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Genetics in Otorhinolaryngology

Editors
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Contents

Preface

Molecular genetics has been developing rapidly, and the molecular basis of many diseases has been discovered. Identification of the relevant genes has led to more precise diagnosis and has provided clinicians and researchers with important clues as to the identity of previously unsuspected molecules whose function is critical for the development and maintenance of the disease. This includes diseases in the field of otorhinolaryngology. A knowledge of genetics has already become important for the otorhinolaryngologist when examining patients at their outpatient clinic. Research is in progress to identify the genes for deafness and craniofacial anomalies as well as the development of novel therapeutic modalities, including gene therapy for head and neck cancer.

Otorhinolaryngologists have been active participants and have made significant contributions to advancing clinical as well as basic scientific knowledge in the molecular characterization of the above diseases. In many cases, the suspicions of an otorhinolaryngologist have led to the identification of underlying genetic abnormalities and clues toward making a diagnosis have been identified. Many patients have highlighted the need for otorhinolaryngologists to be constantly alert to the possibility of diagnosing hereditary disorders and to obtain genetic consultations for a complete evaluation. However, genetics, and particularly molecular genetics, remains an unfamiliar subject for most otorhinolaryngologists. Therefore, we present a volume on this subject in this Karger series.

We have organized this volume around the molecular analysis of diseases as well as emphasizing the clinical significance of this molecular analysis in the field of otorhinolaryngology. The purpose of the volume is to explain to general otorhinolaryngologists how genetics can be useful to them and to their patients, and to summarize the information about genetic diseases that is relevant to their clinical activity.

In this volume, we do not include a detailed description of the basic methodology of genetic studies. You will find information about the positions of the various loci on chromosomes, and, in cases where the gene has been identified, details of the gene and the effects of the mutations. The clinical aspects of diseases are described with attention to particularly distinguishing features that may be diagnostic and to any variability observed in the phenotype. We focus on how this information will be useful to the clinician and to the patient, particularly with reference to enabling accurate genetic counseling, with a list of references if more details are needed.

A basic and general review of genetics, which is requisite for readers to be able to understand the content of this volume, is presented in the first part of the volume. More pages are allocated to hearing impairment than to other aspects of otolaryngology because great progress has been made over the last few years in identifying the genes associated with hearing impairment. One chapter focuses on for the mouse model for hearing impairment because the mouse is the most useful animal system for advancing research in hereditary hearing impairment.

The application of molecular analysis to head and neck carcinoma has been one of the fundamental breakthroughs in understanding the cell biology of the carcinoma. In relation to these breakthroughs, two chapters are devoted to a discussion of tumor suppressor genes and oncogenes.

Understanding of the molecular basis of the disease will lead to develop new modality in treating patients with that disease. Although it seems unlikely that gene therapy will be clinically useful for a long time, gene therapy, ultimately, is expected to become the treatment of choice for curing patients with diseases related to genetic abnormalities. One chapter is therefore devoted to gene therapy.

The field of molecular genetics has rapidly evolved from basic scientific research to having diagnostic and therapeutic clinical applications. Today's laboratory discoveries will undoubtedly lead to major advances in the way clinicians diagnose and treat diseases. We do hope that this volume can review the molecular and clinical features of different types of genetic disorders facing the otorhinolaryngologist and can create a productive interplay between geneticists and otorhinolaryngologists.

Finally, I thank Prof. Karen P. Steel for her great editorial work as an editor and all contributors for their commitment to this volume.

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General Review of Medical Genetics

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Medical genetics is one of the most rapidly developing fields in medicine, since it has been recognized that genetic factors are related to many disorders as a core role of its etiology. Our genetic background determines not only the cause of catastrophic single-gene disorders, but also predisposition to cancer, heart disease, psychiatric disorders and even to some infectious diseases. Knowledge about the structure and function of genes is accumulating at an accelerating pace. An increasing number of diseases can now be understood by genetic analysis. This contributes to improved precision in diagnosis and prognosis, helps to counsel patients and their relatives, and provides an outlook toward treatment. Now, medical genetics is one of the fundamentals not only for basic scientists but also for any kind of clinician, including ENT doctors.

The Load of Genetic Disease

Although each genetic disorder is not frequent, the total of genetic disorders is not small and they are an important cause of illness and death. It is generally said that 3–4% of all pregnancies result in the birth of a child with birth defects including genetic disorders. According to a survey in British Columbia, more than 5% of liveborn persons under 25 years of age had a genetic disorder. Moreover, all humans may suffer from diseases related to genetic factors before their death, because susceptibility to almost all diseases is genetically determined [1].

Classification of Genetic Disorders

Genetic disorders have often been classified into three major categories: chromosomal disorders, single gene defects and multifactorial diseases. However, recent studies request two more genetic categories: mitochondrial diseases and somatic cell genetic defects. Each genetic category will be briefly discussed.

Chromosomal Disorders

Chromosomal disorders are quantitative abnormalities of genes caused by the addition (trisomy) or deletion (monosomy) of entire chromosomes or parts of chromosomes. As each chromosome contains several thousands of genes, and each chromosomal band contains several hundreds of genes, patients with those quantitative abnormalities have severe conditions such as mental retardation, growth failure and multiple congenital anomalies. Nearly 1% newborn babies suffer from chromosomal disorders. Chromosomal disorders occur in 15% of recognized pregnancies and in 50% of conceptions, most of those end in a miscarriage.

Trisomies of entire autosomes observed in newborn babies are only trisomies 13, 18 and 21. Patients with trisomies 13 or 18 die in early infancy. Down syndrome caused by 21 trisomy is the most frequent chromosomal disorder and occurs in about 1 in 1,000 liveborn infants and increases in frequency with advancing maternal age. It is characterized by mild to moderate mental retardation and physical abnormalities including ear anomalies.

Chromosome banding analysis enables us to make a precise diagnosis of partial trisomy and partial monosomy syndromes. Several hundreds of partial monosomy or partial trisomy syndromes have been reported. The most important advance in cytogenetics in recent years is the development of the FISH (fluorescence in situ hybridization) technique. If useful DNA probes are available, FISH enables us to detect very tiny structural chromosomal abnormalities (microdeletion syndromes or contiguous gene syndromes). FISH increases the accuracy of cytogenetic detection by 100 times compared to the conventional banding methods. Now, we can make diagnoses of the following malformation syndromes by using FISH analyses: Wolf syndrome (4p16.3), cat-cry syndrome (5p15.2), Williams syndrome (7q11.23), Prader-Willi/Angelman syndrome (15q11.2), Miller-Dieker syndrome (17p13.3), Smith-Magenis syndrome (17p11.2), CATCH22 (DiGeorge syndrome, Velocardiofacial syndrome, 22q11.2), and Kallmann syndrome (Xp22.3).

Single Gene Disorders

Single gene disorders are caused by single mutant genes that have large effects on phenotypes. As single gene disorders are inherited in simple Mendelian fashions, they are also referred to as Mendelian diseases. More than 4,000 disorders are known as single gene disorders inherited in autosomal dominant, autosomal recessive or X-linked fashion. Most of the diseases described in this book are classified into this category. Single gene disorders are relatively rare, with a frequency that may be as high as 1 in 500 but which is usually much lower. However, there are many different kinds of single gene disorders and their combined impact is significant. Recent molecular genetics research mainly has focused on the single gene disorders; mapping and cloning of the responsible gene, and elucidating the basic biochemical defect, have been performed in several hundreds of the single gene disorders.

Multifactorial Diseases

Multifactorial diseases are not caused by a single error in the genetic information, but result from the interaction of multiple genes and environmental factors. These diseases are responsible for congenital malformations such as cleft lip and palate, congenital heart diseases and pyloric stenosis, and for many common disorders of adult life such as diabetes mellitus, hypertension, coronary heart disease and schizophrenia. The clinical impact of multifactorial diseases is important in both the neonatal period and in adult life. Recent molecular genetics research is elucidating susceptibility genes in several common diseases, and prevention method will hopefully be developed.

Mitochondrial Diseases

Mitochondrial diseases, caused by mutation of mitochondrial DNA, often affect energy production in nerve and muscle, and may play a role in cellular aging. Some deafness is thought to be a mitochondrial disease. As mitochondrial diseases are inherited in a characteristic maternal fashion, special attention is necessary in genetic counseling.

Somatic Cell Genetic Disorders

One of the representatives of somatic cell genetic disorders is cancer. It is now clear that all human cancer results from mutations in DNA. However, these mutations are restricted only in specific somatic cells and are never transmitted to subsequent generations except for several familial cancers. Other than cancer, some developmental disorders such as McCune-Albright syndrome are classified into this category.

Molecular Genetics Research

The purpose of the human genome project is to determine the complete genetic information carried in the human genome. The human genome is composed of approximately 3,000,000,000 nucleotides. The first step of the human genome project is determining, deciphering and recording the chain of ATGC (adenine, thymine, guanine, cytosine) that makes up the human genome. According to recent expectation, the first step will be completed in 2,003. The information from human genome will elucidate the approximately 100,000 genes existing in the 60 trillion cells of the human body and their characteristics and functions.

In the study of genetic disorders, we have to clone the gene responsible for the genetic disease. The following four strategies are used in this research:

1) *Functional cloning:* If we know the function of the gene, we can clone it by purifying the gene product and determining aminoacids. However, there are not many genes for which this strategy is available.

2) *Positional cloning:* At first, gene locus is determined by linkage analysis or analysis of a patient with both chromosomal rearrangement and genetic disorder. Then narrowing the gene locus by constructing the physical and genetic map leads to cloning the gene. This strategy is useful in many disorders, but it needs tremendous amount of effort, labor and a large budget.

3) *Position-independent candidate gene approach:* If there is a similarity of phenotype between human and animals, the gene responsible for the phenotype in the animal is a candidate of the human gene. However, there are few genes for which this strategy is useful.

4) *Positional candidate gene approach:* We can know many candidate genes at each chromosomal location by genome data base. We can clone a gene by combination of location information and candidate gene information. This is the main strategy to clone a gene.

In several disorders described in this book, the responsible gene has been cloned and its functional research has begun. However, in the many other disorders especially in nonsyndromic deafness, we can know only the gene localization. We have to recognize that there is a great distance between mapping the gene and cloning the gene. After cloning the gene, we have to prove that this gene is the cause of the disease by finding mutations among the patients, recovery of the gene function through gene introduction, or creating model animals (knockout or transgenic).

Although we are still standing in the middle of the medical genetics research, medical genetics must deal with human disease at the fundamental level and its development has had profound implications for clinical medicine and the magnitude of those implications will continue to grow.

Instructions:
— Key should contain all information relevant to interpretation of pedigree (e.g., define shading)
— For clinical (non-published) pedigrees, include:
  a) family names/initials, when appropriate
  b) name and title of person recording pedigree
  c) historian (person relaying family history information)
  d) date of intake/update
— Recommended order of information placed below symbol (below to lower right, if necessary):
  a) age/date of birth or age at death
  b) evaluation (see Figure 5)
  c) pedigree number (e.g., I-1, I-2, I-3)

	Male	Female	Sex Unknown	Comments
1. Individual	b. 1925	30 y	4 mo	Assign gender by phenotype.
2. Affected individual				Key/legend used to define shading or other fill (e.g., hatches, dots, etc.).
				With ≥2 conditions, the individual's symbol should be partitioned accordingly, each segment shaded with a different fill and defined in legend.
3. Multiple individuals, number known	5	5	5	Number of siblings written inside symbol. (Affected individuals should not be grouped.)
4. Multiple individuals, number unknown	n	n	n	"n" used in place of "?" mark.
5a. Deceased individual	d. 35 y	d. 4 mo		Use of cross (†) may be confused with symbol for evaluated positive (+). If known, write "d." with age at death below symbol.
5b. Stillbirth (SB)	SB 28 wk	SB 30 wk	SB 34 wk	Birth of a dead child with gestational age noted.
6. Pregnancy (P)	P LMP: 7/1/94	P 20 wk	P	Gestational age and karyotype (if known) below symbol. Light shading can be used for affected and defined in key/legend.
7a. Proband	P	P	P	First affected family member coming to medical attention.
7b. Consultand			Individual(s) seeking genetic counseling/testing.	

Fig. 1. Common pedigree symbols, definitions, and abbreviations.

Genetic Testing, Genetic Counseling and Clinical Genetics

Recent advances in molecular genetics give us several new tools, such as genetic testing, in the field of clinical practice. Genetic testing is the analysis of a specific gene, its product or function, or other DNA and chromosome analysis, to detect or exclude an alteration likely to be associated with a genetic

Instructions:
— Symbols are smaller than standard ones and individual's line is shorter. (Even if sex is known, triangles are preferred to a small square/circle; symbol may be mistaken for symbols 1, 2, and 5a/5b of Figure 1, particularly on hand drawn pedigrees.)
— If gender and gestational age known, write below symbol in that order.

	Male	Female	Sex Unknown	Comments
1. Spontaneous abortion (SAB)	male	female	ECT	If ectopic pregnancy, write ECT below symbol.
2. Affected SAB	male	female	16 wk	If gestational age known, write below symbol. Key/legend used to define shading.
3. Termination of pregnancy (TOP)	male	female		Other abbreviations (e.g., TAB, VTOP, Ab) not used for sake of consistency.
4. Affected TOP	male	female		Key/legend used to define shading.

Fig. 2. Pedigree symbols and abbreviations for pregnancies not carried to term.

disorder. By using genetic testing, presymptomatic diagnosis, carrier detection or prenatal diagnosis is now available in nearly one hundred genetic disorders. In many cases, genetic testing is different from other forms of medical tests, and contains ethical legal and social issues. Thus, genetic testing must be looked at as an overall process including genetic counseling, not simply as a laboratory activity. According to the definition by the American Society of Human Genetics, genetic counseling is a communication process that deals with the human problems associated with the occurrence, or the risk of occurrence, of a genetic disorder in a family. This process involves an attempt by one or more appropriately trained persons to help the individual or family to (a) comprehend the medical facts, including the diagnosis, probable course of the disorder, and the available management; (b) appreciate the way heredity contributes to the disorder, and the risk of recurrence in specified relatives; (c) understand the alternatives for dealing with the risk of recurrence; (d) choose the course of action that seems to them appropriate in view of their risk, their family goals, and their ethical and religious standards, and to act in accordance with that decision, and (e) make the best possible adjustment to the disorder in an affected family member and/or to the risk of recurrence of that disorder [2].

We sometimes call the genetics supported by recent molecular technique 'new genetics' [3]. Usefulness of new genetics will expand to all medical and clinical fields. However, the fundamental of clinical genetics is still the taking of a comprehensive family history. I would like to introduce the standardized human pedigree nomenclature recommended by the American Society of Human Genetics (figs. 1–5). This nomenclature reduces the chances for incorrect

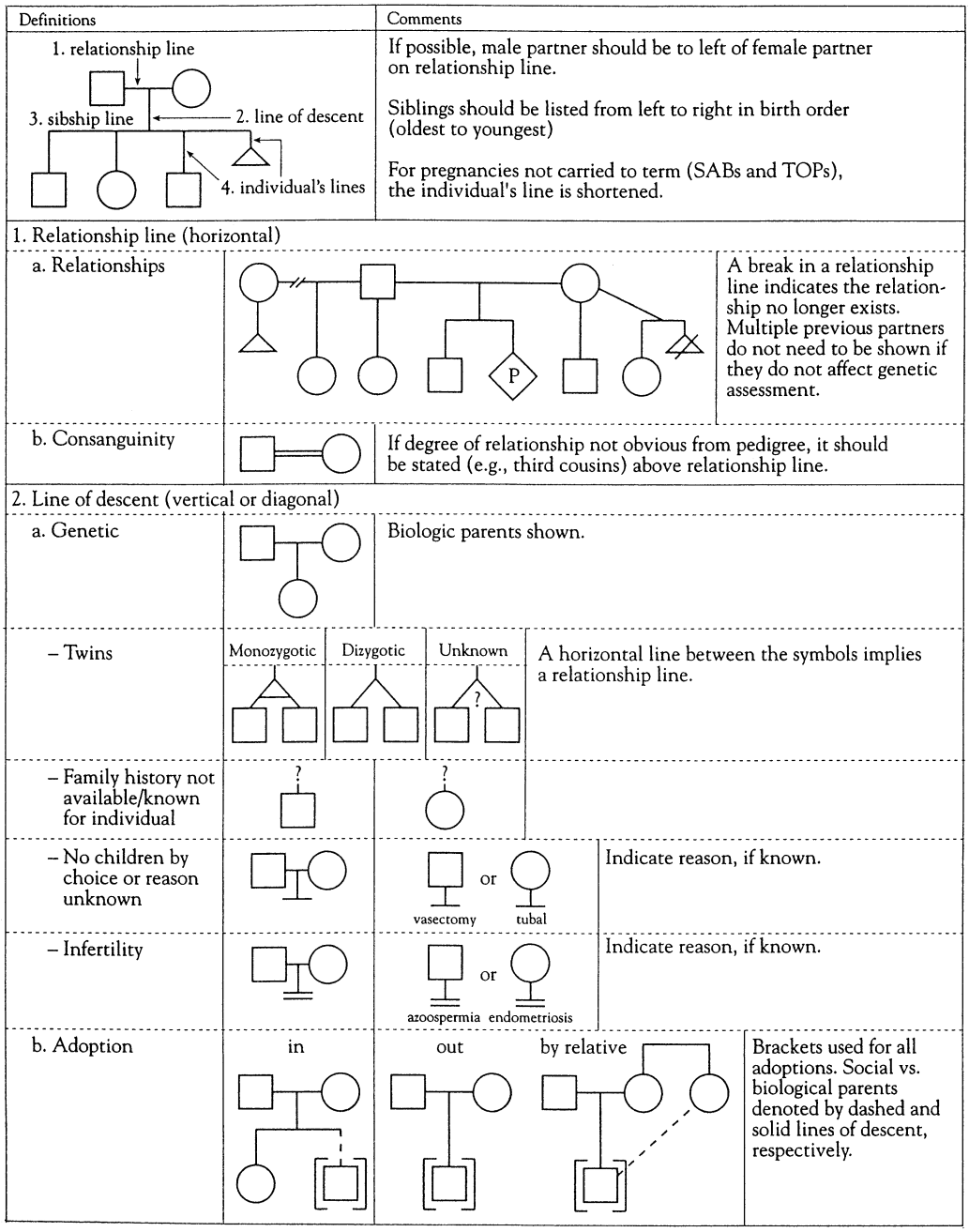


Fig. 3. Pedigree line definitions.

Definitions:
— Egg or sperm donor (D)
— Surrogate (S)
— If the woman is both the ovum donor and a surrogate, in the interest of genetic assessment, she will only be referred to as a donor (e.g., 4 and 5)
— The pregnancy symbol and its line of descent are positioned below the woman who is carrying the pregnancy.
— Family history can be taken on individuals, including donors, where history is known.

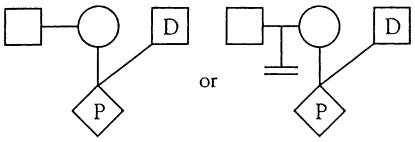
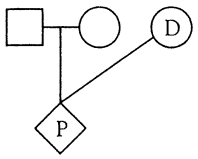
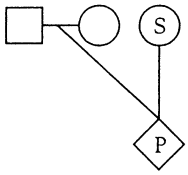
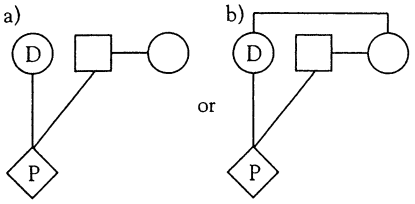
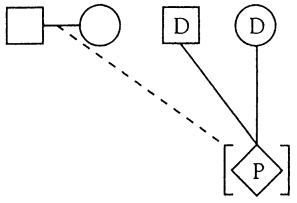
Possible Reproductive Scenarios		Comments
1. Sperm donor		Couple in which woman is carrying pregnancy using donor sperm. No relationship line is shown between the woman carrying the pregnancy and the sperm donor. For a lesbian relationship, the male partner can be substituted with a female partner.
2. Ovum donor		Couple in which woman is carrying pregnancy using donor egg(s) and partner's sperm.
3. Surrogate only		Couple whose gametes are used to impregnate another woman (surrogate) who carries the pregnancy.
4. Surrogate ovum donor		Couple in which male partner's sperm is used to inseminate a) an unrelated woman or b) a sister who is carrying the pregnancy for the couple.
5. Planned adoption		Couple contracts with a woman to carry a pregnancy using ovum of the woman carrying the pregnancy and donor sperm.

Fig. 4. Assisted-reproductive-technologies symbols and definitions.

Instructions:
— Evaluation (E) is used to represent clinical and/or test information on the pedigree.
  a. E is to be defined in key/legend.
  b. If more than one evaluation, use subscript (E_1, E_2, E_3) and define in key. May be written side by side or below each other depending on available space.
  c. Test results should be put in parentheses or defined in key/legend.
  d. If results of exam/family study/testing not documented or unavailable, may use a question mark (e.g., E?).
— Documented evaluation (*)
  a. Asterisk is placed next to lower right edge of symbol.
  b. Use ***only*** if examined/evaluated by **you** or ***your*** research/clinical team or if the outside evaluation has been personally reviewed and verified.
— A symbol is shaded only when an individual is clinically symptomatic.
— For linkage studies, haplotype information is written below the individual. The haplotype of interest should be on left and appropriately highlighted.
— Repetitive sequences, trinucleotides and expansion numbers are written with affected allele first and placed in parentheses.
— If mutation known, identify and place in parentheses.
— Recommended order of information:
  1) age/date of birth or age at death
  2) evaluation information
  3) pedigree number (e.g., I-1, I-2, I-3)

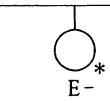
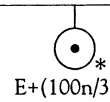
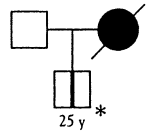
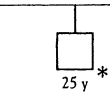
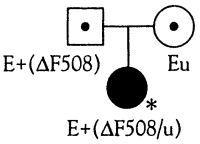
Definition	Symbol	Scenario	Example
1. Documented evaluation (*)		Woman with normal physical exam and negative fragile X chromosome study (normal phenotype and negative test result).	 E−
2. Obligate carrier (will **not** manifest disease).		Woman with normal physical exam and premutation for fragile X (normal phenotype and positive test result).	 E+(100n/35n)
3. Asymptomatic/presymptomatic carrier (clinically unaffected at this time but could later exhibit symptoms)		Man age 25 with normal physical exam and positive DNA test for Huntington disease (symbol filled in if/when symptoms develop).	 25 y E+(45n/18n)
4. Uninformative study (u)	 Eu	Man age 25 with normal physical exam and uninformative DNA test for Huntington disease (E_1) and negative brain MRI study (E_2).	 25 y E_1u(36n/18n) E_2^-
5. Affected individual with positive evaluation (E+)	 E+	Individual with cystic fibrosis and positive mutation study, although only one mutation has currently been identified.	 E+(ΔF508) Eu E+(ΔF508/u)
		18 week male fetus with abnormalities on ultrasound and a trisomy 18 karyotype.	 18 wk E+(tri 18)

Fig. 5. Pedigree symbolization of genetic evaluation/testing information.

interpretation of patient or family genetic information, and thus improves the quality of patient care provided by genetic professionals. All clinicians should be aware of the importance of family history. Without family history, we cannot use new genetics properly in clinical practice. The family history often gives us a correct diagnosis, an accurate prognosis and an important clue for the prevention or avoidance of genetic disorders. If a clinician finds a large familial case of etiology-unknown genetic disorders from the chance of seeing only one patient, he can contribute to the basic research of mapping and cloning the gene responsible for the genetic disorder. Then the etiology of the disorder will be elucidated and suitable treatment for the disease will be possibly developed.

Molecular genetics must be useful as a tool for diagnosis, prevention and even treatment of genetic disorders. However, it must be supported by conventional clinical genetics including genetic counseling and the consideration of ethical, legal and social issues.

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Genetic Heterogeneity of Usher Syndrome

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Usher Syndrome (US) is an autosomal recessive disorder characterized by congenital hearing loss and retinitis pigmentosa. Variation between families corresponds to three clinically distinct clinical syndromes, called types I, II and III. These are distinguished by differences in severity and progression of the hearing loss and by presence or absence of vestibular dysfunction. At least six genes have been found to be responsible for type I US: *USH1a* (14q), *USH1b* (1q), *USH1c* (11p), *USH1d* (10q), *USH1e* (21q), and *USH1f* (10q). Three genes have been hypothesized as responsible for the milder Usher II: *USH2a* (1q41), *USH2b* (3p), *USH2c* and another as yet unlinked locus (USH2d). The Usher type III gene (*USH3*) has been localized to 3q. While the existence of nine Usher genes has been demonstrated through linkage, only two Usher genes have been identified, cloned, and characterized. The gene causing Usher Ib has been identified as myosin VIIa and the gene for US type IIa has recently been identified as a novel gene sharing partial homology with the laminin gene family. This extreme heterogeneity is almost unparalleled and has important implications for diagnosis and genetic counseling of the families involved.

Incidence

Usher Syndrome is an autosomal recessive disorder characterized by deafness and retinitis pigmentosa (RP). Although first recognized by Von Graefe (1858), it was named after Usher (1935) who emphasized its hereditary nature.

The frequency of US has been estimated at 3.0/100,000 in Scandinavia [1, 2] and at 4.4/100,000 in the United States [3]. The prevalence of US among

Table 1. Usher syndrome subtypes

Clinical type	Sub-type	Chr.	Audiologic	Vestib	Retinal
I	Ia	14q			
	Ib	11q			
	Ic	11p	Congenital and profound	Congenital and severe	Prepubertal
	Id	10q			
	Ie	21q			
	If	10q			
II	IIa	1q41	Congenital, stable, sloping mild to profound		Early teens
	IIb	3p			
	IIc	5q			Late teens
III	III	3q	Progressive	Progressive	Variable

deaf individuals has been reported to range from 0.6 to 28% [4] making it the most common form of syndromic hearing impairment. Conversely, the frequency of deafness in the RP population is estimated to range between 8.0 and 33.3%, suggesting that the Usher genes play a significant role in causation of RP. Overall, there are about 16,000 deaf and blind people in the United States, of which more than half are believed to have US.

Clinical Heterogeneity

Clinically, the three types of US are distinguishable from each other on the basis of severity of hearing loss, the extent of vestibular involvement, and onset and progression of hearing loss (see table 1). Type I patients are profoundly deaf while type II patients are hard of hearing and have a sloping audiogram going from mild to severe in the low frequencies to profound impairment in the higher frequencies. The audiograms are so characteristic that there is seldom any significant deviation away from their expected patterns. Vestibular function is another reliable discriminator between Usher types I and II since type I patients lack vestibular function [2, 5, 6] and type II patients have normal vestibular reflexes. Type III patients show a progressive hearing loss which is mild to moderate in the young patient and can be profound in the adult. Consequently, type III patients can have an audiological phenotype that is similar to type II as children but which appears more like type I as adults. Further complicating the clinical picture is the observation that type III patients also appear to show a progression to vestibular areflexia.

Retinitis pigmentosa causes gradual loss of peripheral vision as well as nightblindness. Electrophysiological studies provide the earliest and most reliable diagnosis of retinitis pigmentosa. Several studies have shown that the electroretinography (ERG) is subnormal as early as $2\frac{1}{2}$ to 3 years of age, before abnormalities can be seen functionally or on fundoscopic examination [7–9]. Responses extinguish totally as the degeneration progresses. The visual evoked responses also show decreased amplitudes and longer latencies [7]. An absent or diminished ERG in at least one affected sibling is a necessary condition for the diagnosis of US in a family. A history of nightblindness and measured restriction of visual fields are also required for the diagnosis to be accepted. The age at onset/diagnosis of the RP, although more consistent within sibships than between, nonetheless overlaps between all types of US [10]. However, characterization of the visual phenotype within and between the ten subtypes of US has not been undertaken yet and there is reason to believe that some subtypes may show certain distinguishing attributes.

Audiologic evaluations are important to confirm the expected severity of hearing involvement. For example, patients with vestibular involvement are expected to have a profound loss across all frequencies; patients with normal vestibular responses are expected to have a milder hearing loss with a greater loss in the higher frequencies. Unexplained deviations from this rule would bring the diagnosis into question. The lack of a history of progressive hearing loss is also an important criterion to differentiate both types I and II from type III [6, 11].

Sophisticated vestibular studies (including rotary chair, EOG, platform testing) are essential in distinguishing between the two major Usher types. In addition, a history of late walking (at or after 18 months of age) is almost an invariant finding in person who have Usher type I. The 'ataxia' described originally by Hallgen is of purely vestibular origin and there is no evidence of any more central neurological degeneration in US. The nature of the vestibular involvement in Usher type III patients is still unresolved although preliminary reports on families from Sweden (Moller, pers. commun.) indicate that type III cases develop a vestibular problem as they age.

Genetic Heterogeneity

The Usher II gene was the first Usher gene to be localized [12, 13]. It was found to lie on chromsome 1q41. It was also determined at that time that the clinical heterogeneity was due to the fact that types I and II were due to different genes but the true extent of the genetic heterogeneity was not yet appreciated.

The first localization of Usher type I was to markers on 14q [14]. While this linkage was not confirmed in either the general USA sample nor in the Louisiana Acadians [15], the French sample was significantly heterogeneous with only a fraction showing the 14q linkage; the families showing linkage came from the Poitou-Charentes region in western France. This type of Usher I was given the designation, Usher type Ia, and the gene is called *USH1A*.

The most common form of US type I was found to be linked to 11q13 [16] and the Usher gene in the French Acadians was linked to 11p13 [17]. These two genes are called *USH1b*, and *USH1c*, respectively. Recently, three new Usher I localizations, two on 10q and one on 21q have been reported [18, 19]. Type III has been localized to chromosome 3q [20]. While types I, II and III are clearly different with no clinical overlap, no distinguishing phenotypic differences have been reported to exist between the different subtypes. Thus, there are six different Usher type I genes, as based on linkage and gene mapping reports. Of these, the most common is Usher type Ib, comprising 50 to 75% of all Usher I patients. Usher Ic seems to be mostly limited to the French Acadians and Ia appears to be limited to persons who trace ancestry back to western France. The relative frequencies of types Id, e and f is not known.

An Irish-Scottish family was reported to have typical type II symptoms but it failed to show linkage with the 1q41 markers [21]. Several type II families unlinked to 1q41 have been subsequently observed and two genes have been found, one localized to 3p (*USH2B*) [22] and another localized to 5q (Pieke-Dahl et al. 2000). It is estimated that the proportion of Usher type II patients with type IIa is about 80% and that most of the remainder are type IIc linked to chromosome 5. A small fraction of families with the type II phenotype are unlinked to either Usher II locus. Interestingly, the phenotype of Usher type IIc appears to have a milder retinal component.

Usher type III is distinguished from the other two types by the presence of a progressive hearing loss. While there is one report of a progressive hearing loss associated with a myosin VIIa mutation [14], progressive hearing loss is seldom encountered in either type I or II patients. Progressive hearing loss can sometimes be a complaint of a type II patient but when investigated by collecting serial audiograms, the hearing is seldom found to be changing. It may be that the gradual loss of vision is reducing the utility of visual cues and lessening their understanding of speech. Type III patients are often diagnosed as type II because when seen by the audiologist, they have an audiogram typical of that type. The gene for type III has been localized to chromosome 3q. Type III is frequent in Finland but is observed infrequently in European and American families.

Usher Type Ib and Myosin VIIa

USH1B is caused by mutations in an unconventional myosin (*MYO7A*) [23]. Similarly, mutations in the orthologous mouse myosin VIIa gene cause the shaker-1 (*sh*1) deafness phenotype [24]. *MYO7A* mutations have been demonstrated for the non-syndromic deafness gene, DFNB2 [25, 26], deafness previously linked to 11q13 [27]. Likewise, dominant non-syndromic deafness (DFNA11) linked to 11q [28] has been shown to be caused by a novel *MYO7A* mutation acting in a dominant negative fashion [44].

The human *USH1B* gene encodes myosin VIIa [23], a newly identified member of the myosin subfamily of actin-based motors [29–31]. Recently, the complete genomic organization of myosin VIIa was characterized, the exon-intron structure of the gene was determined [32, 33]. To date, 55 unique mutations within the coding region of this myosin segregate with Usher syndrome type Ib [23, 25, 34]. It has been estimated that about 75% of all the *MYO7A* mutations are detectable [34]. The remaining mutations are expected to be found in the introns (as activated splice sites) or in the 5′ regulatory region(s) or in the unscreened tail domains of the protein.

Hypothesized functions of the myosin VIIa protein include acting as structural component of specialized cellular microvilli and junctional complexes, and associating with synaptic vesicles in sensory epithelial cells in the cochlea, retina, and olfactory system [30, 35]. Myosin VIIa has been localized to the apical stereocilia and cytoplasm of hair cells in the cochlea and vestibular system [29, 36] and to the apical processes of pigment epithelium cells and the connecting cilia of rod and cone photoreceptor cells in the retina [43].

Myosin VIIa mRNA and protein expression is spatially regulated during early embryonic development of the ear and eye [33, 35, 38]. In early embryos, expression in the otocyst is limited to the cochlear and vestibular neuroepithelium in the inner ear. Later, expression in the inner ear is limited to cochlear and vestibular hair cells. Phenotypes of mutations in gene transcriptional regulatory elements and mutations that cause truncation of the protein product are often similar [39]. Examples of other regulatory element mutations that cause deafness include an upstream chromosomal inversion in the mouse *Snell's Waltzer* recessive deafness gene, which encodes myosin VI [40], and in humans, a duplication/paracentic inversion in a regulatory region upstream of the *POU3F4* gene that causes X-linked nonsyndromic deafness (DFN3) [41].

Usher Type IIb and Laminush

The identity of the Usher IIa gene on chromosome 1q41 was recently discovered [42]. The gene appears to be novel but contains LE repeats seen in laminin as well as F3 domains observed in fibronectin genes. The protein has a definite signal for transport across the cell membrane. It seems likely that the protein is either a component of the basement cell membrane or is a cell adhesion molecule, perhaps anchoring the cell to the basement membrane. The full sequence of the cDNA is known and the genomic structure has been uncovered. It has 21 exons spread over approximately 120 Kb of genome DNA. Usher II families have been screened for mutations and several mutations have been found. All found to date are deletions causing a frame shift, early stop codon, and a predicted premature truncation of its protein. One mutation, 2299delG, probably represents the single most common form of mutation known to cause Usher syndrome and perhaps RP as well.

Molecular Diagnostics

Both genetic and clinical heterogeneity are a hallmark of US. It seems reasonable to assume that we will eventually be able to find clinical differences between types that could represent important prognostic information to be transmitted to the patient and family. The observation that Usher type IIb has a milder RP than IIa is one good example. Consequently, the issue of diagnosis for US subtype becomes an important consideration. DNA-based diagnostics is the only practical approach. At the present time, a molecular diagnosis can be made in a majority of instances by combining mutation detection of *MYO7A* and *USH2A* with linkage analysis of markers flanking the other 7 Usher genes. As the other genes are identified, the fraction of patients who can be unequivocally diagnosed will increase. No clinical molecular diagnostic programs have yet been established and the clinician must rely upon collaboration with one of the research groups studying US.

Summary

Progress towards the understanding of the molecular basis of US has been substantial. Nine different loci have been found to be responsible and two have had the specific gene identified. This information is expected to lay the foundation for the eventual development of new treatment strategies. Usher syndrome is the combined loss of both of humans most important two senses

and a better understanding of the genes involved should not only help the families with US but will also provide much needed basic information about the hearing and visual systems.

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Alport Syndrome

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In 1902, Guthrie reported a family in which some members suffered from hematuria [1]. Twenty five years later, Dr. Arthur Cecil Alport (1880–1959) reexamined this family and found that individuals with hematuria had hearing impairment [2]. Since then several hundred cases of hereditary/familial nephritis with intermittent hematuria and progressive sensorineural hearing impairment have been reported. In honor of Dr. Alport this type of disease entity was named Alport syndrome. Gene frequency of this syndrome is 1:5,000 to 1:10,000, and it represents 0.6–3% of end-stage renal disease (ESRD). The Alport syndrome has attracted the attention of otolaryngologists since this syndrome is indeed the first hereditary deafness whose cause was elucidated at the gene level; the syndrome accounts for approximately 1% of individuals with hereditary deafness. In this review, advances in the understanding of Alport syndrome are presented with a special interest on the otological aspect. For other aspects, readers are encouraged to refer to other excellent reviews [3–25].

Clinical Symptoms

The main organs affected by Alport syndrome are the kidney, cochlea and eye. Occasionally the smooth muscle and platelets are affected. Diagnosis criteria of the syndrome involve family history, electron microscopic findings of kidney biopsy, ophthalmologic findings, and hearing test findings.

Flinter [4] proposed that at least three of the following four criteria were required for diagnosis of Alport syndrome:

1. Positive family history of hematuria with or without chronic renal failure (i.e. more than one individual affected);

2. Electron microscopic evidence on renal biopsy of characteristics change, i.e. thickening and splitting of the glomerular basement membrane with electron-lucent areas containing small, dense granulation;
3. Characteristic eye signs seen on slit-lamp ophthalmoscopy (i.e. anterior lenticonus and perimacular flecks);
4. High-tone sensorineural deafness, which is usually progressive during childhood.

In juvenile (i.e. progressive) type Alport syndrome, renal insufficiency reaches ESRD before the age of 31. In the adult (i.e. non-progressive) type, prognosis is unpredictable since the time course of the disease varies depending on each individual; ESRD usually occurs around 40 years old.

Hearing impairment is observed in a majority of Alport individuals. Audiometric findings are consistent with cochlear sensorineural hearing loss; Bekesy's audiogram is classified as type II, SISI (short increment sensitivity index) score is high, recruitment phenomenon is positive, tone decay is not observed and speech discrimination test score is high. Standard audiograms are usually symmetric: right to left difference of hearing loss is minimal except at 4 kHz and 8 kHz. Audiograms may be classified into three types: horizontal, saucer and slope [26]. Since the average ages of cases showing these three types were 8.5, 13.7 and 17.8 years old, respectively, the three types likely represent a natural history of hearing impairment. Audiometric tests, such as audioscan method, found hearing impairment in some carriers, i.e. heterozygous females [27]. Some individuals complain of vertigo and dysequilibrium, while abnormalities of other individuals are found only after equilibrium tests. Ocular defects are restricted to the juvenile type; the typical ocular associations are a dot-and-fleck retionopathy and anterior lenticonus [3].

Alport Syndrome as a Disease of Basement Membranes

Alport syndrome is characterized by an alteration of glomerular basement membranes (GBMs). Electron microscopic studies revealed that the typical trilaminar appearance of the GBMs, consisting of a central homogenous lamina densa and two adjacent laminae, is destroyed in Alport syndrome. These GBMs exhibit irregular thickening of lamina densa, which is replaced by a network of interlacing electron-dense strands interspersed with granular electron-dense bodies [28, 29]. In the early stages, segments of thinned GBMs can also be seen [30, 31].

Goodpasture syndrome is an autoimmune disease cause by anti-GBM autoantibodies, which recognize the noncollagenous region of type IV collagen, the major constituent of GBMs [32]. Goodpasture autoantibody, however,

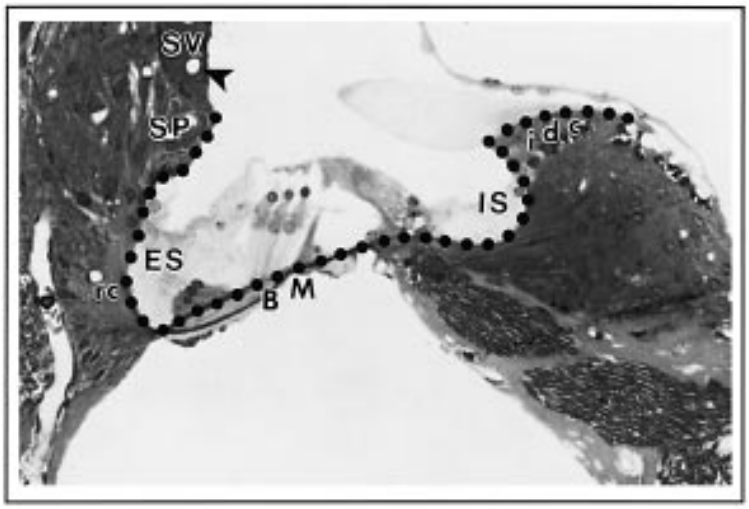
fails to recognize Alport GBMs, which lack peptides of Goodpasture antigen [33, 34]. When Alport individuals receive renal transplantation, they occasionally generate anti-GBM antibody [35].

These electron microscopic and immunochemical studies indicate that Alport syndrome is caused by anomaly of GBMs, most likely caused by mutations of genes encoding type IV collagens.

Biology of Cochlear Basement Membranes

Basement membrane (BM) is a continuous and amorphous sheet-like extracellular structure secreted from adjacent cells with diverse biological roles, which include embryonic development, tissue remodeling during development and wound healing, tissue architecture maintenance, mechanical stress absorption, and blood or air filtration [11]. BMs consist primarily of type IV collagen (a type of collagen found only in BMs), together with type XV, XVIII and XIX collagens, and other glycoproteins such as fibronectin, laminin, heparansulfate proteoglycan and nidogen. A type IV collagen molecule consists of a triple-helical protomer with three α chains. To date, six different α chains, α1-6(IV), have been identified. The α1(IV) and α2(IV) chains (i.e. classical chains) are the common one, and occur in most BMs as [α1,α2,α2](IV). The α3(IV), α4(IV) and α5(IV) chains (i.e. novel chains) are less common, and are specific to certain tissues. For example, the [α3,α4,α5](IV) heterotrimer has been localized in BMs of kidney glomerulus and lung alveolus; the [α5,α5,α6](IV) heterotrimer, in BMs of skin epidermis and kidney Bowman's capsules [18, 36].

Occurrence of classical chains of type IV collagen in the cochlear BMs were shown in adult human [37], guinea pig [38], and mouse [39]; they are present primarily in the perineural BMs of the osseous spiral lamina, spaces between spiral ganglion cells and vascular BMs of spiral ligament and stria vascularis. A developmental study of the murine cochlea revealed that the classical chains were widely expressed at birth, while the novel chains were completely absent [40]. At 4 days of age, novel chains were activated and thus expressed in most of the cochlear cells. From day 8 through day 14, they are restricted to specific areas: structures bounding the interdental cells, the inner sulcus, the basilar membrane, a fibrous bed around the root cells, and membranes surrounding the capillaries of stria vascularis [39, 40] (fig. 1). The classical chains were expressed in these regions only transiently (from day 6 to day 10), after which a gradual developmental inactivation led to the adult expression pattern described above. The developmental switch from [α1,α2,α3](IV) to [α3,α4,α5](IV) is also shown in GBMs, and failure of this switching is shown in GBMs of the X-linked Alport syndrome. Intriguingly,



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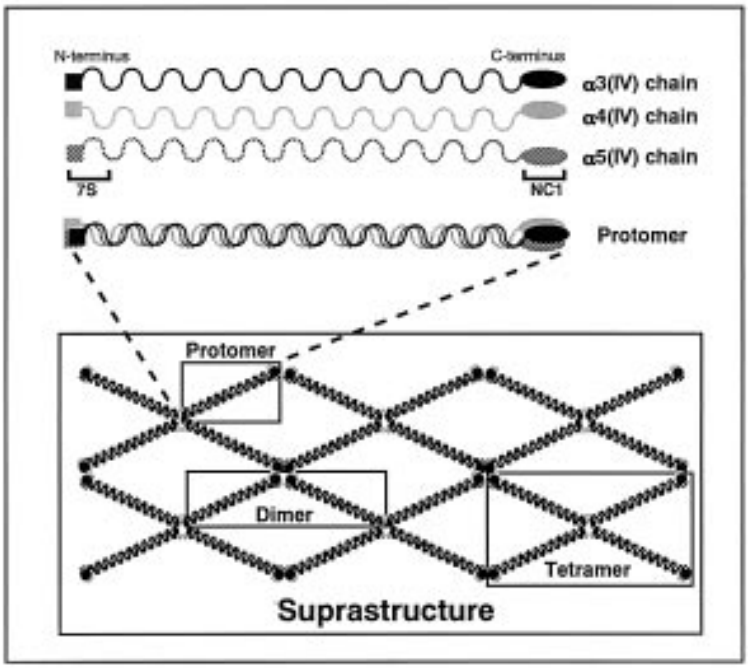

b

Fig. 1. (*a*) Schema of the localization of basement membranes (BMs) in the mouse cochlea. The track of BM (dotted line) was drawn based on the results of Cosgrove et al. [39, 40]. The track runs along the boundary structures of interdental cells (idc), inner sulcus (IS), basilar membrane (BM), external sulcus (ES), fibrous beds surrounding root cells (rc), and spiral prominence (SP). BMs are also localized around the capillary of stria vascularis

the [α1,α2,α3](IV) chains are cysteine-rich and thus more resistant to collagenase digestion than [α3,α4,α5](IV) chains [41].

Existence of several kinds of protomers confers a level of diversity to the suprastructure of type IV collagen since several different combinations of protomer-to-protomer interactions are possible [11, 36]. Each type IV α collagen chain is composed of approximately 1,400 amino acids consisting typically of Gly-Xaa-Yaa repeats, where Xaa and Yaa are often proline or hydroxyproline, and each chain has a collagenous domain with (7S domain at the amino terminus) and non-collagenous (NC1) domain at the carboxy terminus. The type IV collagen exists as a supramolecular network composed of protomers that interact by end-end association [18].

Structure and Chromosomal Localization of Type IV Collagen Genes

In situ hybridization techniques localized genes for α1-5(IV) chains, i.e. *COL4A1-5*, to three chromosomes: *COLA4A1* and *COL4A2* to chromosome 13q34 [42], *COL4A3* and *COL4A4* to chromosome 2q35-q37 [43], and *COL4A5* to Xq22 [44, 45]. Two genes on a chromosome are localized in a head-to-head manner (fig. 2) and share a bidirectional promoter. For example, *COL4A1* and *COL4A2* are separated by intervening 127 bp containing symmetrical functional elements such as CAAT boxes and binding sites for Spa, but no TATA box [46].

Molecular Genetics of Alport Syndrome

In a majority of Alport families patients are males, but in some families equally severe symptoms may be seen in females, indicating the genetic heterogeneity. Over 200 mutations of IV genes [14] found in Alport families unequivocally show the genetic heterogeneity of Alport syndrome.

X-Linked Dominant Trait

This form is most prevalent and accounts for more than 85% of Alport cases. In this trait, affected fathers transmit the disease to all daughters but none of

(SV) (arrowheads). (*b*) Schematic representation of the molecular structure of type IV collagen in BM of adult cochlea. The α3(IV), α4(IV) and α5(IV) chains are the main constituents of the adult cochlear BM. Two protomers associate in a head-to-head (NC1-to-NC1) fashion to form a dimer. Two dimers cross-link in tail-to-tail (7S-to-7S) to form a tetramer, after which tetramers cross-link with each other to produce the characteristic 'chicken-wire' scaffolding network.

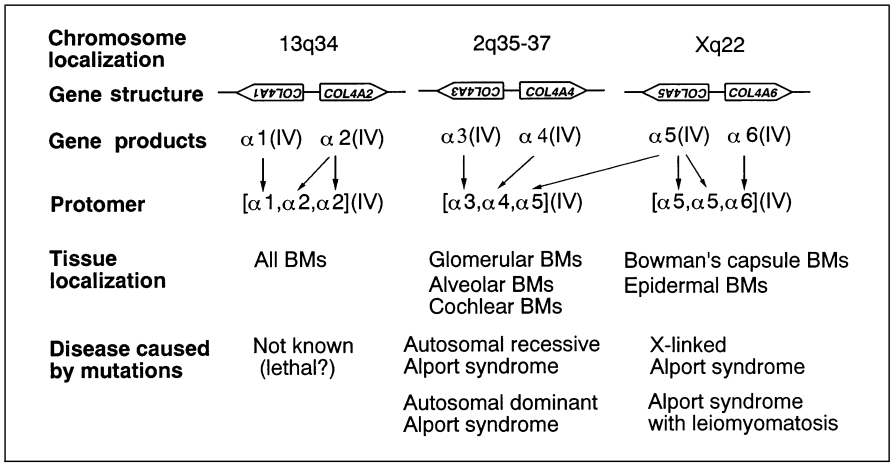

Fig. 2. Chromosomal localization and structures of type IV collagen genes, and tissue localization of the gene products. The genes encoding the six type IV collagen chains are located in pairs in a head-to-head fashion on three different chromosomes. Paired genes were driven by a bidirectional promoter in the different directions (indicated by pencil-like boxes). Their gene products are assembled in heterotrimer protomers and localize in a tissue-specific manner.

the sons, while affected mothers transmit the disease to either sex. Linkage analysis of these families mapped an X-linked Alport locus to Xq22 [47–49], where the *COL4A5* gene is located. Since the first set of mutations was found in this gene [50], 38 large- and medium-size deletions, 62 amino acid substitution mutations, 69 nonsense mutations, 6 inframe deletions, 1 inframe duplication, and 26 splice-site mutations were identified [14]. Among the 62 amino acid substitutions found, 55 were detected in the collagenous domain (49 substitute glycine in Gly-Xaa-Yaa repeats) and remaining 7 substitute an amino acid in NC1 domain. The α5(IV) chain with Gly substitution presumably results in abnormal folding of the [α3,α4,α5] heterotrimers, which may be poorly secreted or excessively susceptible to degeneration. The α5(IV) chain lacking NC1 domain cannot form heterotrimers with α3(IV) and α4(IV); the free α3(IV) and α4(IV) chains presumably will be degenerated [13].

Major rearrangements of *COL4A5* are consistently associated with the juvenile Alport syndrome. Large deletions, extending from within the *COL4A5* gene to intron of *COL4A6* gene, cause Alport syndrome with esophageal/genital leiomyomatosis [51–53]. A large deletion involving *COL4A6* exon 2 and 1, the entire *COL4A5* and an adjacent gene encoding long-chain acyl-CoA synthetase 4 has been reported in a family with the X-linked semidominant Alport syndrome, elliptocytosis and mental retardation [54].

Autosomal Recessive Trait

Equally severe case of the disease are present in men and women and tends to appear after consanguineous marriage [7]. The first set of mutations was found in the *COL4A3* genes in homozygous individuals of two Alport families; the *COL4A4* genes, in homozygous individuals of two others [55]. Including these mutations, six *COL4A3* mutations and three *COL4A4* mutations have been reported. While most of *COL4A3* mutations were homozygous, two mutations were compound heterozygous. All *COL4A3* mutations created a premature stop codon and caused Alport syndrome. One *COL4A4* mutation was nonsense, and the other was missense mutation.

Autosomal Dominant Trait

In autosomal dominant Alport families from Utah, despite the clinical evidence of father-to-son transmission of nephritis, molecular studies have not shown genetic linkage to *COL4A1* or *COL4A2* genes in 13q34 region, nor to *COL4A3* or *COL4A4* genes in 2q35-37 region [7]. However, recently a family of the autosomal dominant Alport syndrome was linked to *COL4A3* and *COL4A4*. Interestingly, a missense mutation of *COL4A4* was detected in the unaffected mother of this family [56]. One *COL4A4* mutation is associated with autosomal dominant familiar benign hematuria [14].

Animal Models of Alport Syndrome

Spontaneous hereditary nephritis in Samoyed dogs resembles the X-linked Alport syndrome [57]. English cocker spaniel nephritis is a model for the autosomal recessive Alport syndrome [58], and bull terrier hereditary nephritis is a model for the autosomal dominant Alport syndrome [59]. Although these dogs show nephritis symptoms, they do not show eye and ear anomalies.

Transgenic mice with an insertional mutation of a gene for a peroxisomal protein, named Mpv17, developed progressive glomerular nephritis and sensorineural deafness; full development of these changes required up to 18 months [60]. Recently a murine model for the autosomal Alport syndrome was generated by targeted mutagenesis of the murine *COL4A3* gene [61]. This 'Alport mouse' reaches ESRD at 8 to 9 weeks of age.

Otopathological Findings of Alport Syndrome

Human temporal bone studies of Alport individuals have disclosed a wide range of changes. These include collapse of Reissner's membrane, hair cell

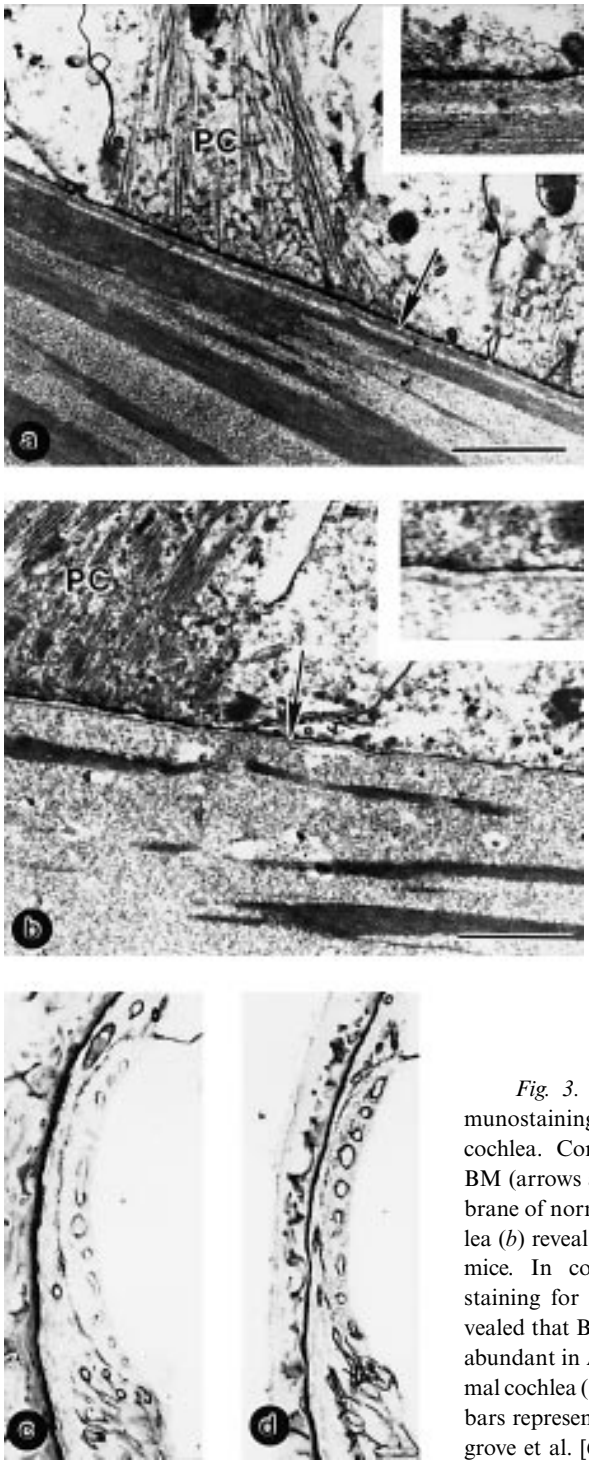

Fig. 3. Ultrastructure (*a, b*) and immunostaining (*c, d*) of normal and Alport cochlea. Comparison of ultrastructure of BM (arrows and insets) of the basilar membrane of normal (*a*) and Alport mouse cochlea (*b*) reveals the thinning of BM in Alport mice. In contrast, comparison immunostaining for type IV collagen α1 chain revealed that BM of strial capillaries are more abundant in Alport cochlea (*d*) than in normal cochlea (*c*). PC: Pillar cell. Magnification bars represent 1 μm. Reproduced from Cosgrove et al. [68] with permission.

degeneration, loss of cochlear neurons, and atrophy in stria vascularis [62–65]. This wide variety probably reflects the results of aging, prolonged uremia, and post-mortem autolysis. Observation at the light microscopic level showed that gross anatomy of stria vascularis was not deteriorated [65], while another observation at the electron microscopic level revealed the degeneration of stria vascularis with thickening of capillary BMs [66].

Gross morphological changes were observed in the cochlea of Alport model mice, Mpv17; these included changes of strial capillary BMs and loss of hair cells and spiral ganglion cells [67]. In contrast, no apparent gross morphological change was detected in the cochlea of other Alport mice, i.e. *col4a3*$^{-/-}$ mice [68]. Electron microscopic study of *col4a3*$^{-/-}$ mice cochlea, however, revealed the thin 'BM track' running from the spiral limbus, down the inner sulcus, across the basilar membrane and up to spiral prominence. Moreover, BMs that normally ensheathe the root cells were not visible (fig. 3a). Immunohistological study revealed that the novel collagen IV chains (α3, α4 and α5) were not present in the track, while classical chains (α1 and α2) persisted. In contrast to the BM track in the organ of Corti but similar to Alport GBMs, BMs surrounding the strial capillaries were significantly thickened. Classical chains (α1 and α2) were more abundant in these BMs when compared with normal mice (fig. 3d). Although two of novels chains, i.e. α3, α4, were not present in these BMs, other one, i.e.α5, persisted. Diameter of the capillary was reduced and the endothelial cells of the capillary swelled. Strial marginal cells of *col4a3*$^{-/-}$ mice were affected as evidenced by swelling of their nuclei and loss of basolateral infoldings. Auditory-evoked brainstem response measurements of *col4a3*$^{-/-}$ mice between 6 and 8 postnatal weeks suggested a small increase in thresholds across all frequencies [68].

No apparent evidence for genotype-phenotype correlation in respect to hearing was observed in individuals with *COL4A* mutations; of 116 families 83 were afflicted with deafness and the other 33 were not. Mutations in the same exon can result in a phenotype with or without deafness: a Gly1143Ser substitution mutation was associated with deafness, while the Gly1143Asp mutation was not. Intriguingly, individuals with *COL4A3* mutations were afflicted with deafness, while those with *COL4A4* mutations were not [14].

Treatment

The prognosis of Alport syndrome nephropathy is poor. The male sex and the presence of hearing impairment and ocular defects have been frequently associated with a poor prognosis. Proteinuria, morphologic changes in glomerular, tubular, and interstitial zones, severe thickness and splitting of GBM,

and the presence of the defects in GBM antigenicity are indicative of severe disease [10]. No treatment has been found to prevent the progression of the renal disease, but general management is indicative to ameliorate hyperfiltration and hypertension [7]. Management includes salt restriction, phosphate restriction, and avoidance of excessive protein intake. When ESRD occurs, dialysis and renal transplantation are effective. However, anti-GMB nephritis occurs in 3 to 4% of transplanted Alport individuals, most often occurring in men with deafness and onset of renal failure before 30 years of age. Family history of anti-GBM nephritis is frequent, and in 76% of the cases, the complication occurs within the first year after transplantation. Anti-GBM nephritis may respond to immunosuppressive therapy, but the rate of recurrence after retransplantation is high [13]. Alport syndrome is a potential target for gene therapy [69, 70].

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Waardenburg Syndrome

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Auditory-Pigmentary Syndromes

Waardenburg syndrome is a clinical label applied to a heterogeneous group of human auditory-pigmentary syndromes. Such syndromes are known from a wide variety of mammalian species, and constitute one of the principal categories of syndromic hearing loss. Steel [1] has divided causes of genetic hearing loss into three groups: morphogenetic (with structural abnormalities of the bony or membranous labyrinth), neuroepithelial (where a structurally intact cochlea fails to transduce the sound signal into a nervous impulse) and cochleo-saccular (with a primary defect in the stria vascularis). Auditory-pigmentary syndromes fall into the cochleo-saccular group.

The combination of hearing loss and pigmentary abnormalities is caused by a physical absence of melanocytes from the affected areas. In the skin and hair the absence is usually patchy, producing areas of leukoderma, white forelock and, in animals, white spotting of the coat. Sometimes there is a uniform dilution of pigmentation instead. In the eyes the manifestations are heterochromia or sometimes characteristic brilliant blue eyes with a hypoplastic stroma of the iris. Melanocytes are also required by the stria vascularis to maintain the ionic composition of the cochlear endolymph. Melanin is not required - albinos hear normally - but without melanocytes, no endocochlear potential is generated, and there is secondary collapse of Reissner's membrane and degeneration of the inner ear [2].

All melanocytes, except those in the retinal pigmented epithelium, originate in the embryonic neural crest. The neural crest gives rise to many other tissues, including the craniofacial bones, limb muscles and enteric neurons, and so defective neural crest function can produce defects in these other tissues along with auditory-pigmentary features.

