurs (*panels 3 and 4*), the distal right femurs (*panels 5 and 6*), both knees (*panels 5 and 6*), and ankles (*panel 5*). Ankles are seen on MIBG views only. A large left abdominal mass is seen on MIBG images (*panel 3*)

10.4 Prenatally Diagnosed Neuroblastoma

With the advent of routine prenatal ultrasonography, the diagnosis of fetal (Ho et al. 1993; Toma et al. 1994; Jennings et al. 1993) or congenital (Granata et al. 2000; Forman et al. 1990) neuroblastoma has become more common. The tumor may be solid or cystic (Atkinson et al. 1986; Hamada et al. 1999) and may be localized (stage 1) or associated with liver metastases (stage 4S; Toma et al. 1994). Subcutaneous nodules are frequently present. At birth, these findings can be confirmed by ultrasonography. In addition to NB, the differential diagnosis of an adrenal mass detected prenatally or at birth includes extralobar pulmonary sequestration (Curtis et al. 1997) and adrenal hemorrhage (Strouse et al. 1995; Burbige 1993). Many adrenal hemorrhages show shrinkage within 2 weeks after birth; however, shrinkage of an adrenal hemorrhage may sometimes take weeks to months. These masses therefore may be confused with tumor. On the other hand, regressing NB in a neonate can also mimic adrenal hemorrhage (Croitoru et al. 1992). In the absence of large tumor masses and organ compromise, observation rather than surgery is acceptable in an asymptomatic infant with a small adrenal mass (see Chap. 11). Follow-up ultrasonography is important, especially if there is liver involvement. If clinically indicated, the distinction between NB and adrenal hemorrhage can usually be made with MIBG scintigraphy.

10.5 Stage-4S Neuroblastoma

In the absence of adverse biological factors infants with stage-4S disease have a high incidence of spontaneous regression and an excellent prognosis (see Chaps. 7 and 10). In stage-4S disease the extent of disease is initially evaluated with CT or MR of the head, neck, chest, abdomen and pelvis, and MIBG scintigraphy; however, once the diagnosis is made and if good biology is confirmed, ultrasonography is a useful method of follow-up. The liver may show persistent heterogeneous echo texture, although focal nodules should eventually resolve (Fig. 10.5). If the mass

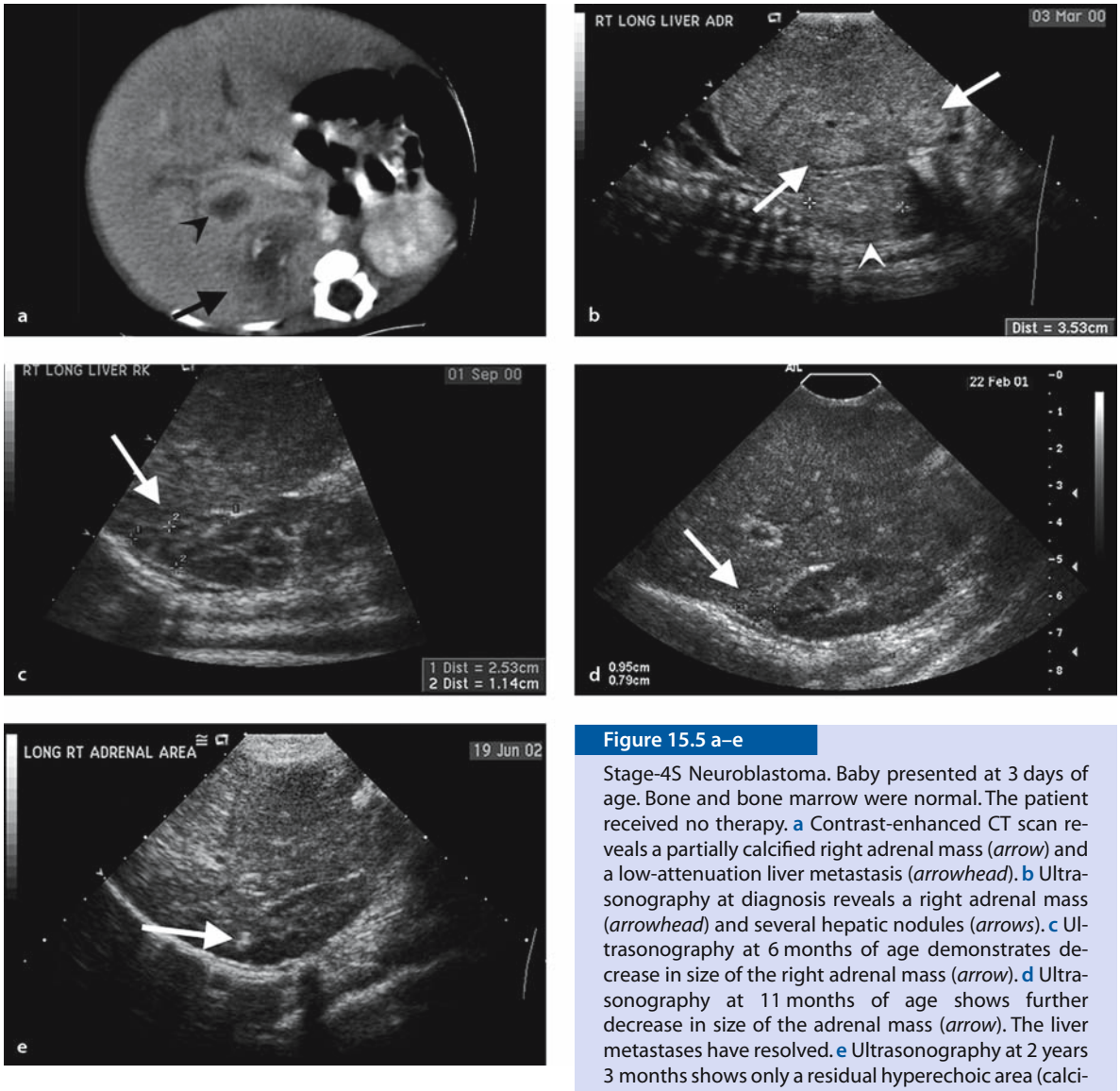


Figure 15.5 a–e

Stage-4S Neuroblastoma. Baby presented at 3 days of age. Bone and bone marrow were normal. The patient received no therapy. **a** Contrast-enhanced CT scan reveals a partially calcified right adrenal mass (*arrow*) and a low-attenuation liver metastasis (*arrowhead*). **b** Ultrasonography at diagnosis reveals a right adrenal mass (*arrowhead*) and several hepatic nodules (*arrows*). **c** Ultrasonography at 6 months of age demonstrates decrease in size of the right adrenal mass (*arrow*). **d** Ultrasonography at 11 months of age shows further decrease in size of the adrenal mass (*arrow*). The liver metastases have resolved. **e** Ultrasonography at 2 years 3 months shows only a residual hyperechoic area (calcification) in the region of the right adrenal gland (*arrow*)

increases in size or liver lesions progress, patients should be re-evaluated, and treatment may be indicated. Efforts to limit exposure to repeated ionizing radiation from follow-up CT examinations and routine MIBG studies in this group of young infants should be made.

10.6 Evaluation of Disease Response

According to the INSS, disease response should evaluated CT/MRI of the primary and metastatic sites, MR of epidural or CNS involvement, bone scan, and MIBG scan; however, it must be remembered that occasionally ganglioneuromas are detected by MIBG scintigraphy. Prior to second-look surgery, a repeat CT scan of the primary site is important for surgical planning. In addition to the primary tumor, adjacent lymph nodes in the thorax, retroperitoneum, and pelvis should be evaluated. Small lymph nodes are better identified on CT than MR, and may be too small to characterize on MIBG scanning.

For patients with intermediate- and high-risk disease, CT/MRI examinations are typically performed in conjunction with MIBG scans at 3- to 6-month intervals during treatment and for 1–2 years following the completion of therapy. Routine scanning after this time should be continued only for those patients with persistent abnormalities. Routine follow-up bone scans do not yield much extra information, unless progression is suspected and or local radiation is planned. Equivocal findings can be further evaluated with FDG-PET scans (Fig. 10.2).

10.7 Conclusion

Imaging plays a critical role in evaluating the extent of disease at diagnosis and in assessing response to therapy. A variety of imaging modalities are available, and each can provide unique information. Radiation exposure, scanning protocols, and sedation times are significant factors that should be taken into account in determining which studies should be used. Minimizing radiation exposure is of paramount importance in the follow-up of neonates and young infants with favorable biology NB. At the present time, several different imaging studies are needed to optimally evaluate the primary tumor, and the presence and location of metastatic disease. New imaging modalities may improve our ability to evaluate NB with less risk of radiation exposure.

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Treatment of Neuroblastoma

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Contents

11.1 Low-Risk Neuroblastoma

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11.1.1 Introduction

Low-risk neuroblastoma is defined as disease that is curable with no or minimal cytotoxic therapy and is strongly associated with spontaneous regression. Approximately 40% of neuroblastoma patients have low-risk disease. According to the Children's Oncology Group (COG) Neuroblastoma Risk-Group Schema criteria, this category includes patients with completely resected localized tumors (stage 1), unresectable unilateral tumors (stage 2A), localized tumors with ipsilateral regional lymph node spread (stage 2B), and infants with unilateral primary tumors with distant disease limited to the bone marrow, liver, and skin (stage 4S; see Chap. 7). Numerous studies have demonstrated excellent survival rates for infants with favorable biology stage-4S neuroblastoma with minimal to no therapy (Guglielmi et al. 1996; Hero et al. 2000; Katzenstein et al. 1998; Nickerson et al. 2000; Schleiermacher et al. 2003), and most patients with INSS stage-1 and stage-2 disease can be cured with surgery alone (Alvarado et al. 2000; de Bernardi et al. 1995; Evans et al. 1996; Kushner et al. 1996b; Matthay et al. 1989; Nitschke et al. 1988; Perez et al. 2000); however, the resectability of local–regional tumors, which is a defining criterion for INSS stage, is dependent to some extent on subjective factors such as the surgeon's experience and the treating team's commitment to avoiding cytotoxic therapy. Prognostic uncertainty also applies to at least two very rare subtypes currently included in the low-risk category, namely, stage-1 neuroblastoma with *MYCN* amplification (Cohn et al. 1995) and biologically favorable stage-2 tumors in adolescents (Franks et al. 1997; Gaspar et al. 2003).

11.1.2 Clinical Presentation

The majority of low-risk neuroblastomas are discovered incidentally. An asymptomatic abdominal neuroblastoma may be palpated during a routine physical examination or revealed in utero by prenatal ultrasonography. A posterior mediastinal neuroblastoma may be serendipitously seen on a chest film performed in a child with suspected pneumonia. Low-risk neuroblastomas may also cause signs and symptoms that prompt medical investigations. Relatively common direct mass effects in low-risk cases include progressive abdominal distention, cervical adenopathy, Horner's syndrome, and acute paraplegia. Systemic symptoms associated with low-risk cases include watery diarrhea from vasoactive intestinal peptide release by neuroblasts, tachycardia from excessive catecholamine production, and opsoclonus–myoclonus–ataxia, which is thought to result from an autoimmune phenomenon mediated by antibodies cross-reacting with antigens on neuroblasts and on cells in the cerebellum (see Chap. 13).

In contrast to the marked preponderance of abdominal primaries in high-risk disease, up to 50% of stage-1 and stage-2 neuroblastomas are extraabdominal. Adrenal primaries and liver lesions occur in 80–90% of stage-4S cases, while morphologic evidence of bone marrow involvement is seen in ~35% of cases and subcutaneous nodules are present in 15% of cases (DuBois et al. 1999).

11.1.3 Clinical Staging

Aspects of staging worthy of note include the post-operative presence of microscopic residual disease with stage 1 and the imprecision of "incomplete gross excision" for separating stage 2A from stage 1; however, since all stage-1 and stage-2A neuroblastomas are grouped into the same low-risk category, the practical implication of exact stage classification (using the extent of post-operative disease) for these two entities is nil. In contrast, risk-group assignment and major management decisions are dependent on staging distinctions that have an uncertain biologic basis in several clinical settings: (a) stage 2A and one subtype of stage 3 (intermediate risk) both involve lack of resectability and gross residual post-operative disease; (b) stage 2B and one subtype of stage 3 (intermediate risk) both involve regional lymph node spread with laterality as the sole distinguishing factor; and (c) widespread disease in infants without osseous or extensive bone marrow involvement can be low-risk stage 4S or intermediate-risk stage 4 depending on the size and resectability of the primary tumor and the presence or absence of contralateral or distant nodal involvement.

11.1.4 Biologic Prognostic Markers

The prognostic value of biological markers in low-risk neuroblastoma is controversial. In part, this is because few events are observed in this cohort of patients and few studies have been performed in which *MYCN* copy number, DNA index, and histology have been analyzed in large numbers of patients. According to the current COG Risk-Group Schema, stage-4S disease is classified as low risk if all three of these biologic factors are favorable; however, while several studies have indicated that *MYCN* amplification and unfavorable histology are associated with poor outcome in infants with stage-4S disease (Hachitanda and Hata 1996; Katzenstein et al. 1998; Shimada et al. 1995), the prognostic impact of diploidy in the absence of *MYCN* amplification is not clear. Although Bourhis et al. found that diploidy correlated with poor outcome in a small study (Bourhis et al. 1991), DNA content was not found to be predictive of outcome in stage-4S infants in other studies (Bowman et al. 1997; Look et al. 1991).

Currently, stage 1 in all age groups is considered low risk regardless of biologic markers and the same holds for infants with stage-2 disease. In older patients, lack of *MYCN* amplification is the only biologic finding needed for classifying stage 2 as low risk. Large Pediatric Oncology Group (POG) and Children's Cancer Group (CCG) studies have demonstrated that *MYCN* amplification occurs in less 5% of children with INSS stage-1 and stage-2 disease (Alvarado et al. 2000; Perez et al. 2000). In the series reported by Perez et al. 7 patients had *MYCN*-amplified tumors (Perez et al. 2000). Two of 4 patients with stage-1 disease remain disease free following surgery alone or treatment with surgery and chemotherapy. Of the 3 patients with stage-2B disease, 2 have died of progressive disease. Similarly, *MYCN* amplification strongly predicted lower EFS and S rates in the POG study (Alvarado et al. 2000); however, the 5-year estimated S rate for this group of patients was 64±27%. Four of 11 patients with *MYCN* amplification remain disease free after surgical resection alone, and 4 of the 7 patients with relapsed disease were successfully saved with additional therapy. Additional factors are still needed to distinguish those patients with *MYCN* amplification who will achieve long-term remission with surgery alone from those who will develop recurrent disease.

11.1.5 Treatment

The focus of this chapter is on the subset of patients with neuroblastoma who can do well without cytotoxic therapy and lack clinical characteristics that connote a poor prognosis, namely, highly locally invasive unresectable primary tumor, cortical bone metastases, and extensive bone marrow involvement. Osteomedullary involvement is an objective finding, but resectability is partly dependent on subjective, nonquantifiable factors. An aversion to perform major surgery might be strengthened by imaging studies showing extensive disease. Resectability, however, can only be definitively assessed during surgery. Low-risk tumors are frequently characterized by a firm consistency that makes resection a fea-

sible option, even if the tumor extends across the midline. By contrast, primary tumors in patients with high-risk disease are often impossible to mobilize and resect due to hemorrhagic friability and adhesiveness to neighboring tissues. These disparities in tumor consistency likely reflect biological differences. Although low-risk neuroblastomas might *potentially* be resectable in their entirety, complete resections sometimes entail the risk of significant morbidity such as brachial plexopathy with a cervical tumor; thus, a treatment plan that includes chemotherapy regardless of the extent of tumor resection may make it unjustifiable to undertake a difficult procedure needed to achieve a gross total excision. Alternatively, a partial resection followed by chemotherapy or observation alone may be reasonable, given the very small risk that residual biologically favorable tumor might evolve into lethal metastatic disease.

11.1.5.1 Localized Tumors with No Regional Spread

Since the 1980s, surgery alone has been deemed adequate treatment for the 10% of patients with neuroblastoma whose tumors have no nodal or distant spread and are grossly excised (stage 1). This approach, even in the presence of microscopic residual disease, has been accepted because retrospective studies (Adam and Hochholzer 1981; Castleberry et al. 1979; Coldman et al. 1980; Hayes et al. 1983; Le Tourneau et al. 1985; Zucker 1974) and prospective studies (Berthold et al. 1986; de Bernardi et al. 1987; Evans et al. 1976, 1984; Kushner et al. 1996b) of patients with well-circumscribed neuroblastoma have shown near 100% survival regardless of post-operative management (Table 11.1.1). For example, in an early prospective group-wide study in which surgery alone was used for Evans stage I (equivalent of INSS stage 1), there was only one (late) death among 26 patients followed for a minimum of 45 months (Evans et al. 1984). In another cooperative group study noteworthy for its large size, prospective design, and use of staging criteria identical to those of INSS stage 1, there were only 3 deaths with surgery alone among 101 patients (Nitschke et al. 1988). Subsequent large prospective group-wide studies in the 1990s confirmed that outcome for this subset of patients following surgery alone is excellent (Alvarado et al. 2000; Perez et al. 2000).

Newborns with small adrenal masses constitute a particularly favorable cohort of patients (Acharya et al. 1997; Ho et al. 1993; Holgersen et al. 1996; Nishihira et al. 2000; Sauvat et al. 2002; Saylors et al. 1994; Yamamoto et al. 1998). Recently, trials of expectant observation have been reported for newborns with adrenal masses, and to date, all tumors decreased in size or resolved spontaneously (Holgersen et al. 1996; Nishihira et al. 2000; Yamamoto et al. 1998). These observations suggest that newborns with small or cystic localized neuroblastomas can be safely observed with low-risk of progression to advanced-stage disease. The COG is currently testing this hypothesis in an ongoing clinical trial in which newborns with small adrenal masses clinically consistent with stage-1 neuroblastoma will be treated with close observation; thus, these infants may be spared surgery and the risks associated with adrenal resection. Yamamoto and co-workers have reported spontaneous regression of localized tumor in infants diagnosed with neuroblastoma by screening in Japan, suggesting that infants with neuroblastoma detected beyond the first month may also be safely observed (Sauvat et al. 2002; Yamamoto et al. 1998).

Surgical resection alone remains the current recommended treatment for *MYCN*-amplified stage-1 disease because occasional patients with this rare entity have become long-term event-free survivors with little or no therapy (Cohn et al. 1995). In addition, mild chemotherapy regimens (such as those currently used for intermediate-risk neuroblastoma) are considered unlikely to be effective for preventing evolution into advanced-stage (high-risk) disease, and there is a reluctance to subject a clinically disease-free infant or child to the aggressive, highly toxic multi-modality therapy that is only partially effective against advanced-stage disease. Close clinical monitoring of these patients is warranted.

Table 11.1.1 Localized neuroblastoma with no regional spread (INSS stage 1): selected series. *CT* chemotherapy, *Cy* cyclophosphamide, *Pepti* peptichemio, *RT* radiotherapy, *S* surgery, *Vcr* vincristine

Reference (by chronology)	Period of study	Clinical stage	Treatment	Survival
Evans et al. (1976)	1970–1974 Prospective	Evans stage I	S±RT±Cy	27 of 27
Evans et al. (1984)	1975–1978 Prospective	Evans stage I	S alone	25 of 26
Adam and Hochholzer (1981)	1944–1978 Retrospective	Evans stage I	S alone S+RT±CT	18 of 18 18 of 19
Hayes et al. (1983)	1962–1980 Retrospective	Stage I and IIA by St. Jude system	S alone S+RT+Cy/Vcr S+RT	15 of 15 12 of 12 6 of 6
Berthold et al. (1986)	1979–1985 Prospective	Evans stage I	S alone	27 of 27
De Bernardi et al. (1987)	1979–1984 Prospective	Stage I by Italian system	S alone S+Pepti±RT	15 of 17 19 of 19
Nitschke et al. (1988)	1981–1986 Prospective	POG stage A	S alone	98 of 101
Perez et al. (2000)	1989–1995 Prospective	Evans stage I	S alone, plus CT and/or RT in 10% of patients	140 of 141
Alvarado et al. (2000)	1990–1997 Prospective	POG stage A	S alone (CT in 6 patients)	313 of 323

11.1.5.2 Regionally Invasive Unilateral Localized Tumors

Through the early 1990s, chemotherapy and/or radiotherapy were routinely used in patients whose neuroblastomas involved regional lymph nodes apart from the main mass and/or in whom gross total resection of tumor was not achieved (stages 2 or 3). A number of considerations led to a reassessment of that approach and to the emergence of a minimal therapy approach:

1. An analysis of published reports suggested that cytotoxic therapies were having little impact on cure rates of unselected patients with neuroblastoma: most patients ultimately died even after myeloablative regimens, while the remainder did well, often with little or no cytotoxic therapy.
2. A limited potential for malignant progression of non-stage-4 neuroblastoma was indicated in early studies by the survival with minimal therapy of many stage 4S patients (Evans et al. 1981; Nickerson et al. 1985; Stephenson et al. 1986) and of patients left with microscopic residual disease after surgery (stage 1) (Adam and Hochholzer 1981; Castleberry et al. 1979; Evans et al. 1984; Hayes et al. 1983; Nitschke et al. 1988).
3. Conflicting reports on the prognostic value of regional lymph node invasion by neuroblastoma (Hayes et al. 1983; Le Tourneau et al. 1985; Ninane et al. 1982; O'Neill et al. 1985; Rosen et al. 1984). The variable prognosis of extensive but localized neuroblastomas (Berthold et al. 1986; de Bernardi et al. 1987; Evans et al. 1984; Hayes et al. 1983; O'Neill et al. 1985; Rosen et al. 1984; Zucker 1974) could be accounted for by the inclusion of patients whose tumors had unfavorable biology (Chaps. 7

and 11) and of patients whose stage-4 disease was missed because of suboptimal staging studies.

4. There was increasing concern over the late effects of cytotoxic therapy in patients with long projected survival (Chap. 18). It was becoming clear that patients with non-stage-4 neuroblastoma who developed recurrent disease could be saved (Adam and Hochholzer 1981; Carachi et al. 1983; Castleberry et al. 1979; McGuire et al. 1985; Nitschke et al. 1983).

   Taken together, the above observations and uncertainties undermined the logic of treating stage 2 differently from stage 1 or stage 4S.

Approximately 15 years ago, a retrospective CCG study demonstrated excellent outcome for patients with Evans stage II (which included a substantial number of patients with INSS stage 2) disease without systemic therapy (Matthay et al. 1989). In that series, 75 of 156 patients received no post-operative therapy while 66 received local radiotherapy and no systemic therapy. Long-term survival was excellent independent of the extent of residual disease and whether the patient received radiation therapy. Single-institution studies were also showing excellent outcome without the routine use of cytotoxic therapy (Castleberry et al. 1979; Evans et al. 1996; Kushner et al. 1996a), with one group questioning the efficacy of adjunctive therapy following partial or complete surgical excision of the primary lesion in [Evans] stage-I or stage-II neuroblastoma (Castleberry et al. 1979). Chemotherapy, however, has a role in the initial treatment of patients with stage-2 tumors who present with spinal cord compromise from a paraspinal mass or airway compromise from a tumor in the superior mediastinum. Once such patients are clinically stabilized, which usually occurs with a few cycles of chemotherapy, successful surgical resection can often be accomplished after which no further cytotoxic therapy need be administered.

Biologic findings reinforce arguments against the use of cytotoxic therapy in localized disease. The striking differences in chromosomal features of lethal vs low-risk forms of neuroblastoma constitute a biologic basis for the radical dichotomy in prognosis (see Chaps. 4 and 5). Furthermore, progression of non-stage-4 tumors with low-risk biologic features (triploidy, unamplified *MYCN*) to lethal stage-4 disease is a rare event. Neuroblastoma screening studies provide independent evidence that supported the concept that non-stage-4 neuroblastoma without *MYCN* amplification rarely, if ever, evolves into lethal disease (see Chap. 2).

Local–regional neuroblastoma (stages 1 and 2) is diagnosed in very small numbers of adolescents/adults. Unfortunately, the outlook appears to be much worse in this older cohort of patients compared with younger children with stage-1 and stage-2 disease (Franks et al. 1997; Gaspar et al. 2003); thus, this small subset of patients warrants close clinical monitoring. It is not known why, given identical biologic markers, infants with bone marrow involvement and large tumors in soft tissues (stage 4S) are readily curable with little or no cytotoxic therapy (see below), while adolescents/adults, including those with localized disease, are rarely cured (Franks et al. 1997; Gaspar et al. 2003).

11.1.5.3 Stage 4S

The clinical entity known as stage 4S is unique in its unusual pattern of involvement (including bulky distant tumors) combined with a waxing and waning clinical course regardless of whether surgery, chemotherapy, and/or radiotherapy are used (Coldman et al. 1980; Evans et al. 1981; Guglielmi et al. 1996; Katzenstein et al. 1998; Nickerson et al. 1985, 2000; Schleiermacher et al. 2003; Stephenson et al. 1986). An unequivocal distinction from stage 4 is not always possible given subjective factors relating to the stage of the primary tumor (as discussed above) and given the uncertain significance for involvement of distant lymph nodes and of atypical soft tissue sites of disease (e.g., pleura) (Coldman et al. 1980). The presence of atypical sites is accepted as indicating stage 4 by some investigators but is considered compatible with stage 4S by others (Hero et al. 2000). It is possible that some cases of infant stage 4 without metastatic involvement of cortical bone and without *MYCN* amplification might exhibit the benign natural history of stage 4S, were they not treated with chemotherapy.

Biologically favorable (low-risk) stage-4S disease resolves spontaneously in the preponderance of cases, and surgical resection of primary tumors at diagnosis is no longer recommended since these are likely to regress (Guglielmi et al. 1996); however, some stage-4S tumors with the low-risk prognostic markers of non-amplified *MYCN*, hyperdiploidy, and favorable histopathology, can cause life-threatening cardiopulmonary compromise and coagulopathies due to extensive liver involvement. This dire situation, which is largely confined to the neonatal period and represents a medical emergency, may abate following treatment with one to two cycles of low-dose chemotherapy and/or modest doses of radiotherapy (e.g., 150 cGy/fraction, times three fractions, using lateral fields in an attempt to spare the kidneys and spine). Despite persistence of liver lesions, once clinical improvement has occurred, additional cytotoxic therapy is not needed (and may be more risky than beneficial), since the residual disease, even if extensive, is likely to regress.

11.1.6 Future Directions

Greater reliance on biologic findings, including some not currently used in risk-stratification schemas, combined with less emphasis on precise stage, may further the decade-long trend towards reduction of cytotoxic therapy. This may lead to an increased number of patients currently classified as having intermediate-risk disease being managed with surgery or observation alone, rather than with chemotherapy or radiotherapy (see the present chapter). Improvements in prognostication are foreseen for the very small subsets of patients with *MYCN*-amplified stage-1 or stage-2 disease and for adolescents with stage-2 tumor via refinements in biologic characterization of neuroblastomas.

11.1.7 Conclusion

Most patients with low-risk neuroblastoma are cured with surgery alone, while a subset of low-risk infants with small adrenal tumors can be safely observed without surgery or other treatment. The excellent outcome is due, in part, to a high incidence of spontaneous tumor regression observed with this group of tumors. The identification of biologic markers associated with favorable prognosis has facilitated treatment reduction for ever greater numbers of neuroblastoma patients. While gross total resection of a localized neuroblastoma remains the current treatment recommendation for most patients, it is now well recognized that such a procedure is not justified if it entails acute risks such as loss of a major organ (e.g., kidney) or damage to important nerves (e.g., brachial or sacral plexus) as the residual biologically favorable disease will likely remain stable or even regress spontaneously. Ongoing biologic studies will hopefully lead to a refinement in the risk-group schema as additional factors may identify the rare patient, currently classified as low risk, who is destined to fail treatment with surgery alone.

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11.2 Intermediate-Risk Neuroblastoma

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11.2.1 Introduction

Intermediate-risk neuroblastoma is a clinically and biologically heterogeneous entity. According to the current Children's Oncology Group (COG) Neuroblastoma Risk Stratification System (see Chap. 7), this grouping includes infants with INSS stages 3 or 4 tumors that lack *MYCN* amplification, infants with stage-4S disease with normal *MYCN* copy number and either unfavorable histology or diploidy, and children >1 year of age with favorable histology stage-3 tumors that lack *MYCN* amplification (Table 11.2.1). Based on these clinical and biologic criteria, approximately 15 % of all patients diagnosed with neuroblastoma are classified as intermediate risk (Table 11.2.2). Previous clinical trials have shown that >85 % of these patients can be cured with moderate-dose chemotherapy and surgery (Bowman et al. 1997; Garaventa et al. 2002; Matthay et al. 1998; Rubie et al. 2001; Schmidt et al. 2000; Strother et al. 1997) (Table 11.2.2 and Figure 11.2.1); thus, these patients stand apart from those with high-risk disease, who have long-term survival rates of <30 % even with intensive multi-modality therapy (see the present chapter), and from children with low-risk neuroblastoma, who are usually cured with surgery alone (see the present chapter).

11.2.2 Clinical Presentation

Intermediate-risk neuroblastomas usually present with symptoms and signs from mass effects of the primary tumor or of the metastatic deposits; however, unsuspected cases of intermediate-risk neuroblastomas may be detected during routine physical examination, by measuring catecholamine levels in urine (as in neonatal screening programs), or when imaging studies are performed for other reasons, e.g., X-ray for suspected pneumonia or antenatal ultrasonography (Sauvat et al. 2002).

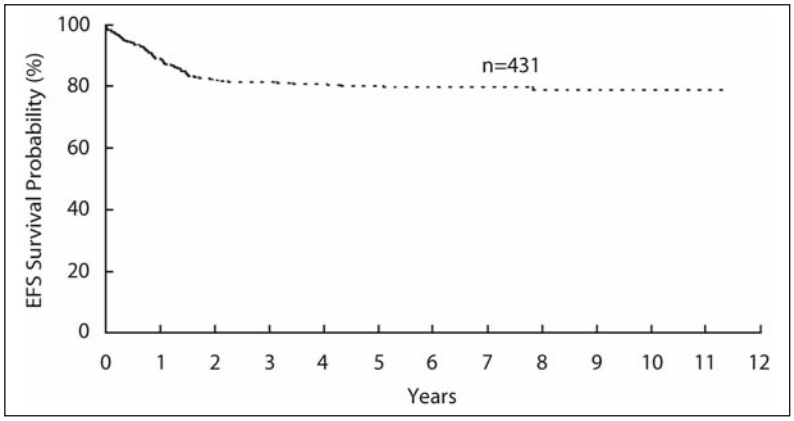


Figure 11.2.1

Kaplan-Meier analysis of survival for 431 intermediate-risk neuroblastoma patients enrolled on the Pediatric Oncology Group Neuroblastoma Biology Study 9047 between 1990 and 1999. (Survival curve provided by W. London, COG Statistics and Data Center. Risk groups were determined by INSS stage, age, *MYCN* status, tumor cell ploidy, and available histology.)

Table 11.2.1 The Children's Oncology Group Intermediate-risk Protocol A3961. Treatment is stratified by biology subgrouping

INSS stage	Age (days)	Biology
Favorable biology		
3	0 to <365	*MYCN* non-amplified, FH,DI>1
3	≥365	*MYCN* non-amplified, FH
4	0 to <365	*MYCN* non-amplified, FH,DI>1
Unfavorable biology		
3	0 to <365	*MYCN* non-amplified, but either DI=1 and/or UH
4	0 to <365	*MYCN* non-amplified, but either DI=1 and/or UH
4S	0 to <365	*MYCN* non-amplified, but either UH and any ploidy or FH and DI=1

Table 11.2.2 Estimated accrual and survival according to risk-group. (Data provided by W. London from the COG Statistics and Data Center)

Risk group	Estimated accrual (%)[a]	No. of patients	5-year EFS±SE (n)[b]	5-year S±SE(n)[b]
Low	40	413	89.9±2.0	95.7±1.3
Intermediate	16	170	87.6±3.1	92.8±2.4
High	44	458	27.6±2.7	33.5±2.7
Overall	100	1041		

[a] Relative proportions are based on data from POG 9047 for whom risk group was known
[b] The EFS and S rates are based on all patients from POG 9047 for whom risk group was known

The primary site in infants and children who have widespread disease is the retroperitoneum in at least 70–80 % of cases, whereas the primary site is extraabdominal (mainly thorax and pelvis) in up to 50 % of children with stage-3, favorable biology tumors (Matthay et al. 1998). Infants with widespread neuroblastoma typically come to medical attention because of abdominal distention from large liver tumors or because of periorbital ecchymoses from metastatic involvement of cranial bones. Subcutaneous tumor nodules are another characteristic, though less common, presenting sign in infants. The distribution of distant sites of disease – bone, bone marrow, liver, skin – differs greatly between infants and older patients, as evidenced in a large Children's Cancer Group (CCG) study of 648 patients (DuBois et al. 1999). In stage-4S patients (n=81), bone marrow, liver, and skin were involved in 34.6, 80.2, and 13.6 % of cases, respectively. With stage-4 disease, osteomedullary involvement was significantly less frequent in infants (n=133) than in older patients (n=434): 48.9 vs 68.2 % for bone, and 57.1 vs 81.3 % for bone marrow. Conversely, liver involvement with stage 4 was significantly more common in infants than in older patients: 53.4 vs 12.9 %. Thus, some infants who are classified as having stage 4 by virtue of large primary tumors may actually have a pattern of distant disease that more closely resembles stage 4S.

11.2.3 Clinical Staging

As with other cancers, clinical staging of neuroblastoma is useful for prognostication and for comparing results of treatments carried out by different groups. Staging is a particularly important factor for classifying cases as intermediate risk. The issue is straightforward for infants with classic stage 4, which is one of the most obvious and dramatic clinical pictures in oncology: a previously well baby becomes irritable and is found to harbor a large abdominal tumor, sizeable metastatic deposits in bones, and numerous syncytial clumps of tumor cells in bone marrow. Other major phenotypes within the intermediate-risk category, however, are dependent on features of the primary tumor. For example, tumor resectability is a key factor in distinguishing stage 3 (intermediate risk) from stage-1 or stage-2 disease (low risk, no chemotherapy). Yet, tumor resectability is hard to determine by imaging studies and can be influenced by subjective factors such as a surgeon's experience and the acceptance by many oncologists and surgeons of the necessity to use chemotherapy to shrink a large tumor.

11.2.4 Biologic Prognostic Markers

The absence of cortical bone and extensive bone marrow metastatic involvement in a young neuroblastoma patient should cause a shift in attention to biologic prognostic markers, including the three currently used to denote intermediate risk (Table 11.2.1). *MYCN* amplification is a particularly reliable predictor of aggressive disease. This chromosomal aberration is, therefore, not present in any subset of intermediate-risk neuroblastoma; however, the presence of three to nine copies of this proto-oncogene can result from whole chromosome gains (i.e., hyperdiploidy). At the present time the clinical significance of the gain of *MYCN* genes by this mechanism remains unclear.

The role of the Shimada histopathology system in defining intermediate-risk cases is limited to stage 4S and to stage 3 in patients more than 1 year old; thus, unfavorable histopathology places non-*MYCN*-amplified stage-4S disease in the intermediate-risk, rather than the low-risk, category, while favorable histopathology places children with non-*MYCN*-amplified stage-3 tumors in the intermediate-risk, rather than the high-risk, category. The DNA index is relevant to the intermediate-risk category for non-*MYCN*-amplified stage-4S tumors, with diploidy separating intermediate-risk from low-risk disease. Within the intermediate-risk category, histopathology and DNA index distinguish the favorable vs the unfavorable biology subsets, with the former treated with fewer cycles of chemotherapy than the latter in the current COG study.

11.2.5 Treatment

A major aim in the management of intermediate-risk neuroblastoma is to reduce acute and late toxicity risks while maintaining the current high rate of cure. It is noteworthy that while metastatic involvement of

bones or bone marrow denotes a dismal prognosis for most children older than 1 year of age with neuroblastoma, infants with a similar clinical picture of neuroblastoma have an excellent outlook with modest doses of chemotherapy. Also worthy of note is the excellent prognosis when local–regional neuroblastoma cannot be entirely excised by surgery or completely sterilized by radiotherapy, i. e., long-term survival ensues despite the presence of residual disease. Neuroblastoma is one of the rare, well-defined oncologic entities that regresses or remains quiescent without further treatment when disease is left behind after surgery, or when the disease process involves multiple sites (stage 4S); yet, current group-wide studies call for additional chemotherapy and/or radiotherapy for residual disease, an approach that might entail more risk (toxicity) than benefit (antitumor effect).

11.2.5.1 Treatment for Stage-3 Neuroblastoma

Through the early 1990s, the uniformly excellent survival rate of stage-1 and stage-2 patients (95–100 %) stood in marked contrast to the variable survival rates (50–75 %) reported for patients with extensive but localized neuroblastomas [Evans stage III, Pediatric Oncology Group (POG) stage C] (Castel et al. 1995; Castleberry et al. 1991; Garaventa et al. 2002; Haase et al. 1989; Tsuchida et al. 1992; West et al. 1993). These patients were, therefore, considered to be at intermediate risk for poor outcome. At least two major advances allowed more accurate prognostication for this group of patients: firstly, clinical staging using both more sensitive bone marrow studies and improved imaging modalities eliminated unsuspected cases of high-risk (stage-4) disease, and, secondly, biologic evaluation of the tumor cells using the aforementioned chromosomal and histopathologic findings provided insights into the natural history of a given case.

Infants with stage-3 neuroblastoma lacking *MYCN* amplification have survival rates approaching 100 %. In multi-institution studies in North America and Europe, these patients have been treated with various combinations of platinum compounds, etoposide, cyclophosphamide, doxorubicin, and/or vincristine in modest dosages (Bowman et al. 1997; Garaventa et al. 2002; Rubie et al. 1998, 2001). In single-institution studies, these patients have often been initially managed by surgery alone, with no cytotoxic therapy (Cheung et al. 1997). The overall results favor efforts to reduce or even to eliminate cytotoxic therapy entirely in infants with non-*MYCN*-amplified stage 3. In the current COG study, infants with stage 3 continue to receive chemotherapy, but only four cycles if disease is hyperdiploid vs eight cycles if disease is diploid, and carboplatin is used, rather than cisplatin, in an attempt to reduce toxicity.

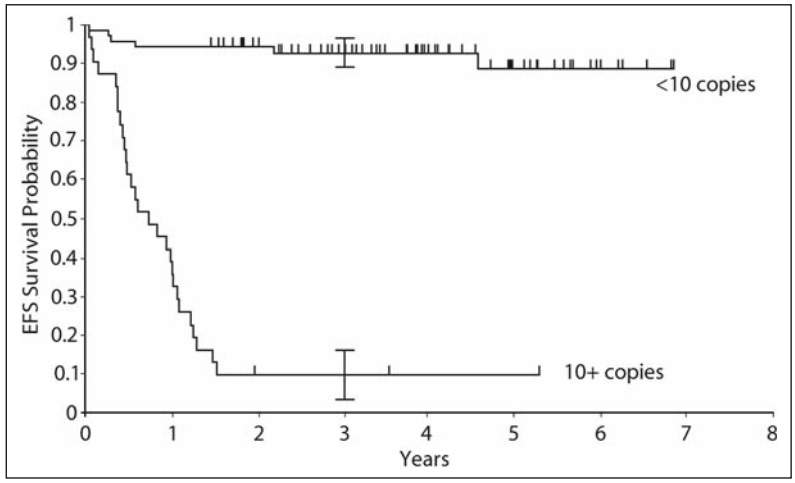
Older patients with intermediate-risk stage-3 neuroblastoma, i. e., no *MYCN* amplification and favorable histopathology, have event-free survival rates exceeding 90 %, similar to infants (Garaventa et al. 2002; Matthay et al. 1998; Rubie et al. 1998; Strother et al. 1997). The French Society of Pediatric Oncology achieved this result using alternating cycles of carboplatin/etoposide and cyclophosphamide/doxorubicin/vincristine (maximum of three cycles of each combination), in moderate doses (Rubie et al. 1998). In a large CCG study, treatment included 9 months of combined usage of cisplatin, etoposide, cyclophosphamide, and doxorubicin (Matthay et al. 1998). In one large POG study, cycles of high-dose cisplatin/etoposide alternated with low-dose cyclophosphamide/doxorubicin, and in a successor POG study, patients received cycles of cyclophosphamide, etoposide, vincristine, plus either cisplatin or carboplatin (Strother et al. 1997). In the current COG study, these patients receive only four cycles of chemotherapy.

11.2.5.2 Treatment for Stage-4S Neuroblastoma

Stage 4S neuroblastoma is a well-defined clinical entity that often resolves with minimal or no cytotoxic therapy, but can be lethal from complications of enlarging liver tumors or from progression to classic stage 4 with bone and extensive bone marrow metastases (Berthold and Hero 2000; de Bernardi et al. 1992; DuBois et al. 1999; Evans et al. 1981; Guglielmi et al. 1996; Hachitanda and Hata 1996; Hero et al. 2000; Martinez et al. 1992; Nickerson et al. 2000; Strother et al. 1995; Schleiermacher et al. 2003; van

Figure 11.2.2

Kaplan-Meier event-free survival (EFS) for infants with stage-4 neuroblastoma according to *MYCN* amplification status treated on the CCG 3881. (From Schmidt et al. 2000)



Noesel et al. 1997). This highly variable natural history has complicated management decisions, as has the risk of major sequelae from cytotoxic therapies in these very young patients. In published reports, approximately 50 % or more of infants with stage 4S received cytotoxic therapy.

Clinical features of possible prognostic import include age <2–3 months (unfavorable) and skin nodules (favorable). With stage 4S, *MYCN* amplification is an independent marker of poor outcome and diploidy is considered an adverse risk factor (Bowman et al. 1997; Hachitanda and Hata 1996; Katzenstein et al. 1998; van Noesel et al. 1997); however, the significance of diploidy in the absence of *MYCN* amplification has not been systematically studied in this subset of infants, and the same holds for unfavorable histopathology. Biologic markers predictive of uncontrollable hepatic enlargement have not, to date, been found. COG uses the presence of diploidy and unfavorable histopathology to confer intermediate-risk status on stage 4S with the implication of a need for treatment with chemotherapy (up to eight cycles in the current COG study); however, this approach is not universally accepted. For example, the German Society of Pediatric Oncology and Hematology recommends observation alone in the absence of *MYCN* amplification or clinical deterioration (Berthold and Hero 2000; Hero et al. 2000). For symptomatic hepatomegaly, low-dose chemotherapy and/or radiotherapy (e.g., 150 cGy/fraction, times three fractions) have been used with variable success (see the present chapter; Schleiermacher et al. 2003).

11.2.5.3 Treatment of Infant Stage-4 Neuroblastoma

Cure rates of infants with stage-4 neuroblastoma have increased from 10 to 50 to >70 % (Bowman et al. 1991, 1997; de Bernardi et al. 1992; Paul et al. 1991). When the small number of infants with *MYCN*-amplified disease are excluded, the cure rates are even higher: nearly 100 % in the recent CCG study (Fig. 11.2.2) (Schmidt et al. 2000). This change suggests that these patients comprise the one neuroblastoma subgroup for whom chemotherapy has clearly had a major impact on prognosis. The improved outlook holds even if patients who in retrospect may actually have had stage-4S disease are excluded from the analysis. This improvement has been noted with several different chemotherapy regimens and stands in marked contrast to the failure of intensive and myeloablative therapies to have a significant impact on the long-term prognosis of older patients with stage-4 neuroblastoma involving bone and bone marrow. In the current COG study, infants with non-*MYCN*-amplified stage 4 are treated with modest-

dose chemotherapy, four cycles if tumor is hyperdiploid and has favorable histopathology, and eight cycles if tumor is diploid and/or has unfavorable histopathology.

11.2.6 Future Directions

Genetic abnormalities and molecular markers not utilized in the current COG risk-group classification schema have been shown to have prognostic value (Maris and Matthay 1999). Prospective studies investigating the clinical significance of genetic abnormalities, such as deletions of chromosomes 1p, 11q, and 14 q, and gain of chromosome 17q, are ongoing. A number of investigators are also examining whether telomerase activity or the expression of neurotrophin receptors, such as TRK-A, will prove to have prognostic significance, independent of the factors currently used to define risk. In addition, microarray studies are being utilized to evaluate the pattern of gene expression in biologically distinct subsets of neuroblastoma. These studies may result in modifications of the risk-group classification system, and may thereby lead to a further improvement in the current risk-group-based treatment strategies.

Recently, some of the clinical criteria currently used in the risk-group schema have been questioned. For example, although 12 months has heretofore been a critical maker in defining stage 4S and in estimating risk for stage 4, recent data from a CCG study suggest that 12- to 18-month-old toddlers with non-*MYCN*-amplified stage 4 have estimated 6-year survival rates of >85% when they are treated with intensive multi-modality therapy (Schmidt et al. 2003). Similarly, a report from the POG suggests that children 12–18 months of age with hyperdiploid, non-*MYCN*-amplified stage 4, have an estimated 4-year event-free survival rate of >90% following intensive multi-modality therapy (George et al. 2003). These observations suggest that children 12–18 months of age with non-*MYCN*-amplified metastatic neuroblastoma, traditionally considered at high risk for treatment failure, may in fact benefit from a reduction of currently prescribed intensive induction and consolidation therapeutic approaches. Patients >12 months old who are classified as stage 4 by virtue of distant lymph node involvement, and do not have adverse biologic prognostic markers, may also warrant re-classification as intermediate risk rather than high risk. This informally called stage "4-N" group of patients may do as well as favorable biology stage 3 or stage 4S following treatment with chemotherapy and surgery (Rosen et al. 1985).

Other patients assigned to the intermediate-risk category may not require any cytotoxic therapy for cure, and may, therefore, be currently subjected to toxicities of therapy needlessly. For example, some infants with stage 4 by virtue of either a large primary tumor or distant nodal involvement but without bone or extensive bone marrow invasion may have disease that is biologically similar to stage 4S. Also, it may be reasonable to question the validity of using bilateral regional lymph node involvement as a feature defining advanced-stage disease and as a finding that places a patient in the same stage-3 grouping as a patient with a large tumor that infiltrates and encases (not just displaces) midline structures. For example, a midline primary tumor with bilateral inguinal nodal involvement (stage 3) might actually be more appropriately managed like a low-risk localized tumor with regional (unilateral) lymph node involvement; however, prospective clinical trials are needed to test if the high cure rates currently observed in these subsets of patients will be maintained with a reduction in therapy.

11.2.7 Conclusion

Intermediate-risk neuroblastoma constitutes a clinically and biologically heterogeneous subset of tumors, which is highly curable with moderate-dose chemotherapy and surgery; thus, current therapeutic strategies are aimed at reducing treatment in an effort to minimize treatment-related toxicities. While on one hand a subset of patients in this group have survived with less or even no therapy, some "high-risk" patients may be safely "down-staged" and managed under the intermediate-risk category (George et al. 2003; Schmidt et al. 2003). Furthermore, achievement of complete remission, i.e., elimination of all evidence of disease, may not be a necessary goal of treatment in intermediate-risk cases. This possibility

is based on the limited potential for malignant progression of low- and intermediate-risk neuroblastoma as evidenced by the survival with conservative management of many patients with non-*MYCN*-amplified stage 4S and of patients with localized tumors who have gross residual disease following attempted tumor resection (Evans et al. 1996; Matthay et al. 1989; Nickerson et al. 2000). Prospective evaluation of these clinical parameters and further analysis of additional genetic and molecular prognostic variables may enhance our ability to identify children at high vs low risk for disease relapse. Such studies are likely to lead to a further refinement in the current risk-group schema, and hopefully will result in treatment strategies that are optimally tailored for individual children with neuroblastoma.

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11.3 High-Risk Neuroblastoma

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11.3.1 Treatment Approach for High-Risk Disease

The high-risk group in neuroblastoma is comprised primarily of children (>1 year of age at diagnosis) with stage-4 disease and stage 3 with tumor *MYCN* amplification or unfavorable histopathology. Although there is no worldwide consensus, stage 2 plus *MYCN* amplification, or stage-3, stage-4, and stage-4s infants plus *MYCN* gene amplification are currently treated in the COG with high-risk treatment protocols. Recent data suggest that this must be further qualified, as analysis of the most recent high-risk study in the Children's Cancer Group showed that toddlers between 12 and 18 months of age with stage-4 *MYCN*-non-amplified disease have an improved outcome compared with those >18 months (Schmidt et al. 2003). Similarly, studies from the Pediatric Oncology Group demonstrate that hyperdiploidy and non-amplified *MYCN* confers a favorable prognosis in children 12–18 months of age with disseminated neuroblastoma (George et al. 2003). The 4-year survival for stage-4 patients >1 year at diagnosis in the CCG studies from 1978 to 1985 (n=507) near tripled by 1991–1995 (n=675; p<0.001; Matthay 1997), although the projected cure rate remained <25%. The most recent phase-III studies indicate that the event-free survival and overall survival of this group has now increased a little further, with myeloablative therapy becoming standard and with more widespread use of treatment of minimal residual disease (Matthay et al. 1999; Reynolds et al. 2002; Ladenstein et al. 1998; Grupp et al. 2000; Cheung et al. 2001a; Villablanca et al. 1998).

Therapy for high-risk neuroblastoma is currently divided into four phases: intensive induction treatment; primary site local control; high-dose marrow ablative therapy; and management of minimal residual disease. The goal of induction therapy is to achieve maximum reduction of tumor burden, including reduction of bone marrow tumor (in vivo

purging), within a time frame which will minimize the risk of developing resistant tumor clones and clinical progression. Surgical resection of primary tumor, with addition of local radiotherapy either at the time of resection or later, is essential in preventing primary site relapse. Subsequently, very high-dose marrow ablative therapy may be used to try to overcome residual and potentially resistant tumor, followed by hematopoietic cell transplant (HCT). The high relapse rate even after such treatment (Ladenstein et al. 1998; Matthay et al. 1993); has led to the approach of using tumor-targeted therapies following myeloablative treatment, to try to eliminate microscopic resistant clones [minimal residual disease (MRD)] (Matthay 1999; Cheung et al. 1998a; Ozkaynak et al. 1998).

11.3.2 Induction Therapy

The introduction of platinum drugs into the combination chemotherapy and dose-intensive application of several agents into the combination chemotherapy, with better supportive care, may be largely responsible for improving the remission induction rate in recent years. The importance of dose-intensity in neuroblastoma has been contested (Cheung and Heller 1991; Pinkerton et al. 2000). Although overall response rate and median survival may be improved (Cheung and Heller 1991), there is general agreement that the overall cure rate does not appear to be substantially changed. The advent of improved surgical techniques, as well as second-look and delayed-resection strategies, have also improved the overall response status (see 11.4). Induction regimens used in recent large studies have shown overall response rates, including complete and partial remission (CR+PR), ranging from about 60 to 90% at the end of 5–6 months of treatment (Matthay et al. 1999; Castleberry et al. 1994; Coze et al. 1997; Kaneko et al. 1999; Tweddle et al. 2001) (Table 11.3.1). Results from some of the most recently completed studies have not yet been published, including that from the ENSG (1990–1999) using alternating cycles of OPEC and OJEC, vincristine, cyclophosphamide, etoposide, and either cisplatin or carboplatin, or the rapid COJEC, using eight cycles spaced at only 10-day intervals of combinations of vincristine, cisplatin, etoposide, cyclophosphamide, and carboplatin (Tweddle et al. 2001). Results are also pending from the POG study P9341 using five cycles of alternating high-dose pairs of chemotherapy, including cisplatin/etoposide, cyclophosphamide/doxorubicin/vincristine, ifosfamide/etoposide, and carboplatin (Grupp et al. 2000).

Some more recently approved single agents have also been tested in newly diagnosed neuroblastoma, using the "up-front phase-II window" approach. Following two courses of single-agent therapy prior to induction treatment, response rates (CR+PR) for effective agents (>30% response) were easily detectable, including ifosfamide, carboplatin, iproplatin (Castleberry et al. 1994),, and topotecan (Kretschmar et al. 1995). Two agents that were less effective in this setting were epirubicin (Castleberry et al. 1994)and taxol (Kretschmar et al. 1995). Although there was no evidence that such a design adversely affected the subsequent outcome of patients, phase-II windows in the context of more standard induction regimens should always be done with careful early stopping rules. Other novel approaches to enhance induction therapy include non-myelosuppressive agents interspersed with the chemotherapy, such as anti-GD2 antibody, anti-angiogenic agents, or differentiating agents (see Chaps. 14–16).

