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Volume 8

Tetsuichiro Muto
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Editors

TUMOR BUDDING *in* COLORECTAL CANCER

Recent Progress in Colorectal Cancer Research

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TUMOR BUDDING IN COLORECTAL CANCER: RECENT PROGRESS IN COLORECTAL CANCER RESEARCH

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TUMOR BUDDING IN COLORECTAL CANCER: RECENT PROGRESS IN COLORECTAL CANCER RESEARCH

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CONTENTS

FOREWORD

Colorectal cancer is one of the most prevalent malignancies in the world, and its metastatic disease has remained a challenge in clinical practice. The mechanisms of tumor cell invasion and metastasis have been extensively studied using surgically resected specimens, animal experiments and in vitro experiments, and outstanding results have been witnessed in the last twenty years, even at the molecular level. Tumor budding, the phenomenon at the tumor-stroma interface, has been paid special attention, and is discussed extensively in this issue by a group of authors from institutions scattered across the world, under the direction of Drs. Muto, Mochizuki and Masaki. Articles by authors from Poland, Canada, Switzerland, Germany, and numerous institutions in Japan display extraordinary insights in this field. Drs. Muto, Mochizuki and Masaki are to be congratulated for finding contributors with extensive experience, a critical view, and a broad perspective to write these articles.

The abundant findings from basic research described here will help us make more sophisticated research protocols in the future. Furthermore, this issue will be an important step to the rational management of patients with colorectal cancers, because evidence-based medicine is now urgently demanded in clinical practice. I foresee that the treatment of patients with colorectal cancer will be determined scientifically in the next decade.

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PREFACE

The depth of tumor penetration and the regional lymph node involvement have been thought to have the biggest influence on the postoperative outcome of patients with curatively resected colorectal cancer. From this standpoint, prognostic grading systems adopting these two parameters have been widely used; i.e., Dukes' classification, TNM Classification of UICC, Classification of Colorectal Carcinoma by the Japanese Society for Cancer of the Colon and Rectum, etc. However, patients with the same stage do not always have the same outcome after potentially curative surgery. This prognostic difference is thought to derive mainly from variation in the biological aggressiveness of primary tumors classified as having the same stage.

Many studies have demonstrated that some pathological features of primary tumors indicate tumor aggressiveness and are useful for more accurate assessment of patients' prognosis. For example, the histological type of cancer, growth pattern, and lymphocytic infiltration at the invasive front have been shown to be significant and accurate predictors of postoperative outcome, independent of tumor depth and nodal involvement. These pathological factors reflect the biological aggressiveness of colorectal cancers and are regarded as useful predictors of postoperative outcome. However, grading systems using these parameters have not been widely used in routine practice. There seems to be much interobserver and intraobserver variation in the evaluation of these parameters.

Tumor "budding", which is the focus of this book, is generally defined as an isolated single cancer cell or a cluster composed of a very small number of undifferentiated cancer cells in the stroma of the actively invasive front. The correlation of tumor budding with clinical outcome in colorectal cancer was first reported in the 1980s by Japanese surgeons. Since then, the clinical usefulness of this finding in colorectal cancer has been recognized in many countries. Its usefulness has been clarified not only as a prognostic factor and a potential parameter for a grading system, but also as an indicator for therapeutic decision making. Recently, more objective evaluation of tumor budding has been advocated, and the underlying mechanisms of tumor budding formation have been investigated at the molecular level.

In this book, tumor budding in colorectal cancer is discussed from various aspects. It is the editors' hope that this book will provide useful information for all investigators trying to deepen the understanding of colorectal cancer and develop evidence-based therapeutic strategies.

We would like to thank each author for taking valuable time from their practices and families to help us edit this book. We could not possibly have hoped to produce this work in a timely and outstanding manner without the invaluable suggestions of Mr. Frank Columbus, President and Editor-in-Chief of Nova Science Publishers, Inc.

Tetsuichiro Muto, M.D.
Hidetaka Mochizuki, M.D.
Tadahiko Masaki, M.D.
Editors

PART 1: HISTORICAL ASPECTS OF TUMOR BUDDING

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Chapter I

IN THE FIELD OF GASTROINTESTINAL PATHOLOGY

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Introduction

"Zokushutsu" is a Japanese term widely used in the context of destruction of duct formation accompanying cancer infiltration, but its precise definition is unclear. Broders (1920) classified this structural degradation into 4 levels, grades 1 – 4, according to the proportion of occupation by undifferentiated cells in 533 cases of squamous cell carcinoma of the lip [1]. Imai (1949) proposed the CPL classification. In this classification, destruction, including not only "sprouting" but also desmoplastic reaction, and vessel invasion, is grouped into C (cirrhotic form), P (progressive sprouting) and L (lymphatic or blood vessel permeation), and each of them is graded into 4 levels, i.e. C (0 – III), P (0 – III) and L (0 – III) [2]. In cases with high progressive sprouting, period to death due to recurrence is short, as reported by Akazaki et al. (1953), Ohta et al. (1957), Enjoji et al. (1958) and Majima et al. (1967). Experimental studies using rats and cultured cells have supported these clinical observations: Araki (1979) showed a correlation between progressive sprouting at the invasive front of cancer and active motility in N-methyl-N'-nitro-N-nitrosoguanidine-induced

gastric cancer of rats, as did Gabbert (1985) for 1,2-dimethylhydrazine-induced colon cancer of rats and Carr (1986) for tissue culture cells.