Table 1. The principal human auditory-pigmentary syndromes

Syndrome	OMIM number	Inheritance	Distinguishing features	Comments
WS1 (Type 1 Waardenburg)	193500	Ad	Dystopia canthorum	Nearly all have PAX3 mutations
WS2 (Type 2 Waardenburg)	193510	Ad	No dystopia; melanocyte-specific	Heterogeneous; 10% have *MITF* mutations
WS3 (Klein-Waardenburg)	148820	Ad (most cases sporadic)	Hypoplasia of limb muscles; contractures of elbows, fingers; rare	Variant presentation of WS1; mostly *PAX3* heterozygotes; some may be homozygotes
WS4 (Shah-Waardenburg)	277580	Ar or Ad	WS2 + Hirschsprung's disease; rare	Heterogeneous; includes homozygotes for *EDN3* or *EDNRB* mutations and heterozygotes for *SOX10* mutations
Tietz-Smith	103500	Ad	Uniform dilution of pigmentation in skin, hair, eyes; profound hearing loss	Dominant negative *MITF* mutations in some cases

Ar = Autosomal recessive; Ad = autosomal dominant. OMIM is the online version of McKusick's Mendelian Inheritance in Man [12]. It is freely accessible at http://www3.ncbi.nlm.nih.gov/Omim/, and is the primary reference for all human mendelian conditions.

Human Auditory-Pigmentary Syndromes

Four types of Waardenburg syndrome have been described (table 1; [3]). Of these, only Type 1 (WS1) is a single well-defined genetic entity. Type 2 (WS2) is defined negatively by the absence of dystopia canthorum. The label WS2 should be reserved for patients with a patchy absence of melanocytes without involvement of any other neural crest derivatives, but even with this restriction, the clinical condition is certainly genetically heterogeneous. WS3 or Klein-Waardenburg syndrome describes WS1 with additional involvement of the upper limbs. Generally the involvement is minor and is simply a variant presentation of WS1, but some severely affected cases are WS1 homozygotes, and there may be other causes as well; it is very rare. WS4 or Shah-Waardenburg syndrome comprises a heterogeneous set of rare major neurocristopathies, where both melanocytes and enteric neurons are defective, producing the combination of WS2 with Hirschsprung disease.

Table 2. Penetrance (%) of clinical features of Type I and Type II Waardenburg

Type	Source	n	SNHL	HetI	HypE	WF	EG	Skin	HNR	Eyb
WS1	Liu et al.	60	58	15	15	48	38	36	100	63
	Literature	210	57	31	18	43	23	30	52	70
WS2	Liu et al.	81	78	42	3	23	30	5	0	7
	Literature	43	77	54	23	16	14	12	14	7

Data from Liu et al. [4]. SNHL = Sensorineural hearing loss; HetI = heterochromia irides; HypE = hypoplastic blue eyes; WF = white forelock; EG = early greying; Skin = white skin patches; HNR = high nasal root; Eyb = medial eyebrow flare.

Diagnostic Criteria and Clinical Features of WS Types 1, 2 and 3

The clinical manifestations of WS1 and WS2 are generally very similar and include:

(1) hearing loss – this is sensorineural, congenital and non-progressive. The degree of loss is quite variable, ranging from slight to profound, and it can be symmetrical or asymmetrical, with varying shapes of audiogram.

(2) white forelock – varying in size from a few hairs to a large tuft. Sometimes the forelock is present at birth, and it may persist or disappear; it may reappear or appear for the first time later, usually as a prelude to early greying (scalp hair mainly white before age 30 years). Cases have been described with forelocks of different colours, or with a tuft of white hair on a different part of the head.

(3) pigmentary disturbances of the iris – heterochromia (two different coloured eyes, or two different colours within a single eye, usually in sharply demarcated radial segments), or sometimes both eyes are brilliant blue, with hypoplasia of the iris stroma – a feature that is very striking when it occurs in people from dark-skinned, brown-eyed races.

All these features are highly variable, both between and within families. Table 2 shows the frequency of each feature in a series of patients personally examined by Prof. V.E. Newton and Dr. X.Z. Liu in Manchester [4], with figures from the literature for comparison.

The distinction between WS1 and WS2 is made by the presence or absence of dystopia canthorum. Dystopia is a specific displacement of the inner canthi and lacrimal puncti, which is not the same thing as hypertelorism or wide-spaced eyes. Together with dystopia, there is often a broad nasal root, syno-

phyrys and hypoplastic *alae nasi*, all of which give rise to a WS1 face; all these facial features are absent in WS2.

For a diagnosis of WS1 an individual should have at least two of dystopia, congenital sensorineural hearing loss, white forelock, heterochromia or an affected first-degree relative. For WS2 the criteria are more arbitrary since the condition is so heterogeneous, but reasonable criteria would be at least two of the clinical features listed above plus a first-degree relative who also has at least two features.

The label WS3 is usually applied to occasional WS1 patients who have some minor contractures of the hands or fingers, or some underdevelopment of the arm and shoulder muscles. Two patients have been described with an altogether more severe syndrome of hearing loss, dystopia, extensive depigmentation and a severe amyoplasia of the arms and shoulders. The reason for the association of WS1 with limb defects is described below.

PAX3 and Type 1 Waardenburg Syndrome

Mutations in the *PAX3* gene at chromosome band 2q35 are the cause of WS1 [3]. Most *PAX3* mutations are private to a particular family, so that unrelated individuals with WS1 usually have different mutations, and if a new family is being studied it is necessary to screen the entire *PAX3* gene sequence for mutations. We know quite a lot about the function of the PAX3 protein, mainly from studies of its mouse homologue, *Pax3* and of the *Splotch* mouse that has mutations in *Pax3*. The protein is a transcription factor that is active mainly in the embryonic neural crest, and among the genes it controls are *Met*, *MyoD* and *MITF*, all of which are discussed below.

The diversity of mutations is not the cause of the clinical variability of WS. Even within a family, where everybody has the same mutation, the presentation is very variable, and no correlation between type of mutation and clinical features has been found that is strong enough to be predictive for individuals. This has the important consequence that although prenatal diagnosis is possible in a family with a known mutation, it is quite impossible to predict how severely affected a fetus would be, and in particular, we cannot predict whether or not it will be deaf.

WS1 is dominant, and affected people are heterozygotes with one normal and one mutant copy of PAX3, but there is haploinsufficiency – that is, a 50% dosage level of PAX3 protein is not sufficient for completely normal embryonic development. All the mutations are believed to have the same effect: they destroy the ability of the PAX3 protein to bind DNA There are many different ways of stopping a protein working, and so we see many different mutations.

Some mutations cause translation of the mRNA to come to a premature stop (nonsense, frameshift and splice-site mutations) and these can occur anywhere within the gene. Others simply replace one amino acid by another at one point in the protein (missense mutations) and these changes are concentrated in the DNA-binding elements (paired domain and homeodomain) of the protein. Because PAX3 protein interacts with other proteins and DNA sequences, variations in its partners can modify the pathogenic effect of a 50% dosage level, hence the clinical variability.

Among these partners are *Met* and *MyoD*, the two master genes for development of the limb muscles [6]. Limb muscle development usually proceeds normally with a 50% dosage of PAX3, but occasionally through bad luck or variants in the partners, there is some minor effect, and this produces the mild contractures of the hands or fingers that are seen in some 'WS3' patients. The two very severely affected WS3 patients described by Klein [5] and Zlotogora [7] are quite different. Zlotogora's patient has been shown to be homozygous for a *PAX3* mutation. It is quite surprising that homozygotes can live, because *Splotch* homozygous mice have lethal neural tube defects and often also lethal heart defects, and the only other known *PAX3* homozygote was an anencephalic and grossly abnormal fetus [8; A.P.R., unpublished].

MITF and Type 2 Waardenburg Syndrome

WS2 comprises a heterogeneous group of defects that affect melanocyte proliferation or survival. Only one causative gene has been identified, the *MITF* transcription factor that maps to chromosome 3p12 [9]. *MITF* appears to be a master gene for melanocyte development, and interestingly, it turns out that *MITF* is itself controlled by *PAX3* [10]). This probably explains the very similar auditory and pigmentary phenotypes of WS1 and WS2; dystopia canthorum is the result of an independent action of *PAX3* on development of the craniofacial bones. As with *PAX3*, the mutations are quite heterogeneous [3] and the clinical result is variable even within families, pointing again to haploinsufficiency as the pathogenic mechanism. No human *MITF* homozygotes have yet been found, but by analogy with the *microphthalmia* mouse [11], we would expect them to be strikingly depigmented, maybe with microphthalmia or retinal degeneration as well as hearing loss.

Relatively few *MITF* mutations have been found. It appears that in most families WS2 is not caused by *MITF* mutations, but the other WS2 genes remain to be discovered. Families with *MITF* mutations are not clinically distinguishable from WS2 families with no detectable mutation, although it

is noticeable that large families with a clear dominant pattern of inheritance and many affected cases tend to have *MITF* mutations; the non-*MITF* families tend to be smaller with only one or a few affected people, and often with rather minimal signs.

The MITF protein binds its DNA targets as a dimer. Whilst most mutant forms simply do nothing, one can imagine an interesting class which in heterozygous people do positive harm by sequestering the normal protein in non-functional dimers. Such dominant negative mutations would be expected to produce a more severe and less variable clinical result than simple loss of function mutations. Examples are known in the mouse [11], and interestingly, two families with the Tietz-Smith syndrome (see table 1) have MITF mutations that are predicted to function as dominant negatives. In keeping with this, the Tietz-Smith phenotype is more severe and more constant than WS2.

Genes Underlying Waardenburg-Hirschsprung Disease

The association of WS with Hirschsprung disease (HSCR) is well established in the literature, but almost certainly this is not an association with WS1 or WS2, but a separate set of neurocristopathies. Three genes have been identified, based on studies of homologous phenotypes in the mouse, that can cause a WS-HSCR or WS4 phenotype in humans. These are the endothelin 3 gene *EDN3* on chromosome 20q13, the endothelin receptor B gene, *EDNRB* on 13q22 and the *SOX10* gene on chromosome 22. Presumably in all these cases the gene defect is affecting a pluripotent population of cells that can give rise to both melanocytes and enteric neurons; or alternatively, the genes are required by two different cell lineages for correct development.

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Branchio-Oto-Renal Syndrome

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Clinical Delineation

The term Branchio-Oto-Renal (BOR) syndrome (MIM113650) was introduced in 1975 to describe individuals presenting with varying combinations of branchial, otic (affecting the outer, middle and inner ear) and renal anomalies [1]. The branchial anomalies are observed in approximately 60% of affected individuals and consist of latero-cervical fistulas, sinuses and cysts [2, 3]. The hearing loss can be conductive, sensorineural or mixed [4]. The outer ear anomalies consist of preauricular pits (82% of individuals), auricular malformations (36%), as well as preauricular tags, malpositioned ears, microtia and atresia to stenosis of the external auditory canal (less than 20% of individuals) [5]. Various anomalies of the middle ear ossicles have been documented, including underdevelopment, displacement or absence of the three ossicles, fusion and fixation of two or more ossicles (such as together, to the attic of the tympanic cavity or, in the case of the stapes, to the oval window) and absence of the oval window (either with absence or presence of the stapes) as well as reduction in size and malformation of the middle ear cavity [6, 7]. The inner ear anomalies, as detected by computer tomography, affect both the auditory and vestibular apparatus [6, 8]. Documented anomalies of the auditory portion are a small, absent or underdeveloped cochlea showing only 2 turns. Absent or hypoplastic semicircular canals comprise the anomalies of the vestibular apparatus. The renal anomalies affect both the excretory (renal agenesis, hypoplasia and dysplasia) and collecting (duplication or absence of the ureter, megaureter, blunted or distorted calyces, extra or bifid pelvis) system [2, 4, 9].

Localization and Identification of the Causative Gene

BOR syndrome is an autosomal dominant defect and the first putative chromosomal localisation for the causative gene was proposed by Haan et al. in 1989 when the authors reported an affected family (hereafter referred to as AUS) with an inherited complex translocation involving the long arm of chromosome 8 [10]. A 10 cM gene interval was subsequently defined, in 1994, by the analysis of an 8q deletion detected on the karyotype of another affected individual [11]. This interval was further reduced to 7 cM by the linkage analysis of BOR affected families.

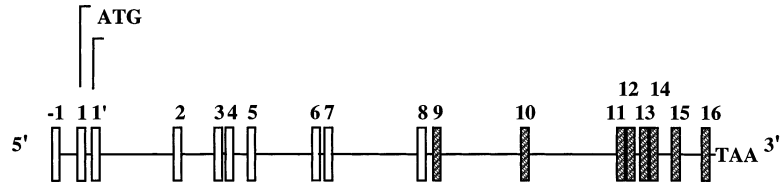
Fluorescent in situ hybridization (FISH) of probes from this gene interval to metaphase chromosome spreads of an affected individual from family AUS, resulted in the detection of a translocation-associated deletion with an estimated size of approximately 500 kb [12]. Large-scale sequencing of this newly defined gene interval resulted in the identification of a gene [13] highly homologous to the *drosophila* developmental gene *eyes absent (eya)* [14]. This gene was named *EYA1* for *eyes absent*-like1 and belongs to a newly identified gene family comprising at least three other members in vertebrates, *EYA2, EYA3* and *EYA4* [13, 15]. The C-terminal end of their predicted amino acid sequences is highly conserved between *drosophila* and man and was named eyaHR for eyes absent homologous region [13].

EYA1 is made up of 16 exons, exons 9–16 encode the eyaHR (see fig. 1), and two alternative transcripts differing in their 5′ ends have also been identified [16]. Mutation screening of *EYA1* in BOR affected individuals has consistently demonstrated a clustering of mutations within or immediately adjacent to the eyaHR, indicating a crucial role for this highly conserved domain [13, 16, 17] (see fig. 1). Mutations in *EYA1* have also been identified in two families exclusively affected by Branchio-Oto (BO) syndrome (characterized by branchial and otic anomalies without the association of renal anomalies), demonstrating that BOR and BO syndromes are allelic defects of *EYA1* [18] (see fig. 1).

Expression Pattern

A study of the expression pattern of the murine homologue of *EYA1, Eya1*, during mouse embryogenesis has allowed a correlation of this pattern with the clinical signs of BOR syndrome and provided an insight into its pathogenesis. The branchial anomalies of BOR syndrome are attributed to faulty obliteration of the branchial arches which normally occurs during development via epithelial cleft interactions. *Eya1* is expressed in the epithelium of the second, third and fourth branchial clefts and pharyngeal pouches [19], the persistence of which

a



b

Case	Location	Nucleotide change	Effect on protein
BO familial[d]	exon 4	297del8	frameshift
BOR familial[a]	exon 8	823C→T	R275X
BOR sporadic[a]	exon 8	755insC	frameshift
BOR familial[c]	exon 8	781C→T	R261X
BO familial[d]	exon 9	870insGT	frameshift
BOR familial[b]	exon 9	del 5.6 kb	deletion
BOR familial[b]	exon 10	ins*Alu*	exon skipping
BOR familial[a]	exon 12	1251T→CC	frameshift
BOR sporadic[a]	exon 13	1360T→C	S454P
BOR familial[b]	exon 13	1359insC	frameshift
BOR familial[a]	exon 13	1372T→AGAGC	frameshift
BOR familial[b]	exon 14	1414T→G	L472R
BOR familial[a]	exon 14	1498+2T→G	aberrant splicing
BOR familial[b]	exon 15	1599+5G→C	aberrant splicing
BOR sporadic[a]	exon 15	1555ins4	frameshift
BOR familial[c]	exon 15	1501del4	frameshift
BOR familial[c]	exon 15	1592delC	frameshift
BOR sporadic[a]	exons 11 to 15	del5.8-7 kb	deletion
BOR familial[b]	exons 11 to 16	del20-37 kb	deletion

Fig. 1. a Schematic representation of the *EYA1* gene. Boxes represent the exons. Exons (-1) and (1′) belong to the alternative transcripts EYA1B and EYA1C [16]. Exons 9 to 16 (grey hatched boxes) encode the eyaHR. The ATG start site (situated in exon 1′ for EYA1B and EYA1C) and the TAA stop codon are indicated. *b* List of all mutations reported to date in BOR and BO affected patients. [a](13); [b](16); [c](17); [d](18).

gives rise to the branchial fistulas (specifically involving the second cleft and pouch), sinuses (external, due to the persistence of the clefts and internal, due to persistence of the pouches) and cysts (due to retention of surface ectoderm).

During early stages of outer ear development, *Eya1* is not expressed in the regions of the branchial arches that will give rise to this organ, however,

at later stages, *Eya1* expression is seen in the mesenchyme immediately adjacent to the entrance of the developing external auditory canal which may account for the auricular anomalies [19]. In contrast, as *Eya1* is not expressed in the epithelium of the first branchial cleft (which develops into the external auditory canal), it is possible that the canal anomalies of BOR syndrome may indirectly result from those of either the auricle and/or middle ear structures. During early middle ear development, as seen for the outer ear, *Eya1* is not expressed in the regions of the branchial arches from where the ossicles arise, whereas, during more advanced stages, *Eya1* is expressed in the mesenchyme surrounding the cartilage primordia of all three ossicles [19] suggesting a direct role for this gene in the development of all three ossicles. Similarly, *Eya1* is not initially expressed in the epithelium of the first pharyngeal pouch (which will become the tympanic cavity) but is expressed later in the tubotympanic recess.

In contrast, to the proposed later role of *Eya1* in outer and middle ear development, this gene is expressed early in inner ear development. *Eya1* expression is observed in both the epithelium of the otic vesicle (precursor of the membranous labyrinth) and the periotic mesenchyme (precartilage of the bony labyrinth) [19]. As reciprocal interactions between these two tissues have been shown to direct the morphogenesis of the mammalian inner ear [20–22], both of these sites of expression could account for the capsular abnormalities of BOR syndrome. *Eya1* is expressed in the ventro-medial wall of the otic vesicle (from where the future neuroepithelia will differentiate) and in the statoacoustic ganglion, and later, in the neuroepithelial sensory and supporting cells as well as in the spiral ganglion; these sites of expression could individually account for the sensorineural hearing loss of BOR syndrome. Interestingly, *Eya1* continues to be expressed in the inner ear after birth and maturation, indicative of an additional role for this gene in the differentiation and/or survival of some inner ear cell populations.

Finally, in the urinary system, *Eya1* expression is limited to the developing metanephros where, in turn, it is restricted to the condensing mesenchymal cells [14, 19]. This single site of expression, due to the reciprocal epithelial-mesenchymal interactions that are also required to direct kidney morphogenesis [23], can account for both the excretory and collecting system anomalies of BOR syndrome.

Role of *EYA1*

The role of the *eyes absent* gene family has mainly been addressed in *drosophila* where it has a role in directing eye specification, however, the clinical signs of BOR syndrome indicate a critical role for *EYA1* in the embryogenesis

of other organs. Recently, a spontaneous mutation in the murine *Eya1* gene was discovered [24]. Analysis of mutant mice revealed morphological anomalies of the semicircular canals and the cochlea, including absence of the organ of Corti and sensory epithelium, and kidney anomalies ranging from undetectable to bilateral agenesis. The discovery of this mouse mutation, named $Eya1^{bor}$, now provides a valuable and long-awaited model for studying the role of *Eya1*/*EYA1* and hence the etiology of BOR syndrome.

In conclusion, the gene responsible for the developmental defect BOR syndrome, *EYA1*, seems to play multiple roles in development and elucidation of its role could provide important information on the cascade of genes required for ear morphogenesis. Furthermore, this gene has also been shown to underly BO syndrome resolving the much-debated question as to whether BOR and BO syndromes can arise due to the variable expressivity of one gene. In contrast, *EYA1* is not necessarily responsible for other syndromes associating branchial arch and otic anomalies. Along this line, a family presenting with autosomal dominant inheritance of first branchial arch and otic anomalies not associated with mutations in *EYA1* has been described [25], arguing for the existence of other branchial arch syndrome genes. Thus the identification of *EYA1* provides a useful tool for classifying the vast number of branchial arch syndromes comprising otic anomalies into distinct genetic and/or clinical entities.

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The Jervell and Lange-Nielsen Syndrome

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Despite the rarity of the Jervell and Lange-Nielsen syndrome (JLNS) in clinical practice, a knowledge of the salient features and phenotype is useful because of the potential seriousness of the condition. Moreover, now that molecular confirmation of the diagnosis is possible in many cases, there is an opportunity to refer families for genetic counselling and mutation detection.

The syndrome was first reported in 1957 by Jervell and Lange-Nielsen who described the combination of profound congenital sensorineural deafness with episodes of fainting and sudden death in childhood in a Norwegian family [1]. The clinical importance of the condition was highlighted by the fact that three of the four children affected died during an attack. No signs of heart disease could be detected by physical or X-ray examination and the only finding was a prolonged QT interval on the ECG, thought to reflect abnormal cardiac repolarization following the action potential, leading to cardiac arrhythmia. In 1964 Fraser, Froggatt and James published a further nine cases from six sibships following an extensive survey of 1,500 deaf-mute children in England, Wales, Scotland and Ireland [2]. They detailed the clinical presentation and the genetics of the condition, both of which merit mention here, before a description of recent progress and the molecular basis of the condition.

Clinical Features

Fraser's cases, and the original report, summarize the important clinical features. All children affected with the condition suffer from severe or profound

bilateral congenital sensorineural deafness requiring special education. The hearing loss affects mainly the high tones with some preservation of the low frequencies. Although no tests of vestibular function were performed in the original descriptions it is now apparent that there is also vestibular failure. Therefore it is not unusual for parents of affected children to give a history of delayed motor milestones in the first year of life (Bitner-Glindzicz, unpublished data).

The fainting attacks are precipitated by exertion or emotion and interestingly, auditory triggers such as ringing telephones or alarm clocks [3]. Swimming also appears to be a triggering factor [4, 5]. Some attacks appear to be mild without loss of consciousness and Jervell remarked that they resemble angina pectoris [6]. Fraser describes the onset of pallor and sweating combined with moaning or crying [2]. More severe attacks resulted in loss of consciousness for 5–10 minutes. It is not uncommon for the patient to be incontinent of urine during unconsciousness which is why such attacks may be misdiagnosed as epilepsy. However, recording of an ECG during one of these episodes has shown the presence of ventricular fibrillation or ventricular asystole [6]. There is intrafamilial variation in severity of attacks such that some individuals may be only ascertained after diagnosis following a more severe attack in a sibling. Indeed some children present with cardiac arrest and mortality is high.

Prevalence

The prevalence of the syndrome has been estimated at between 1.6 and 6 per million births in Britain, with the caveat that this was likely to be an underestimate [7]. This is because some children with severe disease will die before they are ascertained (stillbirth, death before deafness is diagnosed, failure to recognize the syndrome) and others may continue to live without a correct diagnosis ever being made.

Genetics

There is a high incidence of consanguinity among the parents of children with the syndrome [3, 7–10] and this observation together with segregation analysis provided good evidence for autosomal recessive inheritance. Parents of affected children were clinically normal, with normal hearing, although occasionally they were noted to have a prolonged QT interval on the ECG [2, 6]. Fraser considered that JLNS and the Romano Ward syndrome (RWS)

might be allelic (caused by mutation in the same gene). RWS is an autosomal dominant condition in which syncope is associated with a prolonged QT interval but there is no deafness. Fraser considered that RWS individuals might be heterozygous for a mutant allele and those with JLNS, homozygous. Against this hypothesis was the observation that the complete cardiac manifestations, including sudden death, had not at that time been reported in cases with and without deafness coming from the same family [11]. Such occurrences have now been reported [3, 10] and that the two conditions are indeed allelic has now been confirmed by mutation detection [3, 9, 10, 12].

Linkage analysis of large families with the more common, dominant RWS together with positional cloning and candidate gene strategies showed that RWS is due to mutation in one of several ion channel genes; *KVLQT1* on chromosome 11 (which probably accounts for over half the cases of long QT syndromes), *HERG* on chromosome 7, *SCN5A* on chromosome 3 and *KCNE1* (also called *ISK*) on chromosome 22 (reviewed in [13]). In addition there is evidence for the existence of other loci, one of which maps to chromosome 4 and at least one other unmapped locus. In the heart, the *HERG* gene encodes a potassium channel, which underlies I_{kr}, the rapidly activating component of the delayed rectifier current [14]. *SCN5A* encodes an α-subunit of a cardiac sodium channel, *KVLQT1* encodes an α-subunit of a cardiac potassium channel and *ISK* (minK) encodes a unique transmembrane β-subunit. KvLQT1, the product of the *KVLQT1* gene has been shown to associate with IsK, to form a channel reproducing the properties of I_{ks}, the slowly activating component of the delayed rectifier current [15, 16]. The C-terminal end of minK appears to interact directly with the pore region of KvLQT1 [17, 18] and the minK-HERG complex also regulates the cardiac potassium current, I_{kr} [19]. Individuals heterozygous for mutations in these genes may suffer from RWS, although it has been known for some time that penetrance is incomplete and the phenotype variable within families. It has now been shown that homozygosity for mutations in *KVLQT1* results in the much more severe phenotype of JLNS i.e. congenital, profound deafness and cardiac syncope. *KVLQT1* as well as being expressed in the heart has also been demonstrated in the mouse in the marginal cells of the stria vascularis, the tissue responsible for maintaining a high concentration of potassium in the endolymphatic fluid bathing the hair cells of the organ of Corti [10]. Analysis of a number of families with JLNS has shown that most are due to mutation in *KVLQT1* but that homozygous mutation in the gene *ISK* may cause the same clinical picture, although this is much rarer [9, 20]. This is not surprising since ISK is also expressed in the marginal cells of the stria, coassembles with *KVLQT1* to form a functional

potassium channel, and when knocked out in the mouse causes profound deafness and vestibular dysfunction and an inner ear pathology similar to that reported in post-mortem specimens of patients who have died from JLNS [21].

If RWS and JLNS are allelic, why are symptoms rarer in carriers of JLNS mutations? Symptoms such as syncope and sudden death have now been reported in obligate carriers of JLNS mutations [3, 10] but are rare even allowing for the fact that penetrance of mutations in RWS is incomplete [22]. A careful comparison of the mutations described to date in RWS with those found in JLNS shows some differences. Most mutations in *KVLQT1* that underlie dominant Long QT syndrome (RWS) are missense mutations [23–31]. This means that the mutation involves substitution of one amino acid for a different one. Since KvLQT1 polypeptides coassemble into multimers, the creation of a localized mutation may mean that mutant subunits may still combine with normal KvLQT1 subunits into ion channels, greatly reducing the number of fully functional channels. Indeed, in vitro studies of mutant ion channels injected into Xenopus oocytes shows that all RWS mutations expressed so far result in the dominant negative suppression of channel activity [12, 25]. Of the 30 different *KVLQT1* mutations reported to date associated with LQT1, 26 are missense. Of those that remain, the effect is likely to be localized and in-frame [32, 33].

In contrast, most mutations reported in JLNS thus far appear to be nonsense or frameshift mutations, and cause premature truncation of the encoded protein [3, 9, 10, 33]. Such mutations do not necessarily act in a dominant negative manner as they may be unable to coassemble with normal subunits, particularly if they are truncated near the N-terminal end of the protein. Thus all the channels that are present may be fully functional but they may be present in much reduced numbers. Expression studies of these mutations will reveal whether or not these mutations act in a loss of function manner and thus differ at a functional level from the missense mutations underlying the RWS. Loss of function mutations might be expected to cause a milder phenotype in heterozygous carriers in line with previous observations [2, 6, Tranebjærg, pers. commun.]. This would suggest that the penetrance of these mutations is somewhat less than the 60% suggested for RWS [4, 22].

However, trying to correlate genotype with phenotype may be an over simplification as some missense mutations in *KVLQT1* have been reported in JLNS [26, 32]. It may be that mutations located in the C-terminal end of the protein (which some of the JLNS mutations are) as opposed to the transmembrane and pore-forming regions of the predicted protein, produce a less severe phenotype in heterozygotes [34].

Clinical Significance of Recent Work in JLNS

Although JLNS is an extremely rare condition and most practitioners will not see a case during the course of their professional career, making the diagnosis in a child is likely to be lifesaving, since long-term studies have shown the untreated mortality to be in excess of 50%. A high index of suspicion is therefore necessary in order to diagnose JLNS. In our center all deaf children have an electrocardiogram (ECG) and in some countries all deaf children see a cardiologist with this diagnosis in mind. Any suspicion of a prolonged QT interval or a history of fainting in a deaf child warrants immediate referral to a cardiologist for a specialist opinion. Certainly syncope or seizures precipitated by 'fight, flight or fright' in anyone (deaf or not) should be LQTS until proven otherwise [4].

Although Fraser reported no history of syncope and sudden death in the study in 1964 in relatives of those with JLNS, such events have now been documented. As a result, mutation carriers should also now be referred to a cardiologist for clinical assessment, ECG and possibly exercise testing because of the risk of sudden death, albeit small. We are still a long way from being able to predict which mutation carriers will suffer from symptoms and which won't but expert opinion and testing may reveal those with prolonged QT intervals who may benefit from drug treatment.

Recent advances in molecular genetics also means that accurate genetic counselling based upon mutation detection is now available for some families. Molecular genetic analysis may also be valuable in the future for directing drug therapy which may be tailored towards mutations in particular genes [4].

From the wider clinical viewpoint, the finding of nonsense and frameshift mutations in ion channel genes, which may occasionally become penetrant, in addition to the previously identified missense mutations, and the recent description of 'recessive' isolated long QT syndrome without deafness [35], is most interesting in the light of recent data on Sudden Infant Death Syndrome (SIDS) [36]. The finding of prolonged QT_c intervals in infants subsequently dying of SIDS may be explained by molecular genetics; some infants may have new missense mutations in *KVLQT1* i.e. sporadic cases of RWS, but some may be heterozygotes for low penetrance mutations (possibly nonsense and frameshift) which are not clinically manifest in their parents. Some may be homozygous for recessive mutations, with or without deafness, which may again be clinically inapparent in heterozygous parents but which may recur in siblings, a phenomenon noted in SIDS. In the future it may be possible to offer molecular screening of high-risk infants, those with a family history of SIDS or LQTS or 'near miss' cases

[37], which may become more feasible with the advent of DNA microchip technology.

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Treacher Collins Syndrome

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Clinical Features

Treacher Collins syndrome (TCS) is named after the ophthalmologist E. Treacher Collins, who first described the essential features of the syndrome in 1900 [1]. The first complete description of the condition was detailed by Franceschetti and Klein, who used the term mandibulofacial dysostosis to describe the facial appearance [2]. TCS is the most common of the mandibulofacial dysostoses and affects in the region of 1 in 50,000 live births. The condition is inherited in an autosomal dominant fashion. The main criteria used in the diagnosis of TCS include bilaterally symmetrical midfacial hypoplasia (89%), downward slanting of the palpebral fissures (89%), notching of the lower eyelids, with sparse eyelashes (69%), mandibular retrognathia (78%), microtia (77%) and other deformities of the external ears, which often lead to conductive hearing loss (50%) and cleft palate (28%) [3]. The typical facial appearance is depicted in figure 1. Although the mutated gene is highly penetrant, there is marked phenotypic variation. In some cases the features are so mild that it is difficult to make an unequivocal clinical diagnosis while in others perinatal death may result from an inability to maintain a patent airway secondary to the hypoplasia of the facial bones. In cases where expression of the syndrome is complete, TCS can be readily diagnosed on the basis of the clinical appearance alone. However, diagnosis may not be as straightforward for mildly affected individuals. Given that approximately 60% of cases of TCS arise without a previous family history, usually as the result of a de novo mutation [4], this may lead to severe difficulties in the provision of accurate genetic counselling, for example where the diagnosis of an affected child's parents is in doubt. In such cases, radiological examination may prove to be

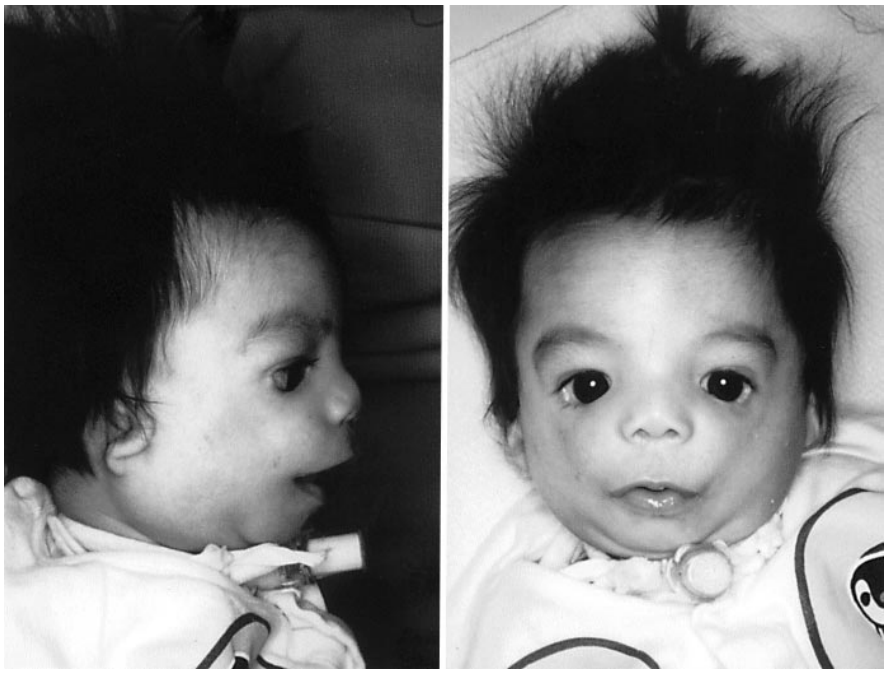

Fig. 1. Clinical features of a child with TCS. (*a*) Lateral view to show hypoplasia of mandible and zygomatic complex and severe anomalies of the external ear. (*b*) Frontal view to show down-slanting palpebral fissures, colobomas of the lower eyelids and a paucity of lid lashes. The features are bilaterally symmetrical.

a useful adjunct in the management of the patient [5]. Given that TCS may also cause significant morbidity, prenatal diagnosis may be requested by a subset of families (see below).

Mapping of the Treacher Collins Syndrome Locus and Isolation of the TCOF1 Gene

On the basis that the tissues affected in TCS arise during early embryonic development from the first and second pharyngeal arches, it has been proposed that the condition may arise from abnormal neural crest cell migration [6], improper cellular differentiation during development [7], or an abnormality of the extracellular matrix [8]. More recent studies on murine embryos exposed to 13-*cis*-retinoic acid at 9.0–9.5 days post-fertilization have described features similar to TCS [9] and have suggested that cells derived from both the neural

crest and the ectodermal placodes are involved in the development of mandibulofacial dysostosis. Nevertheless, none of these studies suggested a strong candidate gene or an animal model for TCS. Under these circumstances, positional cloning strategies were used to isolate the mutated gene. The TCS locus (*TCOF1*) was initially mapped to human chromosome 5q31-q34 via genetic linkage studies in well-characterized families. Although initial studies used restriction fragment length polymorphisms (RFLPs) [10], highly informative short tandem repeat polymorphisms (STRPs) were subsequently used to refine the location of *TCOF1* to 5q32-q33.1 [11–13]. The development of a combined, high-resolution genetic linkage and radiation hybrid map of human chromosome 5q31-q33 [14], allowed STRPs closely flanking *TCOF1* to be identified and facilitated the creation of yeast artificial chromosome and cosmid contigs across the *TCOF1* critical region [15, 16].

In order to isolate genes that could be screened for TCS-specific mutations, a combination of exon amplification and cDNA library screening was used to create a transcript map of the candidate region [17, 18]. One of the genes that was isolated using these techniques [18] was ultimately shown to contain an open reading frame of 4,233 bp encompassed by 26 exons, which encodes a low-complexity 144 kDa protein, named treacle. Mutation analysis of this gene has resulted in the identification of over 70 predominantly family-specific mutations, the vast majority of which introduce a premature termination codon into the protein suggesting that the mechanism underlying TCS is haploinsufficiency [18–21]. The most 5′ mutation identified is the nonsense mutation Q36X, whereas the 3′-most mutation is a five bp deletion, nt4135 del(GAAAA), which occurs in exon 24, 94 bp from the termination codon. Genotype-phenotype correlations have not emerged. Unfortunately, the mutational spectrum has provided little clue as to the function of treacle.

The first potential clue as to the function of treacle was derived from database sequence comparisons that revealed weak similarity to a family of nucleolar phosphoproteins [21, 22]. Subsequently, a number of repeated units were identified within treacle, each of which mapped onto an individual exon. Each repeat unit consists of clusters of acidic amino acid residues, containing numerous consensus sites for casein kinase II phosphorylation, which are separated by basic amino acid stretches comprising a majority of lysine, alanine and proline residues. In addition, the carboxyl terminus of the protein is lysine-rich and encodes a number of potential nuclear localization signals (NLS). These sequences are essential for the transport of proteins into the nucleus. Although no single consensus sequence has emerged, NLSs are usually short, up to 12 amino acids long, and they contain a high proportion of positively charged amino acids [23]. Within treacle, the potential NLSs, which are located towards the carboxyl terminus, are of the consensus K-K/R-X-R/K [24]. The

low complexity amino acid composition, the nature of the repeated units and presence of nuclear localization signals are features which treacle shares with the nucleolar phosphoproteins [21, 22]. Moreover, these features are all conserved in the murine homologue of *TCOF1* [25].

Indirect evidence provided by database analyses therefore suggested that treacle may function as a nucleolar phosphoprotein. These proteins are phosphorylated and exhibit nucleocytoplasmic shuttling. Although their precise function is not known, it has been suggested that they may play a role in nucleocytoplasmic transport for example in the import of ribosomal proteins and export of preribosomal particles [26]. Direct evidence that treacle may function in a similar fashion has been provided by subcellular localization studies. Immunocytochemical studies performed using polyclonal antibodies raised against the amino terminus of treacle revealed bright staining of the nucleoli [27]. This result was confirmed by creating a fusion protein between treacle and green fluorescent protein, which also was found to localize to the nucleolus. Interestingly, fusion proteins lacking putative nuclear localization signals, or those containing mutations observed in individuals with TCS, were mislocalized within the cell [27]. This further supports the hypothesis that, like the nucleolar phosphoproteins, an integral part of treacle's function involves shuttling between the nucleolus and the cytoplasm.

Interestingly, *TCOF1* has been shown to be expressed in a wide range of adult and embryonic tissues. While these observations would appear to be at variance with a disease that causes only craniofacial malformations, the expression patterns obtained from whole-mount in situ hybridization studies performed on developmentally staged murine embryos have indicated that the highest levels of expression are observed in the crests of the neural folds immediately prior to fusion and in the first branchial arch [25]. These observations are consistent with a role for the gene during craniofacial morphogenesis and also with the possibility that TCS results from haploinsufficiency. Nevertheless, the precise mechanism by which disruption of treacle leads to the pathogenesis of TCS is unknown. It is interesting to speculate on the possible mechanism by which haploinsufficiency of a widely expressed protein can cause the features of TCS, which are restricted to the face. Since treacle is found within the nucleolus, it is possible that the protein is involved in ribosome assembly [28] and functions at a critical, rate-limiting step during development, when high levels of translational activity are essential. Any reduction in the maximum synthesis rate of treacle would likely be deleterious, leading to the morphological defects observed in TCS. Alternatively, perhaps the proteins with which treacle interacts, or which it shuttles, are important specifically in craniofacial embryogenesis. Such proteins will also be important in explaining the extremely variable phenotype that is observed between individuals even

with the same mutation. Other reasons for a lack of genotype/phenotype correlations for TCS may include environmental influences, or the effects of different chromosomal backgrounds.

Implications for Diagnosis

Prior to the commencement of molecular studies on TCS, prenatal diagnosis was only possible via fetoscopy or ultrasound imaging. Both methods have been used to make positive predictions [29–32]. While the quality of ultrasound imaging has improved markedly in recent years, allowing non-invasive prenatal diagnosis to be performed, it may still be difficult to make a positive diagnosis, particularly where the fetus is mildly affected. Given these circumstances the procedure is usually not diagnostic for apparently unaffected fetuses. Moreover, prenatal diagnosis using either fetoscopy or ultrasound imaging is not possible until the second trimester of pregnancy. Conversely, molecular diagnosis can be undertaken in the first trimester of pregnancy. Chorionic villus samples can be analyzed at around the 12th week of pregnancy, much earlier than detailed information can be gained from ultrasound imaging. Previously, diagnostic predictions were made on the basis of linked STRPs, in families showing significant evidence of linkage to 5q31.3-q32 [33]. More recently, in light of the identification of *TCOF1*, these predictions are now more reliable, since the presence or absence of a particular mutation can be assessed. However, genetic counselling of families with TCS is complicated by the fact that, in general, mutations between families are different. Hence, the specific mutation must first be identified within a family, before any counselling or pre-natal diagnosis can be performed. Since this may require analysis of all 26 exons of *TCOF1*, this can be a time-consuming process. In addition, even if the fetus is found to carry the disease-causing mutation, no conclusions can be drawn about the severity with which the child will be affected. Therefore, parents may opt to delay any decision-making until ultrasound can be used to provide further information on the extent of the abnormalities. However, for those cases in which the fetus does not carry the mutation, parents can be reassured that their child will be unaffected. The isolation of *TCOF1* also makes possible post-natal diagnosis of individuals who are so mildly affected as to make clinical diagnosis difficult. This will also be useful in being able to counsel accurately apparently unaffected parents of a child in whom TCS is thought to have arisen as the result of de novo mutation.

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Non-Syndromic Dominant DFNA1

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Introduction

The high degree of structural complexity of the auditory system [1–4] must have a very high price in terms of specific regulatory and structural genes. Hence, the large number of different types of mutations that cause deafness in mice and humans [5–8]. During the last decade more than 40 non-syndromic deafnesses were mapped, as well as many deafness syndromes with complex phenotypes. Most non-syndromic hearing losses are transmitted as recessive traits, but many dominant mutations are also known, as these can be easily detected spreading through large kindred [7]. Most types of mutations affect initially the high frequencies of the auditory scale, but a few damage the low frequencies at the time of onset [3, 5, 6]. Low frequency onset indicates that the mutation first affects hair cells in the cochlear apex, advancing centrifugally towards the basal turn with progressive loss [3, 5].

A dominant, low frequency, early-onset progressive sensorineural deafness was described in the M-kindred of Costa Rica [9] and initially classified by McKusick [10] along with a family from Tennessee as Low Frequency Hearing Loss I (LFHL1, McKusick #124900). The M-kindred mutation, later designated DFNA1, leads to severe losses and is fully penetrant. The Tennessee family has a non-syndromic progressive loss, incompletely penetrant, involving the low frequencies at the time of onset, progressing to all frequencies but never becoming severe. Recently the mutation in the Tennessee family-designated DFNA6- has been mapped to chromosome 4p16.3 [11] and McKusick (MIM on line) has assigned separate categories for these two deafnesses. The DFNA1 mutation in the M-kindred is described in this chapter. It was mapped to 5q31 in 1992 [12] and recently cloned and sequenced [13], in a decade-long effort between US and Costa Rican research teams.

Clinical Phenotype of the DFNA1 Mutation

Early studies of the clinical phenotype were based on medical histories, complete medical examinations, pure tone audiometries and blood tests in a family sample (N = 34) with both affected and unaffected sibs. Thyroid hormone levels (T_3 and T_4) as well as other blood parameters were within normal range in affected members and indistinguishable from values in unaffected members of the M-kindred [14]. Medical histories and neurologic examinations did not reveal vestibular impairment or any other abnormality. Pure tone audiometry demonstrated bilateral and symmetrical deafness, with initial loss of low frequency perception at 250 and 500 Hz, progressing to all frequencies with age [9, 15]. In older family members all frequencies were abolished, although a few individuals retain some perception at high intensities. The ratio of affected to unaffected sibs in the family is 1:1; males and females are equally likely to inherit DFNA1.

Recently, three affected members of the family underwent extensive clinical, audiologic, vestibular, and radiologic evaluations at the UCSF Medical Center [16]. Vestibular evaluations of the three affected individuals, two adults and a child, demonstrated absence of nystagmus and good balance without evidence of cerebellar dysfunction. There was no gaze, spontaneous or positional nystagmus on electronystagmography. The child with a moderate hearing loss presented normal auditory evoked potentials indicating functional central auditory pathways. The presence of distortion-product evoked otoacoustic emissions implicated functional outer hair cells. High resolution computerized tomography showed normal cochlea, semicircular canals, vestibular and cochlear aqueducts and internal auditory canals; thus the inner ear abnormality is limited to the membranous labyrinth. The presence in the child of an elevated summating potential-to-action potential ratio on electrocochleography suggested endolymphatic *hydrops*.

Most affected members of the M-kindred report a tonal tinnitus that disappears with progressive hearing loss. In one specific instance a father and child reported a 'click' tinnitus at the time of onset, that could be objectively detected [16]. Despite the prevalence of tinnitus none of the affected members report vestibular disturbances. A large variability in the age of onset was originally detected, subjectively reported by family members between 5 and 30 years of age [fig. 2, ref. 9]. Heritability of onset age is not clustered in the family; siblings may show marked differences (i.e. family indicated by an arrow in fig. 1, with onset in one male in late teens whilst in two female siblings and one female niece showed losses before age 10y). The M-kindred phenotype is defined, for linkage analysis, as a loss of at least 50 dB by age 30 at 250 and 500 Hz in pure tone tests. This level of auditory loss, however, can be present in very early-onset children by age 15y [9, 15, 16].

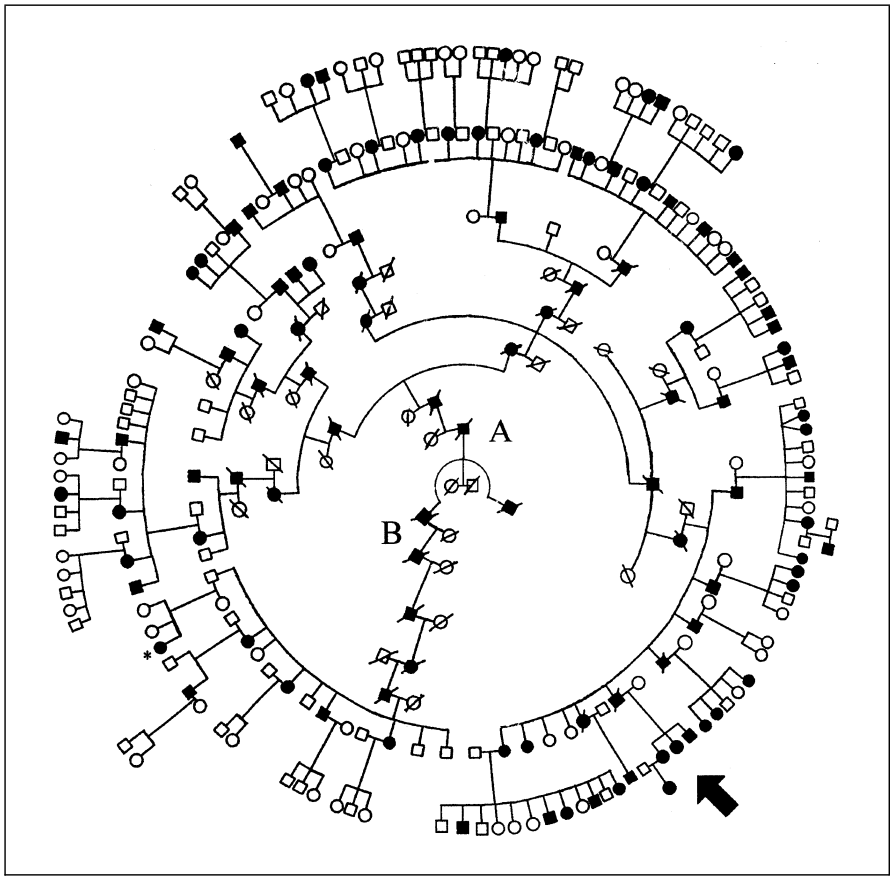

Fig. 1. Updated geneology of the M-kindred with descendents from FM, previously the last common ancestor [12]; branch A in this figure. A second branch of the family has since been analyzed (branch B). The affected status of FM and his two brother derives from *Testamentos* (Wills) recovered from late 17th and early 18th century archives, which explicitly mentioned their deaf status. The big arrow points at a sibship mentioned in the text, which presents different ages of onset within the cluster. The asterisk shows a congenitally deaf child, presumably due to maternal viral infection during pregnancy, who lacks the affected haplotype and the mutation.

Loss of auditory feedback mechanisms with progressive deafness eventually may alter vocalization, so in many deaf adults speech deteriorates. In the M-kindred a high pitched intonation often develops presumably as the feedback shifts with progressive loss to the higher frequencies of human vocalization.

Mapping the DFNA1 Mutation to Chromosome 5q31

Early attempts to map the DFNA1 mutation with classical immunologic blood markers did not provide significant evidence of linkage [17]. A site on chromosome 22 was suggested with an erythrocyte marker P1, but this was rapidly discarded with DNA polymorphic markers. DNA was obtained from cell lines established with Epstein Barr virus, with peripheral lymphocytes from 147 family members. RFLPs (restriction fragment length polymorphisms) were used first but later STR (short tandem repeat) markers based on PCR technology were exclusively used. Linkage of deafness in the M-kindred to markers in chromosome 5q31 was established with great statistical confidence (LOD scores >13). The 7 cM region originally reported [12] was reduced to a 1 cM stretch between markers D5S658 and D5S1979, with several key recombinants in the linked-haplotype and with many additional STR markers from Genethon and other sources [13, 18]. The presence of the linked DNA haplotype in all affected member and its absence in all unaffected siblings indicated that the DFNA1 mutation is totally penetrant; therefore the affected phenotype appears sooner or later in all members with this mutation.

No couples between two deaf members of the family are known, hence no individuals homozygous for the mutation have been found. Construction of a physical map of the 1 cM critical region covered a sequence of about 800 Kb of DNA [13].

The DFNA1 Mutation in the M-kindred Affects the Human *Diaphanous* 1 Gene (*hDIA1*) Involved in the Organization of the Actin Cytoskeleton of Cells

The identification of a disease causing mutation in a linked region can be broken down into three stages. First the region itself needs to be cloned into some vector which allows the propagation of pure DNA fragments from the region in microgram quantities for sequence analysis. Second, the cloned DNA fragments from the linked region are analyzed to identify all of the genes in the region, their relative locations, and their sequences. Third, the sequences of the genes in the linked region are compared in affected and unaffected individuals. Any differences in gene sequences are further analyzed to determine if they are disease associated.

For the cloning of the DFNA1 region we settled on using the Bacterial Artificial Chromosome (BAC) vector system. Human BAC clone libraries are commercially available as DNA pools which can be rapidly screened using PCR technology. Once identified, large amounts of DNA from each clone can be

obtained easily using common reagents and techniques. To start building the set of overlapping clones of the DFNA1 region we used the PCR-based genetic markers that defined the linked region. The ends of BAC clones that were shown to contain the linked markers were sequenced. These sequences were then used to rescreen the BAC DNA pools to find overlapping BACs. This process was repeated until the set of overlapping BACs from each end of the region met and overlapped in the middle of the adjacent region. Each of these clones contained on average 140 kilobases of DNA and the linked 800 kilobase region was covered by a minimum set of six overlapping BAC clones [13].

To find the genes in the DFNA1 region each of these BAC clones was randomly sheared into one kilobase fragments. Several hundred fragments from each BAC were sequenced, and each of these sequences was compared with the Genbank database of known genes and proteins and with the Expressed Sequence Tags (dbEST) using the Basic Local Alignment Search Tool (BLAST) [13]. Because these databases are nearly comprehensive, this technique of identifying genes by direct sequence analysis is becoming common practice. With the completion of the Human Genome Project, this step and the prior one will become unnecessary. Once a linked region is defined by genetic markers, researchers will simply search the databases of sequence information to identify all the genes of interest to their project.

Having identified a set of genes from the DFNA1 region we compared the sequence of each of these genes in the affected and unaffected individuals. Rather than sequence each gene, we chose to use a technique known as Single-Strand Conformation Polymorphism (SSCP) detection. This rapid technique allows the identification of sequence changes on 300–400 basepair DNA fragments. The observation of a change in the electrophoretic migration rate of each DNA fragment through an acrylamide gel is a consequence of differences in the sequence of the DNA fragment. While it is not as accurate as direct sequencing of the DNA, it is much faster and less expensive and, when done correctly, the accuracy of identifying mutations approaches 95% [19].

The analysis of patient DNA using SSCP was performed on eight genes out of the fourteen identified in the DFNA1 region. The eighth gene analyzed was the human ortholog of the *diaphanous* gene in *Drosophila*, so named because the phenotype of mutations in this gene included a ruffled or diaphanous appearance at the leading edge of *Drosophila* cells [20]. Initially *diaphanous* was not considered to be a strong candidate as the literature surrounding this gene indicated that it was widely expressed and was critical to the basic cellular process of cytokinesis in cell division. The gene is a member of the formin gene family, which includes the limb deformity gene in mice, the *Drosophila* gene *cappuccino* and the yeast gene *Bni1p* [13, 21]. The human diaphanous 1 (*hDIA1*) gene is composed of 26 exons that code for at least three

well-defined domains: a RHO-binding domain near the amino end, a polyproline stretch in the middle referred to as the Formin Homology 1 (FH1) domain; and a highly conserved Formin Homology 2 (FH2) domain near the C-terminus [22]. The RHO-binding site suggests that hDIA1 is an effector of the RHO signal-transduction pathway [22, 23]. The polyproline region is a binding site for profilin which when bound catayzes the formation of actin filaments from actin monomers. The FH2 domain does not have any function associated with it yet. Its sequence indicates it may interact with proteins which aid in the localization of the RHO-hDIA1-profilin complex to sites in the cell undergoing cytoskeletal formation or reorganization.

A point mutation involving a guanine-to-thymine substitution in the donor splice site of the penultimate exon was detected by SSCP in all affected members of the family. This substitution was absent in all nonaffecteds and in a sample of 330 unrelated hearing, control individuals. This point mutation disrupts the canonical splice donor sequence AAGgtaagt ⇒ AAG**T**TAAgt, shifting the splice site 4 base pairs into the intron sequence, hence the mutated messenger RNA contains the additional nucleotides TTAA. This results in a frameshift encoding 21 aberrant amino acids, followed by protein termination that truncates 32 amino acids.

Proposals on the Possible Mechanism of the Pathology

Human *diaphanous* protein has three distinct functional sites mentioned above shared by many formins. Thus, it appears likely that hDIA1 is part of a multiprotein signaling complex involved in polymerization, scaffold organization and anchoring of the actin cytoskeleton [23–25]. Given the importance of the actin cytoskeleton in hair cells, we hypothesized [13] that (i) the DFNA1 mutation affects amplification of sound reception by outer hair cells, that relay kinetic energy to inner hair cells based on an actin cytoskeleton and on membrane motors [1–3, 26]. An alternative (ii) might be that DFNA1 prevents the repair of the paracrystalline actin array of steriocilia, which can become disordered upon acoustic stimulation [27]. Failure to repair this actin array or its anchorage sites in the cuticular plate could lead to uncoupling of steriocilia from the transduction channels and hence to the absence of depolarizing potassium ion currents moving into hair cells.

The mutation has no apparent impact on other tissues, not even in the semicircular canals, given the absence of associated abnormalities [14, 16]. Alternatively, subtle effects on other tissues might only become evident under stress situations, such as intense muscular activity. The DFNA1 mutation does not abolish the function of hDIA1 during early development either, as affected chil-

dren have normal hearing. The obvious conclusion is that the mutation is a partial loss of function that affects *repair* of the actin cytoskeleton during constitutive expression of the gene in early childhood and adolescence. Obviously, other cochlear structures could be altered, but further characterization will require an animal model, since histological samples from the M-kindred are not available.

This impact of the DFNA1 mutation on *diaphanous* gene function is confounded by the fact that humans have at least two other *diaphanous* copies, one in the X-chromosome and an autosomal copy on chromosome 17q12-13 [13, 28, unpubl. observations]. The X-chromosome diaphanous gene *hDIA2*, is implicated in premature ovarian failure which is a phenotype seen in *Drosophila diaphanous* mutants. The mild phenotype of the DFNA1 mutation could be related to functional redundancy with these two other *diaphanous* genes in many tissues. Understanding the pattern of gene expression of the formin family in humans will undoubtedly help clarify these questions.

We consistently encounter older members of the family not exposed to hearing aids at the time of onset refuse to adopt them, claiming that the high noise levels are stressful. This may be partly due to the effect of loudness recruitment, which tests positively in the M-kindred [12, 15, 16]. Longitudinal audiometric studies might provide a test for a repair hypothesis if exposure to sound increases the rate of loss, by retesting individuals that only wear one hearing aid. A practical application of this knowledge would be to tell members with hearing loss to avoid exposure to intense sound to protect their hair cells.

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DFNA 2, 5, 8, 12

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DFNA2

In 1994, we analysed an extended Indonesian family with autosomal dominant non-syndromic hearing loss (ADNSHL) [1]. The pedigree contained more than 140 family members with hearing loss, all living in a relatively isolated community and descending from a common ancestor 7 generations ago. Their hearing loss started in the high frequencies in the second or third decade of life and progressed with age to include the middle and low frequencies. By age 40, the hearing loss was moderately severe to profound. A large number of children below the age of 10 from an affected parent were tested audiometrically, and all of them had normal hearing, indicating that the hearing loss started after the first decade.

Using a subset of the family, genome-wide linkage analysis was performed, and linkage was found to the short arm of chromosome 1, in band 1p34 [1]. The locus was named DFNA2, reflecting the fact that this was the second ADNSHL locus to be discovered. A number of American families with dominantly inherited hearing loss were immediately tested for linkage to DFNA2, and a single linked family was found [1]. The phenotype in the US family was similar to that in the Indonesian family, with progressive, initially high frequency, hearing loss that later affected lower frequencies. However, age of onset was lower, as several children under the age of six had moderately severe hearing loss. Most of the affected persons in the American family had profound hearing loss by the age of 40. The penetrance of the hearing loss in both the Indonesian and the American family was complete. The combined genetic information from the two linked families placed the DFNA2 gene in an interval of 6 cM or approximately 6 million basepairs.

Since the initial report describing two DFNA2 families, linkage to this locus has been found in five additional families [2–5]. One family, with more than 27 affected family members, originates from Belgium [4]; three families, with respectively 41, 24 and 18 affected family members, originate from the Netherlands [2, 3, 5]; and one additional family, with 51 patients, comes from the USA [6]. In all of these new families, the hearing impairment is progressive, starting in the high frequencies and ultimately affecting the middle and lower frequencies.

Analysis of the additional DFNA2 families with numerous polymorphic markers in the DFNA2 region reduced the critical interval to ~ 1.25 megabases [4], a size amenable to positional cloning. Several candidate genes from this region have been analysed, but no disease-causing mutations have been identified (unpublished results).

Statistical analysis of the audiograms has been performed for the Indonesian and Dutch families. The Indonesian family shows great intrafamilial variability in the severity of the hearing loss and in the configuration of the audiogram [7]. The average rate of progression of the hearing loss is approximately 1dB per year for all frequencies (fig. 1). Variability in the three Dutch families is less pronounced in comparison to the Indonesian family, and the Dutch families also show a remarkably similar phenotype. The progression rate is close to 1dB in two of these families [2, 3], and in the third, slightly slower (0.7 dB/year) [5]. In all of the Dutch families, the statistical analysis suggests a possible congenital high frequency impairment. It is noteworthy that in one of the Dutch families [5], many affected persons attended schools for the hearing impaired.

The possibility that some DFNA2 families may be distantly related has been investigated by comparing disease haplotypes in five of the pedigrees [4]. This analysis indicates that it is unlikely that the families share a common ancestor, implying that mutations in the DFNA2 gene have arisen on separate occasions and are likely to be different. Of all autosomal dominant non-syndromic loci, DFNA2 has the most linked families. The evidence that these families are unrelated implies that the DFNA2 gene is a major cause of ADNSHL.

DFNA5

During the last four decades, a large Dutch family with more than 100 persons with ADNSHL have been extensively studied clinically [8–13]. The hearing impairment is sensorineural and bilateral, and starts in the high frequencies between 5 and 15 years of age. Initially, the hearing loss is confined

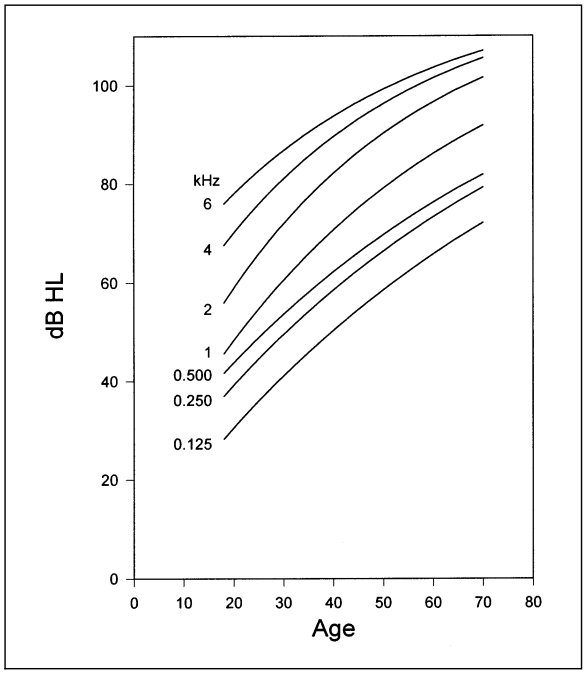

Fig. 1. Progression of hearing impairment in the Indonesian DFNA2 family. For different frequencies, non-linear regression lines were fitted for hearing thresholds on the basis of 66 patients. (from [7].)

to the high frequencies but with increasing age, the lower frequencies become affected. Progression rate differs between individuals, with rapid and slow progression patterns typically related to age of onset. If hearing loss begins at age 5, progression is rapid and by age 15, the lower frequencies are affected and by age 25, the end stage is reached. If hearing loss begins at age 15, progression is slower. The low frequencies are affected before age 40, and the end stage is reached at age 60. Audiograms of typical patients from both categories are represented in figure 2.