A small subgroup of "ultra-high-risk" patients are resistant to induction therapy, with up to 10–20% of children either developing progressive disease or having less than partial response to induction therapy. Several tests to detect such patients early have been suggested. A study from the Children's Cancer Group showed that patients with residual bone marrow tumor >0.1% by immunocytology after three cycles of induction therapy was highly prognostic for relapse (Seeger et al. 2000). Another technique has been early response by semi-quantitative scoring of MIBG scans. Evaluation of 75 stage-4 patients showed that a relative reduction in MIBG scan score of 0.5 after two cycles of induction therapy predicted a significantly higher likelihood of response at the end of induction and of EFS after myeloablative therapy (Matthay et al. 2003). Novel approaches need to be developed to treat such patients, either incorpo-

Table 11.3.1 Induction regimens for high-risk neuroblastoma since 1985 (>50 patients). (Modified from Matthay et al. 2000). *NA* not applicable

Group/reference	Year	Regimen	Number	CR+PR (%)
POG 8742 (regimen 1) (Castleberry et al. 1994)	1987–1991	Days 1–5, CDDP 40 mg/m^2 day^{-1} Days 2–4, VP16 100 mg/m^2 day^{-1} This alternates q 21 days with days 1–7, CPM PO 150 mg/m^2 day^{-1} Day 8, DOX 35 mg/m^2	111	77
POG 8742 (regimen 2) (Castleberry et al. 1994)	1987–1991	Day 1, CDDP 90 mg/m^2 Day 2, VP16 100 mg/m^2 Days 3–10, CPM 150 mg/m^2 day^{-1} postoperatively Day 11, DOX 35 mg/m^2 Repeat q 21 days	115	68
SFOP CADO/PE (Coze et al. 1997)	1987–1992	Days 1–5, CPM 300 mg/m^2 day^{-1} Days 1 and 5, VCR1.5 mg/m^2 day^{-1} Day 5, DOX 60 mg/m^2/d Alternates q 21 days for two cycles each with Days 1–5, CDDP 40 mg/m^2 day^{-1} Days 1–5, VP16 100 mg/m^2 day^{-1}	183	64
Study Group of Japan (Kaneko et al. 1999)	1985–1997	Day 1, CPM 1200 mg/m^2, VCR 1.5 mg/m^2 Day 3, THP-ADR 40 mg/m^2 Day 5, CDDP 90 mg/m^2 Repeat q 28 days × six cycles	168	92
CCG-3891 (Matthay et al. 1999)	1991–1996	Day 1, CDDP 60/m^2 Day 3, DOX 30 mg/m^2 Days 3 and 6, VP16 100 mg/m^2 day^{-1} Days 4 and 5, CPM 900 mg/m^2 day^{-1} Repeat q 28 days × five cycles	539	78
POG-9341 (Grupp et al. 2000)	1993–1996	Five cycles at 21-day intervals; A, B, C, D, A A: Days 1–5, CDDP 40 mg/m^2 day^{-1} Days 2–4, VP16 100 mg/m^2/dose q 12 h B: Day 1, 8, 15, VCR 1.5 mg/m^2 Day 1, 2, CPM 1000 mg/m^2 Day 1, DOX 60 mg/m^2 C: Day 1–3, VP16 75 mg/m^2/dose q 12 h Day 1–5, IFOS 2000 mg/m^2/day^{-1} D: Day 1, VP16 175 mg/m^2 Day 1, CaP 500 mg/m^2	150	NA
N-6, N-7 MSKCC (Kushner et al. 2003)	1990–2002	Five to seven cycles at 21-day intervals; A, A, B, A, B, A, B A: Days 1 and 2, CPM 70 mg/kg day^{-1} Days 1–3, VCR 0.067 mg/kg day^{-1} Days 1–3, DOX 25 mg/m^2 day^{-1} B: Days 1–4, CDDP 50 mg/m^2 day^{-1} Days 1–4, VP16 150 mg/m^2 day^{-1}	90	96
ENSG OPEC/OJEC (Tweddle et al. 2001)	1990–1999	Alternating courses of OPEC and OJEC: VCR 1.5 mg/m^2, VP16 200 mg/m^2, CPM 600 mg/m^2 with either CDDP 80 mg/m^2 (OPEC) or CaP 500 mg/m^2 (OJEC) Alternate to total of seven cycles	130	NA

Table 11.3.1 Continued

Group/reference	Year	Regimen	Number	CR+PR (%)
ENSG COJEC	1990–1999	A: start days 0, 40: Day 1, CaP 750 mg/m^2 Days 1 and 2, VP16 175/m^2 day^{-1} Day 1, VCR 1.5 mg/m^2 B: start days 10, 30, 50, 70: Day 1, VCR 1.5 mg/m^2 Day 1, CDDP 80 mg/m^2 C: start days 20, 60 Day 1, VCR 1.5 mg/m^2 Days 1 and 2, VP16 175/m^2 day^{-1} Days 1 and 2, CPM 1050 mg/m^2 day^{-1}	125	NA

rating new chemotherapeutic agents, targeted radiotherapy with ^{131}I-anti-GD2 (Cheung and Miraldi 1988) ^{131}I-MIBG (Mastrangelo et al. 2001), or biologic therapies.

Peripheral blood stem cell harvest can be performed as soon as clearance of circulating tumor cells and reduction of bone marrow tumor has been achieved. The optimal timing with respect to minimal residual disease (MRD) has not yet been determined, but in practice, a harvest after only two to three cycles has been shown to eliminate circulating tumor cells (Seeger et al. 2000) and results in tumor-free peripheral blood stem cells down to a sensitivity of 1 tumor cell per 100,000 in almost all cases (Kreissman et al. 2000). Quantitative studies suggest that tumor content in the blood is 100-fold less than that of marrow, and if marrow is negative for tumor by immunocytology ($<1/10^6$), peripheral blood contamination is generally $<1/10^8$ (Faulkner et al. 2000). While rare tumor cells may continue to be present by sensitive testing methods, such as immunocytology and RT-PCR (Burchill et al. 2001; Cheung et al. 2003), the ability of these rare cells to cause relapse after infusion is unknown. An ongoing randomized Children's Oncology Group study of stem cell purging with measurement of MRD in stem cell products and serial samples of peripheral blood and bone marrow by immunocytology and RT-PCR may help to resolve this question. Harvesting stem cells earlier in induction rather than later permits a better collection of CD34 cells, before stem cells have been depleted by repeated courses of intensive chemotherapy, but risks contamination by tumor cells.

11.3.3 Local Control

Surgical resection of the primary tumor or other bulky soft tissue disease is recommended during or at completion of chemotherapy induction, in order to remove residual viable tumor that presumably harbors resistant cells or tumor inaccessible to chemotherapy due to incomplete vascularization and necrosis. Some data suggest that with intensive induction, the maximum decrease of tumor volume has occurred after completion of three cycles of therapy (Wheatley et al. 1995). Although gross total resection of the tumor in the primary site was previously irrelevant when the majority of patients succumbed to distant relapse, improved survival in the last decade has revived this debate (Shorter et al. 1995; Matsumaura et al. 1988; La Quaglia et al. 1994). Local–regional recurrence in primary site is a component of relapse in a large proportion of children with high-risk neuroblastoma, in rates ranging from 20 to 80% in reports which often include local radiotherapy and myeloablative therapy (Matthay et al. 1993, 1999; Ikeda et al. 1992; Villablanca et al. 1999). Analysis of the Children's Cancer Group experience in the most recently completed study of high-risk disease showed that the timing of the resection, whether at diagnosis

or after induction chemotherapy, did not appear to affect overall EFS or, interestingly, the extent of surgical complications and normal organ resection. There was a slight advantage in EFS among 539 high-risk patients, for those patients with complete gross resection of the primary tumor, although this was not significant (Adkins et al. 2004). In the context of a more dose-intensive induction, plus 2100 cGy radiation to the primary site, and post-induction immunotherapy, gross total resection was highly significant in reducing local relapse and prolonging long-term survival over the span of two decades at Memorial Sloan-Kettering Cancer Center (MSKCC; La Quaglia et al. 1994).

Good local control is likely the result of more complete resection plus effective radiation. Using 2100 cGy hyperfractionated radiation, after intensive chemotherapy and gross resection, local relapse was <15% since 1987 at MSKCC (Wolden et al. 2000). These results were confirmed by a multi-institution pilot study utilizing myeloablative consolidation with radiotherapy (21 Gy) administered to the primary tumor bed after gross total resection (Villablanca et al. 1999). Analyses of the results with and without local radiation from the large cooperative study of high-risk disease in CCG-3891 showed better local control from the combination of local radiation (10 Gy) and BMT with TBI (10 Gy), compared with patients treated with local radiotherapy (10 Gy) and standard dose of chemotherapy, without the additional 10 Gy of the TBI (Haas-Kogan et al. 2003). A dose effect from the higher dose of radiation could not be separated from the better results with myeloablative chemoradiotherapy. Pilot studies have also been reported using higher focal radiation via intra-operative radiotherapy in order to spare normal organs (Haase et al. 1994; Haas-Kogan et al. 2000).

11.3.4 Consolidation Therapy

Beginning in the 1980s, investigators have tested the use of consolidation with high-dose myeloablative therapy supported by hematopoietic cell transplantation for patients achieving some response to induction therapy. The observed linear-log relationship between drug dose and tumor cell cytotoxicity for alkylating agents suggested that if drug dose could be increased without dose-limiting extra-medullary toxicity, that log increments in tumor cell killing could be achieved (Frei et al. 1988; Keshelava et al. 1998). The ability to restore hematopoiesis with autologous hematopoietic cells allowed the use of much higher doses of chemotherapy, while the demonstration that bone marrow tumor cells could be eliminated using immunomagnetic purging (Reynolds et al. 1986; Kemshead et al. 1986) allowed the use of autologous marrow support in neuroblastoma, a tumor commonly metastatic to bone marrow.

Initially, single-arm pilot studies suggested improved outcome, with EFS ranging from 24 to 50% (Matthay et al. 1993; Philip et al. 1987; Seeger and Reynolds 1991; Graham-Pole et al. 1991; Dini et al. 1989, 1991) (Table 11.3.2). Caution must be used in comparing these studies, as patient populations may differ with respect to stage, EFS varies in whether it is calculated from time of diagnosis or from time of transplant, and inclusion in some cases was restricted to patient in complete remission only.

Several cooperative pediatric groups reported statistical non-randomized comparisons of early outcomes for groups of patients treated either with conventional doses of chemotherapy or myeloablative chemotherapy, total body irradiation, and purged autologous bone marrow transplant, with differing conclusions (Stram et al. 1996; Shuster et al. 1991; Philip et al. 1991; Hero et al. 1997). Philip et al. compared the LMCE1 protocol (1983–1988) to the previous Lyon cooperative study, LMCE (1978–1983), and showed a difference in 2-year progression-free survival of 39 vs 12% for patients treated with myeloablative therapy and ABMT vs standard chemotherapy (Philip et al. 1991). A CCG comparison of 167 stage-4 patients showed a similar improvement, with an EFS of 40% for the patients treated with ABMT vs 19% for those continuing for 1 year of chemotherapy (Stram et al. 1996). In contrast, a POG study of 116 patients showed no significant prognostic benefit of switching in remission from a chemotherapy protocol to a transplant protocol (Shuster et al. 1991). A smaller study by the German cooperative group evaluated 39 patients undergoing megatherapy and myeloablative therapy

Table 11.3.2 Event-free survival for high-risk neuroblastoma in first remission using myeloablative therapy and HCT for studies of >20 patients. Unless otherwise stated, EFS measured from time of transplantation. (Modified from Matthay and Yamashiro 2000)

Reference	Regimen	Number	Toxic death	3-year EFS (%)
Hartmann et al. (1987)	BCNU, teniposide, melphalan [total of one (*n*=15) or two (*n*=18) courses]	33		49 (2-year EFS)
Pinkerton (1991)	Melphalan	24	1	40
Pole et al. (1991)	Melphalan, TBI	54	7	32
Stram et al. (1996); Seeger et al. (1991)	Cisplatin, VM-26, Doxorubicin, melphalan, TBI Cisplatin, VP16, melphalan, TBI Carboplatin, VP16, melphalan, TBI	45 54 48	7 5 4	42 50 41
Philip et al. (1991)	Vincristine, melphalan, TBI	62	13	30
Dini et al. (1991)	Vincristine, melphalan, TBI	34	1	29
Kushner et al. (1991)	Cisplatin, BCNU, melphalan (or thiotepa), VP16	25	6	40
Ohnuma et al. (1995)	Etoposide, melphalan or cisplatin, etoposide, THP-Adriamycin, melphalan, with (*n*=6) or without TBI	31	3	50
Kamani et al. (1996)	VM26(or VP16), thiotepa, TBI	27	4	41
Hero et al. (1997)	Melphalan±VP16, vincristine, cisplatin, BCNU	39	7	35
Ladenstein et al. (1998)	European Bone Marrow Registry Data	439	60	24 (5-year EFS)
Kletzel et al. (1998)	Cyclophosphamide, thiotepa	51	1	48
Matthay et al. (1999)	Carboplatin, VP16, melphalan, TBI	129	12	43
Villablanca et al. (1999)	Carboplatin, VP16, melphalan, local radiation	77	4	62
Hartmann et al. (1999)	Busulfan, melphalan	116	7	47
Castel et al. (2001)	Cyclophosphamide, carboplatin	49	4	33

with either allogeneic or autologous bone marrow, all with a melphalan "backbone," compared with 49 patients receiving continued chemotherapy by investigator choice. All were patients who achieved complete or partial remissions. The EFS was significantly better in the transplanted patients compared with the chemotherapy group (p=0.005), although the curves nearly converged by 6 years (Hero et al. 1997).

The first randomized study performed by the European Neuroblastoma Study Group (ENSG) from 1983 to 1985 suggests a progression-free survival advantage for myeloablative therapy (Pinkerton 1991); however, only 50 of 84 patients (59%) were randomized, for a variety of reasons ranging from toxic death to parental or physician preference. Furthermore, overall survival and EFS advantage diminished greatly after the first 2 years.

In 1991 CCG launched a randomized study in the U.S. comparing high-dose chemoradiotherapy with purged ABMT to an intensive non-myeloablative chemotherapy intensification (Matthay et al. 1999). The results showed a significant improvement in 3-year EFS for the patients randomly assigned to ABMT, both by an intent-to-treat analysis and also by treatment received (Fig. 11.3.1). As in the previous CCG non-randomized comparison, the highest-risk patients, those with *MYCN*-amplified tumors or those older than 2 years at diagnosis, had the most significant benefit; however, there was no significant difference in survival. A follow-up analysis 4 years

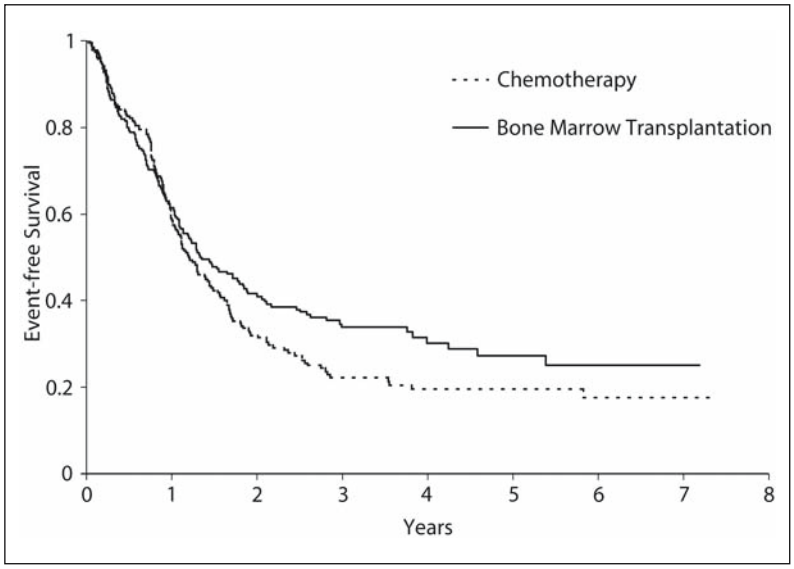

Figure 11.3.1

Results of CCG-3891, a randomized study of myeloablative chemoradiotherapy with purged autologous bone marrow transplantation vs intensive non-myeloablative therapy. A second randomization was performed on all consenting patients completing the consolidation therapy without progression to test the efficacy of 13-*cis*-retinoic acid for minimal residual disease. (From Matthay et al. 1999). **a** Improved EFS with myeloablative therapy compared with standard dose chemotherapy. The difference in EFS for the 379 randomized patients was 34 vs 22 % at 3 years (P=0.034).

later continues to show a significant difference in EFS (Reynolds et al. 2002). Further follow-up will be required to see if high-dose therapy with hematopoietic support has truly made an impact on long-term cure rate in this disease (Matthay et al. 1999).

At present, pilot studies have led to the approach currently used in ongoing studies both in Europe and the U.S. of further increase in chemotherapy dose intensity by eliminating total body irradiation, and instead using higher doses of chemotherapy and local irradiation, with an EFS of 62 % at 3 years (Villablanca et al. 1999). Intensification may also be achieved using a tandem transplant approach, with two or three consecutive myeloablative courses (Grupp et al. 2000; Philip et al. 1993; Frappaz et al. 2000; Kletzel et al. 2002). These studies have demonstrated feasibility with good engraftment, although toxicity prevented proceeding to the subsequent myeloablative course in some cases. Further cooperative randomized studies are necessary to determine the relative risks and benefits of the tandem approach.

Allogeneic hematopoietic cell transplant may obviate the possible risk from tumor cell contamination of autologous peripheral blood stem cells or bone marrow, but presents other problems, including graft-vs-host disease, difficulty finding an HLA match, and much higher toxic death rates in the previously reported studies.

11.3.5 Therapy of Minimal Residual Disease

Early and late relapse continue to occur at a high rate, although the newer, dose-intensive induction and myeloablative therapies have considerably prolonged survival and improved EFS (Matthay et al. 1999). Relapse is most common in bone and bone marrow, and sites of hypoxic and microscopic residual disease (DuBois et al. 1999). For this reason it has become increasingly important to find new approaches to eliminate minimal residual disease with agents that will be tolerable following myeloablative therapy, when disease is likely to be minimal. This provides the ideal window of time to eradicate resistant clones that are still present using novel therapies not dependent upon standard cytotoxic mechanisms (see Chaps. 14–16); these include differentiating agents such as 13-*cis*-retinoic acid (Matthay et al. 1999), fenretinide (Delia et al. 1993; Maurer et al. 1999; Garaventa et al. 2003; Basniewski et al. 1999), anti-GD_2 monoclonal antibodies (Ozkaynac et al. 2000), immunocytokines (Lode et al. 1997), genetically engineered vaccines (Bowman et al. 1998; Davidoff et al. 1999), anti-an-

giogenic therapy (Stern et al. 2001), small molecule inhibitors of tyrosine kinase genes (Evans et al. 1999; Smith et al. 2004), or histone deacetylase inhibitors (Huang et al. 2002).

11.3.6 Conclusion

High-risk neuroblastoma presents a continuing therapeutic challenge. Progress thus far in combination chemotherapy, local control, myeloablative consolidation therapy, and treatment with differentiating agents for microscopic residual disease has improved the overall prognosis. Although the 3-year event-free survival for children with stage-4 disease has improved from <10 to >40% in the past two decades, late relapses continue to be a challenge such that the overall cure rate for stage-4 patients diagnosed at age greater than 18 months with neuroblastoma remained less than 25%.

Dose-intensive induction protocols coupled with gross total resection are expected to achieve near complete remission (CR or VGPR) rates in excess of 70% of patients. In addition, primary site recurrence can now be effectively reduced with surgery combined with ~20 Gy hyperfractionated radiation. Although tumors cannot be detected by histologic examinations or functional nuclear imaging, MRD remains the final hurdle. Myeloablative therapy while prolonging progression-free survival may only have a small effect on the long-term cure rate. Relapses in the CNS plus secondary leukemia can be other serious adverse events. Although their prevalence is <10% among survivors, these late effects of intensive chemotherapy and radiation therapy are expected to surface as patients live longer.

The challenges therefore are multiple. Since treatment induced cancer (e.g., leukemia) is a function of dose (Le Deley et al. 2003), can CR/VGPR rates be maintained by reducing dose without sacrificing dose intensity for subsets of patients? Can new cytotoxic drugs be incorporated into induction therapy for the subset of patients whose tumors are resistant to standard agents? Should myeloablative therapy be applied to only those at higher risk for relapse? New methods to detect MRD suggest that this often persists even when patients are in clinical complete remission, and may predict relapse. Can MRD measurement provide surrogate markers of disease such that different consolidation strategies can be objectively compared, and earlier treatment intervention be initiated? Finally, other approaches to eliminate MRD need to be explored urgently. Novel strategies to overcome drug resistance and to attack sanctuary sites of disease are necessary. Such approaches may include targeted radiotherapy, differentiating agents, immunologic therapies, agents to inhibit important transduction pathways, or drugs to inhibit tumor angiogenesis and invasion.

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11.4 The Role of Surgery in the Treatment of Neuroblastoma

Michael P. La Quaglia

11.4.1 Introduction

The surgeon has a crucial role in the management of neuroblastoma. Initial diagnosis and assessment of *MYCN* amplification, histopathology, DNA index (ploidy), and other parameters are dependent on close collaboration between the surgical oncologist, pediatric oncologist, and pathologist. In particular, the surgeon must obtain an adequate tumor sample for both histopathology and molecular studies. During surgical resection of the primary tumor, efforts should be made to preserve normal organs, such as the kidney. Furthermore, the surgeon must evaluate the status of both ipsilateral and contralateral lymph nodes and accurately describe the extent of primary tumor resection to ensure accurate assignment of stage and risk status. Finally, supportive procedures, including institution of reliable vascular access and management of treatment-related complications like typhlitis, bowel obstructions, and others are important in the surgical management of neuroblastoma patients. This chapter describes the role of surgical intervention in the management of this heterogeneous and challenging tumor.

11.4.2 History

In 1953 Robert E. Gross noted for neuroblastoma in infancy and childhood that "extensive and radical surgery has a definite place under certain circumstances and can lead to permanent cure" (Gross 1953). In 1955 C. Everett Koop described the positive effect of tumor de-bulking on outcome in a book he authored entitled, "Neuroblastoma in Childhood. Survival after Major Surgical Insult to the Tumor" (Koop et al. 1955). These were among the first publications in which a role for surgical resection in neuroblastoma was reported. In 1968 Koop analyzed the impact of surgical interventions depending on whether the tumor was resectable, non-resectable, or

Table 11.4.1 High-risk neuroblastoma: review of the surgical literature

Positive effect of primary site resection		Little or no effect of surgical resection	
Reference	**Number/stages**	**Reference**	**Number/stages**
Koop and Schnaufer (1975)	X/1–4	Kiely (1993)	80/3,4
Rosen et al. (1984)	136/1–4	Losty et al. (1993)	57/1–4
Le Tourneau et al. (1985)	130/1–4	Shorter et al. (1995)	79/1–4
Carlsen et al. (1986)	253/1–4	Kaneko et al. (1997)	14/4
Moss et al. (1987)	21/4	Kaneko et al. (1998)	36/1–4, 4s
Matsumara et al. (1988)	320/4	Castel et al. (2002)	98/4
Haase et al. (1989)	58/3	Von Schweinitz et al. (2002) (surgery effective only in MYCN amplified disease)	878/4
Hata et al. (1990)	76/1–4	Olgun et al. (2003)	1–4, 4 s
Nakadate et al. (1990)	25/4		
Haase et al. (1991)	118/2–4		
Shamberger et al. (1991)	60/3, 4		
Philip et al. (1991)	62/4		
Tsuchida et al. (1992)	121/3, 4		
Berthold et al. (1992)	308/4		
La Quaglia et al. (1992)	70; 4		
Chamberlain et al. (1995)	24/4		
De Cou et al. (1995)	99/4		
Strother et al. (1995)	88/4		
Yokoyama et al. (1995)	8/4		
Mugishima et al. (1995)	36/4		
Powis et al. (1996)	202/3		
Matthay et al. (1998)			
Kaneko et al. (1999)	157/3, 4		
Kawa et al. (1999)	66/3, 4, 4s (all *MYCN* amplified		
Wolden et al. (2000)	47/4		
Castel et al. (2002)	72/3, 4		
Tsuchida et al. (2002)	66/3, 4, 4s		
Kuroda et al. (2003)	33/3, 4		
La Quaglia et al. (2004)			
Adkins et al. (2004)			

metastatic (Koop 1968a), essentially an early attempt to define risk status. During the 1960s workers in both Japan and Europe also described their experience with neuroblastoma resection (Kasai and Watanabe 1968; Menjyo 1968; Schenga 1969; Zittel and Wuttke 1969). With the introduction of adjuvant chemotherapy in 1965 (James et al. 1965), plus major advances in pediatric imaging, surgery, anesthesia, blood banking, and critical care (Harrison et al. 1974; Hollmann and Lampert 1975; Stephen 1977; Tsunooka 1972), and the establishment of the pediatric oncology cooperative groups in the 1970s, multi-institution data became available and the number of surgical reports on neuroblastoma has steadily increased. Retrospective studies from the Children's Cancer Study Group (CCSG) on the role of surgery in disseminated neuroblastoma and localized neuroblastomas were published in 1983 and 1985, respectively (O'Neill et al. 1985; Sitarz et al. 1983). In 1988 investigators from the Pediatric Oncology Group (POG) published a prospective study (Nitschke et al. 1988) showing that certain localized neuroblastomas could be effectively treated with surgery alone despite regional nodal involvement. Furthermore, the authors noted that overall survival was excellent, even in patients who developed relapses; however, comparisons between cooperative group experiences were hampered by lack of a uniform staging system. This was remedied by the establishment of the International Neuroblastoma Staging System (INSS) in 1988 and its revision in 1993 (Brodeur et al. 1988, 1993).

During the 1980s and early 1990s controversy arose as to the efficacy of primary tumor resection in patients with advanced-stage disease. Table 11.4.1 compares surgical reports supporting surgical resection of the primary tumor in high-risk neuroblastoma with those that do not. Despite doubts as to the feasibility, safety, and efficacy of surgical resection in high-risk neuroblastoma, the present consensus in the Children's Oncology Group (COG), and European and Japanese cooperative groups is that an aggressive resection of loco-regional disease should be attempted. Surgery has an even more important role in low- and intermediate-risk disease.

11.4.3 Staging

Staging systems for pediatric solid tumors have traditionally placed great weight on surgical removal of the primary tumor and regional nodal involvement (Evans et al. 1976a) The present INSS was devised as a synthesis of the previous systems (Kiely 1993; Evans et al. 1990). This staging system has been adopted by the Children's Oncology Group USA (COG), as well as cooperative groups in Europe and Japan. It is worthwhile to review each staging system to illustrate the impact of surgery on stage and consequently risk status and treatment.

11.4.3.1 Stage 1

The classification of a stage-1 tumor is dependent not only on primary tumor resection, but also on microscopic evaluation of regional nodes. It is imperative that the surgeon seek and biopsy lymph nodes in the main draining lymphatic echelons at the time of primary tumor removal. For adrenal primaries the ipsilateral peri-caval nodes on the right, or peri-aortic nodes on the left, should be sampled. The surgeon should also evaluate interaortocaval lymph nodes located in the space between the abdominal aorta and inferior vena cava, as well as those located either supra-renally or infra-renally, or both. In assessing these nodes, the surgeon should separate the aorta and vena cava and visualize the spine posteriorly. Finally, an assessment of contralateral lymph nodes and those at the base of the mesentery should be performed. For a right-sided adrenal primary, examination and biopsy of the contralateral peri-aortic nodes should be done. Conversely, contralateral peri-caval lymph nodes should be sampled for left-sided primaries. If these nodes cannot be identified, the surgeon must comment on this in the operative note documenting that they were actively sought.

For thoracic primaries, peri-aortic nodes on the left and peri-azygous nodes on the right should be assessed, and any abnormal nodes running along the intercostals vessels should be excised. It is helpful to biopsy normal-appearing nodes in these regions when feasible. In the case of pelvic primaries the lymph nodes running along the iliac vessels should

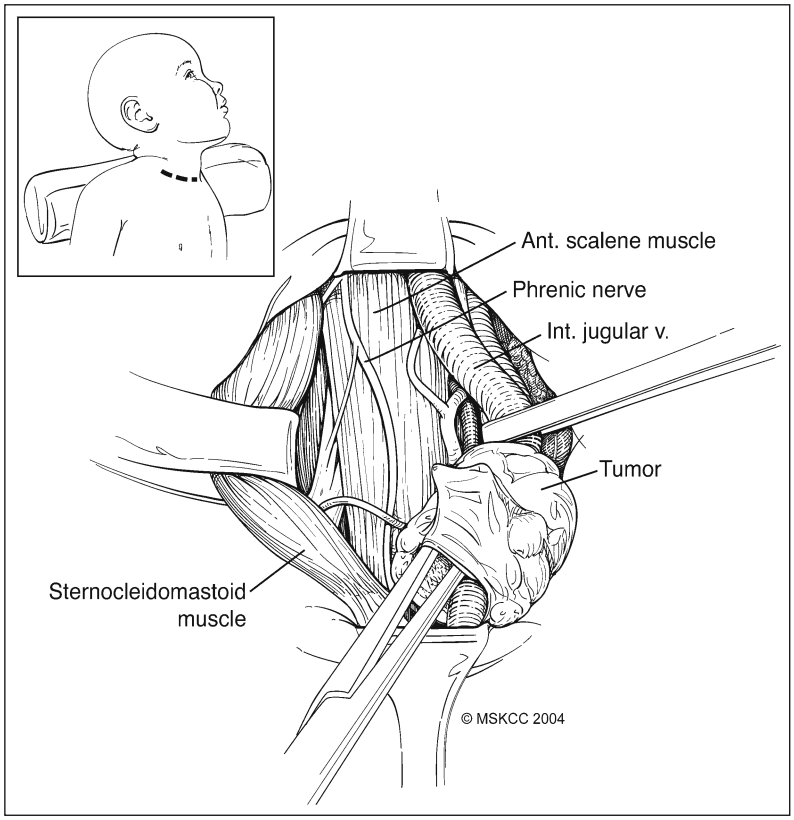

Figure 11.4.1

Resection of a cervical neuroblastoma. A transverse neck incision followed by dissection of the carotid sheath contents was done. Division of the tumor over blood vessels is a characteristic of neuroblastoma surgery

be sampled as well as those in the lower peri-aortic and peri-caval regions. The level II–IV jugulo-digastric lymph nodes are sampled with cervical primaries(Figs. 11.4.1–11.4.3).

11.4.3.2 Stage 2

The criteria for stage 2 focus on the extent of primary tumor resection and on the microscopic assessment of ipsilateral lymph nodes. The contralateral region is explored and identifiable lymph nodes are sampled. If thorough review of pre-operative imaging and intraoperative exploration fails to identify contralateral lymph nodes, then this should be documented in the operative note.

11.4.3.3 Stage 3

Stage-3 disease crosses the midline and is usually associated with encasement of the great vessels. In the abdomen, the aorta and/or vena cava, as well as the celiac axis, superior mesenteric artery, and renal arteries, may be involved. In the mediastinum encasement of the thoracic aorta or azygous vein can occur. Vascular encasement prevents or complicates gross total resection; however, on occasion, tumors that appear to be stage 3 by imaging studies obtained pre-operatively can at times be completely resected (gross total resection), thus down-staging the patient to stage 1, thereby improving prognosis and eliminating the need for further therapy. The ability for tumor surgery to change risk classification, should not be underestimated. Haase et al. (1989) reported an improved survival in Evans stage-III

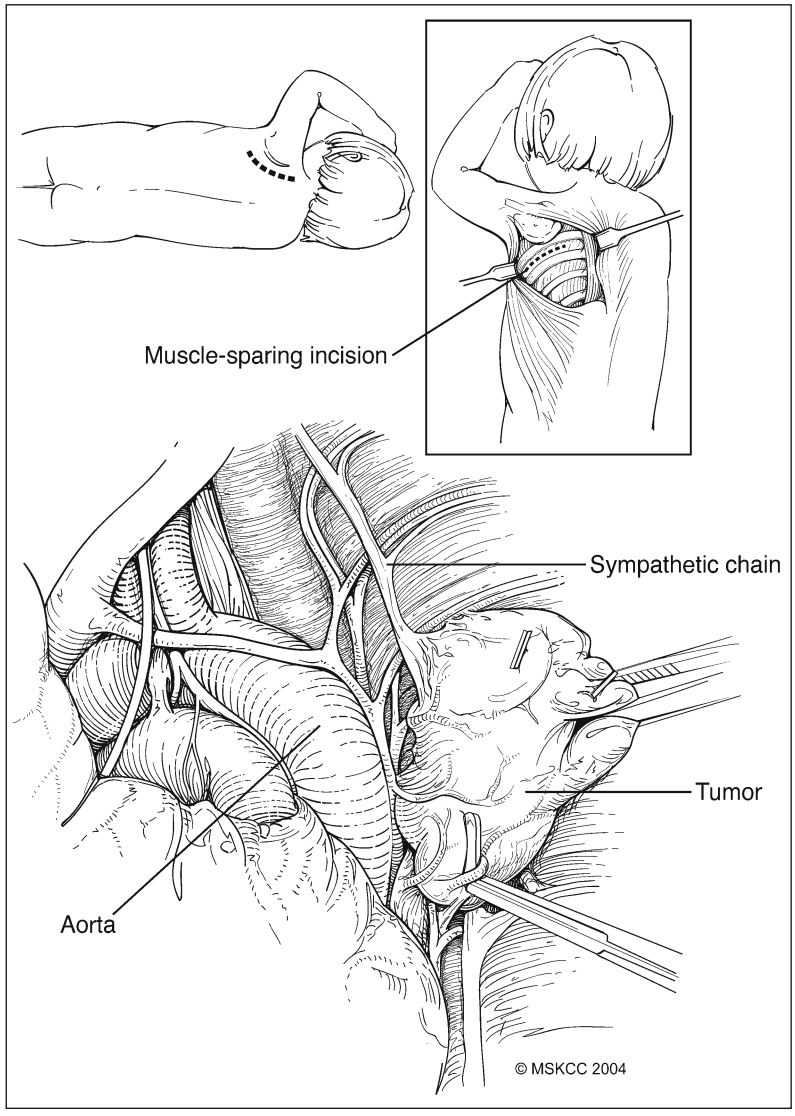

Figure 11.4.2

The approach for posterior mediastinal tumors. A muscle-sparing technique can be used for small lesions. Infiltration through spinal foramina may require foraminotomy.

patients who underwent gross total resection. A similar finding was noted by Matthay et al. (1998).

11.4.3.4 Stage 4

In the past, the role of surgery in stage-4 disease was limited. Presently, besides playing a key role in establishing the diagnosis, the surgical oncologist can ensure the procurement of adequate tissue for assessing relevant biologic parameters, even in cases where the diagnosis can be made solely based on urinary catecholamines plus bone marrow studies. It is recommended that at least 1 cm^3 of viable tumor tissue be obtained at initial biopsy, although this volume requirement is likely to be substantially reduced with future refinements in various molecular techniques. Foremost among biological parameters is determination of the *MYCN* proto-oncogene copy number. Tu-

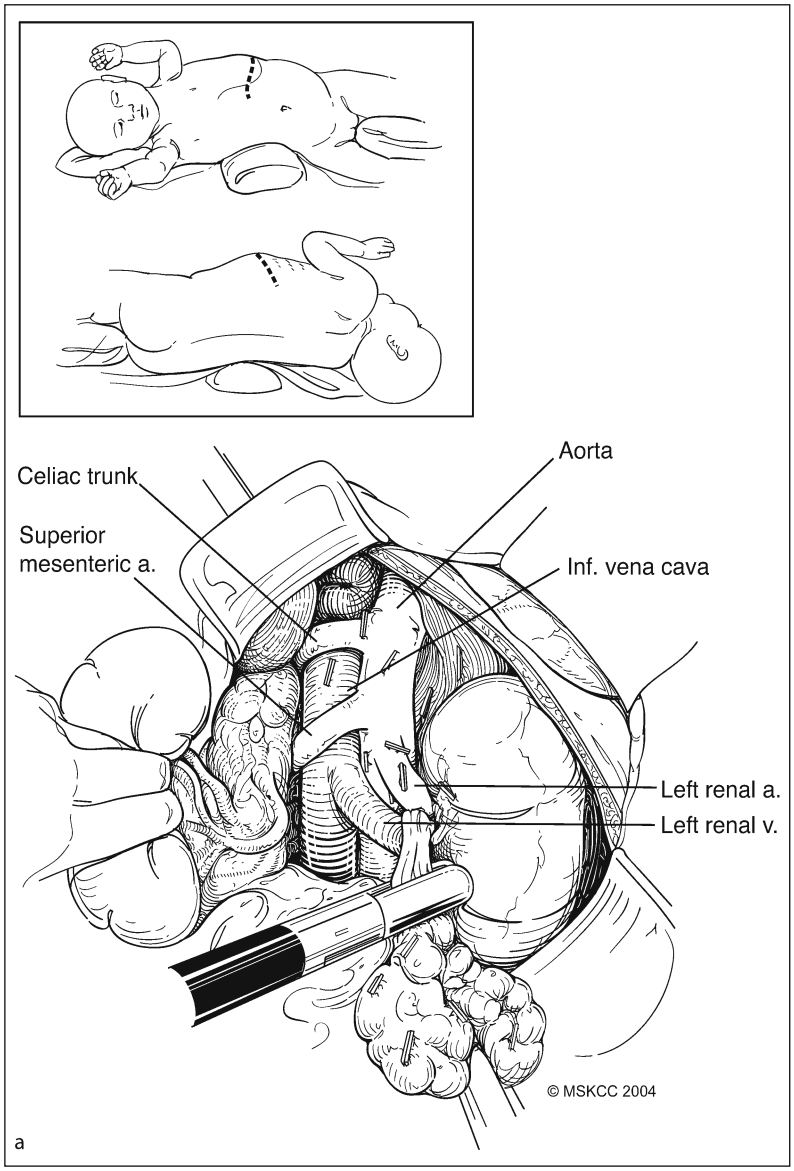

Figure 14.4.3 a

a A thoracoabdominal approach.

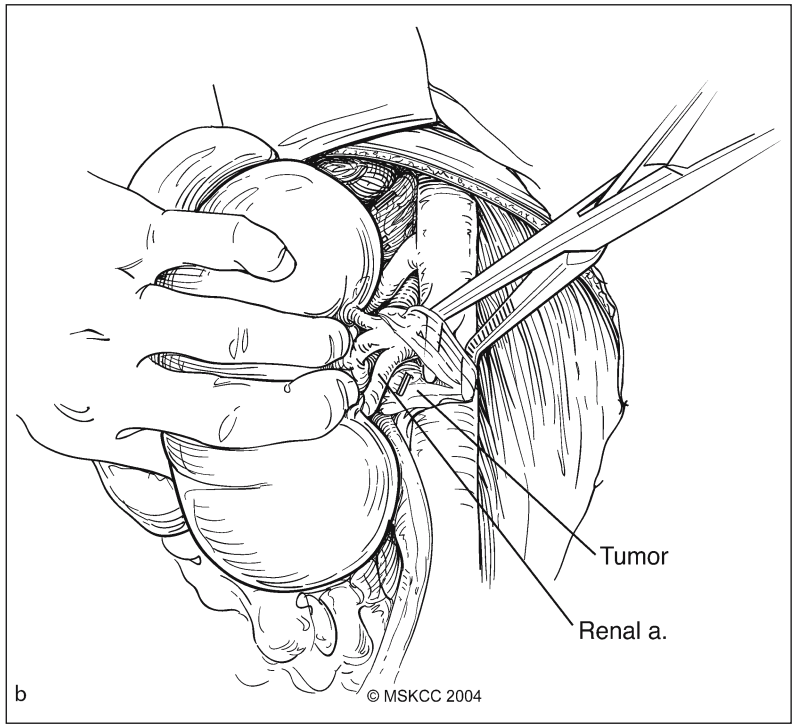

Figure 11.4.3 b

b Transection of a tumor mass that circumferentially encases the renal vessels. This maneuver is often necessary in neuroblastoma resection.

mor tissue is also needed for histologic classification and tumor cell ploidy analysis (Joshi et al. 1992; Joshi et al. 1996; Shimada et al. 1984; Bowman et al. 1997). Unfortunately, outcome for patients with high-risk stage-4 neuroblastoma remains poor, with overall long-term survival rates <30% (Olgun et al. 2003; Frappaz et al. 2002). Progress in this disease will require basic investigations requiring fresh or fresh frozen tissues. The surgeon should make every effort to obtain extra tissue that can be used for these purposes. The role of gross total resection in stage-4 neuroblastoma remains controversial and is dealt with later in the section on high-risk tumors.

11.4.4 Risk Status and Surgical Intervention

Neuroblastoma patients are presently classified as low, intermediate, or high risk by clinical and biological criteria (see Chap. 7). The neuroblastoma committee of the COG recommends complete tumor removal in each risk group when feasible. In the final analysis, a decision to attempt complete tumor resection must be dependent on the consulting surgeon in collaboration with the attending pediatric oncologists and after careful review of the clinical situation as well as imaging studies. It is strongly recommended that these patients be reviewed at a tumor board or treatment planning conference with oncologists, diagnostic radiologists, and surgeons in attendance. Some of these resections may be technically difficult and the surgeon should not hesitate to obtain consultation from experienced colleagues if he or she is unsure as to the appropriate course. Web-based resources including clinical guidelines for neuroblastoma surgery based on the risk-dependent COG protocol are available through both the COG and American Pediatric Surgical Association (APSA) websites. In addition, the surgical principal investigator assigned to a specific COG neuroblastoma therapeutic protocols can be contacted through the COG website. The timing of definitive resection is based on risk status.

11.4.4.1 Low-Risk Patients

The present COG low-risk protocol prescribes surgery alone as treatment for low-risk tumors (see the present chapter). The goal is complete primary tumor removal, accurate staging by biopsy of non-adherent nodes, and adequate tissue sampling for biologic studies. The reported overall survivals in low-risk disease are >90% with almost all patients receiving only surgery (de Bernardi et al. 1995; Kushner et al. 1996b; Evans et al. 1996).

11.4.4.2 Intermediate Risk

The goals of surgery for patients with intermediate-risk disease are to establish the diagnosis, to resect as much of the primary tumor as is safely possible, to accurately stage the disease through sampling of non-adherent lymph nodes and previously unsuspected metastatic sites, and to obtain an adequate amount of tissue for diagnostic studies. In the current COG intermediate-risk clinical trial, patients with unresectable intermediate-risk tumors are treated with chemotherapy (see the present chapter). Using COG guidelines, resection may be performed at diagnosis and/or after the fourth and eight cycles. Cooperative group data is pending, but in single-institution studies surgery alone has been an effective treatment of loco-regional disease with intermediate-risk characteristics (Cheung et al. 1997; Kushner et al. 1996a). Since adjuvant chemotherapy is recommended only for patients with unresectable tumors, it can be avoided if the tumor can be removed.

11.4.4.3 High Risk

The goal of surgery in high-risk tumors is an initial diagnostic biopsy to obtain an adequate amount of tissue for biologic studies (1 cm^3; see the present chapter). Complete resection of the primary tumor is usually done after neoadjuvant chemotherapy and is the current COG recommendation. Following chemotherapy, imaging studies (computerized tomography or magnetic resonance imaging) are obtained prior to surgery and post-operatively to assess the extent of resection. Some studies suggest that the completeness of resection will have a major impact on local recurrence and ultimate survival (La Quaglia et al. 1994, 2004; Tokiwa et al. 2003). These operations are typically difficult even in the best of hands, and require careful presurgical planning and consultation among colleagues with appropriate expertise. In patients with progressive disease, surgery is generally not recommended.

11.4.4.3.1 Gross Total Resection

Neuroblastomas are infiltrative tumors that usually involve the retroperitoneum or mediastinum. Except for small adrenal primaries, none can be resected with a negative microscopic margin as is done with epithelial tumors and some sarcomas; however, in some cases all grossly visible and palpable disease in the primary site and regional lymphatics can be removed leading to the term "gross total resection." The microscopic margin is always assumed to be positive. It is noteworthy that all high-risk patients are also treated with radiation therapy. There are no studies that dissect the individual role of radiation vs surgery in the local control of neuroblastoma; however, radiation therapy alone is generally unable to induce a complete remission in soft tissue sites of gross tumor involvement in advanced-stage neuroblastoma. In general, patients receive both modalities and any analysis should account for this colinearity.

11.4.4.3.2 Rationale for Gross Total Resection in High-Risk Patients

High-risk patients often have extensive primary tumors as well as large metastatic deposits in regional lymph nodes and/or in bone and bone marrow. In view of this, many pediatric surgeons have rightly questioned the efficacy and safety of primary tumor resection and regional lymphadenectomy. Table 11.4.1 lists reports that refer to the role of surgery in neuroblastoma, including patients with high-risk disease. None of these reports are prospective and only a few define resection by criteria other than the operative report. Overall, the majority of studies support a role for gross total resection in the treatment of high-risk neuroblastoma. Most authors have ana-

Table 11.4.2 Local control

Reference	Number	Local progression or recurrence with in complete resection (%)	Probability of primary site relapse with gross total resection (%)
La Quaglia et al. (1994)	70	61 (19 of 31)	6 at 5 years[a]
Wolden et al. (2000)	47	25 (1 of 4)	16 at 5 years[a]
Kushner et al. (2001)	99	43 (3 of 7)	3.3±3.0 at 12 months[a] 5.7±4.4 at 24 months 7.2±5.5 at 36 months
Castel et al. (2002)	98	19 (3 of 16)	15
Haas-Kogan et al. (2003)	539	26±15	20±18[a,b]
La Quaglia et al. (2004)	141	55	5[a]

[a] Primary site radiation given
[b] Autologous bone marrow transplant

lyzed the effect of primary tumor resection on survival. An equally important measure of the efficacy of gross total resection is its effect on local tumor progression.

11.4.4.3.3 Local Control

Most high-risk patients have significant amounts of metastatic disease not amenable to surgical treatment except in unusual circumstances (e.g., epidural disease, isolated bony metastases). A more appropriate measure of the efficacy of gross total resection is its effect on *local* tumor progression. Local control rates of >80% have been reported with a combination of gross total resection and local–regional irradiation to approximately 2000 cGy (La Quaglia et al. 1994; Kushner et al. 2001; Kuroda et al. 2000; Tokiwa et al. 2003). A recent report focusing directly on the issue of local progression confirmed that this could be minimized with gross total resection (La Quaglia et al. 2004). This contrasts with a 30% local recurrence rate in patients undergoing incomplete resection (Hans-Kogan et al. 2003) (Table 11.4.2). In summary, the available data supports use of gross total resection of the primary site and regional lymphatics in high-risk neuroblastoma.

11.4.5 Surgical Complications and Mortality

In high-risk patients, neuroblastomas tend to involve and/or encase major vascular and neural structures in their sites of origin or surrounding nodal echelons. Major surgical complications following neuroblastoma resection are listed by organ system in Table 11.4.3; most serious among them are massive hemorrhage, major vascular injury, and respiratory failure requiring mechanical ventilation after major surgery. Cervical and upper mediastinal resections are often associated with a permanent post-operative Horner's syndrome. Excision of epidural tumors or those heavily involving spinal foramina can result in paralysis (Shimada et al. 1995). Nephrectomy or renal infarction may occur with removal of retroperitoneal neuroblastomas (Shamberger et al. 1998) (Table 11.4.4). An increased frequency of complications, including foot drop, can occur after removal of pelvic tumors despite their overall good prognosis (Cruccetti et al. 2000). Operative death is quite rare despite massive resections. In high-risk neuroblastoma, complications following resection of the primary tumor are reduced by giving neoadjuvant chemotherapy (Shamberger et al. 1991) that reduces tumor volume (La Quaglia 2001; Medary et al. 1996). Typically with dose-intensive induction, surgery for high-risk neuroblastoma can be done after the administration of three to five cycles of chemotherapy.

Table 11.4.3 Surgical complications

System	Complications
Vascular	Arterial or venous laceration: primary repair Arterial laceration: graft Renovascular hypertension Lymphatic ascites
Genitourinary	Nephrectomy Renal infarction (arterial or venous occlusion or thrombosis) Ureteral transection or fibrosis Neurogenic bladder Bladder perforation Urinary tract infection
Gastrointestinal	Intussusception Chronic diarrhea Gastric atony Motility disorders
Nervous	Spinal cord injury with paralysis Horner's syndrome Recurrent nerve injury Brachial or lumbosacral plexus injury Sensory loss

11.4.6 Surgical Technique

It is almost never possible to obtain a clear microscopic margin; thus, dissection generally proceeds along the pseudocapsule of the tumor. Sectioning of the tumor overlying vital structures or its partial removal allows better visualization in order to achieve a gross total resection. The use of titanium surgical clips can improve hemostasis and lymphostasis while marking involved areas for subsequent radiotherapy (Tokiwa et al. 2003; Ikeda et al. 1998; Weiser et al. 2003).

11.4.6.1 Initial Biopsy

Initial tumor biopsy is extremely important in determining biologic aggressiveness, as discussed previously. The surgeon should obtain at least 1 cm^3 of viable tumor tissue, or more if possible. Often the mass is enclosed by a pseudocapsule which can be exploited for hemostasis. The capsule is opened and multiple biopsies are taken. Hemostatic agents can then be used to pack the capsule. Central line placement and staging bone marrow aspirations and biopsies can be conveniently done at the same time.

Table 11.4.4 Complication rates

Reference	Number	Complication rate (%)	Nephrectomies/ renal infarction (*n*)	Operative mortality
Shamberger et al. (1991)	42	19	3	0
La Quaglia et al. (1994)	70	27	4	0
Cruccetti et al. (2000)	17[a]	35[b]		
Von Schweinitz et al. (2002)	2112	19	2.3%	0.9%
Castel et al. (2002)	76	12	5	1.3%
Tokiwa et al. (2003)	47	15	3	0
La Quaglia et al. (2004)	141	8	5	0

[a] All pelvic tumors
[b] This was a series of pelvic tumors and complications included permanent sciatic nerve injury, urinary and fecal incontinence, neuropathic bladder, and leg weakness or L4–S1 nerve root injury

11.4.6.2 Cervical Lesions

Extension of the tumor into the thoracic inlet must be determined prior to surgery as the exposure is much different for these tumors. Most pure cervical lesions have favorable histologic characteristics, a good pseudocapsule, and occur in patients <1 year of age at diagnosis. Very large lesions may require partial or complete division of the sternocleidomastoid muscle. Grossly involved jugulodigastric lymph nodes should be removed in a systematic way using a modified neck-dissection technique. Parents should be forewarned that removal of cervical lesions almost always results in Horner's syndrome.

11.4.6.3 Cervico-Thoracic Lesions

Tumors that are primary to the neck or chest may extend into and through the thoracic inlet. The best surgical exposure for lesions in this area is a cervicothoracic incision. The neck is exposed as outlined in Fig. 11.4.1 and the sternum is then divided either completely, or down to the fourth interspace and then extended laterally. Nerve stimulation is useful when dissecting close to the brachial plexus.

11.4.6.4 Mediastinal Tumors

Figure 11.4.2 illustrates the approach for posterior mediastinal primary tumors that do not involve the thoracic inlet. A muscle-sparing approach is often feasible for tumors that are not large. Access to the ipsilateral upper extremity allows nerve stimulation of the T1 nerve root of the brachial plexus which may dip down into the thoracic cavity. Injury to the sympathetic fibers near the stellate ganglion may result in postoperative Horner's syndrome. The recurrent and phrenic nerves are also at risk.

11.4.6.5 Lesions in the Upper Abdomen and Retroperitoneum

Adrenal primaries often involve regional lymph nodes in the ipsilateral para-aortic or pericaval chains as well as interaortocaval lymph nodes. Indeed, the primary tumor bulk may actually be confluent, enlarged nodal metastases rather than extension from the primary tumor. As a consequence of this retroperitoneal origin with lymphatic infiltration, the great vessels may be partially or completely encased but not invaded by tumor. Adequate vascular control and retroperitoneal exposure is best obtained using an ipsilateral thoraco-abdominal incision except for very small lesions with minimal regional nodal involvement. A midline extension may be necessary for lesions extending into the lower abdomen. On the left side the spleen and tail of the pancreas are rotated medially to expose the supra-celiac aorta. The celiac axis is the first major vessel identified followed by the superior mesenteric artery about 1 cm below. The left lateral surface of the aorta is cleared to the origin of the left renal artery which can then be followed toward the renal hilus. When a vessel is encased, division of tumor tissue over a clamp is necessary. Vascular injury is possible when the aorta and visceral vessels are cleared. Small side vessels can be controlled with finger pressure and the placement of fine monofilament sutures that approximate the adventitia or superficial media. If the aortic wall is weakened or there is a larger injury, the aorta should be clamped or compressed both proximally and distally. Supra-celiac aortic clamping is usually well tolerated for short periods of time and the aortic pressure must be reduced before sutures are placed or the vessel may tear. Monofilament sutures with reinforcing Dacron pledgets should be used and larger tears may require a patch angioplasty. These maneuvers are rarely required but may be lifesaving.