Later, Hayashida et al. (1987) and Morotomi et al. (1988) proposed that this pattern of invasion should be called "budding". In 1992, Gabbert (Mainz University, Germany) used the term "tumor-cell dissociation (TCD)" for budding at the invasive front of gastric cancer [3]. Hase (1993) studied the prognostic value of budding in operated colon cancer, using a classification of mild (BD-1) and moderate to severe (BD-2), and reported significant differences in recurrence rate, 5-year-survival rate and other parameters [4]. However, Kobayashi (1994) indicated that the definition of this budding was obscure. Ono (1996) and Kaihara (2003) used the term "dedifferentiation" for budding or sprouting [5, 6]. Ito (1998) used budding as a prognostic factor for rectal cancer in the lower region, and showed its correlation with lymph node metastasis. In 2001, Brabletz demonstrated the correlation between dedifferentiation and expression of β-catenin [7]. Kirchner examined the role of dedifferentiation in gastric cancer, and furthermore Masaki examined the correlation between budding and expression of MMP-7 in submucosal invasive colon cancer and emphasized its clinical significance [8, 9].

Although the clinicopathological significance of cancer cell dissociation in submucosal layer is clear, consensus on its terminology in English or definition has not necessarily been established. It seems that the term, "dedifferentiation", should be used differently from destruction of duct formation.

For cancer cell dissociation in the submucosal layer, "sprouting" or "budding" can be used depending on individual preference, but purposive definition of the term is important. Since "budding" means continuous growth by budding from the main glands (used for protruding lesions such as polyps, regardless of malignancy, by Nakamura and Morson) and also interstitial scattering of dissociated cancer cells (Jass et al.), Imai's classification uses the term "sprouting", specifically "progressive sprouting". Budding includes step-wise transition, as pointed out by Shirouzu et al., and can be a term expressing growth patterns ranging from benign to malignant proliferation. Consequently, "budding" can be distinguished progressive type (developing or progressive sprouting) from initiating type (beginning or/and growing). The important process of dissociation and dispersion of cancer cells from the main glands in during metastasis is also indicated by them. When the highest priority is to be given to that, a term limited to budding-progressive type becomes necessary. From this point of view, "progressive sprouting" can be used for "Zokushutsu", but it is difficult to distinguish from budding. Hence, for the purpose of expressing the concept of dissociating infiltration of cancer cells into the submucosal layer ("Zokushutsu"), we follow Gabbert in useing "tumor cell dissociation (TCD)" in our studies. In this context, "TCD" is defined as occurrence of tumor cell dissociation from the main cancer nest in a range from small nest to single cell infiltration.

This article is a revised and extended version of our previous works as for the relationship between TCD and lymph node metastasis in esophago-gastrointestinal carcinoma (EGIC) [10,11].

TCD in Invasive Squamous Cell Carcinoma

Material and Methods

Patient Selection

We obtained 239 samples of superficial esophageal squamous cell carcinoma (SESCC) from 239 patients who underwent surgery at Dokkyo University Hospital between 1987 and 2003. Among these, we investigated 37 cases of SESCC defined as carcinoma with invasion limited to the submucosa. The resected specimens were fixed in a neutral aqueous phosphate-buffered 4% solution of formaldehyde and sectioned at intervals of 5 mm, then embedded in paraffin and stained with hematoxylin and eosin (H&E). Additionally, we performed elastica van Gieson (EVG) and Victoria blue staining to detect vessel invasion.

We examined the histopathological findings according to the Japanese Guidelines for Clinical and Pathological Studies on Carcinoma of the Esophagus [12]. Infiltrating growth (inf) was classified into three types as follows: expansive growth pattern (inf α), infiltrating growth pattern (inf γ), and intermediate pattern between infα and infγ (inf β).

On the basis of the depth of tumor invasion, we classified all carcinomas into four groups. Carcinomas not invading as far as the muscularis mucosa were classified as group A; carcinomas invading to the muscularis mucosa or to less than one-third of the upper submucosa were classified as group B; those invading to the middle layer of the submucosa were as group C; and those invading to one-third of the lower submucosa were classified as group D.

Evaluation of TCD

We selected the section of each carcinoma with the greatest dimension. We examined these sections for TCD at the invasive front and measured the longitudinal length of the TCD region. Furthermore, we measured the maximal longitudinal length of the area of invasion into or beyond the lamina propria with an optical micrometer (U-OCM 10/100, Olympus, Tokyo, Japan). Silver staining was used to enable easy identification of the basement membrane, and to determine whether the carcinomas were limited to the epithelium or had invaded beyond the basement membrane. TCD score was calculated by dividing the length of the TCD region by the maximal longitudinal length of the area of invasion into or beyond the lamina propria, and multiplying by 100 (Figure 1).