In 1995, DNA was collected from the members of one branch of this family and a genome-wide search was performed. The hearing loss gene was localised to chromosome 7p in a linkage interval of 15 cM. The locus was designated DFNA5 [14]. Further linkage studies in two other branches of the family refined the candidate region, initially to approximately 2 cM [15], and finally to 600–800 kb [16]. Subsequent positional cloning experiments were facilitated by ongoing sequencing efforts as part of the Human Genome Project. A mutation that leads to a premature truncation of the protein was found

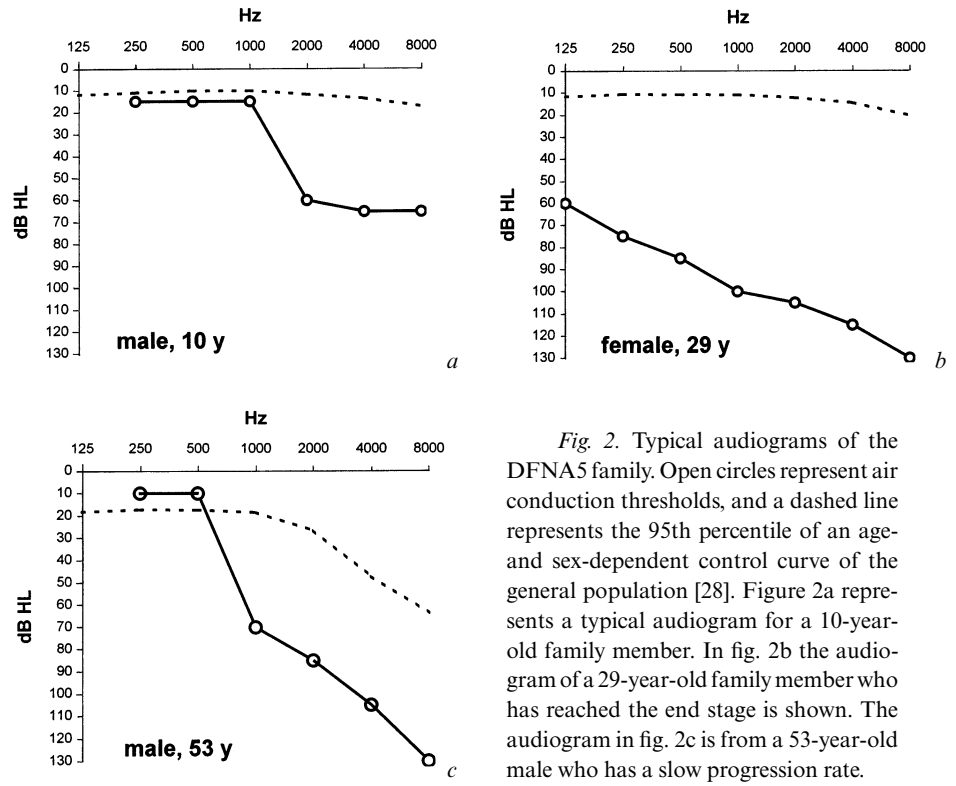

Fig. 2. Typical audiograms of the DFNA5 family. Open circles represent air conduction thresholds, and a dashed line represents the 95th percentile of an age- and sex-dependent control curve of the general population [28]. Figure 2a represents a typical audiogram for a 10-year-old family member. In fig. 2b the audiogram of a 29-year-old family member who has reached the end stage is shown. The audiogram in fig. 2c is from a 53-year-old male who has a slow progression rate.

in a gene within the candidate region. The mutation cosegregated with the hearing loss in the family and was never found in controls. Several further lines of evidence indicated that this mutation causes the hearing loss. Firstly, only a few genes are located in this apparently gene-poor region, and the mutation clearly inactivates the gene. Secondly, the gene is expressed in the cochlea (with low levels of expression also in the placenta, heart, brain and kidney).

Extensive computational analyses have not provided any information regarding the subcellular localization or function of this gene, which therefore has been designated *DFNA5*. While it has been possible to classify all previously identified non-syndromic deafness genes by function or protein family (i.e. transcription factors, unconventional myosins, extracellular matrix proteins, gap junctions or channel proteins), *DFNA5* is the first exception to this rule. It may take several years to unravel the function of this gene and to gain a better understanding of the pathogenesis of this type of hearing loss. In the

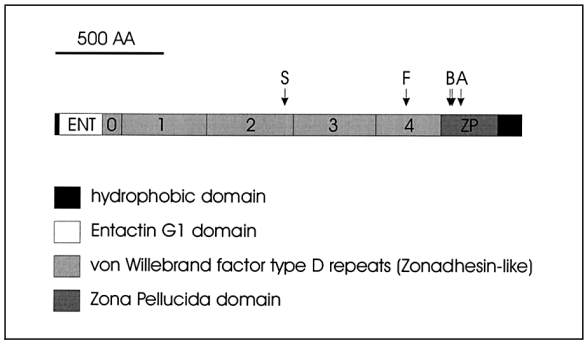

Fig. 3. Domain structure of the human α-tectorin protein. Arrows indicate the position of the different missense mutations. A: mutation in Austrian family, B: mutations in Belgian family, F: mutation in French family, S: mutation in Swedish family.

long term, this knowledge may lead to some form of therapy for persons affected with this type of deafness.

DFNA8/12

In 1997, we localized the gene for the ADNSHL segregating in a Belgian family to chromosome 11q22-24; the locus was named DFNA12 [17]. Shortly thereafter, an Austrian family with autosomal dominant hearing loss was linked to the DFNA12 region [18]. Interestingly, the disease gene in the Austrian family originally had been mapped to chromosome 15 and designated DFNA8. However, further analysis revealed that the 15q linkage was erroneous, and true linkage to chromosome 11 was found. The locus name, DFNA8, has been retained for this family, and the chromosome 11q22-24 locus now is referred to as DFNA8/12.

The hearing loss in the Austrian and Belgian families is very similar in configuration, affecting all frequencies, although in the mid-frequencies the loss is more severe [18, 19]. The degree of impairment in the Belgian family is moderate to moderately severe, with a median hearing loss of 51 dB [19]; in the Austrian family, it is moderate to severe, ranging from 60 to 80 dB [18]. Onset in both families is prelingual, and subsequent deterioration of the hearing is not in excess of normal age-related threshold increases. Phenotypically, the DFNA8/12 families are different from the majority of dominant families linked to other loci, in which the typical pattern of hearing loss is postlingual and progressive. Computed tomography of the temporal bones of persons in the Austrian family and

high resolution magnetic resonance imaging of the inner ear in persons in the Belgian family have not shown any morphological malformations [20].

Recently, two additional families with ADNSHL, one from France and the other from Switzerland, have been linked to DFNA8/12 [21, 22]. However, the hearing impairment in these families is different. In the French family, the hearing loss mainly affects the high frequencies, with onset prelingually or before the age of six, and progresses from a mild to a moderate loss at an average rate of 0.7 dB/year [22]. In the Swedish family, the mean age of onset of the hearing loss is 9 years [21]. The high frequencies (6–8 kHz) are preferentially affected, with most persons showing limited or no hearing loss at the low frequencies (< 500Hz). Thresholds in the high frequencies progress from moderate to severe at a young age to more than 80 dB at the age of 50. Interestingly, in the Swedish family a suggestion of linkage also has been found to DFNA2, and the authors therefore suggest that the inheritance in this family may be digenic [21].

The DFNA8/12 gene was identified using a positional/functional candidate strategy. An integral component of the cochlea is the tectorial membrane, an extracellular matrix composed of collagens and noncollagenous glycoproteins and into which are embedded the tops of the stereocilia bundles of the hair cells [23]. The most important noncollagenous glycoproteins are α- and β-tectorin, which account for 50% of the total protein content of the tectorial membrane. The mouse α-tectorin gene was cloned [24] and localized to a region of mouse chromosome 9, which shows evolutionary conservation with human chromosome 11q [25]. This localization and the specific function of α-tectorin in the inner ear made the human α-tectorin (*TECTA*) an ideal positional and functional candidate gene for DFNA8/12.

In order to analyse the human *TECTA* gene for mutations in DFNA8/12 families, we determined the complete sequence and intron/exon structure of the *TECTA* gene [20]. The *TECTA* protein has three distinct domain structures (fig. 3) [24]. The N-terminus shows homology to the G1 domain of entactin, a protein that is a major component of basement membranes; the central core contains von Willebrand factor (vWf) type D repeats, homologous to zonadhesin (ZA), a sperm membrane protein; and the C-terminus encodes a zona pellucida (ZP) domain. The latter was first identified in proteins from the extracellular matrix surrounding the unfertilized egg (zona pellucida) and hence the name. α-Tectorin is processed into three different polypeptides, each encompassing one of the three domain structures [24]. It is assumed that these three polypeptides are crosslinked to each other by disulphide bridges and interact with β-tectorin to form the non-collagenous matrix of the tectorial membrane. The way in which they interact is not known, but various possibilities exist. These include homophilic and heterophilic interactions between ZP

Table 1. TECTA mutations in DFNA8/12 families

Family	Mutation			Phenotype			Reference
	DNA	protein	domain	onset	progression	frequencies	
Belgian	5459C→T	L1820F	ZP	prelingual	stable	mid	[20]
	5472G→A	G1824D	ZP				
Austrian	5610A→G	Y1870C	ZP	prelingual	stable	mid	[20]
French	4857G→C	C1619S	ZA	pre- or postlingual	progressive	high	[22]
Swedish	3170T→A	C1057S	ZA	postlingual	progressive	high	[27]

ZP = Zona pellucida domain; ZA = zonadhesin-like domain.

domains of α- and β-tectorin as has been suggested for other ZP domain containing proteins, and interactions between the ZA-like domain of α-tectorin and ZP domains of α- and/or β-tectorin, similar to the interaction between sperm and egg.

To date, five different missense mutations have been found in the four DFNA8/12 families (table 1). In the Belgian family, two different mutations were found only 12 basepairs apart. Although it is possible that only one of them is disease causing, it also is possible that both are required to produce disease. In the Belgian and Austrian families, the identified mutations change highly conserved amino acids from the ZP domain, while in the Swedish and French families, cystein residues from the ZA-like domain are substituted. The mutation in the French family changes one of the vicinal cystein residues of the fourth vWf-type D repeat. These highly conserved cystein residues, separated by two amino acids, play an important role as a catalytic site for disulphide-bonded multimer assembly of vWf [26].

It is possible that the TECTA missense mutations reduce the amount of functional protein in the tectorial membrane, thereby interfering with its structure and function. A deficient tectorial membrane might be expected to lead to inefficient transmission of sound to the mechanosensitive stereociliary bundles of the hair cells resulting in hearing loss. However, it is also possible that mutations in α-tectorin have dominant-negative effect by disrupting the interactions between the different tectorin polypeptides thereby precluding normal function of the tectorial membrane. This possibility appears more likely since all known mutations to date are of the missense type.

Because there are phenotypic differences in the DFNA8/12 families, it is tempting to make phenotypic-genotypic correlations. Mutations in the ZP

domain (in the Austrian and Belgian families) appear to lead to prelingual stable hearing loss across all frequencies, while mutations in the ZA-like domain (in the French and Swedish families) result in progressive hearing loss starting in the high frequencies. However, the identification of more mutations in additional families is needed to determine the validity of this putative genotype-phenotype correlation.

Conclusions

To date, the clinically important consequence of gene localization and identification has remained diagnostic. In families where the mutation has been identified, an easy and fast diagnostic test is available by direct mutation detection. However, even in families for which the mutation is not known, if linkage has been proven (i.e. the DFNA2 families), a reliable genetic test can be performed on the basis of linkage analysis using closely linked genetic markers. A predictive test can be performed in children or adolescents, which may be helpful for career planning. Because only a small fraction of the genes for hereditary hearing loss have been identified, the diagnostic possibilities are limited. Expanding diagnostic capabilities and developing new forms of therapy for progressive hearing loss will be dependent on the identification of more genes and the elucidation of their function.

Acknowledgment

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Editors' Note Added in Proof

Two genes have been identified as being involved in DFNA2 since this chapter was written: *GJB3* and *KCNQ4*. Furthermore, the *TECTA* gene involved in DFNA8/12 has been shown also to underlie the recessive deafness DFNB21.

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DFNA3

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In 1994, the autosomal dominant form of sensorineural deafness DFNA3 was mapped to the centromeric region of human chromosome 13, by linkage analysis of a large French affected family. The causative gene, *CX26*, also named *β2* or *GJB2*, encodes the gap junction protein, connexin26. This gene, isolated in 1997, was definitively recognized as underlying DFNA3 in 1998.

Clinical Aspects

The clinical delineation of the DFNA3 form of deafness is based on the observations of the large family (LY1) in which the locus was initially identified [1]. The deafness is nonsyndromic, and appears before 4 years of age. Pure-tone audiometry shows a bilateral and symmetrical sensorineural hearing loss, which affects all frequencies. Air conduction pure-tone average may be moderate to profound. Low-tone thresholds (0.25–0.5 kHz) vary between 15 and 90 dB. The hearing loss is more important in the high frequencies (4–8 kHz); thresholds are over 70 dB and a large proportion of the affected individuals have no hearing at 120 dB. According to the classification of Liu [2], audiometric shapes are either gently or sharply sloping (see fig. 1). The deafness is either slightly or nonprogressive. Click-evoked oto-acoustic emissions are absent in affected ears at the frequencies corresponding to a hearing loss superior to 30 dB. Information on vestibular function and inner ear computerized tomography (CT) scans are not yet available for this form of deafness.

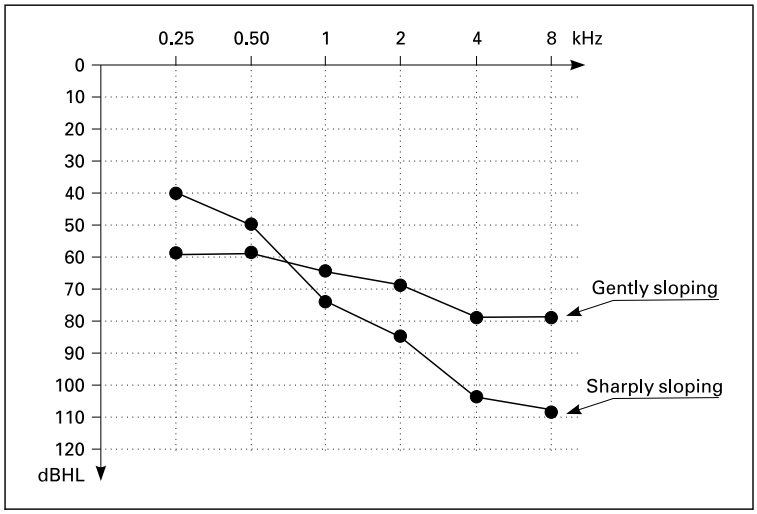

Fig. 1. Example of audiogram shapes in deaf individuals affected by DFNA3.

The DFNA3 Locus

In 1994, Chaïb et al. showed that the dominant form of deafness DFNA3 was linked to the loci D13S175, D13S1316, D13S141 and D13S143 at chromosome band 13q12 [1], i.e. in the same chromosomal interval as that previously defined for the autosomal recessive form of deafness DFNB1 [3]. This was the first example of a co-localization of a dominant and recessive form of nonsyndromic deafness. It indicated that either (i) two tightly linked deafness genes were present in this chromosomal region or (ii) different mutations within the same gene could give rise to both forms. It has been subsequently proven that the latter hypothesis holds true (see below).

The Causative Gene: *CX26* (*GJB2*)

In parallel to the identification of the gene encoding connexin26, *CX26*, as responsible for DFNB1, a mutation, M34T, was detected in a small family affected with an autosomal dominant form of deafness [4]. However, the involvement of *CX26* in the dominant form was rapidly questioned as the single mutation reported, M34T (which converts a methionine to a threonine), was detected in several normal hearing individuals in one family [5]. Thereafter, in two large French families (including LY1) showing a significant linkage to

the DFNA3 locus, all deaf individuals were exclusively shown to carry the same mutation in *CX26*. This mutation consists of a G > C transversion which creates a tryptophan to cysteine substitution at codon 44 (W44C); it was not observed in 340 normal hearing individuals. This finding established that *CX26* underlies the dominant form of deafness DFNA3 [6].

Connexin26 is one of the thirteen connexins known in mammals. These proteins are involved in the formation of intercellular channels, called gap junctions, that allow adjacent cells to share cytoplasmic components having a molecular weight inferior to 1 kD. A complete intercellular channel results from the association of two half channels (connexons), each connexon being an assembly of six connexin subunits. The topology of connexins is highly conserved, consisting of 4 transmembrane domains, linked by one cytoplasmic and two extracellular loops, with cytoplasmic C- and N-termini. Connexin26 is widely found in several organs and by many cell types, among others, hepatocytes, keratinocytes, intestinal, pancreatic acinar, endometrial, and cochlear cells [7, 8]. In the cochlea, connexin26 is detected in most of the supporting cells and fibrocytes of the cochlear duct, but is absent from the sensory hair cells. The connexin26 cellular distribution within the cochlea suggests that it might be involved in the recycling of K^+. The concentration of this ion is particularly high (140 mM) in the endolymph, the liquid which bathes the stereocilia, and it carries the mechanotransduction current through the hair cells.

In the DFNB1 form of deafness, most of the *CX26* mutations, including the most frequent mutation, 30delG or 35delG, are expected to lead to a protein truncated at its very beginning [9–11]. According to the absence of hearing loss in patients heterozygous for a null mutation in *CX26* (DFNB1 carriers), the W44C mutation present in DFNA3 affected patients is expected to have a dominant negative effect. Connexin26 comprises 226 amino acids. The tryptophan at position 44 is located in the first extracellular loop. This residue is likely to play a crucial role since it is present in 12 out of the 13 mammalian connexins characterized so far, and the last harbors at this position a tyrosine, i.e. an amino acid residue with some similar biochemical characteristics [12]. In addition, the two extracellular loops exhibit a strikingly conserved pattern of three cysteines. These cysteines have been shown to be involved in interloop disulfide bonds, and their requirement for normal protein activity has been demonstrated, by site-directed mutagenesis [13] (for a review, see [14]). Accordingly, the presence of an additional cysteine in the mutated connexin26 is likely to interfere with the formation of normal disulfide bonds. The W44C mutation is expected to have a dominant negative effect due to the incorporation of the mutated connexins into intercellular channels, which would disrupt channel activity.

Coming back to the M34T mutation, two types of data argue for its pathogenicity. When expressed in *Xenopus* oocytes, this mutated form does not sustain electrical coupling between two adjacent cells and coexpression of M34T mutant with wild-type *CX26* result in marked inhibition of intercellular coupling [15]. Moreover, in families affected with the X-linked form of Charcot-Marie-Tooth disease, two different substitutions have been found at the same amino acid position (M34V and M34W) in the causative gene, *CX32*, which encodes connexin32 [16, 17]. In contrast, several genetic data argue against the pathogenicity of M34T. The M34T mutation has been reported in two small families affected with recessive deafness [18]; in one family, the mutation did not segregate with the deafness. Moreover, normal hearing in heterozygous carriers of M34T has been repeatedly observed [5, 9, 18] and estimated to occur in up to 3% of the normal hearing population in the USA. If this mutation is responsible for deafness even in the recessive state, a very large proportion of DFNB1 families should be heterozygote composite or homozygous for this mutation. However, a possible effect of this mutation on hearing function will be definitively excluded when enough normal hearing and deaf individuals will have been examined for the presence of this mutation. Today, DFNA3 seems to be a rare form of autosomal dominant deafness: the search for mutations in the causative gene, *CX26*, in 30 unrelated subjects affected by an autosomal dominant deafness failed to detect a mutation in this gene [6].

Two other *CX26* mutations have been reported as associated with autosomal dominant syndromic forms of deafness. One is a missense mutation, R75W, present in an Egyptian family also affected by palmoplantar keratoderma [19]. However, the effect of this mutation remains uncertain, as the mutation has been found in a nonaffected control individual. More recently, a D66H *CX26* missense mutation was described in 3 families affected by Vohwinkel syndrome, characterized by deafness associated with diffuse mutilating keratoderma [20]. In these families, the phenotype clearly cosegregated with the D66H mutation. This mutation is also likely to have a dominant negative effect. Other connexins, namely connexin31 [21], connexin30 and connexin43 [22] are coexpressed in the cochlear cells, but failed to compensate for the lack of connexin26. Connexin31 has also been shown to underlie autosomal dominant deafness. This suggests that each connexin might have a distinct role in this sensory organ.

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Editors' Note Added in Proof

Since this chapter was written, a further gene, *GJB6*, at the DFNA3 locus has been found to be involved in deafness.

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Characterization of Autosomal Dominant Non-Syndromic Hearing Loss Loci: DFNA 4, 6, 10 and 13

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Non-syndromic hearing loss (NSHL) is the most common neurological disorder in the elderly [1]. Both environmental and genetic factors are known to contribute to hearing loss. Although epidemiological data have not yet established the relative prevalence of simple Mendelian inheritance as a cause of age-related hearing loss, many families have been described with postlingual progressive autosomal dominant NSHL (ADNSHL). Classical linkage analysis with these families has shown that ADNSHL is a markedly heterogeneous disease. To date, fifteen different loci have been identified, and in five cases, the responsible genes have been cloned (table 1) [2–20].

The first ADNSHL locus was identified by Leon et al. in 1992 [2]. Since that time, detailed genetic maps and advances in molecular analysis have heralded the localization of 14 additional loci each prefixed by DFNA (DFN = deafness; A = dominant) and numbered sequentially in order of date of discovery. Five of the responsible genes also have been cloned (DFNA1/*HDIA1*, DFNA3/*GJB2*, DFNA8 & 12/*TECTA*, DFNA11/*MYO7A*, and DFNA15/*POU4F3*) [3, 6, 12, 16, 20, 71]. In this report, we describe the DFNA4, 6, 10 and 13 loci, each of which is characterized by postlingual progressive hearing loss.

Table 1. Autosomal dominant non-syndromic hearing loss (ADNSHL) loci characterization

Locus	Location	Gene	Frequency	Onset	Type	Reference
DFNA1	5q31	*HDIA1*	low	postlingual	progressive	[2, 3]
DFNA2	1p32	–	high	postlingual	progressive	[4]
DFNA3	13q12	*GJB2*	high	prelingual	stable	[5, 6]
DFNA4	19q13	–	mid/all	postlingual	progressive	[7]
DFNA5	7p15	–	high	postlingual	progressive	[8]
DFNA6	4p16.3	–	low	postlingual	progressive	[9]
DFNA7	1q21-q23	–	high	postlingual	progressive	[10]
DFNA8	11q	*TECTA*	mid/all	prelingual	stable	[11, 12]
DFNA9	14q12-q13	–	high	postlingual	progressive	[13]
DFNA10	6q22-q23	–	mid/all	postlingual	progressive	[14]
DFNA11	11q12.3-q21	*MYO7A*	mid/all	postlingual	progressive	[15, 16]
DFNA12	11q22-q24	*TECTA*	mid/all	prelingual	stable	[12, 17]
DFNA13	6p21.3	–	mid/all	postlingual	progressive	[18]
DFNA14	4p16	–	low/mid	postlingual	progressive	[19]
DFNA15	5q31	*POU4F3*	mid/all	postlingual	progressive	[20]

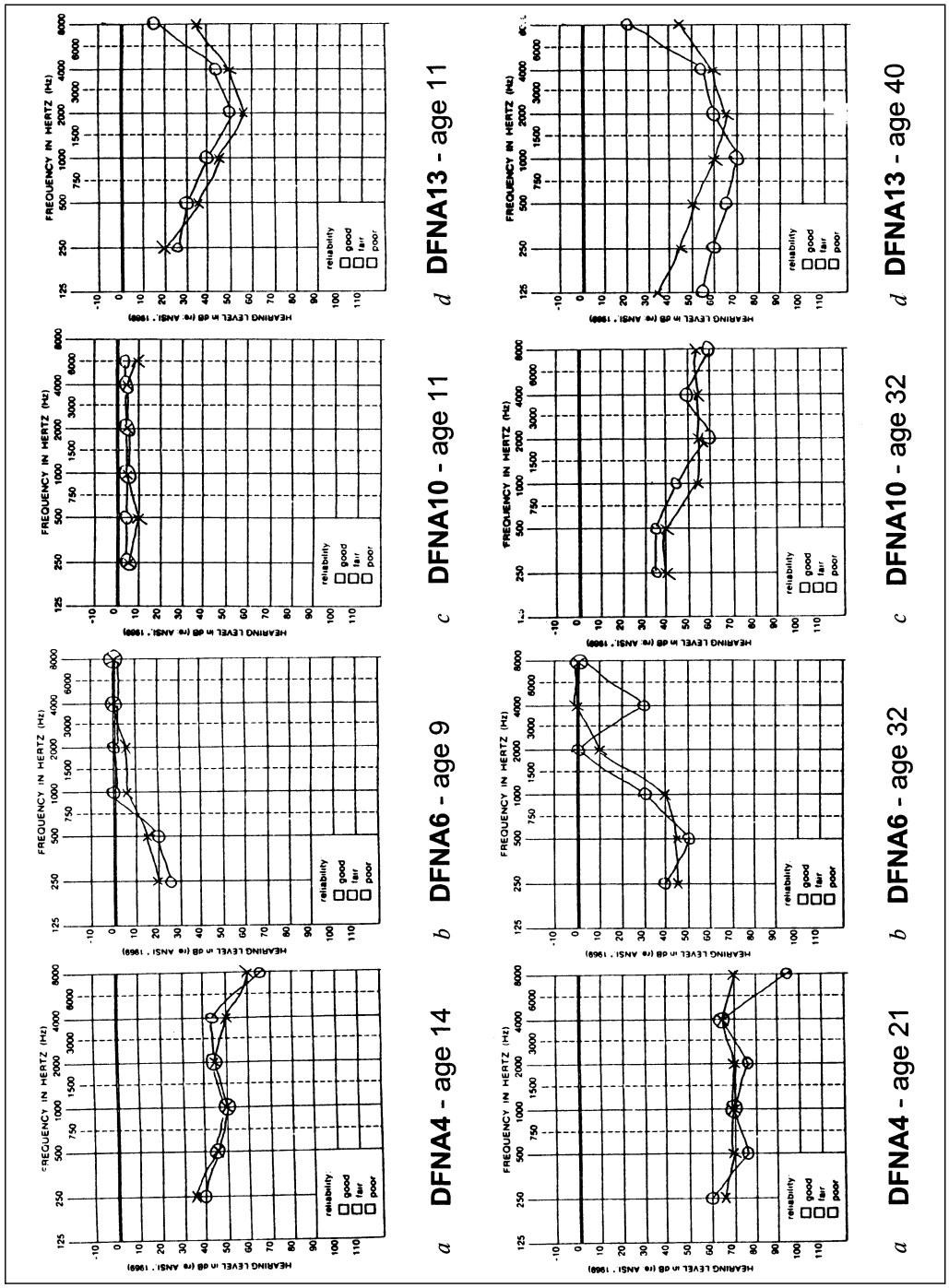
DFNA4

Family Data

A large multigenerational family from the midwestern United States with ADNSHL was ascertained through the University of Iowa Department of Otolaryngology – Head and Neck Surgery [7]. Affected persons have a fluctuating, though inexorably progressive, sensorineural hearing loss (SNHL) that begins in the second decade and leads to severe-to-profound impairment by the age of 40 (fig. 1).

Linkage Analysis

Exclusion of known deafness loci was initially performed by two-point linkage analysis using MLINK (version 5.10) of the LINKAGE software package [21]. Regions of high GC content were then selected to search for areas of exclusion. Using polymorphic markers for the ApoC2 locus on chromosome 19q13, a two-point lod score of 4.2 was obtained. Additional polymorphic markers were analyzed in the region, resulting in a maximum lod score of 4.22 using reported intermarker sex-averaged distances and analyzing the disease locus against three polymorphic loci (four-point analysis with no more than 9 alleles total for the polymorphic loci) [22, 23]. No recombination events were observed in the D19S425-to-ApoC2 interval. An additional family, also from the midwestern United States but unrelated to the original family, has since been identified that maps to the same genomic region.


a DFNA4 - age 14
b DFNA6 - age 9
c DFNA10 - age 11
d DFNA13 - age 11
a DFNA4 - age 21
b DFNA6 - age 32
c DFNA10 - age 32
d DFNA13 - age 40
FREQUENCY IN HERTZ (Hz)
HEARING LEVEL in dB (re ANSI, 1969)
reliability
good
fair
poor

1

Discussion

The interval which defined DFNA4 is relatively large and contains several genes on the long arm of chromosome 19 (19q13), including the apolipoprotein E/CI/CII gene cluster [24], an amyloid precursor protein [25], several zinc finger proteins [26] and the myotonic dystrophy kinase (DM kinase) gene [27]. The reported association between myotonic dystrophy (DM) and auditory dysfunction makes DM a strong candidate gene for DFNA4 [28, 29]. DM is the most prevalent inherited neuromuscular disorder in adults [28]. Its phenotype is highly variable, and although the classic presentation includes progressive muscular degeneration and frequent involvement of the ocular, cardiovascular and central nervous systems, audiometric studies also have demonstrated a high-frequency SNHL in excess of that expected for age ('precocious presbycusis') [28, 29].

The DM kinase gene encodes a serine-threonine protein kinase that contains 15 exons within a 14-kb genomic region [27]. It likely plays a role in a wide range of biochemical and cellular pathways, including regulation of excitation-contraction coupling and maintenance of cellular physiology through protein-protein interactions in ion channels [30, 31]. The disease-causing mutation is an amplified trinucleotide CTG repeat in the 3′-untranslated region [32]. Although the significance of this type of mutation on auditory function is unknown, animal studies using homozygous DM-kinase knockout mice have demonstrated a higher resting intracellular calcium concentration in muscle cells, resulting in slower and smaller responses than seen in the wild type [33]. Because higher intracellular calcium levels have been shown to lead to cell death of spiral ganglion neurons in vitro [34], one can speculate that this process may effect hair cell responsiveness and lead to a 'precocious presbycusis' phenotype.

DFNA6

Family Data

The large multigenerational family from the southeastern United States first described in 1968 by the Vanderbilt University Hereditary Deafness Study Group [35] was used to identify the sixth ADNSHL locus, DFNA6, in 1995

Fig. 1. Representative audiograms from ADNSHL loci DFNA 4, 6, 10 and 13 demonstrating progressive sensorineural hearing loss: (*a*) DFNA4 – same individual at age 14 and age 21; (*b*) DFNA6 – same individual at age 9 and age 32; (*c*) DFNA10 – same individual at age 11 and age 32; (*d*) DFNA13 – father (below) and son (above) at ages 40 and 11, respectively.

[9]. Hearing loss measured by audiograms over a 25-year period demonstrates a progressive, symmetric deficit which initially affects the lower frequencies (250, 500, 1,000 Hz) (fig. 1). This pattern is relatively uncommon among the ADNSHL loci (DFNA1, 6 and 14; table 1).

Most DFNA6 family members do not require hearing aids and none has required cochlear implantation. Mean age of onset is in the second decade, with variable progression thereafter, generally following one of three patterns: (i) confined to the low frequencies, (ii) involving all frequencies, producing a flat audiogram, and (iii) involving low and high frequencies with sparing of the middle frequencies (2,000 Hz) [9]. The severity of hearing impairment varies from mild to severe, related to age.

Linkage Analysis

After exclusion of known loci for ADNSHL, a genome-wide screen was performed with over 300 polymorphic microsatellite markers dispersed over the entire genome [36–38]. Initial data analysis using LINKAGE software version 5.01 [21] confirmed linkage to D4S432 on 4p16.3. Additional markers linked to D4S432 were used to reconstruct haplotypes. Based on established marker order, D4S43-D4S127-D4S412-D4S126-D4S432, a multipoint map was produced, yielding a minimum lod score of 6.5 in the interval between D4S126 and D4S432. Four recombination events were identified during haplotype analysis. A novel locus (DFNA14) for ADNSHL with a similar phenotype to DFNA6 has been recently reported by Van Camp et al. [19]. This locus maps in close proximity to DFNA6 but the two regions do not overlap.

Discussion

The DFNA6 interval is located on chromosome 4p16.3 and spans a genetic distance of approximately 5 cM in the relatively well-studied region just centromeric to the Huntington gene, *IT15* (important transcript 15) or *huntingtin* [39]. The number of recombination events observed in this family is not unusual. The 4p telomere is a member of isochore family H3, a group of chromosomal regions known to have a high gene concentration and an increased rate of recombination [40]. These recombinations facilitate fine mapping, and using the latest physical maps of the area, the candidate interval approximates 2 Mb.

DFNA6 is fully linked to D4S126, which is located roughly 500 kb from the 3′ end of *huntingtin* [9]. The possibility of an allele of *huntingtin* causing hearing impairment seems unlikely, however, as hearing loss has never been recognized as a clinical feature of Huntington disease. Genes in the DFNA6 interval known to be associated with hearing loss include alpha-L-iduronidase (*IDUA*) [41] and fibroblast growth factor-3 (*FGFR3*) [42]. Mutations in the former are seen in Hurler and Scheie syndrome [41], where the accumulation of

glycosaminoglycans in the cochlea and infiltration of the cochlear nerve result in chronic serous otitis media and mixed hearing loss [43, 44]. Mutations in *FGFR3* lead to achondroplasia [51]. Achondroplasia is the most frequent form of short-limb dwarfism and can be associated with sensorineural hearing loss and chronic otitis [42, 45, 46]. Mice homozygous for a targeted disruption of *FGFR3* demonstrate inner ear defects that result in profound deafness and abnormal endochondral ossification causing skeletal overgrowth [47]. *FGFR3–/–* mice exhibit failure of pillar cell differentiation in the developing organ of Corti and reduced innervation of outer hair cells [47]. A recent study by Hollway et al. [48] found a specific *FGFR3* mutation (C749G, Pro250Arg) in a family with craniosynostosis and moderate sensorineural hearing loss. The low penetrance of symptomatic craniosynostosis in affected individuals suggests that some families with the Pro250Arg mutation could present with isolated hearing loss [48].

Interesting also because of an association with hearing loss is Wolf-Hirschhorn syndrome, a contiguous gene deletion syndrome of 4p classically characterized by growth retardation, mental retardation, microcephaly, dysmorphic facies and cleft lip-palate deformity [49, 50]. Although the Wolf-Hirschhorn critical region is $\sim$2 Mb, a larger deletion of $\sim$8 Mb from the p-telomere to D4S62 has been described in a patient with an additional phenotype that included hearing loss and an external ear deformity [51]. A second patient with Wolf-Hirschhorn syndrome and sensorineural hearing loss without external ear deformity also has been described [52]. The DFNA6 interval falls within this ~8 Mb phenotypic map, and suggests that the DFNA6 phenotype may be due to a gene dosage effect.

DFNA10

Family Data

The family in this study was ascertained through the University of Iowa Department of Otolaryngology – Head and Neck Surgery and lives in the midwestern United States [14]. Affected persons exhibit progressive SNHL beginning in the second to fifth decades and leading to severe to profound hearing impairment that necessitates amplification (fig. 1). Due to the variable age of onset of hearing loss in affected individuals, in the initial linkage analysis family members under the age of 50 years were excluded. Since the original study, however, 16 additional affected individuals have been ascertained.

Linkage Analysis

After excluding known loci for ADNSHL, a genome-wide screen was completed and areas of exclusion were calculated by two-point linkage analysis

using MLINK (version 5.0) of the LINKAGE software package [21]. Maximum lod scores of 4.44 and 4.00 were obtained with markers D6S472 and D6S407, respectively, and multipoint mapping generated a lod score of 5.27 with D6S407.

Discussion

Fine mapping with the expanded pedigree has narrowed the critical region to less than 1 cM on chromosome 6q22-q23 between D6S472 and D6S975. Thirteen expressed sequence tags (ESTs) have been mapped to this interval (The Human Gene Map at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/msrch2)) and seven have been shown to be expressed in the cochlea. However, direct sequencing of affected and non-affected individuals has failed to demonstrate disease-causing mutations in 5 of the 7 EST-derived genes. The two remaining ESTs are currently being evaluated and encode a human connective tissue growth factor and a novel gene, which has homology to a glucose transporter.

Evaluation of the NCBI Human/Mouse Homology Relationships (www.ncbi.nlm.nih.gov/Homology) for the DFNA10 region revealed two additional candidate genes, connexin 43 and phosphodiesterase I/nucleotide pyrophosphatase I. Connexin 43 is a ubiquitously expressed gap junction protein similar in structure to other connexin family proteins [53]. Although mutations in another connexin gene, connexin 26, result in DFNB1 [54] and DFNA3 [6], no disease-causing mutations have been found in affected persons from two different DFNA10 families. Phosphodiesterase I/nucleotide pyrophosphatase I is a bone/cartilage enzyme which modulates physiologic mineralization and pathologic chondrocalcinosis by generating inorganic pyro- phosphate [55, 56]. Current work to identify the DFNA10-causing gene involves completion of a physical map over the critical region and mutation screening of the remaining candidate genes. If necessary, additional coding sequence will be identified by exon trapping, cDNA selection or direct sequencing.

DFNA13

Family Data

DFNA13 was localized using multigenerational American kindred of northern European descent [18]. Hearing impairment in affected individuals begins in the second to fourth decades, involves all frequencies, and ultimately leads to a moderate to severe deficit that requires amplification for effective communication (fig. 1). Additional family members have been ascertained to permit pedigree expansion over five generations.

Linkage Analysis

After excluding known loci by two-point analysis using markers from the Hereditary Hearing Loss Homepage (http://dnalab-www.uia.ac.be/dnalab/hhh/index.html), a genome-wide screen of approximately 180 evenly dispersed polymorphic markers (Research Genetics) was performed [57]. Lod scores were calculated with the FASTLINK software program [58], with evidence for linkage to D6S422 ($Z_{max} > 3.0$). Additional polymorphic markers in this area generated a maximum two-point lod score of 6.409 with D6S299. Expansion of the pedigree and fine mapping of the DFNA13 interval has narrowed the critical region to < 1 cM bounded by D6S1285 and D6S1666.

Discussion

The DFNA13 critical region is located on chromosome 6p21.3, tightly linked to the MHC class II region. Fine mapping and physical mapping with development of polymorphic markers from P1-derived artificial chromosome (PAC) clones has narrowed the location of the gene to an ~ 600 kb area that overlaps and extends centromeric to the end of the MHC class II region. This area contains at least one gene every 30 kb and has been intensely studied [59]. Interestingly, an association between hearing impairment and certain HLA class II alleles has been suggested for several types of auditory dysfunction, including otosclerosis, Meniere's disease, sudden sensorineural hearing loss, and progressive sensorineural hearing loss [60].

Although several genes have been identified within the DFNA13 region that may have a role in auditory function, three are especially interesting. The first is collagen type XI (*COL11A2*), mutations in which are associated with a unique Stickler syndrome phenotype [61]. Classically, the Stickler phenotype is characterized by arthropathy, ocular signs, cleft palate, micrognathia, flat facies and sensorineural hearing impairment, and is caused by mutations in the collagen type II (*COL2A1*) gene [62]. Mutations in *COL11A2* lead to a variant Stickler syndrome in which ophthalmologic findings are absent [61]. This phenotypic variability suggests that an allelic mutation in the *COL11A2* gene could cause ADNSHL.

The second gene of interest, *HSET*, is a kinesin family protein that functions as a microtubule-based motor necessary for mitotic spindle activity and possibly vesicle transport [63]. Kinesins form a heterotetramer of two heavy and two light chains with a conserved motor domain that possesses ATPase activity [64]. Ultrastructural evaluation of the kinesin motor domain reveals striking similarity to the myosin motor domain, suggesting an evolutionary relationship [64]. Mutations in myosin genes have been implicated in both syndromic [65] and non-syndromic [16, 66] forms of sensorineural hearing

loss, and screening of a fetal cochlea cDNA library with EST from *HSET* has confirmed that this gene is expressed within the cochlea.

The third gene of interest is *RING1*. Physical mapping of the centromeric end of the class II MHC region with a 220 kb yeast artificial chromosome (YAC) allowed construction of a single cosmid contig which produced 42 kb of genomic sequence [59]. Contained within this sequence is *RING1*, a transcription repressor [67] that is expressed in the cochlea. The association between transcription factors and hearing loss has been demonstrated in ADNSHL (DFNA15) [20] and X-linked NSHL (DFN3) [68], both of which are caused by mutations in *POU* genes. Generation of additional polymorphic markers to further narrow the interval and mutation screening of candidate genes are ongoing.

Conclusion

The presentation of ADNSHL is typically postlingual in onset, although with some DFNA loci, prelingual hearing loss can occur (DFNA3, 8/12) [5, 11, 17]. Studies to determine the frequency and mode of inheritance in postlingual hearing loss are not available, and environmental factors have a significant impact on hearing loss as we age. Mitochondrial mutations that lead to postlingual NSHL also have been described [69, 70]. The identification of four ADNSHL loci (DFNA4, 6, 10 and 13) that share the phenotype of postlingual progressive hearing loss is the prologue to cloning these genes and providing new insights into the mechanisms responsible for normal auditory function and the aberrations which cause hereditary hearing impairment.

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Editors' Note Added in Proof

Since this chapter was written, the authors have confirmed the involvement of the *COL11A2* gene in DFNA13.

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DFNA7

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We report the audiological and genetic investigation of one large Norwegian family with autosomal dominant progressive sensorineural hearing loss (DFNA7). Thorough long-term follow up demonstrated considerable audiological variation in the progression of the hearing impairment. The disease gene has been regionally mapped to chromosome 1(q21-q23) [1]. No further DFNA7 families have been reported. Exclusion of linkage to the DFNA7 locus in two other Norwegian deafness families indicates that this might be of low prevalence in causing deafness. The genetic interval is about 22 cM which is too large to initiate positional cloning. Future studies will concentrate on refining the genetic region and testing of the potential candidate genes of the region. Linkage and preliminary mutation studies seem to exclude two candidate genes, P_0 and connexin 50.

Materials and Methods

Hearing impaired individuals in a large Norwegian family were followed for maximum 34 years with audiological examinations. Forty-two family members of whom 22 had hearing impairment were included in our study. Selected individuals have been examined more extensively with tympanometry, auditory brainstem response (ABR) and bithermal caloric tests [2].

For the genetic linkage studies, 42 family members participated. Extracted DNA from peripheral leukocytes was prepared and standard methods were applied as described [1]. In the linkage analysis only individuals above the age of ten were included, and this classification was based on the natural history of hearing impairment in this family. Since the report by Fagerheim et al. in 1996 [1], additional family members have been classified as hearing impaired and additional affected and unaffected individuals have been included in studies pending.

Results and Discussion

The hearing loss was in all cases postlingual. Transmission of the impaired hearing was clearly autosomal dominant with complete penetrance (MIM 601412, fig. 1a, and fig. 1b) and with male-to-male transmission, strongly pointing against mitochondrial or X-linked recessive inheritance to be considered. In the linkage analysis we applied age-dependent liability classes in concordance with the observed age of onset in the family.

One big branch of the family remained living within the same region for over 30 years and could be followed with audiological studies, which provided a solid basis for the natural history of the hearing loss. Often, the audiometric data regarding the large number of deafness genes have been sparse which compromises any audiological comparison between genetically distinct types of hearing loss. Those patients followed from infancy showed normal audiograms for the first 4–5 years of life. One affected male only had minor hearing problem at the age of 20. At the age of 22, he experienced subjective hearing trouble and at age 26 an audiogram showed a 60 dB pure tone average hearing loss.

The hearing loss was in some instances asymmetrical in periods of rapid progression. Maximum difference was 35 dB HL at the same frequency, but in the majority of cases the difference in hearing between the two ears was less than 10 dB. Progression of hearing loss measured as change in pure tone average (PTA) at 0.5, 1, 2 and 4 kHz, both ears together, was highly variable between individuals and through different periods of life in the same person. The maximum speed of progression of hearing impairment was 35 dB HL, PTA observed during one year from age 5 to 6 in a boy. After age 20 the calculated progression of hearing loss approached 1 dB HL, PTA, per year.

In general, high-freqency hearing was first affected with preservation of useful hearing in the low and middle frequencies until old age. Hearing loss at 1 kHz was delayed 5 to 10 years compared with 2 and 4 kHz, and never reached the same degree, while 0.5 kHz was even less affected. All individuals retained acceptable social hearing and good spoken language.

There was no evidence for tinnitus, delayed motor skills, neurological symptoms or dysmorphic features in any hearing-impaired person. Caloric responses were tested and found normal in five affected members.

Linkage was first excluded for the loci DFNA1–DFNA6 and DFNA8 as well as DFNB1–DFNB8. A genome search using a set of polymorphic microsatellite markers with 20 cM resolution was then started, starting with chromosome 1. A total of 13 markers were analyzed before linkage was obtained. We identified thereby [1] a locus for hearing impairment of non-syndromal autosomal type. The candidate region is approximately 22 cM

large, and with the centromeric border being defined by D1S104 and the telomeric border being at D1S466. No further narrowing of the region has been obtained since our report [1]. To the best of our knowledge no other reports of further families with linkage to that region of chromosome 1 has since been published (Hereditary Hearing Loss Home page World Wide Web URL: http://dnalab-www.uia.ac.be/dnalab/hhh/). Our studies of two additional large Norwegian families with autosomal dominant deafness have excluded that region in both (Fagerheim and Tranebjærg, unpublished data), indicating that the DFNA7 locus is not frequently involved in impaired hearing in Norway.

Several genes are located in 1(q21-q23) including a transcription factor gene, called *OCT1* which is expressed in cochlea [3, 4]. Also the P_0 (myelin protein zero gene) mutated in Charcot-MarieTooth 1B is located within that region, and must be considered as a candidate gene because of its location and the fact that hearing loss is sometimes associated with the sensorimotor neuropathy in that disease [5, 6]. A mutation search in P_0 by means of SSCP analysis was negative, but we cannot entirely exclude this gene (Eva Nelis). *OCT1* gene has not been studied sufficiently to be excluded. Future strategy will first attempt to narrow the genetic region before reconsidering which candidate genes should be examined in molecular details. After the disclosure of gap junction genes – Connexins (Cx) – being frequently mutated in deafness it is of great potential interest that Cx 50 is located to 1q21 [7]. From our present genetic information about the relative location of the microsatellite markers it seems that Cx 50 is centromeric to one of the most proximal markers we used, D1S426, which probably means it is not a candidate gene but final conclusions can only be drawn after further refinement of the genetic region encompassing the deafness gene in this family (http://linkage.rockefeller.edu/chr1 and http://gdbwww.gdb.org).

Genetic Counselling

At risk individuals from this family can be counselled after establishment of a haplotype with the closest flanking microsatellite markers. Because of the large genetic region involved there is considerable uncertainty in such a prediction. In the absence of an identified responsible gene, it is impossible to identify sporadic cases of hearing impairment caused by the DFNA7 gene.

Even if the audiograms from different members in this family show considerable similarities and the affection primarily is of high frequencies, it is not possible from the audiograms in unrelated sporadic or familial individuals with dominant hearing impairment to define if the DFNA7 gene is mutated.

The present family contributes by means of long-term audiometric data and genetic linkage studies to the major undertaking of better understanding of the biology underlying development of hearing loss.

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Mutations in *COCH* (formerly Coch5b2) Cause DFNA9

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DFNA9 presents as a non-syndromic, dominant, late-onset, progressive sensorineural hearing loss; more than one family member might have received a cochlear implant. Histologic examination of cochlea from affected individuals demonstrates a unique pathology. The gene encoding DFNA9 maps to chromosome 14 and has been identified as *COCH*. *COCH* is expressed in the cochlea and has limited homology to the limulus factor B gene.

Family studies and histologic analyses of individuals with DFNA9 were initially carried out at the Massachusetts Eye and Ear Infirmary. Dr. Saumil Merchant noted an unusual histologic deposit of eosinophilic ground substance in cochlear and vestibular channels of an affected individual [this volume, p. 200]. Because DFNA9 patients develop late-onset deafness which can be treated by cochlear implantation, Dr. Joseph Nadol managed several members of the same family with this condition. Subsequent genetic analyses of DFNA9 was based on the collection of blood samples and clinical evaluation of this large family by Dr. Michael McKenna.

Individuals begin to lose hearing between 16 and 28 years (mean 21 years) [2]. Hearing loss is slowly progressive, about 3 dB/year. Initially, the hearing loss is more pronounced in the high frequencies; the loss eventually progresses to anacussis in mid-life. In the third decade, word recognition also drops sharply causing reduced benefit from amplification and impaired communication. Four affected family members had undergone cochlear implantation by the fifth decade with successful results. Although systematic laboratory

vestibular testing has not been performed, several affected individuals had clinical signs of vestibular defects.

The gene responsible for this clinically non-syndromic, postlingual, progressive sensorineural hearing loss was mapped to chromosome 14q12-13 by a random genome-wide search using polymorphic short tandem repeat loci. A maximum lod score of 6.19 was achieved at locus D14S121 ($\theta = 0$) indicating that the odds of linkage are greater than 1:1,000,000. Haplotype analysis demonstrated a 9 cM interval between loci D14S252 and D14S49 that must contain the disease gene [3].

Recently, we demonstrated that mutations in *COCH* cause DFNA9 in affected members of three unrelated families [4]. These families were identified because a family member's temporal bone was stored in a temporal bone library. All three mutations are missense mutations in a cysteine-rich portion of the protein. How the missense containing protein leads to the death of cochlear and vestibular cells remains uncertain. The mechanism by which these mutations cause DFNA9 and the role of *COCH* in the hearing process should be elucidated in future studies.

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Sensorineural Hearing Impairment, Non-Syndromic, Dominant DFNA11

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Recent advances in molecular analysis for hereditary hearing impairment have shed some light on the molecular bases of phenotypic similarities and differences. One of the most intriguing findings is that distinct clinical forms of hereditary hearing impairment can be caused by different mutations in the same gene. The gene at the eleventh locus for a dominant form of non-syndromic sensorineural hearing impairment, DFNA11 (entry number of Online Mendelian Inheritance in Man: 601317) [1], has been identified as the same gene responsible for both syndromic and non-syndromic forms of recessive hearing impairment. This review focuses on the molecular basis of clinical features caused by a mutation identified in the gene at the DFNA11 locus.

Clinical Features of the DFNA11 Family

Figure 1 shows the pedigree of a Japanese family with a dominant form of non-syndromic sensorineural hearing impairment. Most affected individuals noticed hearing impairment in their teens after complete speech acquisition, with subsequent gradual progression. All affected individuals suffer from bilateral sensorineural hearing impairment without any other associated symptoms. They had symmetric, gently sloping or flat audiograms with hearing loss at

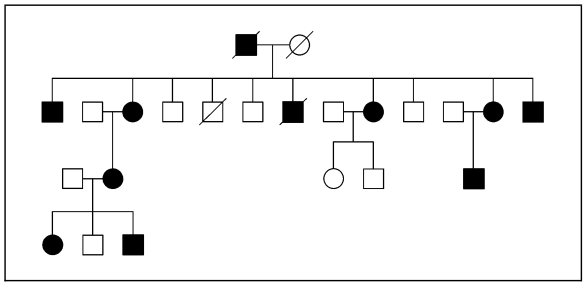

Fig. 1. Pedigree of the Japanese family with non-syndromic sensorineural hearing impairment. Filled symbols indicate affected individuals. (Modified from Tamagawa et al. [1].)

all frequencies. Most affected individuals had moderate hearing loss. Acoustic reflex thresholds and short increment sensitivity index tests on selected patients demonstrated the presence of recruitment phenomena, indicating cochlear involvement. They had no detectable otoacoustic emissions, known as signs suggestive of dysfunction in the outer hair cells. Although no family members presented with vertigo, three of five affected members who underwent caloric testing revealed vestibular dysfunction. Two affected members, ages 29 and 76 years, underwent electroretinograms and showed normal results.

Mapping of the DFNA11 Locus

Linkage and haplotype analysis on the Japanese family mapped the DFNA11 locus to the region of chromosome 11 (11q12.3-q21) [1]. This region contained the other two loci for hereditary hearing impairment; the second locus for a recessive form of non-syndromic sensorineural hearing impairment (DFNB2) [2], and a locus for Usher syndrome type Ib (USH1B) [3]. These three forms of hearing impairment show vestibular dysfunction in varying degrees. In 1995, unconventional myosin VIIA gene (*MYO7A*) was identified as the gene responsible for Usher syndrome type Ib [4]. Overlapping chromosomal regions and phenotypic similarities suggested that the *MYO7A* gene was also a candidate for the gene at the DFNB2 and DFNA11 loci. Sequence analysis in two families from China and a large DFNB2 family from Tunisia revealed that mutations in the *MYO7A* gene indeed result in non-syndromic recessive hearing impairment [5, 6].

A Mutation in the *MYO7A* Gene in the DFNA11 Family

Mutation analysis of the *MYO7A* gene in genomic DNA from affected members of the DFNA11 family revealed an inframe nine-base-pair deletion in exon 22 resulting in loss of three-amino-acid residues (Ala886-Lys887-Lys888). All affected members were heterozygous for the mutation, while unaffected members were homozygous wild type. The mutation was not detected in 60 other control DNA samples [7].

A Dominant-Negative Effect in the DFNA11 Family

The deletion mutation in the DFNA11 family occurs in the coiled-coil domain of myosin VIIA, affecting the fourth and fifth heptad repeats. This was the first mutation identified in the coiled-coil domain of myosin VIIA. There is some evidence from yeast 2-hybrid studies that myosin VIIA forms homodimers through the coiled-coil domain [5]. Assuming that the mutated myosin VIIA is stable, the mutants might dimerize with the wild-type molecules, resulting in heterodimers without function. These heterodimers could have a dominant-negative effect, thus leading to a dominant form of hearing impairment in this family.

The amount of myosin VIIA with normal function might explain phenotypic differences in the severity of hearing impairment. Affected members of the DFNA11 family, in which the amount of presumptive homodimers of normal myosin VIIA is estimated to be less than 50% of the normal value, have postlingual moderate hearing impairment with gradual progression. In contrast, USH1B patients and affected members of the Chinese families carrying mutations in the *MYO7A* gene have congenital profound hearing impairment, with the absence of normal myosin VIIA. The phenotype of the DFNA11 family may suggest that the residual amount of normal myosin VIIA homodimers may be sufficient for the development and primary function in the inner ear, but not enough to maintain its function for a long period of life.

The identification of the mutation in the DFNA11 family revealed that the *MYO7A* gene underlies a wide clinical spectrum of hereditary hearing impairment, including both dominant and recessive forms of non-syndromic hearing impairment as well as syndromic hearing impairment of Usher syndrome type 1b. So far, this family is the only one carrying a dominant-negative mutation in the *MYO7A* gene. We suggest that the other families with a dominant form of non-syndromic sensorineural hearing impairment should be screened for mutations in the coiled-coil domain of myosin VIIA.

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DFNA15

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Progressive hearing loss, resulting from both genetic and environmental insults, affects a significant proportion of the population [1]. Despite its prevalence, very little can be done to alleviate hearing loss today, and so defining the molecular events leading to deafness may have dramatic consequences in the future for the hearing-impaired population. The genetic basis of progressive hearing loss in an Israeli Jewish family, named Family H, was studied with the intention of mapping and eventually identifying the mutated gene associated with hearing loss. This was a project that could potentially take years, but due to advantages offered by the mouse as a research tool and technical advances in human genomics, the *DFNA15* gene was cloned in just one year [2].

The Israeli Family H

The first individual reported to lose his hearing prematurely came from a Jewish family with roots in Italy, although by the time he was born in 1843, his family had moved to Libya (fig. 1). Other family members migrated through Tunisia and Egypt, eventually settling in Israel, Belgium and the United States. The founder of the family had four children, one of whom inherited progressive hearing loss. Of his seven children, four developed progressive hearing loss. In subsequent generations, 14 out of 30 children with one deaf parent exhibited hearing loss prematurely, a pattern consistent with dominant inheritance. The linkage and subsequent cloning of the Family H gene was done on 12 affected and 11 unaffected individuals from this generation [2].

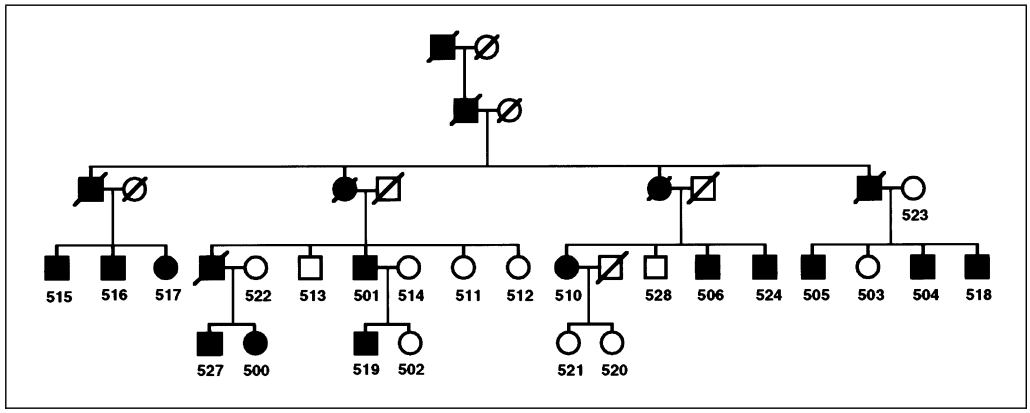

Fig. 1. Pedigree of Israeli Family H. Individuals with hearing loss are indicated by solid symbols, unaffected by open symbols. Individuals younger than 40 were omitted from the pedigree. All hearing-impaired individuals except for 505 harbor the *POU4F3* mutation.

Identification of the *DFNA15* Locus

A positional cloning approach, which involves the isolation of a gene based on its map location in the genome, was used to identify the gene responsible for deafness in Family H. This approach requires no functional information. More than 50 loci for non-syndromic hearing loss (NSHL; deafness with or without vestibular impairment, but no additional symptoms) have been mapped so far. These loci were identified in families from all over the world with dominant, recessive, X-linked or mitochondrial non-syndromic hearing loss. Since mutations in the same genes may be associated with deafness in several families from different origins, deafness loci already identified may be candidates for the family currently being studied. Therefore, the first step in mapping *DFNA15* was to determine if deafness in Family H mapped to a chromosomal region known to harbor a gene for hearing loss. Members of Family H were genotyped for markers near thirteen (at that time) known dominant deafness loci. (Markers near each known locus were straightforward to identify, because this information is updated regularly on the website [3].) Genotypes of markers on chromosome 5, near *DFNA1*, the first locus mapped for dominant, non-syndromic deafness [4], suggested that deafness in Family H was coinherited with a gene near *DFNA1*, but not *DFNA1* itself. Further genotyping confirmed linkage of deafness in Family H to markers on chromosome 5 distal to *DFNA1*. The locus for deafness in Family H was named *DFNA15*, as it was the fifteenth dominant locus identified. *DFNA15* mapped to chromosome 5q31-q33 (fig. 2).

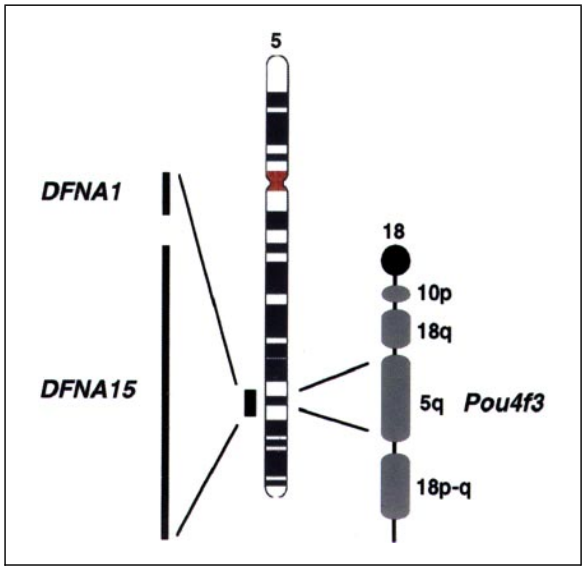

Fig. 2. Human chromosome 5 and mouse chromosome 18 homology. *DFNA15* was localized to a 25 cM region on 5q31 distal to *DFNA1*. The mouse *Pou4f3* gene is located on mouse chromosome 18, in a region of homology to human chromosome 5q. Other segments of mouse chromosome 18 are homologous to human chromosomes 10 and 18. Chromosomes are not drawn to scale.