A right-sided thoraco-abdominal exposure is focused on control of the supra-and infra-renal vena cava. The cava is identified just below the liver and dissection proceeds along its right lateral wall. It is usually best to identify the right renal vein and then move superiorly. The Trendelenburg position may reduce the pressure in the vena cava as well as the chance of air embolism.

11.4.6.6 Pelvic Tumors

Pelvic tumors usually have favorable biologic characteristics but are complicated by encasement of iliac vessels or infiltration of the lumbosacral plexus. A

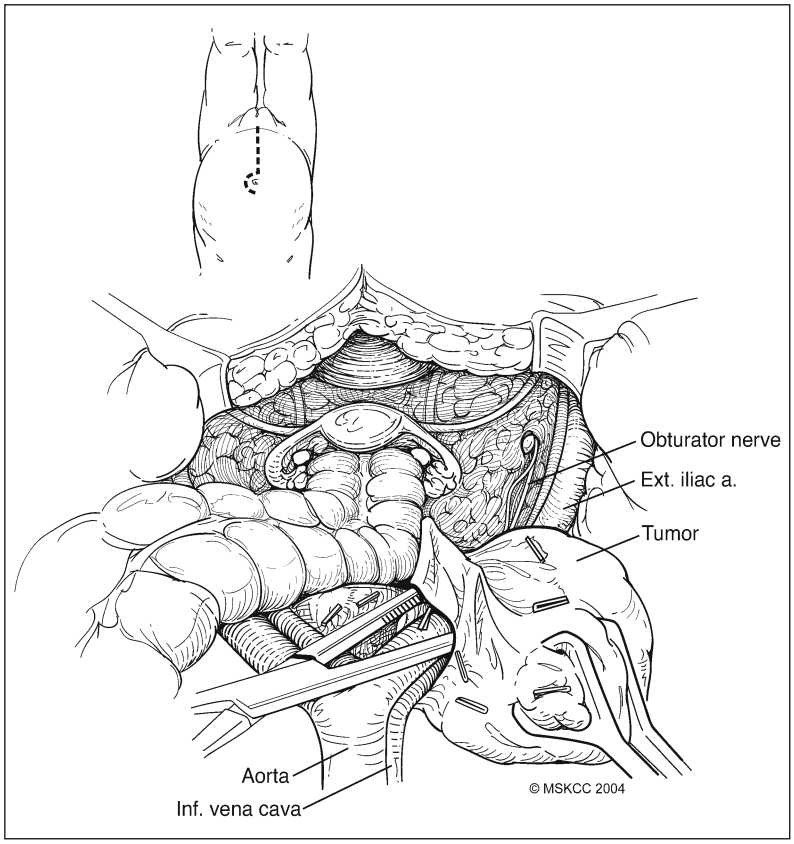

Figure 14.4.4

A lower mid-line incision gives good exposure for resection of pelvic neuroblastomas. Proximal identification of the iliac vessels and ureters is facilitated. Resection of pelvic tumors is associated with a high rate of permanent nerve injury and nerve stimulation should be done with these dissections.

low midline incision down to the pubic symphysis gives good exposure and allows control of the distal aorta and vena cava. Both lower extremities should be prepped into the field and covered with clear plastic so that nerve stimulation may be done. The ipsilateral internal iliac vessels, if encased, may be ligated and resected. Foot drop is a common complication after resection of large pelvic lesions and should be discussed with the family pre-operatively. One study reported a 35% rate of permanent neurologic injuries after pelvic neuroblastoma resection. Also, incomplete resection does not preclude long-term remission (Shamberger et al. 1998).

11.4.7 Conclusion

As part of the worldwide effort to reduce toxicity of neuroblastoma treatment while improving overall survival, the role of surgery continues to evolve. The necessity, timing, and the extent of tumor resection should be critically evaluated. Although much progress has been made, surgical intervention is still required to establish the stage, and therefore risk status, of most neuroblastoma patients. A reduction in therapeutic intensity is now possible in intermediate-risk neuroblastoma as surgery assumes a larger role. For specific neuroblastomas diagnosed in the pre- or neonatal period, elimination of primary tumor surgery is being prospectively evaluated by the COG. Finally, as systemic therapy is being refined, local control and possibly survival in high-risk tumors

have been correlated with gross total resection. The pediatric surgeon's input will continue to be a vital component of the disease management team effort.

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11.5 Radiation Therapy

Suzanne L. Wolden, Daphne A. Haas-Kogan

11.5.1 Background

Precise delineation of the role of radiotherapy in the treatment of neuroblastoma is hampered by the paucity of studies that evaluate radiation in a prospective, randomized fashion. Data from decades-old retrospective studies are currently used to guide radiation practices. Nevertheless, new studies, although preliminary, may provide insights into the optimal use of radiation therapy in a risk-based treatment approach of this disease.

Historically, radiation therapy was administered to the vast majority of neuroblastoma patients, with the exception of stage-I tumors (Evans 1980). The dose of radiation ranged from 10 to 45 Gy according to patient age rather than stage of disease (Evans et al. 1984). During that era in which patients with early-stage disease received radiation, two studies examined the radiation dose of neuroblastoma (Jacobson et al. 1983, 1984). Doses below 20 Gy were deemed sufficient to achieve local control; however, the majority of patients included in both studies would likely not receive radiation therapy today. Nonetheless, the adequacy of <20 Gy dose was widely adopted for all stages of disease; thus, this dose of radiation that has become the gold standard for high-risk disease was derived from patients now known not to require radiation.

11.5.2 Radiation Approach According to Risk Stratification

11.5.2.1 Low- and Intermediate-Risk Disease

The standard of care for irradiation in neuroblastoma began to evolve in the 1980s, when studies indicated that patients with early-stage disease did not benefit from post-operative radiation to the primary site. In the 1970s and 1980s two large randomized trials focused on localized and regional disease, and although neither study examined radiation in a prospective manner, mounting evidence suggested that adjuvant radiation therapy did not influence survival of patients with stage-2 neuroblastoma (Evans et al. 1976, 1984; Matthay et al. 1989).

The CCG recently reported on the outcome of Evans stage 1–2 neuroblastoma treated with surgery as primary therapy. Chemotherapy and radiation were reserved for progressive or recurrent disease or local disease-related symptoms such as spinal cord compression or respiratory distress. The event-free survival (EFS) and overall survival (OS) rates were 93 and 99%, respectively, for stage-1 patients, and 81 and 98%, respectively, for stage-2 patients. Additional therapy (radiation, surgery, and/or chemotherapy) was needed in only 10% of stage-1 patients and 20% of stage-2 patients (Perez et al. 2000).

Based on these results, the current standard of care is to reserve radiation therapy only for those low-risk patients whose disease is not adequately controlled with surgery and chemotherapy. In the current COG protocol for low-risk neuroblastoma (#P9641), a dose of 21 Gy is recommended for stage-1 and stage-2 patients who require radiotherapy.

11.5.2.2 Intermediate-Risk Disease

Intermediate-risk patients are defined as infants with stage-4 disease without *MYCN* amplification, favorable biology stage 3, or INSS 4s with unfavorable histology or DNA index. Three-year survival rates for this group of patients are 75–98% and recent studies have focused on minimizing treatment-related side effects while maintaining high event-free and overall survival rates. The current approach to intermediate-risk patients consists of four to eight cycles of standard doses of chemotherapy and primary tumor resection.

Studies that have addressed the use of radiation for intermediate-risk disease combine INSS 2B and 3 patients and use various outdated staging systems precluding conclusive recommendation regarding when and how to incorporate radiation into the treatment of intermediate-risk patients (Evans et al. 1980; Rosen et al. 1984; McGuire et al. 1985; de Bernardi et al. 1987; West et al. 1993). For patients residing in the more favorable portion of the intermediate-risk

group, radiation therapy is not indicated in their initial management. Matthay et al. reviewed the Children's Cancer Study Group (CCSG) experience of stage-2 disease from 1978 to 1985 and found excellent 5-year progression-free survival (PFS) and OS rates of 90 and 96%, respectively. Germane to the current discussion is the finding that radiation therapy did not influence clinical outcome. Six-year survival was 98% for those treated initially with surgery alone compared with 95% for those receiving radiation and/or chemotherapy (Matthay et al. 1989); thus, intermediate-risk patients with stage-2B disease do not require routine radiation as part of their initial treatment.

The role of radiation therapy is better established for a subgroup of patients with stage-3 disease. Older studies, before the era of biologic staging, reported an advantage to radiotherapy in patients with Evans stage 3 and/or with positive lymph nodes (POG stage C) neuroblastoma. In a small series, Koop and Johnson found that postoperative irradiation improved survival, since 6 of 7 who were irradiated were alive compared with only 1 of 9 patients who did not receive postoperative radiation (Koop and Johnson 1971). A randomized trial addressed the role of radiation in patients with unresectable non-metastatic disease. Stage-C patients older than 1 year were randomized to receive postoperative chemotherapy or chemotherapy plus regional RT (24–30 Gy, 1.6–2.0 fractions). Of those receiving chemotherapy alone, 45% achieved complete remissions and 31% were disease free at a median of 35 months. For patients receiving radiation in addition to chemotherapy, 67% achieved complete remission and 58% remained disease free at a median of 23 months (Castleberry et al. 1991). Conclusions drawn from this study should be applied to current management with great caution since neuroblastoma treatment now utilizes a different staging system, biologic tumor characteristics, and more intensive chemotherapy regimens. Incorporation of biologic factors (hyperdiploidy, favorable histology, absence of *MYCN* amplification) in the management of stage-3 patients suggests that radiation is not essential (Matthay et al. 1998). Survival and local control for patients with amplified *MYCN* are lacking and the role of more aggressive irradiation in such patients is a testable question.

In the most recent COG study for intermediate-risk patients (COG Protocol A3961), including those with INSS 3 with favorable biology and infant with INSS 4, surgery provides diagnostic material at diagnosis and maximal safe resection of the primary tumor after chemotherapy. The duration of chemotherapy, consisting of cyclophosphamide, doxorubicin, carboplatin, and etoposide, is based on the biologic risk factors. Radiation therapy is indicated for patients with clinical deterioration despite chemotherapy and surgery or those with persistent tumor after chemotherapy and second-look surgery.

11.5.2.3 Stage-4S Disease

A unique use of radiation therapy is for infants with stage-4S disease who have respiratory distress or compression of abdominal viscera from massive liver involvement (Paulino et al. 2002). A very low dose of radiation, three fractions of 1.5 Gy each, is extremely effective in reversing these life-threatening problems without a significant risk of long-term complications. While radiation may not be necessary immediately, it is advisable to consult a radiation oncologist as soon as possible because the child's condition can deteriorate rapidly and emergency treatment may be warranted. These infants can be treated without anesthesia or simulation because a papoose provides adequate immobilization and a clinical setup is generally possible for their palpable livers. Complex shielding is not necessary or appropriate because the liver generally fills the entire abdomen and the dose is low.

11.5.2.4 High-Risk Disease

Although reports in the 1980s and early 1990s suggested a benefit to radiation in patients with more advanced disease, the applicability of such conclusions to modern treatment has been confounded by evolving staging systems and risk groups that take into account modern molecular and genetic tumor features, introduction of myeloablative chemotherapy, and other changes in the approach to neuroblastoma. Nonetheless, for patients with advanced-stage disease, studies indicated a potential advantage to

radiation delivered to the primary site (Castleberry et al. 1991; Halperin 1996). Currently, COG recommends that patients with high-risk disease receive radiation to the primary disease site regardless of the extent of surgical resection, as well as to sites of metastatic disease that display persistent ^{131}I-metaiodobenzylguanidine (MIBG) avidity on the pre-stem cell transplant scans.

Several contemporary studies have examined relatively uniform cohorts of patients and have used modern indications for radiation therapy. Such results strongly support the administration of radiation to the primary site in high-risk disease. These single-institution and small consortium studies have reported excellent local control rates after treatment regimens that consist of induction chemotherapy, delayed primary surgery with attempted resection of primary and bulky metastatic lesions, external beam radiation to the primary tumor site, and persistent metastatic areas, with or without myeloablative chemotherapy and infusion of stem cells. Such a series of patients with stage-4 neuroblastoma received 1.5 Gy twice a day to 21 Gy to the pre-chemotherapy, pre-surgery primary tumor volume and regional lymph nodes and had an actuarial locoregional control rate of 84% at 5 years (Wolden et al. 2000).

An update of this single-institution experience reported a 10.1% probability of primary-site failure among 99 patients, most of whom (92 patients) had no evidence of disease in the primary site at the time of irradiation (Kushner et al. 2001). Among seven patients with disease at the primary site at the time of irradiation, three had disease that recurred locally. A similar treatment regimen was used by the German multicenter neuroblastoma trial in which 14 of 26 patients with advanced disease had disease that relapsed, four of which (29%) included the primary sites (Kremens et al. 1994). Similar regimens have resulted in decreased local relapse rates, ranging from 0 to 17% (Ikeda et al. 1992; Sibley et al. 1995; Villablanca et al. 1999).

Such favorable local-control rates notwithstanding, direct comparisons between these results and those of large multi-institutional studies are limited by the considerably higher rate of complete total resections and the various chemotherapy regimens used in single-institution and small consortium studies. A case in point is the largest, modern multi-institutional trial carried out by the Children's Cancer Group (CCG). This randomized study (CCG-3891) showed superior clinical outcomes for patients with high-risk neuroblastoma who were treated with myeloablative chemotherapy and total body irradiation (TBI) with transplantation of purged autologous bone marrow, followed by treatment with 13-*cis*-retinoic acid. External-beam radiation therapy (EBRT) was prescribed for all patients with gross residual disease after induction chemotherapy and surgery. Patients randomly assigned to the transplantation arm received additional TBI as a component of the ablative regimen (Matthay et al. 1999).

In CCG 3891, relapse at the primary disease site was a major component of unsuccessful treatment. Among 539 patients, 349 had recurrences, including 31 with isolated locoregional relapses, 148 with simultaneous local and distant recurrences, and 150 with distant relapses. At 5 years the estimated locoregional recurrence rate was 51±5% among patients who received continuation chemotherapy compared with 33±7% among patients who received transplantation. The difference in local relapses between the continuation chemotherapy and autologous bone marrow transplantation groups was most pronounced in patients with *MYCN*-amplified tumors. Among patients with *MYCN* amplification the estimated 5-year local recurrence rate was 70±10% for those who received continuation chemotherapy compared with 25±15% for patients who received autologous bone marrow transplantation (Haas-Kogan et al. 2003).

The high rate of local recurrence in CCG 3891 prompted an examination of whether addition of EBRT improved local control rates. Although this question could not be answered directly by this study because EBRT was not randomly assigned, several conclusions emerge from the analyses. For patients who received 10 Gy of EBRT to the primary, the addition of 10 Gy of TBI and autologous bone marrow transplantation decreased local recurrence compared with continuation chemotherapy. The benefit for radiotherapy is particularly evident when sys-

temic treatment is optimized with myeloablative therapy and 13-*cis*-retinoic acid. The data further suggest a dose-response relationship with local EBRT, although the optimal dosage to primary tumor sites has not been established (Haas-Kogan et al. 2003). For patients with high-risk neuroblastoma COG recommends EBRT to the primary tumor site in the context of a myeloablative regimen that does not include TBI. Radiotherapy is best administered following myeloablative chemotherapy and resection, when the volume of disease is minimal. This eliminates the potential problem of acute toxicity from radiotherapy interfering with optimal administration of systemic therapy.

According to the current COG A3973 high-risk study, the tumor volume measured prior to surgical resection should be treated with a minimum dose of 21.6 in 1.8 Gy daily fractions. It is anticipated that this dose will be adequate for local control in patients with a complete surgical resection; however, poor local control rates are observed with this dose or radiation in patients with subtotal resection. Prospective studies should, therefore, be developed to test whether this subset of patients may benefit from a higher radiation dose.

11.5.3 Radiation Techniques

11.5.3.1 General Technical Considerations

Over the past decade, dramatic technological advances have revolutionized radiation planning and delivery. Three-dimensional CT scan simulation and treatment planning have allowed physicians to better target areas at risk while sparing healthy tissues. Further advancements, including proton beam, radiosurgery, intraoperative radiation therapy (IORT), and intensity-modulated radiation therapy (IMRT), have given physicians multiple options for highly conformal therapy (Swift 2002). Magnetic resonance imaging (MRI) and posi-

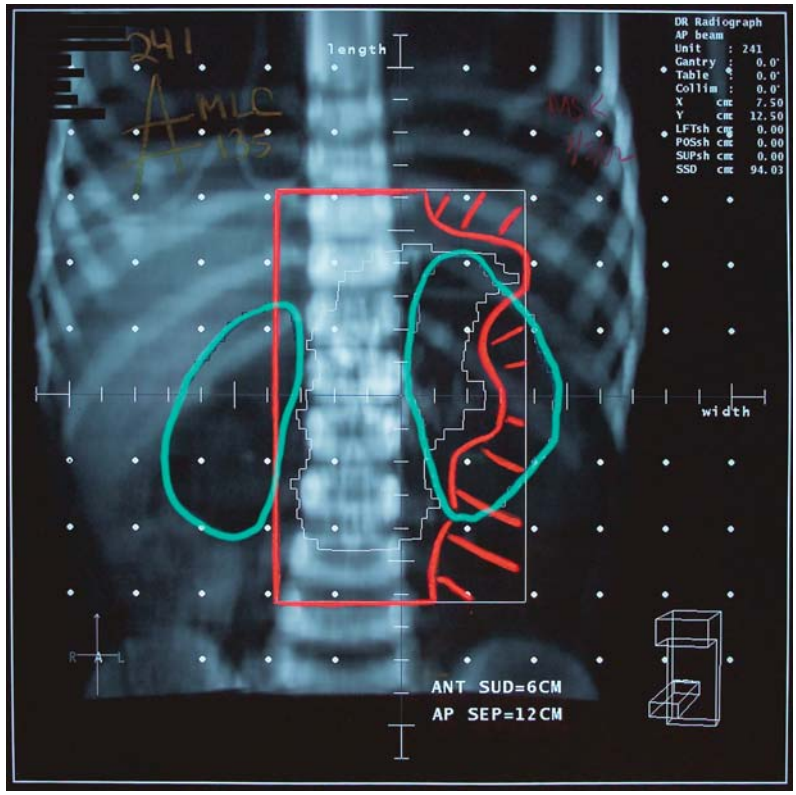

Figure 11.5.1

Example of a radiotherapy portal for an adrenal primary tumor following chemotherapy and gross total resection. The field encompasses the tumor bed and para-aortic lymph nodes. The treatment field is outlined in *red* and the kidneys are in *green*. It is necessary to spare at least one-third of each kidney (preferably more) to maintain renal function. Each patient's field is individualized based on three-dimensional outlines of the target on a planning CT scan.

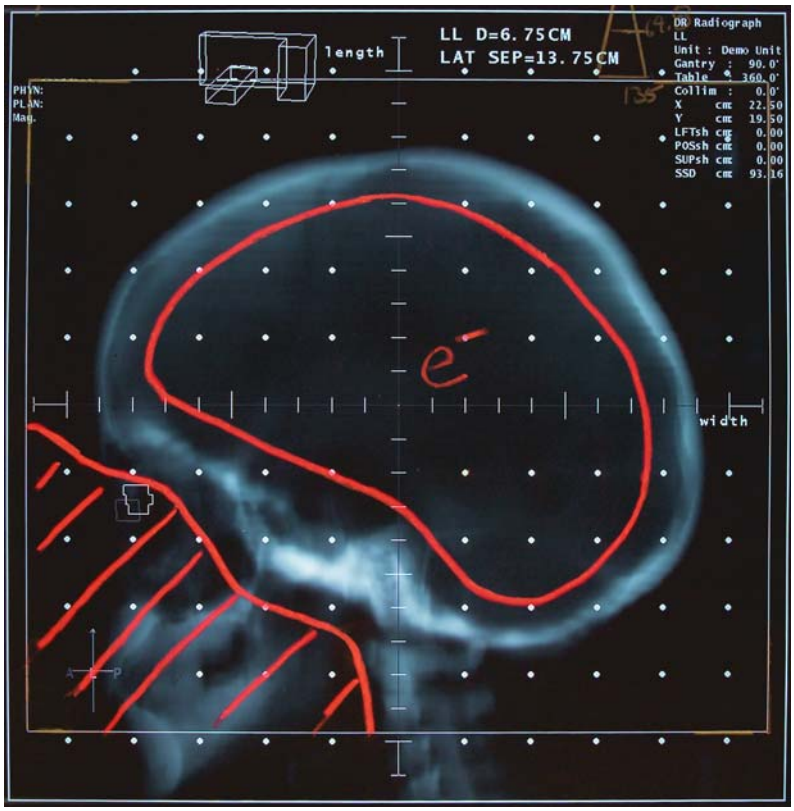

Figure 11.5.2

Combined photon and electron treatment for diffuse skull metastases. The photon field can be seen around the periphery of the skull, including the skull base. This field is deeply penetrating. The electron fields (e^-) are in the middle, treating the lateral skull superficially. This allows radiation to the entire bony calvarium and skull base with relative sparing of the brain.

tron emission tomography (PET) scans can be fused with treatment planning computed tomography (CT) scans to aid in defining disease sites in challenging cases (Hevezi 2003; Krasin et al. 2004).

The radiation technique employed depends upon the site being treated, the planned dose, patient age, and whether there has been prior radiation. Most commonly, patients with high-risk disease receive a relatively low dose of 21 Gy to the primary site, often in the adrenal gland. Based on patterns of failure, it is important in these cases to cover the para-aortic lymph nodes (Wolden et al. 2000). CT planning is imperative to precisely delineate the target region as well as normal tissues including the kidneys and liver. Simple anterior and posterior beams, as demonstrated in Fig. 11.5.1, are often the best solution, but IMRT may be helpful if standard techniques would not provide adequate sparing of critical organs.

Bone metastases are also often best treated with simple opposed beams; however, more sophisticated approaches are needed when treating sites in the head and neck because of the complex anatomy and critical structures. For instance, IMRT may be useful for metastatic disease in the paranasal sinuses (Fig. 11.5.2). For high-risk patients with diffuse metastases throughout the calvarium, orbits, and skull base, we have employed a relative "brain-sparing" radiation technique that allows treatment of bones without full exposure of the brain in young children (Fig. 11.5.3). Photons are used to treat the outer skull, posterior orbits, and skull base. These fields are matched to low-energy electron beams to treat the lateral skull. Electron beams do not penetrate very deeply beyond the bone, and therefore much of the brain is spared (Hall 2000).

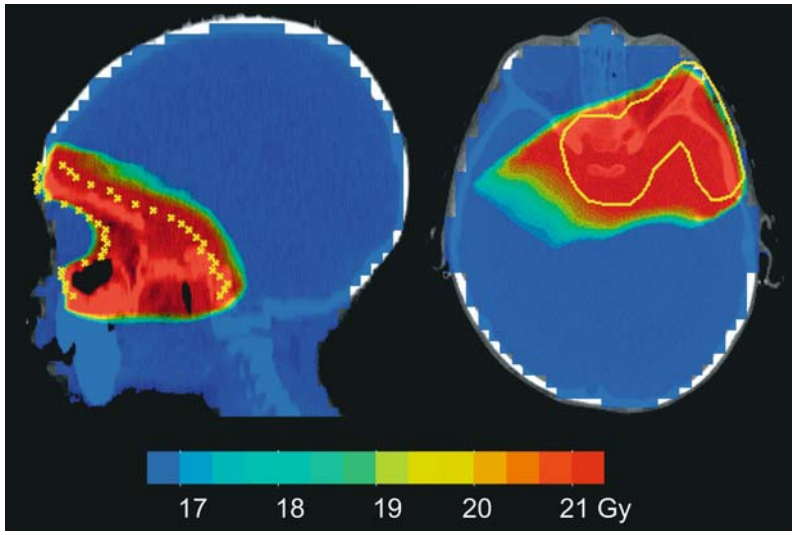

Figure 11.5.3

Intensity-modulated radiation therapy (IMRT plan) for a solitary but extensive skull metastasis. The percentage of the prescription radiation dose is represented by the colored "isodose" lines. This technique maximizes sparing of adjacent critical structures.

Radiation is also an important modality for palliation of patients with progressive neuroblastoma. It is extremely effective for relief of bone pain and neurologic deficits. The appropriate fractionation regimens for palliative therapy depend upon the site of treatment and anticipated survival of the patient. For a high-functioning child, 15 fractions of 2 Gy each may be used while for a patient with end-stage disease, a single fraction of 7 Gy may be considered. IMRT is very useful if a specific site requires a second course of salvage radiation therapy. Parenchymal brain metastases have become an increasing site of isolated failure in high-risk patients (see the present chapter). Investigators at MSKCC have found that even solitary brain metastases are associated with a very high rates of leptomeningeal dissemination, suggesting that prophylactic craniospinal radiation therapy may have clinical utility for patients who develop brain metastases (S.L. Wolden, personal communication).

Neuroblastoma is common in very young children, necessitating the frequent use of anesthesia for radiation treatments. In this case, propofol is safe and well tolerated, even for twice-daily treatments. With proper immobilization devices and input from parents and child-life specialists, some very young children can be coached to receive treatment without anesthesia; however, the precision of our current radiation techniques requires a great deal of cooperation and lack of motion.

11.5.3.2 Intraoperative Radiation Therapy

Radiation for neuroblastoma in most commonly administered to patients with high-risk disease. Multi-institution studies report significant local recurrence rates of 20 % even after myeloablative multimodality treatment and aggressive gross total surgical resections. Furthermore, patients with high-risk disease frequently present with large abdominal primary tumors, abutting or invading many dose-limiting normal tissues. External beam radiation to these tumors often requires treatment of a large volume of normal tissue, including bowel, liver, kidney, bony structures, and spinal cord. Radiation therapy to neuroblastoma occurring at other primary sites, including the thorax and pelvis, similarly exposes normal tissues to the risk of long-term side effects. Long-term toxicities associated with EBRT are particularly severe in children (Meadows 1989; Hawkins 1990; Donaldson 1993) (see Chapter 18). Furthermore, EBRT may decrease renal function, resulting in diminished tolerance to high-dose chemotherapy with stem cell transplant.

Although EBRT plays a key role in the treatment of neuroblastoma, several institutions have explored IORT as an effective radiation modality that may minimize acute and long-term side effects. In contrast to EBRT, IORT allows treatment of high-risk areas at the time of primary resection. Critical structures can be directly visualized and manipulated at the time of surgery, allowing their exclusion from the radiation field provided they are at low risk for microscopic disease. A high radiation dose can thus be delivered to residual tumor and areas at high-risk for microscopic disease, while minimizing the radiation dose to nearby normal tissues. Most reports on the use of IORT focus on adult patients. IORT in these studies is generally used for tumors with a high propensity for local failure, such as colorectal, stomach, and bladder cancer (Abe and Takahashi 1981; Matsumoto et al. 1981; Abe and Shibamoto 1996; Hanks and Lanciano 1996; Kim et al. 1997). Many of these studies have shown improved loco-regional control when compared with standard therapy (Abe and Takahashi 1981; Matsumoto et al. 1981; Abe and Shibamoto 1996; Hanks and Lanciano 1996; Kim et al. 1997). A small number of studies have established the potential for IORT as a treatment modality in pediatric patients, including patients with neuroblastoma (Haase et al. 1994; Aitken et al. 1995; Leavey et al. 1997; Merchant et al. 1998; Nag et al. 1999). IORT in these studies was extremely well tolerated and may have improved local disease control.

A recent update from the University of California, San Francisco reported on a cohort of 28 consecutive patients treated with IORT for newly diagnosed high-risk neuroblastoma. With follow-up ranging from 19 to 200 months (median 45 months), none of the 20 patients who had gross total resections experienced local recurrences. In contrast, three of eight patients who had subtotal resections recurred locally, despite the addition of 20 Gy of EBRT to the primary site post-operatively (DeWitt et al. 2003).

IORT at the time of primary resection achieves excellent local control in patients with high-risk neuroblastoma and is well tolerated. Compared with historical outcome data, IORT achieves comparable control and survival rates while avoiding the use of systematic EBRT. Additional therapy with EBRT may not be warranted in high-risk patients treated with IORT who have undergone successful gross total resection of their primary tumor, although more conclusive evidence requires larger patient numbers and longer follow-up. Higher local failure rates in high-risk neuroblastoma patients after subtotal resection of their primary tumors and multiple positive lymph nodes suggest that additional therapy with EBRT may be warranted.

11.5.4 Side Effects of Radiation

Side effects of radiation therapy depend on the site of treatment and radiation dose. Acute and long-term side effects as well as tumor response may also be enhanced by concurrent use of radiosensitizing chemotherapy. Prior exposure to highly sensitizing chemotherapy may increase radiation toxicity and radiation recall effects are well described with subsequent use of these agents. The most highly sensitizing agents are doxorubicin and dactinomycin; these are generally contraindicated during radiation therapy. Other mild sensitizers include cisplatin, topotecan, and irinotecan, which are usually safe to give during radiotherapy. Little work has been done to attempt to exploit these synergistic relationships in neuroblastoma. Research in this area would be appropriate, especially for patients with unfavorable predictors of local control such as high-risk patients with gross residual disease or *MYCN* amplification.

Abdominal therapy is often associated with nausea and anti-emetics are recommended. Diarrhea and cramping are less common acute effects. When large amounts of bone marrow are treated, blood counts may drop and these should be monitored during therapy.

Long-term sequelae of radiation are important factors to consider when designing treatment for young children and counseling parents (see Chap. 18). The highest risk of growth abnormalities is in very young children (Paulino et al. 2002). Although the anticipated effect on growth for most patients treated with fractionated doses of 21 Gy and less is negligible, when multiple vertebral bodies or growth centers of long bones are treated, a percentage of patients may experience impaired growth

(Roebuck 1999). Radiation doses >25 Gy are expected to cause bone growth arrest, and in young children, may cause significant abnormalities in skeletal development (Roebuck 1999). Growth centers of bones should be shielded whenever possible. It is also important to irradiate vertebral bodies symmetrically in order to prevent radiation-induced scoliosis.

Organ dysfunction may result from radiation exposure if tolerance doses are exceeded (see Chap. 18). Standard whole-organ tolerance doses are as follows: heart 15 Gy; lungs 15 Gy; kidneys 18 Gy; liver 30 Gy; bowel 30 Gy; ovaries 10 Gy; and testes 2 Gy. These doses are only general guidelines. Young children who are heavily pretreated with chemotherapy or who have had surgery may experience organ dysfunction at lower doses. Neurocognitive dysfunction and endocrine abnormalities as a result of brain irradiation are dependent upon the child's age, radiation dose, and volume of brain exposed. These issues are important to consider when treating skull and orbital lesions. Cataracts are common side effects of radiotherapy and thus doses to the lens should be minimized when treating the orbit. Most children will not have permanent alopecia after doses of 21 Gy or less, but a small percentage may have permanent thinning. Doses exceeding 21 Gy do pose the risk of permanent epilation in the radiotherapy portal.

Reliable risk estimates of second malignancies following radiotherapy for neuroblastoma are not available. One may extrapolate from a large body of literature in pediatric Hodgkin's disease survivors and assume that children who are cured after receiving radiation for neuroblastoma will develop an excess number of cancers 10–20 years later (Wolden et al. 1998). Common radiation-related malignancies include breast cancer, sarcomas, lymphomas, and other solid tumors. Data from Hodgkin's disease survivors also indicate that patients receiving thoracic radiotherapy likely have a higher risk of cardiac disease as adults; thus, long-term survivors of neuroblastoma will require lifelong screening for late sequelae of radiation therapy.

11.5.5 Conclusion

The role of radiation therapy in the treatment of neuroblastoma continues to evolve. Past investigations have taught us that the majority of patients with low-risk and intermediate-risk disease do well without radiation therapy. There are several important exceptions where the option of radiotherapy must be considered, such as infants with stage-4S disease requiring rapid reversal of respiratory or gastrointestinal compromise.

Conversely, the majority of patients with high-risk disease do benefit from the addition of radiation therapy to the combined modality treatment paradigm. Cooperative group- and single-institution experiences indicate that this group of patients have excellent local control when the primary site is managed with complete surgical resection followed by approximately 21 Gy; however, this dose of radiation does not appear to be adequate if complete resection is not achieved. Going forward, more aggressive attempts at surgical resection or higher radiation doses must be investigated.

Radiation therapy is an indispensable tool in the management of neuroblastoma metastases, either as part of initial therapy or as palliation. Techniques and fractionation schedules can be tailored to the clinical situation of each individual patient. New technologies that allow highly conformal therapy are often applicable to patients with neuroblastoma; however, survivors must be monitored well into adulthood for potential late effects of therapy.

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11.6 Stem Cell Transplantation

Stephan A. Grupp

11.6.1 Introduction

The role of autologous stem cell transplantation (ASCT) as consolidation therapy for malignancies has been debated, both in the pediatric as well as the adult setting. General design criteria include: (a) a chemo-responsive tumor type, typically with a good initial response to induction therapy, but a poor long-term (i.e., 3- or 5-year) outcome; (b) a conditioning (pre-transplant chemotherapy) regimen that may be dose-escalated safely past marrow tolerance; (c) conditioning agents not utilized in the induction chemotherapy; and (d) optimal supportive care, especially as regards stem cell source and processing techniques. The use of this treatment option, especially in the era of peripheral blood stem cell (PBSC) collection, has special challenges when applied to young patients with neuroblastoma where the median age at diagnosis is 3. Finally, although the vast majority of transplant procedures for patients with NB now utilize autologous PBSC as the stem cell source, allogeneic transplant has been advocated by some investigators, so we briefly explore this issue as well.

The primary source of hematopoietic stem and progenitor cells for use in autologous and allogeneic transplantation has been, until recently, bone marrow. Over the past decade, there has been an increasing use of peripheral blood containing mobilized stem and progenitor cells for transplantation (Table 11.6.1; Kessinger et al. 1986). This product is variously referred to as peripheral blood stem cells, peripheral blood progenitor cells, or given the shorthand designation "stem cells." Although each cell source used for hematopoietic transplantation contains stem cells, when the term "stem cells" is used without a qualifier, it is usually referring to PBSC.

Table 11.6.1 Cellular characteristics of various stem-cell sources. *PBSC* peripheral blood stem cell

Stem-cell source	Bone marrow	PBSC	G-CSF primed bone marrow	Umbilical cord blood
Stem-cell content	++	++	++	+
Progenitor-cell content	++	++++	+++	+
T-cell content	+	++++	+	+/Functionally immature
Risk of tumor cell contamination in autologous transplant	+++	+	+++	Not applicable

11.6.2 Autologous Transplant in Neuroblastoma

Commonly referred to as autologous transplant, the use of the patient's own stem cells to support recovery from high-dose chemotherapy is more properly referred to as high-dose chemotherapy with stem cell rescue (HDC/SCR). The HDC regimen used to prepare the patient is usually myeloablative, meaning that no bone marrow recovery is possible without SCR. There are also submyeloablative HDC regimens, in which the SCR is used to speed recovery, decrease toxicity, and decrease treatment interval without being absolutely required for engraftment (Kletzel et al. 2002; Kreissman et al. 1997).

Compared with autologous marrow, PBSC provides faster hematopoietic recovery from HDC resulting in lower infection risk, shorter duration of mucositis and hospital stay, and lower transfusion requirement, especially of platelets. The use of PBSC, along with other advances in prophylaxis and supportive care, has decreased the treatment-related mortality (TRM) rate in autologous transplant to <5% in many studies. As a result, the use of autologous marrow to support HDC/SCR has very limited indication.

There are several well-established indications for autologous HDC/SCR. In recurrent Hodgkin's disease, 70% relapse again after successfully achieving a second complete remission (CR). Using HDC/SCR in second CR increases EFS to approximately 40–60% (Lazarus et al. 2001). Another established indication for HDC/SCR is high-risk NB. A number of single-arm or retrospective studies suggested that autologous bone marrow transplant might improve EFS (Matthay et al. 1998). A large EBMT retrospective analysis of 1070 HDC/SCR for NB noted overall 49% survival at 2 years. Some relapses were as late as 7 years from transplant, although most events occurred within the first 18 months. Forty-eight of the 1070 procedures were performed after relapse, with no survivors among those undergoing a second HDC/SCR procedure (Philip et al. 1997). In Table 11.6.2, EFS rates at or around 3 years from several major studies are summarized.

11.6.2.1 Children's Cancer Group 3891

The Children's Cancer Group (CCG) 3891 study has provided the largest phase III experience in NB to date. This study employed a 2×2 factorial design. Patients were randomized to consolidation with HDC/SCR vs continuation chemotherapy after induction. Bone marrow purged using immunomagnetic method (see 11.6.3.6) was the stem cell source for HDC/SCR. After completion of consolidation, patients in both groups were randomized to 13-*cis* retinoic acid or no further therapy (Matthay et al. 1999). Patients randomized to HDC/SCR +13-*cis* retinoic acid showed improved EFS compared with those treated with conventional chemotherapy without 13-*cis* retinoic acid. Note that the survival curves (Chap. 15, Fig. 15.5.) start at the time of the second randomization (after HDC/SCR), and not at diagnosis. This study also highlighted the challenges of a 2×2 design and a complex treatment plan: of 579

Table 11.6.2 Results from large studies of HDC/SCR in high-risk neuroblastoma. *C* carboplatin, *E* etoposide, *M* melphalan, *Ctx* cyclophosphamide, *T* thiotepa, *TBI* total body irradiation

Group	Number	Study type	EFS from	EFS (%)	Myeloablative regimen(s)
EBMT	1070	Retrospective	Transplant	2 years, 49 5 years, 33	Various
CCG 3891	539	Phase III	Estimated from diagnosis	3.7 years, 38	CEM/TBI
Grupp et al.	91	Phase II	Diagnosis	3 years, 56	#1 CECtx #2 melphalan/TBI
Kletzel et al.	25	Phase II	Diagnosis	3 years, 57	#1 CE #2 CE #3 TCtx
Villablanca et al.	73	Phase II	Transplant	3 years, 49[a]	CEM

Study populations differed significantly in these five studies. The EBMT analysis included allogeneic transplants and transplants after relapse. Villablanca et al. included only stage 4 >1 year in the group presented

[a] Unpublished data

eligible patients, 379 underwent the first randomization and 258 patients participated in the second randomization, leaving approximately 50 patients in each of the four treatment groups.

11.6.2.2 Experimental HDC/SCR

The HDC/SCR concept has been extended using the more rapid recovery and lower tumor burden afforded by PBSC in studies which use sequential cycles of HDC/SCR. The approach is called tandem transplant and allows for greater dose intensification in the consolidation phase. This approach was initially tried using bone marrow as a stem cell source, and encountered a 24% rate of TRM (Philip et al. 1993); however, the switch to PBSC has allowed more rapid recovery from HDC/SCR, and several groups have tested tandem HDC/SCR supported by PBSC (Grupp et al. 2000b; Kletzel et al. 2002). The largest of these studies was conducted over 6 years at four cooperating institutions (see Fig. 11.6.1 for the schema and an EFS curve as of the most recent update). Important characteristics of the study included early collection of PBSC (generally after the third cycle of induction), use of CD34 selection of PBSC as a purging method, and two fully myeloablative consolidation regimens (carboplatin/etoposide/cyclophosphamide and melphalan/ TBI). The 3-year EFS rate from diagnosis in this sequentially treated group of 91 patients was 56% (see Fig. 11.6.1; Grupp et al. 2000b; S. Grupp, unpublished data). The TRM was 6%, including one death from EBV lymphoproliferative disease (EBV–LPD). EBV–LPD is a very uncommon complication of autologous HDC/SCR and three cases total were observed among 91 patients, suggesting that the combination of CD34 selection and tandem transplant is more immunosuppressive than HDC/SCR using unpurged PBSC (Kanold et al. 2000; Powell et al. 2004). A similar study was conducted by Kletzel et al., using three HDC/SCR regimens in sequence (Fig. 11.6.2; Table 11.6.2; M. Kletzel, unpublished data). Among 26 patients in the published report, 19 completed HDC/SCR #2, 17 went on to HDC/SCR #3, and one late TRM was observed. Eight of the patients received at least one course of anti-GD2 monoclonal antibody following induction chemotherapy and surgery. The EFS in this group of patients at 3 years was 57%.

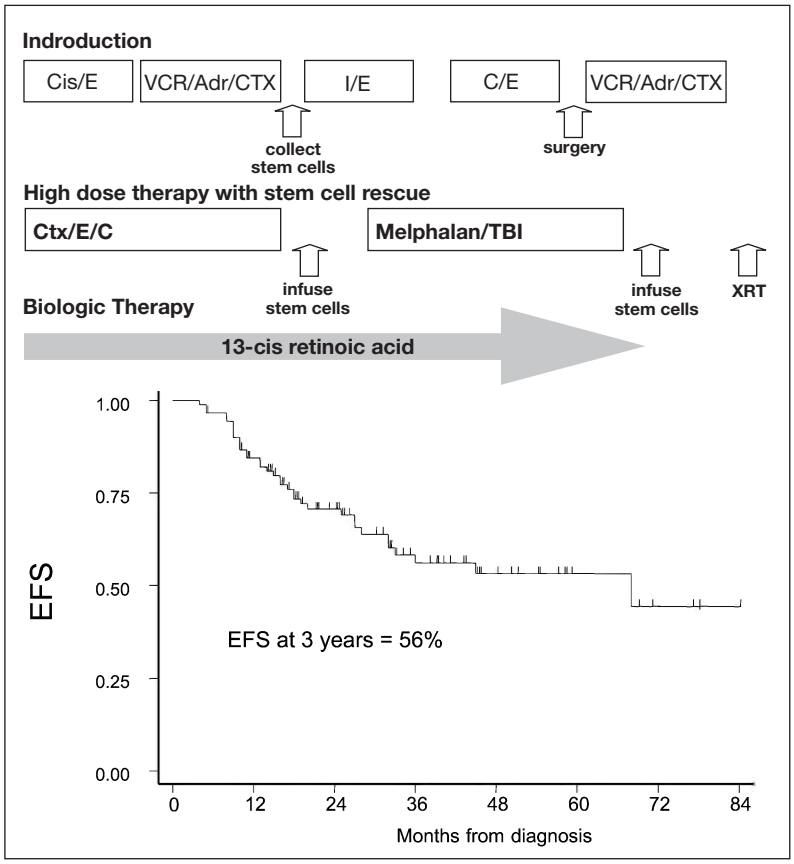

Figure 11.6.1

Top panel. Schema of the CHOP/DFCI tandem transplant study. Bottom panel. Event-free survival (*EFS*) from diagnosis. *C* carboplatin, *E* etoposide, *Cis* cisplatin, *I* ifosfamide, *VCR* vincristine, *Adr* adriamycin, *CTX* cyclophosphamide, *TBI* total body irradiation

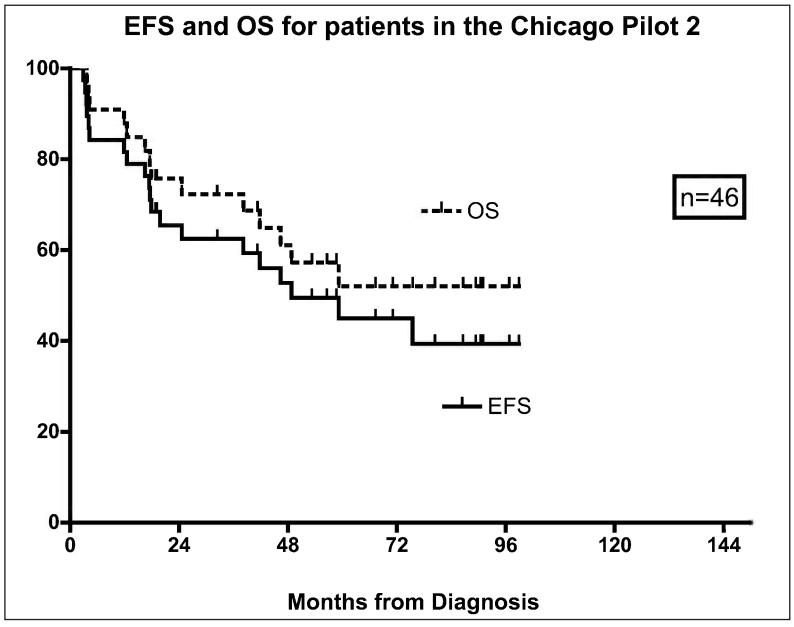

Figure 11.6.2

Event-free survival (*EFS*) from diagnosis on the Chicago "triple tandem" transplant study. *OS* overall survival

Two other experimental approaches to transplantation are worthy of mention. Combining therapeutic doses of [^{131}I-m]IBG with high-dose chemotherapy showed preliminary evidence of efficacy in high-risk patients (Klingebiel et al. 1998; Yanik et al. 2002). Another experimental approach to NB transplant is the use of allogeneic transplant. A graft-vs-tumor effect, thought to accompany the graft-vs-host response, has been demonstrated in leukemias, especially chronic myelogenous leukemia. This effect has not convincingly been demonstrated in the setting of solid tumors (Srinivasan et al. 2004). Retrospective analyses of conventional allogeneic BMT for NB have failed to show any benefit over standard therapy (Matthay et al. 1994; Philip et al. 1997). With the advent of nonmyeloablative transplant regimens, the interest in allogeneic transplant in NB has been revived, with the hope that reduced intensity will reduce TRM in order to detect a therapeutic benefit. At this point, there are no data to justify this approach in children undergoing their primary treatment for high-risk NB.

11.6.3 PBSC Collection

There are a variety of techniques to mobilize more stem and progenitor cells circulating in the peripheral blood. Apheresis separates specific blood components from a patient continuously by centrifugation. Any component can be targeted for relatively specific removal – plasma, red cells, platelets, or white cells. After venous blood is collected and processed through the apheresis device, the nontargeted components are returned to the patient.

11.6.3.1 Vascular Access

To allow continuous blood processing for PBSC collection two ports of vascular access are necessary. In adults this requires two antecubital lines. In 5–10 % of adults and most children, percutaneous antecubital large-bore access is not possible and a pheresis catheter is used instead. A veno-arterial approach, utilizing an arterial line to draw blood and a conventional venous catheter to return it to the patient, has also been described (Takaue et al. 1995). Although there are many configurations, a pheresis catheter is generally a two-lumen catheter with offset proximal and distal ports and side holes along the tip of the catheter. This offset configuration minimizes mixing of processed and unprocessed blood and maximizes the efficiency of the collection. Since apheresis machines can draw 70 cc per min, conventional Broviac-type catheters can be difficult for patients <35 kg, because the lumen collapses under the negative pressure used to draw blood at 2 ml/kg min^{-1}. A pheresis catheter is designed to allow faster draw rates using a combination of larger lumen size, shorter catheter length, and stiffer walls.

Pheresis catheters are available both for temporary and tunneled insertion. Small patients (approx. 10–30 kg) may require an 8 F cuffed tunneled pheresis catheter (MedComp). Smaller patients may require femoral line placement. The concern in smaller patients is threefold: (a) the risk of partial or complete vessel occlusion with the catheter; (b) the risk of vessel erosion and perforation, which may be greater with stiffer catheters in small vessels (Welch et al. 1997); and (c) the difficulty in placing an offset catheter in a short vessel where, if the proximal port is in adequate position, the distal port may be too far advanced. Femoral catheters are short, allowing faster collect rates for a given diameter; however, a patient with a percutaneous nontunneled femoral catheter cannot walk, necessitating admission to the hospital for what is otherwise an outpatient procedure. Also, the perceived risk of complication (especially infection) with a femoral catheter is higher (Merrer et al. 2001). For both of these reasons, femoral catheters are generally only placed temporarily, except in unusual circumstances (Chow et al. 2001). Another approach used at some institutions is to place a single lumen 7F Broviac-type central venous catheter on the opposite side of the patient's existing double lumen catheter. The single lumen line is then used as the draw line and the smaller double lumen catheter is used as the return line.

11.6.3.2 Collection

Even in a mobilized patient, the number of stem cells circulating in the entire blood volume may be inadequate to provide engraftment; thus, processing of multiple blood volumes, often over more than 1 day, is required for some patients (Rowley et al. 2001). This is typical for patients who have been extensively pre-treated with chemotherapy. The minimum required for most patients is one large-volume leukapheresis (LVL), which represents approximately 20 l in an adult or three to four blood volumes in a child. This volume is a typical goal for a single apheresis session, although some physicians will pherese for a total of six or more blood volumes.

There are two issues in PBSC collection that require special consideration in children. First is the issue of priming. Even using devices that minimize extracorporeal volume, smaller children require priming of the apheresis machine with red cells. This prevents unacceptable dilutional anemia. Second is the issue of anticoagulation. In older patients, anticoagulation required for the apheresis procedure is accomplished using ACD. Although rapidly reversible, ACD creates a higher risk of symptomatic hypocalcemia in young patients. These patients are managed with a combination of ACD and heparin to achieve anticoagulation, or receive a calcium infusion in the apheresis return line.

11.6.3.3 Techniques for Stem Cell Mobilization

Large increases in the number of circulating stem and progenitor cells occur during recovery from myelosuppressive chemotherapy, typically when the absolute neutrophil count (ANC) has reached 1000/µl and rapidly rising. The exact point of maximal stem cell mobilization is difficult to predict and highly patient dependent (see 11.6.3.4). Commonly, cyclophosphamide (a total dose of 4 g/m^2 over 2 days) has been used. Multiple-drug regimens as part of the primary treatment can also be used to induce a nadir after which PBSC collection is possible. Because of the concern that the DNA damage may occur in hematopoietic stem cells, and hence increased risk of secondary (treatment-related) leukemia, topoisomerase inhibitors (e.g., etoposide) are often not used to induce the nadir. In one study, use of PBSC collected after etoposide resulted in a 7- to 12-fold relative risk of secondary leukemia (Krishnan et al. 2000), with other studies also demonstrating an increased risk attributable more to the prior chemotherapy than to the preparative regimen used for the stem cell transplant (Kollmannsberger et al. 1998). This effect, together with the observation that multiple cycles of chemotherapy reduce yields of PBSC collection (Jerjis et al. 2000), argue that PBSC should be collected as early in treatment as possible, but after sufficient therapy (usually two to three cycles of chemotherapy) to clear circulating tumor (Faulkner et al. 2000; Moss et al. 1990).

The use of chemotherapy to mobilize PBSC may not be possible or desirable in every patient, has a risk of toxicity during the nadir, and is clearly not appropriate for normal allogeneic donors in whom the chemotherapy has no potential benefit. An alternative approach of using hematopoietic growth factors (HGF) is in widespread use. Donors are placed on a daily regimen of HGF injections, followed by initiation of PBSC collection on day 4–5 of treatment. The HGF treatment continues until the apheresis is complete. There are several choices of HGF doses and regimens (Table 11.6.3). Filgrastim (rhuG-CSF) is the most common HGF used for this purpose. Doses given vary widely. There is a modest dose-response effect between 2 and 16 µg/kg of G-CSF. Although doses as high as 24 µg/kg day^{-1} have been used for mobilization, there is little evidence that these very high doses are more efficacious and they have the disadvantage of greater cost and higher incidence of side effects, especially bone pain.

Sargramostim (rhuGM-CSF) is an alternative. Comparisons of G-CSF and GM-CSF as single agents reveal either no significant advantage of one HGF over the other in terms of PBSC collection efficiency or extent of progenitor cell mobilization (Gazitt 2002), or a modest advantage for G-CSF (Weaver et al. 2001). Laboratory studies have suggested that PBSC collected after G-CSF mobilization may have a polarization in T-cell response toward the more suppressive T-helper lymphocyte type 2 (Th2) response (Sloand et al. 2000). This may have a theoretical

Table 11.6.3 Regimens for PBSC mobilization

Cyclophosphamide 2000 mg/m^2 day^{-1} over 2 days, followed by G-CSF 5 μg/kg day^{-1} SQ from day 3 to the end of pheresis
G-CSF 5 μg/kg day^{-1} SQ for 3–4 days, followed by pheresis on days 4–5 and subsequently
GM-CSF 250 μg/m^2 day^{-1} SQ for 3–4 days, followed by pheresis on days 4–5 and subsequently
Combination of G-CSF 5–10 μg/kg day^{-1} (SQ in AM) and GM-CSF 250 μg/m^2 day^{-1} (SQ in PM) for 4 days, pheresis starting on day 5
Other chemotherapy/HGF combinations

advantage for (a) recovery of cellular immunity after SCT, and (b) the risk of graft-vs-host disease after allogeneic SCT. The combination of G-CSF and GM-CSF may be superior to either alone, although one pediatric study failed to show an advantage for the combination.