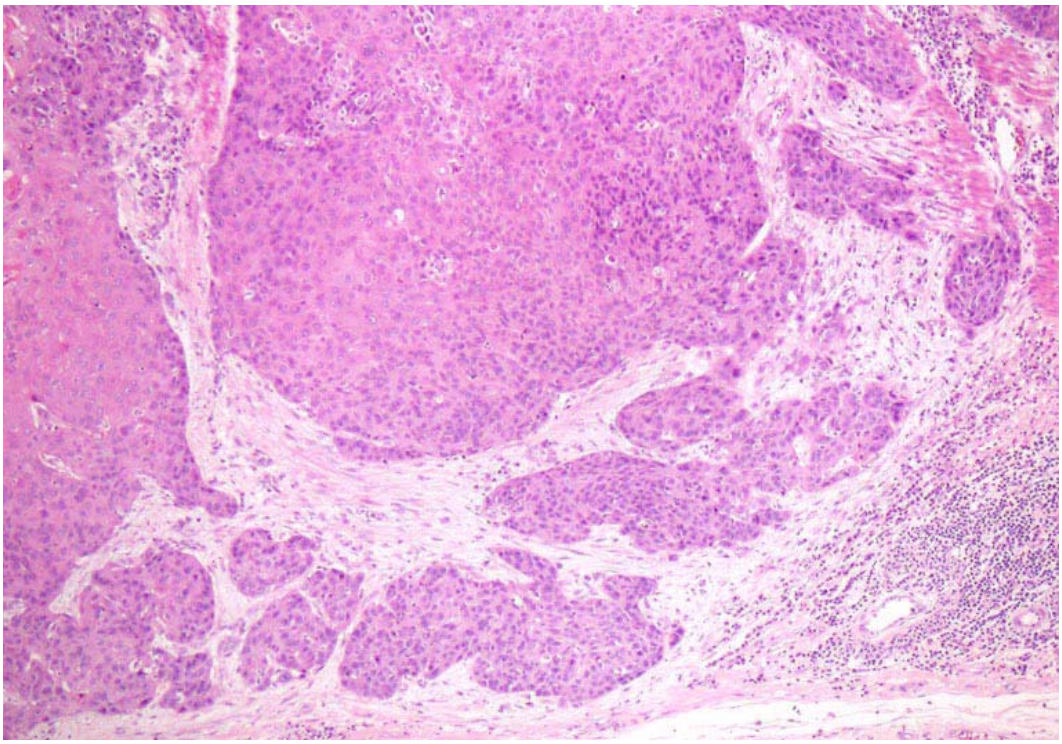

Figure 1. Tumor cell dissociation (TCD) was shown at the invasive front in the squamous cell carcinoma of esophagus.

Immunohistochemistry for E-cadherin

Immunohistochemical analysis was carried out with a primary antibody against E-cadherin (diluted 1:4000; Transduction Laboratories, Lexington, KY, USA) in formalin-fixed paraffin-embedded tissue sections by use of a Labeled Streptavidin-Biotin kit (DAKO, Carpinteria, CA, USA), as described previously [13]. For the primary antibody, normal esophageal epithelium was stained and used as a positive control. Normal epithelium adjacent to the tumor was stained and served as an internal control. Negative controls were duplicate sections stained simultaneously in which the primary antibody was replaced by nonspecific rabbit serum.

The intensity of E-cadherin staining of the carcinomas was compared with that of the normal epithelium of the esophagus. The pattern of E-cadherin expression of the carcinomas was evaluated by analysis of the dominant pattern of E-cadherin-positive cells. Normal esophageal epithelium showed localized and preserved staining of E-cadherin in the cell membrane. E-cadherin expression of the carcinomas was investigated in the TCD region and the successional area of mucosal invasive carcinoma (SAM) located over the TCD area. Tumors with localized and preserved staining of E-cadherin in the cell membrane were classified as having a preserved pattern. Tumors with reduced staining in the cell membrane and staining in the cytoplasm only, or tumors with no immunolabeling, were classified as having a reduced pattern.

Statistical Analysis

The chi-squared test (or Fisher's exact test when the expected number of any cell was smaller than or equal to five cases) was performed to determine any correlations between the clinicopathological findings and the presence of lymph node metastasis. Differences at $P < 0.05$ were considered to be statistically significant.

Student's *t* test was performed to compare TCD score and the depth of tumor invasion or E-cadherin expression, where $P < 0.05$ was considered significant.

Results

Relationships between Clinicopathological Findings and Lymph nOde Metastasis

In total, out of 37 SESCC cases, one verrucous carcinoma was excluded from the analyses, and one case having severe necrosis was excluded because of loss of the mucosal lesion. We also excluded 2 other cases, because small carcinoma nests that had at first appeared to be TCD with H&E staining proved to be blood vessel invasion upon staining with EVG. Lymph node metastases were found in 9 (27.2%) of the 33 SESCC cases. The incidence of lymph node metastasis was 0% (0/5) in group A, 10% (1/10) in group B, 36.4% (4/11) in group C, and 57.1% (4/7) in group D.

Regarding the relationship between lymph node metastasis and vessel invasion, 9 of 18 cases showing lymphatic vessel invasion and 5 of 8 cases showing blood vessel invasion had lymph node metastasis. The incidence of lymph node metastasis was significantly higher in SESCC cases with lymphatic vessel and blood vessel invasion than in those without ($P = 0.0025$, $P = 0.040$, respectively).

Relationship between Lymph Node Metastasis and TCD Score

The TCD scores of the SESCC in each group were shown in Table 1. The mean TCD scores (± SE) of carcinomas with and without lymph node metastasis were 85.3 ± 5.7 and 16.3 ± 3.9, respectively. The mean TCD score for cases with lymph node metastasis was higher than for those without ($P < 0.001$).

All cases without TCD in group A, had no lymph node metastasis. In group B, one case with lymph node metastasis had a TCD score of 51.8. The remaining 9 cases had no lymph node metastasis and had TCD scores of less than 30, except for 1 case. In group C, there were 4 cases with lymph node metastasis, and their TCD scores were 100, 90.1, 89.2 and 68.0. On the other hand, 7 cases had no lymph node metastasis, and all of them had a TCD score of less than 30. In group C, the mean TCD scores (± SE) of carcinomas with and without lymph node metastasis were 86.8 ± 6.7 and 15.9 ± 2.6, respectively, and the mean TCD score of cases with lymph node metastasis was higher than that of cases without lymph node metastasis ($P < 0.001$). There were 4 cases with lymph node metastasis in group D, and their TCD scores were 73.9, 96.5, 98.0 and 100. The 3 cases without lymph node metastasis had widely varying TCD scores: 4.7, 62.0 and 88.3.