A Mutation in the Transcription Factor *POU4F3* is Associated with the *DFNA15* Locus

The critical region defined for *DFNA15* was 25 cM, corresponding to approximately 25 million nucleotides, a vast region in which to search for a single mutation. However, a shortcut was possible, because an excellent candidate gene mapped to this region. We used the method of 'positional-candidate cloning', which combines identification of the chromosomal location of the locus, with knowledge of genes previously mapped in that region. Candidates may be in the form of previously mapped human genes, expressed sequence tags (ESTs) [5], or genes in the mouse, derived from the known large regions of homology between human and mouse chromosomes that contain orthologous genes [6]. Genes on human chromosome 5q31 are homologous to genes on both mouse chromosomes 5 and 18 (fig. 2).

An ideal candidate was identified on mouse chromosome 18 in the region of homology to 5q31. This gene, *Pou4f3* (also known as *Brn3c* or *Brn3.1*), is a developmental regulator [7]. Targeted deletion of the entire *Pou4f3* gene

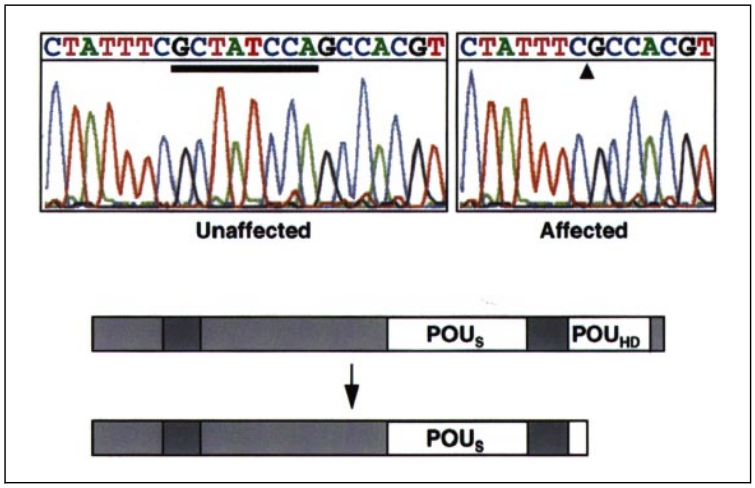

Fig. 3. The *POU4F3* mutation in Family H. The sequence analysis from unaffected individuals shows the 8-bp sequence (underlined in left panel) which is deleted in members of Family H with genetic hearing loss. Panel to the right shows sequence analysis in affected individuals. The schematic diagram below shows the POU4F3 protein, including the POU-specific domain (POU_S), and POU homeodomain (POU_{HD}). The truncated form of the POU4F3 protein formed by the 884del8 mutation in the POU_{HD} is shown.

leads to vestibular dysfunction and profound deafness in the knockout mice [8, 9]. Expression of murine *Pou4f3* is restricted to the cochlear and vestibular hair cells of the inner ear. These three facts were compelling reasons for suspecting that mutations in this gene might cause human deafness. Primers spanning the human gene were designed and DNA from both unaffected and affected Family H members was amplified. In deaf members of Family H, an 8-bp deletion (884del8) was identified in the 3′ portion of human *POU4F3* (fig. 3). This deletion leads to a frameshift, causing a stop codon to be formed at amino acid 299. Presumably, a truncated protein is formed, which still has the capacity to bind to DNA, though at a lower affinity due to the loss of a large portion of the POU homeodomain (fig. 3).

Clinical Aspects of DFNA15

A complete medical history of each affected individual was collected to ensure that the hearing loss was not a result of infection, head trauma, acoustic trauma or ototoxic drugs. Hearing was measured by pure-tone audiometry

on all participating relatives of Family H. The thresholds were compared to the age- and gender-related median of normal hearing standards [10]. Four individuals, 504, 505, 516 and 519, were tested further with speech audiometry and immittance testing (tympanometry and acoustic reflexes), and auditory brainstem response (ABR). 519 also underwent click-evoked otoacoustic emission (CEOAE) and distortion-product otoacoustic emission (DPOAE).

Progressive sensorineural bilateral hearing loss was the common theme in all Family H members with the *POU4F3* mutation above the age of 40 [11]. The clinical history of the family did not reveal any medical problem that could explain the hearing loss. Two members of the family had some conductive hearing loss as well. There was little consistency in the shape of the audiograms between affected individuals, ranging from a flat to sloping curve. The criterion for hearing impairment in Family H is hearing loss below the 95th percentile (p95) of the standard reference curves. Normal ABR suggests a functional central auditory pathway in hearing-impaired individuals with *POU4F3* mutations. More detailed clinical parameters are reported elsewhere [11].

Individual 519 tested positive for the 884del8 mutation in *POU4F3*. Pure tone testing at the age of 38 showed normal hearing in the frequencies 250–1,000 Hz and increasing bilateral sensorineural hearing impairment in the higher frequencies (data not shown). Three years later, the hearing remained unchanged in the lower to mid frequencies and decreased further in the higher frequencies (fig. 4). Abnormal distortion products suggests that the outer hair cells are malfunctioning. His relatively mild impairment in the mid-frequency range with severe impairment only at higher frequencies at the age of 41 demonstrates the variability of hearing levels among individuals with the same mutation or alternatively, that the progression of his hearing loss is still occurring.

Individual 516 tested positive for the 884del8 mutation in *POU4F3*. Pure tone testing at the age of 51 demonstrated bilateral moderate sensorineural hearing loss in the low frequencies and a more severe hearing loss in the higher frequencies (fig. 4).

Individual 504 tested positive for the 884del8 mutation in *POU4F3*. Pure tone testing at the age of 54 demonstrated bilateral moderate to severe sensorineural hearing loss, with a flat audiogram (fig. 4).

Individual 505 does not carry the 884del8 *POU4F3* mutation, although he had a moderate sensorineural hearing loss in the left ear, and moderate to severe mixed hearing loss in the right ear with evidence of otosclerosis when tested at age 53. The speech reception thresholds (SRT) and maximal discrimination were different between both ears. No ABR response was present in the right ear. The ABR of the left ear showed prolonged absolute latencies of the Vth peak and prolonged brainstem transmission time (BTT). The

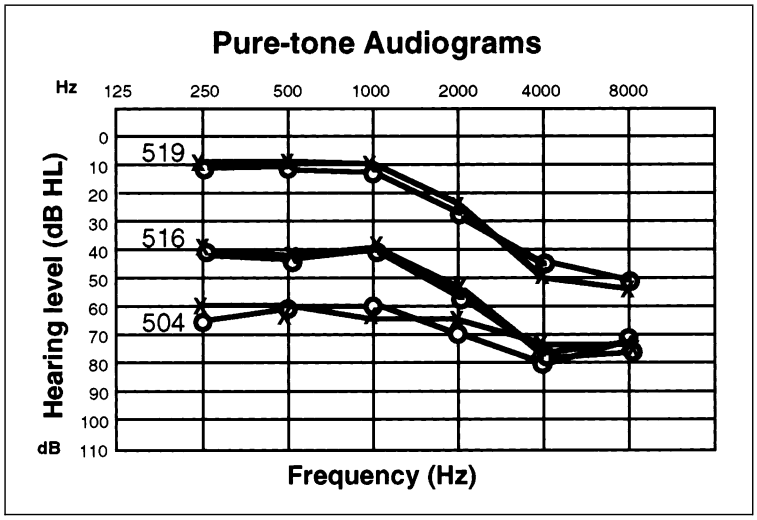

Fig. 4. A composite of audiograms from three individuals harboring the *POU4F3* mutation: 519 (age 41), 516 (age 51), and 504 (age 54). The horizontal axis shows tone frequency (Hz) and the vertical axis displays hearing level (dB). Right ear (O); left ear (X).

presence of conductive hearing loss and lack of uniform hearing loss between both ears suggests that this individual is a phenocopy; that is, an individual with a similar clinical presentation (phenotype), but due to a different cause, either genetic or environmental.

Careful audiological evaluation was essential to distinguish the hearing loss in individual 505 from that of other members of Family H. Had individual 505 been misdiagnosed, *DFNA15* could not have been identified. The critical role of the clinician in consistent, thorough diagnosis involving both audiological evaluations and anamnesis cannot be stated too emphatically.

Presymptomatic Diagnosis

The above individuals claimed that their hearing loss began in their 20s, though there are no audiograms to confirm this. Despite being aware that approximately 50% of children will inherit the *POU4F3* mutation, most individuals in this family expect to suffer from hearing loss. This feeling may be a compensatory one in order to avoid subsequent disappointment. Therefore, several young individuals expressed the opinion that they did not require confirmation of the results; rather, their parents were more eager to know if

individuals in their 20s harbor the mutation. Mutation analysis and pure tone audiograms was performed for multiple young adults, at their request. Several individuals in their 20s carrying the critical *POU4F3* mutation had audiograms indistinguishable from their relatives with normal *POU4F3*. From a clinical point of view, it would be helpful to obtain audiograms at frequent intervals from young individuals with the mutation, in order to observe the progression of the hearing loss.

The Role of the POU4F3 Transcription Factor in the Inner Ear

Mutations in *POU4F3* in both mice and humans suggest a role for POU4F3 during embryogenesis and adulthood. In the *Pou4f3* knockout mouse, no hair cells are present at post-natal day 14, around the time mice begin to hear, indicating that they are required for the terminal differentiation and trophic support of sensorineural cells. But clearly POU4F3 is also involved in the maintenance of hair cells, since the 884del8 mutation affects hearing by adulthood. The deletion in the human family leads to a milder phenotype since this deletion may form a truncation, removing the 3′ portion of this POU transcription factor crucial for high-affinity binding to DNA (fig. 3). This results in a progressive phenotype, possibly due to a dominant-negative effect of the normal versus the mutant allele. A dominant-negative effect occurs when the mutant protein loses its own function and prevents the normal product from functioning properly, most often seen with proteins that work as dimers or multimers. The stochiometry of POU4F3 levels may change over time, so that initially the hearing of these individuals is within the normal range. As mutant POU4F3 accumulates, this transcription factor may cease to function properly. This phenomenon would lead us to predict that severe *POU4F3* mutations may be found in prelingually deaf humans and even milder mutations than that displayed here will lead to elderly hearing loss (presbycusis).

Implications for the Future

In our society, the ability to hear is the norm, whereas those who have difficulty hearing must conform in order to succeed both socially and professionally. There are many sociological implications of progressive hearing loss, including problems of isolation and loneliness. Furthermore, many individuals losing their hearing during adulthood are ashamed of wearing hearing aids and admitting they have a hearing disability. For those individuals with prelingual

hearing loss, a deaf community exists, providing both a work and social structure in which they can thrive. For those that lose their hearing later in life, no such community exists, and not only do they not consider themselves part of the deaf community, but they will always yearn to hear normally again. Although there is more awareness today of the capabilities of hearing-impaired individuals, it remains difficult for them to interact at home, in social relationships, and in the workplace.

Understanding the molecular basis of deafness and the biological function of the proteins involved in auditory transduction will lead toward a new generation of biologically defined treatments of audiovestibular disorders, including sensory hair cell regeneration and gene therapy. As more genes involved in hearing loss are cloned, genes amenable to gene therapy at various stages of development will be identified. Transcription factors, such as POU4F3, are such candidates since their gain- or loss-of-function influence activation or repression of other downstream targets.

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Mapping of the DFNB1 Locus

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The first gene locus (DFNB1) for autosomal recessive non-syndromal sensorineural hearing impairment/deafness (ARNSSNHI/D) was mapped in 1994 to the proximal portion of the long arm of chromosome 13 (13q11-q12) using autozygosity mapping with 2 complex consanguineous families from Tunisia with profound prelingual sensorineural deafness [1]. Confirmation that a gene at this locus caused inherited deafness was provided by reports of linkage of ARNSSNHI/D in a large inbred Bedouin family with profound deafness from Israel [2] and in a complex consanguineous British Pakistani family with moderate to profound deafness [3].

Evidence to suggest that a gene at the DFNB1 locus made an important contribution to ARNSSNHI/D was provided by a study of the segregation of polymorphic DNA microsatellites linked to the DFNB1 locus in 19 families with NSSNHI/D which was likely to be autosomal recessive from Australia and New Zealand. The results suggested that a gene at this locus made a significant contribution to ARNSSNHI/D in a Caucasian population [4]. A similar study of 30 Italian and 18 Spanish families with NSSNHI/D showed that in approximately 80% a gene at the DFNB1 locus was likely to have a role in the hearing impairment/deafness [5].

Identification of *CX26* as the Gene at DFNB1

Identification of the gene at the DFNB1 locus at 13q11-q12 was facilitated by the ascertainment of a family in which an inherited disorder of the skin, palmoplantar keratoderma (PPK), and sensorineural deafness appeared to be inherited as an autosomal dominant disorder and showed linkage to poly-

morphic microsatellite markers at 13q11-q12. A locus responsible for autosomal dominant non-syndromal sensorineural hearing impairment/deafness, DFNA3, had previously been mapped to the same region of the long arm of chromosome 13 [6]. These observations suggested that genes which had been mapped to that region of chromosome 13, which were known to be expressed in the skin, were potential positional candidate genes for the disorder in the family. The gap junction protein beta-2 (GJB2) connexin 26 gene (*CX26*) had been localized to that region of chromosome 13 and immunological studies in the rat and mouse had shown it to be expressed in the suprabasal layer of the epidermis, suggesting it as a possible positional candidate gene.

Analysis of the *CX26* gene in DNA from persons with deafness in the family revealed a T to C missense mutation at position 101 resulting in the substitution of methionine to threonine (M34T) residue in the first transmembrane region of the GJB2 protein. Review of the features in the family showed that this mutation was only present in persons with the skin disorder who were deaf and not in those with normal hearing, suggesting the mutation in the *CX26* gene was only responsible for the hearing impairment/deafness in the family. Samples from individuals with ARNSSNHI/D from three complex consanguineous British Pakistani families in which the deafness was known to be linked to the DFNB1 locus showed affected individuals to be homozygous for two different nonsense mutations in the connexin 26 gene. These two mutations cause premature stop codons in the first and second transmembrane domains of the GJB2 protein, leading to a severely truncated and nonfunctional GJB2 protein, thus confirming the role of *CX26* in causing ARNSSNHI/D [7].

Possible Role of *CX26* in Causing ARNSSNHI/D

The connexins are a large family of proteins widely expressed in different tissues. Six connexin subunits assemble to make a connexon; the association of 2 connexons between adjacent cells form the gap-junctions between cells. Gap junctions are thought to function as intercellular communication channels facilitating the passive exchange of ions and small molecules up to 1 kiloDalton in size. Connexins are highly conserved at the amino acid level and are made up of four transmembrane domains, two extracellular loops, an intracellular loop and a cytoplasmic tail (fig. 1). The amino acid sequence of the loop regions and the length of the cytoplasmic tail distinguish the different connexins.

Immunohistochemical studies of the inner ear have shown that GJB2 protein is expressed in the inner ear in the *stria vascularis*, basement membrane, limbus and spiral prominence of the cochlea [7]. The gap junctions in the

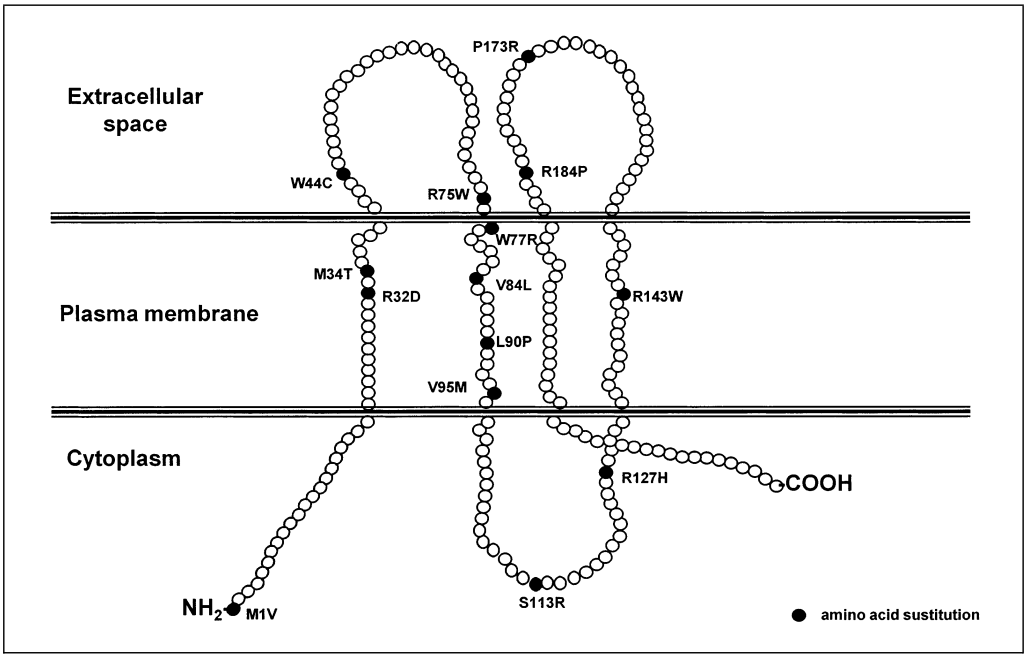

Fig. 1. Diagrammatic representation of the structure of the GJB2 protein coded for by the *CX26* with location and type of mutations identified.

inner ear are the channels through which potassium ions are recycled from the sensory hair cells through their supporting cells back to the endolymph after the mechanoelectrical transduction of auditory stimuli. It is believed that mutations in the *CX26* gene would lead to loss of function of the GJB2 protein interfering with the recycling of potassium ions.

The Range of Mutations in the *CX26* Gene Seen in Persons with ARNSSNHI/D

After the identification of *CX26* mutations in persons with ARNSSNHI/D, a number of groups have screened individuals with non-syndromal sensorineural hearing impairment/deafness from a variety of different populations for mutations in the gene. A deletion of a single guanine (G) residue in a stretch of 6 Gs, at nucleotide position 30–35 (variably known as 30 or 35delG), was found to account for greater than 60% of *CX26* mutations in families with ARNSSNHI/D from the Mediterranean area previously shown to be

Table 1. Mutations identified in *CX26* causing ARNSSNHI/D

Codon	Nucleotide change	Mutation type	Country/population	Reference
10	35delG (30delG)	Deletion/frameshift	UK, Belgium, Spain, Italy, Tunisia, Morocco, Arab-Israeli, Australia, New Zealand, US, Dominican Republic, Puerto Rico	8–12, 14–17
10	31-68del(38)	Deletion/frameshift	New Zealand	9
W24X	74G→A	Nonsense	Pakistan, India	7, 15
M34T	101T→C	Missense	UK	7
E47X	139G→T	Nonsense	Tunisia	9
55	167delT	Deletion/frameshift	Spain, Italy	8, 16, 17
W77R	229T→C	Missense	Arab-Israel	12
W77X	231G→A	Nonsense	Pakistan, India	7, 15
V84L	250G→C	Missense	US	16
V95M	283G→A	Missense	US	16
105	314-327del(14)	Deletion/frameshift	UK/US	9, 16
111	333-334del(AA)	Deletion/frameshift	US	16
S113R	339T→G	Missense	US	16
118	351-354del(3)	Deletion	Australia	9
Q124X	370C→T	Nonsense	India	15
R143W	427T→C	Missense	Ghana	13
R184P	551G→C	Missense	Australia	9
211	631-632del(GT)	Deletion/frameshift	US	16

linked to DFNB1 [8]. This single base pair deletion alters the reading frame (i.e. a frameshift mutation) generating a stop codon at codon 13 of the *CX26* gene resulting in premature termination of translation leading to a GJB2 protein truncated at the first transmembrane domain. The same mutation has also been found in approximately 70% of families with ARNSSNHI/D from Tunisia, France, New Zealand and the UK [9]. In addition, further studies have shown that this mutation is responsible for approximately 50% of sib-pairs and between 1 in 10 to 1 in 3 sporadically affected individuals with NSSNHI/D [10, 11].

To date 18 different mutations in the *CX26* gene have been reported in a variety of different populations as causing ARNSSNHI/D. These include missense, nonsense, deletions and frame-shift mutations (table 1) [12–17]. The majority of mutations other than 35delG have only been observed a limited number of times, although the mutation deletion/frameshift mutation first

reported by Zelante et al. [8], was the second most common mutation seen in the series reported by Kelley et al. [16], and the affected individuals with this mutation in this latter report were of Ashkenazi-Jewish ancestry.

Although a number of individuals with ARNSSNHI/D have been shown to be compound heterozygotes (i.e. to have two different mutations in the *CX26* gene), in a significant number of affected individuals only a single mutation in the *CX26* gene has been identified. It is not yet known if they have a second mutation in the 5′ promoter region of the gene or whether there is a more complex cause, such as an interaction between mutations in two different recessive genes (what are known as double heterozygotes) which interact (termed digenic inheritance), as has been shown in retinitis pigmentosa [10].

Carrier Frequency of *CX26* Mutations

The carrier frequency in the general population of the 35delG mutation varies in studies of different populations, but is as high as 1 in 20 in those of Mediterranean origin [8, 9, 11]. The 35delG mutation has been found on a number of different background haplotypes [8, 12], suggesting that this mutation has arisen independently on many separate occasions. The most likely explanation is that the run of 6 Gs represents a mutational 'hotspot', the mutation arising as a result of slipped mispairing during DNA synthesis.

The 167delT mutation has been found to be carried by 4% of persons of Ashkenazi-Jewish origin [17], making it as prevalent as the 35delG mutation in individuals of Mediterranean origin [8]. Conservation of the haplotype flanking the 167delT mutation suggests it is likely to have arisen on a single occasion.

Autosomal Dominant NSSNHI/D

The M34T *CX26* variant reported in the original family segregating PPK and sensorineural deafness [7] has also been reported in persons with normal hearing. Studies of the frequency of this sequence variant in the general population suggest that it could be a rare polymorphic variant and is not of any functional consequence [16, 18], although in vitro studies in *Xenopus laevis* oocytes of this mutant have shown abnormality of channel activity [19].

In the family with ADNSSNHI/D which had mapped the DFNA3 locus to the same region of chromosome 13 [6], a missense mutation in which a G is substituted by a cytosine (C) leads to a tryptophan to cysteine substitution at codon 44 (W44C) of *CX26* [20]. This amino acid substitution is located in

the first extracellular domain of *CX26* and would interfere with the formation of normal disulphide bonds. This is likely to alter the interaction between connexins of adjacent cells. The likely functional consequence of this mutation would be to disturb ion channel activity and is likely to act in a dominant negative manner. The same mutation in the *CX26* gene was found in a second family with autosomal dominant non-syndromal sensorineural hearing impairment/deafness out of a total 30 autosomal dominant families from France, suggesting that mutations in *CX26* are likely to be an uncommon cause of ADNSSNHI/D.

Genotype-Phenotype Correlations in *CX26*

The hearing impairment in the majority of individuals with ARNSSNHI/D due to mutations in the *CX26* gene reported to date is usually prelingual, bilateral with the degree of sensorineural impairment usually being severe or profound. In a minority, the hearing impairment/deafness is moderate and/or asymmetrical in its severity. There is no apparent correlation of severity of hearing impairment with particular mutations in the *CX26* gene [7, 8–17]. The hearing impairment/deafness in the affected individuals from families with ADNSSNHI/D, although prelingual in onset, was progressive with the high frequencies being more severely affected [6, 20]. More detailed genotype-phenotype studies are underway at present and will be reported in the near future.

Diagnostic Testing/Genetic Counselling/Recurrence Risk Implications

In most families with a child with congenital or childhood-onset non-syndromal sensorineural hearing impairment/deafness, there is no family history of deafness and there is no obvious acquired or environmental cause. Physical examination and the results of conventional clinical investigations such as TORCH titres, thyroid function tests, etc., rarely identify a cause, genetic or acquired. The chance of recurrence of hearing impairment/deafness in subsequent offspring is based on empirical figures, i.e. the observed recurrences of hearing impairment/deafness in such families. These figures range from 1 in 10 to as high as a 1 in 4 chance if the hearing impairment in the affected child is greater than 80 dB [21]. The identification of a single mutation in *CX26* as a common cause of ARNSSNHI/D, means that a significant proportion of families presenting with a child sporadically affected with NSSNHI/D can now be provided with definitive Mendelian recurrence risks [10].

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Sensorineural Hearing Impairment: Non-Syndromic, Recessive DFNB2

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Non-syndromic hereditary hearing impairment is highly heterogeneous. There is a lack of audiovestibular criteria available that will allow the differentiation of the inner ear defects caused by the various genetic loci involved, making it difficult to isolate the underlying genes. Nevertheless, during the last 5 years, over 40 loci involved in non-syndromic deafness have been localized, of which 19 are recessive and 20 dominant [1]. However, only ten genes for non-syndromic hearing impairment have been identified using either positional cloning strategies or candidate gene approaches [1]. In the present section we discuss the *DFNB2* non-syndromic recessive deafness locus on human chromosome 11 that is encoded by the myosin VIIA (*MYO7A*) gene. We also explore the correlation between phenotype and genotype for *MYO7A* mutations.

Identification of the Gene Encoded at the DFNB2 Locus

The *DFNB2* locus was identified following conventional linkage analysis of a large consanguineous Tunisian family affected by a form of sensorineural deafness [2]. The *DFNB2* gene maps to 11q14, a region that also contains one of the loci responsible for Usher syndrome type 1 (*USH1B*). This region of human chromosome 11 is also known to be homologous to a portion of mouse chromosome 7 that contains the mouse recessive deafness mutation shaker1 [2]. Shaker1 belongs to a class of mouse mutations that demonstrate abnormali-

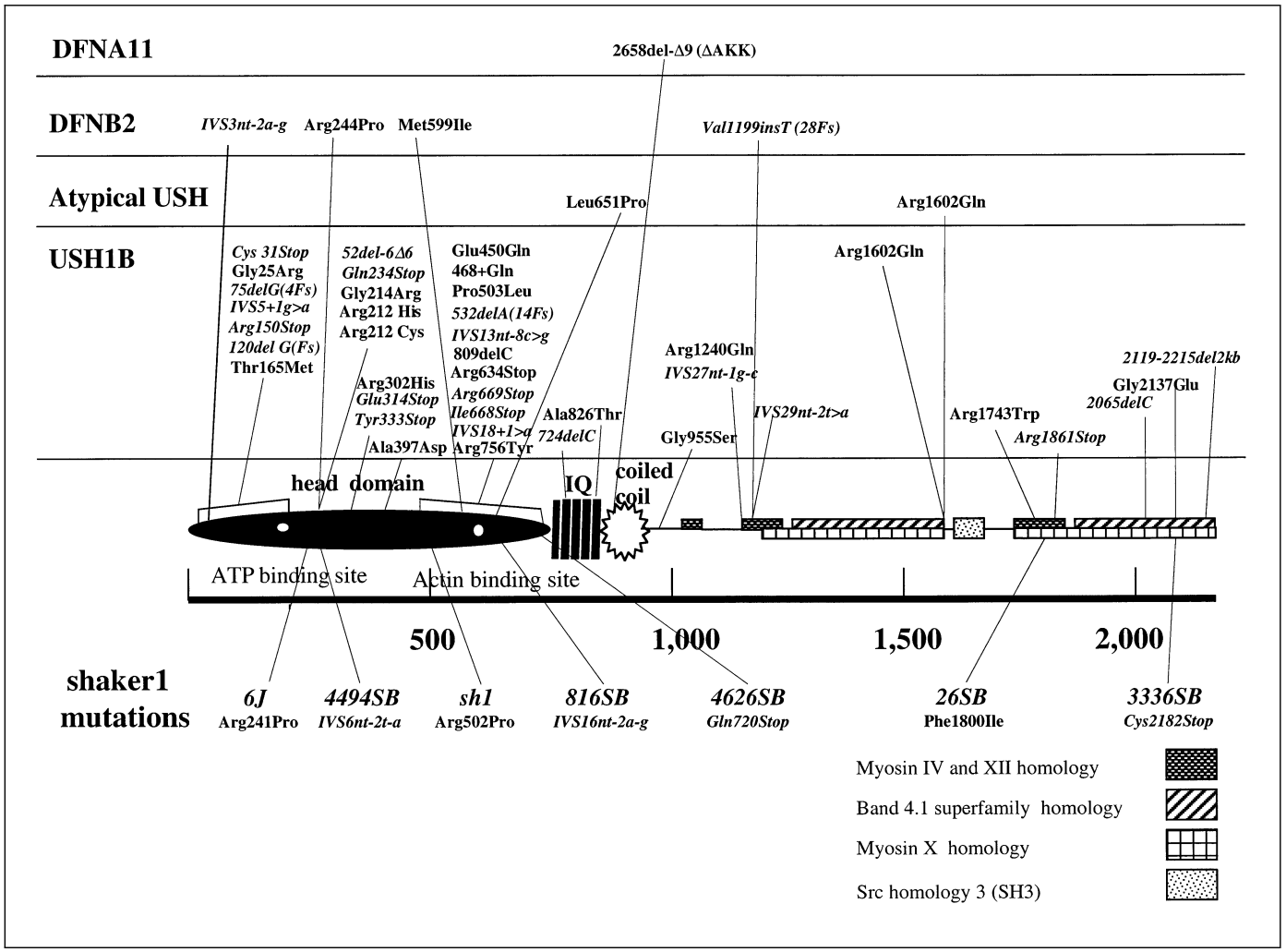
ties of the sensory neuroepithelia in the inner ear. Shaker1 homozygotes show vestibular abnormalities causing hyperactivity, circling and head-tossing as well as neuroepithelial defects resulting in the dysfunction and progressive degeneration of the organ of Corti. Both *USH1B* and the shaker1 locus have been shown to be encoded by the myosin VIIA gene [3–5].

Myosin VIIA is one member of a large superfamily of myosin molecules. All myosins possess a highly conserved head or motor domain containing ATP-binding and actin-binding sites. Phylogenetic analysis of myosin motor domains indicates that there may be at least 13 different classes within the myosin superfamily. Each myosin class has a distinct tail domain that is involved in determining the specific cellular function of the class. The myosin VIIA gene, composed of 49 exons, encodes a predicted protein of 2,215 amino acids (fig. 1 and [5, 6]). In mouse, myosin VIIA has been found to be expressed in the developing hair cells in the cochlea where it appears to be involved with stereocilia positioning on the apical hair cell surface [7]. The expression of myosin VIIA in the neuroepithelium along with its involvement in neuroepithelial-type deafness in the mouse suggests that it should be considered a candidate for non-syndromic deafness in the human population, particularly the gene encoded at the *DFNB2* locus.

By screening families from China with recessive non-syndromic hearing impairment we have identified two families carrying *MYO7A* mutations [8]. At the same time, a mutation in *MYO7A* was reported in the Tunisian family from which the DFNB2 locus was originally defined [9]. It was clear from both studies that mutations in the *MYO7A* gene could indeed lead to non-syndromic recessive hearing impairment.

MYO7A Mutations in DFNB2 Families

We screened eight non-syndromic recessive deafness Chinese families for *MYO7A* mutations. Five patients born to unrelated parents from two families (DFNB.01 and DFNB.05) had congenital bilateral sensorineural hearing loss ($>$90 dB hearing level at 500–8,000 Hz). In family DFNB.01, two of the three affected siblings complained of balance problems from a young age and had absent vestibular function as demonstrated by caloric tests. The third sibling had no balance problems but did have reduced vestibular function. All of the affected siblings underwent electroretinograms (ERG) with normal results. While the mother and an unaffected sibling had good hearing, balance and normal pure tone audiograms, the mother showed decreased bilateral vestibular function, elevated acoustic reflex thresholds (ARTs) and bilateral notches at 2,000 Hz using Bekesy audiometry. Several studies have indicated that elevated


DFNA11
2658del-Δ9 (ΔAKK)
DFNB2
IVS3nt-2a-g
Arg244Pro
Met599Ile
Val1199insT (28Fs)
Atypical USH
Leu651Pro
Arg1602Gln
USH1B
Cys 31Stop
Gly25Arg
75delG(4Fs)
IVS5+1g>a
Arg150Stop
120del G(Fs)
Thr165Met
52del-6Δ6
Gln234Stop
Gly214Arg
Arg212 His
Arg212 Cys
Arg302His
Glu314Stop
Tyr333Stop
Ala397Asp
Glu450Gln
468+Gln
Pro503Leu
532delA(14Fs)
IVS13nt-8c>g
809delC
Arg634Stop
Arg669Stop
Ile668Stop
IVS18+1>a
Arg756Tyr
Ala826Thr
724delC
Arg1240Gln
IVS27nt-1g-c
Gly955Ser
IVS29nt-2t>a
Arg1602Gln
Arg1743Trp
Arg1861Stop
2119-2215del2kb
Gly2137Glu
2065delC
head domain
IQ
coiled coil
ATP binding site
Actin binding site
500
1,000
1,500
2,000
shaker1 mutations
6J
Arg241Pro
4494SB
IVS6nt-2t-a
sh1
Arg502Pro
816SB
IVS16nt-2a-g
4626SB
Gln720Stop
26SB
Phe1800Ile
3336SB
Cys2182Stop
Myosin IV and XII homology
Band 4.1 superfamily homology
Myosin X homology
Src homology 3 (SH3)

ARTs and audiometric notches are associated with obligate carrier status in genetic deafness [10]. The unaffected sibling had normal vestibular function but like the mother showed elevated ARTs and bilateral notches at Bekesy audiometry. The subclinical pathology in the mother and unaffected sibling suggest that the non-syndromic deafness observed in this family may be semi-dominant. In the second family, DFNB.05, two affected siblings had absent vestibular function and normal ERG results. No other members of this family were available for study.

Three mutations in the *MYO7A* gene were identified in these two Chinese families [8]. In family DFNB.01, sequence analysis demonstrated that the three affected siblings were homozygous for a mutation causing an arginine to proline change at position 244 in the *MYO7A* amino acid sequence (Arg244Pro); both the mother and unaffected sibling were heterozygous for the mutation. The Arg244Pro mutation lies within the highly conserved motor domain of the myosin VIIA protein. An alignment of known myosin motor domain sequences shows that this mutation occurs in an area of high conservation but that the residue itself is not conserved in all classes of myosins. It may be significant that in none of the myosins sequenced to date is there a proline in this position. Interestingly, one of the mouse shaker1 mutant alleles, 6J, carries an Arg241Pro change that lies only three codons upstream of the human mutation [6]. This residue is absolutely conserved in all known myosins and is crucial for maintaining the integrity and organization of this region of the nucleotide-binding pocket [11]. In the second Chinese family, DFNB.05, two affected individuals were found to be compound heterozygotes carrying a splice site mutation in intron 3 (IVS3nt-2a-g) and an insertion mutation, Val1199insT (Fs), in exon 28 that leads to a frameshift (Fs) and a stop codon 28 amino acids downstream.

In the large Tunisian family, all 22 affected members showed profound deafness [2]. However, the age of onset varied, both within and between sibships, ranging from birth to 16 years of age. Four affected members presented with vertigo. All 22 affected members were homozygous for a G to A transition at the last nucleotide of exon 15 converting a methionine to an isoleucine residue (M599I). This type of mutation is thought to decrease the efficiency of splicing. It is presumed that the mutation causes aberrant splicing

Fig. 1. MYO7A mutations that lead to *USH1B* [12–15] and atypical USH syndrome [16] as well as recessive [8, 9] and dominant [18] non-syndromic deafness. Position of seven shaker1 mutant alleles are also shown below [6] and the various homology domains in the *MYO7A* tail are indicated [5, 6]. Italicized mutations are truncating (frameshift, nonsense, and splice-site). Non-italicized mutations are non-truncating (missense or small in-frame insertions or deletions).

Table 1. Phenotypic spectrum of mutations in *MYO7A*

Clinical category	Hearing impairment	Clinical features	
		Vestibular function	Retina
USH1B	Congenital profound	Absence	RP*
Atypical USH	Progressive	Variable	RP*
DFNA11	Progessive moderate post-lingual	Variable	Normal
DFNB2	Profound	Variable	Normal

* Retinitis pigmentosa.

and a reduction in correctly spliced myosin VIIA RNA, leading to a significant decrease in the levels of normal myosin VIIA in homozygotes sufficient to cause sensorineural deafness. The normal hearing parents were heterozygous for this missense mutation.

Clinical Heterogeneity of *MYO7A* Mutations

The authors and others have demonstrated that different mutations in *MYO7A* can lead to a wide range of phenotypes including both syndromic Usher type 1b [12–15] and atypical Usher syndrome [16] as well as both non-syndromic recessive (*DFNB2*) and dominant (*DFNA11*) deafness [17, 18]. All patients with *MYO7A* mutations can be classified as four distinguishable clinical types from the least severe *DFNA11* to the most severe *USH1B* (table 1, fig. 1). With the exception of the *DFNA11* mutation there does not appear to be a clear relationship between mutation and phenotype. Mutations in the coiled-coil region may result in dominant hearing loss via a dominant-negative effect [16]. There is often a significant difference in the degree of hearing impairment between non-syndromic dominant and recessive deafness, with dominant hearing impairment being milder than recessive [19]. Indeed, unlike the other three forms of hearing loss caused by *MYO7A* mutations, *DFNA11* patients demonstrate a progressive moderate post-lingual hearing loss [17]. In the three *DFNB2* families with *MYO7A* mutations reported to date, profound hearing impairment with variable vestibular dysfunction is a constant feature (see table 1 and above). But in the family from Tunisia, affected members show a variable age of onset of deafness due to a mutation that is known to decrease the efficiency of splicing. It may suggest that the residual concentration of normal myosin VIIA produced in the homozygous patients is close to a critical threshold of activity for the hair cells [9].

There are many instances whereby different mutations in the same gene can result in diverse phenotypes [20]. Allelic heterogeneity can account for markedly different phenotypes. *MYO7A* mutations in human can lead to both non-syndromic as well as syndromic deafness. It is possible in human that tissue-specific differences in function of *MYO7A* might result in differences in the effect of specific mutations in the eye versus the inner ear. It seems likely, however, given the wide range of *MYO7A* mutations identified for both non-syndromic deafness and syndromic deafness, that genetic background effects are important in determining the development and severity of non-syndromic and syndromic hearing impairment. The present data underline both the genetic and phenotypic heterogeneity that is observed in non-syndromic deafness as well as Usher syndrome, and the difficulties that will face us in using molecular criteria for diagnosis and management.

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DFNB3 Families and *Shaker*-2 Mice: Mutations in an Unconventional Myosin, *MYO 15*

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Reliable and complete phenotypic data are fundamental to a genetic analysis, since incomplete clinical information can severely compromise a study to determine the chromosomal address of a gene. In a family with many deaf individuals, it is essential to distinguish between inherited deafness and acquired deafness and to at least check for the most common syndromes in which deafness is part of a complex phenotype. Even after ruling out common deafness syndromes and obvious other complications, the 'nonsyndromic' designation for mutations of a particular deafness locus should be considered tentative until the function and pattern of expression of the gene is known. There are now cautionary tales [1, 2] suggesting that assignment of the nonsyndromic classification may be premature in the absence of thorough clinical data.

Forty genes for nonsyndromic deafness have been assigned a chromosomal address and more remain to be mapped. No other human neurosensory disorder appears to be so genetically heterogeneous. Thus, mapping these genes is a special challenge. Much of nonsyndromic hereditary deafness occurs in small families with affected individuals all displaying the same phenotype. The hereditary deafness in these families could be due to the effects of mutations at many loci. In the worst case scenario for genetic mapping, pooling genetic

data from many small families each carrying a different deafness gene would effectively prevent identification of the chromosomal localization of any one of these genes. The solution is to ascertain large families or inbred populations with many deaf individuals.

In this chapter we describe the ascertainment of a large remote population in Bali and two consanguineous families from India segregating profound, congenital deafness. We also discuss the genetic mapping and cloning of the genes responsible for *DFNB3* and *shaker-2*, the *DFNB3* mouse homolog. In the early 1990s several research groups began projects to identify mouse and human genes for hereditary hearing impairment (HHI). Identifying mouse genes for deafness was bound to give insight into mammalian auditory function in general. Many strains of mice with hearing loss phenotypes were available and some were likely to be the homologs of defective genes in humans that cause deafness [3]. A particular strength of the mouse as a model system is that the number of parent to offspring transmissions is virtually unlimited permitting the responsible genes to be mapped rapidly to a small chromosomal interval. In contrast to mice, a genetic analysis of human HHI meant ascertaining multigenerational families each containing several hearing-impaired individuals.

High Prevalence of Deafness in Bengkala, Bali

The *DFNB3* story began in 1992 with an effort to map and clone a gene for congenital profound deafness segregating in Bengkala, Bali, Indonesia. A multidisciplinary research team consisting of Indonesian clinicians, an anthropologist and geneticists collaborated on this study of deafness in Bengkala, a village in the north of Bali in a rural farming area. Bengkala is very old, with plaques (prasasti) commemorating its formal incorporation in 1178 AD. The 48 deaf individuals currently living in the village share a unique sign language with one another and with most of the 2,254 members of the hearing population of the village. Subsistence in this highland village is based on farming of rice, vegetables and a variety of fruit trees. The deaf population of Bengkala is one of the very few examples of a relatively large group of deaf people who are well integrated into their local community. The hearing and deaf residents live in residential clusters based on descent from common male ancestors. Babies born to deaf parents are carefully checked for signs of deafness. Adults clap their hands, beat on water buckets or gamelan cymbals, and make other sudden noises in an attempt to startle a child.

Deaf and hearing children are socialized together, and learn sign language from one another. Before they are able to speak coherently, hearing children have been observed signing with their deaf playmates. Bengkala sign language

is complex but totally embedded in the specific cultural context of the village. The deaf are not literate and as a result they are unaware of the official names hearing people have assigned them. Deaf people use a system of name signs for everyone they know, based on some striking feature of each individual. These signs are not necessarily flattering.

Deafness Segregating in Bengkala is Autosomal and Recessive

In Bengkala, there are 24 deaf males and 24 deaf females. Analysis of the pedigree in figure 1 indicates that only deaf children are born to two deaf Bengkala parents. Hearing parents from Bengkala may have both hearing children and deaf children. Seventeen congenitally deaf individuals in Bengkala have two hearing parents. Taken together, this pattern of inheritance suggests a single autosomal recessive gene as the cause of congenital deafness in Bengkala [4]. In a few instances, marriages between a deaf person from Bengkala and a deaf person from another village have produced only hearing children. This indicates that the deafness of individuals from other Balinese villages, if hereditary, results from a mutation in some other gene than the one responsible for deafness in Bengkala.

We performed pure tone audiometry in this population using a portable Belltone audiometer with sound attenuating audiocups, and were able to confirm that the majority of deaf individuals in Bengkala could not detect sound even at 90 dB from 250 Hz to 8,000 Hz. Since the hearing loss was profound at birth the distinction between unaffected and affected individuals was obvious. Of note are two deaf individuals in Bengkala (individuals 46C and 48C, see fig. 1b) who were said to be hearing at birth and hearing as young children but experienced prolonged high fevers, and subsequently acquired profound loss of hearing. These two individuals were excluded from our genetic analyses. In the end, we discovered that one of these two individuals (48C) was homozygous for the mutation causing deafness and likely deaf at birth. Individual 46C carried no copies of the *DFNB3* mutation. Her parents recounted that in childhood she became deaf following protracted high fever.

Vestibular Phenotype

Sophisticated vestibular tests [5] were not available in Bali, so we observed and videotape-recorded the deaf villagers engaged in their normal daily activities. The deaf, like the rest of the village population, are farmers who also participate in many of the cooperative work groups (gotong royong) that carry

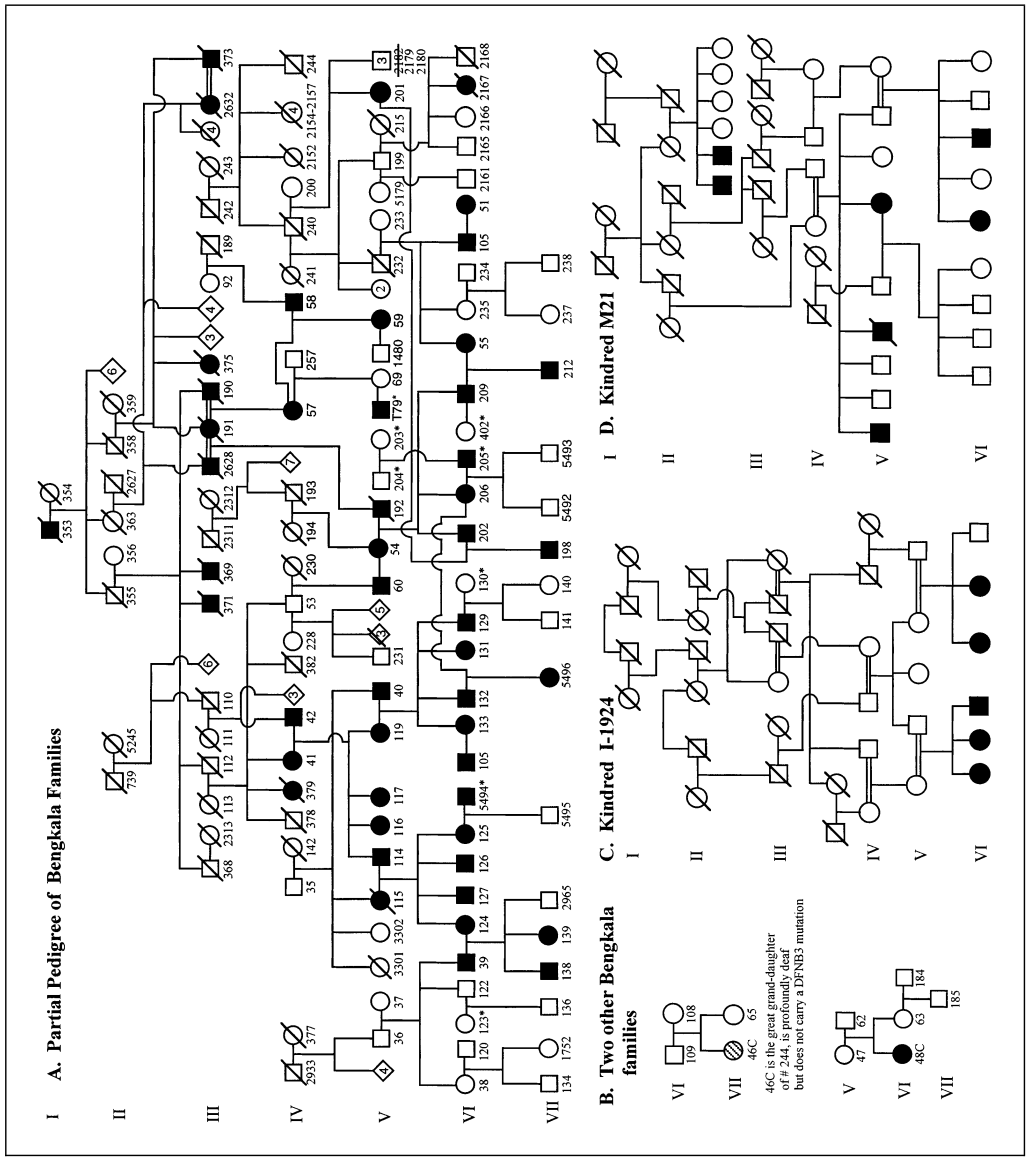

Fig. 1. A Partial genealogy of families with deaf individuals from Bengkala, Bali [12]. Open squares and circles are unaffected males and females, respectively. Blackened symbols are congenitally deaf individuals. An asterisk indicates deaf individuals living in Bengkala but originating in other villages. *B* Individual 46C was not deaf at birth but lost her hearing after a high fever (acquired deafness). *C, D* Two consanguineous Indian families (I-1924 and M-21) with profound congenital deafness linked to *DFNB3* also have mutations in *MYO15* (see fig. 2).

out public works in the village. For example, deaf men lead improvement and repair of the vital water system of the village. Deaf people are often employed in wage labor such as harvesting coconuts from the tops of palm trees, fruit tree spraying and barn building. Beginning in the 1960s, the deaf men were taught both Balinese classical dance and the martial art, silat, by two hearing men and now travel from village to village putting on complicated performances. Some of the deaf men in Bengkala are particularly noted for their martial arts skills, and are recruited to join the all-night village guard (hansip) details. The deaf people of Bengkala do not have an obvious problem with balance when their eyes are open. However, when asked to stand or walk with their eyes closed, several of the deaf individuals in Bengkala (but not obligate carriers of the mutation) gave the sign for the feeling of being drunk or of dizziness. A proper clinical evaluation of vestibular function of deaf individuals from Bengkala is needed.

Among the deaf Bengkala villagers and the two Indian families, M-21 and I-1924 (fig. 1), we ruled out common syndromic forms of deafness. For example, Pendred syndrome is known to be an early-onset deafness syndrome associated with goiter. Although thyroid goiters are common in Bali because of iodine deficiency, we found no statistically significant correlation between goiter and deafness in Bengkala, ruling out Pendred syndrome [4]. We also ruled out Waardenburg or Usher syndromes as the deaf individuals had no obvious craniofacial dysmorphology, pigmentary anomalies or retinopathy [4]. We remain open to the possibility that a less obvious clinical anomaly might be found once we understand more about the function of the gene causing deafness in Bengkala and in the Indian families (fig. 1).

DFNB3 Maps to Chromosome 17 at p11.2

Over many generations the mutation causing deafness has become disseminated in the village by virtue of endogamous marriages while the preference of deaf individuals for deaf mates (assortative mating) in Bengkala has also increased the number of deaf individuals. We originally calculated that one in six hearing individuals in Bengkala carry the mutation causing deafness [4]. Now that we have cloned the gene (described below) we know that ~25% of the villagers carry this mutation.

In isolated populations and in consanguineous families, affected individuals with recessively inherited diseases most likely carry two copies of the very same mutation (homozygous by descent). Based on this observation, a strategy was proposed to map a gene by identifying a region of homozygosity in affected individuals from a founder population or from a consanguineous family. Homo-

zygosity mapping was proposed as a strategy decades ago but has only recently been popularized [6, 7] and now is frequently used to refine the map positions of genes [8–10]. In 1995 we had no information about the chromosomal location of the gene causing deafness in Bengkala. Nevertheless, we decided to employ a homozygosity mapping strategy to localize the gene to a chromosomal interval [11]. This was the third human gene for nonsyndromic recessive deafness to be mapped and was given the name *DFNB3*. *DFNB3* was the first human disease gene to be mapped using a homozygosity strategy in a genome-wide screen. *DFNB3* mapped near the centromere of chromosome 17 [11].

The chromosomal segment in which *DFNB3* was located initially was about 12 cM, roughly corresponding to 12 million base pairs of DNA. Such a large region encompassed hundreds of genes, too many to easily screen for the HHI mutation common in Bengkala. Further work localized *DFNB3* to a 4-5 cM region of the short arm (p) of chromosome 17 in band 11.2 [12]. The 17p11.2 region is rich in genes with a daunting number of good *DFNB3* candidates. Given the complexity of the auditory system and how little we know about the biological function and expression patterns of most genes, it is possible that a mutation in any one of several different genes along 17p11.2 might cause deafness.

Cancer biologists and geneticists have been interested in the genes at 17p11.2 since a deletion of 17p11.2 causes PNETS, a frequent childhood brain tumor [13, 14]. A similar deletion of 17p11.2 causes Smith-Magenis syndrome (SMS, OMIM 182290) [15]. SMS is a disorder with multiple congenital anomalies and mental retardation that results from haploinsufficiency for one or more of the many genes in the 17p11.2 interval [16]. Haploinsufficiency refers to the situation in which one copy of a normal autosomal gene is not sufficient for a normal phenotype. Interestingly, the complex SMS phenotype includes mild to severe neurosensory hearing loss, albeit with reduced penetrance. About 50% of SMS patients have some obvious but mild neurosensory, conductive or mixed hearing loss [16]. A few individuals with SMS have more severe neurosensory hearing loss. We are testing the hypothesis that the more severe hearing loss found among SMS individuals is due to a mutation in *DFNB3* on the intact chromosome 17 and the loss of the DFNB3 gene on the homolog harboring the SMS 17p11.2 deletion.

DFNB3 Families in India

We next pursued two strategies to identify the *DFNB3* gene: (1) clone *shaker-2*, the likely mouse homolog of *DFNB3*, and (2) search for more families with deafness linked to 17p11.2 that might permit us to further refine the

chromosomal region and thus reduce the number of *DFNB3* candidate genes. Additional *DFNB3* families would also help demonstrate a causal connection between a putative *DFNB3* mutation and deafness in each *DFNB3* family. As we were refining the map position of *DFNB3*, consanguineous families from India with profound nonsyndromic recessive deafness were being ascertained by Edward Wilcox, PhD, Anil Lalwani, MD (UCSF) and their research colleagues in India. Two of these families from the district of Kolhapur, Maharashtra State, India proved to be linked to genetic markers in the 17p11.2 region. Audiometry and bone conduction studies were performed in the field. Obligate carriers of *DFNB3* (parents of affected children) in both of the Indian families and in Bengkala had normal hearing. Genetic analyses of the two Indian families did not help refine the *DFNB3* interval. However, as discussed below, these two *DFNB3* families were crucial in establishing a causal connection between mutations of a candidate *DFNB3* gene and deafness segregating in these families [12, 17].

Functional Cloning *DFNB3* and *Shaker*-2, the Mouse Homolog of *DFNB3*

The phenotype of *shaker-2* mouse is circling behavior and profound deafness as measured by ABR. The circling and head-tossing behavior of *shaker-2* mice is indicative of a severe vestibular malfunction. The rationale for initially proposing *shaker-2* as the homolog of *DFNB3* was based on the knowledge that *shaker-2* mapped to mouse chromosome 11 linked to the peripheral myelin protein-22 gene, *Pmp22*. The human homolog, *PMP22*, is located at 17p11.2 very near *DFNB3* [12]. Therefore, *shaker-2* and *DFNB3* are likely to be caused by mutations of a homologous gene. As animals diverge during evolution, genes in close proximity on a chromosome tend to remain linked [18].

The *shaker-2* gene was mapped in a 500-member cross providing a resolution of 0.2 cM ($\sim$400 to 800 kb of DNA). Based on the hypothesis that *shaker-2* would represent a mutation in a gene homologous to *DFNB3*, we utilized markers for human genes from the corresponding human gene map [12] to refine the mouse genetic map. We then identified several mouse bacterial artificial chromosomes (BACs) carrying the genomic DNA that spanned the *shaker-2* region. Following genetic and physical mapping described above, the usual next step is to screen the BACs for genes with a mutation responsible for the *shaker-2* phenotype. There are a variety of techniques available such as exon trapping and cDNA selection methods to find parts of genes (protein coding regions) along a segment of cloned chromosomal DNA. These methods are labor intensive, especially when applied to large genomic regions. To expedi-

tiously clone *shaker-2* we employed a novel strategy involving 'gene therapy' coupled with large-scale DNA sequencing [19]. Each BAC spanning the *shaker-2* region was injected one at a time into a total of fifty pronuclei of homozygous *shaker-2* fertilized eggs. These eggs were then transferred to a foster mother. In doing so we hoped to recover a transgenic *shaker-2* mouse with normal hearing and movement behavior. We expected that a BAC containing the entire *shaker-2* gene would correct the deafness and circling behavior of *shaker-2* even if the BAC was integrated into a chromosomal site other than the normal location (ectopic site) of the *shaker-2* gene on chromosome 11.

One transgenic *shaker-2* homozygous mouse injected with BAC425p24 was born that did not circle and responded to sound with a normal Preyer's reflex. This mouse, named Sebastian, was mated with several *shaker-2* females. Approximately half of Sebastian's offspring could hear and had normal ABRs or a partial hearing loss. DNA analysis confirmed that all of the hearing offspring had inherited the BAC425p24 and all of the deaf offspring (i.e. nontransgenic *shaker-2*) did not. This was a substantial advance in our effort to identify *shaker-2*. The location of *shaker-2* was now refined from ~400–800 kb on chromosome 11 to just 140 kb of DNA of BAC425p24.

The DNA sequence of BAC425p24 was analyzed using computer programs that predict the presence of likely protein-coding regions of genes (exons) (GRAIL, http://compbio.ornl.gov/Grail-bin/EmptyGrailForm and GENSCAN, http://CCR-081.mit.edu/GENSCAN.html). These analyses predicted two genes encoded in BAC425p24. One was the house homolog of a previously described human GTP-binding protein gene, *Drg2* [20]. However, we found no mutations in human *DRG2* in deaf individuals from Bengkala or from the two *DFNB3* Indian families (fig. 1). The second gene was predicted to encode a novel unconventional myosin, named *MYO15* for the human gene and *Myo15* for the mouse homolog.

Shaker-2: Mutations in a Novel Mouse Unconventional Myosin Gene, *Myo 15*

The unconventional myosin on BAC425p24 was a strong candidate for *shaker-2*. There is precedent for mutations in *MYO7A*, an unconventional myosin, causing both dominant and recessive nonsyndromic deafness (*DFNA11* and *DFNB2*) and Usher syndrome 1B [21–24]. Also, mutations of *Myo6* cause deafness in mice [25]. Myosins are motor-proteins that bind cytoskeletal actin and hydrolyze ATP to produce force and movement. There are many myosin heavy-chain genes in fungi, plants and animal cells [26]. Myosin genes have an evolutionarily conserved motor domain at the amino

terminus with a C-terminal tail domain that is divergent between myosin classes (fig. 2). Myosin genes are subdivided into conventional myosins (myosin class II of muscle and nonmuscle cells) and unconventional myosins (classes I and III to XV) [17, 26]. The functions of some unconventional myosins are just now becoming known. Data indicate a role for these molecular motors in intracellular transport along actin filaments of cargo such as specific proteins, RNA and organelles. Unconventional myosins also are involved in the processes of endocytosis, regulating ion channels, localizing calmodulin, and tethering and cross-linking extension of filopodia [26–28].

We determined the complete genomic seguence of mouse *Myo15* which allowed us to screen for the mutations responsible for the *shaker-2* phenotype. Putative exons identified by computer programs were PCR amplified from *shaker-2* genomic DNA and checked for mutations by DNA sequencing. Meanwhile, cDNA libraries were screened to detect *Myo15* transcripts that would corroborate the exons identified by the prediction programs and also to identify exons missed by the computer predictions. In *shaker-2* mice a single nucleotide change was found in codon 676 within exon 19. This G to A transition mutation in the motor domain of myosin-15 causes tyrosine to be substituted for a highly conserved cysteine. The vast majority of all conventional and unconventional myosins have a cysteine at this position (http://www.mrc-lmb.cam.ac.uk/myosin/myosin.html) [29]. There is a second mutant allele of *shaker-2* designated *shaker-2^{J}*. Analysis of the complete structure of the *Myo15* gene revealed a deletion in *shaker-2^{J}* that eliminates the last 5 exons of *Myo15* and beyond [30, 31]. It remains to be determined if the *shaker-2* and *shaker-2^{J}* mutations completely eliminate *Myo15* function.