The one setting in which the combination of G-CSF plus GM-CSF may be superior is when a patient has had inadequate numbers of stem cells collected over several aphereses. In these so-called poor mobilizers, combination HGF regimens may improve the likelihood that adequate PBSC can be collected (Stiff 1999). Other HGF have been tested as PBSC mobilizers, including stem cell factor and thrombopoietin, but there is no evidence to suggest superiority in terms of clinical outcome during transplant over the standard use of G-CSF, even when higher numbers of CD34+ cells are collected. On the other hand, collection of higher numbers of CD34+ cells has the potential to reduce the number of LVL a donor must undergo, which is a benefit in terms of cost, convenience, and potential donor exposure, especially in children. Balanced against this is the high cost of HGF, and the fact that adding a second HGF doubles this cost. In patients who are receiving myelosuppressive chemotherapy, HGF such as filgrastim are often used to improve recovery. The concurrent use of chemotherapy and an HGF improve PBSC mobilization as well (Knudsen et al. 1996; Levine and Boxer 2002), although a randomized trial did not show this improved mobilization to have an impact on survival or engraftment (Narayanasami et al. 2001). Thus, any patient receiving chemotherapy after which PBSC collection is planned should be placed on an HGF, even if similar courses during the treatment are not supported by an HGF.

11.6.3.4 Target Dose for PBSC Infusion

When bone marrow is collected, most operators target a final volume, or more commonly, a volume and a nucleated cell dose. Because of the high variability in stem and progenitor cell content in PBSC, a more direct assay is needed to assure that adequate numbers for reliable engraftment have been collected. There is no well-established assay for human stem cells, although stem cell activity is likely to be found in a portion of cells that are detected by the long-term culture initiating cell assay or the SCID mouse repopulating cell assay. Progenitor cell content can be assessed by the colony-forming unit granulocyte/monocyte (CFU-GM) assay. The presence of 2–10×10^4 CFU-GM/kg of recipient weight is predictive of engraftment, but the assay is laborious, expensive, and difficult to standardize. It also takes 14 days to complete, making it useless to assess PBSC collections in real time. For all these reasons, most centers have moved away from CFU-GM assays.

A major advance in the use of PBSC was the recognition that most (although not all) (Goodell et al. 1997) of the cells in the hematopoietic stem- and progenitor cell compartment bear the antigen CD34, regardless of lineage. Enumeration of CD34+ cells allows for more accurate assessment of engraftment potential provided by a given number of mononuclear cells. There is a threshold for reliable engraftment and a rough correlation between number of CD34+ cells above this threshold and engraftment (Table 11.6.4). The threshold for reliable engraftment is generally thought to be 1×10^6 CD34+ cells/kg (Shpall et al. 1998). Below this threshold, the likelihood of delayed engraftment of neutrophils and especially platelets increases (Weaver et al. 1997). Increasing the minimum acceptable number to 2–2.5×10^6 CD34+ cells/kg decreases this likelihood somewhat further, and this is the threshold that most transplant centers attempt to achieve. Some authors

Table 11.6.4 Choosing doses of PBSC for stem-cell transplantation

Dose level	CD34+ cells/kg of recipient weight	Notes
Minimum	1×10^6	At this dose, there is a risk of prolonged neutropenia and extended platelet transfusion requirements
Optimum	$2–2.5 \times 10^6$	Threshold dose for many centers
Ideal	5×10^6	There is a limited dose-response effect at doses $>2.5 \times 10^6$ cells, and this target may increase number of phereses needed and cost

have advocated a goal (rather than a minimum) of 5 to up to 15×10^6 CD34+ cells/kg (Stiff 1999). This higher goal is unrealistic in a number of patients, especially patients who have been treated with multiple cycles of chemotherapy prior to collection, and has the potential to significantly increase the cost and length of apheresis.

CD34+ cells in the bone marrow can range from 1 to 4%, while CD34+ cells in mobilized pheresis products can range from 0.1% (in a poor mobilizer) to >1%. The assay for CD34+ cells is a flow cytometric assay (Sutherland et al. 1996; Trischmann et al. 1993), and this technique is inherently inaccurate at low percentages. This means that the number of CD34+ cells based on a measured frequency of 0.1% could easily be off by twofold in either the direction of more or fewer cells, and this must be borne in mind when assessing when to stop apheresis in patients with poor collections. In collections that have undergone CD34 selection (see 11.6.3.5), the CD34 purity is generally >60% and these determinations are extremely accurate. Another consideration is that low CD34 PBSC collections often may have a higher granulocyte content, which can complicate freezing/thawing and therefore have an impact on yield of cells actually infused after storage.

There are many different approaches to determining when a donor will be most successfully pheresed for the highest number of PBSC. The goals are to get adequate numbers of PSBC as defined above, preferably in a single collection procedure. When HGF regimens alone are used, timing is simple: the donor is pheresed on either the fourth or fifth day of HGF administration. After chemotherapy, the point at which the best collection can be obtained in more difficult to predict. Peripheral WBC count is a poor predictor of stem cell mobilization (Yu et al. 1999). There is some theoretical concern for donor safety and possible hyperleukocytosis at WBC counts of more than 70×10^9/l, and some advocate G-CSF dose reductions for donors whose WBC reaches this level. Many centers use some variation on the following algorithm: 1–3 days after the rising ANC reaches 1000, at a point where there is some evidence of platelet recovery, stem cell collection begins. Rather than using a rising neutrophil count to trigger apheresis, some centers with access to rapid-turnaround, quantitative (or "absolute") CD34+ cell counts use the rise in peripheral CD34+ cells to time initiation of collection. Detection of <5 CD34+ cells/μl of blood is highly predictive of poor PBSC collection, whereas >10–20 CD34+ cells/μl correlates well with the likelihood of collecting $>2.5\times10^6$ CD34 cell/kg in a single LVL procedure (Yu et al. 1999).

11.6.3.5 Processing and Storage of PBSC

Most PBSC products collected to support transplant procedures are autologous and must be cryopreserved for later use. In the allogeneic setting, products can be collected prior to starting pretransplant conditioning in the recipient or they can be collected on the day of intended infusion. In addition to cryopreservation, other processing options exist, depending on the purpose for which the PBSC will be used. Specific engineering of the graft is possible to remove or expand desired cell populations

After collection, the PBSC product is taken to the stem cell processing lab. This is where procedures to

ensure quality of the product take place, including determination of CD34+ cell content (see 11.6.3.4), viability determinations, mononuclear cell counts, and confirmation of sterility. Stem cell practice has attracted more regulatory attention recently. The Foundation for the Accreditation of Cell Therapy has been established to provide uniform standards for collection and processing of stem cell products, as well as the clinical care of both donors and recipients (Rowley 2002). The various procedures involved in stem cell processing have also attracted more scrutiny from the Food and Drug Administration. Options for stem cell processing include (a) depletion of granulocytes by density gradient centrifugation (Rowley et al. 1990), (b) depletion of potential tumor cells by a direct purging technique or CD34 selection (Civin et al. 1990), and (c) depletion of T cells in an allogeneic product to decrease the risk of graft-vs-host disease. Many of the stem cell processing steps described here and below, with the notable exception of CD34 selection, were developed using marrow products and all are made somewhat more complicated by the considerably higher number of cells found in PBSC compared with bone marrow.

11.6.3.6 Tumor Cell Purging

All of these processing procedures either depend on negative selection (removal of the cell type that is unwanted) or positive selection (selection of stem/progenitor cells, leaving all other cells behind). CD34 selection is the primary positive selection technique available to stem cell labs. CD34 is a cellular antigen that is expressed on stem cells, as well as progenitor cells of all hematopoietic lineages. Automated processes that select the CD34+ cell population (Strauss et al. 1991) away from the 99% of PBSC that are irrelevant for engraftment are available, and one of these technologies, the Isolex 300i device, is FDA approved. An alternative device, the Miltenyi CliniMACS device (Schumm et al. 1999), is approved in Europe and may soon become available in the United States. In general, CD34 selection will result in a product that is 60–95% CD34+, removing more than 99% of T cells (Beelen et al. 2000) and tumor cells (Donovan et al. 2000; Klein et al. 2001; Mohr et al. 2001), providing that the tumor cells do not express the CD34 antigen. Hematopoietic tumors, such as acute leukemias, often express CD34 and are therefore not depleted by CD34 selection. CD34 selection has been used to purge stem cell products in patients with NB, but concerns have been raised that some NB cells or cell lines may express CD34 or express surface epitopes that cross-react with monoclonal antibodies (MoAbs) that recognize CD34 (Hafer et al. 1999; Voigt et al. 1997). Our data have not confirmed expression of CD34 on NB (Donovan et al. 2000), and we and others have shown purging of NB cells from PBSC products in the clinical setting (Kanold et al. 2000). These data suggest that CD34 selection may be a purging alternative for PBSC products obtained from NB patients.

Negative selection procedures, by contrast, are tumor- or cell-type specific. For example, many techniques have been developed to negatively select T cells or T-cell subsets away from stem and progenitor cells in bone marrow and PBSC (see Ho and Soiffer 2002 for a recent review). These include: (a) a variety of monoclonal antibodies directed against T cells; (b) counterflow centrifugal elutriation (Wagner et al. 1990), which separates out lymphocytes based on physical characteristics; (c) sheep red blood cell rosetting (Reisner et al. 1981); and (d) immunomagnetic removal of T cells. Some of these procedures allow for more specific graft engineering by removing specific T-cell subsets such as CD8+ T cells or T cells expressing activation markers such as CD25 or CD69 (Fehse et al. 2000).

It is also possible to deplete tumor cells using specific anti-tumor monoclonal antibodies, relying on complement (Stein et al. 1988) plus cell-mediated cytotoxicity (Cheung et al. 2002), or more often an immunomagnetic depletion method (Reynolds et al. 1986). This approach has been proven to purge tumor cells from stem cell products collected from patients with B-cell lymphomas (Freedman et al. 1999). That tumor-contaminating stem cell products may contribute to relapse was shown in gene-marking studies in NB patients undergoing autologous bone marrow transplant. In these studies, a bone marrow aliquot was transfected with a marker gene and infused after transplant. Tumor cells at sites of relapse were found to con-

tain the marker gene, suggesting that clonogenic tumor had been infused with the graft (Rill et al. 1994). In follicular lymphoma, inability to detect tumor cells in the stem cell product after purging is associated with improved outcome after autologous transplant (Freedman et al. 1999), but no study has shown that purging itself improves outcome. To study this question, the Children's Oncology Group (COG) is conducting a randomized comparison (COG A3973) of purged vs unpurged PBSC given in support of HDC/SCR in NB. Compared with bone marrow, PBSC from a patient receiving HDC/SCR for a malignancy are less likely to contain tumor cells (Ladetto et al. 2002; Moss et al. 1990; Faulkner et al. 1998) and have a lower content of tumor cells if any are present, and are therefore more likely to be purged successfully of tumor cells (Faulkner et al. 1998, 2000; Ladetto et al. 2001).

11.6.3.7 Storage

After processing, PBSC are then cryopreserved for later infusion. Controlled-rate freezing with temperature curve monitoring is typically used. Products are stored in the vapor phase of liquid nitrogen until they are required for infusion. Usually the storage period is weeks to months, but stem cell products have provided adequate engraftment when infused 8–10 years after cryopreservation (Attarian et al. 1996). After thawing, PBSC again are checked for viability. Granulocytes do not survive cryopreservation, so loss of this cell fraction from the collection is expected. In order to allow cells to survive freezing and thawing, they are placed in a medium containing 7.5–10% dimethyl sulfoxide. Stem and progenitor cells lose viability over time in this medium (Rowley and Anderson 1993), so it is important to infuse the cells immediately after thawing.

11.6.4 Conclusion

At this point, an accepted treatment for high-risk NB includes multi-cycle induction, early collection of PBSC, testing of the PBSC product for evidence of NB contamination, as complete a surgical resection as can be accomplished without organ sacrifice, HDC/SCR (without clear evidence of one conditioning regimen being superior to another), and local radiotherapy either before or after HDC/SCR followed by treatment with 13-*cis* retinoic acid. The ongoing COG phase-III trial will help answer the question of whether purging of PBSC will improve the outcome of high-risk NB patients. To determine if intensifying consolidation will further enhance the outcome of high-risk NB patients, a randomized study of single vs tandem cycles of HDC/SCR is under development within the COG.

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11.7 Minimal Residual Disease Measurement

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11.7.1 Introduction

Patients with high-risk NB who are in clinical remission after completing induction chemotherapy are often left with minimal residual disease (MRD), i.e., the presence of microscopic levels of tumor cells not detectable by conventional clinicopathologic methods. MRD in high-risk patients is likely to contribute to the patient's eventual relapse and death. It stands to reason that accurate determination of MRD is crucial in the overall clinical management of NB patients. The bone marrow (BM), a frequent site of tumor recurrence, must be monitored for MRD. Peripheral blood (PB) is another reservoir of MRD, and sampling by venipuncture is less invasive and better tolerated by most patients.

The current multi-modality treatment for high-risk patients includes dose-intensive induction chemotherapy, tumor resection, local radiation, autologous stem cell harvest with or without purging, megatherapy with stem cell rescue, as well as biologic and/or differentiation therapy to eradicate MRD. Although methods to detect MRD are fully capable and useful for measuring large tumor loads, their clinical utility is most evident after the first three to five cycles of induction therapy, when residual NB are no longer detectable by conventional means. By serving as a surrogate end point, the "kinetics" of tumor response can be better defined by examining BM and PB during successive cycles of chemotherapy. MRD can also be used to evaluate the efficacy of various marrow/stem cell purging techniques, and to identify the optimal time to harvest stem cells. Despite positive selection of CD34+ cells from PB stem cells, substantial contaminating tumor cells remain (Moss et al. 1994; Lode et al. 1997). As therapy for NB becomes more effective, being able to quantify tumor cells during follow-up will help define the quality of remission. It is conceivable that patients in solid remission, without evidence of MRD, may be spared further cytotoxic therapy and treatment-related leukemia. Adjuvant therapy, be it mye-

Table 11.7.1 Techniques in the detection of minimal residual disease (MRD)

Method of detection	Sample source	Archived Samples	Turn-around time	Sensitivity
Cytology	BM Aspirate (BMA)	Yes	Hours	10^{-3}
Histology	BM Biopsy	Yes	2 days	10^{-3}
Immunocytology	BMA, PB	No	1 day	10^{-5}
Immunocytology/FISH	BMA, PB	No	2–3 days	10^{-5} to 10^{-6}
RT-PCR	BMA, PB	Yes	Hours to 1 day	10^{-6} to 10^{-7}

loablative therapy with autologous BM/stem cell rescue (Matthay et al. 1999; Pole et al. 1991; Ladenstein et al. 1998), or biologic therapy such as immunotherapy/differentiation therapy (Matthay et al. 1999; N.K. Cheung et al. 1998; Kushner et al. 2001; Yu et al. 1998), is typically applied at the time of clinical remission. An objective evaluation of the efficacy of these distinct adjuvant strategies is only possible when standardized protocols and quality-controlled detection techniques are used (Ambros and Ambros 2001). Ultimately, the rational choice of adjuvant therapies may become individualized depending on the MRD profile. To date, no treatment decision has been based solely on the detection of MRD.

11.7.2 Techniques in the Detection of Tumor Cells in the Hematopoietic System

11.7.2.1 Histology/Cytology

According to the International Neuroblastoma Staging System and International Neuroblastoma Response Criteria (Brodeur et al. 1993), BM studies evaluated by histologic or cytomorphologic examinations of biopsy specimens and of aspirates from bilateral anterior and bilateral posterior iliac crests are part of the extent-of-disease evaluation. It is also important to perform both biopsy and aspirate at different sites to achieve the highest detection sensitivity (Aronica et al. 1998); however, the sensitivity of tumor detection is relatively low, and thus the true prevalence of BM disease can be grossly underestimated (Cheung et al. 1997; Mehes et al. 2003). Nevertheless, histology and cytology are often used as the gold standard against which new detection techniques are measured (Cheung et al. 1998).

11.7.3 Detection Methods for MRD

Numerous diverse techniques have been developed to detect and in some assays to quantify MRD in NB, because there may be an advantage to know the amount of residual tumor cells over simply establishing its presence. Optimal MRD assays must have superior sensitivity and specificity. The sensitivity of MRD detection methods is influenced by the source of tissue being analyzed and is limited by the number of cells available to be assayed. Moreover, the heterogeneity of tumor cell populations can result in some residual disease escaping detection.

11.7.3.1 Immunocytology/ Immunohistochemistry

In this cell surface antigen detection technique, mononuclear cells isolated from marrow aspirates and heparinized PB are incubated with a single or a panel of murine monoclonal antibodies directed against NB surface antigens. Monoclonal antibodies specific for the NB surface disialoganglioside GD2 are commonly used because this antigen is expressed homogeneously on NB cell surface in high density (Wu et al. 1986). The antibody of choice must have high affinity for tumor cells, with little or no cross reactivity to normal hematopoietic cells. Some examples of anti-GD2 monoclonal antibodies that have excellent specificity include 3F8 (Cheung et al. 1997; Faulkner et al. 1998), 14.18 (Mehes et al. 2001), 3A7 (Saarinen et al. 1996), and HSAN1.2 (Smith and Reynolds 1987). Its usual detection limit of 1 tumor cell in 10^5 normal hematopoietic cells may be improved to 1 in 10^6 or even lower by the use of a com-

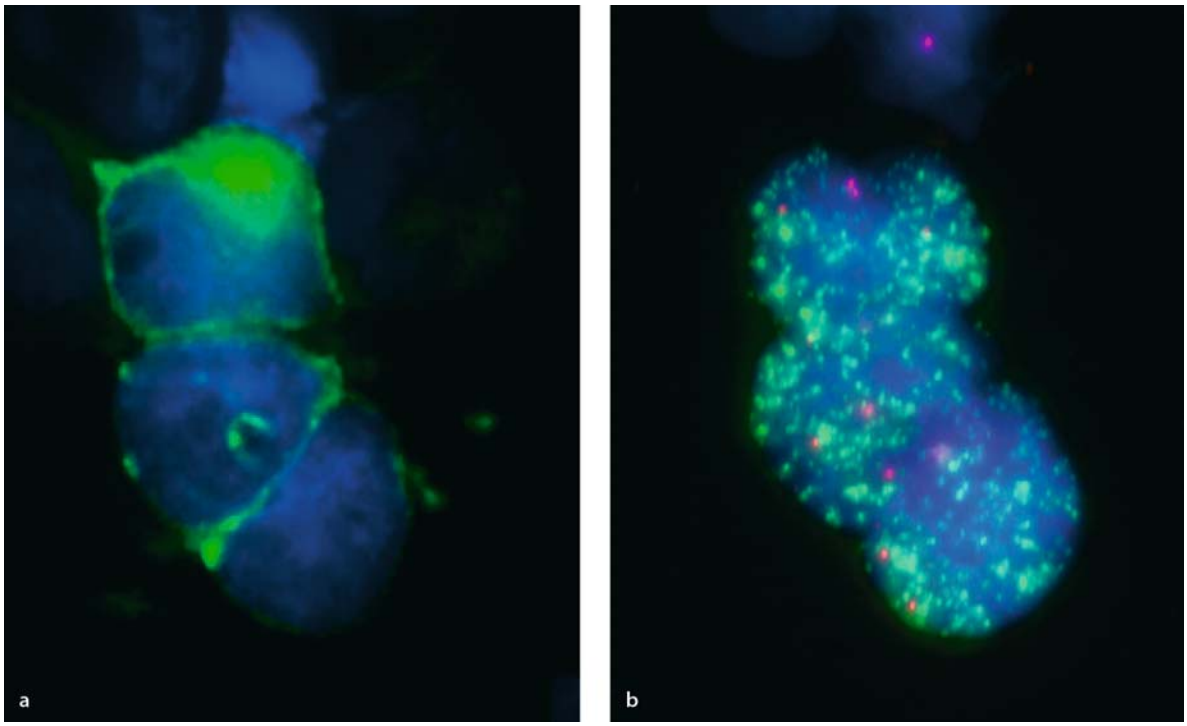

Figure 11.7.1 a,b

a Bone marrow cells from a neuroblastoma patient stained with FITC labeled GD2 antibody. **b** The same cells as in **a** were subsequently analyzed by fluorescence in situ hybridization using a *MYCN*-specific probe (FITC) and a chromosome-2 specific probe (TRITC). All three GD2 positive cells showed *MYCN* amplification.

bined immunofluorescence and genetic approach (Mehes et al. 2001) and to 1 in 10^7–10^8 by the use of immuno-magnetic sorting (Faulkner et al. 2000).

One important advantage of this method is the possibility to quantify the exact number of tumor cells in a specimen. It can exploit a mixture of monoclonal antibodies of different specificities (Moss et al. 1991). More importantly, it allows the genetic confirmation of malignant features of individual cells, and provides insights into the biologic make up of disseminated tumor cells (DTCs); however, this assay requires freshly collected samples which must be processed right away. The technique is also labor intensive because it requires counting and analyzing cells under the microscope.

11.7.3.2 Automatic Immunofluorescence Detection Techniques

The recent development of a fully automated microscopic device allows for the objective screening and quantitation of DTCs, as well as their immunologic and genetic analyses (Mehes et al. 2001a, 2003; Ambros et al. 2001; Ambros and Mehes 2002). The unequivocal identification of the true nature (malignant vs benign) of DTC can be achieved by a sequential fluorescence in situ hybridization (FISH) of the immunologically positive cells (Fig. 11.7.1). Furthermore, the visualization of different antibodies on the same cell can provide vital information on a tumor's aggressiveness. There is a fundamental difference between detecting a proliferative tumor cell vs an apoptotic cell (Mehes et al. 2001b).

11.7.3.3 Reverse Transcription-Polymerase Chain Reaction

In molecular methods to detect MRD, total RNA is first isolated from mononuclear cells. mRNA is reverse-transcribed to cDNA, then amplified by PCR using target gene-specific primers. In some assays, nested PCR is carried out, i.e., an aliquot of the PCR product is subjected to a second PCR reaction, although carryover contamination is a concern. The target gene is identified by the presence or absence of a band of the appropriate molecular size after separation by agarose gel electrophoresis of the PCR product. In some cases, the presence of a gene transcript is further verified using Southern blot, as well as DNA sequencing.

11.7.3.3.1 Real-Time Quantitative RT-PCR

The emergency of real-time quantitative PCR (qRT-PCR) technology permits not just the identification of a target gene, but also its transcript level (Heid et al. 1996; Gibson et al. 1996). Such a development has broadened the potentials in MRD monitoring. With a wide linear dynamic range, superior sensitivity and accuracy, real-time RT-PCR allows good intra-assay and inter-assay reproducibility. Additional attractions include high throughput capacity, speed, and elimination of lengthy post-PCR handling steps, preventing potential carryover contamination. Two major technical variables that need to be addressed are the selection of a reference gene against which the test samples can be normalized, and how to discriminate a positive from a negative result.

11.7.3.3.2 Molecular Targets

Several molecular targets have been studied extensively in the detection of residual NB cells in the BM and PB. Specificity is determined by the absence of gene expression when a series of normal BM and PB is evaluated. Sensitivity experiments are carried out by spiking varying concentration of tumor cells from a NB cell line to normal mononuclear cells, and assess the limit of detection. Depending on the specific gene expression of the cell line, sensitivity can reach as high as $1/10^7$. Tyrosine hydroxylase (TH, tyrosine 3-monoxygenase), being the first and rate-limiting enzyme in the biosynthesis of catecholamine, is a logical choice since most NBs secrete catecholamines. Detection of occult NB cells by RT-PCR of TH mRNA was first reported in BM by Naito et al. (1991), and by Burchill et al. (1994) in PB. Recently, several real-time RT-PCR assays for TH transcript have also been developed (Träger et al. 2003; Tchirkov et al. 2003).

Another useful molecular target is the transcript of GD2/GM2 synthase (β1,4-N-acetylgalactosaminyltransferase). It is the key enzyme required for the synthesis of GD2 (Furukawa et al. 1996), an antigen ubiquitously expressed on NB. Its utility as a molecular marker in the detection of NB cells in the BM was first reported by Cheung et al. (Cheung and Cheung 2001; Lo Piccolo et al. 2001). A highly sensitive and specific quantitative RT-PCR assay which measures GD2 synthase mRNA was developed with transcript levels correlating well with the number of NB cells as measured by immunocytology (Cheung and Cheung 2001).

The cancer testis antigen *GAGE* belongs to a family of genes which encode distinct tumor-associated peptides recognizable by autologous cytolytic T lymphocytes when presented by HLA class-I molecules (Van den Eynde et al. 1995). It is expressed in human tumors of different histologic types including NB, but is silent in normal adult tissues except for placenta and testis (De Backer et al. 1999). *GAGE* was demonstrated to be a potentially useful MRD marker of NB (Cheung and Cheung 1997; Cheung et al. 2000) and melanoma (Cheung et al. 1999).

11.7.3.3.3 Perspectives on Molecular Detection

Molecular-based MRD assays can be hampered by the inherent pitfalls of DNA amplification. False-positive findings may result from "tumor-specific" genes which are occasionally transcribed even in normal tissues. Illegitimate transcription, i.e., the transcription of any gene in any cell type, and pseudogenes, which lack intronic sequences resulting in PCR products indistinguishable from those generated from the mRNA, also pose concerns. False-negative results in molecular assays can be due to degraded RNA, tumor cell heterogeneity, the presence of inhibitors, technical errors, sampling problem, as well as down-regulation of the target gene.

Table 11.7.2 MRD detection with prognostic significance in survival ($p<0.05$)

Study	Number of Patients	Sample	Time from diagnosis	Survival	Detection method	Reference
CCG-3891	242	BM	12 weeks from diagnosis (after three cycles of chemo)	PFS	Immunocytology	Seeger et al. (2000)
	195	BM	BM harvest	PFS	Immunocytology	Seeger et al. (2000)
France	22	BM	~3 months from diagnosis (after three cycles of chemo)	OS	TH qRT-PCR	Tchirkov et al. (2003)
	21	PBSC	PBSC harvest	OS	TH qRT-PCR	Tchirkov et al. (2003)
Japan	21	BM	4 months from diagnosis	PFS	TH RT-PCR	Fukuda et al. (2001)
MSKCC-N7	31	BM	Before 3F8[a] purging	OS	GD2 synthase qRT-PCR	Cheung et al. (2002)
Germany	24	PBSC	PBSC graft	PFS[c]	Immunocytology	Handgretinger et al. (2003)
MSKCC-N7	45	BM	Before ^{131}I-3F8 and 3F8[a]	PFS, OS	GD2 synthase qRT-PCR	Cheung et al. (2003a)
MSKCC-9418	74	BM	Before third cycle of 3F8[a]+GM-CSF (1.8 months from protocol entry)	PFS	GD2 synthase qRT-PCR	Cheung et al. (2003b)
UKCCSG-NB9305	112	PB	Off therapy in clinical remission[b]	PFS	TH RT-PCR	Burchill et al. (2001)
MSKCC-N6, N7	44	BM	24 months from diagnosis	PFS, OS	*GAGE* RT-PCR	Cheung et al. (2000)
MSKCC-N6, N7	44	BM	24 months from diagnosis	PFS, OS	GD2 synthase qRT-PCR	Cheung and Cheung (2001)

[a] 3F8 is a murine anti-GD2 monoclonal antibody
[b] Off therapy defined as after surgery and/or PBSC transplant
[c] Favorable survival with increased tumor cell contamination

11.7.4 Clinical Relevance of MRD

The ultimate utility of MRD detection is to determine the clinical significance of occult tumor cells in relations to patient relapse, survival, and even cure, using progression-free and overall survival as the clinical end points. Prognostic impact of MRD needs to factor in the genetic profile of the tumor. For example, NB patients with stage-4s disease are well known to have marrow disease, and yet they predictably have favorable outcome. This suggests that the presence of marrow disease may not necessarily be clinically relevant to all NB stages. In fact, a Children's Cancer Group study with 374 patients reported no statistically significant difference between stage-1 and stage-2 patients who had immunocytology-positive vs immunocytology-negative marrow disease at diagnosis (Perez et al. 2000). Interestingly, in a compa-

rable patient group, a false-positive rate of >30 % was found after genetic verification of the immunologically positive DTCs (Mehes et al. 2001). Indeed, an unambiguous identification and quantification of MRD must be a requisite for MRD monitoring. For patients with stage-4 NB undergoing multi-modality treatment, the clinical significance of MRD is highly dependent on when the sampling is being carried out.

Several research groups have reported that among stage-4 NB patients older than 1 year at diagnosis, MRD during and after therapy had a statistically significant impact on patient survival (Table 11.7.2). Some studies have further underscored the adverse effect of the presence of NB cells in BM and PB at diagnosis on clinical outcome (Seeger et al. 2000; Burchill et al. 2001). The implication of this conclusion is that emphasis must be placed on improved therapeutic strategies. It is not surprising to find the presence of MRD at the end of therapy to have prognostic importance, as demonstrated by the MSKCC and UKCCSG studies (Cheung and Cheung 2001; Cheung et al. 2000; Burchill et al. 2001). More relevant to the clinical management of NB is likely the impact of MRD during and after induction (Seeger et al. 2000; Fukuda et al. 2001). In BM/PBSC harvest, an adverse effect on survival was demonstrated with ≥100 tumor cells per 10^5 nucleated BM detected by immunocytology (Seeger et al. 2000), >500 TH transcripts in PBSC by qRT-PCR (Tchirkov et al. 2003), and >5 GD2 synthase transcript units in prepurged BM by qRT-PCR (Cheung et al. 2002). In contrast, Handgretinger et al. reported that patients with CD34+ PBSC grafts containing >2000 tumor cells as measured by immunocytology using chimeric anti-GD2 antibody ch14.18 had a lower risk of relapse than patients with fewer contaminating tumor cells (Handgretinger et al. 2003). It was suggested that a threshold number of tumor cells would elicit an anti-tumor immune response after autologous transplant, although false positivity remains a possibility. With the advent of novel post-induction therapies, MRD serves as a sensitive surrogate response marker in comparing the efficacy of different adjuvant therapies. The presence of positive GD2 synthase transcript in patients who did not achieve CR/VGPR before the onset of radioimmunotherapy had a higher risk of relapse and death (Cheung et al. 2003a). For patients undergoing another adjuvant therapy with a combination of anti-GD2 antibody 3F8 and GM-CSF, early molecular response was found to have prognostic impact on progression-free survival (Cheung et al. 2003b). The ability to identify a subset of patients who are unlikely to benefit from this adjuvant therapy and are at a great risk of relapse may provide the rationale for a more timely application of alternative treatment options.

11.7.5 Future Directions

Tumor heterogeneity as well as the occult nature of MRD often lead to failure in detection when only a single method is used; thus, optimal MRD surveillance should utilize multiple independent techniques, such as the inclusion of both immunologic and molecular based assays, as well as serial samplings over time. However, time, cost, and quality-control issues need to be considered. Potential NB targets identified by gene expression arrays will likely enhance detection sensitivity and specificity. The NB research thus far has implicated that, among selected groups of stage-4 NB patients, the presence of minimal residual tumor cells in the BM and PB at specific phases of the multi-modality treatment scheme is likely to portend an adverse survival outcome; however, to fully understand the role of MRD in the overall management of high-risk NB, a large multi-center prospective study with uniformly treated patients using quality-controlled detection techniques is warranted.

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Treatment of Relapsed and Refractory Neuroblastoma

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Contents

12.1 Introduction

This chapter addresses the challenges of treating patients with high-risk neuroblastoma who have failed front-line therapy. Partially resected, incompletely responding, or locally recurrent low-risk neuroblastomas can usually be successful treated with little or no cytotoxic therapy (see Chap. 11.1). By contrast, despite aggressive multi-modality treatment, including dose-intensive and myeloablative chemotherapy, high-risk neuroblastoma eventually progresses and eventually proves lethal in more than 70% of cases (see Chap. 11.3). The prognosis of recurrent intermediate-risk disease is also guarded. This chapter focuses on resistant disease that portends a lethal outcome.

Relapse and recurrence are synonymous and refer to disease that re-emerges after complete or very good partial remission (CR/VGPR) has been achieved. Refractory disease indicates neuroblastoma that is stable or possibly reduced but still evident in macroscopic amounts after several months of adequate therapy, i.e., disease that responds incompletely to treatment. Resistant neuroblastoma encompasses both relapsed and refractory disease. In the International Neuroblastoma Response Criteria (INRC), refractory disease can be partial response, minor response, or no response, while relapsed/recurrent disease is progressive disease (PD). Persistence of metastases is particularly ominous after multiple cycles of intensive chemotherapy, whereas a partial response of the primary tumor may often be rendered CR or VGPR with surgery and radiotherapy. Progressive disease is also present when refractory disease spreads to a new site or

when the volume of a refractory or residual lesion increases more than 25% (Brodeur et al. 1993).

If refractory disease could be detected at diagnosis or very early in induction, then such patients might be treated with novel approaches. Early detection of primary refractory disease is now possible via use of metaiodobenzylguanidine (MIBG) scintigraphy during the induction period as a semi-quantitative response measure (Matthay et al. 2003a; Ladenstein et al. 1998). Measurement of tumor cells in both blood and bone marrow by immunocytology and by the possibly more sensitive technique of reverse transcriptase-polymerase chain reaction may also be a means of early detection of refractory disease (Cheung et al. 2003; Burchill et al. 2001; Seeger et al. 2000) (see Chap. 11.7). In addition, genome-wide screening may lead to the identification of favorable vs unfavorable genetic patterns, which may, in the future, be used to distinguish resistant cases and stratify treatment at diagnosis (Keshelava et al. 2001; Takita et al. 2004; Hiyama et al. 2004).

Depending on the biology of the tumor, resistance to standard therapy is evident in 10–15% of children, resulting in disease that responds incompletely (primary refractory disease) to induction or initially responds but then recurs and progresses rapidly. More frequently, the typical patient with high-risk neuroblastoma will achieve remission, but suffer a relapse later, commonly within 2 years after myeloablative therapy with hematopoietic stem-cell support (Matthay et al. 1999). The approach to therapy of relapse in such patients should assume systemic dissemination. It is rare that even an isolated recurrence will be successfully treated with only local control measures. Bone and bone marrow are by far the most frequent sites of relapse (DuBois et al. 1999; Matthay et al. 1993b). Metastases in sites that are rarely involved at diagnosis, such as the central nervous system (CNS) and lungs, have been reported in up to 8% of relapsed patients (DuBois et al. 1999; Matthay et al. 2003b; Kramer et al. 2001). Regrowth of disease in the primary site has occurred in 10–20% of cases without and up to 50% of cases with distant disease. The incidence of local recurrence may decrease with dose-intensive chemotherapy, total resection, and adequate radiotherapy to the primary tumor bed plus regional nodal groups (Wolden et al. 2000; Haas-Kogan et al. 2002; Ikeda et al. 1992).

Mechanisms of tumor resistance are manifold, ranging from anatomic factors, such as sanctuary sites (CNS and testes), hypoxic conditions (bone or poorly perfused primary tumor), host factors (drug pharmacokinetics), and molecular features of the tumor cells. Examples of molecular changes include emergence of MRP1 and p-glycoprotein-mediated multi-drug resistance (Norris et al. 1996; Blanc et al. 2003; Manohar et al. 2004), altered DNA repair, decreased ability to undergo apoptosis because of p53 mutation (Keshelava et al. 2001; Tweddle et al. 2001, 2003), over-expression of Bcl-2 or Bcl-X_L Dole et al. 1994; Dole et al. 1995), and detoxification of alkylators via various enzymes that conjugate xenobiotics to glutathione (Tew 1994).

Relapse may also result from occult tumor cells admixed with autologous hematopoietic stem cells infused after myeloablative therapy. Support for this possibility comes from (a) the report that after infusion of unpurged autologous bone marrow marked with transduced neomycin-resistance gene, tumor cells in the recurrent neuroblastoma in all three cases showed the genetic marker (Rill et al. 1994), and (b) the occasional reports after autologous bone marrow transplantation of miliary metastases to the lung, a site at risk from infusion of tumor cells through a central venous catheter (Watts and Mroczek-Musulman 1996)and reports that circulating neuroblastoma cells in blood are clonogenic (Moss et al. 1994). The current Children's Oncology Group (COG) protocol for high-risk neuroblastoma is investigating the importance of tumor-free stem cells via a randomized study of ex vivo purging.

Herein we discuss the ever-expanding repertoire of cytotoxic agents (chemotherapy), tumor-targeted agents, and differentiating agents available for resistant neuroblastoma, and suggest how these therapies might best be integrated in an overall treatment plan for different subsets of resistant disease.

Table 12.1. Treatment approaches for different types of relapse (*MIBG* ^{131}I-metaiodobenzylguanidine, *MRD* multiply relapsed disease)

Disease status	Treatment approach
Primary refractory	Novel chemotherapy, MIBG+myeloablative therapy, MRD therapy
Early relapse	Novel chemotherapy, then targeted therapy, myeloablative therapy, MRD therapy
Late relapse	Standard combination chemotherapy, surgery, radiotherapy or MIBG, and novel MRD therapy
Multiple relapse	Low-toxicity oral chemotherapy or outpatient-targeted therapy

12.2 Treatment Strategies for Resistant Disease

The appropriate approach to the patient with recurrent or resistant neuroblastoma depends on the goals of the therapy. Although in previous studies the median survival for patients who relapsed after myeloablative therapy and bone marrow transplantation was only 3 months, with current multimodality approaches and judicious use of established as well as investigational agents, the survival can be prolonged for years (Kushner et al. 2002), and cure may be a possibility in some settings. Whether the goal is symptom palliation, prolongation of life, or complete remission depends on the timing and nature of the relapse, the prior therapy, and the tumor biology. General considerations in the choice of therapy include the use of agents with a different mechanism of action than those tried previously; tailoring therapy to sites of recurrence (e.g., agents with CNS penetration); the availability of stem cells for use with myelosuppressive or myeloablative agents (including ^{131}I-MIBG); and organ status (e.g., impaired renal function from prior cisplatin and/or ifosfamide; Table 12.1).

12.2.1 Primary Refractory Disease

Management of disease that responds incompletely to induction therapy has evolved considerably since the 1980s, which can be considered the beginning of the modern era of combination chemotherapy and myeloablative treatments for neuroblastoma. Early enthusiasm for use of myeloablative chemoradiotherapy for primary refractory neuroblastoma has waned due to the failure to achieve cure. In retrospect, the unsatisfactory results are not surprising, given the presence of a large residual tumor burden comprised of neuroblasts that have survived multiple cycles of combination chemotherapy, and given that the myeloablative regimens usually consist of agents identical to, or in the same drug categories as, those already used in the induction. The addition of total body irradiation does not appear to improve chances for cure, nor does the use of allogeneic stem cells. Myeloablative chemotherapy is ineffective against grossly visible residual soft tissue disease (incompletely resected primary tumor), and the same holds for local radiotherapy (Ladenstein et al. 1993, 1998).

Current treatment strategies for primary refractory disease can apply novel therapies that hold out the possibility of cure. These therapies include either prolongation of induction therapy with intensification of dose if there has been some response to treatment, or else adding chemotherapeutic agents that differ in their mechanism of action from those used in induction, biologic response modifiers, or targeted radiotherapy; thus, when standard induction regimens using alkylating agents (cyclophosphamide, ifosfamide), platinum compounds (cisplatin, carboplatin), and topoisomerase-II inhibitors (etoposide, doxorubicin) fail to achieve CR/VGPR, further cytoreduction is a good possibility by combining the topoisomerase-I inhibitor topotecan with one or two other agents. If there is a satisfactory response, then consideration can be given to myeloablative consolidation, followed by treatments for minimal residual disease such as local radiotherapy and the biologic response modifiers, 13-*cis*-retinoic acid and anti-GD2 antibodies. For morphologically detectable

residual bone marrow disease, the use of 13-*cis*-retinoic acid and anti-GD2 antibodies can achieve CR/VGPR or even cure in a minority of patients, and some of the other biologic response modifiers may prove useful in the future (see Chaps. 14–17); however, for gross residual disease, approaches such as targeted radiotherapy with ^{131}I-MIBG or novel cytotoxic agents may be more appropriate for cytoreduction.

12.2.2 Early Relapse

The development of new disease during induction, although uncommon with the regimens currently in wide use, portends early death since a durable major response to different chemotherapy or to non-chemotherapeutic measures is unusual. The chance for cure is only slightly better when relapse occurs in the 6- to 12-month period after stem-cell rescue. In this situation, management is similar to that of primary refractory disease (see above), except that there is no role for (repeat) myeloablative therapy and organ toxicity may limit chemotherapeutic options. Poor bone marrow reserve post-transplant can be an obstacle to aggressive retrieval therapy, but this problem has lessened considerably with the excellent hematologic recovery that follows use of abundant peripheral blood stem cells (for the initial stem-cell rescue) or with the availability of previously collected stem cells for use with the retrieval program.

12.2.3 Late Relapse

Disease recurrence more than 1 year off therapy is usually responsive to retrieval chemotherapy. Thus, major disease regressions can be expected, even with use of the similar agents or regimens that comprised induction following the initial diagnosis; however, different consolidation measures are required to offer any chance for long-term control, which is still very limited in patients who have already received myeloablative therapy. For patients who achieve a second complete remission, there is a potential role for the same repertoire of treatments for minimal residual disease mentioned above, including retinoids, anti-GD2 antibodies, and local radiotherapy.

12.2.4 Multiply Relapsed Disease

Cure of multiply relapsed high-risk neuroblastoma is virtually unheard of; hence, quality of life becomes a predominant concern and toxic treatments should be avoided. Reasonable chemotherapeutic options include use of topotecan (i.v. or p.o.), irinotecan, etoposide (i.v. or p.o.), or temozolomide, singly or with other agents such as cyclophosphamide, carboplatin, temozolomide, and/or vincristine – all at relatively mild dosages. Widely available cytostatic agents that warrant consideration in this setting may include retinoids, Gleevec, and thalidomide. Phase-I therapies that have expected low toxicity and may be available on an outpatient basis may be reasonable. Local radiotherapy and low-dose ^{131}I-MIBG are useful for palliation of pain or for preventative treatment of heavily involved sites (especially skeletal) likely to become symptomatic.

12.3 Cytotoxic Chemotherapeutic Agents

Agents that may attack resistant neuroblastoma with novel mechanisms to overcome resistance may be divided into cytotoxic chemotherapeutic agents vs those that are targeted to either tumor-specific receptors or biochemical and genetic pathways that are relatively tumor specific. The cytotoxic agents may either be drugs already approved for other cancer indications, or else still under IND, which implies less certain future availability, depending on the overall marketability. In developing a new agent for use in a relatively rare tumor, such as neuroblastoma, it is important to establish efficacy using appropriate tumor models (Houghton et al. 2002). The section below discusses each of these approaches, both for agents that have undergone early clinical trials and those with preclinical data supporting ongoing trials (Table 12.2). In general, efficacy of these agents was based on small phase-I and phase-II clinical studies. Typically, there was substantial heterogeneity in the patient populations, including time from diagnosis and extent of relapse, as such response rates cannot be easily

Table 12.2. New agents with potential in neuroblastoma (*HDAC* histone deacetylase inhibitors)

Cytotoxic	Apoptotic pathway	Immunologic	Anti-angiogenic	Retinoids	Targeted radiotherapy
Topoisomerase inhibitors	HDAC inhibitors	Antibodies	Thalidomide	Fenretinide	^{131}I-MIBG
Alkylators	Demethylating agents	Cytokines	Antibodies	Other retinoids	^{131}I-anti-GD2
Cross-linkers	Tyrosine kinase inhibitors	Vaccines	Small molecules		

compared among these agents. Most would agree that cytotoxic therapy is necessary for large bulky recurrences, while cytostatic or biologic therapy may be most optimally applied when there is small tumor load.

12.3.1 Alkylating and DNA Cross-Linking Agents

12.3.1.1 Ifosfamide and Cyclophosphamide

Initial approaches to treatment of resistant or recurrent neuroblastoma in the previous decade concentrated on use of more intensive combination therapy or use of newer platinum or alkylating agents. Neuroblastoma was proven to be responsive to ifosfamide alone (Castleberry et al. 1994; Pratt 1992; Kellie et al. 1988), and then to combinations of ifosfamide with etoposide (Watts 1992; Kung et al. 1993) or with carboplatin with or without etoposide (Goorin et al. 1995; Alvarado et al. 1997) or with continuous infusion doxorubicin, cisplatin, and etoposide (Campbell et al. 1993; Fernandez et al. 2000). These studies resulted in the incorporation of ifosfamide subsequently into multiple induction regimens (Pinkerton et al. 1990; Olgun et al. 2003; Grupp et al. 2000). Since exposure to alkylating agents at high doses is now widely used in both induction and consolidation regimens for neuroblastoma, and since many patients may already have impairment of renal function due to surgery and prior cisplatin, ifosfamide may be currently less useful than cyclophosphamide as a treatment of relapse.

12.3.1.2 Melphalan Combined with Buthionine Sulfoximine

Since therapy for neuroblastoma relies heavily upon alkylating agents and acquired alkylator resistance likely contributes to recurrent disease, drugs that improve response to alkylating agents may be useful in resistant disease. Glutathione (GSH) is a ubiquitous, intracellular thiol containing tri-peptide that, along with its associated enzymes, plays a critical role in cell growth and metabolism, by maintaining the redox potential of the intracellular environment (Tew 1994; Stokes et al. 2000). Buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis, can enhance alkylator anti-tumor efficacy in a variety of solid tumors. In vitro data have shown BSO to have significant single-agent cytotoxicity against neuroblastoma (Anderson et al. 1997, 1999). Pre-treatment of neuroblastoma cell lines with only 10 μM BSO for 24 h synergistically enhanced the cytotoxicity of 10 μM melphalan by 1–2 logs of cell kill (Anderson et al. 1997). Promising results have been found in adult trials for ovarian cancer, small cell lung cancer, and melanoma, using continuous infusion of BSO and non-myeloablative doses of melphalan, with myelosuppression as the main toxicity (Bailey et al. 1997; O'Dwyer et al. 1992; Yao et al. 1993).

A pilot study of BSO (3 g/m^2 bolus followed by a 72-h continuous infusion (CI) of 0.75–1.0 gm/m^2 h^{-1}) and L-PAM (15 mg/m^2 bolus at hour 48 of BSO infusion) was carried out in 32 patients with recurrent neuroblastoma (Anderson et al. 1998). Of 31 evalu-

able patients, there were 7 partial responses (PR), 2 minor responses (MR), 9 stable disease (SD), and 13 patients with progressive disease (PD). Nearly all patients experienced grade-3 leukopenia and thrombocytopenia, plus grade-2 nausea. There were two toxic deaths on study secondary to renal and CNS toxicity. At autopsy, both children had evidence of mid-brain edema and eosinophilic necrosis of the pons. Other patients on study showed no consistent pattern of impending renal/neurologic toxicity (Anderson et al. 1998). A phase-I study of BSO with melphalan is underway in the New Approaches to Neuroblastoma Therapy (NANT) consortium (N9902), which increases the melphalan to myeloablative doses with peripheral blood stem-cell support, while holding the BSO infusion constant, with close monitoring for possible renal or neurologic toxicity.

12.3.1.3 Platinum Compounds

Newer platinum derivatives with differing toxicity profiles have also been tested. Initially, carboplatin was tested in an attempt to reduce the nephrotoxicity of cisplatin and in the hope of non-cross-resistance. The drug showed excellent activity in phase-I and phase-II trials (Ettinger et al. 1994), in newly diagnosed patients in a phase-II window study (Castleberry et al. 1994), and in combination with etoposide (Frappaz et al. 1992). A recent phase-II trial combined cisplatin with carboplatin in relapsed patients, and showed a 42% response rate (Frappaz et al. 1998). Since myelosuppression is a prominent toxicity of carboplatin, several trials were then done incorporating high-dose carboplatin into regimens utilizing hematopoietic stem-cell infusion (Matthay et al. 1999; Kreissman et al. 1997; Park et al. 2000). Iproplatin produced a 67% response rate in newly diagnosed patients in a phase-II window (Castleberry et al. 1994). Current protocols are open to test oxaliplatin, a derivative with decreased nephrotoxicity, which showed activity in neuroblastoma in preclinical testing, and is apparently non-cross-resistant with cisplatin and carboplatin (Riccardi et al. 1999; Bleiberg 1998).

12.3.1.4 Temozolomide

Temozolomide, an imidazotetrazine prodrug, is an alkylating agent that mediates its cytotoxic effects via O(6)-methylguanine adducts in DNA and their recognition and processing by the post-replication mismatch repair system. Temozolomide is similar to dacarbazine (DTIC) in that they share the same active metabolite; however, activation of the parent drug is spontaneous with temozolomide and not dependent on enzymatic activity, as is the case with dacarbazine. Temozolomide has excellent oral bioavailability with a single-agent MTD of 200–215 mg/m^2 day^{-1} when given to pediatric patients on a 5-day schedule in 28-day cycles, with the dose-limiting toxicity being neutropenia and thrombocytopenia. Temozolomide readily crosses the blood-brain barrier, and has been proven to be active against a variety of brain tumors. Adult phase-II trials have shown response rates as high as 35–50% using temozolomide for recurrent high-grade glioma (Yung et al. 1999), leading to FDA approval for this indication. In addition to activity against brain tumors, temozolomide also appears to have modest activity against mouse models of non-CNS solid tumors, including neuroblastoma (Middlemas et al. 2000; Houghton et al. 2000). In a phase-II study recently reported in abstract form, 27 pediatric patients with refractory non-CNS solid tumors were treated with temozolomide 215 mg/m^2 day^{-1} in 5-day courses. Although no objective imaging responses were observed, two isolated bone marrow responses were noted in 13 evaluable neuroblastoma patients, of whom 10 (77%) had stable disease lasting a median of 7 months (Donfrancesco et al. 2004).

In addition to the single-agent activity, the combination of temozolomide and irinotecan is attractive because of non-overlapping toxicities and the significant therapeutic synergy demonstrated by Houghton et al. (2000) in preclinical experiments. The combination of sub-therapeutic doses of each drug resulted in complete responses in four different xenograft models of neuroblastoma. The proposed mechanism of synergy is temozolomide-induced methylation causing localization and enhancement of topoisomerase I-DNA cleavage complexes, allow-

ing irinotecan to more effectively stabilize the DNA-enzyme complex and cause cytotoxicity after collision with the advancing replication fork (Pourquier et al. 2001). Interestingly, the synergy seen in the mouse models appeared to be partly independent of the DNA repair phenotype of tumor tissue, including *p53* status (Houghton et al. 2000). This observation is important in light of the frequency of acquired *p53* mutations in neuroblastoma cell lines established at the time of relapse (Keshelava et al. 2000a). A recent phase-I study of the combination of intravenous irinotecan and temozolomide in pediatric solid tumors established the MTD as temozolomide 100 mg/m^2 day^{-1} daily for 5 days the first week, with irinotecan, 10 mg/m^2 day^{-1} daily for 5 days × 2 consecutive weeks. Future studies may incorporate oral rather than intravenous irinotecan, and use an oral antibiotic to prevent the usual irinotecan-induced diarrhea (Takasuna et al. 1996; Cosetti et al. 2002; Furman et al. 2003).

12.3.1.5 Tirapazamine

Tirapazamine (TPZ), a benzotriazine di-*N*-oxide anti-cancer drug activated to a toxic free radical under hypoxic conditions, is the first drug of this class to enter clinical testing (Brown 1998). In preclinical models it has been shown to extend the activity of traditional cytotoxic chemotherapy, presumably by selective killing of the hypoxic fraction of tumor cells (Dorie and Brown 1993). It has been shown to be safe and effective when combined with cisplatin in adults with NSCLC, although patients receiving the combination experienced significantly more nausea and vomiting (Olgun et al. 2003). A pediatric phase-I trial of the tirapazamine/cyclophosphamide combination was just completed in POG (Yung et al. 1999). The MTD for tirapazamine when combined with 1.5 g/m^2 of cyclophosphamide was 325 mg/m^2. The DLT was reversible ototoxicity. There were 2 children who experienced grade-3 reversible ototoxicity at 420 mg/m^2. There were three responses (two in neuroblastoma and one in rhabdomyosarcoma; Aquino et al. 2004).