E-cadherin expression in TCD and SAM

Twenty-five of the 33 examined cases had TCD, whereas the remaining 8 cases did not have TCD. E-cadherin expression in the area of TCD was reduced in 18 of these 25 carcinomas (72.0%), and E-cadherin expression in the SAM was reduced in 6 (24.0%). E-cadherin expression was significantly reduced in the area of TCD compared with that in the SAM ($P < 0.001$).

Table 1. Relationship between Tumor Cell Dissociation (TCD) Score and Lymph Node Metastasis in Superficial Esophageal Squamous Cell Carcinoma (SESCC)

Lymph node metastasis	No. of cases	TCD score† (mean ± SE)	*P* value
Group A*			
Positive	0		
Negative	5	0	
Group B*			
Positive	1	51.8	
Negative	9	13.9±3.3	
Group C*			
Positive	4	86.8±6.7	< 0.001
Negative	7	15.9±2.6	
Group D*			
Positive	4	92.1±6.1	0.242
Negative	3	51.7±24.7	

* Depth of tumor invasion, see "Material and method" for details.
† Evaluation of TCD in SESCC, see "Material and method" for details; SE, Standard error.

TCD in Invasive Adenocarcinoma

Materials and Methods

Patient Selection

We obtained 132 samples of submucosal invasive colorectal carcinoma (SICC) from 132 patients. Patients with familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer or inflammatory bowel disease were excluded from the study. One hundred and one patients underwent surgical resection, and 31 patients were treated with endoscopic mucosal resection (EMR) followed by additional surgical resection. Twelve patients initially treated with endoscopic resection were eliminated from the study because the margin of the resected specimen was positive for cancer cells. Thus, 120 SICC cases were included in this study. Twenty cases had lymph node metastasis, and 100 cases had no metastasis.

Evaluation of TCD

Sections stained with H&E were examined by light microscopy at 40-200x magnification. Histological findings of small solid carcinoma cell clusters and groups of dissociated dedifferentiated carcinoma cells at the invasive front were considered to be TCD. When TCD

occupied 5% or more of the total horizontal length of the invasive front, the tumor was evaluated as TCD-positive. When TCD occupied less than 5% of the total horizontal length of the invasive front, the tumor was evaluated as TCD-negative (Figure 2).

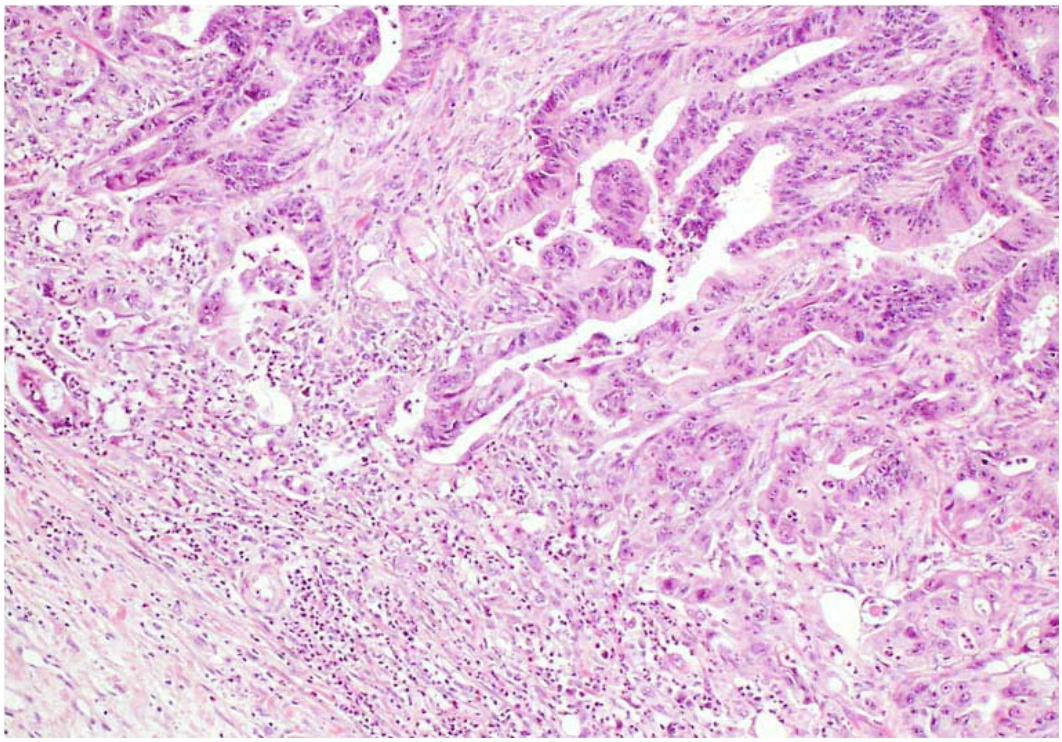

Figure 2. Tumor cell dissociation (TCD) was shown at the invasive front in the adenocarcinoma of colon.

Immunohistochemistry for E-cadherin and β-catenin

Immunohistochemical analysis was carried out with primary antibodies against E-cadherin and β-catenin (Transduction Laboratories, Lexington, KY, USA), diluted 1:4000 and 1:2000 respectively, in formalin-fixed paraffin-embedded tissue sections by use of a Labeled Streptavidin-Biotin kit (DAKO, Carpinteria, CA, USA), as described previously. [13] For each primary antibody, normal colorectal epithelium was stained and used as a positive control. Normal epithelium adjacent to the tumor was stained and served as an internal control. Negative controls were duplicate sections stained simultaneously, in which the primary antibody was replaced by nonspecific rabbit serum.