Mutations of Human *MYO15* Cause Deafness

Identification of the BAC that corrected the *shaker-2* phenotype was a springboard for identification of *DFNB3*. The sequence of the mouse *Myo15* was used to isolate human *MYO15* [17]. Three human genomic cosmid clones containing *MYO15* were isolated and 90 kb of DNA were sequenced. Predicted human *MYO15* exons were identified and confirmed by sequencing human *MYO15* cDNA synthesized from human fetal inner ear mRNA (gift from Dr. C. Morton) and from adult brain and pituitary gland mRNA. *MYO15* DNA was also sequenced from probands of the three *DFNB3* families to identify the human *MYO15* mutations that cause deafness. Three mutations in *MYO15* were found which cosegregated with deafness in the three *DFNB3* families [17] (fig. 2). The mutations of *MYO15* in Bengkala villagers and in the M-21 Indian family result in single amino acid substitutions in exon 28 within the

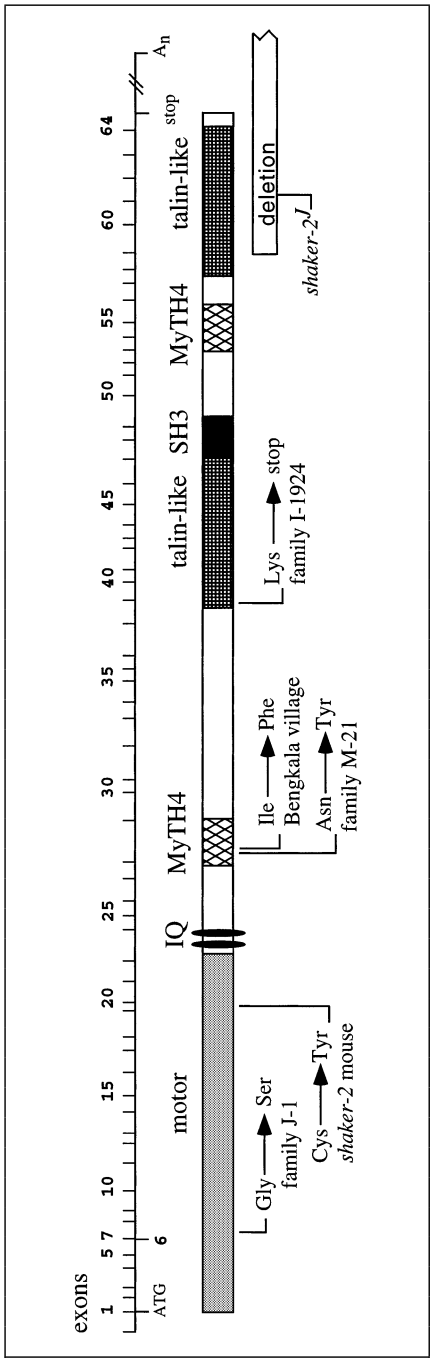

Fig. 2. Human and mouse myosin-15 mRNA structure and protein domains. A diagram of myosin-15 mRNA is shown with the predicted translation initiation and termination signals and the start positions of 63 coding exons. The longest *MYO15* open reading frame encodes a protein of 2,294 amino acids and a predicted molecular weight of ~260 kDa. The length of the longest *MYO15* RNA is about 8,500 base pairs (bp). Exon 6 is a mini-exon of only 6 bp long encoding two amino acids and is alternatively spliced. There are forms of MYO15 with and without exon 6. Exon 23 (not shown on the transcript map) is alternatively included in some transcripts from both human and mice but does not contain an open reading frame (translation stop codons in all three reading frames). Inclusion of exon 23 in a transcript may produce a short myosin-15 protein with a motor and a tail that ends shortly after the two IQ motifs. The domain organization of the myosin-15 protein is shown below the myosin-15 mRNA. Like all myosins, myosin-15 has a highly conserved motor domain that contains actin and ATP-binding sites. Adjacent to the motor domain are two regulatory (IQ) motifs which by precedent with other myosins are predicted to bind calmodulin or related calcium-binding proteins. Based on similarity to regions observed in other proteins, several functional domains are recognizable within the MYO15 tail. These include a protein interaction motif of the src homology 3 (SH3) class and two each of myosin tail homology 4 (MyTH4) and talin-like domains. MyTH4 and talin-like domains are found in a subset of unconventional myosins but their functions remain unknown. Mutations associated with profound hearing loss in humans and mice are indicated.

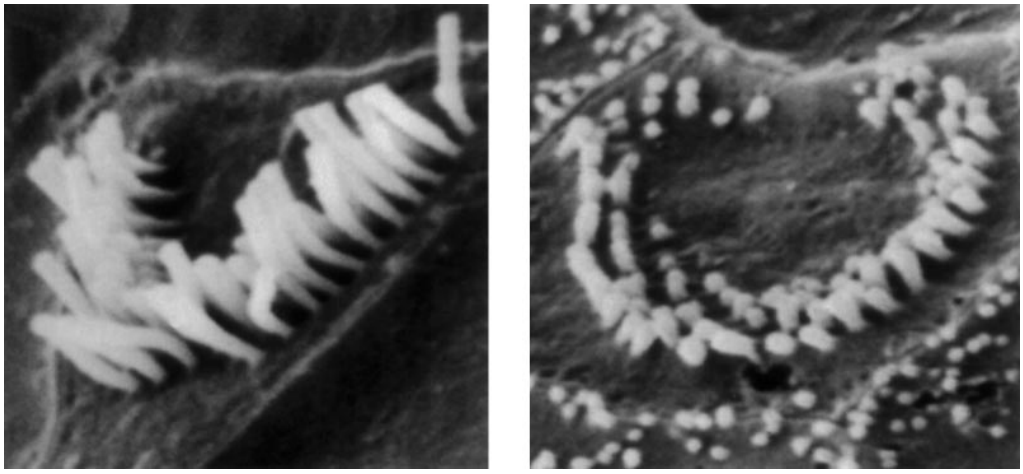

Fig. 3. The stereocilia of *shaker-2* mutants are shorter than normal. The stereocilia on a single outer hair cell from the cochlea of a normal mouse (left panel). A comparable outer hair cell from a *shaker-2* mutant mouse contains very short microvilli or stereocilia that are organized in a similar manner to those of normal mice, yet many microvilli are outside of the normal bundle. ($\times 800$ magnification in both panels)

first of two myosin tail homology 4 domains (MyTH4) (fig. 2). This domain is similar in amino acid sequence to regions found in some other unconventional myosins (human *MYO7A, C. elegans* Myo12, bovine myosin-X and Acanthamoeba HMW myosin-IV) [24]. The function of the Myth4 domain is unknown [26]. The mutation segregating in the Indian family I-1924 introduces a nonsense mutation in exon 38 which truncates the *MYO15* open reading frame resulting in a foreshortened protein or none at all [17].

Hair Cell Stereocilia of *shaker-2* are Abnormally Short

A mouse model of a human hereditary deafness is very helpful for the study of both function and developmental expression of an HHI gene. Some clues about the role of Myo15 in the inner ear can be deduced from histology and microscopy of tissues from *shaker-2* and *shaker-2*^{J} mutant mice. The inner ear tissue was dissected from the bony cochlea, the tectorial membrane was removed, and the remaining tissue containing the inner and outer hair cells and supporting cells were examined whole. The expected specialized hair cells were present, but the stereocilia on the inner and outer hair cells were very short, approximately 1/10 the normal length (fig. 3). In addition

to the short stereocilia in the hair cells of *shaker-2*, we also detected abnormally thick actin bundles within the hair cell cytoplasm by scanning confocal microscopy using rhodamine-conjugated phalloidin to visualize actin and by transmission electron microscopy. The stereocilia of the inner hair cells are essential for sensing the sound pressure applied to the fluid in the cochlea and for transmitting this information to the auditory nerve. The very short stereocilia may explain the lack of hearing in *shaker-2* mice. We hypothesize that deaf humans homozygous for *MYO15* mutations also have shortened stereocilia.

Future Studies

Many questions remain about the role of myosin-15 in the auditory and vestibular systems and in the epidemiology of deafness. To what extend do mutant alleles of *DFNB3* contribute to profound deafness in various populations? Are there 'mild' mutant alleles of *MYO15* that cause mild hearing loss or progressive hearing loss? Does haploinsufficiency (only one gene copy) for *MYO15* present in individuals with the SMS 17p11.2 deletion cause the typical mild hearing loss in SMS patients? What are the other proteins that interact with the myosin-15 tail region or are transported within the cell by myosin-15? The identification of these myosin-15 protein partners will not only be helpful in illuminating the function of myosin-15 in the auditory system, but the genes for these proteins may also be candidates for other hearing impairment genes in both mice and humans.

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The *PDS* Gene, Pendred Syndrome and Non-Syndromic Deafness DFNB4

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Recent advances in human genetics have catalyzed the identification of numerous loci responsible for non-syndromic deafness. In some cases, it has been found that different defects in the same gene can cause either non-syndromic deafness or a syndrome that includes deafness as one of its hallmark features (i.e. syndromic deafness). For example, non-syndromic deafness and Usher syndrome are caused by defects in the myosin VIIA gene [1–3]. Herein we describe another example of this phenomenon, where non-syndromic deafness and Pendred syndrome can arise from defects in a recently identified gene, the *PDS* gene [4].

An association of deafness and goiter was described by Vaughan Pendred in 1896 [5], who published an account of two profoundly deaf sisters with goiters in a single large family. This observation was extended by W. Russell Brain in 1927 [6], who reported five families with congenitally deaf children and normal hearing parents; a strict correlation between deafness and goiter was noted. Brain has thus been credited for the recognition that the disorder is genetic and distinct from other causes of combined goiter and deafness, such as iodine deficiency. These two reports are notable in that they established Pendred syndrome as a clinical entity (OMIM #274600).

Pendred syndrome is a recessive disorder whose hallmark features are deafness and goiter. Because the combination of these features can be seen in other disorders (e.g. congenital hypothyroidism), additional tests can be applied to facilitate the diagnosis. For example, patients with Pendred syndrome almost always have an abnormal perchlorate discharge test, which involves measuring the amount of radiolabeled iodide in the thyroid before and after the administration of perchlorate. In Pendred syndrome, the clearance of iodide from the thyroid is notably higher (15–80%) [7, 8]. However, a positive perchlorate discharge test is not limited to patients with Pendred syndrome [9]. The basis for the abnormal perchlorate discharge test relates to a fundamental defect in the ability to organify iodide in the thyroid [10]. Similar organification defects are associated with autoimmune disease involving thyroid peroxidase (TPO), thyroglobulin (Tg), and the TSH receptor, as seen in patients with Graves' disease or Hashimoto's thyroiditis; more severe defects are seen in patients with mutations in the TPO or Tg genes [11–13]. Unlike patients with defects in the Na^+/I^- symporter [14] (the protein responsible for concentrating iodide in the follicular cells of the thyroid gland), Pendred syndrome patients are able to accumulate iodide in the thyroid in a normal fashion [7].

The deafness in Pendred syndrome is profound (>60 dB) and sometimes progressive, and most often associated with radiologically detectable malformations of the inner ear. The classic type of defect in Pendred syndrome is a Mondini malformation [15–17], which reflects an arrest of development at approximately seven weeks of gestation. Features of a Mondini malformation include both a reduced number of cochlear turns and an enlarged vestibular aqueduct. Partial and complete forms of Mondini malformations have been reported in patients with Pendred syndrome. The clinical features found in several series of Pendred syndrome families are summarized in table 1.

A study aiming to estimate the prevalence of Pendred syndrome involved the examination of 3,535 profoundly deaf individuals from the British Isles and South Australia. 7.8% and 5.6% of adults and school-age children, respectively, were found to have Pendred syndrome [18]. The difference in these numbers likely reflects the fact that the onset of goiter typically does not occur until puberty. Of note, it has been observed that the goiter is significantly more pronounced in females compared to males [18]. Pendred syndrome is not limited with respect to race or ethnic origin; it has been found in those of European, South American, Indian, Middle Eastern, Japanese, and native South African ancestry [18]. The coefficient of inbreeding is much higher among those families with Pendred syndrome (0.00548) compared to the English population as a whole (0.000402), as expected with a recessively inherited disorder [18].

Table 1. Clinical features of Pendred syndrome

	Euthyroid	$^{+}$Per	Goiter	Mondini	EVA	PHL	Patients
Illum et al. [17]	15	15	14	8	NR	14	15
Johnson et al. [40]	8	15/15	17	17	NR	14*	17
Johnson et al. [41]	NR	14	9	12	NR	8	14
Phelps et al. [16]	NR	40	20	8	33	40	40

Various features of Pendred syndrome encountered in four clinical series are summarized. (Euthyroid, euthyroid (remaining patients are hypothyroid). $^{+}$Per, positive perchlorate discharge test. Mondini, Mondini malformation. EVA, enlarged vestibular aqueducts. PHL, prelingual profound hearing loss. Patients, total number of patients reported in the study. NR, not reported*, Some of these patients had a progressive hearing loss or delayed speech development).

The Pendred syndrome gene (*PDS*) was localized to a broad region on chromosome 7q22.3-q31.1 by linkage analysis [10, 19]. Interestingly, a locus for non-syndromic deafness (*DFNB4*) had already been assigned to the same region [20]. Using a positional cloning strategy, Everett et al. identified and isolated the *PDS* gene [4], which was found to contain 21 exons and an open reading frame of 2,343 bp. The predicted protein, pendrin, consists of 780 amino acids (86 kD) and belongs to a family of proteins, all of which appear to be anion transporters.

The two known human proteins closely related to pendrin are the down-regulated in adenoma (DRA) protein [21] and the diastrophic dysplasia sulfate transporter (DTDST) protein [22]; these share 45% and 32% amino-acid identity with pendrin, respectively [4]. Mutations in DRA cause congenital chloride diarrhea (CLD), a recessively inherited intestinal disorder of chloride and bicarbonate transport [22]. CLD is characterized by lifelong watery diarrhea with a high chloride concentration, electrolyte disturbances, and metabolic alkalosis [23]. The DRA protein, which is specifically synthesized in the intestine [24], appears to mediate the transport of sulfate, oxalate, and chloride [25, 26]. The DTDST protein is produced at particularly high levels in cartilage and lower levels in other tissues [27]. Mutations in the *DTD* gene cause diastrophic dysplasia, atelosteogenesis type II, or achondrogenesis type 1B [21, 28, 29]. The DTDST protein has been shown to be a sodium-independent, sulfate/chloride antiporter [21, 27] that operates at the chondrocyte plasma membrane [27]. In patients with diastrophic dysplasia (achondrogenesis type 1B) and (atelosteogenesis type II) [21, 30, 31], there is undersulfation of proteoglycans in cartilage. The phenotypic differences seen in these various

disorders reflect different mutations in the *DTD* gene and correlate with residual activity of the transporter [30]. Surprisingly, despite its high homology to known sulfate transporters, pendrin has been shown to transport iodide and chloride but not sulfate [32].

Numerous mutations have been identified scattered throughout the *PDS* gene [4, 33–36]. Interestingly, four mutations appear to account for 72% of mutant alleles in individuals of Northern European descent [34, 35]. Mutations in *PDS* have also been found in deaf families that do not appear to have classic Pendred syndrome. A distinct *PDS* mutation was found in a large consanguineous Indian family that included 10 profoundly deaf individuals (up to age 38 years old) but no goiter [33]. All three affected members of this family that were examined radiologically had enlarged vestibular aqueducts. In a separate study, *PDS* mutations were found in 12 unrelated individuals with: (i) sensorineural deafness and abnormal inner ear radiology or (ii) sensorineural deafness and goiter, but a normal perchlorate discharge test [35]. Mutations in *PDS* have also been found in families with inherited enlarged vestibular aqueducts and fluctuating sensorineural hearing loss [37]. In the latter study, families were enrolled based on the presence of enlarged vestibular aqueducts rather than goiter or a positive perchlorate discharge test.

It is important to point out that Pendred syndrome has been historically defined as congenital deafness and goiter [5, 6]. Advances in our understanding of this disorder have included the realization that virtually all 'classical' Pendred syndrome patients have an abnormal perchlorate discharge test and that most have evidence of a malformed inner ear (e.g. a Mondini malformation). This information might lead some to argue for a broader definition of Pendred syndrome, a trend that could become even more complicated with the recent identification of the *PDS* gene and its associated mutations. There are clearly deaf individuals with mutations in the *PDS* gene that do not have goiter. Whether such individuals have Pendred syndrome depends on which of the above definitions is applied; to some extent, this is not very interesting. What is important is that there are deaf individuals *without* goiter and with *PDS* mutations that will continue to be evaluated and assigned a diagnosis of non-syndromic deafness.

A further complication in the study of deafness loci on chromosome 7 is the apparent presence of several genes associated with deafness in close physical proximity. For example, *DFNB13* maps to 7q34-36, approximately 30 cM telomeric to *PDS* [38]. *DFNB17* maps to 7q31, approximately 7 cM telomeric to *PDS* [39]. Of interest, a family with profound prelingual, non-syndromic deafness in Madras (India) showed genetic linkage to the PDS gene but not to either the *DFNB13* or *DFNB17* regions; however, no PDS mutations were found in this family [39].

An intriguing aspect of Pendred syndrome is the fact that defects in a single gene and its encoded protein can lead to such divergent pathology as deafness associated with a malformed inner ear and thyroid disease in the form of goiter. A better appreciation of these findings requires characterizing the role of pendrin in these tissues. In each case, pendrin is likely performing a key function by transporting an anion(s) across a cellular membrane. Numerous details remain to be elucidated but represent active areas of investigation.

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Sensorineural Hearing Impairment, Non-Syndromic: DFNB5, 6, 7

Homozygosity Mapping to Localize Genes Causing Autosomal Recessive Non-Syndromic Hearing Loss

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Classification systems of hereditary hearing impairment (HHI) most commonly reflect mode of inheritance and the presence or absence of cosegregating phenotypic features. Based on these criteria, autosomal recessive non-syndromic hearing loss (ARNSHL) is the most prevalent type of hearing impairment among neonates and children [1]. In spite of its obvious clinical and social impact, until recently attempts to study ARNSHL were believed to represent an insurmountable challenge. Epidemiological studies estimated the number of ARNSHL genes at 30–100 [2, 3]. Based on the frequency of the first seven mapped ARNSHL loci (DFNB 1–7; DFN = deafness, B = recessive, digit = sequential order of discovery), we validated these figures and estimated the number of loci in southern India at 57 [4]. This degree of heterogeneity effectively precludes the use of multiple families for linkage analysis.

This limitation means that nuclear families must be used for linkage analysis, although these families are seldom large enough to generate significant lod scores. In fact, no deafness loci have been identified using a single non-consanguineous nuclear family [5]. The identification of rare genes is possible, however, if consanguineous families are studied. At the turn of the century, Garrod noted that an unusually high proportion of persons with alkaptonuria were progeny of first cousin marriages [6]. Capitalizing on this observation, Smith (1953) and Lander and Botstein (1987) proposed homozygosity mapping

(HM) as a method to localize genes that cause rare diseases [7, 8]. Until the 1990s, there were two obstacles that precluded the effective implementation of this strategy, lack of an adequate genetic map and lack of adequate computer algorithms.

Although ARNSHL is not rare, the extreme heterogeneity effectively meant that any given genetic type of ARNSHL could be considered as a rare disease. Realizing this, Fukushima et al. first applied the strategy of HM to ARNSHL in 1995 [9]. To provide a high degree of assurance that hearing loss was the result of homozygosity by descent (HBD), only consanguineous unions with 2 or more affected progeny were studied; linkage analysis was performed on those families with 3 or more deaf progeny.

To generate lod scores above 3, both the degree of consanguinity and the number of affected offspring are important. For first cousin marriages, four affected offspring are required. The first affected person establishes phase and contributes ~ 1.2 to the lod score. Each subsequent affected person increases the lod score by ~ 0.6. For second cousin marriages, only three affected offspring are required. This time, the first affected person establishes phase and contributes ~ 1.8 to the lod score. Each additional affected person increases the lod score by ~ 0.6 [10]. In a non-consanguineous union, at least four grandparents, two parents, and four affected and 12 unaffected children are required to generate a significant lod score (SLINK Monte Carlo simulations using a polymorphic marker with 6 alleles and coding the ARNSHL gene as fully penetrant [11]).

In general, the probability of HBD, α, can be expressed in terms of the coefficient of inbreeding, F, and disease allele frequency, q, as $\alpha = Fq/[Fq + (1–F)q^2]$, where Fq are those who are affected and HBD, and $(1–F)q^2$ are those who are affected due to random meeting of disease alleles (i.e. not HBD). Because $(1–F)q^2$ approximates 0 for rare alleles, α approximates 1; unlined regions are HBD with a probability of F. For second and first cousin marriages, F equals 1/64 and 1/16, respectively [8].

Among regions of the world that deserve special attention for HM are parts of Italy, Andhra Pradesh in India, many areas in the Middle East and some regions of Japan. In southern India, one-third of all Hindu marriages are reported to be between a niece and an uncle ($F = 1/8$) [12, 13]. Fukushima et al. studied this population to identify DNFB5, 6 and 7, and to estimate the number of ARNSHL loci [9, 14, 15].

DFNB5

Two different loci, one on chromosome 7q and the other on chromosome 14q, were reported simultaneously as DFNB4 [9, 16]. The 14q locus was

subsequently renamed DFNB5. This type of confusion can now be avoided by using the Hereditary Hearing Loss Homepage, which lists all hearing loss loci, and by notifying the HUGO/GDB Nomenclature Committee, which assigns numbers to loci sequentially by date-of-notification [5].

The DFNB5 phenotype is prelingual, severe-to-profound deafness. A single family of three affected children has been described [9]. D14S253 and D14S286 flank the region of HBD in the affected siblings. The cytogenic location of this locus is on chromosome 14q, in close proximity to a cochlear-expressed gene, Coch-5B2, which was identified from cochlear-specific cDNA library [17]. Coch-5B2 has now been identified as the DFNA9 gene, a dominant locus that was mapped just centromeric to DFNB5 in 1996 [18]. The DFNB5 gene has not been cloned.

DFNB6

The DFNB6 locus was identified in a consanguineous family of six [14]. Three of the four children were affected with severe-to-profound prelingual deafness. These children were homozygous by descent over a 14–20 cM interval between markers D3S1298 and D3S2400 on chromosome 3p. Telomeric and centromeric boundaries were D3S1619 and D3S1766, respectively. Several candidate genes have been mapped to this interval including zinc finger protein 35, beta-catenin, guanine nucleotide-binding protein, alpha transducing polypeptide-1, laminin S and dystrophin-associated glycoprotein. A possible animal model of DFNB6 is the spinner (sr) mouse mutant, which as a homozygote exhibits signs of defective hearing and shows the typical head tossing, circling and hyperactive behavior of the Shaker-Waltzer mutants [19]. The DFNB6 gene has not been cloned.

DFNB7

Two unrelated Indian families, one from the district of Kolhapur and the other from Tamil Nadu, were used to identify this locus [15]. The phenotype is consistent across families – affected persons have profound deafness. In the family from western India (Kolhapur), HBD was demonstrated between D9S15 and D9S166; flanking markers were D9S319 (centromeric) and D9S276. In the second family, D3S50 and D3S15 bound the region of HBD. The chromosomal assignment of DFNB7 is to 9q13-21.

Another locus, DFNB11, was mapped to the same cytogenic region by Scott et al. in two highly inbred Bedouin kindreds from Israel [20]. Although

initially placed telomeric to the DFNB7 locus, this relationship was based on the reported, but incorrect, location of D9S301 relative to D9S15 and D9S166. Correct placement of D9S301 telomeric to these two markers resulted in reassignment of the DFNB7 locus to a region that overlaps the DFNB11 locus, strongly suggesting that DFNB7 and DFNB11 are due to mutations in a single gene.

The newly defined DFNB7/11 locus is <1 cM and is bounded by D9S1806 (centromeric) and D9S769 [21]. A newly discovered Indian family has confirmed the telomeric boundary. Within the DFNB7/11 interval, Annexin I (WI-7046) and two unknown ESTs (WI-9554 and WI-9511) have been identified as positional candidate genes by PCR screening of a fetal cochlear cDNA library. The two ESTs are transcripts of a novel zinc-finger protein, ZNF216 [22]. Disease-causing mutations have not been found in either of these genes.

Although the DFNB7/11 gene has not been cloned, it remains a potentially more frequent cause of ARNSHL than either DFNB5 or DFNB6. At least four different DFNB7/11 families have been identified of different ethnicity. A murine homolog, the dn/dn mouse mutant, also exists [24]. Affected mice are profoundly deaf and show degeneration of the organ of Corti, stria vascularis and, occasionally, the saccular macula, beginning about 10 days after birth.

Conclusion

Many rare autosomal recessive loci have been identified by HM, including spinal muscular atrophy, Werner syndrome and familial Mediterranean fever [25]. Pedigrees suitable for this type of mapping strategy have come from a relatively small number of populations in North Africa, the Middle East, southern India and Japan. Cultural or religious background has considerably increased the frequency of consanguineous marriages in select populations in these areas.

Although ARNSHL is not rare, because it is a mosaic of dozens of genetic causes of deafness, with the exception of DFNB1, the frequency of each genetic contribution to ARNSHL is low. As such, HM remains a powerful strategy to map ARNSHL loci, a requisite step in the identification of genes relevant clinically to our understanding of the normal auditory process.

The role of clinicians to identify fully informative families cannot be underestimated. Farrall has elegantly described this responsibility in a review article in *Nature Genetics* [25]. 'Linkage analysis to map the human recessive disease cystic fibrosis consumed numerous scientist years and millions of research dollars. It seems that a 'cottage industry' approach to mapping rare

recessive traits is now feasible whereby a researcher armed with a catalogue of PCR primers may reasonably be expected to complete a project provided suitable inbred families can be identified and collected. The initiative rests as heavily as ever on the shoulders of our clinical colleagues.'

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Refined Genetic Mapping of the Autosomal Recessive Non-Syndromic Deafness Locus DFNB8 on Human Chromosome 21q22.3

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Two families with autosomal recessive non-syndromic deafness have previously been independently reported with linkage to the distal chromosome 21q. A Pakistani family (*DFNB8*) revealed a likely localization telomeric to *D21S212* while the disease locus (*DFNB10*) in a large Palestinian family maps to a 12-cM region in 21q22.3 with homozygosity of only the most telomeric marker, *D21S1259*. It is likely these loci are in fact different as, the Pakistani and Palestinian kindreds, while both having recessive non-syndromic deafness, are phenotypically different, having childhood-onset and congenital deafness, respectively.

Genetic, physical and transcript maps of the 21q22.3 region have been developing rapidly and three candidate genes for *DFNB8* have been excluded by mutation analyses. However, the critical region for the *DFNB8* locus is still large and contains many genes and thus identification of the gene by positional cloning will rely on combinations of the following: (i) identification of additional family members and/or families, (ii) use of additional markers on critical meioses; (iii) identification of candidate genes by function, their expression pattern, or from model organisms, and (iv) mutation analyses of all genes in the defined critical region. We are currently pursuing all the above approaches.

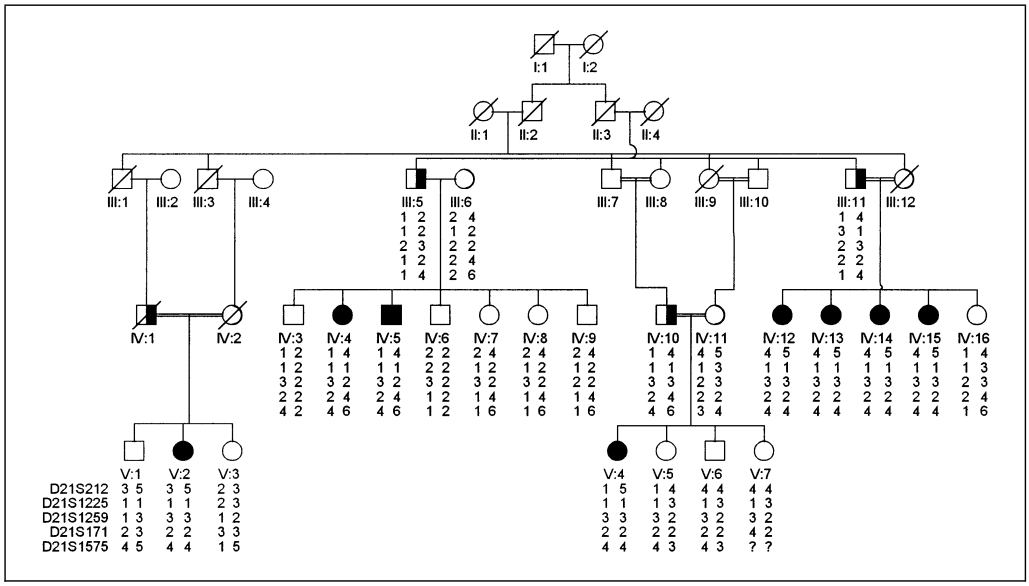

Fig. 1. The pedigree of the DFNB8 family with the most likely haplotypes shown for 21q22.3 markers *D21S212, D21S1225, D21S1259, D21S171* and *D21S1575*. The order is centromeric to telomere. The data is slightly modified from reference [1]. There is a recombinant in the normal individual V1. III6, introduces another mutant allele for the same gene into the otherwise consanguineous family.

Background

An increasingly diverse range of proteins and genes with vital functions in the development of the ear and hearing have recently been identified, mainly from genetic studies of syndromic and non-syndromic deafness.

The authors have previously studied a large family from Pakistan, containing several consanguineous marriages and segregating for a recessive non-syndromic childhood-onset deafness. Linkage analyses mapped the locus (DFNB8) to the distal long arm of chromosome 21 [5]. Due to refined marker orders, we now conclude that the disease locus is telomeric to *D21S212* on 21q22.3 (fig. 1, there is a recombinant in the normal individual V1). The highest lod score of 7.31 ($\theta = 0.00$) was obtained with the most telomeric marker, *D21S1575* [1]. *D21S212* to *D21S1575* are approximately 14 cM apart (http://www.gdb.org).

With the present availability of DNA samples from family members, it will be difficult to further refine the locus telomeric to *D21S1225* as the recombinant is between *D21S1225* and *D21S1259* and given the haplotypes

of the family members, there are unlikely to be further informative meioses (all affected individuals, except for those in branch 2, are homozygous for the markers telomeric to *D21S1225*). For this reason, identification of additional family members and/or additional families with linkage to the same region of 21q22.3 will be vital.

Individual III6 of the DFNB8 pedigree introduces a completely different haplotype into the otherwise consanguineous family (fig. 1). While several explanations for this are possible [1], it may be an independent mutant allele for the same gene. This could be taken as a priori evidence that other familial and sporadic cases of deafness due to the same gene will be found.

Another autosomal recessive non-syndromic deafness locus (*DFNB10*) has been independently reported with linkage to the distal chromosome 21q in a large consanguineous Palestinian family. *DFNB10* maps to a 12-cM region in 21q22.3 with homozygosity of only the most telomeric marker, *D21S1259*, suggesting that *DFNB10* is closest to this locus [2]. It is likely that the *DFNB10* and *DFNB8* loci are in fact different as, the Pakistani and Palestinian kindreds, while both having recessive non-syndromic deafness, are phenotypically different, having childhood-onset and congenital deafness, respectively. Alternatively, the different phenotype could be due to a different mutation in the same gene. Indeed, among the known deafness genes, mutations in the same gene may result in different clinical manifestations as well as in recessive or dominant inheritance [3] (http://hgins.uia.ac.be/dnalab/hhh/).

The genetic, physical, and transcript maps of the 21q22.3 regions are well developed [4]. From physical maps, the distances from *D21S212* to 21q ter is approximately 5.5 Mb [5–10]. There are currently 35 human chromosome 21 genes already known that map between *D21S1225* and 21q ter (for details on these genes see http://www.expasy.ch/cgi-bin/lists?humchr21.txt and [4]). Some are already known to be involved in other genetic diseases. In addition to the *DFNB8* and *DFNB10* loci discussed here, there are loci for another 3 genetic diseases that map to 21q22.3. These are: (i) Knobloch syndrome near *D21S171* [11]; (ii) a holoprosencephaly locus (*HPE1*) telomeric to D21S113 [12], and (iii) a locus for bipolar affective disorder with linkage to *PFKL* [13]. Neither these disorders, or the other genetic disorders with known 21q22.3 genes are likely to be involved in deafness.

Three candidates genes for *DFNB8* have been excluded by mutation analyses (SSCP and direct sequencing). The gene for the cysteine protease inhibitor, cystatin B, is responsible for the recessive progressive myoclonus epilepsy of the Unverricht and Lundborg type (EPM1) [14]. The majority of the mutations are due to the expansion of a 12mer (CCCCGCCCCGCG) repeat in the 5′ flanking region of the gene [15]. A myoclonus epilepsy associated with deafmutism was reported in a family showing other psychological abnormalities

[16]. Thus the 3 exons and 5' flanking region of the cystatin B gene were analyzed; however, no mutations were found.

When the localization of the DFNB8 locus was published in 1996, only one nuclear gene was known to be responsible for a form of non-syndromic deafness [17, 18]. In contrast, mutations in mitochondrial genes were known to be involved in both non-syndromic and syndromic deafness [3, 19]. Two 21q22.3 genes, HES1 and C21orf2, encode mitochondrial proteins of unknown function [20] and were thus analyzed as candidates for *DFNB8* [21, 22]. No mutations were found in either the HES1 or C21orf2 genes in *DFNB8*.

Due to its small size and involvement in Down's syndrome and other genetic disorders, human chromosome 21 serves as a model in the development of 'genomics technologies'. It will probably be the first human chromosome to be completely sequenced. The long arm (21q) is approximately 40 Mb comprising about 1% of the human genome. As of January 1999, there is approximately 20 Mb of genomic DNA sequence in databases (http://chr21.rz-berlin.mpg.de/rec22bis.html; http://www-eri.uchsc.edu/chr21/eridna.html). Despite the interest in 21q22.3 due to the genetic diseases that map there, the majority of this sequence is not derived from this region. However, the genomic sequence for the entire critical region of *DFNB8*, as it is defined today, is likely to be available by the end of the year 2000. Thus the final search for the DFNB8 gene may rely on mutation analyses of all known genes in the critical region. Prioritizing the genes for mutation analyses will obviously depend on the proposed function and expression patterns of the candidate genes. The availability of a Human Cochlear EST Database and cDNA library (http://hearing.bwh.harvard.edu/cochlearcdnalibrary.htm) [23] should facilitate analyses of expression patterns. Model organisms, particularly the mouse, may also be important in identification of candidate genes; however, there are currently no mapped phenotypes in the mouse that show synteny to the *DFNB8* locus.

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DFNB9 and DFNB12

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The progress made in deciphering the molecular basis underlying two forms of sensorineural, non-syndromic, autosomal recessive, profound prelingual deafness, DFNB9 and DFNB12, is described. At first, the respective genes were found to segregate in consanguineous Sunni families living in an isolated village in Northern Lebanon [1] and in Syria [2]. In these families, patients are affected by deafness at birth (or before 2 years of age), which impeded speech acquisition. Audiometry tests showed no response at 100 dB for frequencies $>1{,}000$ Hz and at 80 dB for frequencies $<1{,}000$ Hz. No auditory brainstem response (ABR) was observed up to 100 dB. No associated symptoms, such as balance problems, external ear malformations, mental retardation, eye, renal or integumentary abnormalities, skeletal defects, cardiac or fertilization troubles, were noted.

DFNB9

In a consanguineous family living in Northern Lebanon, we identified an isolated form of sensorineural prelingual deafness which segregated in an autosomal recessive mode and was linked to chromosomal region 2p22-23 [1]. This locus (initially named DFNB6) was then definitively referred to as DFNB9. Three additional Lebanese DFNB9-affected families living in geographically distinct regions were thereafter identified (two from Northern and one from Southern Lebanon) [3]. A DFNB9-affected family originating from Eastern Turkey has also been reported [4].

Using a combination of positional and candidate gene approaches based on the isolation of genes specifically or preferentially expressed in the cochlea [5, 6], we recently identified a novel human gene *OTOF* (GenBank Accession

AF107403), encoding otoferlin, as the causative gene for DFNB9 [3]. The *OTOF* gene extends over 21 kb and contains 28 coding exons, one 5′ untranslated (UTR) exon and one 3′ UTR exon. Otoferlin is a predicted 1230 amino acid protein of 140.5 kD, which contains three C2 domains [7] and a single carboxy-terminal transmembrane domain. We detected the same homozyzous nonsense mutation in exon 18, which is expected to lead to a truncated protein (Y730X), in all the DFNB9-affected individuals from the four unrelated Lebanese families. Expression of the mouse orthologous gene, *Otof*, was detected by RT-PCR in the cochlea, the vestibule and the brain. By in situ hybridization studies of embryonic day (E) 19.5, newborn (P0) and postnatal day (P) 2 mice, the inner hair cells of the cochlea were found to be strongly labeled, and the outer hair cells and spiral ganglion cells, faintly labeled. At P12 and P20, *Otof* labeling persisted only in the inner hair cells. In the vestibule, *Otof* expression was detected in the neuroepithelia of the utricle, saccule and semicircular canals at E19.5, P0, P2 and P20. The vestibular type I cells expressed *Otof*, whereas most of the type II sensory cells did not. No signal was detected in the supporting cells.

Otoferlin is homologous to the *Caenorhabditis elegans* spermatogenesis factor FER-1 [8] and to human dysferlin (encoded by a gene recently identified as underlying two forms of muscular dystrophy [9, 10]). FER-1 has been shown to be involved in membrane vesicle fusion [8]. By analogy, such a function has been assigned to otoferlin [3]. Due to the expression of otoferlin in hair cells, we hypothesize that this protein is likely to be involved in the traffic of the synaptic vesicles. Further studies, including the subcellular localization of the protein, the identification of its ligands and the inactivation of the gene, will help clarify the role of otoferlin in the auditory process.

DFNB12

In a consanguineous Sunni family living in an isolated village in Syria, we demonstrated linkage of an autosomal recessive, non-syndromic form of deafness, DFNB12, to the chromosomal region 10q21-22 [2]. Recombination breakpoints and homozygosity mapping localized the candidate gene to between the markers D10S529 and D10S532, delineating a chromosomal interval of 11 to 15 cM.

The homologous murine region for DFNB12, which extends at least 28 to 37 cM from the centromere of chromosome 10, contains three deaf mouse mutants, *Jackson circler* (*jc*), *Waltzer* (*v*) and *Ames waltzer* (*av*) [11]. Thus, each of these mouse mutants can be considered as a candidate mouse model for DFNB12-associated deafness. The refined mapping of the *av* locus [12],

however, renders unlikely the involvement of the orthologous human gene, in DFNB12-affected individuals.

To the same chromosomal interval as DFNB12, has been localized the gene responsible for one form of Usher syndrome type I, USH1D [13]. Two other examples of the colocalization of a form of isolated deafness and of Usher syndrome have already been reported, (i) DFNB2 and USH1B, which have been demonstrated to be allelic defects of *MYO7A* (encoding the unconventional myosin, myosin VIIA) [14–16], and (ii) DFNB18 and USH1C, which have been proposed to be allelic variants of the same gene [17].

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DFNB11

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Autosomal recessive non-syndromic hearing loss (ARNSHL) is a genetically heterogeneous disorder in which hearing loss result from mutations in any one of approximately 60 different genes [1]. Linkage studies of ARNSHL are difficult to conduct in complex populations since hearing loss caused by different genes are difficult, if not impossible, to distinguished clinically. Our means of overcoming this problem is to perform linkage studies in isolated populations or in large inbred families where fewer ARNSHL genes are likely to be segregating.

Our linkage studies have concentrated on geographically and culturally isolated Israel Bedouin kindreds with ARNSHL [2, 3]. Due to the highly inbred nature of these kindreds, it was likely that hearing loss within each extended family was due to a single ARNSHL allele which had been inherited by affected individuals from a common ancestor.

To identify the location of the ARNSHL genes in these families, a rapid genome-wide search for linkage using pooled DNA was performed [3]. Evidence for linkage to chromosome 9q was found in two Bedouin kindreds and was confirmed by LOD score analysis (Zmax 26.2, $\theta = 0.025$ with marker D9S927). The disease was fine mapped to a region between markers D9S15 and D9S927 which designated as the DFNB11 locus by HUGO Nomenclature [3, 4].

Affected individuals in both kindreds share a common haplotype in the linked region suggesting that they inherited the disease gene from a common ancestor. Representative pure tone audiograms of hearing-impaired individuals from both kindreds showed profound bilateral hearing loss of 90 to 110 decibels in all speech frequencies (550–4,000 Hz). ABR examinations were

also consistent with profound hearing impairment. History and physical examinations of hearing-impaired individuals revealed an otherwise normal phenotype with normal developmental milestones and the absence of external ear abnormalities or other medical problems [3].

Shortly before the identification of the DFNB11 locus, another ARNSHL locus, DFNB7, was mapped to the same cytogenetic region on chromosome 9q13-q21 using two regionally separate consanguineous families from India [5]. Affected individuals in these families had a clinical phenotype of profound, prelingual sensorineural hearing impairment similar to that seen in the Bedouin kindreds. Since the DFNB11 locus mapped telomeric the DFNB7 locus, it was assumed that the DFNB11 locus represented either i) a novel form of ARNSHL in close proximity to DFNB7 or ii) a relocalization of the DFNB7 locus to a region telomeric to its reported location [3, 4].

To distinguish between these possibilities, the order of key short tandem repeat polymorphic (STRP) markers was determined by constructing a radiation hybrid map, a yeast artificial chromosome (YAC) contig, and a P1-derived artificial chromosome (PAC) contig of the DFNB7/11 region [4]. These physical mapping resources indicated that several markers in the DFNB7/11 interval had been placed incorrectly on genetic maps of the region. When genotyping data was reanalyzed using the corrected marker order, the DFNB7 locus mapped telomeric to its originally reported location and overlapped a refined 1.5 Mb DFNB11 interval between markers D9S1806 and D9S769. Based on this data, it seems likely that hearing loss in DFNB7/11 families is caused by mutations in a single gene rather than mutations in two genes in close proximity [4].

Efforts to identify the gene responsible for hearing loss at the DFNB7/11 locus may be aided by similar research in progress on the *dn* (deafness) mouse. The *dn* locus resides in a region of mouse chromosome 19 that is syntenic to DFNB7/11 region of human chromosome 9 [5, 6]. It has been hypothesized that hearing loss at these loci may be caused by mutations in orthologous genes [5]. Linkage analysis and FISH experiments suggest that hearing loss at the *dn* locus may be the result of inversion between markers D19Mit41 and D19Mit14 [7]. If this inversion disrupts the deafness gene or causes a significant decrease in its expression, it may be possible to identify the resulting changes by Northern blot, greatly expediting the search for this hearing loss gene in both mice and humans.

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DFNB15: Autosomal Recessive Non-Syndromic Hearing Loss Gene – Chromosome 3q, 19p or Digenic Recessive Inheritance?

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Hereditary hearing impairment (HHI) affects 1 in 2,000 newborns [1], usually as an isolated finding referred to as non-syndromic hearing loss (NSHL) [2]. Most commonly, it is inherited in an autosomal recessive fashion (autosomal recessive non-syndromic hearing loss, ARNSHL), and although highly heterogeneous, is almost always monogenic. As a result, homozygosity mapping using multiplex consanguineous families has proven a very useful strategy to localize ARNSHL genes. Parental consanguinity assures that all affected offspring are homozygous-by-descent (HBD) over the genomic region that includes the ARNSHL gene [3]. Other intervals of HBD in these families also can be identified, but with families of sufficient size, these regions are not limited only to affected children. Homozygosity mapping was used to identify two regions of HBD, one on chromosome 3q and the other on chromosome 19p, in affected siblings of a consanguineous union, implying that one region (or possibly both) is the site of an ARNSHL gene, DFNB15 [4].

Materials and Methods

Genomic DNA Preparation and STRP Typing

Genomic DNA extracted from whole blood [5] was amplified using polymorphic markers in Short Tandem Repeat Polymorphism (STRP) screening set version 6 [6]. Regions

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of HBD were identified by screening pooled samples of genomic DNA [7], as previously described [4]. Reaction products were resolved on 6% non-denaturing polyacrylamide gels and visualized by silver staining.

LOD Score Calculations

LOD scores were calculated using MAPMAKER/HOMOZ [8]. The frequency of the DFNB15 gene was set at 0.00075 [9], and the disease was coded as fully penetrant and recessive. For STRPs showing HBD, allele frequencies were calculated in unrelated persons fitting similar demographic profiles. By imposing a minimally allowed allele frequency of 10%, a bounded influence calculation was performed to guard against inflated LOD scores.

Results

Clinical Findings

Clinical findings in this family were consistent with the diagnosis of ARNSHL. All affected individuals had a history of prelingual profound auditory impairment, with normal vestibular function as evidenced by developmental motor milestones and physical testing. Pure tone audiometry confirmed the presence of profound sensorineural hearing loss.

Linkage Mapping Using Pooled DNA Samples

After excluding linkage to known DFNB genes (Hereditary Hearing Loss HOMEPAGE, http://alt-www.uia.ac.be/u/dnalab), two genomic DNA pools were used to screen 165 STRPs evenly spaced across the autosomal human genome. Two regions of HBD were shared by affected individuals – a 39-cM interval on chromosome 3q bounded by D3S1290 and D3S1282, and a 32-cM interval on chromosome 19 bounded by D19S209 and D19S411. Lod scores of 2.78 were obtained for both regions (Chen et al., fig. 1, tables 1, 2 [4]).

Discussion

Mapping data identify either chromosome 3q or 19p as the site of a locus for ARNSHL, DFNB15. Linkage analysis at both loci generates a lod score of 2.78, close to the theoretical maximum for this consanguineous family. Independently, each region offers intriguing possibilities as a deafness locus.

The interval on chromosome 3q21.3-25.2 includes the gene for Usher syndrome type III (USH3) [10] raising the possibility that these two types of deafness are allelic. Precedence has been established with the colocalization of DFNB2 [11], DFNA11 [12] and USH1B [13, 14], all demonstrated to be

due to mutations in *MYO7A* [15–18]. An excellent positional candidate is the profilin gene, which interacts with the diaphanous protein in a Rho-mediated fashion to regulate actin polymerization. Recently, mutations in the diaphanous protein have been shown to cause DFNA1 [19].

Murine models based on homologies to human chromosome 3 include the flaky tail (*ft*), deafwaddler (*dfw*), and spinner (*sr*) mutants [20–23]. The flaky tail gene is phenotypically expressed in homozygotes as stretched skin over the feet and hands, early constriction of the tail and pinnae, and hearing defects. By day-of-life 14, the flakiness disappears and with the exception of small ears and an occasional amputated tail, homozygotes look normal. Deafwaddler homozygotes walk with a hesitant and wobbly gait, display head bobbing, and have deafness secondary to progressive degeneration of cochlear hair cells. Spinner homozygotes exhibit head tossing, circling, deafness, and hyperactivity typical shaker-waltzer mutants; inner ear anomalies include degeneration of the organ of Corti and spiral ganglion, reduction in size of the *stria vascularis*, and limited degeneration of the saccular macula.

The interval on chromosome 19p13.3-13.1 includes two genes that may be important for normal auditory function. *MYO1F* [24], an unconventional myosin, is especially intriguing, given the role of unconventional myosins in hair cell architecture and function. *Notch*, the second intriguing candidate gene, encodes a signal receptor in *Drosophila* that interacts with two ligands, *Delta* and *Serrate*, to control cell fate through a process of lateral inhibition [25]. A cell that receives a signal from *Delta* becomes committed to a specific developmental pathway and inhibits its neighbors from doing likewise; ectopic expression of *Serrate* rescues *Delta* mutants [26]. The importance of cell-cell signaling in the inner ear is suggested by the striking pattern of differentiation [27]. Functional roles of the three mammalian homologs of *Notch* have not been determined; however, avian homologs of *Notch, Delta,* and *Serrate* are expressed early in ear development [28]. A murine homolog, based on homologies to human chromosome 19, is the mocha mutant (*mh*) [29]. Homozygotes exhibit postnatal cochlear degeneration, and auditory brainstem responses, which are demonstrable at day-of-life 14, disappear by 6 months of age [30].

Mapping data identify either chromosome 3q or 19p as the site of DFNB15, however the possibility of digenic recessive inheritance remains. Multi-locus inheritance has been reported with other diseases and in other species. In humans, retinitis pigmentosa (RP) can be caused by coinheritance of specific mutations at the unlinked peripherin/*RDS* and *ROM1* loci on chromosomes 6p and 11q, respectively [31]. Heterozygotes for either mutation alone have electroretinograms similar to recessive RP, while double heterozygotes carry the clinical phenotype. In mice, an extreme form of spina bifida that resembles spina bifida occulta in humans has been reported in undulated-

Patch double-mutants recessively homozygous at both loci [32]. Even more complex is the development of overt diabetes in non-obese mice, which requires homozygosis for at least three recessive genes [33].

A model for ARNSHL that involves paired unlinked diallelic autosomal loci in which individuals are hearing impaired only if they are recessively homozygous at both loci has been presented [34], however complex patterns of inheritance of NSHL appear to be rare. Definitive proof of digenic recessive inheritance in the family described in this report must await gene cloning and mutation analysis, although the identification of other families with a similar genotype could provide compelling evidence for this type of inheritance.

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X-Linked Recessive Deafness-Dystonia Syndrome (Mohr-Tranebjærg Syndrome)

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A new type of X-linked recessive progressive hearing impairment with childhood onset was described by Mohr and Magerøy in 1960 [1] (McKusick 304700) and was called DFN-1. A restudy of the original family concluded that deafness defined as DFN-1 was the audiological component of a rare neurodegenerative syndrome characterized by deafness, dystonia, optic atrophy and dementia and caused by mutations in the *DDP* gene with only three families reported worldwide so far [2, 3]. Other syndromes are similar [4–7], and in one case preliminary results have shown a stop mutation in the *DDP* gene [6–8] indicating that Jensen syndrome (McKusick 311150) is allelic to Mohr-Tranebjærg syndrome. The condition must be suspected in all male patients with simultaneous occurrence of childhood onset of progressive sensorineural hearing loss associated with dystonia and suspicion increases with X-linked recessive inheritance. Optic atrophy of slowly progressive nature and psychiatric abnormalities with paranoid symptoms indicate that this disorder is a general encephalopathy with normal life span but a very disabling end stage of deaf-blindness associated with CNS affection.

Materials and Methods

The Norwegian family consists of six generations with information about 16 affected males of whom ten were still alive at the time of the detailed restudy [2]. They range in ages from 17–63 years. After the report in 1995 two affected males have died and autopsies are pending. Detailed results from ophthalmological examinations have been published [6] and showed no retinal involvement except in one male who had abnormalities similar to central choroidal atrophy. As part of the delineation of the phenotype, mutation studies are ongoing on published families with similar clinical affection.

Results

Audiological Features

The males are born at term and in general develop a language before the hearing starts to deteriorate from about age 18–24 months. In one case we have documented normal audiograms before hearing deteriorated [2], and the sensorineural hearing loss is progressive leading to deafness by age 10. The loss of hearing involves all frequencies equally. BAER was abnormal in those tested from age 15. No vestibular dysfunction has been demonstrable. The cognitive dysfunction was too severe to allow any advanced electrophysiological studies like ECOG.

Neurologial and Psychiatric Features

The affected males developed normally in the first years of life. Several were restless, and aggressive with uncontrollable impulsiveness from early teens. The onset of neurological symptoms varied from age 8 to early adulthood. One male who died at age 16 developed rapidly progressive ataxia from age 7. At age 8 he was unable to sit or walk and was assigned a diagnosis of dystonia musculorum performans and for a short while successfully treated with DOPA. He developed dystonic contractures, involuntary dystonic movements and died at age 16. In other family members the progression was less rapid, yet inevitable with spasticity, dystonia, dystonic contractures, mild peripheral neuropathy, and diffuse cerebral atrophy as evaluated by CT or MRI scans in all males above age 40 [2]. In one male, a SPECT scan at age 29 was normal [unpubl. data]. In addition, all affected males developed various degrees of paranoid psychiatric problems such as fear for poisoned food, an imaginary sense of foreign bodies in their eyes, fear of catching infections, repetitive handwashing and psychopathic behavior. The course was always progressive but varied from mainly leading to dystonic contractures or spasticity. Gradually they also deteriorated mentally even if formal assessment was impossible because of their compound severe disablement considered as deaf-blindness severely compromising all communication in a formal test situation.

Ophthalmological Findings

Visual complaints were very gradual in progression and in general started with moderate myopia from childhood, photophobia from about age 15 but with a lot of denial for years. The night and color vision were all preserved, but visual acuity decreased. As described in detail we found normal retinal function [9] except in one male who was for years misclassified as Usher syndrome. Bilateral large scotomas were found, and in one male thought to have Usher syndrome we found abnormalities as seen in central choroidal

atrophy. No evidence of optic atrophy was noted until autopsy of a 53-year-old male showed end-stage atrophic nerves [Tranebjærg L. and Lindal S., unpubl. data]. The lack of ophthalmological detection of optic atrophy could be due to myopia. From the minimal experience we have it seems that visual evoked potential (VEP) is abnormal long before clinical suspicion of optic atrophy presents [Tranebjærg L., unpubl. data] and VEP is recommended in known male patients in order to follow the prognosis.

Other Aspects of DDP Mutations

Several female obligate carriers were examined as a part of the restudy of the Norwegian family in order to assess if they were mildly affected. We found possibly very mild affection with neuropathy and hearing impairment in four females aged 42–72, but it was not possible to conclude firmly if it was a consequence of the mutation of the *DDP* gene or could be ascribed to age [2]. Later on, we investigated the pattern of X-inactivation in DNA from cells from different tissues in female obligate carriers [10]. The X-inactivation was skewed and more extensive studies found evidence for a cell selection phenomenon which is not yet explained [R. Plenge et al., unpubl. data] but which certainly might indicate a crucial function in the cells of the *DDP* protein.

Rehabilitation Aspects

This disorder qualifies to be considered a genetic cause of deaf-blindness and with additional complicating features like mental deterioration, psychiatric symptoms and physical disability which in total leads to an extremely complex disability. Mastering daily life and every contact with the health-care system requires a continuous expert multidisciplinary approach. In Norway, several therapists have undergone special training in order to take care of the affected family members in all stages of life.

Mutations in the *DDP* Gene

The recent identification of this novel gene shows a 1,167 bp cDNA sequence and a predicted polypeptide of only 97 amino acids with a high degree of similarity to a predicted yeast protein [3] indicating a very crucial function, yet completely unknown, in human cells. Preliminary expression studies show surprisingly wide tissue expression in fetal as well as adult brain and other tissues [3]. In the original Norwegian family, the mutation was identified as a one base deletion, 151 delT leading to a frameshift and sub-

sequently to a stop mutation with truncation of the polypeptide at codon 38. After the *DDP* gene was identified [3] it was possible to sequence the *DDP* gene and determine that a frameshift mutation was present in all affected males, and that this mutation segregated with the disease in this family. We could, therefore, design a diagnostic PCR-based analysis for examining the potential female carriers and for prenatal studies. So far only the reports on two additional families have been published, with severe mutations causing complete absence or truncation of the *DDP* protein [3], but the report only contained very minimal clinical description. The normal function of the polypeptide in humans is not known but the strong conservation through evolution [3] indicates a crucial cellular function conserved from yeast to human.

Syndromes Allelic to Mohr-Tranebjærg Syndrome

A stop mutation, 105 G→T, was identified in one affected patient from a previously reported family with Jensen syndrome [6–8]. It remains to be seen whether the mutation segregates with the disorder in that singular family with this syndrome, but it seems very probable that Jensen syndrome (McKusick 311150) is an allelic disease to Mohr-Tranebjærg syndrome.

Studies are pending regarding other published families with X-linked recessive conditions with clinical resemblance to Mohr-Tranebjærg syndrome. It would be intriguing if other mutations of the *DDP* gene might be involved in isolated X-linked recessive deafness, idiopatic dystonia or even frequent neurodegenerative diseases, but no such evidence has been published.

Implications for Genetic Counselling and Diagnostic Approach

The condition of deafness, dystonia and X-linked inheritance seems extremely rare, but it is unknown whether milder phenotypes could be the result of less deleterious mutations than those four reported so far, since they have all been frameshift/stop mutations.

We hypothesize that the family reported by Scribanu [4] and the one recently reported by Hayes [5] are very suspicious regarding *DDP* mutations. The identification of mutations of the *DDP* gene associated with X-linked deafness and dystonia opens more direct diagnostic possibilities in such families too small for linkage analysis [4–6]. From the cases published we urge that all males with the combination of deafness and dystonia are investigated for *DDP* mutations irrespective of the family history since sporadic occurrence might be due to chance or *de novo* mutations in the *DDP* gene. Sequencing

of the small *DDP* gene has become a straightforward diagnostic test in relevant patients. After demonstration of optic atrophy in the Norwegian family and *DDP* mutation in the Jensen syndrome family the clinical presentation of X-linked Deafness Dystonia Syndrome has become easier to recognize clinically.

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DFN2

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The nomenclature of X-linked deafness is confusing since some gene loci were assigned prior to molecular analysis. Thus the symbol DFN1 was assigned to the phenotype of progressive non-syndromic X-linked deafness [1] which has subsequently been shown to be a complex syndrome comprising dystonia, blindness, fractures and mental deterioration and caused by mutation in the gene *DDP* [2, 3]; DFN2 was assigned to a gene locus causing profound congenital sensorineural X-linked deafness (since a number of families with this phenotype had been described in the literature prior to linkage analysis); and DFN3 to mixed deafness with perilymphatic gusher, now known to be caused by mutation in *POU3F4*. However some families mapping to the DFN3 locus with mutations in *POU3F4* in fact may suffer from profound congenital pure sensorineural deafness [4, 5]. More recently other loci, DFN4 and DFN6, have been assigned following genetic linkage mapping of new X-linked gene loci in single families [6].

Families which conformed to the description of the unmapped DFN2 gene (profound congenital sensorineural deafness) included those described by Sataloff et al. [7], Parker [8], Richards [9] and McRae [10]. Sataloff described a five-generation family in which all the affected males had profound congenital nerve deafness, little intelligible speech and attended schools for the deaf. Hearing loss was 60–70 dB in the low frequencies down to 100 dB in the mid-frequencies [7]. The family described by Parker detailed males deaf from birth, with a loss of greater than 70 dB for all frequencies, and again most severe between 1 and 4 kHz. Audiograms of obligate female carriers in this family were reported to be normal [8]. In the family described by McRae audiograms of some of the males showed islands of hearing in the low tones and an upward rise at 4 and 8 kHz. However, two propositi, then aged 5 and 7 years, had different audiograms with a moderately severe hearing impairment with gently

sloping audiograms. Normal hearing was found in all females in this pedigree. In the family described by Richards [9], hearing losses were most severe between 0.5 and 4 kHz in affected males who were deaf from infancy and never acquired speech [10]. It was not known whether all of these families, and others reported in the literature with similar phenotypes under the heading of DFN2, had mutations in the same gene or whether the families were genetically heterogeneous. In fact, it is likely that the family reported by Richards has a genomic rearrangement 5′ of the *POU3F4* gene and is therefore suffering from deafness due to mutation at the DFN3 locus [11, and author's unpubl. data].

However, in 1996 a large British American family with congenital profound sensorineural deafness similar to that ascribed to the unmapped locus DFN2, not mapping to the DFN3 locus [12], was mapped to Xq22 and this is now the designated position of the gene locus [13]. Males in this family suffer from severe to profound prelingual sensorineural deafness and require special education. CT scan of the petrous temporal bone is normal and there is no vestibular abnormality noted on clinical testing [14]. In addition female carriers show mild to moderate hearing losses which are more pronounced in the higher frequencies. However, none of the females complain of any hearing loss clinically.

Linkage analysis of this family mapped the mutant gene between DXS990 and DXS1001, a distance of 25 cM, based on flanking recombinations. A maximum 2 point lod score of 2.91 at $\theta = 0$ was obtained with a fully informative marker at *COL4A5*. Further analysis of a male with a contiguous deletion syndrome mapping to Xq22 but who is not deaf, has narrowed down the candidate region to between markers DXS1231 and DXS1001 [15].

Several candidate genes map to this broad region but none has yet been shown to be mutated in this family [15]. These include the *COL4A5* gene mutated in Alport syndrome (hereditary deafness and nephritis), the *DDP* gene mutated in the syndromic form of deafness DFN1, and a homologue of the *Drosophila* gene, *diaphanous*. The autosomal homologue of the *diaphanous* gene is mutated in an autosomal dominant deafness in humans, DFNA1, in a Costa Rican kindred [16].

It is therefore likely that DFN2 is caused by an unknown gene in Xq22. In order to identify candidate genes for human hearing disorders, an aliquot of an unsubtracted human fetal cochlear cDNA library has been submitted to the IMAGE consortium for the generation of expressed sequence tags (ESTs) by Svorak and colleagues [17]. A number of novel cochlear-specific ESTs map to the DFN2 region and therefore appear to be promising candidates for this disorder. Future work is likely to rely on the provision of suitable candidate genes by strategies such as these given the rarity of this form of deafness and the lack of further recombinations to refine the present candidate region.

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The Ins and Outs of X-Linked Deafness Type 3

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The most frequent form of X-linked deafness, deafness type 3 (DFN3) is characterized by temporal bone abnormalities, stapes fixation, and, in most cases, a mixed type of deafness. The physical fine mapping of (micro)deletions associated with classic DFN3 enabled the positional cloning of the underlying gene, *POU3F4*. Missense mutations identified in the *POU3F4* gene of DFN3 patients are conspicuously clustered in the α3-helix of the POU homeodomain, suggesting that missense mutations outside the POU domains result in a different phenotype. Surprisingly, the *POU3F4* gene was situated outside several microdeletions associated with DFN3 and detailed analysis of the proximal region led to the identification of several DFN3-associated microdeletions 900 kb upstream of *POU3F4*. In addition, we identified a DFN3-associated paracentric inversion (q21.1q21.31) in which one of the inversion breakpoints is located 170 kb proximal to *POU3F4*. The smallest proximal deletion, which is contained in all other proximal deletions except one, merely spans 8 kb and contains non-transcribed but highly conserved sequences. These findings might be explained by the presence of a long-range transcriptional regulation element, possibly an enhancer, 900 kb proximal to *POU3F4*. Spatiotemporal expression studies indicate that *POU3F4* does not only play an important role in otogenesis, but also in brain, kidney and muscle development. In addition, *POU3F4* expression was detected in the adult mouse heart and pancreatic α-cells. The absence of overt clinical features in DFN3 patients outside the temporal bone suggests that there is a high degree of functional redundancy among different members of the POU domain superfamily.