12.3.2 Topoisomerase Inhibitors

Topoisomerase-I and topoisomerase-II inhibitors were the next chemotherapy class to be investigated for treatment of relapsed and newly diagnosed neuroblastoma. Etoposide, a topoisomerase-II inhibitor, as detailed above, has been incorporated into relapse and primary treatment regimens for the past two decades, and is quite effective incorporated into regimens for newly diagnosed neuroblastoma (see Chap. 11). Unfortunately, a significant proportion of cell lines obtained from patients after relapse have demonstrated resistance to this agent, even when cell lines from the same patient obtained at diagnosis were sensitive. Furthermore, cross-resistance with other topoisomerase inhibitors, the camptothecins topotecan and irinotecan, was demonstrated in these cell lines (Keshelava et al. 2000b). Nonetheless, up to 10–15% of children with relapsed neuroblastoma could achieve disease stabilization or partial remission with chronic administration of oral etoposide (Davidson et al. 1997; Kushner et al. 1999; Ng et al. 2000; Schiavetti et al. 2001). Camptothecin is a naturally occurring cytotoxic alkaloid that targets topoisomerase I, a nuclear enzyme that reduces the torsional stress of supercoiled DNA during the replication, recombination, transcription, and repair of DNA. Topotecan and irinotecan are synthetic analogues developed for parenteral administration of the active lactone form of the compound (Garcia-Carbonero and Supko 2002).

12.3.2.1 Irinotecan

Other camptothecins under investigation include irinotecan, a prodrug that undergoes enzymatic conversion to the biologically active metabolite 7-ethyl-10-hydroxy-camptothecin (Garcia-Carbonero and Supko 2002). Irinotecan has shown efficacy in preclinical studies (Thompson et al. 1997; Komuro et al. 1994; Vassal et al. 1996; Shitara et al. 2003) and produced responses in refractory neuroblastoma (Shitara et al. 2003; Furman et al. 1999). Whereas the predominant toxicity of topotecan is myelosuppression, that of irinotecan is diarrhea (Furman et al. 1999; Rowinsky and Verweij 1997). It is usually adminis-

tered intravenously, but trials of oral irinotecan are currently underway. As mentioned above, laboratory studies in neuroblastoma cell lines suggest that lines which are resistant to either etoposide or topotecan are also likely to be resistant to irinotecan (Keshelava et al. 2000b), although another study showed that irinotecan was effective in a xenograft model of neuroblastoma that was resistant to etoposide (Vassal et al. 1996). This drug has also been extensively studied in adult tumors, both alone and in combination with other chemotherapy. It has not yet been shown in human studies of neuroblastoma whether it is non-cross-resistant or in any way more advantageous than topotecan. Other camptothecin analogues are in various stages of clinical development, including 9-aminocamptothecin, 9-nitrocamptothecin, 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, exatecan mesylate, and karenitecin (Garcia-Carbonero and Supko 2002).

12.3.2.2 Topotecan

Multiple phase-I and phase-II single-agent studies have tested topotecan in neuroblastoma, using either a continuous 72-h infusion regimen (Pratt et al. 1994; Blaney et al. 1998) or a daily 1-h infusion for 5 days (Tubergen et al. 1996; Kretschmar et al. 1995; Nitschke et al. 1998; Langler et al. 2002). Responses were reported with both schedules in the phase-I studies, but in the phase-II studies there were no responses with the 72-h continuous infusion, though the response rate was significant in both relapsed patients (10–20%) and in a phase-II window using the 5-day schedule in newly diagnosed patients (37%). Oral topotecan has also been tested with some modest responses (Garcia-Carbonero and Supko 2002; Kramer et al. 2003; Zamboni et al. 1999). Topotecan in combination regimens with other agents active in neuroblastoma, such as cyclophosphamide, cisplatin, and etoposide, have been reported. A phase-I study of topotecan with cyclophosphamide showed that the maximum tolerated dose of topotecan was 0.75 $mg/m^2\,day^{-1}$ when given with a daily dose of 250 $mg/m^2\,day^{-1}$ of cyclophosphamide for 5 days (Saylors et al. 1998). A phase-II window study showed a significant response rate in newly diagnosed patients, and the results are pending for a recently completed randomized study (P9462) in the Children's Oncology Group in relapse patients, comparing topotecan alone at 2 $mg/m^2\,day^{-1}$ to the combined regimen of cyclophosphamide with topotecan (Frantz et al. 2004). Preclinical studies suggest that other cytotoxic agents, such as vincristine (Thompson et al. 1999) or MGI114 (iludin; Weitman et al. 2000), might provide synergistic cytotoxicity with topotecan. A few studies have investigated high-dose regimens incorporating topotecan with thiotepa and carboplatin using stem-cell support, but the dose of topotecan could not be substantially escalated in this regimen beyond standard dose due to mucositis (Park et al. 2000; Kushner et al. 2001). It is possible that further escalation of topotecan in combinations with other agents with less extra-hematopoietic toxicity, such as cyclophosphamide, may be possible with autologous stem-cell support.

12.3.2.3 Pyrazoloacridine

Pyrazoloacridine (PZA), a DNA intercalator, is a rationally synthesized acridine derivative that has been shown to have a broad spectrum of activity against tumor cells in vitro and in vivo (Jackson et al. 1990; LoRusso et al. 1990; Sebolt et al. 1987). Pyrazoloacridine binds nucleic acids (preferentially ribonucleic acid) and inhibits the activity of topoisomerase I and II, causing DNA fragmentation, and thus DNA strand breaks. It is effective against cells in a hypoxic environment as well as the normal oxygen environment, and it is equally effective in cycling and non-cycling cells (Cole 1990; Adjei et al. 1998). It has the further advantage of activity in cells that over-express P-glycoprotein or MRP, as well as in cells that have lost topoisomerase I or II (Cole 1990; Adjei et al. 1998; Sebolt et al. 1989). Preclinical data suggest that prolonged exposure to PZA may increase cytotoxicity (Grem et al. 1996). A recent in vitro study of resistant neuroblastoma cell lines showed that PZA effectively induced cytotoxicity in multi-drug-resistant neuroblastoma cell lines, as well as in drug-resistant p53 non-functional neuroblastoma cell lines. Pyrazoloacridine sensitivity could be shown even under hypoxic conditions, but only with PZA exposure

times that exceeded those tested in previous clinical trials. The in vitro data demonstrated that PZA cytotoxicity is dose- and time dependent (Keshelava et al. 2003). Phase-I and phase-II clinical studies have been conducted, mostly using a short infusion schedule of <6 h, with hematologic toxicity as the main complication. Despite occasional reports of responses, the overall results in pediatric phase-II trials have been disappointing and failed to confirm the anti-cancer activity found in preclinical models (Berg et al. 2000). A possible explanation is that adequate PZA dose levels in humans were not achieved (Berg et al. 1998; LoRusso et al. 1995; Rowinsky et al. 1995). In children and young adults where 640 mg/m^2 of PZA was administered as a 1- or 24-h infusion (Berg et al. 1998), myelosuppression was the dose-limiting toxicity, in contrast to neurotoxicity in the adults.

12.3.2.4 Rebeccamycin

Indolocarbazoles are another group of topoisomerase-I inhibitors, of which rebeccamycin, a naturally occurring anti-tumor antibiotic, derived from an actinomycete. In addition to their action on DNA, rebeccamycin analogues may inhibit the SR kinase activity of topoisomerase I and therefore constitute a unique family of topoisomerase-I poisons quite different from the well-known camptothecins (Prudhomme 2000). In vitro studies support activity in neuroblastoma (Weitman et al. 1998; Marminon et al. 2003). Dose-limiting toxicity in both adults and children in phase-I studies was myelosuppression (Dowlati et al. 2001; Langevin et al. 2003). A phase-II study in pediatric solid tumors is currently ongoing at MSKCC and in the COG.

12.4 Tumor-Targeted Biologic Agents

12.4.1 Retinoids

For information on tumor-targeted biologic agents, including retinoids, see Chap. 15.

12.4.2 Tyrosine Kinase Inhibitors

c-kit has been shown to be expressed in some neuroblastoma cell lines, preferentially those with *MYCN* amplification, and therefore growth inhibitory activity of Gleevec has been demonstrated, both in vitro (Vitali et al. 2003) and in xenograft models (Beppu et al. 2004), leading to testing in pediatric phase-II studies. Another tyrosine kinase inhibitor in clinical testing is CEP-701, a selective inhibitor of several cell-surface receptor-linked tyrosine kinases, with highest affinity and specificity for the Trk receptors. The BDNF/TrkB signaling pathway is a key autocrine survival mechanism for neuroblastomas in patients with high-risk disease, where TrkB is over-expressed (Suzuki et al. 1993; Nakagawara et al. 1994). CEP-701 has high oral bioavailability and potently inhibits all three Trk tyrosine kinases with IC50 values of 3±1 nM (George et al. 1999). CEP-701 also inhibits VEGFR and PDGFR kinase activity, and inhibits the hematopoietic receptor, FLT-3, but has little inhibitory activity against other receptor tyrosine kinases (e.g., EGFR kinase). Targeted inhibition of this pathway has been proven efficacious in preclinical models of human neuroblastoma (Ho et al. 2002; Evans et al. 1999). Ongoing clinical trials of CEP-701 in adult patients with refractory acute myeloid leukemia have shown the drug to be relatively well tolerated, an MTD of 60 mg twice daily has been defined, and biologic and clinical activity has been shown (Smith et al. 2004). A phase-I trial in children with refractory neuroblastoma is ongoing in NANT.

12.4.3 Modulators of Apoptotic Pathway and Angiogenesis

Resistance of tumors to treatment with cytotoxic drugs, irradiation, or immunotherapy may be due to disrupted apoptosis programs.

12.4.3.1 Anti-Angiogenic Agents

Preclinical data have been published supporting the importance of angiogenesis and invasion in progression and prognosis of neuroblastoma (see Chap. 16). An initial report on 50 human tumor samples pro-

vided data that increased tumor vascularity correlated with a poorer prognosis (Meitar et al. 1996), though this was contradicted by a later Spanish report (Canete et al. 2000). High-risk neuroblastomas over-express the $\alpha_v\beta$ integrin, an endothelial transmembrane receptor for neovascular proliferation, which can be blocked with a monoclonal antibody, vitaxin, or an RGD peptide, resulting in increased ceramide production and endothelial cell death (Erdreich-Epstein et al. 2000). Phase-I trials of both the monoclonal antibody and the peptide, cilengitide, have shown promise in adult cancers (Eskens et al. 2003; Tucker 2003). Matrix metalloproteinases, MMP-2, and MMP-9, have a key role in invasion and metastasis, and are over-expressed in the stroma of high-risk neuroblastoma (Sugiura et al. 1998; Ara et al. 1998; Bjornland et al. 2001). In vivo xenograft studies in neuroblastoma have shown that inhibitors of MMPs can decrease angiogenesis and prolong survival (Chantrain et al. 2004). A number of inhibitors are currently in clinical testing, with a few positive results in adult cancers (Ramnath and Creaven 2004). Thalidomide is another agent with antiangiogenic actions in neuroblastoma (Kerbel et al. 2000; Kaicker et al. 2003) currently in clinical cancer trials (Fine et al. 2000, 2003). To date, no large-scale trial of anti-angiogenic or anti-invasion agents has been accomplished in neuroblastoma, although as some of these agents become available and have completed phase-I testing in adults, they will be appropriate for further trials in children.

12.4.3.2 Arsenic Trioxide

Multiple in vitro studies suggest activity of arsenic trioxide against neuroblastoma cell lines, through activation of apoptosis, and possible by differentiation and other mechanisms (Akao et al. 1999; Karlsson et al. 2004; Ora et al. 2000; Carre et al. 2002; Wang 2001). Clinical trials have shown good activity in acute promyelocytic leukemia, and are ongoing in neuroblastoma.

12.4.3.3 Demethylating Agents

Caspase-8 expression acts as a key determinant of sensitivity for apoptosis induced by death-inducing ligands or cytotoxic drugs. Caspase 8 has been shown to be preferentially silenced in neuroblastoma (Teitz et al. 2000). In tumor cell lines resistant to TRAIL, anti-CD95 or TNF-alpha, caspase-8 protein and mRNA expression was decreased or absent without caspase-8 gene loss. Methylation-specific PCR revealed hypermethylation of caspase-8 regulatory sequences in cells with impaired caspase-8 expression. Treatment with the demethylation agent 5-Aza-2'-deoxycytidine (decitabine) reversed hypermethylation of caspase-8 resulting in restoration of caspase-8 expression and recruitment and activation of caspase-8 for drug-induced apoptosis (Fulda et al. 2001; Eggert et al. 2001). The use of a demethylating agent in combination with chemotherapy is being tested in a phase-I trial in the COG.

12.4.3.4 Histone Deacetylase Inhibitors

A dynamic equilibrium of histone acetyltransferase and histone deacetylase (HDAC) controls the level of acetylated histones in nuclear chromatin. By inducing acetylation of the nuclear histones H3 and H4, HDAC inhibitors (HDACI) alter chromatin structure, affecting transcription of several genes, and resulting in growth arrest, differentiation, and apoptosis of tumor cells (Kuo and Allis 1998). Deregulation of histone acetylation has been implicated in the development of several types of cancer. Genes that encode HAT enzymes are translocated, amplified, over-expressed, and/or mutated in various cancers. Two closely related HATs, CBP and p300, are altered in some tumors by either mutation or translocation. A number of new compounds have been developed which inhibit HDAC activity. The best-known examples of this class of agents are the butyrates; however, the butyrates have a short plasma half-life and it is difficult to achieve therapeutic concentrations of butyrates in the plasma. Other compounds that have been developed more recently include depsipeptide (FK-228; Furumai et al. 2002), CBHA, suberoylanilide hydroxamic acid (SAHA), oxamflatin, depudecin, and MS-275 (Yoshida et al. 2001; Marks et al. 2004).

12.5 Immunologic Therapy

12.5.1 Anti-GD2

Multiple approaches are currently under investigation in phase-I, phase-II, and phase-III studies based on antibody targeting of the GD2 ganglioside expressed in >95% of neuroblastoma (see Chap. 14). Treatment with either murine or chimeric anti-GD2 antibody, with or without cytokines, or conjugated to iodine-131, has shown promise for minimal residual and bone marrow disease in refractory and newly diagnosed patients, with responses in phase-I and phase-II studies of 10–20% (Frost et al. 1997; Uttenreuther-Fischer et al. 1995; Handgretinger et al. 1995; Hank et al. 1994; Cheung et al. 1987, 1989; Saleh et al. 1992; Yu et al. 1997; Ozkaynak et al. 1998). A phase-III randomized trial is currently underway in the COG for treatment of minimal residual disease following myeloablative therapy. Current trials in refractory disease are testing the humanized anti-GD2, Hu14.18, covalently linked to interleukin-2 (IL-2; immunocytokine), or use with an immunologic enhancer such as glucan (Cheung and Modak 2002). An anti-idiotype vaccine to anti-GD2, using the monoclonal antibody 1A7 with adjuvant, has completed phase-I testing in neuroblastoma, with evidence of immunologic response.

12.5.2 Interleukins

Interleukin-2 is the most extensively investigated cytokine in clinical use at present. Interleukin-2 enhances the proliferation, cytokine production, and cytolytic activity of T and NK/LAK cell populations, various aspects of monocyte/macrophage function and global measures of immune responsiveness in vivo (Hladik et al. 1994; Foa et al. 1992; Higashi et al. 1991; Verstovsek et al. 1995; Nishimura et al. 1992; Cox et al. 1992). IL-2 has demonstrated anti-tumor activity in neuroblastoma (Lode et al. 1997) and may also have synergy when used in combination with IFN-γ to treat murine tumors, including neuroblastoma (Sigal et al. 1991). A total of 15 studies have reported on the use of IL-2 to treat pediatric patients with solid tumors, including over 100 patients with neuroblastoma (Frost et al. 1997; Negrier et al. 1991; Pardo et al. 1996; Valteau-Couanet et al. 1995; Bauer et al. 1995; Chien and Hsieh 1990; Nasr et al. 1989; Pais et al. 1992; Favrot et al. 1990; Truitt et al. 1992; Ribeiro et al. 1993; Roper et al. 1992; Toren et al. 2000; Pession et al. 1998). The majority of these early studies evaluated the use of chronic, intermittent dosing of IL-2 delivered as an intravenous bolus over periods ranging from 15 min to 2 h. Fever, vascular leak, and hypotension are common side effects, seen more frequently at the higher intravenous dosing schedules.

IL-2 alone has had little activity in relapsed bulky disease in any solid tumor other than renal cell carcinoma (Bauer et al. 1995), and has therefore been more extensively tested in the post-transplant setting. For relapsed disease, IL-2 may be more effective in combination with other immunotherapeutic agents, such as the anti-GD2 antibody or other cytokines. Recent preclinical evidence suggests that in combination, IL-12 with IL-2 may possess potent immunomodulatory and anti-tumor activity that exceeds the effect of either agent alone. IL-12 and IL-2 reciprocally upregulate the expression of their respective receptors (Bacon et al. 1995; Desai et al. 1992; Yanagida et al. 1994) and can greatly enhance T and/or NK cell proliferation, cytokine production, and cytolytic function. Systemic administration of IL-12 in combination with intermittent, weekly doses of IL-2 (pulse IL-2) is not only well tolerated, but can induce rapid and complete regression of established primary and/or metastatic tumor in several murine models (Wigginton et al. 1996, 2001a,b). Several reports have also demonstrated that IL-12 gene therapy administered alone or in conjunction with tumor-targeted IL-2 possesses anti-tumor activity in transplantable murine neuroblastoma tumor models (Lode et al. 1999; Davidoff et al. 1999). Based on preclinical data of the combination therapy in murine neuroblastoma models and the adult human trial data, a phase-I dose escalation trial of IL-12 combined with IL-2 is now underway in the NANT consortium.

12.5.3 Vaccines

Preclinical and clinical trials are in progress to try to further enhance the specificity and efficacy of cytokines by using autologous tumor cells transfected with cytokines such as IL-2 (Bowman et al. 1998a,b), IL-12, GM-CSF, interferon gamma (Bausero et al. 1996; Yoshida et al. 1999), or lymphotactin as vaccines to stimulate the host immune response to the neuroblastoma. Other vaccine approaches include the use of DNA vaccines (Pertl et al. 2003), or dendritic cell vaccines (Chen et al. 2003).

12.6 ^{131}I-Metaiodobenzylguanidine

^{131}I-Metaiodobenzylguanidine (MIBG) is a guanethidine derivative that is structurally similar to norepinephrine, and therefore concentrates in the neurosecretory granules of catecholamine-secreting cells. Radiolabeled MIBG provides very sensitive and specific visualization of primary and metastatic neuroblastoma by scintigraphy (Shulkin and Shapiro 1998). In an attempt to deliver higher doses of tumor-specific radiotherapy and avoid normal organ toxicity, iodine-131 MIBG therapy has been used in pilot trials since the mid 1980s, with more than 500 children reported in the literature. Initially, it was shown to induce 30–40% response rate in highly refractory relapsed patients, without significant non-hematologic toxicity (Klingebiel et al. 1991; Matthay et al. 1998; Voute et al. 1991). At low and moderate doses, up to 12 mCi/kg of ^{131}I-MIBG, the main toxicity has been thrombocytopenia, usually self-limited. Phase-I dose escalation studies showed that higher doses, up to 18 mCi/kg, could be administered with bone marrow or peripheral blood stem-cell support to mitigate the neutropenia and thrombocytopenia, but without clinical organ toxicity, excepting a 10–15% incidence of hypothyroidism due to uptake of some free iodide by the thyroid gland (Matthay et al. 1998; Lashford et al. 1992). There are a few reports of patients with secondary leukemia developing after MIBG therapy, but the estimated risk of this problem at 5 years post-therapy is only 4%, lower than with some chemotherapy regimens (Garaventa et al. 2003; Weiss et al. 2003). Despite a number of clinical studies worldwide, dose response to ^{131}I-MIBG has not been firmly established. Recent studies are investigating the use of low dose ^{131}I-MIBG at diagnosis prior to surgical resection (Troncone et al. 1995), or in combination with standard (Mastrangelo et al. 2001)or high-dose myeloablative chemotherapy (Yanik et al. 2002; Klingebiel et al. 1998). New phase-I studies are currently open in the NANT to test the use of double infusion of ^{131}I-MIBG with stem-cell support or further combination with myeloablative chemotherapy and stem cells. Further investigations are required to determine the optimal timing and use of this targeted approach.

12.7 Conclusion

Refractory and relapsed neuroblastoma is very difficult to eradicate, due to multiple mechanisms of drug resistance. Above all, compassion and consideration for quality of life must be incorporated into the therapeutic goals for the child with refractory neuroblastoma. Currently established therapies include only a few chemotherapeutic and targeted agents, and of these, only the chemotherapy agents are commercially available. Future success depends on rational preclinical in vitro and in vivo testing of new agents and combinations in panels of appropriate neuroblastoma cell lines and tumors, followed by careful clinical trials. Judicious selection of trials depending on the type of relapse, and willing cooperation of patients and parents after careful explanation of the goals of the study are essential to improve survival in this disease.

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Management of Neurologic Complications

Kim Kramer, Michael R. Pranzatelli

Contents

13.1 Introduction

An estimated two-thirds of all cancer patients develop some type of neurologic problem during the course of their illness (Tasdemiroglu et al. 1998), and up to 15% develop a serious neurologic complication (Posner 1995). Such complications in patients with neuroblastoma can be caused directly by involvement of metastatic tumor, indirectly by treatment, or by a paraneoplastic syndrome. Early diagnosis and intervention may prevent long-term neurologic sequelae in some patients.

13.2 Epidural Neuroblastoma

Neuroblastoma is the most common malignancy to cause spinal cord or nerve root compression in children (Conrad et al. 1992; de Bernardi et al. 2001) and may be a complication of loco-regional or metastatic disease. Extension into neural foramina and/or the spinal canal occurs in 10–15% of cases (Plantaz et al. 1996; Massad et al. 1985). Cord compression may manifest as radicular pain (back, neck, leg), weakness or gait disturbance, subacute or acute paraplegia, bowel or bladder dysfunction, sensory abnormalities, or scoliosis, although one-quarter of children with documented epidural disease are asymptomatic at presentation (Sandberg et al. 2003). Prompt resolution of spinal cord compression may prevent the development of permanent neurologic impairment. Several studies have indicated that the rate of neurologic recovery is related to both the severity and duration of neurologic symptoms at the time of diagnosis (Plantaz et al. 1996; Hoover et al. 1999; Plantaz et

Table 13.1. Treatment and neurologic outcomes for neuroblastoma patients with metastatic (group 1) or locoregional (group 2) epidural tumor

Reference	Institution	No. of patients	No. of patients with neurologic deficits	Initial treatment for group 1	Initial treatment for group 2	Outcome
Sandberg et al. (2003)	MSKCC (1987–1998)	46 (31 group 1, 15 group 2)	18 (group 1); 6 (group 2)	12 of 18 symptomatic patients: chemotherapy; 6 of 18 symptomatic patients: surgery; 13 asymptomatic patients: chemotherapy	4 of 6 symptomatic patients: surgical decompression; 2 of 6 chemotherapy and surgery; 7 of 9 asymptomatic–surgical decompression; 2 of 9 chemotherapy	Spinal deformities occurred in 12.5% of patients treated non-operatively and 30% of patients treated operatively
Hoover et al. (1999)	(1967–1994) Multi-center	26 (20 patients group 2, 6 patients group 1)	17 (group 2); 6 (group 1)	4 of 6 symptomatic patients: surgery; 2 of 6 symptomatic patients: chemotherapy	16 of 17 symptomatic patients: surgery; 1 of 17 symptomatic patients: chemotherapy	Patients with partial deficits recover function regardless of treatment approach; long-standing deficits are unlikely to improve
Plantaz et al. (1993)	Institute Gustave Roussy (1982–1987)	25	15 (group 2)	14 symptomatic patients: surgery; 10 patients: preoperative chemo therapy; 1 patient died before thoracotomy		Chemotherapy can improve neurologic deficits in the majority of patients; most patients can be spared neurosurgical intervention; surgery should be reserved for patients with progressive neurodeficits
de Bernardi et al. 2001)	Gaslini Children's Hospital (1979–1998)	76 (19 group 1; 54 group 2; 3 stage 4s)	76	33 patients: chemotherapy; 32 patients: laminectomy; 11 patients: radiotherapy		Laminectomy is more common in group-2 patients with neurologic deficits; chemotherapy reserved for group-1 patients; regardless of treatment, no improvement in paraplegic patients; chemotherapy is preferred for symptomatic patients; sequelae are seen in 44% of surviving patients

Plantaz et al. (1996)	French Society Pediatric Oncology (1990–1994)	42 (all group 2)	27	32, including 20 of 27 with neurodeficits: chemotherapy; 7, including 6 of 27 with neurologic deficits: surgery		Serious neurologic sequelae occurred in 15%; first-line chemotherapy was effective in eliminating sequelae in 77% of patients with initial deficits
Katzenstein et al. (2001)	Pediatric Oncology Group (1990–1998)	83 (21 group 1; 61 group 2; 1 stage 4s)	43	3of 4 patients with severe neurologic deficits treated with surgery; 1 patient with chemotherapy	4 of 11 patients with severe deficits treated with chemotherapy; 7 with surgery	Treatment modality was not predictive of neurologic outcome; severity of pre-existing symptoms was the factor associated with neurologic outcome

al. 1993; Gutierrez et al. 1983; Katzenstein et al. 2001). In addition, the prognosis for recovery in children appears to be better than that in adults, and children who develop paraparesis slowly are more apt to recover than when the deficit evolves rapidly (Antunes 2000). Current therapeutic strategies to relieve spinal cord compression include surgical resection with or without laminectomy, chemotherapy, and radiation therapy; however, because each of these treatment modalities has inherent short- and long-term associated morbidities the optimal initial treatment approach for cord decompression remains unknown.

Most reports of epidural involvement in neuroblastoma have retrospectively reviewed small numbers of patients over many years, with most pre-dating MRI availability and current chemotherapy protocols (Table 13.1). Furthermore, while improvement or resolution of neurologic deficits are often described following initial treatment, most studies focus on survival data alone, and not on the long-term sequelae. In general, patients with symptomatic spinal cord compromise whose diagnosis has not yet been determined require surgery to obtain an adequate specimen for histologic and biologic studies. In patients with known high-risk neuroblastoma and metastatic epidural tumor with spinal cord compression, chemotherapy alone often prevents neurologic deterioration (Sandberg et al. 2003; Hayes et al. 1989). Katzenstein et al. (2001) reported complete neurologic recovery following treatment in 6 of 15 severely affected patients, and 2 of 5 patients with moderate deficits, and 17 of 22 patients with paresis alone. In this Pediatric Oncology Group (POG) study a higher incidence of spinal deformities was found among children with intraspinal neuroblastoma who had undergone laminectomy compared with those managed without laminectomy. The rate of neurologic recovery was similar for patients treated with chemotherapy compared with those managed with laminectomy, and the authors concluded that laminectomy should be reserved for patients who demonstrate progressive neurologic deterioration after the initiation of chemotherapy. Radiation alone (7.5–30 Gy) or in combination with laminectomy has also been used to rapidly reduce cord compression (Punt et al. 1980). Patients treated with this approach

are also at high risk for subsequent development of spinal deformity. The incidence of spinal deformity after multilevel laminectomy is related to age and to the spinal level of the laminectomy (Plantaz et al. 1996). In a recent review of 76 patients with symptomatic spinal cord compression patients from the Italian Cooperative Group for Neuroblastoma (ICGNB), de Bernardi et al. (2001) found scoliosis to be the most common late effect, affecting 31% of surviving patients. This was more frequently observed in patients treated with laminectomy or radiotherapy compared with those treated with chemotherapy alone. In a review of 46 patients with epidural tumor at Memorial Sloan-Kettering Cancer Center, 70% of high-risk patients treated initially with chemotherapy alone improved or remained stable (Sandberg et al. 2003). In this series, 11 of 15 patients with low-risk neuroblastoma were initially treated with decompressive surgery and all remained stable or improved neurologically (Sandberg et al. 2003); however, spinal deformities occurred in 2 of 16 patients (12.5%) who did not undergo laminectomy and in 9 of 30 (30.0%) who did. Low-risk neuroblastoma patients with spinal cord compression may be offered surgery only, but the risk of scoliosis needs to be weighed against those of cytotoxic chemotherapy.

13.3 Metastatic Disease to the Central Nervous System

Central nervous system (CNS) neuroblastoma, involving brain parenchyma or leptomeninges at the time of diagnosis in all published series is rare. This must be distinguished from dural or bone based metastases without invasion into the parenchyma. In a review of 251 patients with metastatic neuroblastoma treated at Memorial Sloan-Kettering Cancer, no patient had brain parenchymal or leptomeningeal disease at the time of diagnosis (Kramer et al. 2001). Although CNS neuroblastoma may result from direct extension of spread of neuroblasts from bone or bone marrow, the cerebrospinal fluid (CSF) appears an equally efficient route of neuraxis dissemination (Banerjee et al. 1995). Autopsy findings suggest that the tumor may penetrate the spinal meninges and disseminate through the CSF. In patients with no obvious breakdown of the blood-brain barrier, leptomeningeal neuroblastoma is presumed to occur by hematogenous spread. As treatment for high-risk neuroblastoma has become more intensive, the pattern of disease relapse has changed and the neuraxis appears to be an important sanctuary site (Kramer et al. 2001). The incidence of CNS relapse in large series ranges from 1 to 16% (Kramer et al. 2001; Blatt et al. 1997; Shaw 1992; Kellie et al. 1991; Rohrlich et al. 1989), with the median time to CNS relapse from initial diagnosis ranging from 13 to 20 months.

The CNS is increasingly recognized as an isolated site of relapse in patients with no evidence of recurrent systemic disease. No consistent prognostic marker predicts which patients are at risk for development of CNS disease (Table 13.2). In two large series, diagnostic lumbar punctures in patients with known bone marrow disease was associated with relapsed disease in the CNS (Kramer et al. 2001; Matthay et al. 2003), raising the possibility that this procedure may enhance the ability of circulating or epidural microscopic tumor cells to seed the craniospinal axis.

Patients with CNS disease may present with altered mental status, headache, seizures, paresthesias, dysarthria, visual disturbance, vomiting, or ataxia; thus, any new cerebral neurologic symptom should provoke a search for brain metastases (Lassman and DeAngelis 2003). The brain should also be evaluated for disease in the presence of an unexplained rise in urine catecholamines (Kramer et al. 2001). The diagnosis is made by radiographic imaging studies, most commonly CT or MRI. Cerebrospinal fluid cytology is positive in approximately one-third of the patients with disease detected by CT or MRI (Kramer et al. 2001).

By the time neuraxis metastases are clinically evident, limited palliative options exist. The median survival from the time of CNS disease detection in most series is 4–14 months (Kramer et al. 2001; Shaw 1992; Kellie et al. 1991; Rohrlich et al. 1989). Corticosteroids often provide a dramatic, albeit temporary, benefit from brain metastases associated with vasogenic edema (Lassman and DeAngelis 2003). Surgery for solitary metastases and radiotherapy are used for palliation. Stereotactic radiosurgery for small, single

Table 13.2. Statistically significant prognostic factors predicting the development of central nervous system (CNS) metastases in patients with neuroblastoma

Reference	No. of CNS events/patients	Prognostic factor(s) identified
Kramer et al. (2001)	11 of 251 patients with metastatic disease (4.4%)	Lumbar punctures performed at diagnosis Elevated serum LDH (>1500 U/ml)
Matthay et al. (2003)	23 of 434 patients with metastatic disease (5.2%)	Lumbar punctures performed at diagnosis *MYCN* amplification
DuBois SG et al. (1999)	17 of 549 patients with metastatic disease (3.1%)	MYCN amplification

metastases (<4 cm diameter) appears to have a better control rate than whole-brain radiotherapy (Flickinger 2001). New techniques including preoperative functional imaging, image-guided neurosurgery, intraoperative ultrasound, and cortical mapping have improved the success of aggressive surgical resection, and lowered the associated surgical morbidity and mortality (Weinberg et al. 2001). Although systemic chemotherapeutic agents currently used for relapsed neuroblastoma are generally unable to adequately cross the blood-brain barrier, aggressive multi-modality treatments may result in a longer median survival for some patients. Unlike the beneficial effect of prophylactic CNS treatment in survival for patients with leukemia and small cell lung cancer (Vines et al. 2003), the rarity of CNS neuroblastoma makes prophylactic treatment difficult to justify; however, molecular detection of tumor-associated gene products by reverse transcriptase-polymerase chain reaction (RT-PCR) may identify patients at risk for leptomeningeal disease. Ongoing studies are investigating whether the CSF measurements of GD2 synthase, a key enzyme involved in the regulatory expression of complex gangliosides at the cell surface of neuroectodermal-derived tumor cells, including neuroblastoma, has clinical utility as it appears to have in the blood and bone marrow of neuroblastoma patients (LoPiccolo et al. 2001; Cheung and Cheung 2001). In addition, novel tumor-selective radioimmunotherapeutic strategies may have potential in inhibiting leptomeningeal tumor growth (Bergman et al. 1999; Kramer et al. 2000; Bigner et al. 1998). Intraventricular administration of 131-I-3F8 targeting disialoganglioside GD2 achieves a favorable cerebrospinal to blood ratio and may have clinical utility in the treatment of patients with GD2-positive leptomeningeal cancers (Kramer et al. 2000).

13.4 Opsoclonus–Myoclonus

Opsoclonus–myoclonus syndrome (OMS), also called Kinsbourne syndrome, dancing eyes syndrome, and myoclonic encephalopathy, is a rare neurobehavioral paraneoplastic disorder found in <4% of patients with neuroblastomas (Rudnick et al. 2001a; Gambini et al. 2003). The true incidence of OMS is unknown, with mild cases often being misdiagnosed (Everson and Cole 1956). Peak age of onset is 18–24 months, but the disease does appear across the age spectrum (Pranzatelli 2000). Children from all races, major socioeconomic groups, and geographic regions are affected (Pranzatelli 1992); males and females are affected equally. Opsoclonus and myoclonus are obligate features of the disease, but ataxia and other abnormalities are common. Tumors are often histologically more mature, less aggressive, have favorable biology, and are associated with excellent rates of survival (Gambini et al. 2003). Most tumors are occult and may require repeated investigations for detection. Tumor removal is usually not therapeutic; indeed, some children worsen after surgery.

The clinical course is associated with a prodromal phase marked by extreme irritability, inconsolability, sleeplessness. During the acute neurological phase, the child suffers from incoordination and falling,

with progressive neurological deterioration, inability to sit or stand, slurred speech rage attacks, hypotonia, head tilt, Horner's syndrome, deep tendon reflex abnormalities, or seizures. The chronic phase is associated with variable impaired cognitive function, IQ loss (Papero et al. 1995), attention deficit disorder with or without hyperactivity, obsessive compulsive disorder, mood and conduct disorders (Koh et al. 1994), speech articulation and fluency problems, ataxia (Mitchell et al. 2002), ocular flutter, and strabismus. Children with moderate and severe symptoms at the onset of OMS will not improve on their own and require immunotherapy. The "wait and watch" approach in this group following tumor resection is ill-advised (Blaes 2002).

13.4.1 Immunology

Opsoclonus–myoclonus syndrome is a putative autoimmune disorder, a "friendly fire" attack of the immune system on the brain (Pranzatelli 2000). Onconeural antigens have not been identified. Tumors from children with OMS are more highly infiltrated with lymphocytes than those from non-OMS counterparts (Martin and Beckwitz 1968); both B cells and T cells congregate in immune nodules (Cooper et al. 2001) and are recruited to the CNS (Pranzatelli 2000). B-cell and T-cell expansion correlates with neurological severity. Histopathologically, inflammatory cells, cerebellar vermian atrophy (Pranzatelli et al. 2002a), and cerebellar cell loss (Hayward et al. 2001) are sometimes identified (Clerico et al. 1993). Non-motor functions of the cerebellum, particularly language acquisition, are well accepted (Lieberman 2002); however, the anatomic substrate of opsoclonus appears to be the brain stem, with mesencephalic and pontine ocular gaze centers containing the burst and omnipause cells that control saccadic eye movements (Fuchs et al. 1985). Also, the seat of myoclonus can be wide-ranging within the CNS, the nucleus gigantocellularis reticularis in the caudal medulla, is the closest to a "myoclonus center" (Pranzatelli 1992). A diffuse neural network originating in the cerebellum with brain stem and frontal connections may become dysfunctional. Purkinje neurons, the main cerebellar outflow to deep cerebellar nuclei, may play a crucial role.

13.4.2 Pharmacology

Several different neurotransmitters, such as serotonin, γ-aminobutyric acid (GABA), and glycine, have been implicated in myoclonus; however, myoclonus and other neurologic features of OMS are unlikely to be due to single neurotransmitters. Steroids (adrenocorticotrophic hormone and prednisone appear to be most effective in restoring neurological function but have multiple trophic effects on brain (Pranzatelli 1994). Antiepileptic drugs and an array of neuroceptor-active drugs are not effective in treating myoclonus or opsoclonus (Pranzatelli 1992). A subgroup of children with OMS has low CSF concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) and the dopamine metabolite homovanillic acid (HVA), but treatment with ACTH may further lower 5-HIAA (Pranzatelli et al. 1998a). It has been speculated that serotonin receptors which are found in neuroblastoma (Pranzatelli 1992), may be one target of immunologic injury. Mood problems and obsessive-compulsive disorders in OMS could relate to serotonin also; however, neuroblastoma is replete with other neurotransmitter receptors as well. The level of free choline in the CSF has not been found to be different in OMS patients than in controls (Pranzatelli et al. 1998b).

13.4.3 Laboratory Testing

There is no confirmatory laboratory test. Finding serum antibodies is helpful (Fisher et al. 1994), but children with OMS are usually seronegative by commercial testing for the paraneoplastic autoantibodies described in adults (Pranzatelli et al. 2002b). Low-serum titers of anti-Hu antibodies occur as often in neuroblastoma without OMS (Antunes et al. 2000) and do not correlate with neurologic outcome (Rudnick et al. 2001a). Autoantibodies to post-synaptic densities or other unidentified brain antigens can be seen on Western blots, and both IgG and IgM antineurofilament antibodies have been reported in OMS (Connolly et al. 1997); however, these antibodies are not specific and are also found in widely divergent disorders (Bataller et al. 2003; Stubbs et al. 2003).

Table 13.3. Therapeutic approaches to (opsoclonus–myoclonus syndrome) OMS

Agent	Pros	Cons
Biologic factors		
ACTH (corticotropin)	Most potent agent for inducing neurologic remission	Must be given by injection; steroid side effects
IVIG	Immunomodulation; decreases infections	Flu-like post-infusion symptoms; potential blood product risks that can trigger relapses
Rituximab	Selective against B cells	Low risk of allergic reaction to murine component; long-term effects unknown
Drugs		
Corticosteroids	May be appropriate for mild cases; ease of administration; well known drug profile	Cushingoid side effects; ineffective in many cases
Azathioprine	Immunosuppressant; easy to use and well tolerated; drug levels can be monitored	Takes several months to see effect
Mycophenolate	Newer inhibitor of lymphocyte proliferation; may be more effective than azathioprine	Long-term effects unknown
Cyclophosphamide	Useful treatment for neuroblastoma; well-known drug profile	Dose-dependent reproductive side effects; not effective in some OMS
6-Mercaptopurine	Established role as "steroid sparer" in other disorders; low toxicity; ease of administration	No data in OMS
Methotrexate	A useful immunotherapy in some chronic autoimmune neurologic disorders; well-known drug profile	No data in OMS
Pheresis		
Plasmapheresis	Therapy directed at autoantibodies	Technically not feasible in infants and toddlers; may cause antibody rebound; should not be used as monotherapy; limited data
Immunoadsorption	More effective at removing antibody load	Expensive; technical expertise required; limited data
Leukocytopheresis	Allows selective removal of lymphocyte populations	No data in OMS

Treatments from different classes or within the same class may be combined

13.4.4 Treatment

Treatment strategies are still being optimized, although early intervention may not necessarily alter the clinical course (Rudnick et al. 2001a; Mitchell et al. 2002) (Table 13.3). Early treatment guided by CSF lymphocyte immunophenotyping as performed at the National Pediatric Myoclonus Center (Pranzatelli et al. 1998a, 2002b) may improve outcome. In that approach, an attempt is made to achieve fast and complete neurologic remission using immunologic markers as surrogates for disease monitoring. Children with OMS may require long-term therapy.

13.4.4.1 Neuromodulation

ACTH, which binds to CNS melanocortin receptors (Wilberg et al. 2000), and steroids, can induce a neurologic remission (Pranzatelli 1996) A high-dose protocol is quite efficacious (Pranzatelli et al. 1998b). Relapse on withdrawal from ACTH or steroids is common unless other immunotherapies have been in-

stituted. Symptomatic treatment with neuropsychotrophic drugs for ADD, rage attacks, and sleep disturbance is usually required for severe OMS and can be used in combination with immunotherapy. A study from the Pediatric Oncology Group (Russo et al. 1997) suggests that chemotherapy may decrease the likelihood of long-term neurologic deficits, while another recent report from the Children's Cancer Group (Rudnick et al. 2001b) noted that children with more advanced stage disease had better outcomes with regard to late neurologic sequelae. Based on these series, the Children's Oncology Group is designing a prospective protocol for OMS patients whereby all patients will receive steroids and chemotherapy and treatment with gammaglobulin will be randomized.

13.4.4.2 Adjunctive Therapy

Neuropsychological testing and IQ monitoring should be performed in all children with neuroblastoma and OMS. Supportive treatment includes speech therapy, early intervention programs, and contact with normal healthy children of the same age and physical therapy.

13.4.4.3 Precautions

Immunizations are considered hazardous in OMS because it activates T cells, which are already acti-vated (Pranzatelli et al. 2002). Caution is taken to avoid live-virus immunizations and groupings of multiple vaccines. Children exposed to varicella while on immunotherapy should be treated accordingly. It is noted that sedatives, such as midazolam, fentanyl, chloral hydrate, or diphenhydramine may cause paradoxical excitation, and sometimes worsen symptoms (Tate et al. 1994). Intravenous propofol is often a short-acting efficacious alternative.

13.5 Treatment-Related Neurologic Complications

All multi-modality treatments for neuroblastoma can be complicated by a number of potential acute and chronic neurologic complications that may warrant immediate attention or long-term therapy; these include metabolic abnormalities (Antunes 2000), CNS changes and peripheral neuropathies (Reddy and Witek 2003; Yu et al. 1998; Cheung et al. 2001), infectious complications resulting in brain abscesses, meningitis, or encephalitis (Tasdemiroglu and Patchell 1997), or vascular events as a consequence of surgery, radiation therapy, or chemotherapy. The reader is referred to Chap. 18 on quality of life and late effects for further references.

13.6 Conclusions

The management of patients with neuroblastoma mandates an understanding of the unique biology, anatomic distribution, and the signs and symptoms that can ensue. The tumor, its treatment, and the host response to the tumor can result in a myriad of neurologic complications, both acute and long term. Isolated CNS relapses are increasingly recognized as a complication of metastatic neuroblastoma occurring in the first 1–2 years after diagnosis. Because of the poor reversibility of neuronal damage, early detection and intervention of neurologic complications using an interdisciplinary approach are critical in minimizing late sequelae, implementing effective palliation, and maximizing quality of life. Clearly, treatment decisions must be based on the patient's age and long-term prognosis.

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Immunology and Immunotherapy

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Contents

14.1 Introduction: The Case for Immunotherapy

Although proposed as a potential therapy for cancer over a century ago, immunological-based strategies have only become a reality in the past two decades. Both the innate and adaptive immune responses are believed to play key roles in tumor surveillance (Diefenbach and Raulet 2002). While innate immunity relies on phagocytes, natural killer (NK) cells, natural antibodies, and complement proteins, adaptive immunity recruits antigen-presenting cells (APC), T cells and B cells (Janeway et al. 2001). As a primeval system of defense, innate immunity depends on invariant receptors recognizing common features of tumor cells, although it has no immunological memory and can often be evaded. The ability to recognize tumors specifically and to prevent their regrowth becomes more efficient with the emergence of adaptive immunity. This property of specificity is based on clonal selection of lymphocytes bearing antigen receptors. A broad range of effector cells, cytokines, chemokines, antibodies, and their recombinant products have been tested in passive immunotherapy in pre-clinical and clinical settings. In addition, tumors and tumor-derived products can be rendered highly effective in stimulating an active immune response.

Like most metastatic solid tumors, stage-4 neuroblastomas (NB) diagnosed after 18 month of age are often incurable with standard multi-modality therapy. Many of these patients achieve near-complete remissions, only to succumb to tumor recurrence within 4 years from diagnosis. Most pre-clinical models testing various forms of immunotherapy document

the greatest efficacy when immunotherapy is applied at the time of small tumor load; thus, application of immunotherapeutic interventions for patients with high-risk NB is likely to be most effective if utilized at the time of minimal residual disease.

14.2 The Immunobiology of Neuroblastoma

To recognize human NB, antibodies, T-cell receptors, and receptors of the innate immune system (e.g., NK, NKT, and γ/δ+T cells) (Jameson et al. 2003) have been explored. Antigens recognized by antibodies on NB are summarized in Table 14.1. Several antigen systems recognized by cytotoxic T lymphocytes (CTLS) have also been demonstrated in NB; these include cancer–testes antigens [*MAGE* (Ishida et al. 1996; Cheung et al. 1998),[5] *GAGE* (Cheung and Cheung 1997), NY-Eso-1 (Soling et al. 1999)], *MYCN* oncogene (Sarkar and Nuchtern 2000), kinase (ALK; Passoni et al. 2002), and others. The activation of antigen-specific T lymphocytes is a multi-step process requiring antigen-specific triggering of the T-cell receptor (TCR) complex on the T cell, and additional signaling via the costimulatory molecules CD28 that interact with CD80 (B7–1/BB1) and CD86 (B7–2/B70)

Table 14.1. Neuroblastoma antigens recognized by antibodies

Biochemical nature Antigen	Target antigen	Antibody forms		Immunoconjugates
GD2	Ganglioside	Human:	OFA-1-2 (Tai et al. 1984)	
		Humanized:	KM8138 (Nakamura et al. 2001)	
		Chimeric:	ch14.18 (Yu et al. 1998)	IL-2 (Hank et al. 1996)
			KM1138 (Nakamura et al. 2001)	GM-CSF (Batova et al. 1999)
		Mouse:	3F8 (Cheung et al. 1987)	^{131}I (Cheung et al. 1986, 2001b)
			14.G2a (Yu et al. 1997)	^{124}I (Larson et al. 1991)
			BW625 (Berthold et al. 1989)	^{225}Ac (Miederer et al. 2004)
			AI (Kawashima et al. 1988)	
		ScFv (Moutel et al. 1997; Tur et al. 2001a)		scFv-CIR (Krause et al. 1998; Rossig et al. 2001; Cheung et al. 2003c)
				scFv-PE-toxin (Tur et al. 2001b)
GD3	Ganglioside	Humanized:	KM8871 (Nakamura et al. 2001)	
		Chimeric:	KM871 (Nakamura et al. 2001; Scott et al. 2001)	I-131 (Scott et al. 2001)
		Mouse:	R24 (Houghton et al. 1985)	
GM2	Ganglioside	Humanized:	KM8969 (Nakamura et al. 2001)	
		Chimeric:	KM966 (Nakamura et al. 2001)	
NCAM (CD56)	Glycoprotein	Humanized:	HuN901 (Roguska et al. 1996)	Maytansinoid (Tassone et al. 2004)
		Mouse:	UJ13A (Lashford et al. 1987), N901 (McGarry et al. 1988)	I-131 (Goldman et al. 1984) blocked-Ricin (Lynch et al. 1997) CC-1065 (Chari et al. 1995)
L1-CAM	Glycoprotein	Chimeric:	chCE7 (Amstutz et al. 1993)	I-131 (Hoefnagel et al. 2003) Cu-64 (Novak-Hofer et al. 2003) Cu-67 (Carrel et al. 1997)
		Mouse:	CE7 (Schonmann et al. 1986)	
		ScFv (Carrel et a. 1997)		
GP58	Glycoprotein	Mouse:	8H9 (Modak et al. 2001)	scFv-Fc (Cheung et al. 2002) scFc-CIR (Cheung et al. 2003c)
GP95	Glycoprotein	Mouse:	BW575 (Berthold et al. 1989)	
NB-p260	Protein	Natural IgM (Ollert et al. 1996)		

ligands on the APCs, plus CD40-CD154 (CD40L) interactions. Adhesion molecules, such as leukocyte function antigen (LFA)-1, LFA-3, and intercellular adhesion molecule (ICAM)-1, are also important in the initial binding of CTLs to APC and to tumor targets. The CTLs must receive the appropriate help before expansion can occur. Antigen-specific T-helper cells with T-helper 1 (TH1) activity must be coactivated by the APCs. Th1 cells release Th1 cytokines, such as interferon (IFN)-γ and interleukin (IL)-12, which are also necessary for CTL activation, and interleukin-2 (IL-2), which is necessary for CTL expansion (Cheung and Rooney 2002). A tumor cell that induces the secretion of T-helper 2 (TH2) cytokines, such as IL-4 and IL-10, may promote antibody instead of CTL responses.

Because of the propensity for NB to undergo "spontaneous regression," many have implicated an endogenous anti-NB immune response. The recent discovery of a natural IgM anti-NB antibody in children suggests that innate immunity may have a potential role in surveillance against this tumor (Ollert et al. 1996). Besides IgM, lymphocytes of the innate immune system (NK, NKT, γ/δ-T cells) (Jameson et al. 2003) interact with tumors through unique activation and inhibitory receptors. The NK cells can lyse human NB in vitro and inhibit xenograft growth (Colucci et al. 2003; Cheung and Modak 2002). Specific ligands on tumor cells trigger activating or inhibitory receptors on NK cells (Schilbach et al. 2000). The NKT cells bear NK markers (CD161, CD122) and are thymus dependent, expressing CD3 plus TCR (Kronenberg and Gapin 2002). They recognize α-galactosyl-ceramide as well as ganglioside GD3 presented on CD1d, a nonclassical major histocompatibility (MHC) molecule (Wu et al. 2003). These NKT cells are effective anti-tumor vehicles in preclinical cellular targeting strategies (Metelitsa et al. 2001; Smyth et al. 2002). Human γδT cells isolated from PBL and expanded with IL-2 in vitro also mediate effective cytotoxicity against NB cells (Schilbach et al. 2000; Carding and Egan 2002).

14.3 How Neuroblastoma Escapes the Innate and Adaptive Immune Systems

Neuroblastoma employs a variety of tactics to evade the immune system (Table 14.2). It downregulates "immune-activating" while overexpressing "immune-inhibitory" receptors. By repressing the expression of class-I and class-II MHC (Lampson et al. 1983) as well as CD1d (Metelitsa et al. 2001), they interfere with both the afferent and efferent arms of adaptive immune response. In addition, NB cells can avoid immune recognition and destruction by releas-

Table 14.2. Neuroblastoma can evade the immune system

Immune function	Modulated Ags	Escape mechanism used by NB
Adaptive immunity		
T cells	↓MHC Class I	↓β2m, ↓TAP1, ↓TAP2, ↓LMP2, ↓LMP7*
	↓Adhesion	↓ICAM2, ↓ICAM3, ↓LFA3
Innate immunity		
NK cells	↓Activation	↓MICA (cytoplasmic), ↓MICB (cytoplasmic) (Raffaghello et al. 2004)
	↑*Inhibition*	Soluble MICA (Doubrovina et al. 2003; Raffaghello et al. 2004)
NKT cells	↓Activation	↓CD1d
Complement	↑Inhibition	CD46, ↑CD59
	↓Inhibition	↓CD55
Inhibition of immune cells		
Lymphocytes	↑Apoptosis	↑GD1a, ↑FasL, ↑GD2
APC	↓Function	↑GD2

* N.K. Cheung and W. Gerald, microarray gene expression analysis of human NB, unpublished results
LMP low molecular weight protein, *TAP* transporter-associated protein

ing the ganglioside GD1a which is directly toxic to human lymphocytes while uncoupling cell signaling through the NFκB pathway (Shen and Ladisch 2002), as well as disialoganglioside GD2 which interferes with T-cell (Li et al. 1995) and APC functions (Shurin et al. 2001; Heitger and Ladisch 1996). Furthermore, some NB cells have been shown to express FAS-ligand (Fas-L) (Shurin et al. 1998), which may act as a death signal causing apoptosis of effector cells, such as T cells or NK cells, which themselves express the surface Fas death receptor (Takamizawa et al. 2000; Li et al. 2002). On the other hand, when confronted with antibodies, despite their low levels of CD55 (decay accelerating factor), some neuroblastomas cells have increased CD59 (homologous restriction factor) and sufficient CD46 (membrane cofactor protein) expression to render them resistant to terminal complement pathways (see 14.4.1). Effective in vivo immunotherapy must circumvent these protective mechanisms of NB in order to ultimately maximize clinical benefit.