Sections were examined by light microscopy, and staining was assessed with respect to localization (nuclear, cytoplasmic, and membranous) and intensity. The adjacent normal colonic mucosa was used as an internal control. Distinct nuclear staining in less than 30% of cancer cells was considered absent and in 30% or more of cancer cells was considered present. Intense cytoplasmic staining in less than 30% of cancer cells was considered normal and in 30% or more of cancer cells was considered increased. Distinct membranous staining in at least 30% of cancer cells was considered normal and in less than 30% of cancer cells was considered decreased.

Statistical Analysis

The chi-squared tests were performed to determine correlations between lymph node metastasis and TCD, lymph node metastasis and expression of E-cadherin or β-catenin, and TCD and expression of E-cadherin or β-catenin. Yates' correction was performed as necessary. *P* values < 0.05 were considered statistically significant.

Results

Relationship between TCD and Lymph Node Metastasis

Of 120 SICC cases, 58 cases were positive for TCD. Eighteen of 20 (90%) cases with lymph node metastasis were TCD-positive, and 40 of 100 (40%) cases without lymph node metastasis were TCD-positive. There was a statistically significant correlation between TCD and lymph node metastasis ($P < 0.0001$; Table 2), as TCD was identified more frequently in cases of SICC with lymph node metastasis.

Table 2. Relationship between Tumor Cell Dissociation (TCD) and Lymph Node Metastasis in Submucosal Invasive Colorectal Carcinoma (SICC)

Lymph node metastasis	TCD*		*P* value
	Negative	Positive	
Negative	2	18	< 0.0001
Positive	60	40	

* Evaluation of TCD in SICC, see "Material and method" for details.

Expression of E-cadherin and β-catenin in SICC with or without Lymph Node Metastasis

Membranous expression of E-cadherin was decreased in 18 of 20 (90%) SICC cases with lymph node metastasis and in 61 of 100 (61%) SICC cases without lymph node metastasis. Nuclear expression of β-catenin was present in 4 of 20 (20%) SICC cases with lymph node metastasis and in 5 of 100 (5%) SICC cases without lymph node metastasis. There was a statistically significant relationship between membranous expression of E-cadherin and lymph node metastasis of SICC ($P < 0.05$), as membranous expression of E-cadherin was significantly decreased in SICC cases with lymph node metastasis. Although it was not statistically significant, there was a tendency for nuclear expression of β-catenin to occur in SICC cases with lymph node metastasis ($P = 0.063$).

Expression of E-cadherin and β-catenin in SICC with or without TCD

Decreased membranous expression of E-cadherin occurred in 46 of 58 (79.3%) SICC cases with TCD and in 33 of 62 (53.2%) SICC cases without TCD. There was a statistically significant relationship between decreased membranous expression of E-cadherin and TCD ($P < 0.01$), as SICC cases with TCD displayed significantly decreased membranous expression of E-cadherin.

Discussion

TCD is the first step of malignant tumor invasion and the most important step in the metastatic process [3]. TCD refers to the histological finding of small solid carcinoma cell clusters and groups of dissociated dedifferentiated carcinoma cells at the invasive front. In a previous study, we conducted an evaluation of TCD in EGIC and indicated that this mode of invasion was highly reflective of the invasive and metastatic potential of invasive squamous cell carcinoma and adenocarcinoma [13-17].

In the present study, we performed an evaluation of TCD in SESCC and SICC, using an appropriate criterion for each tumor type (see Material and Methods for detail). The results indicated a relationship between TCD and lymph node metastasis in SESCC and SICC. Thus, evaluation of TCD would be a useful marker for predicting lymph node metastasis after EMR treatment of EGIC, assisting practitioners in deciding whether to carry out additional surgical resection. However, since the tumor characteristics of malignant potential, such as invasion and metastasis, are different between squamous cell carcinoma and adenocarcinoma, the criteria for evaluation of TCD should be adjusted according to histological type of EGIC.

E-cadherin is known to be one of the major molecules mediating adhesion between epithelial cells, and its expression is reduced in accordance with the progress of cancer [18,20]. Several immunohistochemical studies of E-cadherin expression in EGIC have been undertaken [5, 21-24]. In tumor cell lines, a clear correlation between reduction of E-cadherin expression and diminution of intercellular adhesion has been described [25-27]. Our previous study also showed that lymph node and liver metastasis in EGIC was highly correlated with reduced expression of E-cadherin [6]. These reports suggest that dysfunction of E-cadherin mediated cell-cell adhesion results in the acquisition of invasive and metastatic potential. Thus, reduced membranous expression of E-cadherin might play a crucial role in the formation of TCD and may be associated with invasion and metastasis of EGIC.

In conclusion, TCD and E-cadherin may be important predictive markers for lymph node metastasis in EGIC. Furthermore, evaluation of these markers in EMR specimens might enable accurate prediction of lymph node metastasis, extend the indications of EMR treatment and contribute to avoiding redundant additional surgery.

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Chapter II

IN THE FIELD OF COLORECTAL SURGERY

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Introduction

In comparison with gastric cancers, whose histologies vary, colorectal cancers are known to be histologically monotonous. However, when cancers of the colon and rectum are observed carefully, a subtle intratumoral variety is recognized by examining the locus in a monotonous histology. Since the invasive front of a colorectal cancer represents a struggle between the host's normal tissue and the tumor tissue, it is considered that the tumor's original characteristics of malignancy and invasiveness, as well as its biological attitude, tend to be most apparent at the lesion, where we can grasp the subtle variety in a seemingly monotonous histology. Tumor budding is a histological finding at a cancer's invasive front, and every pathologist observes it in routine pathological examinations. Actually, tumor budding can be examined by the hematoxylin and eosin staining technique for resected specimens without any additional procedure.