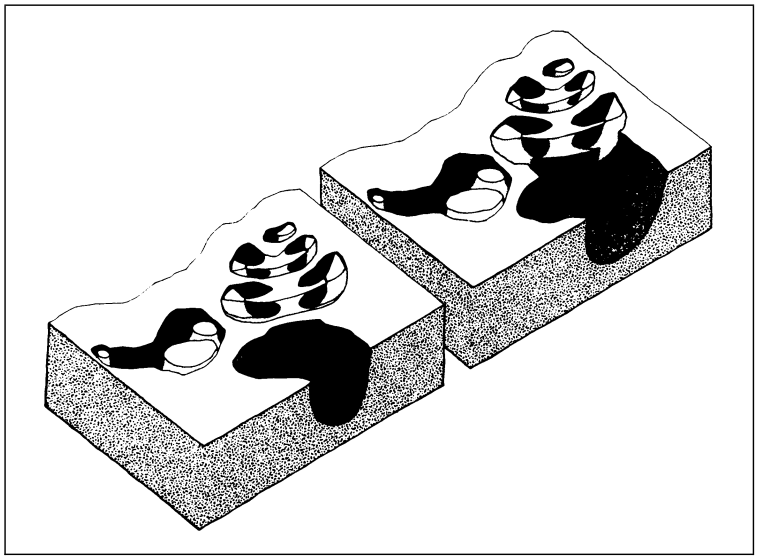

Fig. 1. Three-dimensional picture of part of the normal temporal bone (left side) and a temporal bone observed in a typical DFN3 patient (right side). In the DFN3 patient the inner ear canal as well as its connection to the inner ear are substantially widened (reprinted with permission from the Annals of Otology, Rhinology and Laryngology and Drs. A. Tang and L.S. Parnes [7]).

Clinical Features of DFN3

X-linked deafness type 3 (DFN3) is the X-linked mixed deafness syndrome with congenital fixation of the stapedial footplate and perilymphatic gusher [1]. Perilymphatic gusher refers to heavy flow of perilymph after the stapedial footplate has been surgically opened to replace a fixed stapes. The gush of perilymph actually is cerebrospinal fluid which can enter the vestibule as a result of the lateral widening of the bony internal acoustic canal [2–7] (see fig. 1). Stapes gusher is a dreaded complication because it can cause dizziness in the patient and increase the level of hearing loss [2–8]. Two family studies have presented detailed audiometric findings in affected males [1, 3, 9] and in female carriers [1, 10]. The hearing loss in the affected males has a conductive and a sensorineural component. In case there is a large conductive component of over 30–40 dB, this could be the result of a stapes fixation. Smaller conductive losses are considered to be the result of a widened vestibule leading to a loss of energy of fluid waves on their way to the cochlea. The sensorineural component is progressive; affected males will become profoundly deaf. It is

assumed that changing cerebrospinal fluid pressures are conducted along the internal auditory canal and this will effectively damage the inner ear. Trauma to the head may accelerate the progress of the hearing loss. Stapes surgery should be avoided in affected males and probably also in affected female carriers.

Mutation Spectrum in DFN3

Cloning of the POU3F4 Gene

The DFN3 gene was mapped to the proximal part of Xq by genetic linkage analyses of large families [11, 12] and by the identification of microscopically visible Xq21 deletions that are associated with complex phenotypes consisting of choroideremia, non-specific mental retardation and DFN3 [13–16]. Subsequently, smaller sized deletions were found in patients with classic DFN3 [17, 18], and the underlying gene, encoding a POU domain transcription factor, class III, factor 4 (*POU3F4*), was identified by a positional candidate gene cloning approach [19].

Mutations in the POU3F4 Gene

Mutation analysis of the intronless *POU3F4* gene can be efficiently performed by direct sequence analysis of five overlapping polymerase chain reaction (PCR) fragments spanning the 1,083-bp open reading frame (ORF) [19]. POU transcription factors share a common bipartite DNA-binding domain that comprises an N-terminal POU-specific domain and a C-terminal homeodomain [20]. Mutation analysis in 33 patients revealed 15 small mutations in the *POU3F4* gene (fig. 2), consisting of five protein truncation mutations, nine amino acid substitutions, and one deletion of two amino acids in the POU-specific domain [19, 21–24] [Y.J.M. de Kok and F.P.M.C., unpubl. results]. As expected, the protein-truncating mutations were found throughout the ORF. In contrast, the missense mutations are conspicuously clustered in the α3-helix of the POU homeodomain. According to nuclear magnetic resonance and crystallographic studies, the mutated amino acid residues at positions 230 (located in α3-helix of the POU-specific domain), 328, 330 and 334 (all located in α3-helix of the POU homeodomain) contact the major groove or backbone of the octamer-binding sequence [25]. Pro303, Ala312 and Arg323 are strictly conserved residues among members of the six classes of the POU domain superfamily [26]. Leu317 and Arg329 are conserved in at least four of the six classes of POU proteins [26]. The Phe201 and Lys202 residues are located in the α1-helix of the POU-specific domain and are highly conserved among the six classes of POU proteins [26]. These data indicate that most if not all

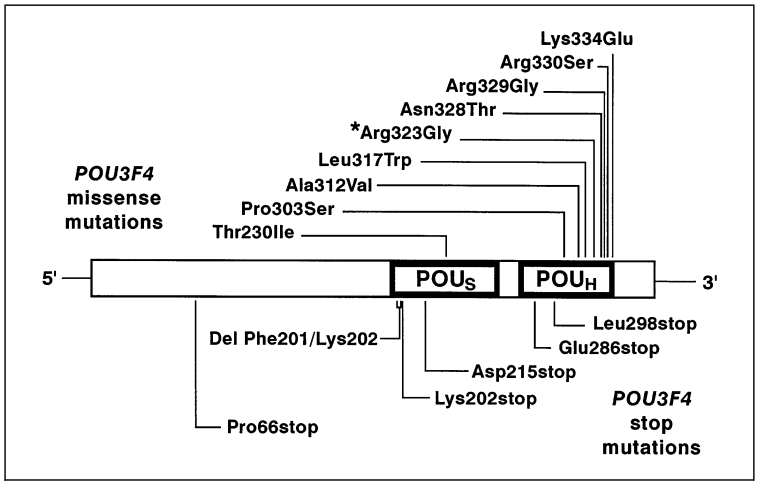

Fig. 2. Small mutations identified in the *POU3F4* gene. The POU domains and the positions of the mutations are drawn to scale. Above the predicted POU3F4 protein we depicted the missense mutations, below the truncating mutations and a 6-bp deletion resulting in the deletion of Phe201 and Lys202. The missense mutations are clustered in the POU homeodomain. The asterisk marks a mosaic de novo mutation. Fifty percent of this patients' peripheral blood cell DNA was mutant and 50% showed the normal sequence.

missense mutations as well as the deletion of Phe201/Lys202 have a severe effect on the proper binding of *POU3F4* to its target sequence(s).

Deletions, Inversions and Duplications Proximal to POU3F4

The construction of a 1,500-kb cosmid contig in Xq21.1 enabled us to identify three DFN3-associated deletions that span the *POU3F4* gene, and nine located upstream of the gene (fig. 3). The largest deletion (patient EP) is estimated to span 5 megabases (Mb) of Xq21.1 [18, 27]. Remarkably, eight proximal deletions, one of which associated with a paracentric inversion (G8314; inv(q13q21.2); ref. 28), overlap in a small chromosomal segment 900 kb proximal to *POU3F4* [27]. The smallest of these deletions comprises 8 kb (patient 2540) and was identified in a male patient belonging to one of the two DFN3 families that were previously used for the genetic linkage mapping of the gene defect [11]. The deletion in patient II/7 is situated between the *POU3F4* gene and the proximal 8-kb segment. In another DFN3 patient, a paracentric inversion (q21.1q21.31) associated with 150-kb duplication (patient 5086), dislocated the *POU3F4* gene approximately 3–5 Mb away from the proximal DNA element [29].

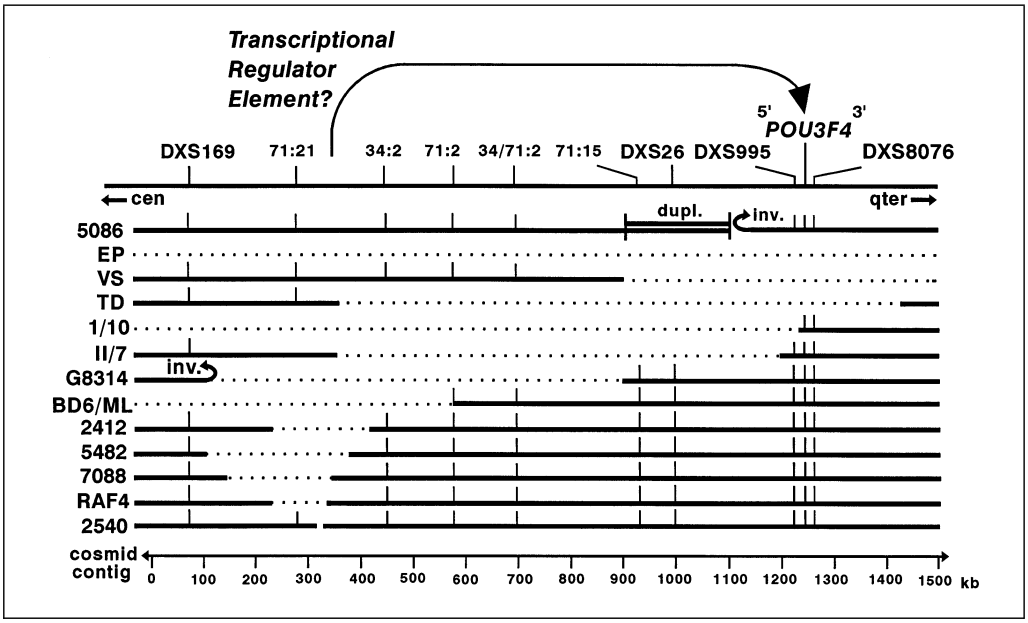

Fig. 3. Genomic map of the DFN3 critical region and chromosomal abnormalities associated with classic DFN3. Except the deletion observed in patient II/7, deletions either span the *POU3F4* gene or overlap 900 kb proximal to the *POU3F4* gene. Patient 5086 carries a paracentric inversion (q21.1q21.3) associated with a 150-kb duplication [29]. Deletions are indicated by stippled lines.

Southern blot analyses of the minimally deleted 8-kb region in man, mouse, hamster and cow revealed significant evolutionary conservation. Sequence analysis of the 8-kb DNA segment in man and mouse revealed a stretch of at least 2 kb of sequence that was 80% identical between these species [F.P.M.C. and Y.J.M. de Kok, unpubl. data]. The authors did not find a sizable ORF and extensive cDNA library screening and database comparisons did not reveal any transcribed sequences arguing against the presence of another gene in this region.

The Diverse Roles of *POU3F4* in Mammalian Embryonic Development and Homeostasis

Spatiotemporal Expression of POU3F4

POU domain proteins are expressed from the early mammalian embryo to adulthood in a defined chronological and topological pattern. Oct-3/4 is

expressed in germ cells and quickly disappears at the early embryo stage. In contrast, the genes coding for the class III and IV POU proteins display specific and restricted expression patterns in the central nervous system. *POU3F4* orthologs have been identified in the mouse (*Brn-4*), the rat (*RHS2*), the amphibian *X. laevis* (*XLPOU2*), but thus far not in the fish [30–34]. The high evolutionary conservation of POU3F4 is illustrated by the fact that *X. laevis* XLPOU2 shows 92% identity with the human POU3F4 at the amino acid level [33]. The nomenclature indicated above is not used consistently. *Brn-4* is mostly used to designate the mouse ortholog of *POU3F4*, while *Pou3f4* is the officially approved mouse gene symbol. In one rat study, *Brn-4* is mistakenly used instead of *RSH2* [35].

Because of its homology to POU domain proteins from class 3 and 4, which play important roles in brain development, early studies concentrated on *Brn-4*/*RSH2* expression pattern in the developing brain of mice and rats [30–32, 36]. The diverse roles of all four intronless class 3 POU domain proteins, i.e. Brn-1, Brn-2, Brn-4 and Tst-1, in the developing rat brain were extensively studied by Alvarez-Bolado and coworkers [35]. In situ hybridization analysis indicates that in the ventricular, subventricular and mantle layer of the forebrain neural tube, class 3 POU proteins display a unique expression pattern. Within each particular region, or layer within a region, none to four of the mRNAs may be detected, and this pattern changes during development. Expression of *Brn-4* is initially broad throughout the neural tube and becomes much more restricted in the adult, where its expression is confined to the striatum (telencephalon), the medial habenula, the paraventricular and supraoptic nuclei, and the subcommissural organ.

POU3F4 expression outside the developing central nervous system was found in human fetal kidney, the rat and mouse otic vesicle, the mouse fetal muscle, and *X. laevis* pronephros [32, 33, 37]. Surprisingly, *Brn-4* was also found to be expressed in pancreatic α-cells of adult mice [38]. Although formal proof for *POU3F4* expression in these tissues in humans has not been established, it seems clear that this gene has a much larger functional repertoire than previously thought.

Phippard et al. [39] investigated the subcellular expression of *Brn-4* in the ontogeny of the mouse ear. Employing immunohistochemistry, Brn-4 was localized in the nucleus of ventral mesenchymal cells in the earliest developmental stages of the otic capsule (10.5–11.5 days postcoitus), correlating with the onset of mesenchymal condensation. Subsequent events in formation of the bony labyrinth require extensive remodeling of mesenchyme to form openings in the temporal bone. Strikingly, during the further differentiation of the otic capsule, the subcellular localization of Brn-4 changes. The Brn-4 protein remains nuclear in those regions of the otic capsule that give rise to the cellular

regions of the mature bony labyrinth, while it shifts to perinuclear/cytoplasmic in those regions of the otic capsule that will cavitate to form acellular regions in the temporal bone, such as the scala tympani, scala vestibuli, and the inner auditory canal.

Transcriptional Regulation of the POU3F4 Gene

Although the POU homeodomain is sufficient for recognizing specific DNA sequences, the POU-specific domain enhances the binding affinity [40]. Most POU factors bind as monomers to a canonical octameric DNA-binding site, 5′-ATGCAAAT-3′. However, interaction is not limited to this DNA sequence, and sequences flanking this motif can also be important. Malik and coworkers identified the 5′-CAATATGCTAAT-3′ nucleotide sequence as the preferred DNA-binding site of Brn-4 [41]. By using DNase I footprinting analysis they also revealed that recombinant Brn-4 was able to protect five sites between –457 and +22 of its own promoter, suggesting autoregulation. Autoregulation was previously observed for the class 1 POU protein Pit-1 [42, 43]. The five protected sites are well conserved between the human and mouse *POU3F4* promoters [Y.J.M. de Kok and F.P.M.C., unpubl.].

In early *X. laevis* development, the POU3F4 ortholog XLPOU2 is induced by noggin [33]. Noggin a polypeptide secreted by Spemann's organizer in amphibians [44], a region which induces the formation of neural tissues from the overlying dorsal ectoderm [45]. XLPOU2 is initially expressed in Spemann's organizer and then in the involuting mesoderm and neuroectoderm of gastrula stage embryos. It appears that XLPOU2 is an early response gene of the endogenous neural inducer noggin and it regulates early events in neurogenesis. Whether this is also the case for human POU3F4 has not yet been investigated.

In the developing mouse skeletal muscle, myogenin-dependent *Brn-4* expression was observed together with other POU genes, i.e. *Emb* (POU class 4) and *Oct-1* (POU class 2), but, contrary to *Brn-4*, the latter two genes are also expressed in postnatal and adult muscle.

Downstream Target Genes of POU3F4

So far, three different target genes of POU3F4 have been identified, the proglucagon, the D_{1A} dopamine receptor, and the involucrin genes. In the mouse, the proglucagon gene is specifically expressed in the α-cells of the islets of Langerhans, the intestines, selected ganglia of the brainstem and the hypothalamus. Its promoter contains several AT-rich motifs which prompted Hussain and coworkers [38] to test the possibility whether POU domain genes are expressed in pancreatic α-cells. By means of RT-PCR, *Brn-4* mRNA could be readily identified. Reporter gene studies showed Brn-4-dependent activation of proglucagon transcription [38]. Okazawa and coworkers [36] reported the

regulation of striatal D_{1A} dopamine receptor gene transcription by Brn-4 and found two Brn-4-binding sites in the intron of this gene. In situ hybridization revealed colocalization of *Brn-4* and D_{1A} mRNA at the level of a single neuron in the rat striatum where this dopamine receptor is most abundantly expressed. Human involucrin, a precursor of the keratinocyte cornified envelope, is specifically expressed in the suprabasal epidermal layers. Welter et al. [46] demonstrated that several POU homeodomain transcription factors, including Brn-4/Pou3f4, suppress transcription of human involucrin in an octamer-binding site-independent manner. Probably, POU proteins interact with other proteins in the vicinity of the TATA box.

Discussion

The compact genomic structure of the *POU3F4* gene and the construction of a detailed genomic map of the DFN3 critical region enabled the detection of 28 mutations in 33 unrelated DFN3 patients. *POU3F4* mutations or small deletions were found in 15 patients, deletions encompassing the entire *POU3F4* gene in three, and structural rearrangements involving sequences proximal to *POU3F4* were found in 10 DFN3 patients. In five patients, no mutations were found, but not all DFN3 patients were investigated for structural abnormalities outside the *POU3F4* gene and the 8-kb proximal DNA element. The deletion observed in patient II/7 resides between the *POU3F4* gene and the proximal hotspot for deletions, indicating that other important sequences are disrupted. Why and how proximal deletions and inversions give rise to the DFN3 phenotype remains to be elucidated.

Several human genetic diseases have been found to be associated with structural rearrangements outside the transcription units, and are generally referred to as 'position effect mutations' [ref. 47 and references therein]. Most of these genes, such as *POU3F4*, display tissue-specific fetal expression patterns rendering the analysis of transcriptional activity very difficult. As defined by small deletions located 900 kb upstream, the distance between the *POU3F4* gene and its putative control region is the largest reported thus far [47]. Strikingly, an inversion/duplication with a breakpoint 170 kb proximal to *POU3F4* is also associated with full-blown DFN3. The relatively small size of some of the proximal deletions, as well as the absence of structural rearrangements up to 250 kb distal to *POU3F4*, argue against a role for position effect variegation-like heterochromatin spreading from nearby chromosomal regions. More likely, a specific transcriptional regular element, maybe a so-called 'locus control region' is situated in the proximal region. It will be of interest to study the presence of DNAse I hypersensitive sites

in this region and to investigate the effect of disruption of the orthologous region in the mouse.

The paucity of missense mutations in the POU-specific domain and their absence outside the POU domains cannot be due to chance. Possibly, missense mutations in these regions lead to a completely different phenotype or, alternatively, are embryonic lethal. Based on the octamer site-independent suppression activity of POU3F4 in human involucrin transcription, it can be hypothesized that POU3F4 segments outside the POU domains not only function as transactivation domains but are also implicated in POU domain heterodimerization of interactions with other transcriptional regulators. Missense mutations may have a dominant negative effect on the proper function of other (POU domain) proteins that play important roles in embryonic development.

The lack of clinically apparent abnormalities outside the human inner ear suggests that the absence of POU3F4 can be compensated for by POU domain proteins, most likely member of the class III (POU3F1, POU3F2, POU3F3) and class IV (e.g. POU4F1, POU4F3, POU4F2) POU proteins. In the developing inner ear, expression of POU3F4 and POU4F3 probably does not overlap since inactivation of the mouse ortholog of *POU4F3*, *Pou4f3* (also named *Brn-3c* or *Brn-3.1*) leads to sensorineural deafness and vestibular impairment [48]. In addition, mutations in human *POU4F3* are associated with a progressive autosomal dominant form of non-syndromic sensorineural hearing loss, DFNA15 [49].

Since all DFN3 patients display the hallmarks of this disease, a genotype-phenotype correlation cannot be made. Affected males in one family in which a 1-Mb deletion spanning *POU3F4* is inherited (family of TD; fig. 3) apart from DFN3 also show hypogonadism and thickening of the cranium [50].

None of the genes described above that are thought to be regulated by POU3F4 are likely to be involved in temporal bone modeling during otogenesis. More than 50 genes have been reported to be expressed in the inner ear [51], and it is likely that this list will continue to grow. To unravel the precise role of POU3F4 in inner ear development it will be necessary to identify further proteins acting upstream or downstream of POU3F4.

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DFN4: Non-Syndromic Autosomal Dominant X-Linked Sensorineural Hearing Impairment

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DFN4 is an X-linked dominant non-syndromic hearing impairment locus segregating to Xp21.2, a region containing the dystrophin gene. To date, deafness in two families has been mapped to this locus: the original American DFN4 family [1] and a Turkish family [2]. In both families, affected males have a profound congenital sensorineural hearing loss. On the other hand, females in both families have a milder auditory dysfunction. Affected females in the American family have a delayed onset with mild to moderate sensorineural hearing impairment beginning at the age of 30 to 35; in the Turkish family, the females have a delayed mild hearing loss affecting all frequencies [2].

Extensive evaluation of the family was performed to exclude syndromic hearing impairment and muscular dystrophy. There was no clinical or laboratory evidence of muscular dystrophy in any of the affected members. Comprehensive evaluation of the family, including examination of the neurological status, electromyography, electroneurography, high resolution temporal bone computerized tomography, and vestibular testing were all normal in both families.

Ophthalmological examination was normal in both families. Electroretinography (ERG) was performed, as some patients with Duchenne Muscular Dystrophy and Becker's Muscular Dystrophy have been shown to demonstrate stereotypical abnormalities. Interestingly, an abnormal b-wave in the ERG was identified in an affected male in the Turkish family strongly implicating dystrophin as the responsible deafness gene in the Turkish family. The ERG

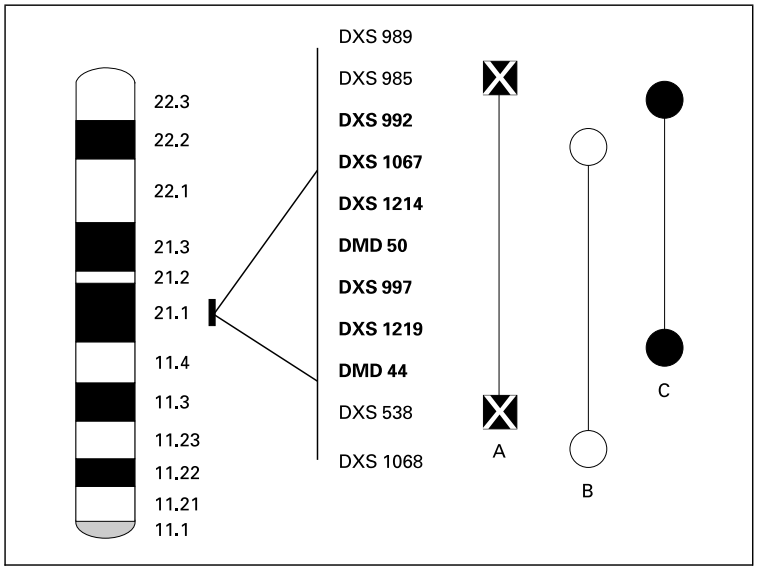

Fig. 1. Map of the X-chromosome, showing the position of the markers, the *DMD* locus as well as the disease locus of DFN4 and the Turkish family. The markers denoted in bold and italic style represent the narrowed region, in which the mutation is potentially located. 'B' represents the reported locus of DFN4, 'C' the region segregating with the disease of the Turkish family and 'A' represents the DMD locus.

is a recording of the summed electrical signal produced by the retina in response to stimulation with a light flash. This type of ERG is also reported in patients with DMD and BMD with mutations distal of exon 29 [3, 4]. Two specific dystrophin isoforms present in the retina, DP260 as well as DP71, have been implicated to play an important role in retinal physiology [5–7].

Genetic mapping of both families, the American and the Turkish family, demonstrated maximal LOD scores (2.98 and 2.59) for markers inside the dystrophin gene. The disease haplotype of the original DFN4 family spans from *DXS1068* to *DXS992* and contains the dystrophin gene as well as 1.5 Mb centromeric to the dystrophin gene (fig. 1). In the Turkish family, the linked region was entirely within the *DMD* locus (fig. 1); the disease locus could be refined to a region between *DXS1219* and *DXS985* which is distal to intron 44 spanning [2] a physical region of 1.5 Mb.

It is tempting to speculate that dystrophin or a cochlear specific isoform is involved in the hearing loss of DFN4 based upon the observation of the ERG abnormality clinically and the genetic mapping of the disease locus to *DMD*. Dystrophin is a large gene spanning 2.8 Mb at Xp21.2 and contains

at least 79 exons. There are at least 7 isoforms of dystrophin and it is likely that additional splice variants exist. Alternatively, it is quite possible that dysfunction of a yet unidentified gene in one of the large introns of the dystrophin gene is the culprit of DFN4 auditory dysfunction.

A potential role for dystrophin in hearing has been implicated by expression studies demonstrating dystrophin in the organ of Corti [8], as well as detection of significant sensorineural hearing impairment in *mdx* mice harboring a stop codon mutation in exon 23 of dystrophin [9]. Moreover, there are reports of SNHL in patients with mutations affecting the DGC complex (Dystrophin-Glycoprotein-Complex) such as secondary adhalinopathy [10], and facioscapulohumeral dystrophy [11]. Interestingly, SNHL has not been reported in patients with Duchennne or Becker Muscular Dystrophy [13], however, a systematic clinical study using contemporary audiologic testing has not been performed.

Dystrophin is linked to this DGC complex via a CC rich region. Therefore, a possible function of dystrophin in the hair cells may be to stabilize the cytoskeleton during active movements of these cells during sound exposure as shown by Brownell [14] and Zenner [15, 16]. Other possible functions of dystrophin include: a contribution to the structural organization of cell membranes; controlling calcium flux; or not yet specified functions in signalling as seen in the retina.

In summary, the clinical and genetic studies of DFN4 suggest that dystrophin is the most likely candidate gene for the disease locus DFN4.

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X-Linked Non-Syndromic Sensorineural Deafness: The DFN6 Locus

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The DFN6 type X-linked hearing loss was first described from the study of a five-generation Spanish family with non-syndromic deafness [1]. Clinical examination revealed ten affected males and seven affected females. For all patients, environmental factors were excluded as the cause of the hearing loss. No syndromic features were revealed by ophthalmologic, tegumentary, and renal exploration. The audiological study included otoscopic examination, tympanometry with acoustic reflex testing, and tuning fork tests (to detect a putative conductive component of the hearing loss), as well as pure-tone audiometry. Vestibular functions were normal and there were no complaints of tinnitus.

The hearing loss was shown to be bilateral, sensorineural and progressive, with a postlingual onset (at 5–7 years of age in males, and at the fourth decade of life in females). In males, the auditory impairment first affects the high frequencies, and later it becomes severe or profound, involving all frequencies for adulthood. In females the hearing loss is moderate or severe.

By assuming an X-linked dominant pattern of inheritance with a penetrance of about 70% for females (seven out of ten obligate carrier females were shown to be affected), DFN6 was found to be linked to markers DXS8036 and DXS8022 (maximum lod score of 5.30 at $\theta = 0.000$), in the Xp22 region. The study of two key recombinants defined a 15-cM interval for DFN6, flanked by DXS7108 on the distal side and DXS7105 on the proximal side [1]. It

should be noticed that there is another key recombinant in the family, a 4-year-old male with a recombination between markers DXS8022 and DXS987, that divides the critical interval in a distal part of 7 cM, and a proximal part of 8 cM [1]. In a recent clinical evaluation, no symptom of hearing impairment was found. Further clinical examinations in the next years should ascertain his clinical status, and therefore it should allow us to locate DFN6 proximal to DXS987 or distal to DXS8022 [1].

The hearing impairment observed in this family differs from other X-linked clinical variants in audiological features, age of onset, progression and severity. The DFN6 type is purely sensorineural, with no conductive component, and so it differs from most of the DFN3 cases (MIM 304400), which show a mixed type of deafness [2]. The DFN6 type is not congenital like the DFN2 (MIM 304500) [3] and DFN4 (MIM 300030) [4] variants. This is well illustrated by the clinical evolution of a little boy carrying the haplotype associated with deafness in the DFN6 family. When examined at the age of 4 years, no hearing loss was detected. However, further examination at the age of 7 demonstrated a significant loss in the high frequencies. On the other hand, the audiograms of the DFN6 patients in the early stages of the hearing loss do resemble those of patients with the MIM 304590 clinical variant (high-frequency sensorineural deafness whose genetic locus remains unmapped) [5]. However, the latter is nonprogressive, whereas the DFN6 type is progressive, evolving to affect all frequencies in adulthood. Finally, the DFN6 type is severe to profound in males, which contrasts with another clinical variant (moderate sensorineural hearing loss, MIM 304600, genetic locus unknown) [6, 7].

Some candidate genes have recently mapped to the DFN6 critical interval [8, 9]. Among them, the PPEF gene, which encodes a serine-threonine phosphatase with an EF-hand motif, is expressed in sensory neurons of neural crest origin, and, interestingly, in the inner ear [8]. DFN6 patients are going to be tested for mutations in the PPEF gene. If this gene were excluded, other positional candidates will be tested in order to identify the gene responsible for the DFN6-type X-linked hearing loss.

Acknowledgments

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Sensorineural Hearing Loss Associated with the Mitochondrial Mutations

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Mitochondrial DNA and Hearing Loss

Human mitochondrial (mt) DNA is a 16,569 bp-long double circular genome which encodes 13 subunits of the inner-membrane respiratory complexes present in mitochondria. As sperm cells lose their mitochondria upon fertilization, mtDNA is always maternally transmitted.

Mitochondrial disorders more often occur in tissues such as muscles and nerves which have high energy requirements. Hearing loss is often seen in mitochondrial disease, most likely because the cochlea is also highly dependent on respiratory chain metabolism [1].

Recently, mitochondrial mutations have been found to cause syndromic or non-syndromic sensorineural hearing loss (SNHL) (for references, see Hereditary Hearing Loss Homepage [2]). Syndromic hearing loss with the 32343A→G mutation has been reported to be associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) as well as diabetes mellitus. The 8344A→G and 8356T→C mutations have been reported to be associated with myoclonic epilepsy and ragged red fibers (MERRF). The 7472 ins C mutation has been reported to be associated with late-onset neurologic complaints. In addition, several large deletions were demonstrated to be associated with progressive external opthalmoplegia (PEO) and Kearns-Sayre syndrome (KSS). The 4,977-bp mtDNA deletion, the most common large-scale mitochondrial deletion found in PEO/KSS, is frequently

Table 1. Hearing loss associated with mtDNA mutations

Mutations	Associated symptoms
1555A→G	none
3243A→G	MELAS, diabetes
7445A→G	palmoplantar keratoderma
7472 ins C	late-onset neurological symptoms
8344A→G, 8356 T→C	MERRF
Heteroplasmic large deletion	PEO, KSS

found in SNHL patients as well as in the temporal bone of the patients with presbycusis [3, 4]. Two point mutations, 1555A→G and 7445A→G, were originally described as causing non-syndromic hearing loss. However, palmoplantar keratoderma was reported in the patients with the 7445A→ G mutation, therefore only the 1555A→G mutation causes 'pure' non-syndromic hearing loss. In these patients, associated clinical abnormalities other than hearing loss have never been reported. Table 1 summarizes the mutation sites which are thought to cause hearing loss. There are many mutations that are responsible for hearing loss. However, the present chapter focuses on two point mutations (3243A→G, 1555A→G) which may be more frequently encountered in comparison with other mutations or deletions.

3243A→G Mutation

Background

The 3243A→G mutation was first reported at a high rate in patients with clinical features of MELAS [5]. This mutation has also been found in diabetes mellitus patients [6, 7] and is known to be highly associated with hearing loss [8].

Heteroplasmy and Threshold Effect

In the patients with this mutation, each cell/tissue has a mixture of mutant and normal mtDNA, which is called 'heteroplasmy'. A minimum number of mutant mtDNA must be present before oxidative phosphorylation is impaired causing clinical symptoms, and this is called a 'threshold effect'. It is thought that this threshold is lower in tissues with higher oxidative demands. Since the proportion of mutant/wild mtDNA varies in different cells/tissue of an individual and in different members of the same family, the clinical (pheno-

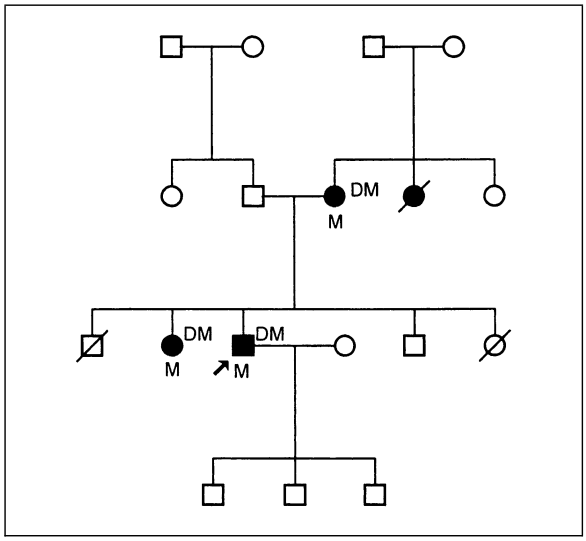

Fig. 1. Pedigree with the 3243A→G mitochondrial mutation. Filled marks indicate hearing loss. M = mitochondrial mutation; DM = diabetes mellitus.

typical) variability may be explained by the degree of heteroplasmy. It has been reported that the age of the onset of diabetes and hearing loss is correlated with the level of heteroplasmy for the 3243A→G mutation [9].

Frequency

This mutation has been reported to be present in 80% of MELAS patients [10] and in from 0.27–6% of diabetes patients [8, 11]. Hearing loss was seen in 61% of the patients with diabetes and the 3243A→G mutation [8].

Clinical Features

Hearing Loss. The common hearing loss associated with 3243A→G mutation was found to be adult onset, bilateral, symmetric and affecting the high frequencies [12–16]. Figure 2 contains overlapped audiograms from 13 previously published audiograms in addition to 2 audiograms from our cases. Some subjects reported progression of the hearing loss [13–16], and hearing loss was sometimes associated with permanent tinnitus.

Concerning the localization of the pathological site in the auditory pathways, it is generally accepted that hearing loss is primarily due to damage of the cochlea, but in some cases the cochlear nerve and its central connections are also involved in advanced stages.

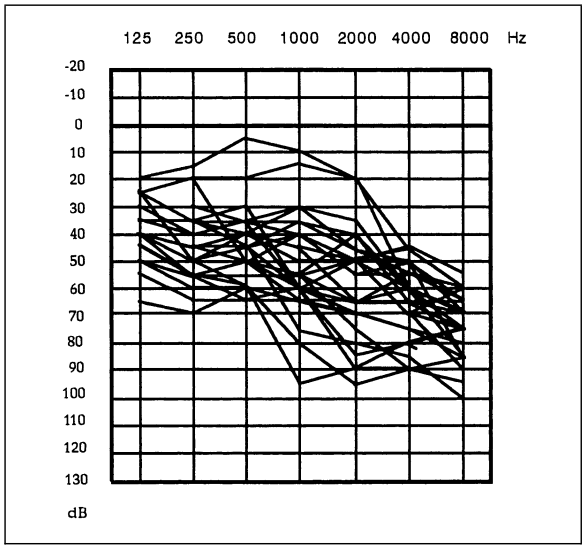

Fig. 2. Overlapped audiograms showing hearing levels with the 3243A→G mutation (audiogram data was collected from the references [12–16] and our 2 cases). Flat to high frequency involved hearing impairment ranging from mild to severe is seen.

The cochlear origin of the hearing loss is supported by the following observations [13, 14, 16]: (i) Békésy audiograms were reported to be either type I or type II pattern and recruitment phenomenon assessed by SISI testing was commonly found; (ii) no EOAEs or DPOAEs were obtained in reported cases; (iii) no elongation of intervals between the I–V waves were found in auditory brainstem response; and (iv) speech discrimination was usually normal. In some cases, however, there was evidence of brainstem involvement, in which latency delay of waves III and V was seen in auditory brainstem response and/or low speech discrimination was observed [15].

Vestibular Involvement. Although there are some cases in which the caloric response was diminished [15], the majority of cases reported in the previous literature have not demonstrated any significant abnormalities by vestibular testing, including caloric test and/or abnormal nystagmus [13–15].

Therapy. Although the use of steroids has been proven effective in one patient for a short period [13], permanent recovery was not obtained. Well-fitted hearing aids which utilize residual hearing are useful. Cochlear implantation has recently been reported to be a therapeutic option in deaf patients with the 3243A→G mutation [16].

1555A→G Mutation

Background

The 1555A→G mutation was first reported in patients with aminoglycoside-induced hearing loss, and known to be associated with a susceptibility to aminoglycoside antibiotics [17, 18]. The 1555A→G mutation also causes hearing loss even without aminoglycoside injection [19, 20], and consequently may be related to inner ear susceptibility. Recent wide screening in our department of hearing-impaired populations revealed a high rate of 1555A→G mutation (see below).

Homoplasmy

The 1555A→G mutation was found to be homoplasmic meaning that all mtDNA carry the causative mutation. However, abnormalities commonly found in other mitochondrial diseases, such as myopathy, were not found and hearing loss was the only clinical manifestation. Such tissue specificity cannot be explained simply by the high energy requirements of the inner ear, and multiple factors may contribute to this mitochondrial disorder.

Frequency

Our ongoing screening study (33 families, 128 patients, including probands as well as family members, as of September, 1998), has revealed that approximately 3% of the subjects who visit our clinic because of hearing loss have the 1555A→G mutation [21], suggesting that there may be a great number of subjects with this mutation within the Japanese population. It should be noted that this mutation is more frequently found in the patients with aminoglycoside injection history, and special attention should be paid to those patients.

Clinical Features

Hearing Loss. Although individual hearing levels varied widely, ranging from normal to severe, common features were that hearing loss is usually bilateral, symmetric, and mainly affecting the high frequencies [19, 22]. Average hearing levels are shown in overlapped audiograms (fig. 3). A progressive nature was sometimes seen [19, 22]. Individual variations were found within families and hearing loss was usually associated with permanent tinnitus [19, 22].

In patients with a history of aminoglycoside injection, the age at the time of injection had no relation to the severity of the hearing loss. Hearing loss usually occurred within three months after injection, and in some cases it occurred after only 1 injection [19].

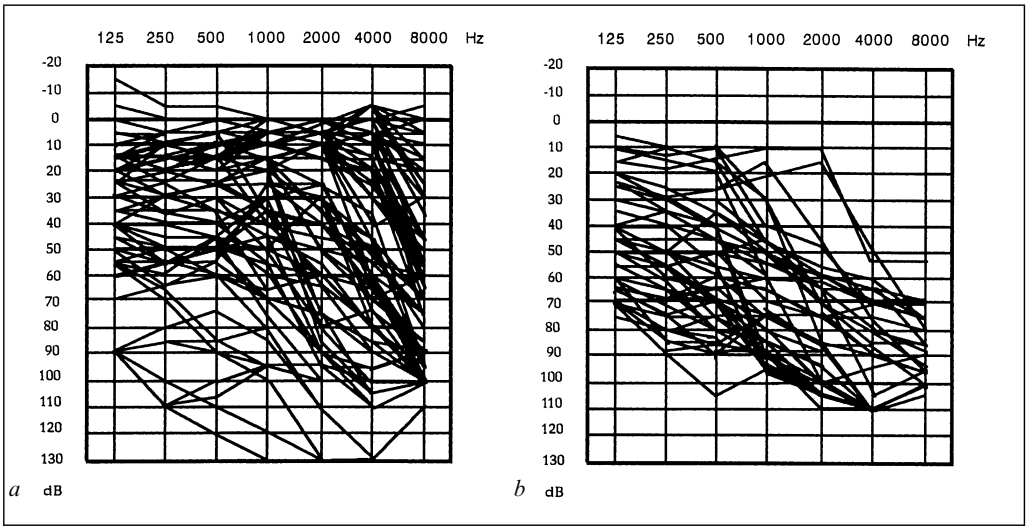

Fig. 3. Overlapped audiograms showing hearing levels with or without aminoglycoside injection. *a* Audiograms collected from 62 subjects who have 1555A→G mutation but no history of aminoglycoside injection. A wide variety of hearing impairment from normal to high frequency involved mild to severe hearing loss is noted. *b* Audiograms from 24 subjects with aminoglycoside exposure. A more profound hearing loss is noted.

Hearing loss was sometimes seen in family members with no known history of aminoglycoside exposure (fig. 4) but in these cases the hearing loss was usually milder (fig. 3). As these individuals had the 1555A→G mutation, subjects with this mutation may be genetically sensitive to other environmental factors. Therefore they may be at risk of hearing loss, even in the absence of exposure to aminoglycosides. Based on detailed anamnestic evaluation the hearing loss of these patients is considered not to be congenital.

It was suggested that the SNHL originated in the inner ear since most cases showed type II pattern in Békésy audiograms. Elongation of intervals between the I-V waves in auditory brainstem response were not found, suggesting no brainstem involvement [19]. The normal speech audiometry also indicated that the hearing loss was not of central origin. One patient bearing the 1555A→G mutation recently showed excellent auditory performance with a cochlear implant [23]. This finding supports the theory that hearing loss is due to damage of the inner ear rather than of the cochlear nerve and its central connections.

Vestibular Involvement. In patients with the 1555A→G mutation, no significant abnormalities were found in vestibular testing, such as the caloric test, the rotatory test, and dynamic posturography [19].

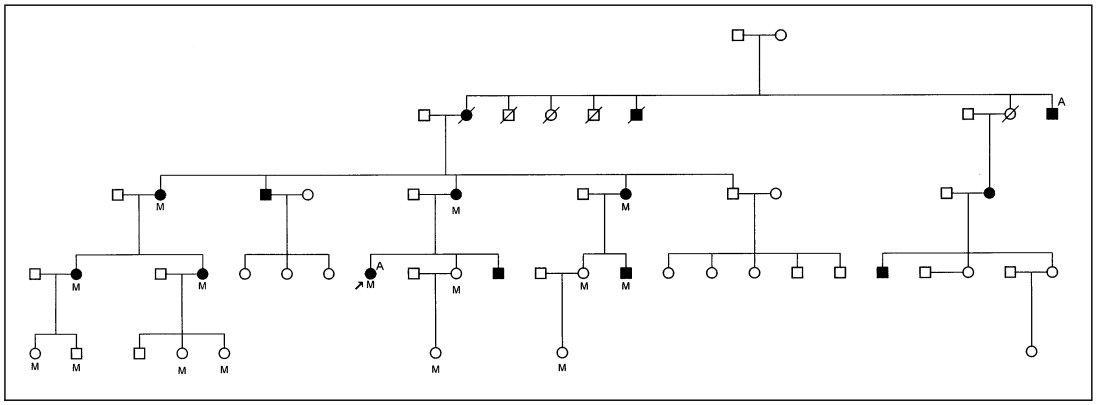

Fig. 4. Pedigree with the 1555A→G mitochondrial mutation. Filled marks indicate hearing loss, A = known history of aminoglycoside injection. M = Mitochondrial mutation.

Therapy. No effective treatments for SNHL due to aminoglycoside exposure have been clinically approved yet. These patients should receive prescriptive, well-fitted hearing aids to utilize any residual hearing. Cochlear implantation was recently successfully done for a profound hearing loss patient with the 1555A→G mutation [23].

Counseling. In Japan, as in many other countries, aminoglycoside usage has decreased due to the serious side effects and the development of viable alternatives. However, care should also be exercised with the new generation of aminoglycoside antibiotics with more minor side effects, which are now in use in Japan and Europe, as we recently experienced two cases of hearing loss after short-term exposure in patients with the 1555A→G mitochondrial mutation [24]. Before the use of aminoglycoside antibiotics for individuals in high-risk populations, a genetic background check should be performed. We now counsel our patients with 1555A→G mutation about the risks of aminoglycoside antibiotic exposure for them and their families [25].

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Histopathology of the Inner Ear in DFNA9

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DFNA9 is characterized by adult-onset, progressive sensorineural hearing loss with vestibular dysfunction that is caused by missense mutations in a novel cochlear gene, *COCH* (see article by Eavey et al. q.v.). The histopathological changes in the inner ears in this disease are somewhat unique (degeneration and acellularity of the spiral ligament, spiral limbus and stroma of the cristae and maculae) and to date, have not been observed in any other disorder affecting the auditory or vestibular systems. Indeed, it was the uniqueness of the pathologic changes that led to the initial recognition of this disorder. In 1986, one of the authors (S.N.M.) made the observation that the temporal bones acquired seven years apart from two individuals bearing the same last name showed this unique inner ear pathology. Both sets of temporal bones had been obtained through the former National Temporal Bone Banks (NTBB) donor program (now the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry). Further inquiry soon established that the two individuals were brothers and belonged to a kindred (designated 1Su) with autosomal dominant, adult-onset sensorineural hearing loss. The acquisition and study in 1987 of a set of temporal bones (again, via the NTBB program) from another individual with the same unique pathology led to the identification of a second kindred (designated 1W; many family members are patients of author J.B.N.) with similar clinical features of genetic hearing loss. A third kindred (designated 1St) was also discovered (author F.H.L.) by examination of temporal bones at the House Ear Institute, again, based on the unique nature of the inner ear changes. Descriptions of the clinical, au-

diometric and histopathologic features of these three unrelated kindreds [1–4] were followed by linkage studies establishing the locus for the disorder [5], and finally, identification of the gene and its mutations [6, 7].

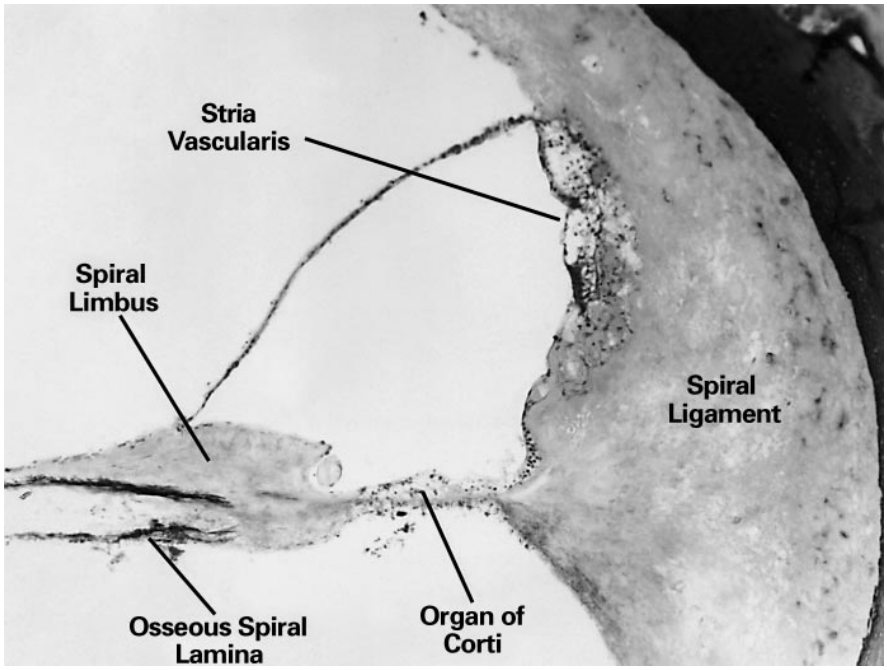
Clinical Features

In the three kindreds examined, DFNA9 is characterized by hearing loss beginning in adult life that slowly progresses to profound deafness over 20–40 years. The hearing loss is sensorineural, bilateral, generally symmetric, worse for the higher frequencies and slowly progressive. Amplification is helpful initially, and cochlear implantation has helped many patients in late stages who have developed profound hearing loss. Postimplant performance has generally been above average. Affected individuals also have symptoms of vestibular dysfunction such as unsteadiness, mild imbalance and ataxia in the dark. Spinning-type vertigo is rare.

Histopathology

We have examined the temporal bones from two affected individuals from kindred 1W, two affected individuals from kindred 1Su and one affected individual from kindred 1St. All individuals were profoundly deaf at the time of death and all inner ears show changes which are remarkably similar. The inner ear is fully developed. The cochlea shows widespread and diffuse degeneration of the spiral ligament (including the spiral prominence) and the spiral limbus, with marked loss of cellularity and replacement by an eosinophilic, uniformly staining, acellular material (fig. 1a). The same eosinophilic material also involves the proximal portion of the basilar membrane near the end of the osseous spiral lamina. The stria vascularis is generally preserved and shows patchy areas of mild to moderate atrophy. The organ of Corti shows variable losses of outer and inner hair cells. There is a severe and diffuse loss of the peripheral processes of the spiral ganglion cells. The cell bodies of the spiral ganglion show a variable degree of loss, which is severe in some bones. The loss of spiral ganglion cells is greater in the basal turn. Mild to moderate endolymphatic hydrops is an inconsistent feature and is seen in some cochleae.

The vestibular system shows diffuse, severe degeneration of the stroma of all maculae (fig. 1b) and cristae (fig. 1c) with marked loss of cellularity and replacement by an eosinophilic, uniformly staining, acellular material, similar to that seen in the cochlea. There is severe loss of sensory hair cells within all the vestibular end organs and a severe loss of the peripheral processes of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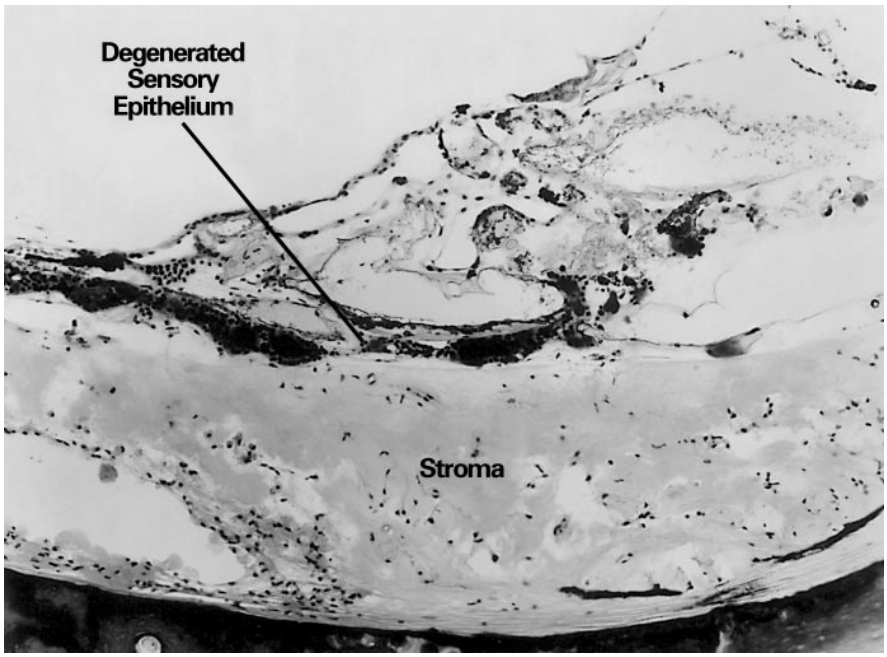
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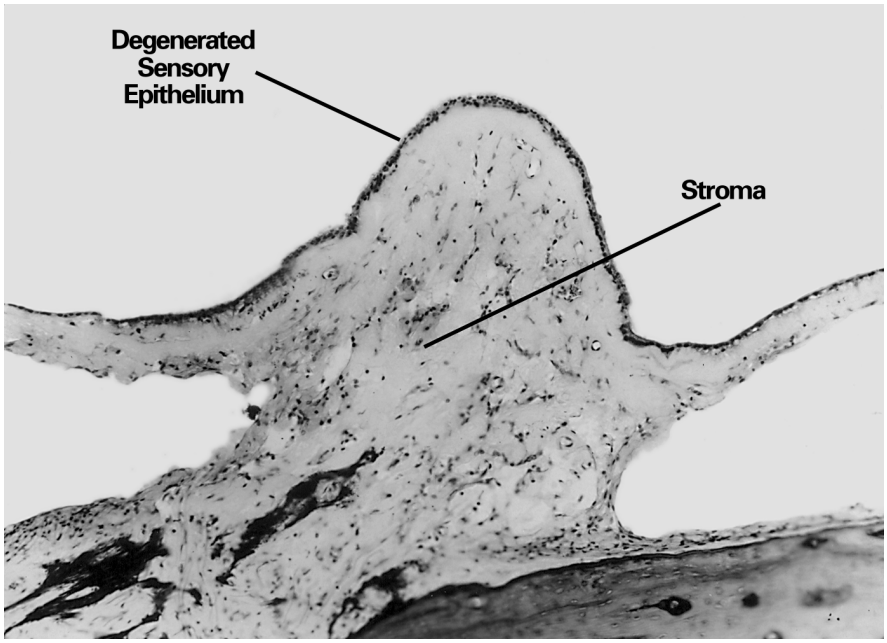

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Fig. 1. Showing the histopathology of the inner ear in DFNA9. *a* Cochlea: basal turn from affected member of kindred 1St, male age 71 years. There is severe loss of cellularity of the spiral ligament and spiral limbus with replacement by an eosinophilic-staining acellular material. The stria vascularis shows partial atrophy. There is no endolymphatic hydrops. The organ of Corti shows loss of hair cells and no nerve fibers are evident within the osseous spiral lamina. × 130. *b* Saccule: macula of saccule from affected member of kindred 1W, female aged 86 years. The stroma shows marked loss of cellularity and severe loss of nerve fibers. The sensory epithelium is degenerated. × 130. *c* Lateral canal: crista from affected member of kindred 1Su, male age 81 years. There is severe loss of hair cells within the sensory epithelium with only supporting cells remaining. The stroma shows diminished cellularity with replacement by an eosinophilic staining acellular substance, similar to that seen in the saccular macula and in the cochlea. There is also severe loss of nerve fibers. × 130.

Scarpa's ganglion. There is also marked thickening of the walls of the vestibular membranous labyrinth and perilymphatic trabecular meshwork. In some temporal bones, there is degeneration and encapsulation of the otolithic membrane of the saccule, collapse and distortion of the walls of the membranous labyrinth and formation of new bone and fibrous tissue within a semicircular canal. Examination of a temporal bone from an unaffected member of kindred 1W who died at age 59 years showed cochlear and vestibular sense organs to be normal for age without any degeneration of the kind observed in affected individuals.

Pathophysiology

Analysis of *COCH* indicates that its structural features are consistent with that of a secreted protein [6, 7]. In situ hybridization studies on the chicken inner ear have shown that this protein is expressed in areas which correspond to the human spiral ligament, spiral limbus, and stroma of the cristae and maculae [7]. These are also the areas where there is marked loss of cellularity and replacement by an eosinophilic, uniformly staining, acellular material. No signal was detected in the chicken inner ear in areas equivalent to the human organ of Corti, stria vascularis or the spiral ganglion cells. Based on these observations, we hypothesize that the *COCH* mutations may initially result in dysfunction of the spiral ligament of the cochlea, thereby interfering with fluid and ion regulation. The spiral ligament is known to be richly endowed with a wide variety of enzymes and other proteins that are critical for ion homeostasis and normal cochlear function [8, 9]. Spiral ligament dysfunction would lead to a loss of endolymphatic potential and would be predicted to result in a downsloping sensorineural hearing loss, similar to that observed in the initial stages of the disorder. The authors also hypothesize that dysfunction of the ligament and related supporting tissues (spiral limbus and the tissue within the spiral lamina) leads to secondary losses of hair cells and peripheral processes of the spiral ganglion cells, further aggravating the hearing loss. This hypothesis could be tested by studies on a knockout mouse model, and also by examination of temporal bones from individuals (carrying the mutation) prior to onset of hearing loss, and from affected individuals in the early stages of the disease. An alternative hypothesis for the hearing loss [1] is that the deposition of the eosinophilic material in the spiral lamina near the habenula perforata may have a direct toxic effect on the peripheral processes of the spiral ganglion cells.

Our temporal bone studies suggest that all individuals with hearing loss would have some degree of vestibular dysfunction. Such dysfunction may not be apparent clinically since the vestibular degeneration probably occurs very slowly and allows for central compensation to occur. Objective vestibular testing has not generally been done on these individuals but would be predicted to show abnormalities consistent with bilateral hypofunction of the semicircular canals and the otolithic organs.

Future Studies

Identification of the gene mutations now allows for genetic testing and counseling for families with the disease, and will also enable epidemiological

studies to be done to determine the prevalence of DFNA9. Much remains to be discovered about the precise location and functional role played by the *COCH* protein. The relationship between the protein and the apparent deposition of acellular, eosinophilic material in the inner ear needs to be clarified. Additional human temporal bone studies including immunostaining and in situ hybridization, as well as studies using mouse models can potentially provide some answers. Elucidation of the pathophysiology of DFNA9 may also permit gene therapy in the future to prevent the onset or progression of hearing loss and disequilibrium.

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Histopathologic Findings of the Inner Ears with Alport, Usher and Waardenburg Syndromes

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Alport syndrome [1], Usher syndrome [2] and Waardenburg syndrome [3] are characterized by hereditary sensorineural hearing loss. Recent studies have provided detailed information about genetic mutations associated with these syndromes. However, an understanding of the mechanisms that produce sensorineural hearing loss requires detailed information about histopathologic changes in these conditions that produce hereditary sensorineural hearing loss. This chapter presents both the histopathological findings of the inner ear in temporal bones from cases of Alport syndrome, Usher syndrome and Waardenburg syndrome and a review of similar cases in the literature.

Temporal Bone Histopathologic Findings

Alport Syndrome (*39-year-old male case*)

Right Ear. The organ of Corti, the cochlear nerve and the spiral ganglion were within normal limits. There was a partial atrophy of the stria vascularis, with basophilic calcium-like deposits in the basal and middle turns of the cochlea. There was also mild atrophy of the spiral ligament. Reissner's membrane was collapsed. There was a slight inner ear hemorrhage in the scala tympani of the basal turn, but no red blood cells were seen in the cochlea aqueduct. The utriculus, the sacculus and the semicircular canals were within normal limits. However, the vestibular aqueduct and the endolymphatic sac were hypoplastic and tubular, rather than showing the flattened, funnel shape

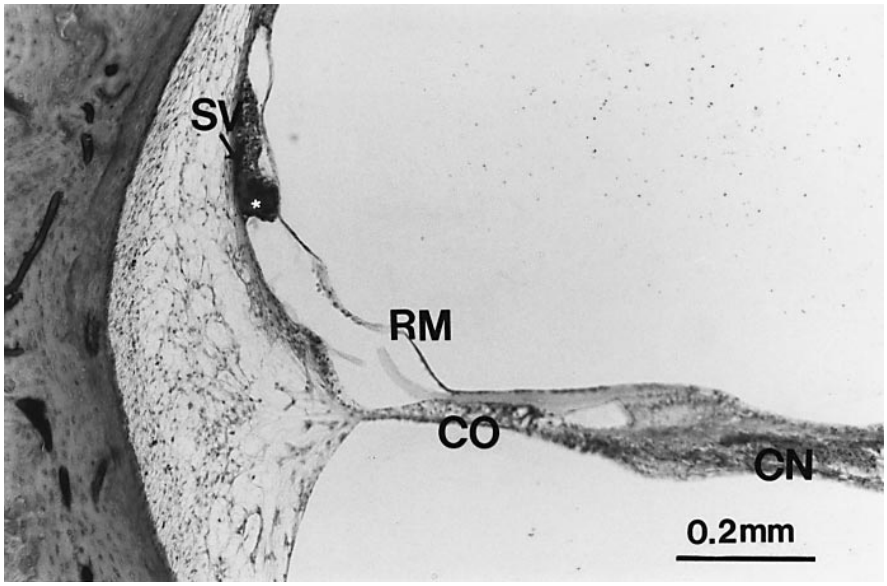


Fig. 1. The middle turn of the left cochlea of 39-year-old male with Alport syndrome is present. Basophilic calcium-like deposits (*) were seen in the atrophic stria vascularis (SV). Reissner's membrane (RM) was collapsed. The organ of Corti (CO) and the cochlear nerve (CN) were noted to be almost normal. Bar indicates 0.2 mm in length. H & E stains.

in normal specimens. The rugose portion of the endolymphatic sac was less rugose than normal.