14.4 Humoral Immunotherapy

With the introduction of the hybridoma technique in 1975 by Koehler and Milstein (Koehler and Milstein 1975), and the more recent emergence of recombinant technology, monoclonal antibodies have transformed the original "serum therapy" concept of Emil Behring and Shibasaburo Kitasato into a pharmaceutical industry. Monoclonal antibodies (MAb) have generated excitement on many frontiers and will likely play an important role in the future of cancer medicine.

14.4.1 Effector Mechanisms of MAb

Anti-tumor MAb can carry out highly effective tumoricidal functions both in vitro and in vivo; these include signaling through receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-mediated cytotoxicity (CMC) (Cheung 2004). MAb vary in their ability to induce downstream effects. For example, MAb 3F8 is unique among anti-GD2 antibodies in its ability to induce apoptosis among EL4 murine lymphoma cells (Tomlinson et al., unpublished results). MAb can also block receptor functions (e.g., EGF-R) (Mendelsohn 2003), and vascular endothelial growth factor receptor (VEGF-R) (Prewett et al. 1999) by interfering with binding of the natural ligands.

There are three types of IgG Fc receptors (FcγR): FcγRI (CD64); FcγRII (CD32); and low-affinity FcγRIII (CD16; Ravetch and Bolland 2001). Most FcγRs are of the activating type except for the inhibitory receptor FcγRIIB. Recent correlation of FcγRIIIA polymorphism with clinical response to rituximab suggests that IgG affinity for Fc receptor can influence anti-tumor response in patients (Cartron et al. 2002; Kimberly et al. 2002). Neuroblastoma cells are effectively killed by NK lymphocytes, granulocytes, and activated monocytes in vitro in the presence of specific MAb. Chimeric hIgG1 specific for GD2 (ch14.18) fused to GM-CSF depends on FcγRII in neutrophil ADCC (Metelitsa et al. 2002). In contrast, 3F8 (murine IgG3 specific for GD2) utilizes both FcγRII and FcγRIII for ADCC (Kushner and Cheung 1992). In addition to FcR, adhesion molecules including CR3 (CD11b/Cd18) (Metelitsa et al. 2002; Kushner and Cheung 1992; Ottonello et al. 1999) and CD66b (Ottonello et al. 1999) for neutrophils, and LFA-1 (CD11a/CD18) for lymphocytes (Edwards et al. 1992), are important in modulating tumor cytotoxicity. When their expression is increased by granulocyte macrophage colony stimulating factor (GM-CSF) or IFN-γ, granulocyte ADCC can be enhanced (Metelitsa et al. 2002; Kushner and Cheung 1989; Vaickus et al. 1990; Masucci et al. 1990). Similarly, IL-2 can increase lymphocyte ADCC (Munn and Cheung 1987; Sondel and Hank 1997). Furthermore, since both GM-CSF and IL-2 expand the effector cell pools, they have potential clinical benefits in tumor therapy when combined with MAb (Fig. 14.1).

Most NB cell lines are sensitive to CMC; however, some are resistant to complement because of anti-complement surface proteins, including CD55 (Cheung et al. 1988; Gorter and Meri 1999), CD59 (Gorter and Meri 1999; Cheng et al. 2000b) and CD46 (Gorter and Meri 1999). The effect of complement activation extends beyond direct tumor lysis. C3b deposited on tumor cells is rapidly cleaved by plasma protease factor I to iC3b. Through CR3 (Mac-1

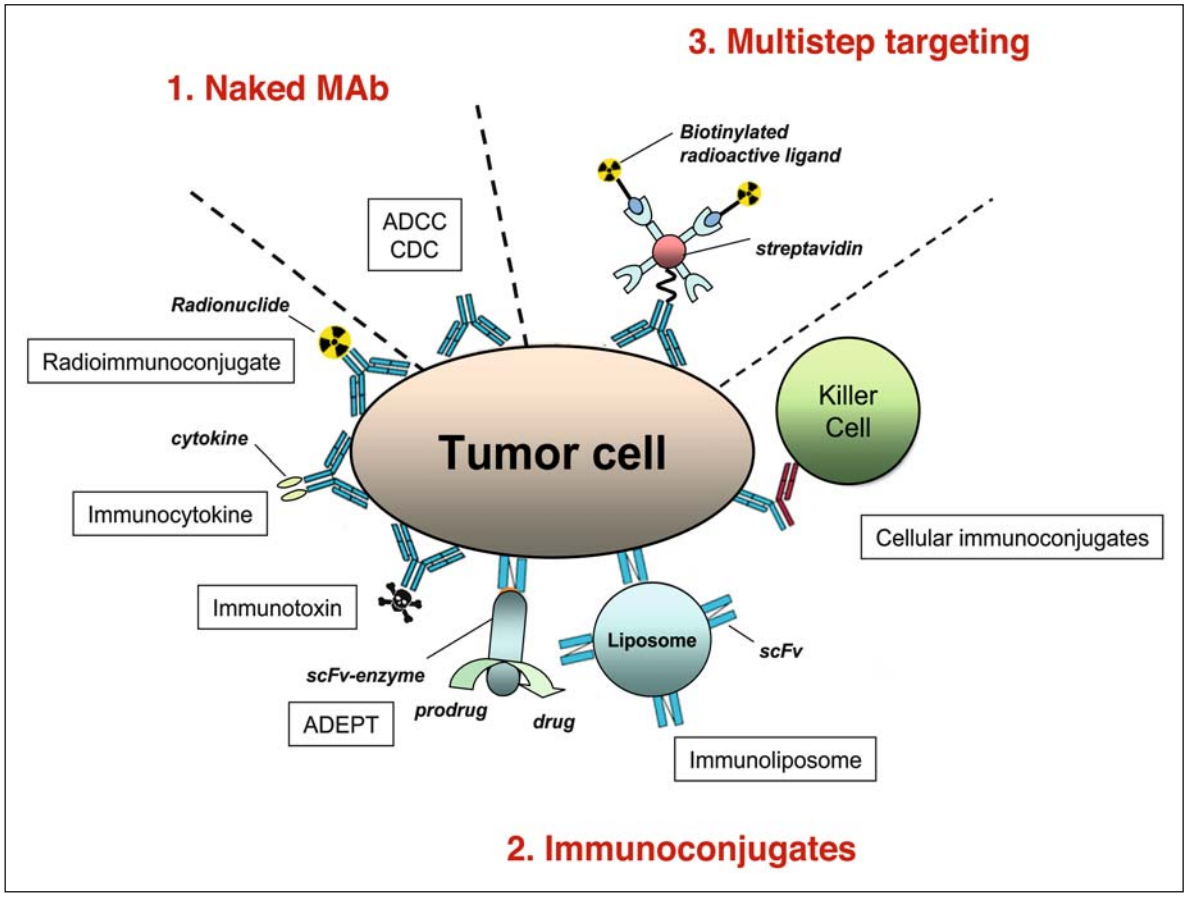

Figure 14.1

Effector mechanisms of monoclonal antibodies. *ADEPT* antibody directed enzyme prodrug therapy; *ADCC* antibody-dependent cell-mediated cytotoxicity, *CMC* complement mediated cytotoxicity, *MAb* monoclonal antibody, *scFv* single-chain Fv fragment. (From Cheung 2004; Carter 2001).

or alpha$_M$beta$_2$-integrin), and CR4 (CD11c/CD18, alpha$_X$beta$_2$-integrin) receptors on leukocytes, tumor cells are opsonized (Ross et al. 1999). C3a and C5a, byproducts of complement activation, are potent mediators of inflammation (Hugli 1978) and are chemotactic for phagocytic leukocytes, drawing them to the tumor sites. C5a can also induce secondary cytokines to increase vascular permeability for both MAb and effector cells.

14.4.2 Clinical Application of MAb

14.4.2.1 Naked MAb

Among the ganglioside antigens, GD2 is particularly relevant for the treatment of NB. Neuroblastoma cells express GD2 at high density, with relatively little heterogeneity within tumors or among patients (Schulz et al. 1984; Kramer et al. 1998). GD2 is not lost from the cell surface when bound to antibodies. In normal serum, GD2 is found in low concentrations typically bound to lipoproteins. This may explain why circu-

Table 14.3. Use of MAb for clinical diagnosis and therapy of neuroblastoma[a]

Radiolabeled antibody for imaging	Sensitivity
^{131}I-UJ13A[a] (Goldman et al. 1984)	89%
^{131}I-3F8 (Miraldi et al. 1986; Yeh et al. 1991)	90%
^{131}I-14.G2a (Podoloff et al. 1991)	85%
^{99m}Tc-BW575 (Smolarz et al. 1989)	90%
^{131}I-CE7 (Carrel et al. 1997)	–

Radiolabeled antibody for therapy	Overall response[b]	BM CR[b]
^{131}I-UJ13A (Kemshead et al. 1985)	–	1/4
^{131}I-3F8 (Larson et al. 2000)	2/10	2/10
^{131}I-3F8 (IT) (Kramer et al. 2000b)	–[c]	–

Naked antibody for therapy[d]	Cytokine	Total dose mg/m^2	Overall Response			BM CR	Bone CR/PR	Masses CR/PR
			N	CR/VGPR	PR			
Phase I								
3F8 (Cheung et al. 1987)	–	5–100	8	0	1	1/8[e]	1/8	0/4
14.G2a (Huang et al. 1992)	–	25–200	15	1	0	2/8	0/12	0/11
14.G2a (Handgretinger et al. 1992)	–	100–400	6	2	2	–	–	–
14.G2a (Murray et al. 1994)	–	50–200	5	0	2	–	–	–
ch14.18 (Yu et al. 1998)	–	10–200	9	0	1	3/7	0/9	1/8
ch14.18 (Handgretinger et al. 1995)	–	150–250	9	2	2	–	–	–
14.G2a (Frost et al. 1997)	IL-2 +/– GMCSF	10–100	31	0	1	–	–	1/1
Phase II								
3F8 (Cheung et al. 1998c)	–	50	16	1	0	3/8	2/7	0/8
3F8 (Kushner et al. 2001)	GM-CSF	100[f]	43[g]	11	–	31/42	–	–
ch14.18 (Simon et al. 2004)	–	100	166	–	–	↓marrow relapse, ↑survival, but no effect on event-free survival		
ch14.18 (Yu et al. 1997)	GM-CSF	200	27	1	3	5/13	0/9	0/7

[a] Antibodies were injected intravenously unless otherwise stated. *IT* = intrathecal
[b] BM CR was proportion of patients clearing marrow disease. Overall response = CR + PR, unless otherwise stated
[c] 1 radiographic and 2 CSF cytologic responses from the first 12 patients
[d] I=phase I, II=phase II
[e] No. of responses/no. of evaluable patients with neuroblastoma
[f] Patients without HAMA after 400 mg/m^2 (4 cycles of 10 days/cycle, 10 mg/m^2 day) were treated q1–2 months (100 mg/m^2/cycle) until 24 months from the first day of enrollment on the protocol
[g] Only patients with evaluable disease (primary resistance to induction or secondary resistance to retrieval therapy) were included in this analysis

lating GD2 in most patients has not interfered with antibody targeting which typically achieved high percent injected dose per gram (%ID/g) with high tumor-to-normal-tissue ratio, and unusually low liver and spleen uptake in patient studies (Yeh et al. 1991; Larson et al. 2000).

Clinical Trials

The first IgG anti-GD2 MAb to undergo clinical testing was 3F8 (murine IgG3; Table 14.3; Cheung et al. 1987, 1998a), followed subsequently by 14.G2a (murine IgG2a; Handgretinger et al. 1992; Frost et al. 1997). and a chimeric form ch14.18 (Yu et al. 1998). In clinical trials (Yu et al. 1998; Handgretinger et al. 1995; Ozkaynak et al. 2000), ch14.18 had a more prolonged serum half-life and lower immunogenicity compared with its mouse counterpart. Most of the responses noted in phase I and II clinical trials of unconjugated anti-GD2 MAbs involved metastatic disease in bone marrow, with less certain effects on bulky tumors. The predominant toxicity was pain, attributed to cross-reactivity of antibodies with peripheral pain fibers (Lammie et al. 1993; Xiao et al. 1997; Yuki et al. 1997). Other side effects included tachycardia, hypotension, fever, anaphylactoid reactions, nausea, vomiting, diarrhea, and transient neuropathy. Most side effects were dose dependent, rarely noted at dosages of $<10\ mg/m^2$ (14G.2a or ch14.18) or $<1\ mg/m^2$ (3F8), and compatible with outpatient treatment. Over a 15-year period, more than 350 patients with NB have been treated with >10,000 infusions of 3F8 at Memorial Sloan-Kettering Cancer Center. There was no treatment-associated mortality. Similarly, the COG (and its component organizations) have been administering 14.G2a or ch14.18 anti-GD2 MAb since 1989 with good safety record. Patients have been followed for up to 15 years. No long-term neurological complications have been noted. The clinical development of anti-GD2 MAb was partly hindered by its pain side effects which have precluded dose escalation.

Although the anti-tumor effect of anti-GD2 MAb was modest, response of microscopic marrow disease was more consistent (Cheung et al. 1998b, 2001a, 2003a,b; Kushner et al. 2001). An association of human anti-mouse antibody (HAMA) response and favorable patient outcome, plus the induction of anti-idiotypic Ab2 and anti-idiotypic Ab3/antibodies through the idiotype network, implicate the potential role of the host immune response in maintaining clinical remission (Cheung et al. 1994, 2000). In a recent update of 98 patients with stage-4 NB newly diagnosed after 18 month of age, who received anti-GD2 MAb 3F8 as part of their combined modality therapy at MSKCC, the induction of a HAMA response and a lower number of tumor cells in the diagnostic bone marrow were the most significant independent prognostic variables for both progression-free and overall survival (N.-K.V. Cheung et al., unpublished results).

14.4.2.2 Antibody in Combination with Cytokines

While cytokines can induce proliferation and activation of effector cells, in the absence of cytophilic MAb, they lack tumor selectivity. Antibody-dependent cellular cytotoxicity (ADCC) is greatly augmented in vitro by cytokines (Kushner and Cheung 1989; Munn and Cheung 1987; Hank et al. 1990; Barker and Reisfeld 1993). Clinical trials of anti-NB antibody combined with GM-CSF (Ozkaynak et al. 2000; Kushner et al. 2001) or combined with IL-2 (Sondel and Hank 1997; Frost et al. 1997) have shown modest antitumor effects. While large tumor masses rarely responded to these combinations, microscopic marrow disease showed consistent response in over 50% of patients whether measured by conventional histology (Kushner et al. 2001) or by RT-PCR (Cheung et al. 2003b). In addition, response may translate into improved survival (Cheung et al. 1998a, 2003b). To prospectively evaluate the clinical efficacy of antibody in combination with cytokine therapy, ch14.18 is being tested in combination with GM-CSF and IL-2 following autologous stem-cell transplant in a phase-III U.S. Children's Oncology Group (COG) randomized trial (A. Yu et al., unpublished results).

14.4.2.3 Antibody Immunoconjugates

The clinical utility of naked MAb has been limited by both host (number and activity of effector cells, FcR polymorphism, and interference by inhibitory FcR) and tumor factors (antigen heterogeneity and com-

plement regulatory proteins). While the CMC and ADCC functions of naked MAb (Fig. 14.1) can be improved by altering the Fc protein structure or by modifying Fc-glycosylation, substantial gains in the clinical potentials of MAb can be achieved using immunoconjugates (Cheung 2004) (Fig. 14.1); these include (a) radioimmunoconjugates to deliver β- and α- emitters (Goldenberg 2003), (b) immunocytokines to deliver cytokines to tumor sites while minimizing systemic toxicities (Davis and Gillies 2003), (c) immunotoxins (Pastan 2003), (d) immunoliposomes to deliver drugs or toxins (Allen et al. 2002), and (e) bispecific MAb (pretargeted to tumor or by ex vivo arming) to direct cells or ligands selectively to tumor (van Spriel et al. 2000).

Radioimmunoconjugates

MAb have the potential to target and ablate tumors in radioimmunotherapy (RIT) (Cheung 2004; Goldenberg 2003). In preclinical models, ablation of established xenografts is possible (Cheung et al. 1986), although radiation damage to the marrow remains dose limiting. Unlike naked antibodies, the bystander effect of RIT from cross-firing of the radioisotopes accounts for most of the toxicities of radioimmunoconjugates, hence limiting their efficacy. Most clinical applications of RIT utilize β-emitting radioimmunoconjugates. β-particles have a relatively long range (0.8–5 mm) and low linear energy transfer (approximately 0.2 keV/μm), resulting in radiation to both antigen-negative tumors as well as innocent bystanders; thus, β-emitters (^{131}I or ^{90}Y) can treat bulky diseases effectively but are not optimal for the killing of single cells or micrometastasis. Because of its γ-emission, ^{131}I also permits dosimetry studies, although it also poses a radio-hazard at high treatment doses, necessitating patient isolation. ^{131}I is also dehalogenated in vivo with potential to damage the thyroid gland. ^{90}Y is a pure β-emitter; its lack of γ-emissions allows outpatient treatment. However, ^{90}Y, which is a pure β-emitter, requires more extensive chemical modification of the MAb than ^{131}I and is deposited in bone when dissociated from the complex. Alpha particles are helium nuclei. When compared with β-particles, they have a shorter range (50–80 μm) and a higher linear energy transfer (approximately 100 keV/μm) (McDevitt et al. 1998). As few as one or two α-particles can destroy a target cell. RIT using α-emitters should result in less nonspecific toxicity to normal bystanders as well as more efficient single cell killing, an ideal setting for controlling minimal residual disease. Alpha-particle-emitting isotopes, such as astatine-211 and bismuth-213, have been tested in clinical trials with minimal extramedullary toxicities (Zalutsky and Vaidyanathan 2000; Jurcic et al. 2002).

UJ13A (anti-NCAM) was the first antibody to undergo clinical testing for radioimaging and radioimmunotherapy (Lashford et al. 1987). ^{131}I-3F8 (anti-GD2, 6–28 mCi/kg) achieved responses in both soft tissue masses and bone marrow (Larson et al. 2000). The use of myeloablative ^{131}I-3F8 (20 mCi/kg) to consolidate remission was tested in patients (>1 year of age) newly diagnosed with stage-4 NB (Cheung et al. 2001a). Extramedullary toxicities were limited to hypothyroidism, which occurred despite aggressive thyroid protection using potassium iodide, liothyronine (T3), and potassium perchlorate. ^{131}I-MAb was also tested in RIT of leptomeningeal cancers in children by intraventricular administration (Lashford et al. 1988; Kramer et al. 2000). Estimated radiation doses of 14.9–56 cGy/mCi to the cerebrospinal fluid were achieved with ^{131}I-3F8, with less than 2 cGy/mCi to blood and other organs outside the CNS (Kramer et al. 2000).

Multistep Targeting (MST). In order to improve tumor uptake and reduce systemic toxicity, a multistep procedure which pretargets the antibody before the binding of the cytotoxic ligand to the tumor has been successfully employed (Cheung 2004; Goldenberg 2003; Boerman et al. 2003; Goldenberg et al. 2003; Paganelli et al. 2001; Cremonesi et al. 1999; Cheung et al. 2004) (Fig. 14.1). In the first step, an antibody-streptavidin conjugate or fusion protein is allowed to localize to tumors in vivo, and any excess is cleared from the blood. A small radiolabeled biotinylated ligand is then injected intravenously. By virtue of the high-affinity interaction, the ligand penetrates tissues rapidly and is strongly taken up by the antibody conjugate at the tumor site. Because of the short transit time of the toxic ligand (radionuclide or tox-

in), a substantial improvement in the therapeutic ratio is achievable without sacrificing the percent injected dose per gram in tumor. Neuroblastoma is uniquely suitable for MST because of its abundance of surface ganglioside GD2. Anti-GD2 5F11-single-chain Fv-fragment (scFv)-streptavidin is a homotetramer with improved avidity and highly favorable tumor-to-nontumor ratios in MST, achieving >50% improvement in radiation dose ratio of tumor to blood. In addition, because biotinylated polypeptides can achieve selective tumor targeting when MST is applied, a large repertoire of agents can potentially be explored for targeting to NB (Cheung et al. 2004).

Immunocytokines

Cell-mediated cytotoxicity can be highly effective against tumors in vitro and in animal models. Immunocytokines (Davis and Gillies 2003; Lode and Reisfeld 2000) have shown remarkable success in activating and redirecting effectors to human tumors. The majority of these studies have focused on NK, NKT, T cells (Davis and Gillies 2003), and granulocytes (Metelitsa et al. 2002). They are active in ADCC in vitro activating effector cells appropriately through their cytokine receptors. In vivo administration of the ch14.18-IL-2 fusion protein induces long-term anti-tumor immunity (Davis and Gillies 2003; Lode and Reisfeld 2000), and provides greater protection against localized or metastatic murine neuroblastomas than does treatment consisting of the identical amounts of ch14.18 antibody and IL-2 given as separate molecules (Lode et al. 1997). Following initial successes with IL-2 and GM-CSF immunocytokines, constructs containing other cytokines have also been tested with encouraging results (Davis and Gillies 2003); these include IL-12, tumor necrosis factor (TNF)-α, and lymphotoxin. Clinical testing of the humanized form of this immunocytokine hu14.18-IL-2 is underway in adults with melanoma and children with NB (King et al. 2002). Immune activation was evidenced by increased serum IL-2 receptor levels, lymphocytosis, and induction of an antibody response against the hu14.18-IL-2. Clinical efficacy is yet to be established.

Immunotoxins

Ribosome inactivating toxins can be potent cancer drugs. One major limitation is the lack of tumor selectivity (Reiter 2001). Two-chain toxins [e.g., ricin and diphtheria toxin (DT)] utilize their B-chain for cell binding and their A chain for inhibition of protein synthesis, while other toxins [Pseudomonas exotoxin (PE), Pokeweed antiviral protein (PAP), gelonin] have a built-in cell attachment site. When conjugated to MAb, they become immunotoxins. In recombinant toxins (e.g., PE40, PE38, or diphtheria toxin DAB_{486}), the cell-binding domains are replaced by scFv. Anti-GD2 monoclonal MAb have been conjugated to different toxins: ricin toxin A chain (Wargalla and Reisfeld 1989; Manzke et al. 2001), DT (Thomas et al. 2002), PE (Fur et al. 2001a), and gelonin (Mujoo et al. 1991). A common toxicity is the vascular leak syndrome, characterized by fluid overload, dyspnea, and sensory-motor neuropathies. Other natural compound toxins have also been explored as immunoconjugates including cobra venom factor (Juhl et al. 1997) and staphylococcal enterotoxin A (SEA; Holzer et al. 1995). Anti-GD2 immunoliposomes have also been explored in vitro for delivering adriamycin (Ohta et al. 1993) and fenretinide (Raffaghello et al. 2003).

Cellular Immunoconjugates (Bispecific Antibodies)

Tumor-selective MAb can be rendered cytophilic by conjugation with MAb specific for trigger molecules on T lymphocytes, NK cells, and granulocytes. These molecules include CD3 (Manzke et al. 2001), CD28 (Bauer et al. 1999), Fc receptors (CD64, CD16) (Michon et al. 1995a,b), and FcαRI (CD89) (van Spriel et al. 2000). While one binding site of the bispecific antibody (van Priel et al. 2000; Friedrich et al. 2002; Scheffold et al. 2002) engages effector cells, the other binding site determines tumor specificity. Since serum IgG competes for FcR, MAb made to recognize the FcR outside its Fc-binding site have been developed to circumvent this concern. Although bispecific MAb have potential in targeting small ligands (e.g., in MST), their clinical application in cellular immunoconjugates has been complicated by the generalized cytokine release from leukocytes and the inherent limitations of trafficking of effector cells into tumors (Friedrich et al. 2002).

Alternative Targets for Anticancer Antibodies

Besides the ability to block receptors from interaction with their natural ligand, MAb can inhibit receptor dimerization or receptor interaction with co-receptors (Agus et al. 2002). While most of the MAb targeting effort has been focused on individual tumor cells, alternative strategies directed at tumor neovasculature (Halin and Neri 2001) or tumor stroma (Hofheinz et al. 2003) are promising approaches. MAb can be made to neutralize the angiogenic factor VEGF (e.g., bevacizumab, Avastin) (Presta et al. 1997), or to block the VEGF-R2/KDR (e.g., IMC-1C11, chimeric anti-KDR; see Chap. 16) (Zhu et al. 2003; Posey et al. 2003). Targeting tumor vasculature may have significant advantages over direct tumor targeting, in that endothelial cells, unlike tumor cells, are less likely to acquire resistance. Another angiogenesis target is αVβ3 integrin which initiates endothelial proliferation, migration, and matrix remodeling. Based on the preclinical antitumor activity of MAb specific for αVβ3 (Gutheil et al. 2000), and the involvement of αVβ3 in NB (Lode et al. 1999), the chimeric IgG1 (MEDI-522) currently in clinical trial may have potential in treating NB.

14.4.3 Humoral Vaccines

14.4.3.1 Ganglioside-KLH Vaccines

Most antigens are able to induce antibodies only with the help of T cells. Other antigens induce immunity without T-cell help, but the antibody response is generally restricted to IgM class, and without persistent antigen, it is usually short-lived with weak memory. The antibody response to tolerated antigens resembles that to T-independent antigens. Many tumor antigens (e.g., carbohydrates or glycolipids) are believed to lack helper T-cell epitopes and thus often behave as tolerogens or poor immunogens. To enhance immunogenicity of carbohydrate antigens (GM2, GD2, and GD3), covalent attachment to carrier proteins (e.g., keyhole limpet hemocyanin) has been highly successful, especially when used in conjunction with adjuvants (e.g., saponin QS-21) that activate antigen-presenting cells (e.g., macrophages) and T lymphocytes (Ragupathi et al. 2003). An alternative to using natural gangliosides is the employment of peptide mimics which can induce strong anti-ganglioside antibody responses in preclinical models (Tsao et al. 2002).

14.4.3.2 Anti-Idiotype Vaccine

Anti-idiotypic (Ab2) antibodies are potential tumor antigen surrogates (Kennedy et al. 1987). Ab2 can induce anti-anti-idiotypic antibodies (Ab3) that cross-react with the original target tumor antigen. As tumor vaccines, Ab2 antibodies have advantages over native antigens (e.g., carbohydrates) because they induce better T-cell help and stronger antibody response. Since they can be easily manufactured, and modified by genetic engineering, they are preferable to difficult chemical synthesis (e.g., complex carbohydrates). Anti-GD2 anti-idiotypic vaccines have been used successfully in tumor models (Cheung et al. 1993; Sen et al. 1998; Zeytin et al. 2000) and are being evaluated in patients with NB and melanoma (Foon et al. 2000; Batova et al. 2002).

14.5 Cellular Immunotherapy

14.5.1 Activation of NK and NKT Cells

The observed spontaneous tumor regressions seen in episodes of sepsis prompted Coley to test bacterial toxins as a form of immunotherapy. While occasional anti-tumor effects were observed using Bacillus Calmette Guerin in the early 1970s, this "non-specific immune activation" strategy has evolved into the application of highly purified recombinant human cytokines such as IFN-γ (Evans et al. 1989), and IL-2 (Handgretinger et al. 1987). As single agents, their anti-tumor effect was modest whether they were used alone (Bauer et al. 1995), or in combination with autologous stem cell transplantation (Favrot et al. 1990; Toren et al. 2000; Bonig et al. 2000; Pession et al. 1998; Marti et al. 1995), despite evidence of immune activation (increase in NK cells, CD8+ cells, and soluble IL-2-Rα) (Vlk et al. 2000). Other NK and NKT-activating cytokines explored in NB therapy include IL-12 (Shimizu et al. 2001), IL-15 (Satoh et al. 1998), and IL-18 (Heuer et al. 1999), as single agents or in combination with IL-2 in murine models (Wigginton and Wiltrout 2002). Dendritic cells have also been

used to activate NK cells via CD40 (Valteau-Couanet et al. 2002; Turner et al. 2001). Galactosyl-ceramide is a potent stimulator of NKT cells with potential for clinical applications (Wu et al. 2003; Metelitsa et al. 2001; Smyth et al. 2002). Alternatively, NK/NKT cells can also be gene modified with scFv chimeric immune receptor (CIR) to be redirected to human tumors (Koehne et al. 2003).

14.5.2 Activation of MHC-Restricted T Cells

Pre-clinical research, particularly in murine models, has identified four conceptually distinct strategies for inducing T cells capable to destroy tumors in vivo through MHC-restricted recognition by αβ+ T-cell receptors expressed by the majority of T cells. They include the following:

1. Administration of systemic or locally injected immunostimulants to tumor-bearing animals to effectively expand already activated endogenous tumor-reactive T cells.
2. In vivo administration of a tumor vaccine, either purified or crude/complex, containing antigenic components of the tumor itself, designed to induce and expand endogenous tumor-reactive T cells to mediate tumor selective destruction.
3. In vitro activation, selection, manipulation, and/or expansion to generate a population of tumor-reactive autologous T cells able to mediate anti-tumor destruction in vivo upon adoptive transfer.
4. Infusion of allogeneic T cells, either directly obtained ex vivo, or potentially modified in vitro, to induce a "graft-vs-tumor" response which takes advantage of genetic or physiological differences between the tumor-bearing host and the healthy allogeneic lymphocyte donor.

14.5.3 Pre-clinical and Clinical Testing of T-cell Based Therapy in Neuroblastoma

14.5.3.1 Immunostimulants

In vivo administration of systemic IL-2 and IL-12 can result in anti-tumor effects against syngeneic NB tumors and is mediated, at least in part, through CD8+ T cells (Wigginton and Wiltrout 2002; Siapati et al. 2003). Similarly, Flt-3L is a cytokine known to activate APC directly. It can induce NK cell expansion and enhance APC function to provide better specific activation of T cells. Mice bearing weakly immunogenic NB can develop anti-tumor responses following 10–17 consecutive days of Flt-3L treatment. Following tumor eradication, these animals demonstrate protective T-cell-mediated immunity to tumor re-challenge (Neal et al. 2003). The specific antigens recognized by T cells in these tumor models have not been characterized.

Effective endogenous T-cell expansion and immunization can be induced by direct injection of immune stimulants such as IL-2, GM-CSF, CpG (Sandler et al. 2003), or B7 (Todo et al. 2001) directly into sites of tumor. While this cytokine injection approach for activating T cells has been demonstrated in murine models for other tumors, it has not yet been evaluated for patients with NB. The administration of fusion proteins consisting of monoclonal antibodies linked to cytokines (IL-2 or GM-CSF) may target cytokines to tumor sites and be alternatives to direct intratumor injection of immune stimulants (Davis and Gillies 2003).

14.5.3.2 Tumor Vaccines

Detailed analyses of more common tumors have identified immunodominant peptides recognized by either antibodies (SEREX) (Scanlan et al. 2002) or cytotoxic T lymphocytes (van der Bruggen et al. 2002). When combined with adjuvants, these purified proteins or peptides can be presented on APC in vitro or in vivo as vaccines to T cells. Alternatively, direct transfer of DNA coding for the immuno-dominant peptide or protein has also been successful (Pertl et al. 2003). While these approaches are underway for treatment of melanomas and carcinomas, they have not yet been pursued for NB. An alternative vaccine approach utilized whole tumor cells to provide a "cocktail" of antigenic targets. Pre-clinical analyses have utilized vaccines comprised of autologous or allogeneic tumor cells. Crude cell lysates have been superseded by in vitro expanded dendritic cells that have been "pulsed" with crude cell lysates. As APCs,

these pulsed dendritic cells activate T-cells in vivo. In children with NB this approach is safe (Geiger et al. 2001). Although NB-specific T cells were stimulated, no anti-tumor effect was observed.

Immunization with tumor cells genetically modified to express immuno-stimulatory proteins (e.g., IL-1 and TNF [Coze et al. 2001], Fas [Shimizu et al. 1999], IL-2 [Bowman et al. 1998], IL-12 [Yoshida et al. 1999; Davidoff et al. 1999; Pertl et al. 2001], GM-CSF [Yoshida et al. 1999], MHC class II [Hock et al. 1995], B7 [Enomoto et al. 1997]) are effective strategies in murine models. CD8+ tumor-specific T cells, induced by these vaccines, specifically and effectively recognize the non-transfected parental tumor cells, both in vitro as well as in vivo, demonstrating protective immunity against subsequent tumor challenge. This approach was tested in patients with NB where a vaccine of allogeneic NB cells transfected with IL-2 and lymphotactin was used (Haight et al. 2000; Brenner et al. 2000). While lymphotactin acts to attract lymphocytes to the site where it is released, IL-2 induces expansion of those T cells stimulated by the irradiated allogeneic tumor cells (Brenner et al. 2000; Rousseau et a. 2003). Immunization with up to 10^7 transfected cells per kilogram body weight subcutaneously at weekly intervals was well tolerated. Following this immunization strategy, lymphocytes from the peripheral blood of immunized patients showed a specific in vitro immune reactivity against the immunizing tumor cell line, documenting the efficacy of in vivo immunization by this approach. Most patients generated antibody responses that showed specific reactivity against the immunizing line, confirming the efficacy of immunization. Three of 21 patients showed major responses (1 PR and 2CR). Additional phase-II testing is in progress.

14.5.3.3 Adoptive Therapy Using Autologous Cytotoxic Lymphocytes

In vitro expansion of tumor-reactive T cells, followed by their in vivo re-infusion, is an effective approach in several murine models; however, this approach remains complex for clinical translation, particularly in smaller pediatric patients where obtaining adequate numbers of autologous T cells for in vitro expansion remains somewhat problematic. This method does allow in vitro manipulation of the T cells prior to their re-infusion. One manipulation is to use bispecific antibodies (see section 14.4.2.3) to target T cells more efficiently. Although successful in preclinical models, clinical efficacy has been difficult to achieve partly because of insufficient cell dose, inefficient homing, and poor survival in vivo. Another development is to transfect these T cells with cell surface Fab or scFv chimerized with cytoplasmic activation (e.g., CD3ζ or ɣ chain) or survival (CD28) proteins. While the antibody fragment provides tumor recognition, the signaling domains activate downstream pathways for optimal T-cell activation (Cheung et al. 2003; Rossig et al. 2001). Since these genetically modified T-cells are not restricted by MHC and can be expanded in vitro, they are potentially useful for adoptive therapy of NB (Cheung et al. 2003; Rossig et al. 2001). An alternative approach grafts the Fab recognition components of the tumor-reactive antibody directly to the TCR alpha and beta receptors to enable signal transmission directly through the intact TCR. Both of these approaches have shown success in pre-clinical models and scFv-CIR directed to least three antigen systems [L1CAM; (Jensen et al., unpublished data); GD2 (Rossig et al. 2001); gp58 (Cheung et al. 2003)] are under investigation in NB.

14.5.3.4 Adoptive Therapy Using Allogeneic Lymphocytes

Infusion of allogeneic immune cells, although effective in certain hematopoietic malignancies, has not been tested extensively for NB. In contrast to the advantage of allogeneic BMT over autologous BMT for several hematopoietic malignancies, several studies have shown equivalent or inferior progression-free survival probabilities in NB patients receiving allografts when compared with those receiving autografts (Philip et al. 1997; Matthay et al. 1994; Evans et al. 1994). In addition, patients receiving the allogeneic transplants appeared to have more peri-transplant toxicities. With recent improvements in supportive care and GVH prophylaxis, further clinical testing of graft-vs-tumor effect is being considered for NB.

14.6 Conclusions

In vivo destruction of NB cells by T-cell recognition, antibody-facilitated recognition, or recognition via cells of the innate immune system have proven effective in pre-clinical murine models. Anti-tumor benefit is likely to be maximized when applied at the time of minimal tumor burden. Unfortunately, to achieve a minimal disease state, intensely immunosuppressive chemotherapy and radiotherapy are often required, which predictably compromise the patient's immune competence. As such, immunotherapeutic interventions for NB during or immediately after induction therapy may not be able to rely on the host's endogenous immune repertoire. Passive immunotherapy, whether antibodies or adoptive cell therapy, is likely to be necessary during this initial immune recovery period. Following immune recovery, active immunotherapy may stand a better chance to be successful. Finally, to overcome immune resistance, multiple immune interventions may need to be combined.

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Differentiation and Retinoids

Carol J. Thiele, C. Patrick Reynolds

Contents

15.1 Introduction

The clinical behavior as well as the associated histopathologic features of neuroblastomas (NB) have long suggested that tumorigenesis of this pediatric cancer is, at least in part, related to defects in the process of cellular differentiation. Primary and metastatic NBs consist of different cell types, and the cells commonly have different stages of differentiation. The histologically more mature forms of NB, ganglioneuroblastoma and ganglioneuroma, correspond to the normal differentiation patterns seen in the developing sympathetic nervous system. Moreover, the histologic classification of NB into subsets based on the extent of differentiation, the presence of stroma or Schwannian tissue, and the degree of mitotic/karyorrhectic cells when combined with age is highly predictive of outcome (Shimada et al. 1984).

15.1.1 Neural Crest Development

The neural crest is a transient embryonal structure that arises at the interface between the non-neural and neural ectoderm or neural plate. As the neural plate invaginates and the folds approximate to form the neural tube, inductive influences from the underlying mesoderm and the overlying non-neural ectoderm stimulate the dorsal aspect of the developing neural tube to form the neural crest cells. Cells migrate laterally from the neural tube to form or contribute to a variety of cell types including:

1. Neuronal cells; the sensory ganglia of several cranial nerves, spinal ganglia and ganglion of the autonomic nervous system

2. Supportive cells of the nervous system; glial, of the peripheral ganglion; Schwann cells of the peripheral nerves, and meninges of the anterior brain
3. Pigmented cells; except pigmented retina
4. Endocrine and paraedocrine cells; adrenomedullary cells, calcitonin-producing cells and type-I cells of the carotid body
5. Mesectodermal derivatives; visceral and facial skeleton; walls of the large arteries derived from the aortic arches, connective tissue of thymus and parathyroid glands, and dermis of the neck and facial regions (Le Dourain and Kalcheim 1999)

The fate or specialization of neural crest cells depends on their rostro-caudal location along the neural tube; however, in vitro and in vivo studies indicate that neural crest cells retain a high degree of plasticity even after they migrate from the neural tube. Interestingly, these are also features that mark highly malignant NB cells.

15.1.2 NB as a Neural Crest Derivative

Neuroblastomas frequently express both tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH), enzymes involved in the synthesis of the catecholamines. Not only are catecholamines characteristic of sympathetic neurons, but migrating neural crest cells are also catecholaminergic (Smith and Fauquet 1984). Tyrosine hydroxylase and DβH provided the earliest molecular markers that distinguished NB from other small round blue cell tumors such as Askin's tumor, Ewing's sarcomas, and peripheral neuroectodermal tumors, which express high levels of choline acetyltransferase (Thiele et al. 1988). Tyrosine hydroxylase and DβH expression are associated with the secretion of catecholamines and their metabolites (VMH and HVA) into the urine, providing diagnostic (Lopez-Ibor and Schwartz 1985) and screening markers (see Chap. 2) for NB.

Clinically, NBs present in a number of different anatomic locations most frequently reflecting the sites of sympathetic nervous system tissues. These sites include postganglionic neuronal precursors that are found in paravertebral ganglia of the sympathetic trunk, pre-aortic ganglia in the plexus around the branches of the abdominal aorta, and in the medullary and/or ganglionic cells of the adrenal gland (Jaffe 1976). During fetal development, sites of sympathetic nervous system tissue also include neuroendocrine structures with rests of neuroblasts in the adrenal gland and paraganglia adjacent to the sympathetic ganglia.

15.1.3 NB and Adrenal Medullary Development

During development of human adrenal medullary chromaffin tissue, cells express TH, chromogranin A (CgA), and neuropeptide Y (NPY) within the first 10 weeks of gestation (Cooper et al. 1990). By 26 weeks of gestation, cells co-express delta (a ligand for the notch receptor) but lose NPY expression until after birth when they express β2-microglobulin, the light chain receptor of the major histocompatibility complex, and re-express NPY. A second population of fetal ganglionic neuroblasts develops in the medullary region that expresses HNK-1 but not TH, CgA, or delta (Cooper et al. 1990). The NB cell lines also express these patterns of gene expression indicating that some NB tumors may arise from tumorigenic events occurring at different stages of adrenal medullary cell development (Cooper et al. 1990). Furthermore, NB tumor tissue express patterns of chromaffin-related genes that correlate with the patterns of gene expression observed during maturation of adrenal medullary chromaffin tissues (Cohen et al. 1990; Cooper et al. 1992).

In vitro studies showed that NB cell lines treated with compounds that raised intracellular cAMP levels stimulated a more neuroendocrine pattern of gene expression, while signal transduction pathways regulated by retinoid receptors stimulated more neuronal gene expression patterns (Gaetano et al. 1991). Oxygen deprivation, as would occur in a necrotic area may cause a hypoxic response and induction of the transcription factor HIF1α. In vitro, the induction of HIF1α leads to decreased expression of more mature neuronal and neuroendocrine markers such as NPY, CgA, and TH, and increased expression of growth factors such as VEGF, IGF-2, and a bHLH transcription factor inhibitor of differentiation, Id–2. These

genes are more highly expressed in immature neural crest cells (Jogi et al. 2002), and VEGF (Eggert et al. 2000) is expressed in more immature and aggressive NBs, suggesting that the underlying biologic features of tumor cells are influenced not only by genetic events involved in tumorigenesis but also by their environment.

15.1.4 Neural Crest Gene Expression During Development

More recently in animal studies, a number of genes, including *slug, AP-2, HNK-1*, *and Krox-20*, distinguish the earliest neural crest cells from other neighboring epithelial and mesoderm derivatives in normal or genetically modified chicks, frogs, zebrafish, and mice. Many of these genes encode proteins that are thought to regulate gene transcription and neural crest cell determination. These genes are regulated in part by signals induced by bone morphogenic protein (BMP2/4) or Wnts. The BMPs are members of the TGFβ signaling path that utilize serine/threonine kinase receptors to transmit extracellular signals to the cytoplasm where a series of intracellular signaling intermediaries (SMADs) relay signals to the nucleus and regulate gene expression. Activation of the Wnt signal transduction path stabilizes the cytoplasmic protein β-catenin enabling its translocation into the nucleus and activation of neural crest markers (Wu et al. 2003). The BMP alone and in combination with retinoids have been found to induce differentiation of NB tumor cells and control their growth (Y. Nakamura et al. 2003; Sumantran et al. 2003).

15.1.5 *MYCN* in Neural Crest Development

As described in Chap. 4, *MYCN* amplification contributes to the clinically aggressive behavior of NB tumors (Seeger et al. 1985). *MYCN* has also been shown to play an important role in the development of sympathetic neurons. Deletion of the *MYCN* gene in animal models leads to a reduction in the number of mature neurons in the dorsal root and sympathetic ganglia (Sawai et al. 1993; Stanton et al. 1992). In addition, *MYCN* can stimulate post-mitotic sympathetic neurons to re-enter the cell cycle and enhance their survival (Wartiovaara et al. 2002). In avian embryos, migrating neural crest cells express *MYCN*. After cessation of the major wave of neural crest cell migration, *MYCN* expression is heterogeneous in the dorsal root and sympathetic ganglia, being more highly expressed in the nucleus of neuronal cells and decreased in Schwann and glial cells. In explants of neural crest cells in vitro, expression of *MYCN* stimulates an increase in differentiated neural crest cells without affecting proliferation. Implantation of *MYCN*-transduced neural crest cells in vivo leads to a massive migration of cells into the sympathetic ganglia and an increase in differentiated cells; thus, *MYCN* functions at an early phase of neural crest development increasing their migratory potential and a later phase promoting their neural differentiation (Wakamatsu et al. 1997).

15.2 Neurotrophins in Neural Crest Development

Neurogenesis in the sympathetic ganglia is governed by precursor proliferation, followed by a period of apoptosis as neuroblasts that fail to innervate their target tissues die, while others complete functional maturation. Upon innervation of their target tissues, neuroblast survival is mediated by growth/survival factors called neurotrophins (NTs). Neurotrophins exist as precursor proteins (proNTs) that are processed to mature NT proteins. The NT family of secreted growth factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 that bind to their cognate tyrosine kinase receptors TrkA, TrkB, and TrkC (Fig. 15.1). Neurotrophins bind and facilitate dimerization of Trk receptors, activating their intrinsic tyrosine kinase activities that initiate a kinase cascade that transmits signals to the nucleus to regulate genes important in neural survival and growth (Teng and Hempstead 2004). Both proNTs and mature NTs bind to the p75 receptor, which has limited homology to death receptor transmembrane proteins (Fig. 15.1). Recent evidence indicates that proNT interacts solely with p75 and in association with an accessory receptor, sortilin, is a potent death signal (Nykjaer et al.

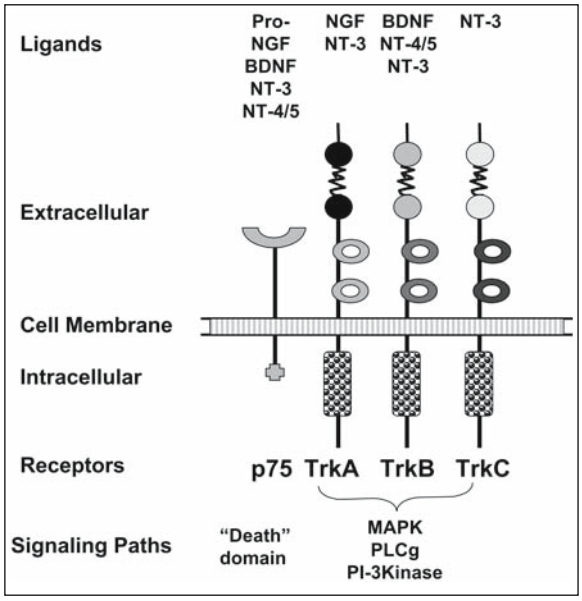

Figure 15.1

The cognate receptors for neurotrophins (NGF, BDNF, NT-3, and NT4/5). The p75 or nerve growth factor receptor (NGFR) binds all pre-processed (i.e., pro-NGF) and processed neurotrophins with equal affinity. p75 shares homology with the death receptor family of signaling receptors and this receptor may transmit either death-inducing or survival signals depending on cell type and context. Different neurotrophins interact selectively with distinct members of the Trk family of tyrosine kinase receptors. The structure of the Trk receptors contains several distinct protein motifs. The *filled circles* represent cysteine-rich regions separated by a fibronectin type-III repeat. The *open circles* to the right of the line contain Ig-like loops and the *sphere-filled rectangle* on the intracellular portion of the receptor contains the catalytic tyrosine kinase domain which is activated upon interaction of the extracellular portion of the receptor with the cognate ligand. Activation of the tyrosine kinase leads to activation of other signaling paths including the MAPK path, the PLCγ path, and the PI-3 kinase paths that ultimately transmit signals to the nucleus regulating gene transcription and ultimately affecting cell survival, growth, and differentiation.

2004). The NT/Trk (Kaplan and Miller 2000), p75 and the membrane bound kinase receptors of glial-derived neurotrophic factors (GDNF/RET), play important roles in the development of the peripheral nervous system.

The majority of migrating neural crest cells express TrkC and p75 and may be mitotically active or post-mitotic. A fraction of these also co-express TrkA. In the adrenal gland. The BDNF is expressed in the adrenal cortex during embryogenesis but is restricted to the interface of the adrenal cortex and medulla in the adult. Another TrkB ligand, NT-4, is highly expressed in the adrenal medulla. The majority of neuronal cells in the medulla express TrkA and p75 but not TrkB, although TrkB is restricted to a small number of ganglion cells. Under certain conditions NT-4 activates TrkA and may serve as a physiologic ligand for TrkA expressing chromaffin cells; however the neurons of the intermediolateral column of the spinal cord express TrkB and BDNF and innervate adrenal medullary chromaffin cells (Schober et al. 1999).

15.2.1 TrkA and NB

In NBs the expression of the neurotrophin receptors, Trks, have prognostic significance (see Chap. 5). TrkA and/or TrkC are more highly expressed in primary NBs tumors from patients who have a good prognosis (Nakagawara et al. 1993;Yamashiro et al. 1997), while expression of $p145^{TrkB}$ and its ligand BDNF is more highly expressed in primary NBs from patients with an unfavorable prognosis (Nakagawara et al. 1994). In NB cell lines engineered to express high levels of TrkA, activation with nerve growth factor (NGF) can decrease MYCN levels (Matsushima and Bogenmann 1993; Woo et al. 2004) by mediating signals transduced through the MAP kinase pathway (Woo et al. 2004). In NB cell lines, interferon gamma (Lucarelli et al. 1995), and to a lesser extent retinoids (Kaplan and Miller 2000), induce TrkA, suggesting that the levels of TrkA may be amenable to regulation in clinically aggressive NBs.

15.2.2 TrkB and NB

Activation of TrkB by its ligand BDNF has been shown to promote survival, alter sensitivity to chemotherapeutic drugs, and stimulate invasiveness – all properties of highly malignant tumors cells (Matsumoto et al. 1995) (see Chap. 5). An interesting aspect of the clinical behavior of poor prognosis NBs is that even though they are initially sensitive to chemotherapeutic agents, they often ultimately become resistant. The basis of this chemoresistance is probably multi-factorial being influenced by traditional chemoresistance factors such as the level of expression of drug efflux pumps such as MRP (Norris et al. 1996) and mutations in *TP53* (Keshelava et al. 2001; Tweddle et al. 2001). In addition, the level of expression of BDNF and its receptor TrkB may also contribute to the escape of NB cells from the effects of cytotoxic chemotherapy. Drug-resistant NB cell lines have increased levels of expression of BDNF and the levels of BDNF increase as the cells become progressively resistant to higher concentrations of cytotoxic drugs (Scala et al. 1996). Additionally, both the concentration of BDNF and the level of expression of TrkB have been shown to diminish the sensitivity of cells to drugs typically used in the therapy of NB (Jaboin et al. 2002). Recent studies have identified two targets of the TrkB pathway amenable to drug development. A drug targeting Trk tyrosine kinases (CEP-701) is in clinical trials and has shown preclinical efficacy against NB mouse xenografts (Evans et al. 1999). Furthermore, a number of compounds targeting the PI-3-kinase pathway and its downstream targets are in pre-clinical development. Such agents may enhance the toxicity of chemotherapeutic agents against aggressive NB.

15.3 Differentiation

Neuroblastoma cell lines are comprised of at least three morphologically distinct phenotypes: neuroblastic cells (N-type); non-neuronal substrate-adherent cells (S-type), and cells with an intermediate phenotype (I type), with distinct morphologic, biochemical, and tumorigenic properties (see Chap. 8). I-type cells can be induced to differentiate into N-type or S-type cells under selective culture conditions. Cytotoxic chemotherapeutic regimens can also induce NB maturation (McLaughlin and Urich 1977; Raaf et al. 1982). These observations led to subsequent studies aimed at evaluating induction of differentiation as a therapeutic strategy. A variety of chemicals and biologic response modifiers, including retinoids, histone-deacetylase inhibitors, agents that raise intracellular calcium levels, activators of protein kinase C, neurotrophins, and cytokines, have been shown to suppress tumorigenicity, control growth, and induce NB differentiation of NB cell lines.

15.3.1 Retinoids

Vitamin A or retinol primarily from the diet plays a critical role in normal neural crest development. The metabolism and storage of retinol is mediated by a number of binding proteins and enzymes. Intracellular retinol is metabolized to all-trans retinoic acid (ATRA). All-trans retinoic acid is a major mediator of the effects of vitamin A via activation of a number of RAR and RXR nuclear receptors that heterodimerize and regulate gene transcription (see below). Exposure of human neuroblastoma cell lines to supra-physiologic doses (micromolar) of ATRA caused a reduction of cell growth and induction of neurite extension and differentiation that was ultrastructurally, biochemically, and electro-physiologically similar to normal neural cells (Fig. 15.2; Sidell 1982; Abemayor and Sidell 1989).