Recent clinicopathological studies on the prognostic factors in colorectal cancer have focused on tumor budding as a potential prognostic factor. Not only are researchers verifying the significance of tumor budding as a prognostic factor in association with lymph node metastasis and postoperative outcome, but they are also utilizing budding examinations as a decision-making aid in establishing treatment strategies for colorectal cancer. Moreover, they are clarifying the mechanism of tumor budding in association with adhesion molecules and the expression of oncogenes. This article reviews tumor budding, mainly from the perspective of colorectal surgery.

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Histological Features at the Invasive Front in Western Countries

The focus on histological features at the invasive margins of tumors in carcinomas is by no means recent. In 1920, Broders [1] reported on undifferentiated cancer cells in the invasive regions of the lip. After classifying these cells into four grades according to the proportion of undifferentiated cancer cells, he reported a correlation between grade and prognosis. In 1932, Dukes [2] noted the altered morphologic features that can be observed along the advancing edge of rectal cancer, and he recommended ignoring these edge features when assigning a tumor grade. On the other hand, in 1939, Grinnell [3] presented eight criteria related to the "manner of growth" of the tumor cells, the cell morphology, and the reaction of the stroma. For the "manner of growth", he demonstrated that the deep infiltrating edge of colorectal adenocarcinoma was often the least differentiated, and that the five-year survival and lymph node metastases were optimally related to the tubular configuration architecture of the adenocarcinoma. Specifically, at the advancing margin of the tumor this architecture featured irregularly folded, distorted, and small tubules or the absence of any tubules at all.

Thus, the advancing edge of colorectal cancer in association with tumor malignancy has received attention since the 1920s. Further research, however, has been thriving again since the 1980s. In 1987, Jass et al. [4] proposed a system of tumor grading based on the nature of the advancing tumor margin (growth pattern), dividing rectal tumors into an expanding type and an infiltrating type. Especially, several multivariate analyses [4-6] have demonstrated that an irregular, infiltrating pattern of growth, as opposed to a smooth-pushing border, is an independent adverse prognostic factor. They noted that glands at the advancing edge of adenocarcinoma were often disorganized and of a higher grade than the adenocarcinoma was predominantly. They concluded that the grade should be based on the worst, rather than the predominant, tubular architectural pattern. Furthermore, in 1995, Shepherd [7] called special attention to the morphologic features along the advancing edges of adenocarcinomas, and he cautioned pathologists to exclude this area in the evaluation of tumor grade. This was similar to Dukes's earlier recommendation.

Histological Evaluation of the Invasive Front in Japan

In 1954, a Japanese pathologist, Imai, proposed a classification of solid carcinoma, including carcinomas of the tongue, larynx, uterine cervix, breast, and stomach, based on variations in the pattern of growth at the advancing margin [8]. He divided the growth pattern into three basic types: nonsprouting, sprouting, and lymphatic- (occasionally blood vessel-) permeating types [9]. In his classification, the nonsprouting type referred to the cancer spreading by forming large cell nests or glandular structures, while the sprouting type referred to the cancer propagating as more or less anaplastic thin cell cords or as individual free cells. In his reports, he mentioned that sprouting was consistent with the finding of the "undifferentiated region" that Broders adopted as a criterion in his classification [8]. Furthermore, the sprouting type was subdivided into reactive sprouting and nonreactive

sprouting according to whether or not the advancing margins are embedded in newly formed connective tissue.

The term "tumor budding" first appeared in a report by Morodomi et al. (1989), which defined it as the presence of isolated single undifferentiated cancer cells or as clusters of five or more cancer cells forming microtubular cancerous glands scattered in the stroma at the invasive margin of colorectal cancer [10]. Later, we redefined tumor budding as isolated single cancer cells or clusters of around five or fewer cancer cells forming a trabecular or microtubular architecture [11], and this definition seems to have become prevalent in the reports from Japan and Western countries [12-16]. The concept of tumor budding is novel and, in terms of the field estimated, apparently differs from the conventional parameters of tumor aggressiveness used in Broders's grading system, Jass's grading system, and Imai's classification. Tumor budding is a histological feature that appears in the more restricted region at the invasive front and should be evaluated in a more highly magnified field than are conventional parameters such as the "undifferentiated region" in Broders's grading system and in Jass's growth pattern, or in sprouting as defined by Imai. For example, tumor budding is reported to be positive in 18% of expanding-type tumors with a growth pattern (Jass's category); the observation of tumor budding in that case was made by low-power microscopic examination and inspection by the naked eye, and was negative in 26% of infiltrating tumors [15]. Tumor budding can be observed at the actively invasive region in a group of differentiated tumors. It should be advantageous to evaluate this feature in colorectal cancers, most of which are classified into the differentiated type.