Left Ear. Pathological changes in the stria vascularis and Reissner's membrane were similar to the right ear (fig. 1). In addition, the following pathologic findings were noted. The number of the cochlear nerve and the spiral ganglion cells were slightly reduced in the lower basal turn of the cochlea (fig. 2). Eosinophilic precipitate was seen in the scala media (fig. 2) and the scala vestibuli of the cochlea and in the endolymphatic fluid spaces of the utriculus and the lateral semicircular canal. The hypoplastic, tubular vestibular aqueduct and the endolymphatic sac were straight (rather than curved) and were located anterior to their normal position. Other structures were within normal limits.

Usher Syndrome (*55-year-old male diagnosed originally as type II*)

Temporal bone specimens were obtained by Dr. A. Kumar, Chicago, Illinois and by his request, processed by us for histopathologic analysis [4].

Right Ear. The organ of Corti was atrophied, especially in the basal turn of the cochlea; atrophy of the stria vascularis and the saccular macula were

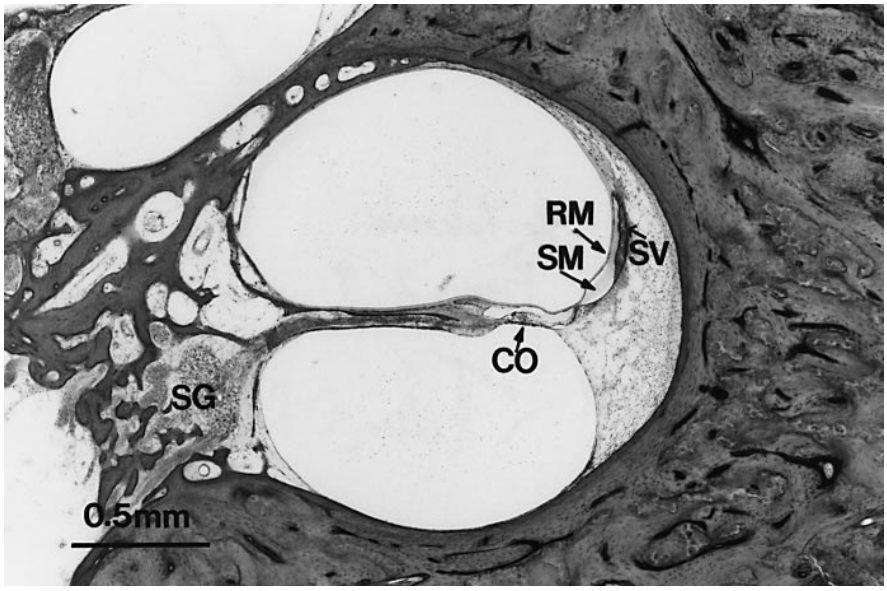


Fig. 2. Anterior part of the lower basal turn of the left cochlea in the same case as fig. 1 is shown. There was slight atrophy of the stria vascularis (SV) with basophilic deposits. Reissner's membrane (RM) was collapsed. Eosinophilic precipitate was seen in the space of scala media (SM). The organ of Corti (CO) was within normal limits but the spiral ganglion cells (SG) were slightly decreased. Bar indicates 0.5 mm in length. H & E stains.

also apparent (fig. 3). The inner and outer hair cells in the organ of Corti could hardly be identified. The number of the spiral ganglion cells was reduced markedly to approximately 30–50% of the normal population in the basal and middle turns of the cochlea (fig. 4). Blood vessels in the modiolus were congestive. The endolymphatic space in the pars inferior was collapsed. Small amount of bleeding was observed in subepithelial space of the endolymphatic sac (rugose portion) within the vestibular aqueduct.

Left Ear. Atrophy of the organ of Corti, the stria vascularis, and the saccular macula and collapse of the endolymphatic space in the pars inferior were milder in the left ear than in the right ear. Other findings were within normal limits.

Waardenburg's Syndrome

This 3-year-old African-American girl was diagnosed originally as a case of type II Waardenburg syndrome with Hirschsprung's disease [5]. In the most recent terminology, though, this case would be regarded as type IV Waardenburg (i.e. Shah-Waardenburg syndrome) [6].

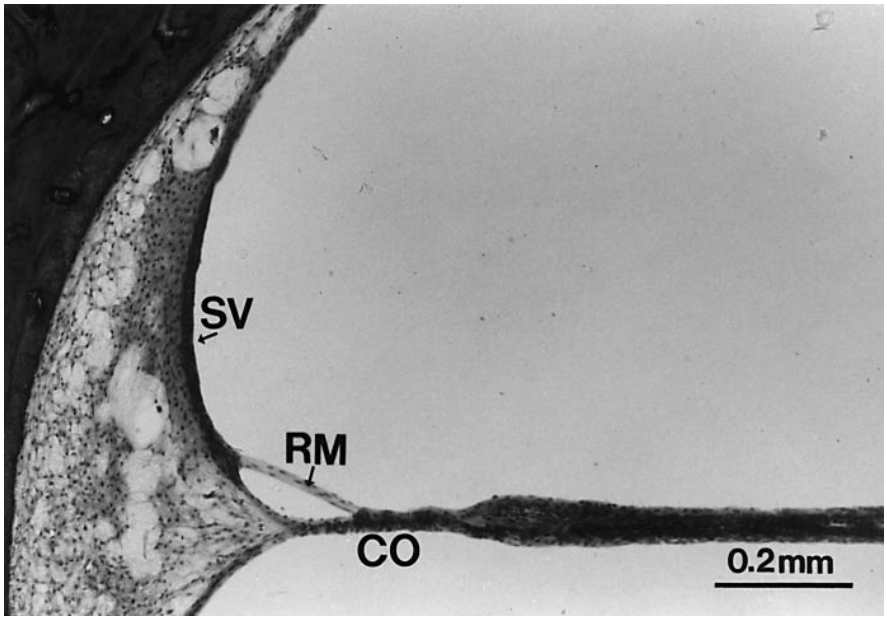


Fig. 3. A photomicrograph shows the anterior part of the basal turn of the right cochlea of 55-year-old male diagnosed as Usher's syndrome type II. Atrophy of the organ of Corti (CO), atrophy of the stria vascularis (SV) and collapse of Reissner's membrane (RM) were observed. Inner and outer hair cells could be hardly identified. Bar indicates 0.2 mm in length. H & E stains.

Right Ear. Melanin pigmentation was absent in all parts of the ear, including the modiolus (fig. 5a, b). In the cochlea, the organ of Corti was atrophic, the supporting cells were barely recognizable, and the hair cells were difficult to identify. The tectorial membrane was either collapsed or rolled and covered with an epithelial lining. Homogenous basophilic substances filled the extracellular spaces of the stria vascularis and there were no blood vessels in the intermediate layer. The blood vessel normally present in the spiral prominence was absent. Reissner's membrane was collapsed and adherent to the organ of Corti and the stria vascularis (fig. 6a, b). Evidence of hemorrhage was also seen in the perilymphatic and endolymphatic fluid spaces at the hook portion of the cochlea. The membrane of the saccule was collapsed, producing stenosis of the endolymphatic space. Evidence of massive hemorrhage, including macrophages, extended into the subepithelial region of the endolymphatic sac. The saccular macula was atrophic; it contained marked intercellular spaces and conglomeration of otoconia was noted.

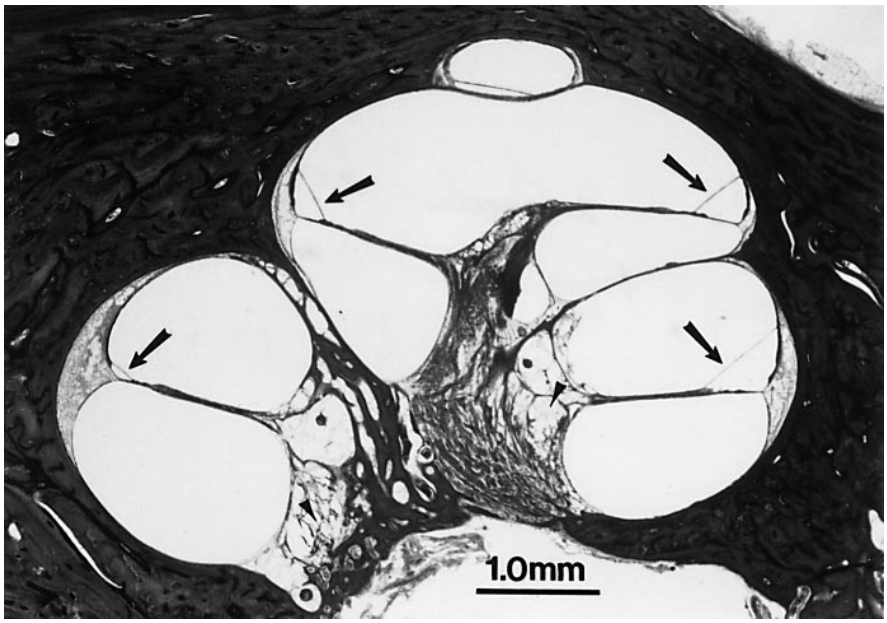


Fig. 4. Midmodiolar section of the right cochlea of the same case as figure 3 is demonstrated. Collapse of Reissner's membrane (arrows) in all turns and decrease in number of the spiral ganglion cells (arrowheads) in the basal and middle turns of the cochlea were observed. Bar indicates 1.0 mm in length. H & E stains.

Left Ear. Pathological findings were similar to the right ear. However, unlike the right ear, there was no pathology of the endolymphatic sac.

Literature Review and Discussion

Table 1 summarizes the histological findings of the inner ear for these 3 syndromes.

Alport Syndrome

Alport syndrome is a group of hereditary diseases that are characterized by progressive defects of capillaries in the glomerular basement membranes of the kidneys (glomerulonephritis), with various nonrenal features including progressive sensorineural hearing loss, ocular manifestations ('dot and fleck' retinopathy in 85% of affected adult males, anterior lenticonus in 25% of affected adult males and posterior polymorphous corneal dystrophy [7]), and

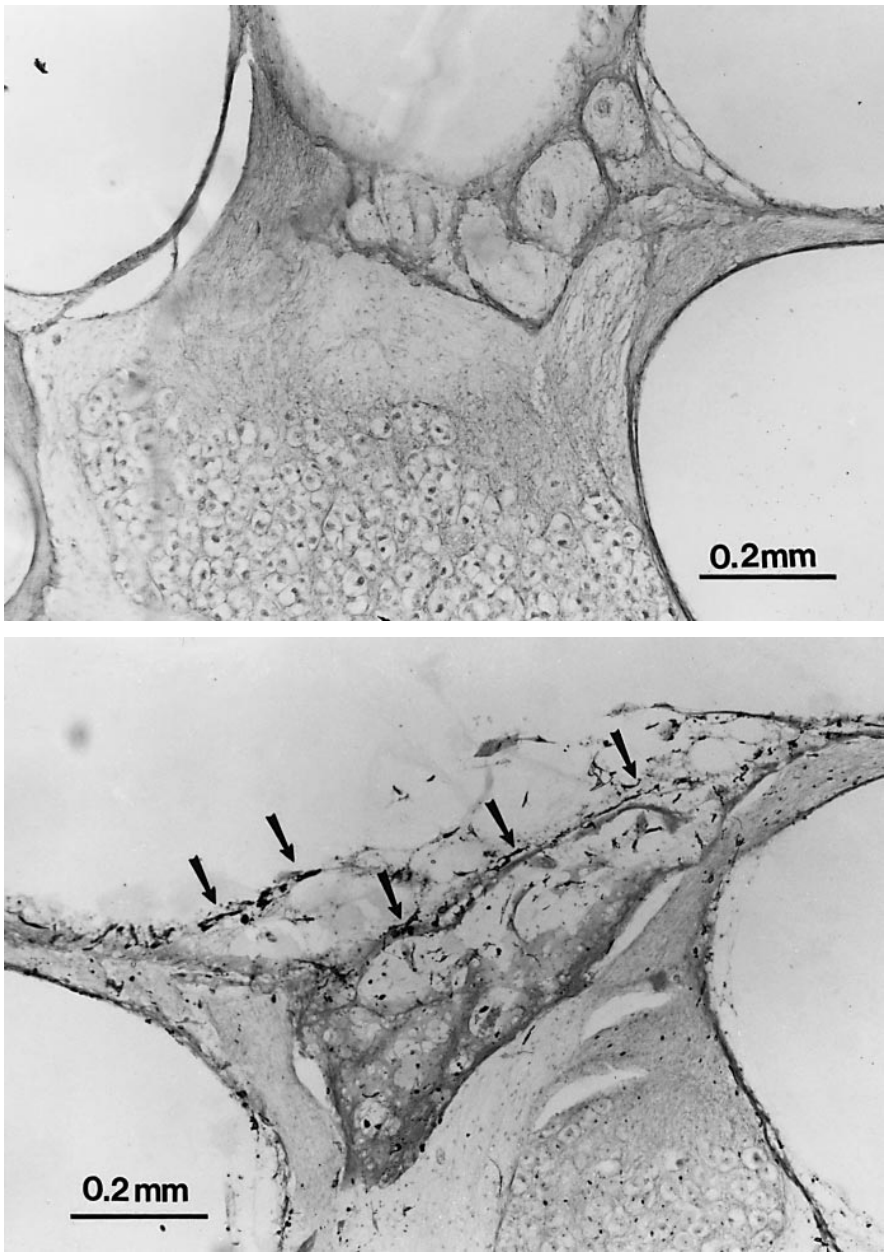
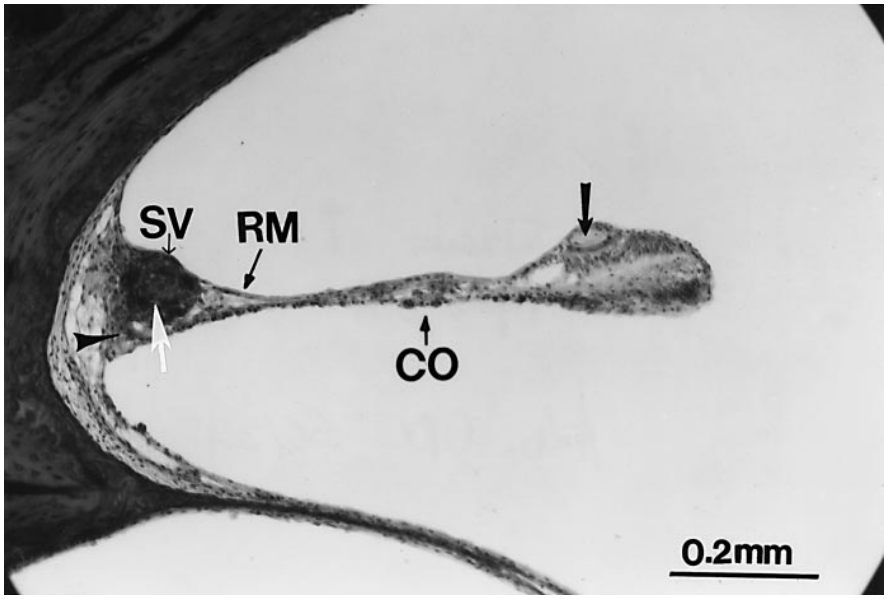


Fig. 5. Figure *a* shows a part of the midmodiolus of the right cochlea of 3-year-old black girl with Waardenburg's syndrome demonstrating no melanin pigment. *b* shows normal cochlear modiolus of 1-year-old black boy as a control case. Rich melanin pigmentation (arrows) is clearly notified. Bar indicates 0.2 mm in length. Fontana-Masson stain specific for melanin.



6a

blood platelet defects [1, 7]. These defects have been attributed to defects in the structure of basement membranes [8, 9], which are caused by genetically heterogeneous defects in genes encoding different alpha chains of type IV collagen [7, 10]. The X-linked dominant Alport syndrome appears to reflect a mutation of the COL4A5 gene on chromosome Xq22, which encodes the alpha 5 chain of type IV collagen. The autosomal recessive form, on the other hand, appears to be produced by mutation of the COL4A3 and/or COL4A4 genes on chromosome 2q36-q37, which encode the alpha 3 and alpha 4 chains of type IV collagen, respectively. It has recently been hypothesized that the heterotrimers of alpha 3-alpha 5 and alpha 5-alpha 6 molecules are important components of basement membranes in different tissues, which would explain both (1) the frequent occurrence of defects in alpha 3, alpha 4 and alpha 6 chains in patients with COL4A5 mutations [10] and (2) common phenotypic pathological features in patients with Alport syndrome.

Type IV collagen is associated with basement membranes of endothelial, epithelial and spiral ganglion cells in the mammalian inner ear [11, 12]. This coincides closely with the loci of histopathologic findings in Alport syndrome, which include loss of hair cells, degeneration of the organ of Corti, atrophic changes in the stria vascularis, basophilic deposits within and beneath the stria vascularis, spiral ligament atrophy and loss and/or degeneration of cochlear neurons [13-17]. The lack of apparent vestibular pathology in these patients

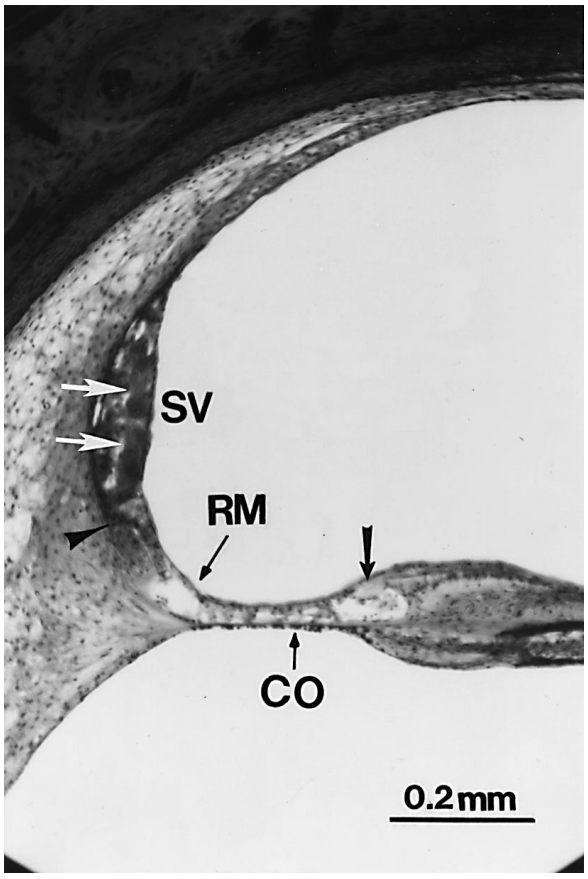


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Fig. 6. a, b The right cochlea in the same case as figure 5a. In both apical (*a*) and basal (*b*) turns, the organ of the Corti (CO) was atrophic, the tectorial membrane (black arrows) was rolled and covered by epithelium, the stria vascularis (SV) was atrophic and had homogenous basophilic substances (white arrows) in the intermediate cell layer, the spiral prominence area lacked blood vessels (arrowheads) and Reissner's membrane (RM) was collapsed. Bar indicates 0.2 mm in length. H & E stains.

is perplexing, though, because basement membrane is also present in these regions.

Histopathologic studies of Alport syndrome are hampered by a number of difficulties. Since the disease is progressive and variable in time course across patients, it is not surprising that the histopathologic findings in the ear vary between cases. Further, it is important to determine whether the cochlear dysfunction occurs concomitantly with the renal disease or is secondary to

Table 1. Summary of histological findings of the inner ear for Alport's syndrome, Usher's syndrome and Waardenburg's syndrome

Syndrome	Age, Sex	Organ of Corti	Cochlea Stria Vascularis	Spiral Ganglion Cells
Alport	39y, m	N	Partly A with basophilic deposits	Decreased in BT
Alport	20y, m	N	N	N
	20y, m	N	N	N
	26y, m	A in HP	N	A in HP
	19y, f	N	N	N
Alport	22y, m	N	N	N
	37y, m	N	N	Decreased in BT
Alport	16y, m	D in HP	A	Slight D
	37y, m	Severe D	A	Slight D
	58y, f	Severe D	A	Slight D
	20y, m	Useless	Useless	Severe D
Alport	48y, f	D	N	N
Usher Type II	55y, m	A, especially in BT	A	Decreased to 30–50% in BT and MT
Usher Type I	84y, m	Severe D, missing throughout most BT	Moderate to severe patchy A	D and severe loss
Usher Type I (?*)	35y, m	A	A and D	Abnormal shape, A
Usher Type I (?)	69y, m	D, hypoplastic, absent in BT	Hypoplastic Lacked melanin	A
Usher Type I	66y, m	A of BT	Anomalous	A
Usher Type I	20y, m	A, absent in BT, higher turns poorly developed	D, absent in BT	D
Usher Type II	65y, m	Severe D, partly missing in BT	A, patchy A in BT	D, severe in BT
Usher Type II	38y, m	D, partly missing in BT		R:18,171, L:21,870 Seg.IV:6,000
Usher Type III	38y, m	A in BT	Scattered A in BT	D in BT
Usher Type III	52y, f	D in BT	N	Severe D in BT
Waardenburg	3y, f	A	A, with basophilic deposits	N
Waardenburg	3y, f	Absence	A	A
Waardenburg	2y, m	Absence or A	A	Sparsity

A = atrophy; BT = basel turn; C = collapse; D = degeneration; HP = hook portion of basal turn; L = left; MT = middle turn; N = normal; R = right; RM = Reissner's membrane; SCC = semicircular canal; Seg. IV, anterior half turn of second turn; SL = spiral ligament; TM = tectorial membrane; m = male; f = female; y = years.

Vestibulum				Note	Author
saccular macula	utriclar macula	canal cristae	scarpa's ganglion cells		
N	N	N		Slight A of SL. C of RM Hypoplastic type of vestibular aqueduct and endolymphatic sac	Our case
N	N	N		Bilateral microscopic otosclerosis	Fujita et al.[13]
N	N	N		Bulging of RM (hydrops) in right ear	
N	N	N			
N	N	N		Numerous globules on the organ of Corti and stria vascularis	
N	N	N			Myers et al. [14]
N				Scattered hair cell loss in HP	Johnsson et al. [16]
N					
N					
N					
			N		Smith et al. [17]
A	N		N	C of the endolymphatic space in the pars inferior	Kumar et al. [4]
N	N	N	N	Distention of RM Pit malformation in utricular macula	van Aarem et al. [27]
A	A	A	A	Bilateral atrophy of nerve VIII Bony defects: vestibular aqueduct and cochlea Posterior SCC protruded into mastoid air space Lack of blood vessels and pigment in inner ear	Alexander [28]
D	D	D	D	Vascular abnormalities in cochlea	Siebenmann et al. [29]
N	D	N	N	Wide endolymphatic duct Atrophy of blood vessels in basal turn of cochlea Atrophy of acoustic and optic centers in brain	Nager [30]
N	N	N	N	Atrophy of labyrinth blood vessels	
N	N	N	N	Decreased cellularity in limbus C of TM throughout MT	Cremers et al. [31] Schmidt [32]
N	N	N	N		Belal Jr [33]
N	N	N	N		Shinkawa et al. [34]
A	N	N	N	No melanin pigmentation in any part of the inner ear including the modiolus Severe C of RM, TM and saccular membrane	Nakashima et al. [5] (Our case)
N	N	N			Fisch [38]
D	D	D	Reduction		Rarey et al. [39]

* included by Lindsay [36], but may not be US.

either the course of renal disease or associated treatment regimens. For example, ototoxic drug administration and hemodialysis can often produce high-tone hearing loss. Hence, histopathologic information on renal and ocular abnormalities should also be considered in each case. Finally, there has not been a systematic effort to correlate histopathologic and genetic findings from the same cases. Despite these caveats, the current evidence appears to be consistent with sequelae of defects in the structure of type IV collagen in basement membranes.

Usher Syndrome

Usher syndrome is an autosomal recessive disorder characterized by bilateral congenital perceptive hearing loss or deafness and progressive retinitis pigmentosa [2]. Two major types have been distinguished on the basis of auditory and vestibular clinical findings. Usher syndrome type I has severe to profound hearing loss and absent vestibular function. It is genetically heterogeneous. Type IA is associated with a mutation in chromosome 14q32, type IB with chromosome 11q13.5 [18], type IC with chromosome 11p14-p15.1 [19–21], type ID with a region on chromosome 10q bounded by D10S529 and D10S573 [22] and type IE with chrosome 21q21 [23]. Usher syndrome type II, by contrast, has normal vestibular function. Usher syndrome type IIA gene has been mapped to chromosome 1q41 between markers AFM268ZD1 and AFM144XF2 [24]. Usher syndrome type III displays progressive hearing loss and has been mapped to a gene on chromosome 3q21-q25 [25, 26]. No genetic information is available for the rare Usher syndrome type IV.

The histopathologic literature indicates that degeneration or atrophy of both the organ of Corti and the spiral ganglion are common features of Usher syndrome. The anomalies are particularly prominent in the basal turn of the cochlea, but the defects often extend throughout the cochlea. For example, Van Aarem [27] reported degeneration of the organ of Corti that was most profound in the basal turn in a case with type I Usher syndrome. However, he also reported moderate to severe patchy atrophy of the stria vascularis, degeneration of cochlear nerve and severe loss of spiral ganglion in all of the turns in both cochleae. Similar features were observed for earlier reports of type I Usher syndrome [28–30], our type II case and other type II cases in the literature [31, 32]. For example, Cremers reported [31] a type II case with a severe congenital hearing loss and onset of retinitis pigmentosa after puberty but no vestibular symptoms. This case showed severe degeneration of the organ of Corti and spiral ganglion with milder atrophy of the stria vascularis. Two histopathologic studies on Usher syndrome type III also showed atrophy [33] or degeneration [34] in the organ of Corti and degeneration of spiral ganglion in the basal turn. Electron microscopy in one of those two patients

showed widespread loss of supporting cell elements of the organ of Corti and dendritic processes of the cochlear nerve [35]. These cochlear findings including ours [4, 27–36] may indicate that atrophy or degeneration of the organ of Corti and degeneration and/or loss of the spiral ganglion (cochlear nerves) are closely associated with all forms of Usher syndrome. Thus, despite the genetic heterogeneity of Usher syndrome, cochlear defects are remarkably consistent between cases.

In contrast with the cochlear data, the histopathologic findings in the vestibular system are variable among cases of Usher syndrome type I. Different case reports have observed atrophy or degeneration of the saccular [28, 29] and/or utricular [28–30] macula, atrophy or degeneration of the cristae ampullaris and Scarpa's ganglion [28, 29], or pit malformation in the utricular macula [27]. Further, we have reported atrophy of the saccular macula in a case of Usher syndrome type II [4]. In the absence of genetic evidence regarding the chromosomal defects in this small number of cases, though, it is impossible to determine whether specific mutations (e.g., Types IA-IE) are associated with specific histopathologic features of the vestibular periphery.

Waardenburg Syndrome

Waardenburg syndrome is an autosomal inherited syndrome which is characterized by dystopia canthorum (lateral displacement of the medial canthi and lacrimal puncta), broad nasal root, confluence of the medial portions of the eyebrows, partial or total heterochromia iridis, circumscribed albinism of the frontal head hair (white forelock), and sensorineural hearing loss (bilateral or unilateral). A common feature of the four types of Waardenburg syndrome is the absence of melanocytes from the hair, eyes, skin or the cochlear stria vascularis. The genetics of Waardenburg syndrome have been reviewed recently [6, 37] and may be summarized as follows. Type I is an autosomal dominant disorder, characterized by mutations of the *PAX3* gene on chromosome 2q35; more than 50 mutations have been identified [6]. Type III, or Klein-Waardenburg syndrome, appears to be a variant presentation of type I in *PAX3* (chromosome 2q35) mutation heterozygotes and some homozygotes. Type II, on the other hand, is genetically heterogeneous, with approximately 15% of cases heterozygous for mutations in the micropthalmia-associated transcription factor gene at 3p12.3-p14.1. Finally, type IV (Shah-Waardenburg syndrome with Hirschsprung disease) is genetically heterogeneous, predominantly autosomal recessive disorder. It includes individuals who are homozygous for mutations in genes for endothelin-3 at 20q13 or endothelin receptor B at 13q22. However, because the clinical features often have incomplete penetrance and variable expressivity [6, 37], it is expected that histopathological features will vary considerably between cases.

Only three cases of Waardenburg syndrome have been studied histopathologically. Unfortunately, the lack of genetic data from the specimens limits the utility of these reports. Fisch [38] examined the temporal bones of a 3-year-old girl and revealed absence of the organ of Corti, and atrophy of stria vascularis and cochlear neurons, but no apparent abnormalities in the vestibular sense organs. The limited clinical description of Fisch's case is consistent with Waardenburg syndrome type I [6]. Rarey and Davis [39] studied the temporal bones of a 1-year-old boy of Waardenburg's syndrome in association with Hirschsprung's disease (probably type IV). They found atrophy or absence of the organ of Corti, atrophy of the stria vascularis, few cells in the cochlear spiral ganglion and degeneration of vestibular sense organs, cell loss in Scarpa's ganglion and reduction of the vestibular nerves. Our type IV case also showed atrophy of the organ of Corti, stria vascularis and saccular macula, but pathologic changes were absent in the spiral ganglion, utricular macula, semicircular canal cristae and the vestibular nerve. The degeneration or atrophy of vestibular organs [5, 39] in these cases may account for the vestibular dysfunction that has been clinically reported in some patients with Waardenburg syndrome [40–42]. However, given that many clinical features of the syndrome have incomplete penetrance and variable expressivity, it is imprudent to generalize from limited histopathologic observations.

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Mouse Homologues for Human Deafness

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Hereditary Deafness and the Mouse as a Model

Studies have shown that approximately 1 in 2,000 children experience permanent hearing loss as a result of a defective gene [1]. Furthermore, researchers have recently found several genes which, when mutated, either cause or underlie a predisposition to progressive hearing loss [2], suggesting that this type of deafness may also have a significant genetic basis. Because genetic deafness is clearly a problem both congenitally as well as later in life it is important that we (i) identify the mutated genes involved and (ii) understand the ear pathology caused by these defective genes. Once accomplished, we can begin to develop ways to therapeutically approach deafness at the molecular level. Since neither objective is easily achieved in humans, animal models are required. For several reasons, the mouse appears to be the best model for investigating genetic hearing loss in humans. Firstly, because large numbers of mice can be produced which all carry the same mutation, the region of the chromosome in which the deafness gene resides can be much more finely pinpointed than in humans. Secondly, the mouse's hearing organ, the cochlea, is structurally very similar to humans in that it shows the same high degree of organization and specialization. Finally, great inroads have been made into the manipulation of the mouse genome, such that it is the most appropriate vertebrate in which to perform transgenesis (transfer of foreign DNA into the genome), a feature which can be useful in both the identification of a mutated gene as well as in the production of potential mouse models.

Types of Deafness

Deafness and ear pathologies can be categorized in several different ways. Firstly, deafness can be broadly categorized based on whether it is associated

Table 1. Identified genes for syndromic deafness

Syndrome (syndromic features other than deafness)	Gene	Protein	Mouse model(s)	Type of deafness (pathology)
Usher IB syndrome (progressive blindness)	*MYO7A*	myosin VIIA	shaker1	sensorineural (neuroepithelial)
Usher IIA syndrome (progressive blindness)	*USH2A*	Usher IIA	–	sensorineural
Waardenburg's syndrome type I[a] (pigmentary disturbancess)	*PAX3*	PAX3	splotch	sensorineural (cochleosaccular)
Waardenburg's syndrome type II[a] (pigmentary disturbances)	*MITF*	MITF	microphthalmia	sensorineural (cochleosaccular)
Waardenburg-Shah syndrome (pigmentary disturbances associated with aganglionic megacolon)	*EDN3,*	endothelin 3	*Edn3* KO and lethal spotting	sensorineural (cochleosaccular)
	EDNRB	endothelin-B receptor	*Ednrb* KO and piebald-lethal	
	SOX10	SOX10	$Sox10^{Dom}$	
Townes-Brocks syndrome[a] (anal, renal and limb anomalies)	*SALL1*	SALL1	–	sensorineural and conductive
Jervell and Lange-Nielsen cardioauditory syndrome (heart abnormalities)	*KVLQT1*	KVLQT1	–	sensorineural
	KCNE1/*IsK*	minK/IsK	*IsK* KO	(cochleosaccular)
Pendred syndrome (thyroid goitre)	*PDS*	Pendrin	–	sensorineural (morphogenetic)
Branchio-Oto-Renal syndrome[a] (cervical fistulas or cysts and renal anomalies)	*EYA1*	EYA1	$Eyal^{bor}$	sensorineural and/or conductive (morphogenetic)
Stickler and Kneist dysplasia[a] (chondrodysplasia)	*COL2A1*	type II (α1) collagen	–	sensorineural and conductive
Alport syndrome (X-linked)[b] (kidney disease)	*COL4A5*	type IV (α5) collagen	–	sensorineural
Alport syndrome[b] (kidney disease)	*COL4A3*	type IV (α3) collagen	*Co14a3* KO	sensorineural (neuroepithelial)
	COL4A4			
Mohr-Tranebjærg syndrome[b] (X-linked; formerly DFN1) (progressive neurodegeneration)	*DDP*	DDP	–	sensorineural
Treacher Collins syndrome[a] (craniofacial abnormalities)	*TCOF1*	Treacle	–	conductive
Norrie disease[b] (X-linked) (ocular abnormalities and mental disturbance)	*NDP*	Norrin disease protein	–	sensorineural

Relevant references can be found at [5] except for SALL1 [26].
– Indicates absence of a mouse model.
[a] Indicates a dominant mode of inheritance.
[b] Indicates progressive hearing loss.
KO = Gene knockout.

with other symptoms (syndromic) or whether it represents the only defect (non-syndromic). A list of some syndromes which include deafness and their causative genes are shown in table 1. It should be noted that many more genes have been found for syndromic deafness than non-syndromic deafness despite the fact that these make up the minority of inherited deafness cases (only about 30% of deafness are though to be syndromic). This is probably due largely to the fact that syndromic cases of deafness are more accurately diagnosed (and thus genetically mapped; see below) due to the additional features of the syndromes.

Non-syndromic types of deafness are further categorized on the basis of the mode of inheritance. The nomenclature for non-syndromic deafness is as follows: DFNA (autosomal dominant), DFNB (autosomal recessive) and X-linked forms are simply DFN. At present 25 DFNB, 19 DFNA, 5 DFN, and 2 mitochondrial loci (genetic locations) have been found. However, these loci may not all represent different genes. For example, cases where more than one locus has been assigned to the same vicinity of the chromosome may in fact represent only one locus or gene. In addition, the same gene may underlie dominant, recessive, syndromic or non-syndromic forms of deafness as in the case of *MYO7A* in which different mutations cause DFNA11, DFNB2, Usher IB, or atypical Usher (see tables 1 and 2).

Deafness can be further classified on the basis of which auditory structures are affected. For example, conductive hearing loss refers to defects found in the outer or middle ear whereas sensorineural hearing loss refers to abnormalities in the sound transmission from the inner ear to the cortex of the brain, although most sensorineural defects are found at the level of the inner ear. Inner ear pathology has been further categorized into three groups including; morphogenetic, cochleosaccular, and neuroepithelial [3]. Morphogenetic defects are characterized by abnormal development of the ear which lead to a malformation. Only a few types of human deafness are known to be due to morphogenetic defects, possibly because important morphogenetic genes are often involved in diverse developmental processes and mutations in these genes can lead to prenatal lethality. Human deafness forms that show morphogenetic defects include Pendred syndrome, which displays a shortened, dysmorphic cochlea (reviewed by [4]) and Branchio-Oto-Renal syndrome, in which defects can range from an absent cochlea to an under-coiled cochlea. Cochleosaccular refers to defects in the stria vascularis, the structure of the ear that is thought to maintain the ionic balance of the endolymph (inner ear fluid). The third type of inner ear abnormality and probably the most prevalent, neuroepithelial, refers to defects in the organ of Corti, the region of the ear in which the hair cells reside, the sensory cells responsible for the first step of the pathway of transducing sound information into neural code. Deafness resulting from neuroepithelial defects is more commonly non-syndromic although some syndromic forms of deafness, such as Usher 1B syndrome, can also be neuroepithelial in nature. In mice, these classifications can be made quite definitely since the pathology can be studied early in development and one can identify the primary structures affected. For example in cochleosaccular deafness, the hair cells degenerate in the cochlea and saccule, although this is in fact a secondary affect of a defect present in the stria vascularis. Hair cells are extremely sensitive to any defects present in the ear and many types of deafness include hair cell degeneration as a secondary feature – thereby highlighting the importance of early developmental studies in discovering the primary defect.

Table 2. Identified genes for non-syndromic deafness

Deafness	Gene	Protein	Mouse model	Type of deafness (pathology)
DFNA1[a]	*DIAPH1*	diaphanous 1	–	Sensorineural
DFNA2[a]	*GJB3* *KCNQ4*	connexin31 KCNQ4	–	Sensorineural
DFNA3[a]	*GJB2*	connexin26	–	Sensorineural
DFNA5[a]	*DFNA5*	DFNA5	–	Sensorineural
DFNA9[a]	*COCH*	COCH	–	Sensorineural
DFNA11[a]	*MYO7A*	myosin VIIA	shaker1	Sensorineural (neuroepithelial)
DFNA8/12	*TECTA*	α-tectorin	–	Sensorineural
DFNA15[a]	*POU4F3*	POU4F3	*POU4F3* KO	Sensorineural (neuroepithelial)
DFNB1	*GJB2*	connexin26	–	Sensorineural
DFNB2	*MYO7A*	myosin VIIA	shaker1	Sensorineural (neuroepithelial)
DFNB3	*MYO15*	myosin XV	shaker2	Sensorineural (neuroepithelial)
DFNB4	*PDS*	Pendrin	–	Sensorineural (morphogenetic)
DFNB21	*TECTA*	α-tectorin	–	Sensorineural
DFN3	*POU3F4*	POU3F4	–	Sensorineural and conductive

Relevant references can be found in [5].
– Indicates absence of a mouse model.
[a] Indicates progressive hearing loss.
KO = Gene knockout.

Identification of a Deafness Gene

Identifying the gene that causes a particular type of deafness is an essential first step in the investigation of hereditary deafness. In addition to improving our understanding of the molecular basis of a certain type of deafness and providing potential models for therapeutic testing, one immediate outcome is that it allows for the development of tests for use in genetic counselling. In identifying the gene, one must first localize the mutation to a particular region

of a chromosome (genetic mapping). In human populations this process is confused by the fact that many types of deafness, particularly non-syndromic forms, are difficult to diagnose as there are few defining features. To minimize grouping cases of deafness together in which the causative gene may be different, genetic mapping must be done either in large consanguineous (intermarried) families, or in populations that have been isolated from immigration. This approach has been successful to a considerable degree in that researchers have been able to localize over 50 non-syndromic forms of deafness [5]. However, the step between localizing the gene to a region of the chromosome and actually identifying the mutated gene is a large and difficult one. Tables 1 and 2 list the genes that have been found for both syndromic and non-syndromic forms of deafness, the majority of which have been discovered in the past several years. The role of the mouse in identifying many of these genes has been a significant one for several reasons. One development over the years that has considerably improved the role of the mouse in finding human genes that cause disease when mutated has been the creation of mouse-human synteny maps. Although the mouse and the human genomes are not strictly conserved – for example they do not contain the same number of chromosomes – large regions of the mouse and human genome are very similar. Thus, once a gene or a mutation is mapped in the mouse it is relatively easy to find the conserved syntenic (corresponding) region of the human genome or vice versa.

The Positional Cloning Approach

Once a deafness locus has been mapped, there are basically three approaches to identifying the causative gene. The first approach, known as positional cloning, involves genetically mapping the mutation to a small interval of the chromosome and then identifying physical pieces of DNA which span the region. This DNA can then be sequenced and compared with the DNA from the deaf patient to ascertain whether any of the genes on this piece of DNA show mutations. Unfortunately, because positional cloning requires large numbers of people which all display the same form of deafness this method has only been successful for the identification of a few deafness genes (mostly syndromic forms) such as *TCOF1*, *PDS*, *USH2A* and *DFNA5*. However, positional cloning is much more easily performed in mice, where large numbers of mice can be bred to all carry the same mutation thereby identifying a smaller region of the chromosome that includes the gene. Due to the genomic conservation between mice and humans the gene can be positionally cloned in the mouse and the homologous human genes can be identified and sequenced for possible mutations. In fact, this was the exact approach used to identify several of the deafness genes listed in table 1 including *MYO7A*, *MYO15*, *PAX3*, and *MITF*. It is likely that this list will grow rapidly as there are quite

a number of mouse mutants that map to an equivalent region of the human genome in which a human deafness lies. A list of potential mouse homologues of human non-syndromic deafness disorders can be found on the Web at the hereditary hearing loss homepage [5]. Because the inner ear is involved in processing balance information in addition to sound information, deafness is often associated with vestibular defects. Fortuitously, mice are particularly sensitive to vestibular defects and display extreme behaviour such as circling and head-bobbing if a vestibular abnormality is present. For this reason quite a number of mouse mutants with inner ear defects have been collected over the years, although mice with deafness alone are likely to be underrepresented since these mice are not easily recognized. For non-syndromic forms of deafness, there are potential mouse homologues for over half of them, suggesting this will be a major avenue for discovering deafness genes in the future.

The great advantage to the positional cloning approach is that it is the only way in which one can identify novel genes, although undertaking this approach can be a long and arduous investigation. To localize the gene to a small region of the chromosome, as many as a thousand mice must be bred and studied. The subsequent physical mapping involves acquiring large pieces of DNA which span the critical region. These pieces of DNA are named by the host organisms in which they can be replicated such as bacteria (BACs; bacterial artificial chromosomes) or yeast (YACs; yeast artificial chromosomes). These BACs or YACs are aligned as overlapping strands called a contig and several are usually required to span the critical region. Traditional methods of physical mapping usually involve various techniques designed to identify genes on the BACs or YACs (methods which are rarely one hundred percent successful) or, more recently, large-scale sequencing – a long, laborious and expensive procedure considering that one YAC, for example, can contain as much as a million base pairs of DNA. A new method has been developed in the mouse, called BAC or YAC rescue, which offers great promise for reducing the time and expense needed to identify the mutated gene. Using the procedure, a YAC or BAC is inserted into the mutant mouse genome. In the case of deafness mutants, one then looks for the otherwise-mutant mouse that can now hear normally or has been 'rescued'. This method can dramatically reduce the amount of sequencing or gene-hunting that must be done since only one BAC or YAC must then be investigated. This method was used recently to identify the shaker2 gene, *MYO15*, and will doubtless be a method used commonly in the future.

The Knockout Approach

The advent of the mouse 'knockout' approach has proven to be another valuable method for identifying deafness genes as well as providing potential

mouse models. This is a powerful technique in which a known gene is rendered non-functional in an effort to discover its purpose. This is done by constructing a 'fake' copy of the gene and introducing it into the mouse genome. This 'fake' copy of the gene contains some of the sequences of the real gene, but much of the gene is removed and replaced with a marker gene. Because some of the real sequences are still there, the 'fake' gene can find its correct place in the genome and replace or 'knock out' the native gene. Several genes causing deafness in both the mouse and the human have been identified this way including *POU4F3*, *KCNE1*/*IsK*, *EDN3*, and *EDNRB*. This method is advantageous because the mouse model is created in the course of identifying the function of a particular gene. Potential drawbacks to this approach are that the gene must first be cloned. Furthermore, removing a particular gene may kill the mouse early in its development or affect structures other than the ear.

The Candidate Gene Approach

The third approach to identifying deafness genes in humans involves mapping the deafness to a region of the genome and then choosing known genes in the region to investigate for possible mutations. However, this method is only effective if there are good candidates in the appropriate region. For example, a gene is a good candidate if it is expressed either in the cochlea or tissues surrounding the cochlea at the appropriate developmental stage. This makes the mouse an important component in using this approach considering expression studies are more easily and more reliably done in animals and mice have the added benefit of a similar ear structure. Several deafness genes have been identified recently using the candidate gene approach including *DIAPH1*, *GJB2*, *TECTA*, and *EYA1*. Similar to the mouse knockout approach, this method suffers from the requirement that the gene must already have been identified. In fact, there is a further requirement that the gene must already have been mapped in order to show it is in the candidate region. Fortunately, because of the human-mouse synteny maps, one can look to both the human and the mouse genetic maps to identify potential candidates. Furthermore, as the human and mouse genome projects become completed and more and more genes are identified, this approach may become the method of choice in discovering deafness (as well as other diseases) genes in the future.

Mouse Models Today and Tomorrow

Once the deafness-causing gene is identified, the next step is to develop a mouse model in which to further study the pathology. Fortunately, in many

cases, the mouse model is created in the process of identifying the gene, either by identifying the gene in a mouse mutant or through the creation of a mouse knockout. Tables 1 and 2 list the mouse models available for the identified deafness genes. Unfortunately, mouse models are available for less than half of the identified genes, although it is likely that this ratio will quickly change considering many of these genes have only recently been identified.

Unconventional Myosins in the Ear

One of the most important insights gained from deaf mouse mutants thus far is in demonstrating the essential role that myosins, a family of motor proteins, play in inner ear function (reviewed by [6, 7]). Myosins, specifically type II or conventional myosins, are most well known for the role they play in muscle. Other types of myosin are considered 'unconventional' and it is these types that have been found to play a role in deafness. Hair cell transduction is a mechanical process so it is therefore not surprising that these molecules play an important role, although the number of different myosins that are involved in deafness has been a startling revelation. The significance of myosins in inner ear function was first discovered with the cloning of the shaker1 gene, *MYO7A*, which, when mutated, also causes four different types of human deafness including Usher 1B, atypical Usher syndrome, DFNA11 and DFNB2. From studies in the shaker1 mouse it appears that a defective *MYO7A* gene leads to disorganized stereocilia, the thin projections or 'hairs' which lie atop the hair cell [8]. Since the stereocilia are critical elements in the transduction process (the transduction channels are thought to reside near their tips), it is not surprising that their disorganization can lead to deafness. More recently, the finding that several other myosins can cause deafness in mice and humans has served to further highlight the importance these molecules play in the inner ear. For example, in the Snell's waltzer mouse, which has a defective *MYO6* gene [9], the stereocilia fuse into one giant stereocilia [27], although as yet there have been no cases of human deafness associated with myosin VI. Shaker2, another mouse mutant whose gene was recently cloned and found to encode yet another myosin, myosin XV, has also been shown to be defective in people who have DFNB3. When the shaker2 mice were examined, it was revealed that hair cells of this mutant display short and stubby stereocilia [10]. Taken together, these mutants reveal the importance of myosins in maintaining the integrity of the stereociliary bundles.

Melanocytes and Deafness

Another important contribution that mouse mutants have made towards understanding inner function is to highlight the importance of the melanocyte in maintaining the high resting potential of the endolymph. The endolymph

is the fluid that bathes the hair cells and a high resting potential is required for normal hair cell function. The stria vascularis, which lies opposite the sensory epithelium containing the hair cells, is the structure of the inner ear that creates and maintains this potential. A number of mouse mutants, including dominant spotting, steel and microphthalmia, have been found to have both deafness and white spotting of the skin – abnormalities similar to those displayed by patients with Waardenburg's syndrome. The skin abnormalities gave a clue as to what might be causing the associated deafness because melanocytes, the pigment cells normally present in the skin as well as a number of different organs, are known to be present in the stria vascularis. Studies of these mice showed that the melanocytes that normally populate the stria vascularis were either reduced or absent [11, 12]. Not surprisingly, studies also showed that the genes responsible for these defects, such as *MITF* [13], *steel* [14, 15], *c-kit* [16, 17], *EDN3* and *EDNRB* [18, 19] are involved in the survival or differentiation of the melanocyte lineage.

Creating New Mouse Models for Human Deafness

Once the gene for a particular form of deafness is known and a mouse model is not yet available, the mouse 'knockout' approach affords a method of quickly supplying a mouse model. In several cases, such as *POU4F3*, *IsK*, and the previously mentioned *EDNRB* and *EDN3*, the gene was knocked out in the course of investigating its function and was only later learned that, when defective, the gene also caused deafness in humans. For example we know from the *POU4F3* knockout that this gene plays an important role in hair cell development. In the knockout the hair cells do not develop stereocilia or many of the other specialized features that hair cells normally display [20, 21]. This finding was particularly exciting as it was initially believed that this gene might be the molecular 'switch' that can turn the average cell into a highly specialized hair cell. Further studies revealed however, that *POU4F3* was more likely to be involved in hair cell maturation and survival, since there were cells which expressed many early hair cell markers such as *MYO7A* and *MYO6*, suggesting hair cells started to form but did not mature [22]. The role of survival is more consistent with the pathology found in humans with DFNA15, a dominantly inherited progressive deafness, suggesting the hair cells may degenerate over time.

However, for several reasons, a total knockout is not always the best approach when developing animal models. As previously mentioned, a knockout is not always feasible as the gene may be involved in important developmental processes and its loss may lead to early prenatal lethality.

Knockouts also normally represent a null allele, in which little or no gene product can be detected. This type of allele does not always faithfully mimic the human mutation, in which the defective gene may encode a protein which retains partial function, or may be acting in such a way as to interfere with the function of the normal protein. Therefore a 'knock-in' approach, in which the exact version of the mutated human gene is inserted in place of the normal gene, may improve the validity of the mouse model. *GJB2*, the mutated gene involved in the highly prevalent deafness DFNB1 [23, 24] as well as DFNA3 (table 1), may be a perfect candidate for the knock-in approach, since mice with this gene deleted die during embryonic life [25].

Clearly, significant inroads have been made into identifying genes which, when mutated, cause deafness. The mouse has played a pivotal role in much of this work and is likely to make a significant contribution in the identification of future deafness genes. The immediate outcome of the identification of deafness genes is that tests can be developed for genetic testing and counselling. A focus must now be made in understanding how these genes function normally in the ear; an aim that has thus far been lagging woefully behind the identification of the genes. The similarity of the hearing apparatus and the rich resource of mutants already available (or the ability to produce one where a relevant mutant does not exist) makes the mouse the most appropriate model in which to investigate that function of these deafness genes.

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Neurofibromatosis 2

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Most patients with neurofibromatosis present head and neck abnormalities and, since such abnormalities are often insidious symptoms, it is important that otorhinolaryngologists monitor such manifestations closely [1]. Although various kinds of lesions occur in the head and neck, the management of neurofibromatosis 2 (NF2), characterized by bilateral eighth cranial nerve schwannomas (acoustic neuromas), is the most challenging problem because it may result in bilateral hearing loss and facial palsy, and bears a poor prognosis. The purpose of this paper is to present the molecular basis of NF2 and discuss the relationship between clinical manifestations and specific mutations of the NF2 gene.

Clinical Manifestations

NF2 is an autosomal dominant disorder characterized by the development of bilateral eighth cranial nerve schwannomas, cranial meningiomas, spinal root and peripheral schwannomas, and presenile lens opacities. According to the criteria proposed by NIH, NF2 may be diagnosed when one of the following is present: (i) bilateral 8th nerve masses seen by MRI with gadolinium, or (ii) a parent, sibling, or child with NF2 and either unilateral 8th nerve mass or any one of the following: neurofibroma, meningioma, glioma, schwannoma, or posterior capsular cataract or opacity at young age [2]. Population-based data from the United Kingdom suggest that one in 35,000 individuals carries the gene for NF2 [2].

On clinical grounds, two major subtypes of NF2 have been suggested: a severe form with early onset and multiple tumors (Wishart type), and a mild form with later onset and usually only eighth cranial nerve schwannomas (Gardner

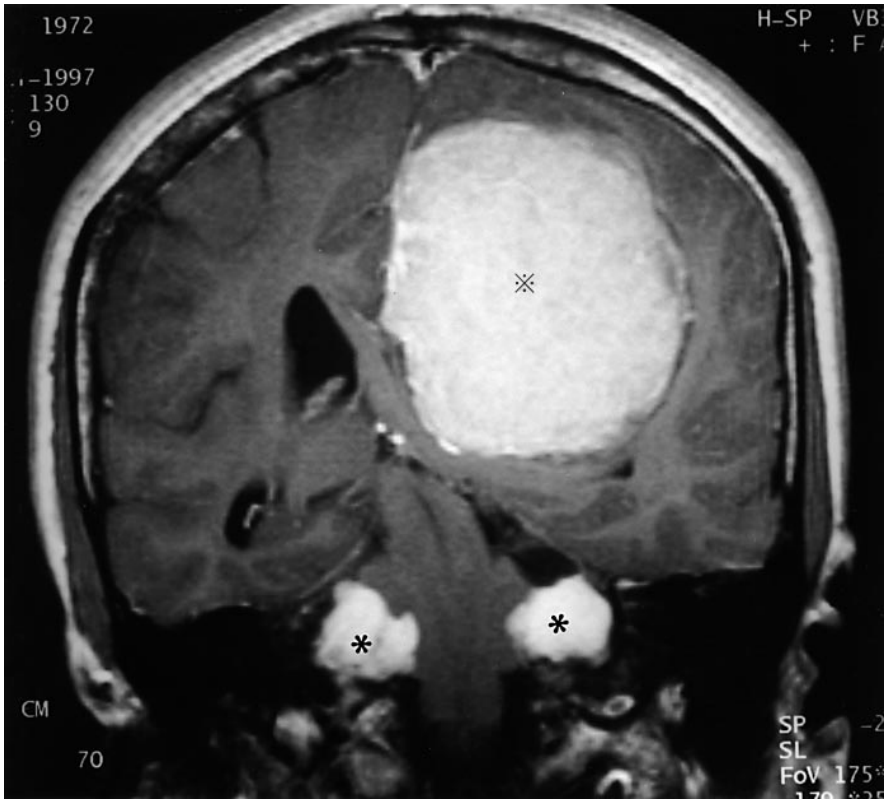

Fig. 1. Bilateral eighth cranial nerve tumor (*) and falx meningioma (※). Coronal T1-weighted MRI with gadolinium shows large tumors in the bilateral cerebelopontine cistern with deafness in the right ear but normal hearing level in the left ear measured by pure tone audiometry.

type) [3]. Further clinical heterogeneity has been observed in patients with NF2. In our experience with 32 patients with NF2, some patients showed clinical characteristics of NF1, such as Lisch nodule in 2 patients, six or more cafe-au-lait macules in 3 patients, and two or more neurofibromas in 5 patients [1].

Auditory symptoms did not occur until late in some patients with NF2. In our experience, four of 32 patients with NF2 did not complain of any auditory symptoms even after bilateral eighth nerve tumors were found. There was no significant correlation between the size of tumor and the degree of hearing loss (fig. 1) [1]. However, the ratio of hearing level deterioration correlated significantly with tumor size [4]. Therefore, treatment should be initiated promptly in patients with NF2.

NF2 Gene

NF2 is known to be caused by the loss of function of a tumor suppressor gene on 22q12 whose deletion or inactivation initiates tumorigenesis [5, 6]. NF2 is thought to carry a single germ-line mutation of the *NF2* gene and sustain the loss of the remaining normal allele as a somatic event in those cells that give rise to the tumor. Thus, the trait is recessive at the cellular level but exhibits a dominant pattern of genetic transmission in families.

The protein product of the *NF2* gene, which is named merlin (moesin-ezrin-radixin-like protein) or schwannomin, has a close relationship to a family of well-studied cytoskeleton-associated proteins [5, 6]. Members of this family are conserved throughout mammalian species and function in a number of roles including maintenance of membrane stability by the binding of integral membrane proteins and the spectrin actin cytoskeleton.

Mutation analysis has revealed various alterations in the *NF2* gene in NF2-related tumors and close genotype-phenotype correlation. Severe disease has been associated with nonsense or frameshift-causing deletion/insertion mutations that lead to truncated gene products which are not expected to function [7, 8]. In contrast, families with the milder form more often have a missense mutation [8, 9]. The corresponding phenotypes of splice-site mutations vary from severe to asymptomatic [10].

The *NF2* gene is the most commonly mutated gene in benign tumors of the human nervous system. Mutations of the *NF2* gene have been found in 30% of meningiomas [11] and in 15% of nonfamilial eighth cranial nerve schwannomas [12]. These results confirmed the importance of the *NF2* gene in the pathogenesis of these tumors.

Diagnosis and Treatment

Early identification of the eighth nerve tumors affords the best opportunity for successful treatment and the preservation of hearing. Because the ratio of hearing level deterioration correlates significantly with tumor size, treatment should be initiated promptly when NF2 is found in patients [4]. Thus, it is important for otorhinolaryngologists to closely monitor any changes seen in patients with NF2. NF2 should be also carefully considered in all patients newly diagnosed as having unilateral eighth cranial nerve schwannomas. Some patients with NF2 have normal hearing in spite of the presence of bilateral eighth nerve tumors, and considerable delay may occur between the onset of tumor development and the appearance of clinical symptoms. Therefore, patients with NF2 and their family members should be closely evaluated even

though they show no auditory symptoms. As well, genetic evaluation and counseling should be provided for all relevant family members once the diagnosis of NF2 is made.

Distinguishing NF2 from sporadic eighth nerve schwannoma is also important for managing the patients and their families. In some patients with NF2, direct analysis of the gene can permit the presymptomatic identification of affected individuals. However, it is often difficult to make a distinction, especially when a patient presents with unilateral eighth nerve schwannoma at an unusually young age, or with minor additional manifestations of NF2 without any family history. The intrafamilial variability associated with splice-site mutation has been reported to make presymptomatic diagnosis especially complex [9].

Patient follow-up should be lifelong in patients with NF2, whose comprehensive management requires the collaboration of many specialists from many disciplines, including neurootology, neurosurgery, the social services and genetics.

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Tumour Suppressor Genes and Head and Neck Cancer

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Introduction and Epidemiology

Head and neck cancer is one of the less common cancers and encompasses a wide range of malignant tumours involving multiple diverse structures and tissues within this region. Collectively, they comprise the eighth most common site for cancer in men and sixteenth for women in the United Kingdom.

Head and neck cancer is increasing in incidence in many parts of the world, although the epidemiological profile of the individual subsites tends to vary. Compared to other more common cancers, it is a disease whose clinical behaviour is more dependent on histology and anatomical site.

By far the most important aetiological factor in cancer of the head and neck is tobacco use. Up to 90% of patients with squamous cancers of the oral cavity, oropharynx, hypopharynx and larynx have a history of tobacco use, typically cigarettes or pipe smoking. Alcohol is also an important aetiological factor and has a powerful synergistic effect when combined with smoking for sites such as the oral cavity, pharynx and supraglottic larynx. The most recent complete figures available from the Cancer Research Campaign reveal, that in 1988, there were 6,752 new cases (two thirds of them males) of head and neck cancers registered in the United Kingdom in a population of 57 million, giving an incidence of 11.8 per 100,000 [1].

Cytogenetics and the Search for Tumour Suppressor Genes

Molecular analysis and allelotyping of squamous cell carcinoma of the head and neck (SCCHN) has revealed a distinctive pattern of chromosomal

changes (LOH – loss of heterozygosity) which is consistent with other models of tumour suppression. Loss of several chromosomal regions are shared, with carcinoma of the lung and other squamous cell carcinomas of different sites, while others are unique to SCCHN. Statistical and molecular analysis suggests that the number of genetic events may be as high as ten, rivalling only prostate cancer for the highest number of molecular events prior to clinical presentation [2]. The evidence is of a high frequency of *p53* mutation in SCCHN with preferential G-T transversions clustered at codons 245 and 248 [3, 4], which is consistent with them being the footprint of tobacco carcinogens. These mutations in the *p53* gene and loss or gain of genetic information on chromosomes 9 and 11, respectively, all involve regulation of the cell cycle and presumably confer a selective growth advantage. The observation of a high frequency of alterations principally in the *p16*/*cdkn2* cyclin kinase inhibitor gene in cell lines of SCCHN [5] only serves to emphasize how critical changes in cell cycle regulation are to the process of conferring a selective growth advantage.

Tumorigeneses has long been thought to be a multistep process [6] and this is clearly observed in the step-wise clinicopathological progression of several tumours, including SCCHN. However, only recently have a combination of cytogenetic and molecular techniques allowed us to look at the process of tumour progression in detail and confirm that, it is indeed, a multistep process in which genetic alterations accumulate. Nowhere has the usefulness of these techniques been better demonstrated than in the case of colorectal cancer [7] where the genetic events associated with key structures in the clonal progression from normal colonic mucosa through to localized malignancy has been identified [7]. These changes involve loss or mutation in genes on chromosome arms 5q, 12p, 17p and 18q. Although these changes occur frequently, they do not occur in every tumour, many other chromosome deletions have been identified [7]. These additional chromosomal deletions probably contain tumour suppressor genes, or oncogenes, in addition to the ones discussed. However, what is clear from these studies is that disease progression in colorectal cancer is the result of mutational activation of at least one dominantly acting oncogene (*K-ras*) and a total or partial inactivation of several tumour suppressor genes (*APC*, *p53* and *DCC*) associated with, as yet largely unidentified, environmental factors [8]. Although these genetic events are associated with specific steps in the progression, it is their accumulation, rather than their order, that is important. Interestingly, the same genes, e.g. *ras* and *p53*, have now been shown to be mutated in many tumours [9, 10] and the lessons learnt from the identification of the genetic events involved in colorectal disease progression are now being applied to SCCHN.