The ATRA treatment of NB cells was accompanied by a decrease in the expression (Thiele et al. 1985) and transcription (Thiele and Israel 1988) of the *MYCN* gene. The decrease in *MYCN* expression preceded the ATRA-induced G1 arrest and evidence of morphologic differentiation (Thiele et al. 1985), and the *MYCN* over-expression blocked differentiation (Peverali et al. 1996). All-trans retinoic acid induced decreases in *MYCN* levels and increases in the cyclin-dependent kinase inhibitor p27 which may mediate the G1 arrest of NB cell cycle (Matsuo and Thiele 1998; M. Nakamura et al. 2003). Pulse therapy with retinoic acid showed a sustained arrest of tu-

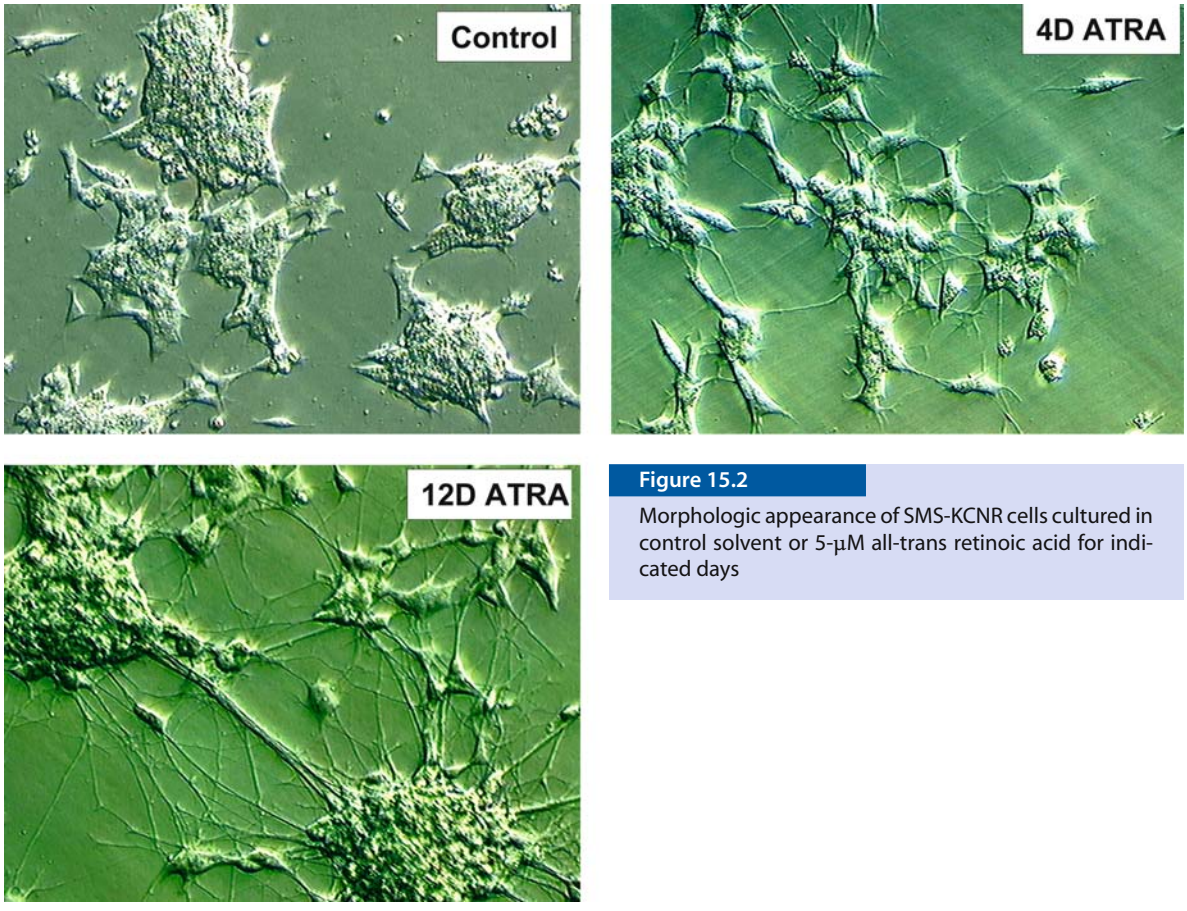

Figure 15.2

Morphologic appearance of SMS-KCNR cells cultured in control solvent or 5-μM all-trans retinoic acid for indicated days

mor cell proliferation in NB cell lines, suggesting that high-dose pulse retinoid therapy (as opposed to the more traditional low-dose continuous retinoid therapy) might be effective in vivo (Reynolds et al. 1991).

15.3.2 Retinoic Acid Receptors

The differentiation and growth arrest of malignant cells produced by retinoic acid are likely mediated by one or more of the two families of retinoic acid receptors (RAR or RXR): RAR α, β, γ; and RXR α, β, γ (Linney 1992); all belong to the steroid/thyroid hormone family of transcription factors and possess discrete DNA-binding and retinoic acid-binding domains. As depicted in Fig. 15.3, retinoic acid binds to the RA receptors, causing conformational changes that promote binding to specific cis-acting DNA sequences, which regulate transcription of certain target genes (Reynolds and Lemons 2001). A study of the RAR and RXR families of RA receptors in NB showed that they were expressed in most NB cell lines and primary tumors (Li et al. 1994). While RAR β was only expressed in 4 of 14 *MYCN* amplified cell lines, it could be induced by ATRA in most of these cell lines (Li et al. 1994). There was no correlation between resistance to ATRA and the level of RAR or RXR expression; however, higher expression of RARβ has been associated with good outcome in NB and RARβ over-expression by transfection in-

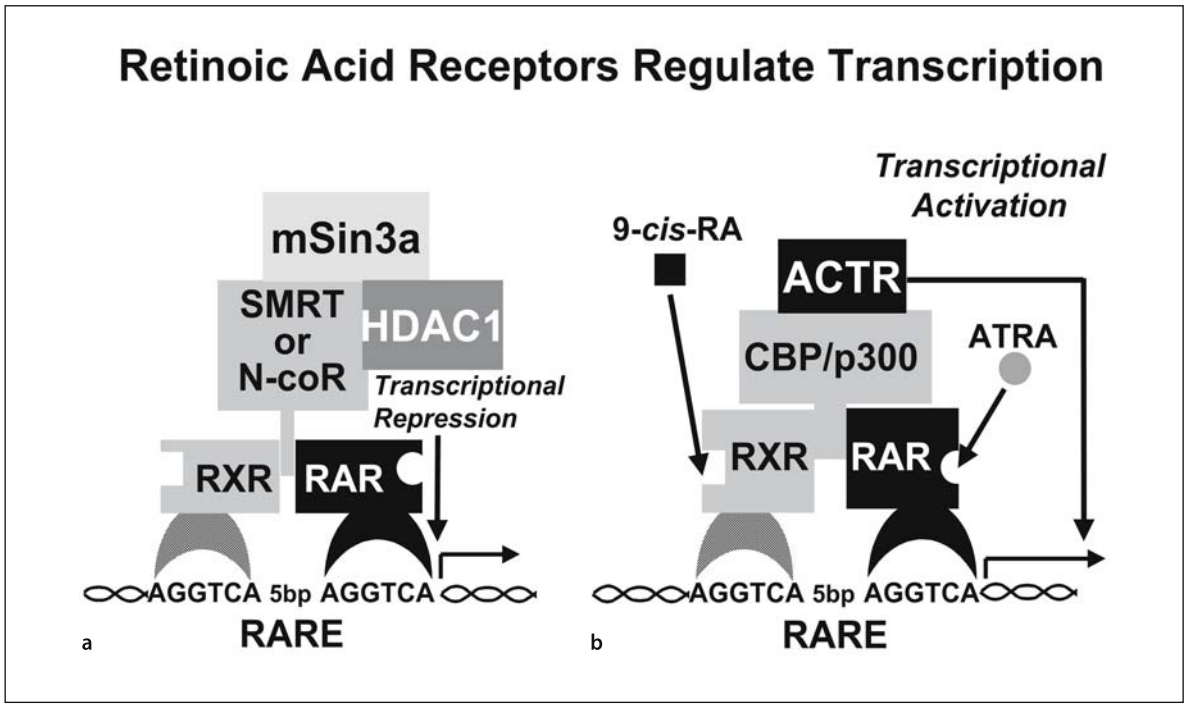

Figure 15.3 a,b

The mechanism of action of retinoids is mediated via zinc-finger transcriptional regulators which function as heterodimers to regulate promoter activity of certain target genes. The RAR and RXR proteins bind to specific direct repeat DNA sequences (AGGTCA are separated by either two or five nucleotides) in gene promoters, known as retinoic acid response elements, or RARE. **a** In the absence of ligand, the RAR/RXR heterodimers interact with nuclear co-repressors including N-CoR and SMRT, which in turn bind to a common adapter protein mSin3 which complexes to proteins with histone deacetylase activity to repress transcription. **b** Retinoic acid binds to the RAR portion of the complex causing a conformational change in the RAR and RXR proteins which releases the co-repressor complex and facilitates binding of 9-cis-RA to the RXR protein (the latter enhances the activation response). The transcriptional co-regulator CBP/p300 then binds to the receptor complex and recruits the coactivator protein ACTR, which contains histone acetyltransferase activity, and promotes transcription (Reynolds and Lemons 2001)

creases the responsiveness of some NB cell lines to RA (Cheung et al. 1998). Furthermore, RARβ selective agonists mediate growth inhibitory signals in NB cell lines (Giannini et al. 1997); thus, while alterations in RAR or RXR do not appear to be a major resistance mechanism, higher levels of expression of these receptors may enhance sensitivity to retinoids.

15.4 13-cis-Retinoic Acid

In the mid-1980s the only retinoid available for clinical use was 13-cis-retinoic acid (13-cis-RA), which induced differentiation in promyelocytic leukemia, and produced objective clinical responses in promyelocytic leukemia, myelodysplastic syndrome, cutaneous T-cell lymphoma (mycosis fungoides), and advanced squamous carcinoma of the skin (Reynolds and Lemons 2001). Although 13-cis-RA has limited

activity against established and progressing solid tumors, it was effective as a single agent in preventing second tumors in patients with head and neck carcinoma and preventing skin cancers in those with xeroderma pigmentosum.

Anecdotal trials of 13-cis-RA in NB showed responses of mass disease and marrow metastases, including a complete response with a 2-year remission in one patient (Reynolds et al. 1991). In a Children's Cancer Group (CCG) phase-II trial of single daily 100 mg/m^2 day^{-1} of oral 13-cis-RA, 2 of 28 patients with refractory NB showed response (Finklestein et al. 1992). At this dose of 13-cis-RA, subsequent pharmacokinetic studies (Villablanca et al. 1995; Khan et al. 1996) demonstrated that drug levels obtained were below the 5- to 10-μM effective levels (Reynolds et al. 1994). The major toxicities of 13-cis-RA at the 100 mg/m^2 day^{-1} dose were dryness of skin, dryness of mucous membranes, cheilitis, fissured lips, conjunctivitis, and hypertriglyceridemia.

15.4.1 High-Dose, Pulse, 13-cis-RA

For 13-cis-RA to be active in NB, effective drug levels (5–10 μM) have to be achieved. A 10-day exposure to 10-μM ATRA produced prolonged arrest of NB cell proliferation in vitro (Reynolds et al. 1991). Sustained growth arrest and down-regulation of MYCN expression in vitro were achieved with sequential -week courses of 5 μM 13-cis-RA (Reynolds et al. 1994).

In a phase-I trial of intermittent 13-cis-RA (divided quarterly 12 h daily for 2 weeks alternating with 2 weeks of mucocutaneous recovery for up to 12 courses) in post-BMT patients, MTD was 160 mg/m^2 day^{-1} with dose-limiting toxicity being hypercalcemia (Villablanca et al. 1995). Peak plasma 13-cis-RA level at MTD was 7.4±3 μM and trough was 4.0±2.8 μM (Villablanca et al. 1995; Khan et al. 1996). Four complete responses were observed in marrow metastases and two had prolonged remission past 2 years (Villablanca et al. 1995). The latter observation suggested that high-dose, pulse 13-cis-RA might delay or prevent tumor recurrence if given in a setting of minimal residual disease after completion of myeloablative therapy.

15.4.2 13-cis-RA vs All Trans-Retinoic Acid

All-trans retinoic acid (ATRA) was used in treating acute promyelocytic leukemia (APL) with excellent results and little toxicity (Warrell et al. 1991). Although 13-cis-RA has never been compared directly with ATRA in APL, most investigators felt that ATRA was superior. The ATRA was more effective then 13-cis-RA against APL cells in vitro when tested at 0.1–1 μM (Chomienne et al. 1990), a dose range achieved at 45 mg/m^2 day^{-1} in patients (Smith et al. 1992a). Dose escalation beyond 60 mg/m^2 in children was limited by pseudotumor cerebri, and ATRA rapidly induces an increase in its own metabolism, such that peak levels and drug half-life significantly decrease after a few days of therapy (Smith et al. 1992b). In contrast, drug levels obtained in the post-BMT phase-I trial of 13-cis-RA were considerably higher (4–7 μM) (Villablanca et al. 1995; Khan et al. 1996) The differences in pharmacokinetic properties of 13-cis-RA and ATRA are summarized in Fig. 15.4.

Because of minimal binding of 13-cis-RA to retinoic acid receptors, it was previously assumed that 13-cis-RA would be less potent then ATRA; however, at clinically achievable drug levels, 13-cis-RA was superior to ATRA in inducing morphological differentiation and growth arrest of NB cell lines. In addition, 13-cis-RA caused down-regulation of *MYCN* gene expression (Reynolds et al. 1994). These data, and the documentation of anti-NB activity for 13-cis-RA in patients (Villablanca et al. 1995; Reynolds et al. 1991; Matthay et al. 1999), suggests that either 13-cis-RA acts via mechanisms that are independent of retinoic acid receptors, or that (more likely) 13-cis-RA serves as a pro-drug for ATRA, resulting in delivery of higher levels of ATRA inside tumor cells then are achievable in vivo with direct ATRA treatment.

15.4.3 Post-Consolidation 13-cis-RA Therapy for High-Risk NB

The efficacy of treating high-risk patients with 13-cis-RA was analyzed in a randomized phase-III trial (Matthay et al. 1999). In CCG-3891, patients received an induction chemotherapy regimen using cyclophosphamide, doxorubicin, cisplatin, and etoposide,

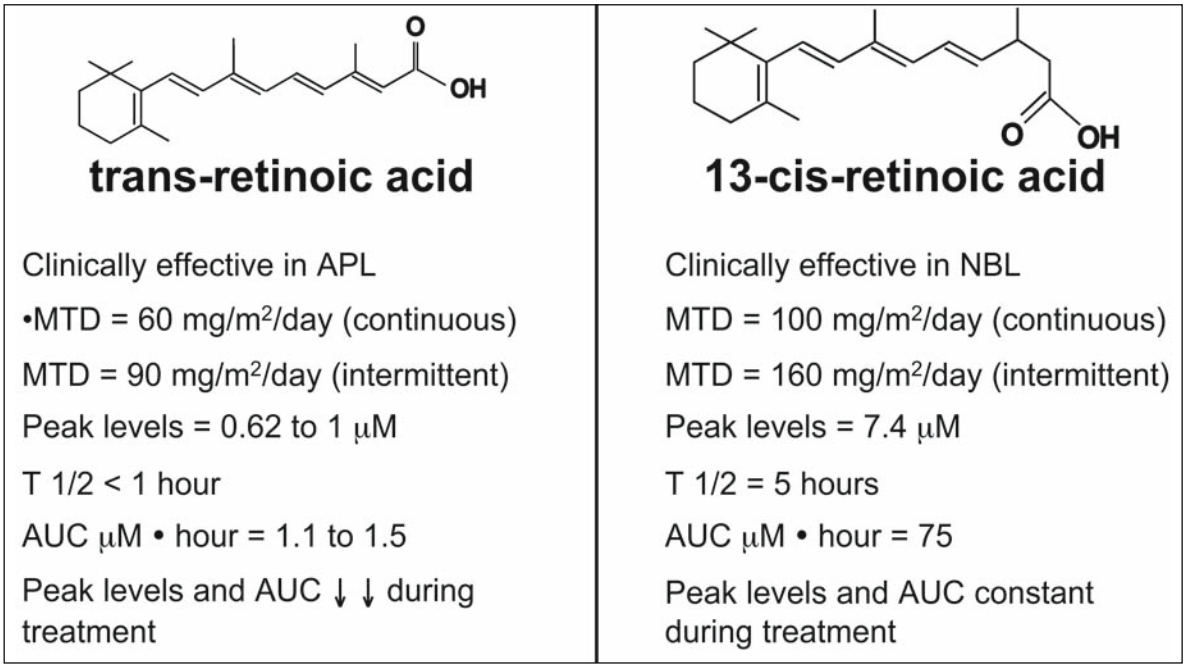

Figure 15.4

Structures of 13-cis-retinoic acid and all-trans-retinoic acid and a summary of the pharmacokinetic properties of these two retinoids in pediatric patients (Villablanca et al. 1995; Khan et al. 1996; Smith et al. 1992a,b)

during which marrow harvest and purging, and surgical resection, were accomplished. Patients were initially randomized to either myeloablative therapy employing melphalan, carboplatin, etoposide, and total body irradiation, or to three cycles of intensive non-myeloablative therapy utilizing cisplatin, etoposide, doxorubicin, and ifosfamide/mesna. A second randomization assigned patients who completed either myeloablative or non-myeloablative consolidation therapy to either no further therapy or to receive 13-cis-RA at 160 mg/m^2 day^{-1} (divided as bid) for 2 weeks each month over a 6-month period. Patients who had documented active tumor by biopsy at the end of consolidation were non-randomly assigned to receive 13-cis-RA. There were 130 patients who were randomized to receive 13-cis-RA, while 128 patients were randomized to no further therapy. Thirty-seven patients were non-randomly assigned to 13-cis-RA for proven residual tumor and 24 patients refused the second randomization, 4 of whom chose to receive 13-cis-RA. The first randomization showed that ABMT achieved a significantly higher 3-year event-free survival (EFS) from time of first randomization of 34±4% compared with 22±4% for those randomized to consolidation chemotherapy (P=0.034; see Chap. 12). As shown in Fig. 15.5a, the 3-year EFS (intent-to-treat analysis) from the time of second randomization for patients randomized to 13-cis-RA was 46±6%, significantly better than the 3-year EFS of 29±5% for those randomized to no further therapy (P=0.027). The positive benefit of 13-cis-RA for those patients with minimal residual disease was not seen for children who were non-randomly assigned to 13-cis-retinoic acid for histologically proven residual disease, as this latter group showed a 3-year EFS of 12±6%. Although the study was not statistically powered to compare the four treatment groups, treatment with 13-cis-RA appeared to be beneficial both

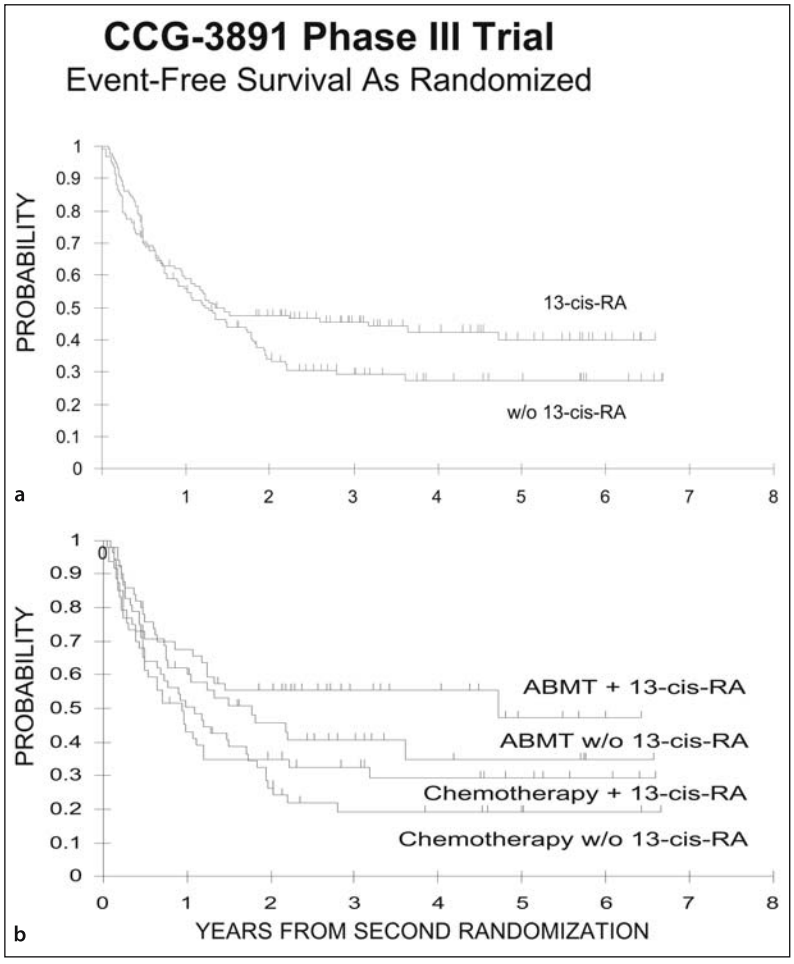

Figure 15.5 a,b

Event-free survival for CCG-3891 showing the second randomization between13-cis-RA vs no further therapy (**a**), and the four groups created by the quasi-factorial design (**b**). The latter curves are limited to only those patients completing both randomizations. All curves are shown from time of randomization (Matthay et al. 1999)

for patients who received either ABMT or non-myeloablative chemotherapy. As shown in Fig. 15.5b, there appeared to be a higher 3-year EFS from time of second randomization in patients undergoing both randomizations for those randomized to both ABMT and 13-cis-RA (55±10%), compared with ABMT alone (41±10%; P=0.28). The 3-year EFS for chemotherapy and 13-cis-RA was 33±7%, compared with chemotherapy alone (19±7%; P=0.17).

In 1989 the European NB Study Group (ENSG) initiated a randomized trial of 13-cis-RA vs no further therapy in children with advanced NB who achieved remission after high-dose therapy (Kohler et al. 2000). Patients randomized to 13-cis-RA on the ENSG study were given a single daily dose of 0.75 mg/kg (22.5 mg/m^2 day^{-1} continuously for 4 years or until relapse). Approximately 175 children were entered into the study with 88 patients randomized to receive 13-cis-RA (3-year event-free survival=37%) and 87 patients randomized to placebo (3-year event-free survival=42%); thus, in contrast to the CCG study, no advantage in event-free survival was shown in this trial for children randomized to receive low-dose, continuous 13-cis-RA. These results emphasize the importance of utilizing adequate dose levels and optimal dosing schedules to achieve pharmacologically efficacious drug levels when employing retinoids as anti-cancer agents.

15.5 Fenretinide

N-(4-hydroxyphenyl) retinamide or fenretinide (4-HPR is a synthetic retinoid; Fig. 15.6) inhibits NB growth in vitro at 1–10 µM (Ponzoni et al. 1995) and was highly active against retinoic-acid resistant NB lines at 5–10 µM (Reynolds et al. 2000). In contrast to 13-cis-RA and ATRA, 4-HPR does not induce maturational changes, but is cytotoxic, causing both apoptosis and necrosis (Maurer et al. 1999). Toxicity of 4-HPR in chemoprevention clinical trials has been minimal. The major clinical toxicity of 4-HPR is decreased night vision, due to decreased plasma retinol levels. No hematologic toxicity has been reported (Reynolds and Lemons 2001). In pediatrics, fenretinide has been well tolerated (Garaventa et al. 2003), and the MTD of oral 4-HPR given for 7 days every 3 weeks is 2475 mg/m^2 day^{-1}, which achieved 4-HPR plasma levels of 6–10 µM (Villablanca et al. 2002).

4-HPR has been shown to achieve multi-log cytotoxicity in NB cell lines resistant to ATRA and 13-cis-RA (Reynolds et al. 2000). Resistance to 13-cis-RA in NB cell lines appears to involve selection for increased expression of *MYCN* or c-*myc*, and such retinoic acid-resistant NB cell lines are collaterally hypersensitive to 4-HPR; thus, pre-clinical data suggest that sequential use of 13-cis-RA, followed by 4-HPR, could be an effective approach to treating minimal residual disease in NB patients after myeloablative therapy.

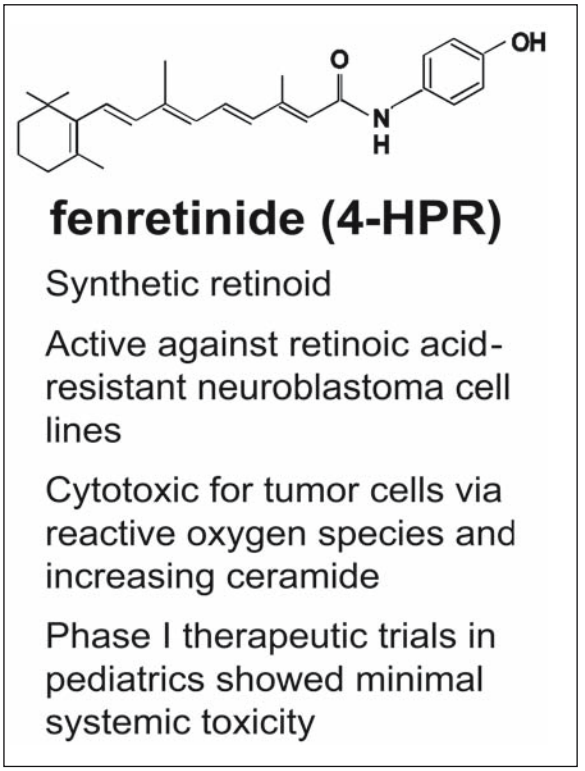

Figure 15.6

Structure of the cytotoxic retinoid N-(4-hydroxyphenyl) retinamide=fenretinide (4-HPR) and a summary of its properties

The mechanisms by which 4-HPR achieves anti-tumor cytotoxicity are not completely understood. One mechanism by which 4-HPR stimulates apoptosis is the induction of reactive oxygen species in NB cells (Maurer et al. 1999; Lovat et al. 2003a). Other possible mechanisms include induction of lipo-oxygenase, the stress-induced transcription factor GADD153 (Lovat et al. 2002; Corazzari et al. 2003), and Bak, a pro-apoptotic member of the bcl-2 family (Lovat et al. 2003b).

A major portion of fenretinide cytotoxicity for NB cell lines at high concentrations (~ 5–10 µM) is via non-apoptotic mechanisms (Maurer et al. 1999). Fenretinide stimulated large increases of ceramide in NB cell lines, which may account for its non-apoptotic cytotoxicity (Reynolds et al. 2004). Agents that modulate ceramide metabolism can increase the anti-tumor activity of 4-HPR. Drugs that inhibit glucosylceramide synthase/1-O-acylceramide synthase or sphingosine kinase, or safingol (L-threo-dihydrosphingosine), which modulate ceramide metabolism and/or action, can significantly increase 4-HPR anti-tumor activity with minimal toxicity to normal fibroblasts or bone marrow myeloid progenitors (CFU-GM; Maurer et al. 2000). Fenretinide has also been shown to inhibit NB-induced angiogenesis (Ribatti et al. 2001), and the anti-angiogenic activity of 4-HPR may be in part mediated via ceramide (Erdreich-Epstein et al. 2002). The latter data suggest that 4-HPR alone or in combination with ceramide mod-

ulators may achieve anti-tumor activity in vivo by both direct effects against tumor and anti-angiogenesis.

One limitation with fenretinide is the need for large administered doses to achieve effective drug levels. Although the currently available oral capsular dose form of 4-HPR is poorly bioavailable and difficult to administer to small children, a phase-II study of the 4-HPR oral capsule formulation in recurrent NB is ongoing in the Children's Oncology Group (COG). Pre-clinical studies have been reported with a liposome formulation of 4-HPR targeted to NB via an anti-GD2 monoclonal antibody looks promising (Raffaghello et al. 2003). New oral and intravenous formulations of fenretinide have been developed via the NCI RAID program and these are entering clinical trials in 2004 (www.nant.org). These new formulations are likely more bioavailable, and will enable the administration of 4-HPR to small children.

15.6 Conclusions

Neuroblastoma tumorigenesis is related in part to defects in cellular differentiation. A number of agents, including 13-cis-RA, are capable of inducing NB differentiation in vitro, and a phase-III trial has definitively shown the clinical benefit of high-dose pulse 13-cis-RA following consolidation therapy; however, there are still tumors that do not respond to 13-cis-RA, even at the time of minimal residual disease, and additional therapies in the post-myeloablative period are clearly needed. Pre-clinical studies have shown that cytotoxic retinoid fenretinide (4-HPR) can achieve multi-log cell kills against NB cell lines resistant to 13-cis-RA, especially when combined with modulators of ceramide metabolism. A challenge with 4-HPR is that the current oral (capsule) formulation is poorly bioavailable and is not suitable for administration to young children. New formulations are currently in development. Randomized clinical trials are needed to determine if incorporating new approaches to treating minimal residual disease in high-risk NB patients, such as use of 4-HPR±ceramide modulators, can improve event-free survival.

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Angiogenesis

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Contents

16.1 Introduction

It is widely accepted that solid tumors must acquire a new blood supply in order to grow beyond a few millimeters in size (Folkman 2002). This concept has stimulated much interest in identifying factors that promote or impede angiogenesis. Proangiogenic cytokines that appear to play a role in human cancer progression include the vascular endothelial growth factor (VEGF) family, fibroblast growth factor (FGF) family, interleukin-8 (IL-8), and platelet-derived growth factor (PDGF) family. Numerous endogenous inhibitors of angiogenesis have also been identified, including thrombospondin-1, angiostatin, and endostatin. It has also been increasingly recognized that genes implicated in malignant transformation, such as the p53 tumor suppressor or the *MYCN* oncogene, may play an important role in the regulation of angiogenesis (Hatzi et al. 2000; Yu et al. 2002). The multiplicity of these factors and their potential interactions, emphasizes the complexity of the regulation of angiogenesis. Patterns of new vessel growth vary in different tumor types, and vary even in tumors of the same type but of different clinical stage or histologic grade. For example, VEGF blockade appears to be less effective in suppressing growth of experimental neuroblastoma tumors than in parallel models of Wilms' tumor (Kim et al. 2001). In addition, expression of angiogenic factors is increased in neuroblastomas of advanced clinical stage; thus, investigating the specific mechanisms by which neuroblastoma tumors acquire a new blood supply may lead to the identification of potential new targets for treatment of this malignancy.

16.2 Vascularity in Neuroblastoma

Folkman and colleagues were among the first to suggest that the intensity of intratumoral angiogenesis correlates with tumor grade and aggressiveness (Brem et al. 1972). Although the majority of published studies have shown a positive correlation between intratumoral microvessel density and prognosis in solid tumors (Hasan et al. 2002), the prognostic role of angiogenesis in neuroblastoma is unclear. Meitar et al. initially reported in a study of 50 primary tumors that high tumor vascularity strongly correlated with widely disseminated disease, *MYCN* amplification, unfavorable histology, and poor survival (Meitar et al. 1996). Ribatti et al. found similar results in a smaller series of patients, with increased microvessel density associated with advanced-stage tumors (Ribatti et al. 2001). In further support, Erdreich-Epstein and co-workers (2000) have recently demonstrated a significant association between high-risk neuroblastoma and high levels of expression of the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are markers of active angiogenesis. In contrast, Canete et al. (2000) in a study of 69 neuroblastoma patients found no correlation of vascular parameters with the prognostic factors of age, stage, histology, TrkA, or *MYCN* amplification or with overall survival. The conflicting results most likely reflect differences in techniques used to measure vessel number, a difficulty encountered in reconciling the results of studies of other solid tumors such as breast cancer (Hasan et al. 2002). Interestingly, all three studies are in concordance, i.e., infants with stage-4S disease have higher levels of vascularity than any of the other stages. The increased vascularity in stage 4S is consistent with the rapid rate of tumor growth in a subset of these patients with widely disseminated disease.

16.3 Expression of Proangiogenic Factors

The ability of neuroblastoma to produce proangiogenic factors was first described by Folkman in 1971, who isolated a "tumor angiogenic factor" from extracts of human neuroblastoma, Wilms' tumor, and hepatoblastoma (Folkman et al. 1971). The tumor extracts caused the formation of new blood vessels in the subcutaneous fascia of rats within 48 h. Since this time, expression of numerous proangiogenic factors, such as VEGF, PDGF, FGF, and angiopoietins, has been found in neuroblastoma.

16.3.1 VEGF and VEGF Receptors

Among the proangiogenic factors VEGF-A has been the best characterized. VEGF-A is a potent mitogen for endothelial cells and can elicit a pronounced angiogenic response in vivo. VEGF-A is also a survival factor for endothelial cells both in vitro and in vivo (Ferrara 2001; Leung et al. 1989). There are numerous isoforms of VEGF-A that are generated by alternative exon splicing that include VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} (Clauss 2000; Ferrara 2001). In addition to the different VEGF-A isoforms, there is a family of VEGF-related angiogenic growth factors VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF; Clauss 2000; Ferrara 2001). The precise function of many of these VEGF-related ligands is currently not known or ambiguous.

VEGF-A is nearly ubiquitously expressed by both neuroblastoma primary tumors and cell lines, with the predominate isoform VEGF-A_{165} (Eggert et al. 2000; Fakhari et al. 2002; Komuro et al. 2001; Meister et al. 1999; Ribatti et al. 1998; Rossler et al. 1999). High expression of VEGF-A has been shown to significantly correlate with advanced stage in several studies (Eggert et al. 2000; Fakhari et al. 2002; Komuro et al. 2001). Elevated serum levels of VEGF-A have also been observed in patients with stage-III tumors (Fakhari et al. 2002). Eggert and co-workers reported that the level of expression of other proangiogenic factors including VEGF-B, VEGF-C, bFGF, angiopoietin-2, transforming growth factor-α (TGF-α), and PDGF-A, was significantly higher in stage-3 and

stage-4 neuroblastomas compared to stage-1, stage-2, or stage-4S tumors (Eggert et al. 2000). In this study, high levels of PDGF-A expression was also significantly associated with decreased survival; however, no correlation between VEGF-C or bFGF and stage was seen in a series of tumors analyzed by Komuro and colleagues (2001). In addition, while Fakhari et al. were able to show correlations between high levels of VEGF-A, VEGF-B and VEGF-C mRNA and advanced stage by real-time RT-PCR, only VEGF-A was detectable in tumor material by Western blotting (Fakhari et al. 2002). These observations suggest that VEGF-A is the major ligand of the VEGF family regulating angiogenesis in neuroblastoma. The role of the other VEGF ligands remains to be elucidated.

VEGF family members bind with differential affinity to three signaling VEGF receptors, VEGFR-1 (*Flt-1*), VEGFR-2 (*flk-1*/*KDR*), and VEGFR-3 (*FLT4*). VEGF binds to both VEGFR-1 and VEGFR-2, while PlGF and VEGF-B bind exclusively to VEGFR-1. VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3, and are mitogens for both vascular and lymphatic endothelial cells. VEGFR-2 appears to be the principal receptor on endothelial cells by which VEGF exerts its angiogenic effects (Ferrara 2001). The role of VEGFR-1 is more complex. This receptor may act not only as a ligand-binding molecule but also aid in the recruitment of bone marrow-derived endothelial precursor cells to newly formed tumor vasculature (Lyden et al. 2001), and in promoting metastases by induction of metalloprotease MMP9 (Hiratsuka et al. 2002). VEGF isoforms that have a heparin-binding site can also bind to the semaphorin receptors neuropilin-1 and neuropilin-2 (Neufeld et al. 2002). Their exact role in angiogenesis is not clear, but neuropilins may modulate binding to VEGFR-2 and subsequent bioactivity (Soker et al. 1998). Recently, expression of neuropilin-1 and neuropilin-2 has been detected in vascular endothelial cells of primary neuroblastoma tumors, but their function remains to be determined (Fakhari et al. 2002).

Expression of VEGFR-2 and to a lesser degree VEGFR-1 in primary tumors has been reported in several studies, consistent with the importance of VEGFR-2 in vascular endothelium (Fakhari et al. 2002; Fukuzawa et al. 2002; Langer et al. 2000; Meister et al. 1999); however, the expression of VEGFR-2 in neuroblastoma tumor cells remains unresolved. VEGFR-2 was detected by RT-PCR in either none (Rossler et al. 1999), few (Langer et al. 2000), or all (Meister et al. 1999) of the neuroblastoma cell lines tested. In four cell lines in which VEGFR-2 was expressed, neutralizing antibody to VEGF did not result in inhibition of proliferation of neuroblastoma cells (Meister et al. 1999). In primary tumors, VEGFR-2 has been detected in tumor cells by immunohistochemistry and in situ hybridization (Fukuzawa et al. 2002); however, VEGFR-2 could not be detected in Western blot analysis of tumor lysates suggesting either a limited expression of VEGFR-2 (e.g., in the vasculature) or a very low/minimal expression in the tumor cells.

16.3.2 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) are a family of endopeptidases that play a key role in maintaining the balance between deposition and degradation of extracellular matrix. Activity of MMP-2 and MMP-9 is associated with tumor progression and metastasis in many cancers, presumably facilitating the invasion of tumor cells and sprouting of new vasculature (Overall and Lopez-Otin 2002). Consistent with these functions, inhibitors of MMPs have been shown to suppress both tumor invasion and angiogenesis. In neuroblastoma, an association between increased levels of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in patients with advanced stage has been reported (Ribatti et al. 2001; Sugiura et al. 1998). In addition, decreased expression of the tissue inhibitor metalloproteinase TIMP-2, a specific inhibitor of MMP-2 and MMP-9, has been significantly related to advanced disease (Ara et al. 1998). Lastly, Sakakibara et al. have demonstrated that higher ratios of gelatinase activation resulting from high expression of membrane-type matrix metalloproteinase-1 (MT-MMP-1) is associated with stage-IV disease and unfavorable outcome (Sakakibara et al. 1999).

16.4 Expression of Angiogenesis Inhibitors

Tumor angiogenesis is regulated by the balance of angiogenesis stimulators and inhibitors produced by tumor cells, the surrounding stoma, and host cells (Bergers and Benjamin 2003). Neuroblastomas are biologically heterogeneous tumors that consist of two main cell populations: neuroblastic/ganglionic cells and Schwann cells. Regulation of angiogenesis by Schwann cells is suggested by the finding that Schwannian stroma rich/stroma dominant tumors are associated with decreased tumor vascularity (Meitar et al. 1996). Further evidence suggests that Schwann cells can influence angiogenesis by producing inhibitors that can induce endothelial cell apoptosis and inhibit angiogenesis in vivo (Huang et al. 2000).

16.4.1 Pigment Epithelium-Derived Factor

One of the factors isolated from Schwann cells is the endogenous angiogenesis inhibitor, pigment epithelium-derived factor (PEDF) (Crawford et al. 2001). PEDF can inhibit angiogenesis both in vitro and in vivo, promote growth and survival of Schwann cells, and induce tumor cell differentiation; thus, PEDF may regulate neuroblastoma growth by inhibiting new blood vessel growth and by supporting the survival of differentiated cell types.

16.4.2 Secreted Protein Acidic and Rich in Cysteine

Recently, another angiogenic inhibitor was isolated from Schwann cell-conditioned media, identified as secreted protein acidic and rich in cysteine (SPARC) (Chlenski et al. 2002). SPARC, also known as osteonectin, BM-40, and 43 K protein, is a highly conserved calcium-binding glycoprotein that plays a modulatory role in cell-matrix interactions and appears to contribute to vascular morphogenesis (Brekken and Sage 2001). SPARC is generally considered an antiangiogenic protein because it blocks VEGF- and FGF-2-induced proliferation of endothelial cells and can inhibit PDGF activity on stromal cells (Brekken and Sage 2001).

SPARC expression is inversely correlated with the degree of malignant progression in neuroblastoma tumors (Chlenski et al. 2002). In favorable histology Schwannian stroma-rich/stroma-dominant tumors, SPARC was detected in Schwann cells as well as differentiating neuroblast/ganglion cells. In contrast, minimal to no staining for SPARC was observed in Schwannian stroma-poor tumors. SPARC was critical for the anti-angiogenic phenotype of cultured Schwann cells, as the addition of anti-SPARC neutralizing antibodies largely reversed the anti-angiogenic activity of Schwann cell-conditioned media. Furthermore, at concentrations found in Schwann cell-conditioned media, purified SPARC inhibited angiogenesis and impaired neuroblastoma tumor growth in vivo.

16.4.3 Thrombospondin-1

Thrombospondin-1 (TSP-1), a well-characterized endogenous inhibitor of angiogenesis, was initially isolated as a constituent of extracellular matrix, and shown to interact with wild-type p53 to regulate angiogenesis (Dameron et al. 1994). More recently, TSP-1 has been shown to modulate mobilization of VEGF directly (Rodriguez-Manzaneque et al. 2001). A number of workers have reported that TSP-1 plays an important role in the differentiation of neuroblasts induced by retinoic acid treatment (Castle et al. 1992; Pijuan-Thompson et al. 1999). Castle and co-workers reported a rapid induction of TSP-1 when cultured human neuroblastoma cells were treated with retinoic acid (Castle et al. 1992). Furthermore, differentiation was partially prevented by anti-TSP-1 antibody. Because TSP-1 is a negative regulator of VEGF, these findings suggest that differentiation of neuroblasts may be linked to a decrease in proangiogenic signaling. The recent development of TSP-1 mimetic peptides with anti-angiogenic activity is therefore intriguing (Reiher et al. 2002) as such agents may promote neuroblastoma differentiation and inhibit VEGF-stimulated angiogenesis.

16.5 Regulation of Angiogenesis by *MYCN*

MYCN amplification is a poor prognostic factor in children with neuroblastoma, and is associated with advanced tumor stage and metastasis. *MYCN* appears to play a significant role in neuroblastoma angiogenesis. Amplification of *MYCN* has been shown to correlate with mean vascular density (Meitar et al. 1996), and the expression of PDGF (but not with VEGF-A) (Eggert et al. 2000). Angiogenic activity of biopsy samples was significantly higher in those with *MYCN*-amplified tumors when tested in a chick embryo chorioallantoic membrane assay (Ribatti et al. 2002). It remains unclear if *MYCN* directly upregulates cytokines that promote neovascular development.

MYCN may also decrease the expression of endogenous inhibitors of angiogenesis. In cultured neuroblastoma cells *MYCN* causes decreased expression of interleukin-6 (IL-6), leukemia inhibitory factor, and activin A (Breit et al. 2000; Hatzi et al. 2000, 2002a,b). Over-expression of IL-6 in neuroblastoma xenografts results in decreased tumor angiogenesis and growth inhibition (Hatzi et al. 2002b); thus, current evidence suggests that *MYCN* promotes angiogenesis in neuroblastoma at least in part by decreasing expression of genes that normally function to restrain new blood vessel growth.

16.6 Preclinical Testing of Antiangiogenic Agents

16.6.1 VEGF Blockade

Inhibition of VEGF has recently been shown to be effective in clinical trials of some human cancers (Glade-Bender et al. 2003). VEGF blockade has also shown efficacy in preclinical models of human neuroblastoma, using agents that target the ligand or its VEGFR2 receptor (Davidoff et al. 2001b; Klement et al. 2000; Rowe et al. 2000). More recent reports demonstrate that neuroblastoma tumors may co-opt host vasculature early in development (Kim et al. 2002a). Partial blockade of VEGF may prolong co-option, which presumably contributes to tumor perfusion and supports continued, although reduced, tumor growth. Use of very high-affinity VEGF-binding molecules, such as the recently described novel fusion construct VEGF-Trap, can cause regression of such co-opted vessels (Holash et al. 2002; Kim et al. 2002a); thus, selection of optimal VEGF blocking strategies for testing in patients with neuroblastoma may require selection of agents based on specific biochemical and pharmacologic properties.

16.6.2 TNP-470

One of the first anti-angiogenic molecules proposed was TNP-470 (AGM-1470), an analog of fumagillin, a naturally secreted antibiotic of the fungus *Aspergillus fumigatus fresenius* (Ingber et al. 1990), identified by its ability to inhibit endothelial proliferation in vitro. Subsequently, its TNP-470 analog was shown to inhibit tumor growth in multiple xenograft models (Ingber et al. 1990; Kurebayashi et al. 1994; Kusaka et al. 1991; Yamaoka et al. 1993a,b). Based on these experiments, TNP-470 aroused much interest as a potential anti-angiogenic agent; however, testing in preclinical models of neuroblastoma has demonstrated relatively modest anti-tumor effects (Katzenstein et al. 1999; Nagabuchi et al. 1997; Shusterman et al. 2001). Some investigators indicate that TNP-470 is most effective when small neuroblastoma tumors are treated (Katzenstein et al. 1999; Shusterman et al. 2001), suggesting that this agent may be more effective in the setting of minimal residual disease.

16.6.3 Endostatin

Endostatin, a peptide fragment of collagen XVIII, was initially purified from a hemangioendothelioma based on its ability to inhibit endothelial proliferation in vitro and tumor growth in vivo (O'Reilly et al. 1997). Endostatin appears to act by disrupting endothelial interactions with anchoring proteins in extracellular matrix (Dixelius et al. 2002; Kim et al. 2000; Rehn et al. 2001). Another report suggests that endostatin may also directly block VEGFR2 signaling (Kim et al. 2002b). Despite these findings, activity of endostatin in murine models of neuroblastoma has not been consistently demonstrated, with groups detecting either modest or no effect on tumor growth

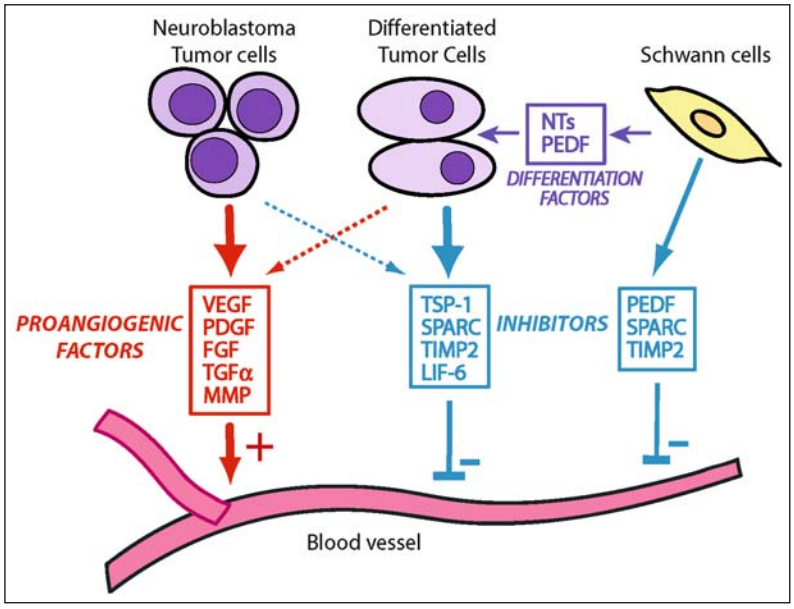

Figure 16.1

Model of angiogenesis in neuroblastoma. Tumor cells produce both proangiogenic factors and inhibitors of angiogenesis. The relative expression of these factors may depend on the differentiation status of the tumor cells. More differentiated tumors may express more angiogenesis inhibitors. Schwann cells produce not only inhibitors of angiogenesis, but also factors, e.g., neurotrophins (*NTs*) and pigment epithelium-derived factor (*PEDF*), that promote differentiation of neuroblastoma tumor cells. *VEGF* vascular endothelial growth factor, *PDGF* platelet-derived growth factor, *SPARC* secreted protein acidic and rich in cysteine, *FGF* fibroblast growth factor, *TGF* transforming growth factor, *MMP* metalloprotease, *TSP* thrombospondin, *TIMP* tissue inhibitor metalloproteinase

(Davidoff et al. 2001a; Jouanneau et al. 2001; Kuroiwa et al. 2001). Davidoff and colleagues report enhancement of a modest anti-tumor effect of endostatin in experimental neuroblastoma by combination of this agent with an immunomodulatory strategy (Davidoff et al. 2001a).

16.7 Conclusions

Investigations of angiogenesis in neuroblastoma to date reflect the complexity that results from the combined influences of genetic and epigenetic factors on tumor vessel formation. Tumors that are clinically aggressive may express higher levels of proangiogenic cytokines, while concurrently expressing decreased levels of factors that function to restrain new blood vessel growth (Fig. 16.1). Conversely, the more benign nature of ganglioneuroblastomas and other relatively indolent, differentiated tumors may reflect the influence of secreted angiogenesis inhibitors. Such factors may be elaborated by Schwann cells in the tumor stroma, or possibly by tumor cells that have undergone further differentiation.

Preclinical studies suggest that neuroblastoma may be susceptible to certain anti-angiogenic strategies. For example, blockade of VEGF in neuroblastoma has shown consistent effectiveness between experimental models and investigators. In addition, this approach has recently been shown to have efficacy in clinical trials of adult human cancers (Glade-Bender et al. 2003); however, even the most promising reports suggest that neuroblastoma may be able to partially evade anti-VEGF agents by co-opting host

vessels (Kim et al. 2002a). Understanding such events will require further dissection of the unique interactions between neuroblastoma cells and developing vasculature. Such studies are essential if patients with advanced neuroblastoma are to benefit from this area of investigation.

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Experimental Therapeutics and Preclinical Models

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Contents

17.1 Introduction

Although the treatment outcome for children with advanced-stage neuroblastoma has improved, 5-year event-free survival in most studies is less than 30% (Castel et al. 2001). In a few studies (e.g., Kaneko et al. 2002) survival reaching 34% for patients with *MYCN* amplification (more than ten copies) receiving intensified regimens of cytotoxic agents has been reported. While the survival rate is significantly increased over that of less intensive regimens, two-thirds of high-risk patients with *MYCN* amplification succumb to their disease. Furthermore, the survival of patients with advanced disease without MYCN amplification (less than ten copies) is not much greater (Kaneko et al. 2002); thus, there is still a need to develop more effective, less toxic therapies that will boost survival probability for these children (Brodeur 2002; Tsuchida et al. 2003).

This chapter focuses on the potential of preclinical animal model systems in development of new therapies. Each model has both limitations and uses; the art is to recognize the model attributes and not to extrapolate results beyond the true useful range for that model, i.e., each type of model has a functional use and it is likely that no one class of model will be useful for all applications. For example, syngeneic animal tumor models may have particular value in development of active immunity approaches, whereas heterotransplant models, such as human tumors in immune-deficient mice, have a more restricted application.

Cytotoxic therapies still play the major role in neuroblastoma treatment inducing high complete response rates even in a subset of patients with stage-4

MYCN amplified disease (Castel and Canete 2004; Donfrancesco et al. 2004). Bone marrow or peripheral blood stem cell transplants appear to have some value increasing relapse-free survival, at least in some studies (Goldsby and Matthay 2004; Imaizumi et al. 2001; Valteau-Couanet et al. 2000; Matthay et al. 1999). Differentiation agents and immunotherapies may also increase survival in patients having a complete or good partial response during induction therapy. It is against this background that we consider the use of preclinical models with particular emphasis on contemporary approaches to therapy with novel cytotoxic agents, "molecularly targeted" agents, antiangiogenic agents, and immunotherapy approaches to treating neuroblastoma.

17.2 Heterotransplant Models

17.2.1 Cytotoxic Agents

Human tumors heterografted into immune-deficient mice have provided the major models for drug identification and development over the past two decades. While such models are often criticized as being inadequate, or for failing to predict responsiveness of human cancers, specifically adult malignancies of epithelial origin (Johnson et al. 2001), the experience with models of childhood cancer has been more positive (Peterson and Houghton 2004). In part this may be because tumors such as neuroblastoma are more chemoresponsive; hence, it may be easier to assess response in the clinical situation. For example, the identification of topotecan and irinotecan, DNA topoisomerase-I poisons, as being highly active in preclinical models of rhabdomyosarcoma and neuroblastoma has translated accurately in clinical phase-II trials (Furman et al. 1999; Houghton et al. 1992, 1996, 2002; Santana et al. 2003; Thompson et al. 1997). Similarly, drugs known to be active against clinical neuroblastoma are identified in these models (Houghton et al. 2002; Tsuchida et al. 1984). Yet despite numerous neuroblastoma xenografts propagated either as subcutaneous implants or as disseminated disease following parenteral inoculation of cells, relatively few therapeutic studies have been reported. One exception is the work of Tsuchida and colleagues (1984), who have characterized the chemosensitivity of subcutaneously propagated neuroblastoma xenografts in athymic nude mice. Consistent with clinical experience, cisplatin and cyclophosphamide were identified as most active in this panel of tumors. One advantage of the xenograft system, compared with syngeneic models, is that tumors can be established from patients either at diagnosis or relapse. Identification of novel agents that retain activity against chemorefractory disease is of particular interest. The biological characteristics and treatment status at the time of tumor establishment for a panel of neuroblastoma xenografts is shown in Table 17.1. The chemosensitivity profile of these neuroblastoma xenografts is shown in Table 17.2. Notably, the camptothecin analog topotecan demonstrated good activity against several of these tumors derived from relapsed samples within the panel, although tumors established at diagnosis were more sensitive to topotecan. Recent clinical results in Japan with irinotecan, another camptothecin analog, and in phase-I and phase-II trials at St. Jude (Furman et al. 1999; Santana et al. 2003), indicate that irinotecan and topotecan have significant activity against neuroblastoma. The DNA methylating agent temozolomide also showed promising activity in both diagnosis and relapse models. Sensitivity to temozolomide has been associated with cells that have functional DNA mismatch repair (MMR), and low levels of the DNA repair protein O^6 methylguanine DNA methyltransferase (MGMT). Determination of whether the MMR/MGMT status of xenografts accurately represents the patient tissue may be important in translating these preclinical results (Middlemas et al. 2000; Wagner et al. 2002).