The Mechanism of Tumor Budding

Tumor budding is thought to be an active invasive form of cancer; specifically, it is the isolation and mobilization of cancer cells from a main tumor mass in the early stage of tumor invasion. In 1985, Gabbert [17] carefully studied 1,2-dimethylhydrazine-induced colon cancer in the rat, and noted that "the first and essential step in tumor invasion is the tumor dedifferentiation and dissociation at the invasive front." He also observed that tumor invasion was supported by an interstitial edema in the host tissue adjacent to the periphery of the tumor, which caused the opening and widening of the host interstitial space and allowed tumor cells to mobilize outside of the main tumor. In 1986, Carr et al. [18] identified single cells and small clusters of cells in the invasive edges of human colorectal carcinomas, which have ultrastructural features suggestive of active cell movement by transmission electron microscopy and tissue culture. These phenomena are supposed to correspond well to the budding of colorectal cancer. In 1999, Nabeshima et al. [19], after studying tumor cell motility and invasion at the invasive front of the lesion in vitro, explained tumor budding as a form of invasion and migration. On the other hand, Morodomi et al. [10], in serial observations of developing cancer, reported that tumor budding consistently precedes lymphatic invasion by tumor. They speculated that budding may be a prelude to lymphatic invasion.

In this way, tumor budding may represent one phase of cancer infiltration, in which cancer cells migrate from the cancer structure, although the precise mechanism of budding has not been clarified. The ample blood supply along the advancing edge and the lack of

secondary degeneration of tumor cells may provide an opportunity for the neoplastic cells to express their full biologic potential [20]. In addition, a dynamic interaction occurs at the interface between tumor cells and the surrounding mesenchymal stroma. The loss of both glandular differentiation and cell cohesion that gives rise to these dissociated elements is probably a crucial event in development of highly invasive and metastatic properties. The altered morphologic features of some neoplasms along their advancing front may be due to a regionally altered immune response, complex growth-repressive phenomena, or altered cell cohesiveness [21]. Various important molecular events in a tumor spread, such as the gain and loss of adhesion molecules, the secretion of proteolytic enzymes, and increased proliferation, are suggested to occur at the invasive front. Recently, some researchers suggested that tumor budding is related to laminin-5 γ 2 chain expression [13,14,22-24], CD44 variant 6 expression [25], syndecan-1 expression [26], Bcl-2 protein expression, [27] p53 protein expression [28], APC mutation and MSI-H status [12], and mismatch repair status [29]. Thus, future studies on the formation of tumor budding should be investigated by molecular or genetic techniques, which may clarify the relationship between budding and the malignant potential of colorectal carcinoma.

Clinical Significance of Tumor Budding in T1 (early invasive) Colorectal Cancer

The widespread use of colonoscopy has enhanced our ability to encounter small, early cancers of the colon and rectum. However, this technology has also created a therapeutic dilemma: Is colonoscopic removal sufficient therapy for tumors that have invaded the submucosa, or should subsequent bowel resection and lymph node dissection be performed? In previous investigations, the incidence of lymph node metastasis varied from 3% to 15% of T1 (early invasive) colorectal carcinomas [20-22, 25, 28, 30-40], and radical surgery with lymph node dissection is known to be necessary with these tumors. It is important to avoid oversurgery while nonetheless completely curing T1 colorectal cancer. The decision for additional bowel resection with lymph node dissection depends on an estimate of the risk factors of lymph node metastasis. In the last 20 years, many investigators have advocated the following histological criteria: massive submucosal invasion, lymphatic invasion, or poorly differentiated histology, when considering additional surgery after endoscopic resection of T1 colorectal carcinomas [30-34, 41, 42]. However, these criteria could not be the definitive risk factors for lymph node metastasis, because of the low incidence of poorly differentiated tumors or the difficulty of judging a lymphatic invasion precisely [33].

Tumor budding has been studied from aspects such as those. First, an excellent advantage of focusing on tumor budding is that it can be judged easily by hematoxylin and eosin staining, with no need for additional special procedures. Next, although a T1 cancer has little absolute quantity of cancer relative to the quantity in advanced cancer, the rate at which tumor budding can be detected in a T1 extraction specimen is said to be high.

Studies on tumor budding in T1 colorectal cancer have mainly been from the standpoint of establishing a correlation with lymphatic invasion and lymph node metastasis. In 1987, Hayashida [43] first reported that tumor budding is related to lymphatic invasion; the rate of coincidence was more than 80% in 30 T1 colorectal cancers. Consequently, tumor budding is

a useful tool to supplement the determination of lymphatic invasion, which is difficult to judge. In 1989, Coverizza et al. [30] reported that adenomas containing well or moderately differentiated adenocarcinoma having a focus of poorly differentiated or undifferentiated histology were at high risk for lymph node metastasis. This finding is supposed to be a part of tumor budding. In 1993, Araki et al. [39] found lymph node metastases in 41% of patients with positive tumor budding.

In 1995, we reported that, among 79 T1 tumors, patients with tumor budding (44% of the population) had a 25% incidence of nodal spread, whereas those with no budding experienced no lymph node involvement [20]. We pointed out five histopathologic risk factors for lymph node metastasis: 1) tumor budding; 2) a poorly demarcated invasive front; 3) moderately or poorly differentiated cancer cells in the invasive front; 4) extension of the tumor to the middle or deep submucasal layer; and 5) cancer cells in lymphatics. In the same study we demonstrated that patients with three or fewer risk factors had no nodal spread, whereas the rate of lymph node involvement with four or more risk factors was 33% or 67%, respectively. In 1999, Goldstein et al. [21] studied histologic features associated with lymph node metastasis in 73 abdominoperineal resection specimens with T1 or superficial T2 adenocarcinomas. They looked especially for the leading edge of the tumor, namely "budding", for undifferentiated cells that were either isolated or in small clusters, or for microacinar structures. The first two features—"budding" and undifferentiated cells—appear to correspond to tumor budding, judging from the photographs in their report. They found a significant association with lymph node metastases when extensive microacinar structures, "budding", or undifferentiated cells existed along the neoplasma's edge. They proposed that when any of these features is present, additional therapy, including adjuvant radiation, chemotherapy, or both—or complete abdominoperineal resection—may be warranted to address potential lymph node metastases in a locally excised rectal adenocarcinoma.