Loss of a specific chromosome region occurs frequently in tumours. Usually, the losses involve only one of the two parental chromosomes present in

normal cells. These chromosomal losses have been interpreted as evidence that the region has affected certain tumour suppressor genes whose products are negative regulators of cell growth and proliferation, and thus, indirectly suppress neoplastic development [11]. Loss or inactivation results in the loss of crucial control on tumour growth. Tumour suppressor genes have been postulated to act recessively, such that both copies of the gene must be inactivated through loss or mutation for the growth suppressor function to be lost [11, 12]. However, the clonal expansion of an altered cell necessary for tumour progression [7] can only occur if the genetic alteration, however small, results in a selective growth advantage. This suggests that some of the tumour suppressor genes need to affect growth, even in the presence of a wild-type (wt) allele; an example of this is *p53*, where the presence of a mutant p53 protein can inactivate the wild-type gene product by binding to it. Thus, the *p53* tumour suppressor gene seems to act in a dominant negative fashion permitting sufficient expansion of the altered cell population to allow a reasonable probability for the second event, i.e. inactivation of the remaining allele, to occur. Indeed, studies have shown that the loss of the protective *wt p53* function allows the evolution of a clonal population of cells with a selective growth advantage that may actually result in the progression of cancer [7, 13].

Knowledge of the clonal chromosomal abnormalities associated with SCCHN derived from the karyotype of tumour cell lines, or the direct karyotyping of primary cultures of tumours has, until recently [14], been rather limited [15]. However, such studies have provided evidence of chromosomal changes principally involving loss of genetic material on the chromosome arm of 3p, 5p, 8p, 9p and 18q and, to a lesser degree, gains in the chromosomal arms 3q, 5p, 7p, 8q and 11q. A significant number of mixed karyotype abnormalities were observed and changes in other chromosomes have also been reported [14]. These chromosome changes were heralded as the site of a putative head and neck carcinoma specific gene consistent with the classical retinoblastoma model of tumour formation.

Molecular Studies

Molecular studies using comparative genomic hybridization (CGH) alleotyping and fluorescent in situ hybridization (FISH) has confirmed many of the genetic events identified by cytogenetic studies and have also found still more abnormalities. CGH has provided much information on structural chromosome alterations including deletions and amplifications [15, 16]. Spiecher et al. [17] studied 13 SCCHN samples using CGH and detected abnormalities,

particularly of chromosomes 3 and 5. A novel copy number again on chromosome arm 3q was also noted [17].

Cytogenetic analysis of cancer cells can yield clues as to the chromosomal basis of carcinogenesis. Its application to lymphomas and leukaemia have lead to the isolation of specific points unique to the various subtypes of these tumours [18, 19].

Retinoblastoma (RB Genes and Knudson's Theory)

Retinoblastoma occupies a pivotal position in cancer cell genetics [20, 21]. Because of the clinical observations that retinal cells from heritable retinoblastoma cases had no heterozygote phenotype, it was suggested that the second event in tumour cell initiation would inactivate the wild-type allele at the retinoblastoma locus. This hypothesis was tested by comparing chromosome compliments at various loci and normal tissues in tumours. DNA probes were developed whose homologous loci matched to chromosome 13 and detected polymorphisms at a restriction endonuclease cleavage site [22]. These are restriction fragment length polymorphisms (RFLPs). An RFLP is a relatively common change in the DNA sequence that either destroys or creates a restriction enzyme recognition site, or alters the distance between them. Such RFLPs are inherited as mendelian traits in a co-dominant fashion [23]. Individuals are heterozygous at a chromosomal location if they have two different alleles at that locus. A person is informative (heterozygous) for a given RFLP when one allele is inherited from the father and one from the mother. Nearly all appear as different bands on a southern blot and can be treated like any other genetic trait in linkage analysis [24]. Deletion is detected as loss of one allele, without a corresponding increase in the density of the signal from the other allele. Heterozygosity and loss of heterozygosity is demonstrated in the examples shown in figures 1 and 2. There are numerous studies which provide evidence that a loss of heterozygosity (LOH) occurs frequently in tumours. Despite the high frequency of chromosomal abnormalities in tumours, wholesale loss of genetic information does not, in general, occur [25].

In the light of recent advances in molecular biology, cytogenetics has been replaced by faster, less laborious techniques, although it still has a role to play in clinical genetics [26]. The application of DNA polymorphisms to the study of genetic alterations in human solid tumours has provided a wealth of information on chromosome inbalance in a large number of cases that would have been difficult to obtain by cytogenetic analysis alone [27]. The approach compares a constitutional genome type at a given marker locus in normal tissues with that of a tumour. Much attention has focused on the phenomenon

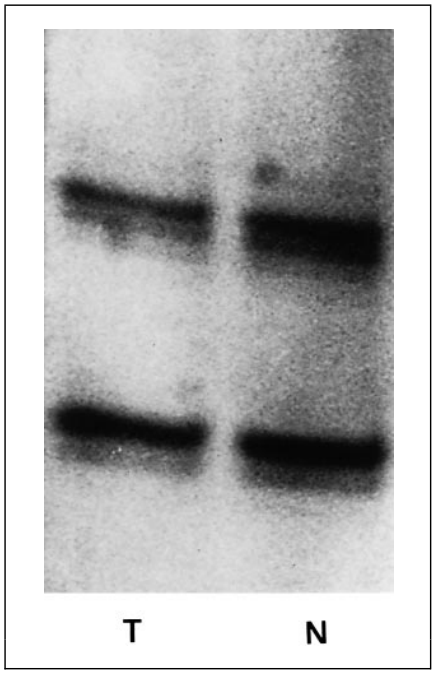

Fig. 1. Two allelic bands in both the tumour (T) and normal (N) specimen lanes indicating that this is a heterozygous sample.

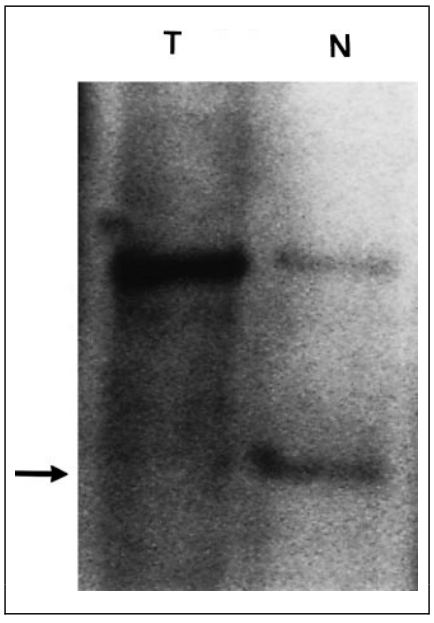

Fig. 2. Loss of one allelic band in the tumour (T) lane, shown by the arrow. This is an example of loss of heterozygosity (LOH).

of LOH at marker loci [24] and specific sites of frequent allele loss have been noted in many types of malignancies [28]. A signal reduction of one allelic band greater than 50% is counted as a loss of heterozygosity [24, 29]. LOH analysis for deletion is exactly analogous to cytogenetic analysis, with the advantages that the resolution may be greater, if closely spaced markers are used. Therefore, the determination of LOH is essentially a refined version of cytogenetic analysis [24]. More recent analysis, using microsatellite primers and the polymerase chain reaction (PCR) in tumours in which the constitutional DNA for the patient is heterozygous has allowed the successful detection, and accurate localization, of chromosomal deletions in several malignancies [30].

Microsatellites are interspersed DNA elements of the form dC-dA (N) bGdT (N) repeats. These specific DNA signatures are polymorphic in length and can, therefore, be used as a pool of genetic markers. Microsatellites constitute one of the most abundant classes of the repetitive DNA families in the human genome, with approximately 100,000 to 150,000 CA (N repeats), they are widely distributed, they can be readily isolated from genomic libraries and have been used extensively in linkage analysis [31, 32]. They are generally more closely spaced than RFLPs, thus analysis using microsatellite markers is thought to be more sensitive than RFLP analysis [31]. These short tandem repeat DNA frequencies may be readily assayed by the polymerase reaction amplification of small amounts of DNA, followed by gel-electrophoresis [31].

LOH and Its Significance

The inactivation of tumour suppressor genes was thought to play an important role in the malignant transformation process. The relevance of chromosome deletions in the development of cancer gained considerable significance with the discovery of tumour suppressor genes [7, 20]. The inactivation of tumour suppressor genes, either by loss or mutation, results in loss of function and other constraints on cellular growth, which may explain the mode of action of the malignant transformation process [32]. The development of new techniques in molecular biology allows the human genome to be mapped. In particular, loss of heterozygosity studies have provided evidence for the presence of normal tumour suppressor genes within the commonly deleted areas of the chromosomes and may indicate molecular pathways involved in the neoplastic process [26]. The repeated observation of LOH of specific chromosome markers in cells from any particular tumour type suggests the presence of a TSG in the vicinity, the loss of which is involved in tumour pathogenesis [24].

Squamous Cell Carcinoma of the Head and Neck

Research into the molecular changes in this disease is relatively recent, although already there are suggestions of lesions on several chromosomes which might be implicated in the malignant transformation process of SCCHN [27]. This has lead to a great deal of interest in the molecular and genetic events that might underline the development of progression of head and neck cancer [33]. In SCCHN a high incidence in loss of heterozygosity has already been reported from the *p53* tumour suppressor gene on chromosome 17 [33]. From the cytogenetic analysis of cell lines of SCCHN suggestions have been made implicating sites of the head and neck cancer gene to be on chromosome 1, 3, 4, 7, 8, 9, 10, 13 and 18; overall over one third of the total number of chromosomes [34]. In certain other types of malignancies, it also appears that several genetic events may be involved in the transformation of malignancy, rather than the deletion of a single gene [35]. Statistical analysis suggests that a head and neck tumour arises from anything from 6 to 10 independent genetic events [35].

Work in my department has shown loss of heterozygosity in SCCHN in chromosome 18 and in chromosome 3. A number of studies suggest that loss of genetic material may be a frequent event on chromosomes 9p, 3p, 11q, 13q, 17p, 18q and 19q in head and neck tumours [36–39]. These studies suggest that multiple areas of allelic loss on several chromosomal arms occurs in such tumours and is consistent with statistical estimates implying multiple molecular steps in the progression of SCCHN.

The *p53* Gene

The best known tumour suppressor gene in head and neck cancers is the *p53* gene. This is composed of 16 to 20 kilobases of DNA on the short arm of chromosome 17 at the position of 17p13.1 [40]. It has been conserved during evolution and is composed of 11 exons, the first of which is non-coding and localized 8–10 kilobases away from exons 2–11 [41]. The *p53* gene was originally regarded as a dominant oncogene because its overexpression resulted in immortalizational of certain cells [42]. Recently, however, several lines of evidence have characterised it as a tumour suppressor gene [42]. Interestingly, *p53* is not the only tumour suppressor gene on chromosome 17 [33].

The *p53* gene encodes for a protein which is 393 amino acids long and is a nuclear phosphoprotein of approximately 53 kilodaltons in molecular weight. It was first identified as a cellular protein in 1979 because it formed a tight complex with the SV40 antigen [43]. The p53 protein may exist in two

forms, a wild-type or normal p53 protein, which is usually found in very low quantities in normal cells and a mutant-type p53 protein. Wild-type *p53* seems to regulate the growth of dividing cells and normally acts as a tumour suppressor gene [44]. Mutations, however, can convert *p53* from a recessive tumour suppressor gene to a dominant oncogene and it is now known that mutant *p53* can transform cells [45]. In addition, some mutant forms of p53 may function to stimulate cell division [44]. Thus, it appears that *p53* can act as a tumour suppressor gene in its normal form but can show gain of function to an oncogene in mutant form [46].

The function of the *p53* gene is unclear in normal cells but its structure suggests that it is a DNA-binding transcriptional activator which acts in conjunction with an accessory protein [47, 48]. The subsequent protein products are believed to exert negative control on the cell-cycle-regulating passage into s phase of DNA replication. The *p53* gene has been implicated in many inherited and sporadic forms of malignancies in humans [49]. The majority of *p53* mutations occurring in human tumours occur in the so-called evolutionary conserved regions [10]. In fact, more than 98% of the base substitution mutations reported so far in malignant tumours are located between exons 5 and 8 of the *p53* gene [10]. Along these conserved regions are at least four loci where mutations occur regularly, these are codons 175, 248, 273 and 282. Mutations in these regions account for approximately 30% of all *p53* mutations [40].

The Role of the *p53* Gene in Squamous Cell Carcinoma of the Head and Neck

Mutations of the *p53* gene have been implicated in a range of malignant tumours to date [50]. Overexpression of p53 protein has also been noted in a wide variety of malignancies including colorectal cancer, lung cancer, breast cancer and oesophageal cancer [51]. *p53* mutations can be found in 33% of human squamous cell carcinomas [42] while *p53* mutations are to be found in some 50% of squamous cell carcinoma cell lines and cell cultures. Both *p53* mutation and *p53* overexpression have also been noted to be frequent in SCCHN [52, 53]. Mutation of the *p53* gene is believed to be the most common genetic alteration detected in SCCHN [53]. This gene locus congregated as a critical site of specific damage by mutagenic carcinogens in tobacco, one of the important risk factors in the aetiology of this disease [54]. In a general review of the role of the *p53* human suppressor gene in squamous cell carcinoma of the head and neck, Field et al. noted that up to 60% of head and neck tumours showed overexpression of the *p53* gene [50]. The overexpression

of *p53* does correlate with a history of heavy smoking and drinking [55, 56]. This helped clarify the findings of previous studies which noted that elevated *p53* expression correlated with the history of smoking in SCCHN [57, 58]. A recent paper suggests that *p53* mutations in SCCHN are even more common than previously thought (42%) and are more frequent in those who smoke and use alcohol (58%) leaving little doubt that *p53* is in someway involved in the progression of these tumours [54]. In a separate study carried out on patients with end-stage SCCHN, *p53* overexpression was found to be correlated with poor clinical outcome [59]. Information from the *p53* database shows that at least 37% of head and neck tumours have *p53* mutations [4, 60]. In addition, a hot spot *p53* mutation in head and neck tumours has been noted in codon position 248 on exon 7 [4]. In their analysis of all the published work on *p53* in head and neck tumours, Field et al. also noted that mutations in the *p53* gene had been found in exons 4–9 with a hot spot at 238, 248 region [61]. Mutations of the *p53* gene are probably important from the therapeutic point of view with increased radiosensitivity of tumours suppressing mutant p53 [62].

Conclusion

p53 is the best known tumour suppressor gene, a derangement of which is implicated in human squamous cell carcinoma of the head and neck. There are almost certainly a large number of tumour suppressor genes and these genes are located on a number of different chromosomes. It is likely, that in the future, the role of these suppressor genes in head and neck cancer will be further delineated and the actual genetic abnormality discerned. Also, the role of *p53* itself, which is highly complex, will be further understood. In therapeutic terms, deletion of a tumour suppressor gene is one of the possible targets for gene therapy. It is possible that such therapy may then lead to apoptosis of the tumour and provide an effective weapon in the fight against cancer.

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Chromosomal Abnormalities in Squamous Cell Carcinoma of the Head and Neck

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During the last decades, cytogenetic and molecular genetic techniques have been successfully used to provide detailed information on the genetic events involved in carcinogenesis and tumor progression. The various techniques that may be applied in these studies each have their advantages and disadvantages, and today it is common to combine two or more methods in the analysis of tumor cells. In order to present a general picture of the genomic changes that may have occurred in a tumor, one of two techniques may be utilized – cytogenetic analysis of cultured cells and comparative genomic hybridization (CGH) of extracted tumor DNA. Cytogenetic analysis has the merit of giving information about both balanced and unbalanced rearrangements and of assessing intratumoral heterogeneity, in contrast to CGH which provides a mean estimate of chromosomal gains and losses only. On the other hand, for CGH analysis no cell culturing is necessary and the results are not obscured by marker chromosomes of unknown origin, which are commonly found in malignant tumors at cytogenetic analysis. However, irrespective of whether CGH or cytogenetic analysis is used as screening method, the recurrent abnormalities detected enable investigators using DNA-level techniques to pinpoint the genes that are crucial for tumor development.

In this presentation we will focus on data achieved by cytogenetics and CGH, and briefly discuss the possible DNA-level consequences of consistently detected rearrangements at the chromosome level.

Cytogenetic Analysis

Chromosomal abnormalities are seen in all human cancer types that have been subjected to cytogenetic analysis [1]. Aberrations have been described in several thousands of hematological malignancies over the last decades. Due to this large amount of data, it has been possible to evaluate the clinical significance of individual aberrations. In certain leukemia types, chromosomal abnormalities are well-established prognostic factors, in particular childhood acute lymphoblastic leukemia [2]. However, for solid tumors such reports have been sparse, initially due to difficulties in establishing reliable culturing techniques. These techniques have improved tremendously over the last years and approximately 70% of the short-term cultures will succeed today. A limitation is that certain nondiploid stemlines are difficult to grow whereas certain diploid clones have a selective growth advantage [3].

Short-Term Culturing and Karyotyping

The techniques involved in obtaining metaphase spreads of good quality from squamous cell carcinomas of the head and neck (SCCHN) have been described in detail earlier [4, 5]. The essential steps in this process may be briefly summarized as follows: The tumor sample is minced mechanically and enzymatically, and the cells are short-term cultured for 5–10 days in a chemically defined medium. The dividing cells are arrested in metaphase by the addition of colcemide. After hypotonic treatment and fixation, the chromosomes are G-banded by, e.g. Wright's stain, whereafter the metaphase spreads can be assessed in the light microscope. The clonal rearrangements, i.e. the same structural changes or gain of a chromosome occurring in at least two cells and the same chromosomal loss occurring in at least three cells, are then combined into a karyotype, according to the recommendations of the ISCN [6].

Cytogenetic Findings in SCCHN

A number of investigations have been published discussing chromosomal abnormalities in squamous cell carcinomas of the head and neck (SCCHN) [7–11]. Abnormal karyotypes have been reported in more than 200 short-term cultured SCCHN [12]. The karyotypes in SCCHN are often complex, revealing a number of structural changes together with numerical changes. However, there seem to be differences between different sites, i.e. carcinomas of the oral cavity, oropharynx and hypopharynx usually have more complex karyotypes than laryngeal carcinomas [11]. Whether these data reflect the clinical observation of a much worse prognosis for hypopharyngeal carcinomas compared to most other sites remains to be elucidated.

The most common chromosomal losses involve arms 3p, 8p, 9p, 11q, 13p, 14p and 15p, and the most frequent chromosomal gains occur in arms 1q, 3q, 8q, and 15q, and band 11q13 [4, 7, 8, 10, 11, 13, 14], indicating possible sites of tumor suppressor genes and oncogenes, respectively. Isochromosomes, deletions, or whole-arm translocations affecting centromeric or near-centromeric regions are frequent recurrent structural changes, while aberrations of bands 1p11-13 and 11q13 are the only common structural changes in euchromatin.

One striking cytogenetic characteristic is the rather high frequency of unrelated clones observed [12]. In general, unrelated clones are more commonly seen in epithelial than in mesenchymal tumors. Whether these observations challenge the current concept of monoclonal carcinogenesis and reflect a polyclonal origin of some SCCHN, as discussed by Jin [15], remains to be elucidated.

The high complexity level, with gain and loss of a variety of chromosomal regions in the majority of the cases suggests that SCCHN development is the result of an accumulation of genetic alterations, a contention which is in line with molecular studies of allelic imbalances [16].

Field Cancerization

According to the 'field cancerization theory', carcinogenic exposure of the epithelium results in multiple, synchronous lesions, finally leading to simultaneous malignant transformation at these sites [17]. If this line of reasoning were correct, one would expect to find genetic damage already in preneoplastic mucosa, at increasing frequencies with increasing genotoxic exposure and susceptibility to develop cancer. However, cytogenetic analysis of normal mucosa has yielded partly conflicting data as only numerical changes were more frequently found in epithelial cultures from SCCHN patients compared to age-matched controls [18].

Categorization of Karyotypic Findings

A large variety of karyotypes, covering the whole spectrum from normal to highly complex ones, have been observed in SCCHN. In order to be able to correlate the karyotypes to clinical parameters, they have to be categorized arbitrarily in a way that take due care of biological as well as clinico-statistical aspects. One way to categorize SCCHN based on such considerations is as follows: Normal karyotype, numerical changes only, simple structural changes (<4 structural changes), and complex karyotype (>3 structural changes) [19]. It has to be noted that at cytogenetic analysis, histologic features of the cells cannot be determined, and one cannot by certainty know whether a metaphase cell belongs to the tumor cell population or to the stroma. Whereas cells with complex karyotypes and normal karyotypes are likely to represent tumor cells

and stromal cells, respectively, metaphase cells showing simple numerical or structural aberrations are less easy to categorize.

Chromosomal Abnormalities as Prognostic Markers

Chromosomal abnormalities, as assessed by cytogenetic analyses, have been correlated to clinical parameters only in a few series of SCCHN. Complex karyotype in general and 11q13 rearrangements in particular have been correlated to poor survival [19]. In this retrospective study of 116 cases these parameters yielded prognostic information independently of clinical variables, such as T- and N-status, demonstrating the limitations of the main system for prognostic evaluation of SCCHN patients, i.e. the TNM-classification system. However, in a previous study comprising 29 patients no correlation between chromosomal aberrations and clinical outcome could be made [10].

11q13 Rearrangements

Cytogenetic abnormalities of chromosome band 11q13 have been reported in a variety of hematologic malignancies and solid tumors. In certain B-cell lymphomas, a breakpoint cluster region (*BCL-1*) [20–22] is affected by the translocation t(11;14)(q13;q32) [23], leading to deregulation and overexpression of the *PRAD1*/*CCND1* gene. Recurrent reciprocal translocations involving 11q13 have been observed also in, e.g. acute myeloid leukemia, chronic lymphoproliferative disorders, and hibernoma [24]. The carcinomas that most frequently show 11q13 rearrangements, often observed as homogeneously staining regions (hsr), i.e. a cytogenetic sign of gene amplification, are breast cancer [25, 26], lung cancer [27] and SCCHN [11].

In SCCHN, hsr is almost exclusively observed in association with complex karyotypes [4, 7, 8, 11, 13]. Of those hsr that have been reported, about half were located in band 11q13, sometimes occurring together with hsr in other chromosomes [4]. Furthermore, in a recently performed fluorescence in situ hybridization (FISH) study, all tumors showing hsr in 11q13 also had a deletion of distal 11q, with breakpoints located close to the amplified region [28], indicating a mechanistic link between amplification and deletion of 11q material. However, it is not clear whether these deletions are of importance, e.g. through the inactivation of a tumor suppressor gene distal to the amplicon, or whether they are simply byproducts of the amplification, without any impact on tumor progression.

Comparative Genomic Hybridization (CGH)

By comparative genomic hybridization (CGH) [29] losses and gains of chromosome segments can be detected. Its ability to screen the entire genome

and its level of resolution is comparable to cytogenetic analysis but it has the advantage of being applicable even in the absence of viable tissue. CGH only requires a reasonably pure source of tumor DNA from the patient. It can detect gains, if overrepresented at least 3–5 times relative to the normal copy number, down to a size of 2 Mb and deletions of less than 10 Mb. False-positive findings are rare. However, centromeres and heterochromatin regions cannot be analyzed, nor can balanced translocations or aneuploidy be detected.

Briefly, the technique is performed as follows: Tumor DNA is extracted from frozen or fixed tissue samples and labeled by nick translation with biotin-dUTP. Normal control DNA is isolated from lymphocytes or other normal tissue and labeled with digoxigenin-dUTP. Tumor and normal DNA are mixed in equimolar amounts and hybridized simultaneously to normal metaphase chromosomes. Chromosomal gains or losses are observed as an increased or decreased color ratio between the two fluorochromes [29].

CGH Findings

Thus far, CGH has been applied to only a limited number of HNSCC. As in cytogenetic analysis, a large variety of gains and losses of chromosomal regions have been reported. In a mixed series of 30 HNSCC (out of which 13 were laryngeal) [30] deletions were found in more than 50% of cases on chromosome/chromosome arms 1p, 3p, 4, 5q, 6q, 8p, 9p, 11q, 13q, 18q and 21q, which is in good agreement with the changes detected by cytogenetic analysis. Gain of genetic material was frequently seen in all or part of chromosome arm 3q (3q24 and 3q27-qter). Increases were also documented in 11q13, 8q, 19q, 19p and 17q. These findings are consistent with the results of four other series, also showing a high frequency of amplification of the regions 3q26-qter and 11q13 and copy number decreases at 3p and 5q [31–34]. Furthermore, amplifications of 11q13 have been observed to occur together with losses of distal 11q [30]. This is in accordance with the above-mentioned cytogenetic and FISH data [28].

Discussion

Despite tremendous technical improvements in surgery, radiotherapy and chemotherapy over the last decades, still approximately 50% of patients with SCCHN will die due to their cancer, a figure unchanged for the last 30 years [35, 36]. The TNM-classification system does not fully predict response to therapy and survival [37]. In the management of these patients, and in order to individualize the treatment protocols, clinicians need more reliable prognostic parameters based on the tumors' biological features, than can be obtained

solely from staging the tumors. Tumors identified as biologically aggressive have certain demands on full-scale multimodality treatment while patients with potentially less aggressive ones could be spared from mutilation through a management based on the principles of organ preservation.

One way to establish tumor markers that reflect primary genetic events, crucial to malignant development and progression, is through studies of chromosomal abnormalities and associated genetic changes of the tumors, and to evaluate their clinical relevance in large homogeneous materials, performed site by site.

Results from cytogenetic analysis and CGH clearly show that rearrangements of 11q13 are recurrent abnormalities in SCCHN, with associations to biological aggressiveness and poor clinical outcome. These aberrations are often represented as amplifications by the two techniques, thus indicating an activation of one or more oncogenes at his locus. Molecular studies have indicated the cell cycle regulating *PRAD1*/*CCND1* oncogene to be such a candidate.

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Kallmann Syndrome

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Kallmann syndrome represents the association of hypogonadotropic hypogonadism, due to GnRH deficiency, and anosmia, accompanied by olfactory bulb hypoplasia. A connection between these two symptoms has become apparent following embryological studies in animals and man, which have established that GnRH-synthesizing neurons undergo migration from the olfactory epithelium to the brain along the olfactory nerve pathway. Familial forms of the disease are unfrequent, and correspond to three different modes of inheritance. The high incidence of mirror movements (3/4 of individuals) and renal agenesis (1/3 of individuals) in the X chromosome-linked form (KAL-1) is noteworthy. The identification of the *KAL-1* gene, which encodes the extracellular matrix protein anosmin-1, has opened the way to the molecular pathophysiology for this developmental disease.

A Definition of the Syndrome

In 1856, Maestre de San Juan, a Spanish anatomist reported the association of the absence of olfactory structures in the brain and the presence of small testes in an individual [1]. The syndrome was identified as a clinical entity in 1944, by an American medical geneticist, Kallmann, who carried out a study on the occurrence of hypogonadism accompanied by anosmia in three affected families [2]. He showed the cosegregation of the anosmia and the hypogonadism in all the affected individuals and therefore established that this syndrome can be hereditary. In 1962, de Morsier, an anatomist from Switzerland, further documented this syndrome. He described the absence of the olfactory bulbs and tracts in several male patients with hypogonadism [3].

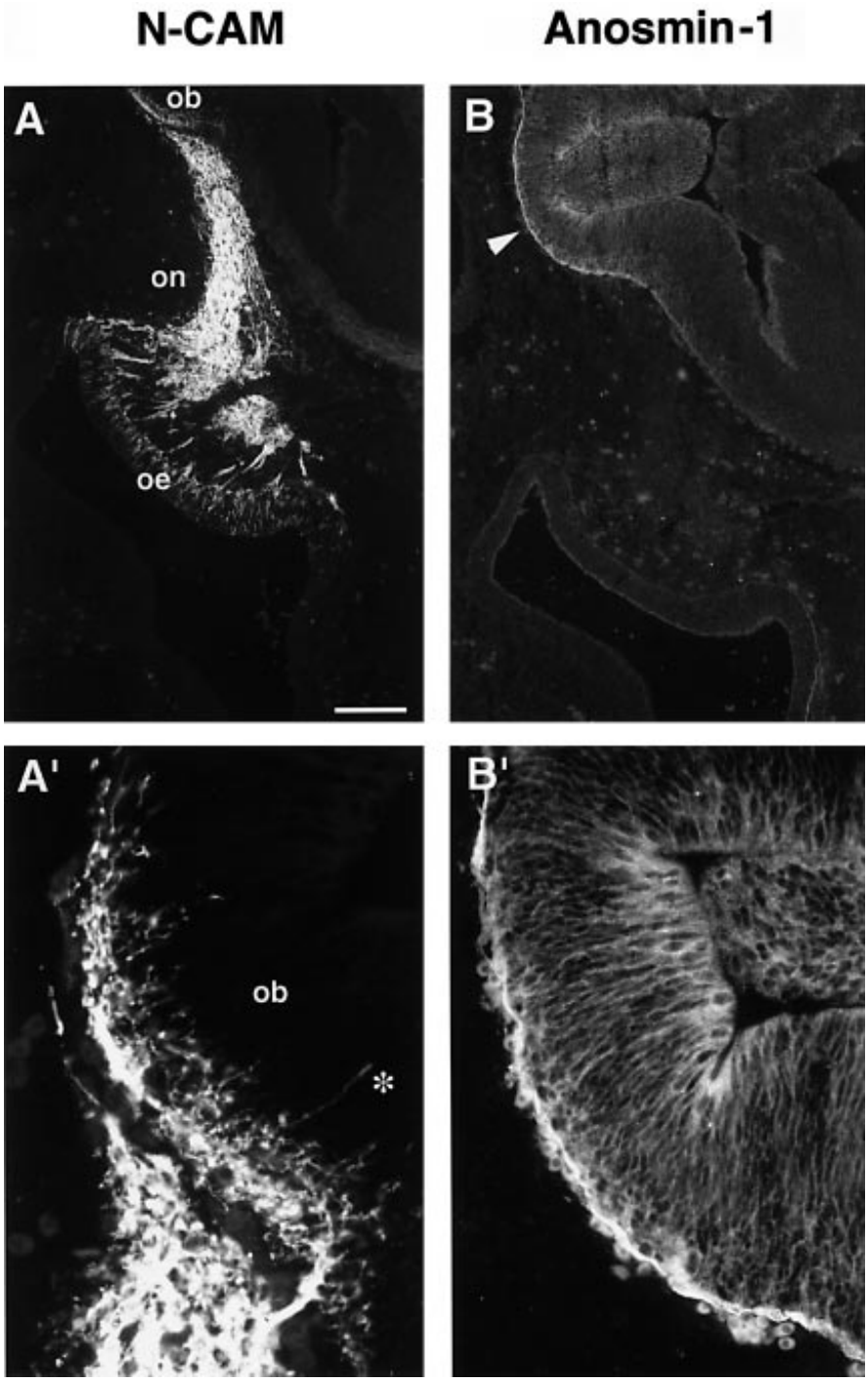
Some years later, the hypogonadism was ascribed to gonadotropin releasing hormone (GnRH) deficiency [4]. However, there is so far no genetic or pathophysiological argument to include isolated hypogonadism of hypothalamic origin into the Kallmann syndrome nosological entity.

The disease affects 1 boy out of 10,000; the prevalence in girls is 5 to 7 times lower. Most cases occur as sporadic cases, and are diagnosed in adolescence, when an absence of spontaneous puberty is found concomitantly to a deficient sense of smell. Confirmation of the latter is to be actively sought after by the means of detailed questioning and smell perception tests as it is rarely spontaneously mentioned. In rare occasions, the diagnosis can be brought up as early as infancy in presence of cryptorchidism or a micropenis in boys. In the rare familial forms of the disease, three modes of inheritance have been described: (i) X chromosome-linked (KAL-1, MIM#308700), (ii) autosomal dominant (KAL-2, MIM#147950) and (iii) autosomal recessive (KAL-3, MIM3244200) [5]. Other symptoms, either neurological (mirror movements, anomalies of visual attention, oculomotor anomalies, ptosis, cerebellar syndrome, sensorineural deafness, pes cavus) or nonneurological (renal aplasia, high-arched palate, labial or palatine cleft, dental agenesis) are occasionally observed. For a number of them, the mode of inheritance to which they are linked is still unknown.

A Disease of Embryonic Development

In the last decade, embryological studies conducted on mammals, birds, amphibians and fish have established the transient existence of a topographical link between GnRH-synthesizing neurons and the peripheral olfactory system. Indeed, GnRH neurons migrate from the medial part of the olfactory epithelium up to the brain along the olfactory nerve pathway. In mammals, they migrate in close association to fibers of the accessory olfactory (or vomeronasal) and terminalis nerves [6]. On reaching the base of the telencephalon, they penetrate into the brain, just caudal to the olfactory bulbs, then make their way within the superficial layer of the medial side of the cerebral hemisphere, to the hypothalamic region where they finally settle. In humans, migration of the GnRH-synthesizing neurons begins on the 6th embryonic week. This period is also when axon terminals of the olfactory sensory neurons first come into contact with the rostral pole of the forebrain; this initial contact taking place just before the emergence of the olfactory bulbs [7] and being a condition of their differentiation.

The histopathological examination of a 19-week-old male human fetus carrying a deletion of the *KAL-1* gene (responsible for the X chromosome-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linked form of the syndrome), revealed both an absence of the olfactory bulbs and an abnormal localization of the GnRH-synthesizing neurons. In this fetus, GnRH neurons had left the olfactory epithelium, but had accumulated outside the brain, in the upper nasal region, along with the axon terminals of the olfactory, vomeronasal, and terminalis nerves [8]. These observations seemed to point towards the noninvolvement of *KAL-1* in the initial stretch of the migrating path of GnRH-synthesizing neurons and axons of the olfactory sensory neurons. On the other hand, they seemed to point towards the involvement of that gene in the final migration stretch travelled by these cells and axonal fibers, that is from their penetration into the brain. Once *KAL-1* was identified (see below), the hypothesis gained in strength thanks to the results of a study on the tissular distribution of the encoded protein, anosmin-1, during human organogenesis. Anosmin-1 is a component of the extracellular matrix, which is transiently present in some basement membranes and interstitial matrices [9]. In the olfactory system, anosmin-1 is expressed as early as the 5th week in the presumptive region of the olfactory bulbs, where it is therefore present at the time of the first contact with the olfactory sensory axons (fig. 1). On the other hand, anosmin-1 is absent in both the olfactory epithelium and the initial stretch travelled by the GnRH cells and the axons of the olfactory sensory neurons on their route from the olfactory epithelium to the brain.

Identification of the *KAL-1* Gene, and Clinical Correlations

KAL-1 has been identified through positional cloning approaches [10, 11], and validated as the actual gene responsible for the disease by the finding of mutations in patients [12–14].

The predicted protein (680 amino acids) comprises a signal peptide and has a compound modular structure which consists of (i) a cysteine-rich amino-terminal region, including a motif with eight cysteines (whey acidic protein-like motif) which is present in several secreted proteins having inhibitory

Fig. 1. Early expression of anosmin-1 in the embryonic olfactory system. Immunofluorescence on parasagittal sections of the fronto-nasal region in a 6-week-old human embryo (A, B), and detail of the olfactory bulb anlage (A′, B′). In (A), an antibody to N-CAM detects the olfactory nerve (on) fibers, which connect the olfactory epithelium (oe) with the olfactory bulb (ob). Some pioneer olfactory axons can be seen entering the depth of the neural wall (asterisk in A′). In (B), an antibody to anosmin-1 detects the protein in the olfactory bulb anlage (arrowhead). The protein is located both in the interstitial matrix and in the lining basement membrane (B′). Scale bar: 200 μm in (A, B) and 50 μm in (A′, B′).

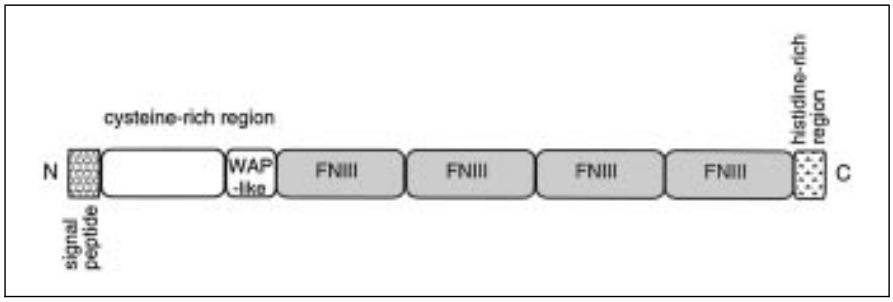

Fig. 2. Predicted modular structure of anosmin-1.

activity toward serine proteases [15–17], (ii) four repetitions of the fibronectin-like type III motif [18], having higher homology with similar motifs of certain axonal adhesion molecules, and (iii) a carboxy-terminal region rich in basic amino acids [19] (fig. 2). Two properties of the protein have been shown in vitro: it is an adhesive substrate for diverse cell types and it allows neurite growth in several neuronal populations [20, 21].

It is interesting to note that the strong prevalence of the disease among males in the general population does not seem to be linked to a frequent involvement of *KAL-1*, as may have been a priori believed. Indeed, a molecular analysis of the gene in the by far more numerous sporadic cases of the disease, only very rarely revealed the presence of a mutated allele. Also, a clinical analysis of individuals carrying mutations in that gene made it possible to ascribe some of the signs observed in this syndrome, i.e. renal aplasia, high-arched palate, synkineses (mirror movements), deafness, pes cavus, to the X chromosome-linked form [13]. Mirror movements and renal aplasia are often met as they affect the three quarters, and a third of patients, respectively [22, 23]. Mirror movements seem to be specific to the X chromosome-linked form of the disease; an abdominal echography, ideally performed in all patients affected by Kallmann syndrome, should soon allow us to know whether renal aplasia too is characteristic of this form of the disease.

Deciphering the part played by anosmin-1 in the development of the olfactory system and the migration of GnRH-synthesizing neurons relies on the generation of animal models of the disease. Still remaining to be identified is, or are, the gene(s) responsible for the autosomal inherited forms of the disease, as well as their role in the framing out of this system.

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Cochlear Gene Therapy

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The introduction or transfer of genetic material into a host to correct or alleviate clinical symptoms is the fundamental goal of gene therapy. Numerous clinical trials, evaluating the efficacy of gene therapy in treating cancer, viral infections, storage diseases, and degenerative diseases of musculoskeletal, cardiovascular, and the central nervous systems, are in progress. Surprisingly, although hearing impairment is one of the most common sensory deficits in humans, the potential application of gene transfer technology in its treatment had not been explored until recently. A lack of effective therapy of most forms of acquired and inherited hearing disorders provides the impetus to explore cochlear gene therapy. Further, the relative isolation of the auditory epithelia within the bony confines of the cochlea makes it an ideal target for organ-specific gene therapy.

The transfer of the therapeutic gene to the host may be indirect, i.e. ex vivo or direct, i.e. in vivo. Ex vivo gene therapy involves introduction of cells into the host that have been genetically altered in vitro while in vivo gene therapy involves the direct introduction of the therapeutic gene to affect genetic alteration within the host. Gene therapy may target either embryonic germ cells or somatic cells. An example of in vivo germline gene therapy producing both morphological and physiological improvements in the auditory system has been recently reported. Absent brainstem auditory-evoked potentials in *shiverer* mice were restored by germline gene transfer of myelin basic protein (MBP) gene [1]. The discussion of the remainder of the manuscript will be limited to studies assessing in vivo and in vitro somatic gene transfer and its potential therapeutic applications for hearing disorders.

Introduction of a gene of interest in the target tissue may be mediated by viral or nonviral vectors. Nonviral-based gene transfer methodology includes

cellular introduction via calcium phosphate method or electroporation, direct injection of nascent DNA or plasmid, and the use of cationic lipids coupled to DNA to facilitate transfection. The ease of production and avoidance of viruses are the major advantages of non-viral gene delivery systems. However, there are several significant drawbacks including inefficient transduction requiring large doses and/or multiple administrations and limited transgene expression due to absence of integration of DNA. Cationic lipids have been recently used to successfully transfer genetic material in the guinea pig cochlea [2]. Transgene expression of the reported gene was noted in a variety of cell types including hair cells, spiral ganglion cells, spiral ligament and spiral limbus and there was absence of cochlear cytotoxicity.

Most gene therapy research utilized recombinant viral vectors to facilitate gene transfer. A variety of viral vectors are available including retrovirus, herpes virus, adenovirus, adeno-associated virus (AAV), and recently lentivirus. Each possess special characteristics useful in particular experimental or therapeutic applications. Retroviruses represent the prototype viral-mediated gene transfer system. They are able to only integrate into the genome of dividing cells, making retroviruses ideal for introducing tumoricidal factors into proliferating neoplastic cells. Therefore, the post-mitotic neurosensory epithelia of the inner ear is not a suitable target for retroviral vector-mediated gene transfer. Lentivirus vector, a relatively new retroviral vector system based on the human immunodeficiency virus (HIV), has recently demonstrated the ability to transduce post-mitotic cells. Lentivirus has been evaluated as a gene transfer vector in the auditory neuroepithelia [3]. Interestingly, while the virus is able to transfect both hair cells and spiral ganglion cells in vitro, the transgene expression is limited to spiral ligament and the epithelial lining of the scala tympani and scala vestibuli in vivo. This limited expression may reflect the inability of the larger lentivirus to enter the endolymphatic space from the perilymphatic space to mediate gene transfer. There was absence of cochlear cytotoxicity.

Herpesvirus, either in its replication-defective recombinant virus form or as plasmid-derived amplicons, can mediate transgene expression in neural and non-neural cells with limited toxicity. Additionally, the recombinant herpes vector can enter a latent state in some neuronal cells and thus potentially mediate stable transgene expression. An in vitro study using a defective HSV-1 vector carrying the BDNF gene demonstrated good transfer and expression of the BDNF gene in auditory neurons with resultant neurite outgrowth from the auditory neurons [4]. Staecker et al. subsequently showed the ability of herpesvirus-mediated expression of BDNF in rescuing spiral ganglion cells from degeneration following ototoxic exposure [5].

Adenovirus is a common human pathogen causing relatively benign syndrome, such as the common cold and conjunctivitis. Adenoviral vectors are

able to infect both dividing and non-dividing cells with high efficiency; however, unlike the retroviral vectors, the adenoviral vectors do not integrate their genes into the genome of the target cell. Instead, the introduced DNA remain as extra-chromosomal elements providing only transient expression. Adenovirus vectors also provoke a strong immune response that may be harmful to the recipient cell as well as limit the benefits of the therapeutic gene by curtailing expression. In vivo and ex vivo studies utilizing adenovirus have demonstrated efficient introduction and expression of a marker gene within the guinea pig middle ear and cochlea with minimal inflammatory response [6–8].

AAV is a linear single-stranded DNA parvovirus that is endogenous to many mammalian species. For lytic growth, AAV requires co-infection with a helper virus, either adenovirus or herpes simplex virus. In the absence of a helper virus, AAV may persist either in an episomal form or as an integrated provirus. AAV is able to infect differentiated, non-dividing cells as well as the dividing cells and integrate into their genome. A major disadvantage of AAV as a delivery vehicle is its small carrying capacity of 4.5 kb. Recent in vivo studies utilizing adeno-associated virus demonstrated efficient introduction and stable expression (6 months) of a marker gene β-galactosidase, within the cochlea and the vestibular labyrinth of the guinea pig [9–11]. Interestingly, expression was also noted in the contralateral cochlea suggesting that the viral vector has the capability of leaving the infused cochlea. AAV-mediated expression of the transgene within cochlear tissues has also been demonstrated using a marker gene encoding the green fluorescent protein (GFP) [12].

The ability to introduce and stably express exogenous genetic material in the peripheral auditory system presents a therapeutic strategy of immense potential in the treatment of hearing disorders that are refractory to existing therapeutic modalities. Identification of genetic factors that underlie hearing disorders and their pathogenic mechanism as well as refinement of techniques in the introduction of foreign genes into target cells represent major advances towards the use of gene therapy as a valuable therapeutic modality in treating hearing disorders.

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Gene Therapy Principles and Strategies for Head and Neck Cancer

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Human cancer continues to be a major and growing cause of death in the world today despite 30 years of new advances in surgery, radiation therapy, and chemotherapy. Head and neck cancer afflicts approximately 52,000 new patients each year in the U.S. and over 500,000 worldwide [1, 2]. The 3–5-year survival rates of patients suffering from advanced T3, T4 squamous cell carcinoma of the head and neck (HNSCC) remains poor, ranging from 20% to 30% [2]. For head and neck cancer as with most solid tumors, surgical excision is the primary mode of treatment and is frequently combined with radiation or chemotherapy for attempted maximal efficacy. Progress with these standard modalities continues at a very slow pace while a new field, 'gene therapy', is rapidly evolving. Gene therapy is founded on the premise that specific genes can be introduced into tumor cells to mediate a direct or indirect antitumor effect.

Current understanding is that head and neck cancer results from a progression of genetic mutations. In this progression paradigm, one cell initially loses normal regulatory control of the cell cycle and proliferates abnormally leading to subsequent clonal expansion of a population of malignant cells with growth advantages [3] (fig. 1). This population of neoplastic cells often continues to evolve into a heterogeneous group of tumor cells, leading to the development of subpopulations of neoplastic cells possessing a particular malignant phenotype allowing regional spread or metastasis [4]. Certainly the concept of replacing one defective gene through gene therapy strategies would therefore be both simplistic and inadequate. On the other hand, replacing all abnormal genes with normal copies would be prohibitively complex. Also, with current gene transfer technology, effective delivery of normal genes to every defective cancer cell is not possible.

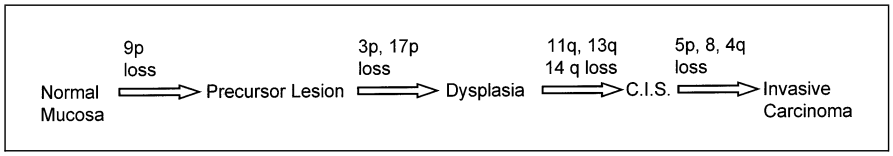

Fig. 1. Current tumor progression model for head and neck cancer. Multiple genetic changes as determined by allelic loss correlate with histopathologic tumor progression.

Head and neck cancer gene therapy is moving in directions that may circumvent these apparent limitations. One area focuses on delivering genes such as cytokines that either stimulate or augment specific antitumor immune responses [5]. Another strategy involves delivery of genes that induce programmed cell death into solid tumors (apoptosis) [6]. A third strategy is 'suicide gene' therapy, which involves delivery of genes that are directly cytotoxic to solid tumor cells [7–9]. As a major advantage for suicide gene therapy, not only are transduced tumor cells killed but also surrounding non-transduced cells are destroyed by a mechanism known as the 'bystander effect' [10].

This review will begin with a general overview on the methods of delivering genes to head and neck cancer. In the following section, the important field of gene delivery vehicle or 'vector' development will be discussed. Finally, the most common genes and gene therapy strategies under investigation for head and neck cancer will be outlined.

Methods for Gene Transfer

The vehicle for gene delivery is commonly called a *vector* and the attributes of an ideal vector for cancer treatment are outlined in table 1. The delivery vehicle must not only carry the gene(s) but must provide other DNA sequences such as long terminal repeats (LTRs), promoters, and enhancers that are necessary for proper gene expression, stability of the DNA and mRNA, as well as integration when applicable. The DNA sequences comprising the target gene, promoter, and associated regulatory elements are collectively called the *expression cassette.*

Viral-Mediated Gene Transfer

Viral-based gene delivery is presently the most common strategy under both clinical and preclinical investigation. Viral vehicles take advantage of the

Table 1. Properties of an ideal vector for gene transfer

- Target cell specificity
- Efficient delivery of expression cassette
- Vector itself is not recognized as foreign by the immune system
- Allows for repeat delivery
- Carries one or more genes and expression or regulatory elements
- Stable and easy to reproduce
- Results in long-term or stable gene expression
- Delivers gene to a wide variety of neoplastic target cells
- Carries no associated direct or indirect toxicity
- Preparation carries no risk of wild-type virus contamination

innate ability of a virus to enter target cells, avoid degradation, and to use host cellular machinery to express viral genes and replicate. To safely apply viral-mediated gene therapy, one must construct a viral vehicle lacking pathogenic functions and reproductive capabilities but contains the appropriate therapeutic gene, promoters, enhancers, and other regulatory elements. Table 2 outlines the commonly chosen viral vectors for both preclinical investigation and human clinical trials.

Retroviral Vectors. The most commonly used viral vector is the retrovirus, especially in immune-based gene therapy strategies [11]. These 10-kb virus integrates into a target cell's genome, enabling stable and long-term expression of the gene product. No major toxic effects have been seen in human clinical trials, although the long-term effects may not be apparent yet [12].

Since the retroviral vectors are capable of inserting their genes into the target cell's chromosomes, they are particularly suited for treating diseases that require permanent gene expression. Recent studies have demonstrated target cells' altered phenotype for 36 months after transduction [13].

A retrovirus will only integrate into actively dividing cells, a principle that may provide selectivity for tumor cells over normal cells but also could be a disadvantage when applied to slow growing cancers. Another disadvantage of the retroviral vector system is the relatively low efficiency of gene transfer into dividing cell populations (1–30%) [14]. It is therefore difficult to generate large numbers of transduced target cells required for effective gene expression. The retrovirus also carries the theoretical risk of insertional mutagenesis since it could conceivably integrate near an oncogene or disrupt a tumor suppressor gene. This event has not been reported to date.

Adenoviral Vectors. The recombinant adenovirus as a vehicle provides the highest degree of gene transfer efficiency in vivo making these large (36 kb)

Table 2. Common viral vectors under gene therapy investigation

	Adenovirus	Retrovirus	Adeno-associated	Herpes simplex
Max. insert size	intermediate (approx. 8 kb)	intermediate (approx. 10 kb)	small (approx. 5 kb)	large (approx. 36 kb)
Infectivity	broad	dividing cells	broad	broad (neural tissues)
Integration	episomal	random chromosomal integration	specific chromosomal integration	episomal
Viral titer	high	low	low	low
Risks	immune response	mutagenesis; tumorigenesis	wild-type virus contamination	cytopathogenicity
Viral production	helper-free	helper-free	helper virus	helper virus and helper-free vectors

DNA viruses very attractive for gene therapy. The adenovirus vector accommodates a 4–8-kb expression cassette and does not integrate into the target cell's genome. Because the transferred gene remains epichromosomal in the host cell, insertional mutagenesis cannot occur. When compared to a retroviral vector, the adenovirus vector demonstrates a significant advantage by infecting a wide variety of both proliferating and quiescent cells with a high level of efficiency [15]. One possible disadvantage and limitation of the adenovirus is that the transferred gene is expressed only transiently [13]. To circumvent this potential limitation, new generation adenovirus vectors are being created that will provide long-term gene expression.

Although current recombinant adenoviral vectors are constructed to be replication-defective, they are not completely 'defective' due to the expression of some viral protein products that may induce an inflammatory response to cells or tissues expressing these proteins [15]. In addition, systemic anticapsid antibodies may be produced which may function as circulating neutralizing antibodies that limit repeat administration of adenoviral vectors [16]. Clinical trials data indicate that there is a high margin of safety in human patients despite possible risks of toxicity due to viral protein-mediated inflammatory responses [12].

Other Viral Vectors. These include the herpes simplex virus (HSV) [17], the pseudotyped retrovirus [18], second-generation adenoviruses [19], adeno-associated virus [20], vaccina [21], human papilloma virus, poliovirus, RNA

virus, baculovirus, and avipox (pox) virus [22]. There are toxicity and safety as well as production issues associated with many of these vectors; however, continued investigation into gene transfer vehicle may enable the use of these other viruses in gene therapy strategies.

DNA-Mediated Gene Transfer

This is a rapidly growing area of gene transfer in both preclinical and human clinical investigation. Liposomes or cationic lipid complexes are the most common vehicles used for DNA-mediated gene transfer [23]. The liposome-DNA complex is capable of fusing with cell membranes of tumor cells and facilitates gene epichromosomal gene uptake and expression. Although eliminating potential toxicity and concerns regarding viral vectors, there are two main disadvantages of DNA-mediated gene transfer. DNA-mediated gene transfer is classically the least efficient means of introducing genes into tumor cells, and it results in gene expression lasting only a few days at best. The transient nature of gene expression, however, may reduce potential toxicity and tends to be less adversely immunogenic. Thus DNA-mediated gene transfer may typically be performed repetitively in attempts to increase in vivo gene transfer efficiency and lasting therapeutic effects.

Specific Gene Therapy Strategies and Clinical Trials

Drug Sensitivity/'Suicide Genes'

In suicide gene therapy, a specific gene is introduced into a tumor cell and converts a relatively nontoxic systemically administered prodrug into a toxic metabolite that directly kills the cell. A major benefit of this strategy is the associated 'bystander effect', which is the cytotoxic effect of transduced cells on neighboring nontransduced cells [10] (fig. 2). This explains why marked tumor regression occurs when only a small fraction of tumor cells actually incorporate the suicide gene.

The bystander effect has been attributed to three general mechanisms. First, toxic metabolic products may be passed between cells via gap junctions that result in killing of nontransduced adjacent normal cells [10]. Second, phagocytosis of apoptotic vesicles arising from the transduced cells may stimulate programmed cell death [24]. Thirdly, the cellular debris and native antigens or even viral proteins released from the dying cell may induce an immune response against the tumor [24].

The most common suicide gene under investigation is herpes simplex virus thymidine kinase (HSV-tk). Upon transfer of HSV-tk into target dividing cells, expression of the viral thymidine kinase renders mammalian cells

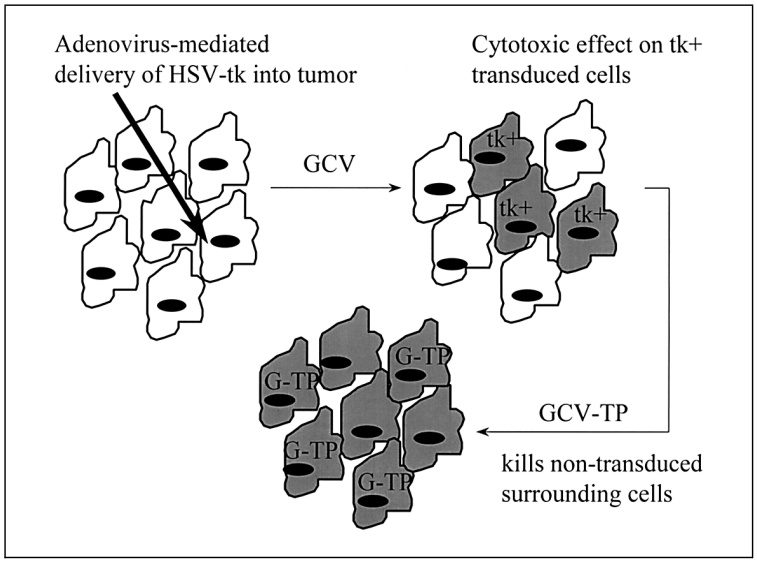

Fig. 2. The bystander effect. The suicide gene HSV-tk is delivered directly to a solid tumor via a recombinant adenovirus vector. Only tumor cells that are exposed to the adenovirus vector are effectively transduced and express the tk protein. Subsequent to systemic ganciclovir (GCV) administration, the dividing transduced tumor cells are killed. Toxic phosphorylated metabolites (G-TP) are transferred to surrounding nontransduced tumor cells through gap junctions that allow intercellular communication. These surrounding tumor cells are killed by the toxic metabolites resulting in the bystander effect.

sensitive to the nucleoside analog ganciclovir (GCV). Dividing tumor cells are therefore selectively killed through the HSV-tk-mediated conversion of ganciclovir into a phosphorylated compound that terminates DNA synthesis [7, 8]. This specificity for dividing cells preferentially targets malignant cells and spares normal surrounding host tissues that are not in the 'S' phase of cell division. Multiple experimental studies with animal models including glioma and head and neck cancer have reported significant regression or eradication of tumors after both retroviral- and adenoviral-mediated HSV-tk gene transfer [8, 9, 25].

Cytokine Gene Therapy

Direct gene transfer of cytokines into head and neck tumors results in a high concentration of cytokine within the local tumor or lymph node environment and a low concentration in the systemic circulation. Not only is this regarded as more physiologically relevant, it also avoids the serious toxicity

associated with high systemic levels of cytokines [26]. Using cytokine gene therapy, one may increase the presentation of tumor antigens and activate tumor-specific T lymphocytes resulting in systemic antitumor immunity against the parental tumor. Two cytokines that have shown some promise in head and neck cancer are interleukin-2 (IL-2) and interleukin-12 (IL-12). Synergistic effects of adenovirus-mediated HSV-tk and IL-2 gene transfer have been demonstrated in a murine head and neck squamous cell cancer model [27]. Also, retrovirus-mediated delivery of IL-12 to the local tumor environment has shown some promise in a squamous cell cancer murine model [28]. Recently, a Phase I human clinical trial has been completed that evaluated the safety of a liposomal IL-2 formulation directly injected into tumors of patients with unresectable head and neck cancer. There were no toxicities noted with single or multidose injections of the IL-2 formulation, and gene expression was identified within the treated tumors (unpubl. data, O'Malley et al., 1998).

Tumor Suppressor Gene Therapy

Enthusiasm for tumor suppressor genes began with the discovery of *p53* and the identification of its inactivation or mutation in approximately 50% of head and neck cancers. The *p53* gene encodes a 53,000-dalton, 393 amino acid phosphoprotein that regulates cell growth and proliferation. In general, *p53* expression results in cell cycle arrest at the G1 checkpoint and induces apoptotic cell death. The wild-type p53 protein may either suppress the expression of genes that drive cell cycling and uncontrolled growth or transactivate other genes that induce cell cycle arrest. Gene transfer to restore wild-type *p53* function may be sufficient to cause growth arrest or apoptosis despite the persistence of many other genetic abnormalities present in a cancer cell [12]. Another potential gene for tumor suppressor gene therapy is *p16*. The p16 protein suppresses cell cycle progression by inhibiting cyclin-dependent kinase 4. Deletions of *p16* have been reported as the most common genetic abnormalities in head and neck cancer [3].

Preclinical studies with adenovirus-mediated gene transfer of *p16* and *p53* have demonstrated growth inhibition in human head and neck cancers [29, 30]. Although presumably the use of *p16* or *p53* gene therapy should be limited to those tumors with mutations or loss, the preclinical animal studies have also shown anti-tumor efficacy in certain human tumors that have wild-type *p16* or *p53*. Tumor suppressor gene therapy investigation has entered human clinical trials. A Phase I human clinical trial evaluating the safety of adenovirus-mediated *p53* gene therapy has recently been completed [31]. Thirty-three patients' tumors have been injected with adeonvirus-*p53* at doses up to 1×10^{11} plaque-forming units. No serious adverse reactions have been reported in spite of antibody responses due to repeated administration. Although this study

had no control population for comparison, tumor regression was reported in 2 patients and 6 patients had stable disease for 3.5 months.

Summary

The ongoing preclinical and now human clinical investigation of gene therapy in head and neck cancer and cancer and cancer overall have provided a few foundation points of information. The first point is that viral and nonviral gene therapy has demonstrated efficacy in a variety of animal models and these successes support consideration and evaluation of gene therapy in human clinical trials. Regarding the retrovirus as a vehicle for gene transfer in humans, it appears to be a safe vehicle. There has been no significant short- or long-term toxicity associated with a wide application of retroviral gene therapy in human patients [12]. Regarding adenovirus as a vehicle, there are less numbers and less advanced trials, but the associated toxicities reported thus far have been both transient and relatively minor. Nonviral cationic lipid-mediated gene transfer also appears safe in human patients. These points are significant because they establish the safety foundation for delivering potentially therapeutic genes to humans. Without this safety data, gene therapy would not have a future in the treatment of cancer.

At the present Phase I stage of human clinical trial investigation for head and neck cancer, the focus remains on patients with advanced or recurrent incurable cancer. Although this patient population is a standard choice for establishing the safety of novel therapies, the greatest chance of eventual success with currently available gene transfer vehicles and gene therapy strategies will most likely be in those patients with less advanced stages of disease. Another future potential for gene therapy in head and neck cancer may be in combination with surgery or radiation or chemotherapy. At some acceptable ratio of efficacy to toxicity, gene therapy may also prove effective against earlier stage cancer either as a primary or adjuvant therapy. In conjunction with the evolution of molecular diagnostics, gene therapy strategies may provide the means for preventing the malignant progression of premalignant head and neck lesions upon early diagnosis.

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