These data suggest that neuroblastoma xenografts respond to known and experimental drugs in a manner similar to that in patients; at least to cytotoxic agents. The potential pitfalls of translating these results have been reviewed recently (Kirstein et al. 2001; Peterson and Houghton 2004). Clearly there are examples of agents that demonstrate very significant activity in such models but fail to fulfill such promise in clinical trials. Retrospective analysis shows that in many instances this disconnect is a consequence of differential host sensitivity: if the mouse is hyper-tol-

Table 17.1. Characteristics of neuroblastoma xenografts

Tumor designation	Clinical stage	*MYCN*	Status/treatment	Doubling time (days)
NB-1382	Local–regional	Amplified	Relapse[a]	11
NB-1643	Stage 4	Amplified	Diagnosis	18
NB-1691	Stage 4	Amplified	Relapse[b]	7
NB-1771	Stage 4	Amplified	Diagnosis	15
NB-EB	Stage 4	Not amplified	Relapse[c]	5
NB-SD	Stage 4	Amplified	Relapse[c]	7

[a] Vincristine, etoposide, cytoxan, cisplatin, carboplatin
[b] Cytarabine, daunorubicin, 6-TG, etoposide, 5-azacytidine
[c] Cytoxan, doxorubicin, cisplatin, etoposide

Table 17.2. Chemosensitivity of neuroblastoma xenografts

Agent	Tumor line NB-1382	NB-1643	NB-1691	NB-1771	NB-EB	NB-SD
Vincristine	++++	++	–	+	–	–
Topotecan	++++++	+++++	+++	++++++	+++++	+++
Temozolomide	++++++	++++++	+	++++++	++++	+++
CPT-11	++++	+++++	++	++++	++++++	++++++
MG1-114	++++++	+++++	++++	++++?	++++	+
VP-16	+++	+++(+)	++(+)	+	++	+/–
CDDP	+	+	ND	ND	–	+/–
Carboplatin	+	ND	+(+)	ND	ND	+(+)
Cytoxan	++	++++	+	+	–	+/–
Doxorubicin	++	+	–		–	++
BMS247550	++++++	++++	++			
ZD1839	++	–	–		–	
SU6668	++		++	+		

Tumor response:
– No growth inhibition
± Transient response, inhibition <Td_2 (=mean time for tumor volume to double)
+ Growth inhibition ≥Td_2
++ Growth inhibition ≥2×Td_2
+++ Growth inhibition ≥3×Td_2
++++ Growth inhibition ≥3 Td_2+volume regression ≥50%
+++++ Complete regression with subsequent growth
++++++ Complete regression with no regrowth of any tumors during the period of observation (≥84 days)

erant to a drug, relative to human, then the mouse models will overpredict activity (Leggas et al. 2002). If the converse holds, then the mouse models may fail to identify potentially useful agents for clinical disease; thus, it is critical to determine whether drug doses inducing tumor regressions in the models achieve systemic exposures that are relevant to clinical exposures at tolerated doses (Boland et al. 1999; Zamboni et al. 1998). In most instances drug pharmacokinetic data are available from phase-I trials in adult patients prior to initiating pediatric clinical trials; hence, these comparisons can be used to prioritize or de-emphasize development of a specific agent in pediatric trials.

17.2.2 Signal Transduction Inhibitors

Perhaps a greater challenge will be identifying agents that are targeted to components of signaling pathways that regulate proliferation and survival. To be of value such models must accurately mimic the cellular metabolic characteristics of the clinical disease, i.e., unless the pathways accurately recapitulate the function in clinical cancer, the models may have limited value in identifying active agents, or in accurately defining the activity of drug combinations. In an attempt to characterize current preclinical models the Cancer Treatment Evaluation Program at the National Cancer Institute has initiated a Project (POPPTAP – Pediatric Oncology Preclinical Tissue Array Project) to molecularly characterize preclinical models by expression profiling and proteomics profiling. The goal of this project is to determine whether expression profiles of xenografts is similar to that of the respective tumor type in children (i.e., do neuroblastoma xenograft samples cluster with clinical neuroblastoma?). The proteomics component will establish tissue arrays that will allow definition of pathways that are activated, and it is anticipated that such data may allow new target identification. This raises a question as to whether orthotopic models will have to be used to accurately recapitulate gene expression profiles found in clinical tumors. (Khanna et al. 2002) have suggested that human neuroblastoma orthotopically grown in the adrenal gland of Beige-SCID mice demonstrates more relevant tumor biology including an angiogenic phenotype and enhanced distant metastases compared with heterotopic (subcutaneous) tumors and tumors at different sites were associated with differences in expression of angiogenesis-associated genes. A similar analysis has not been undertaken with other models of disseminated neuroblastoma, where cells are injected intravenously or into the heart. Although there are many reports of such specialized models (Gilbert et al. 1988; Martinez et al. 1996; Turner et al. 1990), they tend to be labor intensive and few therapeutic studies have been reported (Thompson et al. 2001). A similar concern over the site of tumor growth exists when one considers evaluation of novel signaling inhibitors. For example, the indolocarbazole CEP-751 inhibits Trk receptors expressed on neuroblastoma and medulloblastoma cells. Interestingly, whereas treatment significantly inhibited growth of IMR-5, NBL-S, and CHP-134 when therapy was started against palpable tumor, it was less effective in a setting of preclinical disease where treatment started 4–6 days after inoculation of cells (Evans et al. 1999). Whether such differences reflect expression levels of the receptor is unknown. In other studies it was shown that CEP-751 had greater inhibitory activity against clones of SY5Y neuroblastoma engineered to express TrkB (Evans et al. 2001), suggesting some specificity of the antitumor activity, although clearly there are other targets for CEP-751 other than Trk receptors. That certain kinase inhibitors may have promiscuous activities, however, is demonstrated by the synergistic activity of the ErbB1 inhibitor gefitinib (Iressa, ZD1839) when combined with the topoisomerase-I poison irinotecan (Stewart et al. 2004) against both neuroblastoma and other xenografts that do not express detectable levels of ErbB-family receptors. In this case the enhanced activity of irinotecan appears to be due to potent inhibition of an ABC transporter (ABCG2/BCRP) that confers cellular resistance to SN-38, the active metabolite of irinotecan (Wierdl et al. 2003). Similarly, imatinib mesylate, an inhibitor of c-KIT, PDGFR, and BCR-ABL, potently inhibits the ABCG2 transporter, reversing resistance to topotecan in vitro (Houghton et al. 2004). It will be important to compare gene expression profiles for micrometastases growing in different organs to determine

whether tumor site may ultimately determine the effectiveness of particular signaling inhibitors, adding yet another level of complexity to developing novel therapies for these tumors.

17.2.3 Angiogenesis Inhibitors

Tumor vascularity is highly correlated with outcome. The role for anti-angiogenic, and in vivo angiogenic, activity of neuroblastoma correlates with *MYCN* oncogene overexpression (Ribatti et al. 2002). Consequently, anti-angiogenic agents have been extensively investigated in neuroblastoma xenograft models. Results have been variable. In the study by Katzenstein et al. (1999) initiating therapy with TNP-470 before tumors were clinically evident resulted in 53% of mice being tumor free at 12 weeks. When tumors were staged (<400 mm^3) drug treatment significantly retarded growth of NBL-W-N neuroblastomas, whereas treatment had no effect when tumors were larger (>400 mm^3) at the start of treatment. Similar results were obtained with CHP-134 xenografts (Shusterman et al. 2000, 2001). Other studies (Kim et al. 2002) demonstrated that blockade of VEGF using anti-human VEGF(165) RNA-based fluoropyrimidine aptamer, a monoclonal anti-human VEGF antibody and a VEGFR-1 and VEGFR-2 decoy receptor (VEGF-Trap), inhibited growth NGP-GFP neuroblastomas. Of these the VEGF-Trap approach gave the greatest tumor inhibition; however, in the presence of prolonged inhibition of VEGF function tumors persisted in cooption of blood vessels. This may explain why experimental neuroblastoma may be less sensitive to inhibiting VEGF than another model of Wilms' tumor. Recombinant human and murine endostatin has been evaluated against TNB9 and SKNAS neuroblastoma models, respectively (Jouanneau et al. 2001; Kuroiwa et al. 2001). Both studies initiated therapy when tumors were relatively small, but neither study showed a significant inhibition of tumor growth. Similarly, the VEGFR-2 receptor inhibitor SU6668 demonstrated little activity against several neuroblastoma xenografts (Table 17.2). In contrast, another VEGFR-2 inhibitor SU5416 did inhibit growth of SH-SY5Y tumors by 65% (Backman et al. 2002). A novel approach to controlling angiogenesis through inhibition of hypoxia-inducible factor 1α (HIF-1α) has been tested in various xenograft models including neuroblastoma (Yeo et al. 2003). YC-1, an inhibitor of HIF-1α significantly retarded growth of SK-N-MC PNET xenografts and decreased vascularization. These diverse results reported with different anti-angiogenic agents raises the question of how best to evaluate anti-angiogenic agents in mice; indeed, what constitutes the best model systems? Should orthotopic models be prioritized for these studies, or should established tumors be de-bulked by chemotherapy prior to starting the antiangiogenic treatment? These "secondary" screening models would more readily mimic a clinical situation; however, one still has a hybrid model in which it is murine endothelial elements that are targeted in a human tumor. A more extensive evaluation of anti-angiogenic therapies in a panel of subcutaneous or orthotopic neuroblastoma models is required to address these issues.

17.2.4 Viral-Based Therapies

Adenoviral vectors have been used to deliver angiostatin, an internal fragment of plasminogen containing the first four kringle structures fused to human serum albumin (K3-HAS) (Joseph et al. 2003). Mice bearing IGR-N835 neuroblastomas were administered 5×10^9 PFU (plaque forming units) by intravenous injection when tumors were either early stage, established, or at a state of minimal residual disease. No delay in tumor growth in animals treated with AdK3-HAS was observed compared with control empty virus. K3-HAS was found to be expressed at high levels; hence, it would appear that this approach may not be successful. Instead, IGR-N835 tumors were found to secrete high concentrations of VEGF suggesting that targeting this ligand or its receptor system may be more useful.

A novel approach to treatment of neuroblastoma has been to administer the avian paramyxovirus Newcastle disease virus either by direct intratumoral injection or by intraperitoneal injection (Phuangsab et al. 2001). After a single intraperitoneal injection of 5×10^9 PFU complete regression of IMR-32 neuroblastomas was observed in 9 of 12 mice without recur-

rence for 3–9 months. In half of those mice where tumor recurred complete response was achieved with three additional courses of treatment. Viral-dependent enzyme prodrug therapy (VDEPT) has also been used to selectively purge neuroblastoma-contaminated human or murine bone marrow. Adenovirus encoding a carboxylesterase that efficiently activates the DNA topoisomerase-I poison irinotecan (CPT-11) was used to selectively activate drug in NB-1691 neuroblastoma cells. Purged marrows were bioassayed by injecting cells into SCID mice. Interestingly, marrows having up to 10% tumor cells were successfully purged, as determined by loss of detection of neuroblastoma markers tyrosine hydroxylase and *MYCN*, and failure to establish disease in mice (Wagner et al. 2002).

17.2.5 Immunotherapy and Radioimmunotherapy

Heterotransplant models have also been used to probe the role of antibodies and antibody-conjugates in the treatment of neuroblastoma for both subcutaneous (Cheung et al. 1986; Cheung and Modak 2002), leptomeningeal (Bergman et al. 1999) as well as metastatic models (Raffaghello et al. 2003). Several promising strategies are being tested in the clinic.

17.3 Transgenic Models

The transgenic neuroblastoma model (Weiss et al. 1997), in which the human *MYCN* gene is expressed in neuroectodermal cells under control of the tyrosine hydroxylase promoter, represents a novel syngeneic model of human disease. Because overexpression of the human *MYCN* oncogene in murine neuroblastoma models is one of the relatively few genetic mutations associated with NB tumorigenesis, it is a potential target for manipulation by investigational therapies. The consequences of amplified human *MYCN* expression in the preclinical setting are of particular importance because a recapitulation of histological and pathological aspects of clinical disease has been shown (Hackett et al. 2003; Weiss et al. 1997). Even though these studies suggest that numerous *other* genetic characteristics of clinical neuroblastoma are also present in mice expressing a human *MYCN* oncogene, this particular characteristic (human *MYCN* overexpression) is the only one associated consistently with enhanced tumorigenesis both in the preclinical and clinical settings (Hackett et al. 2003; Weiss et al. 2000). The mechanism by which human *MYCN* overexpression contributes to the pathogenesis of neuroblastoma is not completely understood but likely involves an alteration in potential for malignancy via gene expression regulation [e.g., *MYCN* regulates the expression of the genes that encode the multidrug resistance associated protein 1 (MRP1) and ornithine decarboxylase (ODC) and possibly expression of the type-1 insulin-like growth factor receptor]. Whatever the mechanism, it seems logical that inhibiting expression of the oncogene would ultimately limit the cell proliferation abilities of the tumor. In fact, Burkhart et al. (2003) found that inhibition of the human *MYCN* oncogene in vivo through antisense oligonucleotide administration was associated with decreases in tumor incidence as well as tumor mass. The results appeared to be directly due to human *MYCN* inhibition since expression/activity of the closely related *MYC* gene family was unaffected. Furthermore, because expression of the gene is virtually absent from adult tissues (restricted to stages of embryogenesis), antisense inhibition of the human *MYCN* oncogene would likely be void of serious adverse events. This study not only stresses the significance of the role of human *MYCN* in neuroblastoma, but also highlights its importance as a potential target for future antisense therapies (Burkhart et al. 2003). It is noteworthy that novel therapies, such as targeted oligonucleotide administration, are not without limitation. Applicability in vivo is impaired by significant instability; oligonucleotides in general are highly sensitive to cellular nuclease degradation. Brignole et al. (2003) suggest that lipid encapsulation of the oligonucleotides prior to administration improves stability and enhances antitumor response. Such a formulation showed good cell binding and relatively desirable tumor cell specificity; however, although antisense, or inhibitory, RNA approaches appear useful in vitro, they face significant hurdles before achieving acceptance as

therapeutic modalities for treatment of systemic disease. Instead, at this time they serve as reagents for proof of principle studies in the transgenic mouse models.

17.4 Syngeneic Models

Syngeneic models of neuroblastoma, most frequently derived from the C1300 tumor, have been used to probe the role of immunotherapy. This tumor arose spontaneously in the spinal cord region of a strain-A mouse (Ishizu et al. 1994; Ziegler et al. 1997) and as a subcutaneous implant shows local invasion but rarely metastasizes. A variant of C-1300, TBJ, grows more rapidly and metastasizes extensively, and C-1300 cells inoculated into a subcutaneously translocated spleen gives rise to hepatic metastases. Against C-1300 tumors both rIFN-γ and rIL-2 prolong tumor latency, and enhance tumor lysis in vitro by natural killer cells. The variant neuro-2a tumor has also been used to evaluate the role of expressing both B-7-1 costimulator and IFN-γ. This resulted in upregulation of expression of class-1 MHC and a CD8-positive T-cell response that effectively induced tumor rejection (Katsanis et al. 1996). A variant of C-1300, NX31T28, engineered to express GD2 ganglioside has been used to evaluate biodistribution and activity of a novel fusion protein consisting of mouse chimeric anti-GD2 antibody ch14.18 fused to IL-2 (Lode et al. 1999). This fusion protein, but not the antibody alone or IL-2 plus antibody, was effective in suppressing development of bone marrow and liver disease. The same group showed that CD8+ T cells genetically engineered to produce a single chain IL-12 fusion protein significantly protect syngeneic A/J mice from disseminated neuroblastoma growth (to bone marrow and liver) (Lode et al. 1998a). Local release of cytokines in the tumor microenvironment is the mechanism thought to be responsible for inhibition of malignant growth and thus successful therapy. The NX31T28 model engineered to express IL-12 has also been used to induce CD8 positive-dependent protective immunity, prevented growth of wild-type cells, and eradicated established disease. The same model as been used to evaluate anti-GD2 antibody conjugated to the cytotoxic antibiotic calicheamycin θ^1_1 (Lode et al. 1998b). Other approaches evaluated in syngeneic models include immunotherapy with a modified DNA vaccine where mice were immunized with a tyrosine hydroxylase-based DNA vaccine enhanced with the posttranscriptional regulatory acting RNA element derived from the woodchuck hepatitis virus in combination with an antibody-cytokine fusion protein ch14.18-IL-2. This DNA vaccine was delivered using attenuated *Salmonella typhimurium* and administered by oral gavage (Pertl et al. 2003). This facultative intracellular parasite that colonizes the liver has been shown to accumulate within extrahepatic malignancies (Soto et al. 2004). Other researchers have investigated the use of IL-12 or IL-2 plus IL-18 transduced dendritic cells as vaccines for neuroblastoma treatment (Redlinger et al. 2003a,b), the effect of cytokine expression on neuroblastoma growth (Siapati et al. 2003), and alteration in tumors recurrent after suboptimal dosing of a humanized IL-2 immunocytokine targeted to the GD2-ganglioside. The antitumor effect of retinoic acid on the susceptibility of neuroblastoma to CTL-mediated killing has recently been reported to act through stabilization of MHC class-1 complexes, and independently of IFN-γ or TNF-α (Vertuani et al. 2003).

17.5 Conclusion

There is now an increasing selection of animal models in which to evaluate experimental therapy of neuroblastoma. No one model fits all. Instead, a specific model may have a restricted but valuable role. As yet, there is limited data on the role of the transgenic *MYCN* model in developmental therapeutics, but undoubtedly this will emerge; however, retrospective analysis of why the significant efficacy of cytotoxic or other agents in mice fail to translate into clinical responses suggests that direct translation from any murine model to humans needs considerable caution irrespective of the tumor model being used. Despite the limitations, clearly these models are useful in identifying novel approaches to neuroblastoma treatment.

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Late Effects of Treatment

Caroline Laverdière, James Gurney, Charles A. Sklar

Contents

18.1 Introduction

Neuroblastoma is a cancer with heterogeneous clinical manifestations and behaviors. Localized tumors can usually be cured by surgical resection alone (Kushner et al. 1996b; Perez et al. 2000; Brodeur and Maris 2002). At the other end of the spectrum, aggressive metastatic tumors frequently progress despite intensive chemoradiotherapy (Brodeur and Maris 2002). The overall prognosis of high-risk neuroblastoma patients remains poor, with an overall survival rate of less than 30 % at 5 years (Brodeur and Maris 2002); however, with the use of high-dose chemotherapy regimens, plus new drugs, such as cis-retinoic acid, and new modalities of treatment, such as targeted immunotherapy, a subset of patients with high-risk disease achieve and maintain a complete remission (Kushner et al. 1994; Cheung et al. 1998; Matthay et al. 1999; Cheung et al. 2001). Survivors of high-risk neuroblastoma, therefore, face the long-term consequences of intensive multimodality therapy given, often, at a young age.

Only a limited number of studies have assessed the long-term clinical late effects that are specific to survivors of neuroblastoma. The data are particularly scanty on those who have been treated for high-risk disease (Willi et al. 1992; Olshan et al. 1993; Kaste et al. 1998; Kushner et al. 1998; Hovi et al. 1999; Nève et al. 1999; Barr et al. 2000; Koyle et al. 2001; Hölttä et al. 2002; Van Santen et al. 2002; Weiss et al. 2003).

In this chapter we review the major and most frequent late complications observed in low/intermediate-risk and high-risk neuroblastoma survivors. Since the radiation fields and the chemotherapeutic

agents adopted in neuroblastoma treatment protocols are commonly used to treat other pediatric cancers, published data on late effects in these cancer survivors are highly relevant for children with neuroblastoma. Additionally, we discuss the late effects observed in a cohort of 65 neuroblastoma survivors, predominantly survivors treated for high-risk disease, who are followed in the Long-Term Follow-Up Clinic at Memorial Sloan Kettering Cancer Center (MSKCC).

18.2 Long-Term Complications for Low- and Intermediate-Risk Neuroblastoma Survivors

Recent studies in neuroblastoma have allowed for the development of a risk-stratification system for treatment, based on clinical and biological factors (Brodeur 2003). Low-risk patients have localized tumors with favorable biological features and can be cured by surgical removal of the primary tumor (Matthay et al. 1989; Kushner et al. 1996b; Perez et al. 2000). The intermediate-risk group includes mainly patients with locally invasive tumors with favorable biological features. The current management of these patients is conservative and for most patients overaggressive initial surgery is not preferred (Cruccetti et al. 2000). Instead, moderate-dose chemotherapy is used to render the primary tumor resectable at second-look surgery (Matthay et al. 1998). External radiation therapy is more controversial (Kushner et al. 1996a; Matthay et al. 1998).

In previous decades, these two groups of patients were more heavily treated with combinations of radical surgery, radiation therapy, and chemotherapy (Brodeur and Maris 2002). A few studies discuss the late effects in these survivors who were treated between 1950 and 1990 (Meadows et al. 1975; Mayfield et al. 1981; Pastore et al. 1982; Kajanti 1983; Pastore et al. 1987; Paulino et al. 2002). The most common long-term complications reported were musculoskeletal and neurological problems.

18.2.1 Musculoskeletal

Musculoskeletal effects include scoliosis, kyphosis, hypoplasia, and fibrosis of bone and soft tissues, as well as slipped capital femoral epiphysis (Mayfield et al. 1981; Pastore et al. 1982; Kajanti 1983). Most of the patients who develop scoliosis have been treated with moderate to high doses of orthovoltage radiation therapy (1500–5000 cGy) and received asymmetric irradiation of the spine. Some patients were also irradiated under 6 months of age, while others underwent laminectomy for epidural disease (Mayfield et al. 1981; Pastore et al. 1982; Kajanti 1983).

18.2.2 Neurological

Neurological problems include paresthesias, mild to severe paresis, paraplegia, and neurogenic bladder (Pastore et al. 1982; Pastore et al. 1987). These complications are related to the disease itself (intraspinal tumors) and/or to surgery. Surgical complications in neuroblastoma are reported in 5–25% of these cases, especially when aggressive resections of thoracic or abdominal tumors were attempted (Azizkhan et al. 1985; Nitschke et al. 1988; Cruccetti et al. 2000). The incidence of intraspinal tumors is higher in children with local–regional disease than in patients with widely disseminated neuroblastoma (Katzenstein et al. 2001, De Bernardi et al. 2001, Plantaz et al. 1996). Neurological recovery correlates inversely with the severity of the presenting neurological deficits (Hoover et al. 1999; De Bernardi et al. 2001; Katzenstein et al. 2001). Recent studies suggest that chemotherapy is as effective as laminectomy and radiation therapy for the treatment of spinal cord compression and is associated with less long-term skeletal sequelae than the two other modalities (Plantaz et al. 1996; Hoover et al. 1999; Katzenstein et al. 2001).

18.3 Long-Term Complications for Survivors of High-Risk Neuroblastoma

High-risk neuroblastoma survivors commonly develop long-term complications due primarily to the intensive multimodality therapy they receive. We recently reviewed the data of a cohort of neuroblastoma survivors followed at our Long Term Follow-Up Clinic at MSKCC. Since 1991, 65 neuroblastoma survivors have been seen. The clinical and treatment data on these patients are summarized in Table 18.1. The majority of these individuals were treated for high-risk disease. Long-term complications were noted in 58 of the 65 (89%) patients, and 40 (62%) experienced more than one complication. Sixteen of these 65 patients (25%) had more than three complications. The more frequent late effects observed are listed in Table 18.2. The late effects that can arise following successful therapy for high-risk neuroblastoma, as well as the specific risk factors associated with these adverse outcomes, are discussed by organ systems in the next section.

18.3.1 Audiological

The ototoxicity of platinum compounds is well described and is more common with cisplatin (CDDP) than with carboplatin (Piel et al. 1974; Schell et al. 1989; Skinner et al. 1990). Platinum compounds are key components of the chemotherapeutic regimens for high-risk neuroblastoma, and many protocols included dose-intensive cisplatin or carboplatin (Kushner et al. 1994; Matthay et al. 1999; Cheung et al. 2001). The overall incidence of cisplatin-induced hearing loss in the pediatric cancer population, including neuroblastoma patients, ranges from 20 to 80% (Schell et al. 1989; Skinner et al. 1990; Weatherly et al. 1991; Parsons et al. 1998; Simon et al. 2002). The hearing loss is more pronounced in the high-frequency

Table 18.1. Clinical and treatment characteristics of neuroblastoma survivors followed in the Long-Term Follow-Up Clinic at MSKCC (*n*=65). *ABMT* Autologous bone marrow transplantation, *TBI* total-body irradiation

Characteristic	No. of patients	Percentage
Gender		
Male	33	51
Female	32	49
Stage of neuroblastoma		
Stage 2	1	1.5
Stage 3	10	15
Stage 4	53	82
Stage 4S	1	1.5
Median age at diagnosis	3.8 years (range 0.07–23.5 years)	
Median follow-up	7.3 years (range 1.9–25.5 years)	
Treatment		
Surgery	65	100
Chemotherapy	64	98
Radiation therapy	56	86
ABMT	35	54
TBI	5	8[a]
Immunotherapy (anti-GD2 monoclonal antibody 3F8)	38	58
Radioimmunotherapy (anti-GD2 monoclonal antibody ^{131}I-3F8)	19	29[b]

[a] Five of 35 patients who underwent ABMT received TBI (14%)
[b] Nineteen of 38 patients who received immunotherapy received radioimmunotherapy (50%)

Table 18.2. Frequencies of late effects observed in neuroblastoma survivors followed in the Long-Term Follow-up Clinic at MSKCC (*n*=65)

Late effect	No. of patients	Percentage
Hearing loss	36	55
Ovarian failure	12	38[a]
Primary hypothyroidism	15	23
Musculoskeletal problems	10	15
Dental problems	8	12
Visual problems	8	12
Neurological deficits	7	11
Pulmonary dysfunction	7	11
Neurocognitive problems	6	9
Renal dysfunction	6	9
Gastrointestinal problems	6	9
Growth hormone deficiency	6	9
Second malignant neoplasm	4	6
Hepatitis C	4	6

[a] *n*=32 female patients

range, but speech frequencies can also be affected (Schell et al. 1989; Parsons et al. 1998). It is usually irreversible, but some patients experience partial recovery (Skinner et al. 1990). Risk factors for cisplatin-induced hearing loss include cumulative dose higher than 360 mg/m^2, young age at treatment, previous or concomitant cranial or head and neck irradiation, abnormal renal function, and use of other ototoxic drugs (e.g., aminoglycosides, loop diuretics) (Schell et al. 1989; Weatherly et al. 1991; Parsons et al. 1998). Hearing loss at a young age has a significant impact on the acquisition of speech and contributes to a lessened quality of life in survivors of advanced neuroblastoma (Barr et al. 2000). Early audiological interventions, such as hearing aids and speech and language therapy, are therefore mandatory to minimize the impact of the hearing loss. The use of chemoprotective agents, such as amifostine for platinum-induced ototoxicity, warrants further investigation (Cronin et al. 2000; Fulda et al. 2001).

In our cohort, 55% (36 of 65) of survivors have hearing loss: 10 patients (16% of the cohort) experience losses at high frequencies (500–2000 Hz) and 26 patients (40% of the cohort) at speech frequencies (500–2000 Hz). These results are in accordance with other studies showing that young age at treatment and high doses of platinum compounds are major risk factors for the development of hearing loss.

18.3.2 Endocrine

18.3.2.1 Thyroid Function

Primary hypothyroidism is a common late effect observed in cancer survivors who received head and neck radiation (Kaplan et al. 1983; Halperin et al. 1999). The occurrence of thyroid dysfunction is related, primarily, to the dose of radiotherapy administered but is also influenced by the age and gender of the patient and the time elapsed since the end of treatment (Sklar et al. 2000).

A higher incidence of clinically significant hypothyroidism is observed above radiation doses of 2000 cGy (Kaplan et al. 1983; Constine et al. 1984).

Current radiotherapy doses used for local control in high-risk neuroblastoma patients with thoracic or cervical tumors are often in this range (Halperin et al. 1999; Kushner et al. 2001). Patients who received total-body irradiation (TBI) are also at risk for hypothyroidism (Ogilvy-Stuart et al. 1992). The radiation-related risk persists more than 25 years after treatment (Hancock et al. 1991; Sklar et al. 2000). The addition of chemotherapy to radiation therapy does not seem to increase the risk of hypothyroidism (Van Santen et al. 2003).

Many cases of subclinical primary hypothyroidism have been described in neuroblastoma patients following the administration of ^{131}I-metaiodobenzylguanidine (^{131} I-MIBG; Garaventa et al. 1991; Picco et al. 1993; Picco et al. 1995; Van Santen et al. 2002). The reported incidence is 50–80 % despite thyroid protection with high doses of potassium iodide before, during, and after the ^{131}I-MIBG (Picco et al. 1995; Van Santen et al. 2002). Optimal prophylaxis against the thyroidal damage induced by radio-iodinated substances is still unknown (Van Santen et al. 2002).

In our cohort, 23 % of the patients developed primary hypothyroidism. Of these patients, 66 % received ^{131}I-3F8 antibody (despite protection with potassium iodide and thyroid hormone suppression) and 33 % received chest/mantle external-beam radiation therapy.

18.3.2.2 Reproductive Endocrine Function

Ovarian Dysfunction

Both chemotherapy and radiation therapy can induce ovarian dysfunction, which can be either transient or permanent. In the pre-pubertal state, ovaries are more resistant to chemotherapy-induced damage than in the post-pubertal individual (Rivkees and Crawford 1988; Halperin et al. 1999). Among all the chemotherapeutic agents, alkylating agents, including cyclophosphamide, ifosfamide, busulfan, BCNU, and CCNU, have most consistently caused ovarian damage. High doses of these agents are very toxic even to young ovaries (Thibaud et al. 1998; Sklar 1999). Patients who have undergone allogenic or autologous bone marrow transplant with high-dose alkylator therapy (e.g., busulfan, melphalan, or thiotepa) are at particularly high risk of developing ovarian failure (Thibaud et al. 1998; Sklar 1999). Furthermore, even if female patients recover ovarian function after treatment is completed, a significant proportion of these patients are at risk of experiencing premature menopause in the future (Byrne et al. 1992).

Radiation-induced ovarian failure is also common in female cancer survivors. As with chemotherapy, pre-pubertal ovaries seem to be more resistant to damage from irradiation than post-pubertal ovaries. Radiation doses above 1000–2000 cGy can, however, cause irreversible ovarian damage in young girls (Stillman et al. 1981; Wallace et al. 1989a); therefore, young females who receive abdominal, pelvic, or spinal irradiation for tumors such as neuroblastoma have a high risk of ovarian failure (Shalet et al. 1976; Stillman et al. 1981; Wallace et al. 1989a; Wallace et al. 1989b). Moreover, we can extrapolate that the concomitant use of intensive chemotherapy in neuroblastoma definitely increases the risk of ovarian failure and premature menopause. Patients who received TBI are also at significant risk of developing irreversible ovarian failure (Sklar 1995a). The use of more conformal radiation techniques, shielding of the ovaries, or oophoropexy are strategies used to lessen the occurrence of this complication (Halperin et al. 1999). Female patients who receive abdominal irradiation are also at increased risk for spontaneous abortion, preterm labor, and the delivery of low-birth-weight infants once they reach childbearing age (Li et al. 1987).

In our cohort, 12 of 32 patients (38 %) developed ovarian dysfunction, which was transient in 3 of the patients. All 12 patients had received cyclophosphamide and 75 % had also been treated with abdominal irradiation.

Testicular Dysfunction

Male germ cells are very vulnerable to both radiation and chemotherapy (Aubier et al. 1989; Halperin et al. 1999). Alkylating agents (cyclophosphamide, nitrosoureas) are very toxic to the germinal epithelium (the sperm-producing cells). This effect is more frequent and more severe at higher doses (Aubier et al.

1989; Sklar 1999). The prepubertal state is not always protective (Aubier et al. 1989). Recovery of spermatogenesis has been reported, but the toxicity is often permanent (Halperin et al. 1999; Sklar 1999). The clinical hallmarks of germ-cell damage include reduced testicular volume and an elevated plasma FSH level.

Leydig cell failure with androgen insufficiency has also been described but is seen infrequently and only following high-dose irradiation (>2000 cGy) administered directly to the testicles (Sklar 1999). Compensated Leydig cell dysfunction (i.e., normal testosterone combined with elevated LH levels) is common after chemotherapy with alkylating agents and lower-dose radiation therapy. The patients are usually asymptomatic and usually progress normally through puberty (Sklar 1999).

18.3.2.3 Growth

Three studies assessed the growth of high-risk neuroblastoma patients after autologous bone marrow transplantation (Willi et al. 1992; Olshan et al. 1993; Hovi et al. 1999). When TBI is used as part of the conditioning regimen, the impact on growth can be significant (Hovi et al. 1999). The majority of patients who do not receive TBI have a better growth velocity (Olshan et al. 1993; Hovi et al. 1999). Impaired growth could be partially explained by radiation to the spine or the abdomen at a young age. The growth impairment results primarily in a loss in the sitting height, resulting in disproportionate short stature (Willi et al. 1992; Sklar 1995b; Hovi et al. 1999). Other reasons for poor growth include poor nutrition and hypothyroidism (Sklar 1995b; Hovi et al. 1999). Growth hormone (GH) deficiency has been described in high-risk neuroblastoma patients who received TBI (Olshan et al. 1993; Hovi et al. 1999). Cranial irradiation was common in this group of patients (Olshan et al. 1993). All the patients had a modest response to GH therapy suggesting a state of relative GH resistance (Olshan et al. 1993; Hovi et al. 1999).

In our cohort, 6 patients (9%) had GH deficiency. Three patients received TBI and five received cranial irradiation.

18.3.3 Musculoskeletal Complications and Neurological Deficits

Survivors of high-risk neuroblastoma face the same neurological and musculoskeletal complications as patients treated for low-risk disease in the past (Mayfield et al. 1981; Pastore et al. 1987; Paulino et al. 2002). Risk factors include intraspinal neuroblastoma, laminectomy, spinal irradiation, and overaggressive surgery (Pastore et al. 1987; Cruccetti et al. 2000; De Bernardi et al. 2001; Katzenstein et al. 2001; Paulino et al. 2002).

18.3.4 Dental

Dental abnormalities are common in childhood cancer survivors. Both chemotherapy and radiation therapy can disrupt normal odontogenesis, especially in patients younger than 5 years (Sonis et al. 1990). Head and neck irradiation has been shown to increase dental injury (Sonis et al. 1990; Hölttä et al. 2002; Estilo et al. 2003). Two studies focused on the dental problems observed in neuroblastoma patients. In a cohort of 52 patients, Kaste et al. (1998) reported an incidence of 71% dental abnormalities including microdontia (38%), excessive caries (29%), root stunting, hypodontia, and enamel hypoplasia. Hölttä et al. (2002) studied a group of high-risk neuroblastoma patients treated with autologous stem-cell transplantation. Among patients who received TBI as a part of the conditioning regimen, dental abnormalities were more severe than those receiving non-TBI regimens. These results suggest that intensive treatment at a young age can affect teeth development, resulting in severe malocclusion, as well as other long-term dentofacial problems. Close attention to oral hygiene and regular dental care are, therefore, mandatory in all neuroblastoma survivors.

18.3.5 Pulmonary

Pulmonary complications in cancer survivors can be related to chemotherapy and radiation therapy. Chemotherapy agents most commonly associated with late pulmonary toxicity include bleomycin, nitrosoureas, cyclophosphamide, melphalan, and

busulfan (Mäkipernaa et al. 1989; Nenadov Beck et al. 1995; Nève et al. 1999). A recent study from the Childhood Cancer Survivor Study (CCSS) showed that cisplatin was associated with an increased risk of lung fibrosis in pediatric cancer survivors (Mertens et al. 2002).

In the same study, chest irradiation was associated with a 3.5% cumulative incidence of lung fibrosis at 20 years after diagnosis (Mertens et al. 2002). Three studies reported on the long-term pulmonary sequelae in neuroblastoma patients (Mäkipernaa et al. 1989; Nenadov Beck et al. 1995; Nève et al. 1999). The most common pulmonary function test (PFT) abnormality reported was restrictive ventilatory defect with decreased lung volumes (Mäkipernaa et al. 1989; Nenadov Beck et al. 1995; Nève et al. 1999). More PFT defects were observed in patients younger than 3 years at diagnosis and those with spinal deformities. TBI was not associated with a significant deterioration in pulmonary function, except in patients treated at a very young age (Nève et al. 1999).

18.3.6 Cardiac

Late cardiac toxicity in childhood cancer survivors is most commonly caused by prior administration of anthracycline (e.g., doxorubicin and daunorubicin). These drugs are associated with the late development of a cardiomyopathy that can result in congestive heart failure and arrhythmias. This complication can appear insidiously, without prior symptoms, and at any time during the post-cancer treatment period (Steinherz et al. 1991; Steinherz et al. 1995). Risk factors include cumulative anthracycline dose ≥300 mg/m^2, age younger than 5 years at treatment, female gender, exposure to mediastinal irradiation, TBI, and combined administration of cyclophosphamide (Lipshultz et al. 1991; Steinherz et al. 1991; Steinherz et al. 1995; Lipshultz et al. 1995; Gupta et al. 2003). In one study done at MSKCC among survivors of leukemia and solid tumors, at a median of 7 years after completion of anthracycline therapy (median dose, 450 mg/m^2), the incidence of abnormal cardiac function on an echocardiogram was 23% (Steinherz et al. 1991). A recent study from the same group showed a decrease in cardiac function in 20% of the patients who had received bolus anthracycline (median dose 385 mg/m^2) compared with 11% of patients who had received it via infusion (median dose 345 mg/m^2) at a mean of 7 years after the end of therapy; however, this difference was not statistically significant (Gupta et al. 2003).

Some conditions, such as isometric exercise, pregnancy, labor, and delivery, and viral infections have been reported to precipitate cardiac decompensation following therapy with anthracyclines (Lipshultz et al. 1995). Close follow-up and monitoring of cardiac function is essential for patients who received anthracyclines with or without chest irradiation during their cancer treatment.

18.3.7 Renal

Many chemotherapeutic agents are associated with the development of acute and chronic renal dysfunction. Cisplatin, ifosfamide, and carboplatin are the most nephrotoxic drugs. Cisplatin induces renal magnesium and potassium wasting, which can lead to severe hypomagnesemia and hypocalcemia (Goren 2003). Cumulative cisplatin dose over 200 mg/m^2 and concomitant administration of other nephrotoxic agents (e.g., aminoglycoside antibiotics) are risk factors for therapy-induced nephrotoxicity (Goren 2003). Ifosfamide has been associated with the development of Fanconi's syndrome (proximal tubular acidosis, hypophosphatemia, glucosuria, and aminoaciduria) that can lead to hypophosphatemic rickets. Glomerular impairment is also described with ifosfamide (Loebstein and Koren 1998; Skinner 2003). A cumulative ifosfamide dose ≥60 g/m^2 is the most significant risk factor for the development and the severity of the nephrotoxicity (Loebstein and Koren 1998).

18.3.8 Neurocognitive

Only a small number of studies have focused on the neurocognitive impact of the intensive therapy that received high-risk neuroblastoma patients (Kelaghan et al. 1988; Simms et al. 1988; Kramer et al. 1992; Phipps et al. 1995; Mitby et al. 2003; Notteghem et al. 2003). Risk factors for neurocognitive impairment

include age less than 3 years, high-dose chemotherapy with autologous bone marrow transplantation, and cranial radiation (Phipps et al. 2000). The impact of TBI on the neurocognitive impairment remains controversial (Simms et al. 1988; Kramer et al. 1992; Phipps et al. 1995; Phipps et al. 2000). Notteghem et al. (2003) recently reviewed the neuropsychological outcomes of 46 high-risk neuroblastoma patients with a mean follow-up of 9.1 years. Survivors of neuroblastoma had an overall performance and skills in the normal range; however, patients who were younger than 3 years when they received the treatment had more visuospatial difficulties and a worse visual memory. Furthermore, hearing loss due to cisplatin was associated with defects in verbal performance (Notteghem et al. 2003).

A recent CCSS study evaluated the educational achievement of a large cohort of childhood cancer survivors. Compared with normal siblings, neuroblastoma survivors were significantly more likely to use special education services because of lower tests scores, and were significantly less likely to complete high school (odds ratio 1.7); however, when the neuroblastoma survivors received special education services, risk estimates approximated those of the sibling population. In the same study, age at diagnosis under 6 years and cranial radiation were associated independently with the use of special education services among all the survivors (Mitby et al. 2003). In contrast, another study reported a similar educational achievement between survivors of neuroblastoma and siblings (Kelaghan et al. 1988).

18.3.9 Subsequent Malignant Neoplasms

Second malignancy is an unfortunate consequence of childhood cancer treatment. The cumulative estimated incidence of subsequent malignant neoplasms (SMNs) 20 years after a diagnosis of childhood cancer is 3.2% (Neglia et al. 2001). For neuroblastoma survivors, the relative risk of developing an SMN has been reported as 6.59 when compared with the general population, with a cumulative incidence at 20 years of 1.87% (Neglia et al. 2001). This latter estimate is derived from a historical cohort that includes a large number of survivors treated for low/intermediate-risk disease and may not reflect the true risk for patients who are treated with more intensive contemporary regimens.

The most common secondary malignancies reported after neuroblastoma are myelodysplasia/leukemia, thyroid neoplasm, soft tissue sarcomas, and osteosarcomas (Shah et al. 1983; Meadows et al. 1985; de Vathaire et al. 1989; Tucker et al. 1991; Kushner et al. 1998; Tabone et al. 1999; Schiavetti et al. 2001; Acharya et al. 2003; Garaventa et al. 2003; Le Deley et al. 2003; Weiss et al. 2003). Treatment-related myelodysplasia/leukemia has been well described. The most common chemotherapeutic agents associated with this complication are topoisomerase-II inhibitors (etoposide, doxorubicin) and alkylating agents. The two classes of drugs are associated with specific and different cytogenetic abnormalities (Kushner et al. 1998; Le Deley et al. 2003). Among neuroblastoma survivors treated at MSKCC, Kushner et al. (1998) found a 3-year cumulative incidence of secondary myelodysplasia/leukemia of 7%; therefore, current intensive treatment for high-risk neuroblastoma is associated with a significant risk for treatment-related acute myeloid leukemia and warrants that these patients be monitored closely.

Secondary thyroid neoplasms are also common in neuroblastoma survivors due to chest and spinal irradiation at a young age (Tucker et al. 1991; Le Deley et al. 2003).

Recently, two studies reported on the risk of SMN (leukemia and solid tumors) with the administration of ^{131}I-MIBG (Garaventa et al. 2003; Weiss et al. 2003). In one of these studies, the cumulative risk of developing a second cancer was 20% within 15 years of receiving ^{131}I-MIBG (Garaventa et al. 2003).

18.4 Health-Related Quality of Life

Beyond survival and long-term late effects, health-related quality of life (HRQOL) is increasingly being appreciated as an important outcome measure for evaluating treatment effects in clinical research (Spilker 1996; Staquet 1998; Drotar 1998; Joyce 1999). Health-related quality of life is a subjective term, but it reflects the understanding that health is not only

the absence of disease, but also a complex function of psychological, social, physical, and functional well-being. The very few HRQOL studies conducted on childhood cancer survivors to date have usually been limited to proxy reports from clinicians or parents, without considering the child's own perspective on his or her functioning and well-being. Practically no published HRQOL-related studies are specific to neuroblastoma survivors. One exception was a study of health status conducted by Barr and colleagues (2000) using health utility indexes. They sent questionnaires to parents of children who survived either Wilms' tumor (n=52) or advanced neuroblastoma (n=26), and compared scores related to health attributes and functional capacity. Children surviving high-risk neuroblastoma had a greater overall burden of morbidity, and a significantly higher likelihood of sensory deficits, specifically in speech and hearing, than did children surviving Wilms' tumor. In one measure representing constructs for memory and problem-solving skills, both groups showed indications of cognitive morbidity (Barr 2000). Psychological adjustment and psychosocial functioning among long-term neuroblastoma survivors has yet to be addressed in the clinical literature, although a cross-sectional study on HRQOL and family impact from the cancer experience is currently being conducted through the Children's Oncology Group. This study compares outcomes across neuroblastoma treatment regimens. Results from that study should be available soon.

18.5 Conclusion

Late effects of treatment are common among neuroblastoma survivors, mostly in high-risk patients. These complications are important and must be identified and treated early in order to minimize the impact on quality of life of the survivors. The new treatment paradigms for low- and intermediate-risk patients will probably result in a decrease in treatment-related morbidity. However, for high-risk patients, the intensification of therapy for these patients treated at a very young age can have severe late effects; therefore, close follow-up of these patients after completion of the therapy is strongly recommended. More specifically, ovarian function of female patients should be followed closely. All high-risk patients treated with platinum compounds should be screened for hearing loss, and early audiological interventions, such as hearing aids and speech and language therapy, should be implemented as needed. In addition, neurocognitive development should be monitored with serial psychometric testing and patients should be screened for subsequent malignant neoplasms. Because long-term survivors of high-risk neuroblastoma can have complex medical problems with multi-organ dysfunction, these children are optimally cared for by physicians familiar with the late effects of intensive chemotherapy and radiation. New therapeutic strategies for high-risk neuroblastoma that will lead to higher rates of survival as well as enhanced quality of life are desperately needed.

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Perspectives and Future Directions

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Contents

A hallmark of neuroblastoma (NB) is heterogeneity, with a wide spectrum of clinical behavior which varies according to age at diagnosis, the stage of disease, and tumor biology (Brodeur 2003). This heterogeneity is most evident in the numerous transformation-linked genetic changes identified in cell lines and tumors. Some of these aberrations are predictive of treatment response and outcome (see Chap. 4). Nevertheless, it is increasingly clear that despite such tissue heterogeneity, the clinical biology of NB is generally predictable. By and large, patients with stage-4S and local–regional tumors are curable with minimal or no therapy, whereas children with distant metastatic disease pose an enormous clinical challenge. Only small subsets of patients have elusive risk identities at diagnosis. Modern treatments stratifying patients according to both clinical and biological factors are now the standard (see Chap. 7). At the present time, because of disparities in classification and treatment approaches, it remains difficult to compare the results of clinical trials conducted in different regions of the world; however, efforts are currently underway to develop an International NB Risk Group (INRG) System.

All of the current risk grouping systems utilize age at diagnosis (≤vs >1 year), INSS stage, and tumor *MYCN* status. The COG Risk Classification System also includes tumor histology and ploidy, whereas other cooperative groups have incorporated the pattern of metastatic disease, tumor resectability, and the presence or absence of threatening symptoms (see Chaps. 7 and 11). As currently defined, each of the risk-classification systems has limitations. Small subsets of patients classified as low- or intermediate risk at diagnosis have acted clinically as aggressive

disease, whereas other children, currently classified as high risk, have favorable outcomes and may not require the dose-intensive therapeutic approach presently prescribed. Some genetic abnormalities and molecular markers not utilized in the current classification schemas may help refine the definition of risk groups (see Chap. 4–5), and prospective studies investigating their clinical significance are ongoing. In addition, new techniques, such as comprehensive gene expression profiling, are being utilized to molecularly classify NB tumors (see Chap. 9). These studies are likely to lead to a refinement of the current risk-group classification systems and an improvement in risk-group based treatment strategies.

Although substantial progress has been made in the treatment approach toward patients with low- and intermediate-risk NB, the cure rate for metastatic NB in children remains unsatisfactory. As described in Chap. 11, most low-risk patients are successfully treated with surgery alone, and some infants do not require any treatment because their tumors have a high frequency of spontaneous regression (Chap. 2). Even for infants with stage 4 NB, >90% long-term survival is typical if the tumor *MCYN* oncogene is not amplified. Similarly, among patients with intermediate-risk tumors, >90% survival is expected following moderate-dose chemotherapy and surgery. In contrast, outcome remains poor for children older than 1 year with metastatic NB, with or without *MYCN* amplification, and during the past decade there has been only a modest improvement in cure. This small gain is due to intensification of induction chemotherapy, megatherapy consolidation, biological/immunological therapy and improved supportive care. Several clinical trials, including the large prospective randomized CCG-3891 study which demonstrated superior outcome for patients randomized to myeloablative therapy and bone marrow transplant vs chemotherapy during consolidation (Matthay et al. 1999), support the hypothesis that dose intensification is an important component to achieve successful treatment of metastatic NB (Cheung and Heller 1991). Whether intensification is most beneficial during induction or during consolidation remains controversial. Although promising results have also been observed in recent pilot studies testing tandem cycles of high-dose therapy plus stem-cell rescue (Grupp et al. 2000; Kletzel et al. 2002) (Chap. 11), further dose escalation is likely to be unacceptable. In addition, despite achieving complete clinical remission, the majority of children with high-risk disease will relapse due to drug-resistant residual disease. Eradication of refractory microscopic disease remains the most significant challenge in the treatment of metastatic NB. The paradigm of "more is better" should be questioned and additional high-risk trials testing biological and targeted agents need to be designed (Chap. 11).

Recently, the differentiation agent 13-cis retinoic acid was shown to be clinically effective when administered in the setting of minimal residual disease in the randomized CCG 3891 clinical trial (Matthay et al. 1999) (reviewed in Chap. 15). This seminal study demonstrated that a biological agent was capable of impacting outcome in high-risk NB. The COG is currently conducting a randomized prospective study comparing the efficacy of anti-GD2 ch14.18 antibody plus cytokines and 13-cis retinoic acid vs 13-cis retinoic acid alone in the setting of minimal residual disease. Clinical trials have also been developed in Europe to test immunotherapy in high-risk NB, and a single-arm study investigating the efficacy of the anti-GD2 antibody 3F8 plus GM-CSF, is ongoing at Memorial Sloan-Kettering Cancer Center. Additional phase-I and phase-II studies are testing other targeted therapies (see Chap. 12). As outlined in Chaps. 14–17, preliminary studies suggest that several immunotherapeutic molecules, new retinoids, anti-angiogenic agents, and other experimental therapeutics have activity against refractory disease.

As reviewed in Chap. 18, a variety of acute and late complications from NB and its treatment may occur; these include late effects of chemotherapy, radiation therapy, and surgery. High-risk patients are at greatest risk because of the intensive multi-modality treatment strategies that are currently utilized. Reliable identification of the subset of patients currently classified as high risk who do not require intensive therapy would significantly decrease long-term morbidity and treatment-related mortality for these very young patients. For example, data from both the POG and CCG indicate that toddlers 12–18 months of age

with favorable biology stage-4 tumors may not require the current intensive high-risk treatment regimen to be cured (Schmidt et al. 2003; George et al. 2003); however, ultimate improvements in survival and reductions of late effects may require more targeted therapies. Research aimed at discovering new genes and pathways critical to NB tumorigenesis and drug resistance should be prioritized. It is hoped that these biologically based treatment approaches will prove to be more effective and less toxic than the current regimens.

We have learned important lessons from NB. The clinical biology of stage-4S and local-regional NB, when combined with the findings of the screening study (Chap. 2), have challenged accepted oncological principles. If clinical progression from local regional small NB to metastatic disease does not generally occur, adjuvant cytotoxic therapy is probably not necessary for the majority of these patients. On the other hand, despite general sensitivity of NB to chemotherapy, curing minimal residual metastasis remains difficult. Research focused on its measurement, control, or eradication should be emphasized. Most important of all, with the growing list of promising therapies, efforts devoted to their timely and effective integration into an overall curative strategy should have high priority.

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