In the 2000s, Masaki et al. [22, 25, 35, 37, 44, 45] reported that tumor budding was significantly associated with lymph node metastasis or local recurrence. They concluded that tumor budding was more useful in predicting lymph node metastasis in early invasive colorectal carcinomas than were three histological criteria: massive submucosal invasion, vascular invasion, or poorly differentiated histology. In 2002, Okuyama et al. [36] revealed that the combination of lymphovascular invasion and budding predicted lymph node metastasis in pT1 or pT2 tumors more accurately than lymphovascular alone.

To our knowledge, a total of 13 papers on tumor budding in T1 colorectal cancer have been reported, with the first of these being Araki et al. In those reports, the incidence of positive tumor budding ranged from 21 to 56% [20, 21, 28, 36-40]. In patients with tumor budding, the incidence of nodal involvement varied from 24 to 41%, vs 0 to 7% in those without tumor budding [20, 36, 39]. In 3 of the 13 reports, the incidence of lymph node metastasis differed significantly between positive and negative budding. In univariate analysis, tumor budding was associated with not only nodal involvement but lymphatic invasion [22, 36, 39, 44, 45], absolute grading of the depth and width of submucosal invasion [44], or locoregional failure [22, 25, 35, 44, 45]. Furthermore, in multivariate analysis, tumor budding was associated with lymphatic invasion [25] or locoregional failure (lymph node metastasis or local recurrence) [25, 37, 44, 45].

Clinical Significance of Tumor Budding in Advanced Colorectal Cancer

The TNM and Dukes [2] staging system for colorectal cancer has been the most widely employed classification scheme, since it is simple to apply and provides useful prognostic information. However, it is well known that some cases of cancer of the colon or rectum progress more rapidly than other cases of the disease in the same stage. As for the cause of this difference, it is possible that these staging systems, classified by the extent of the tumor spread at the time of operation, do not account for the degrees of invasiveness and malignancy that the tumor itself has. Concerning the prognosis of colon and rectal cancer, it is considered that the qualitative characteristics of the true biologic activity of the tumor, e.g. tumor budding, are an important key in addition to the staging systems.

The number of potential prognostic factors in colorectal cancer is constantly increasing. This is associated with the biologic diversity of the malignancy for a specific feature of cancer at the same stage of advancement. Tumor budding has been studied for one of these prognostic factors. To our knowledge, a total of 14 reports have been published on the clinicopathological study of tumor budding in advanced colorectal cancer [10-12, 14-16, 27, 40, 46-51]. Two of these reports used terms other than “tumor budding”: Adachi et al. [51] used “sprouting” and Ono et al. [49] used “focal dedifferentiation”.

In 1989, Morodomi et al. [10] was the first to report tumor budding in association with lymph node metastasis in advanced rectal cancer. They divided tumors into three groups according to the degree of tumor budding, by counting the buds in a visual field, which measured 500 μm $\times$ 2500 μm square, at four locations in each slide: squares having an average count of 0 to 4 buds were grouped as negative, those with 5 to 14 were grouped as mildly positive, and those with 15 or more were grouped as strongly positive. By this classification, the rates of tumor budding in 40 rectal cancer cases were 35% negative, 37.5% mildly positive, and 27.5% strongly positive. They also demonstrated strong relationships between the degree of budding and the occurrence of lymphatic invasion, and between the degree of tumor budding and lymph node metastasis.

In 1993, we examined 663 patients who had undergone curative resection of colorectal cancer for tumor budding [11]. We classified tumor budding into two groups according to their degree: none or mild (BD-1) and moderate or severe (BD-2) (Figure 1). BD-1 was revealed in 493 patients (74%), and BD-2 was found in 170 patients (26%). More severe budding was associated with worse outcomes: 71% of BD-2 patients had a recurrence, compared with 20% of BD-1 patients. The five-year survival rate was also significantly worse in BD-2 than in BD-1 (22% vs. 71%). The incidence of BD-2 rose with the Dukes stage. However, the five-year survival rate of Dukes B patients with BD-2 lesions was significantly worse than that of Dukes C patients with BD-1 cancers (29% vs. 66%). Moreover, there was no difference in the five-year survival among BD-1 patients with either Dukes B or C lesions (68% vs. 66%). Therefore, tumor budding was more closely associated with both recurrence in and long-term survival of patients with colorectal carcinoma than the Dukes stage was. We concluded that tumor budding is an important predictor of outcomes in patients with colorectal cancer. However, there was some indication that the criteria by which our grading system judges tumor budding, specifically the way in which it differentiates between BD-1 and BD-2, is not clear. In 2002, Ueno et al. [15] proposed another grading system of tumor

budding in 638 rectal cancers. They divided budding into two groups: low-grade budding was defined as <10 foci in a 250x microscopic field, while high-grade budding was ≥10 foci in a field. With reproducibility and objectivity, the degree of grading agreement was nearly perfect based on their intraobserver study. They observed high-grade budding in 30% of patients with rectal cancer. This category was significantly associated with lower five-year survival rates (41%) than patients with low-grade budding (84%). Furthermore, based on multivariate analysis, tumor budding was selected as a significant independent variable, together with the number of nodes involved, lymphatic invasion, and tumor differentiation. On the other hand, tumor-budding grading systems were also proposed by Ono et al. [49] in 1996 and Guzinska-Ustymowics et al. [27] in 2003. Eventually, the further accumulation of patients in a multi-institutional study may be required.

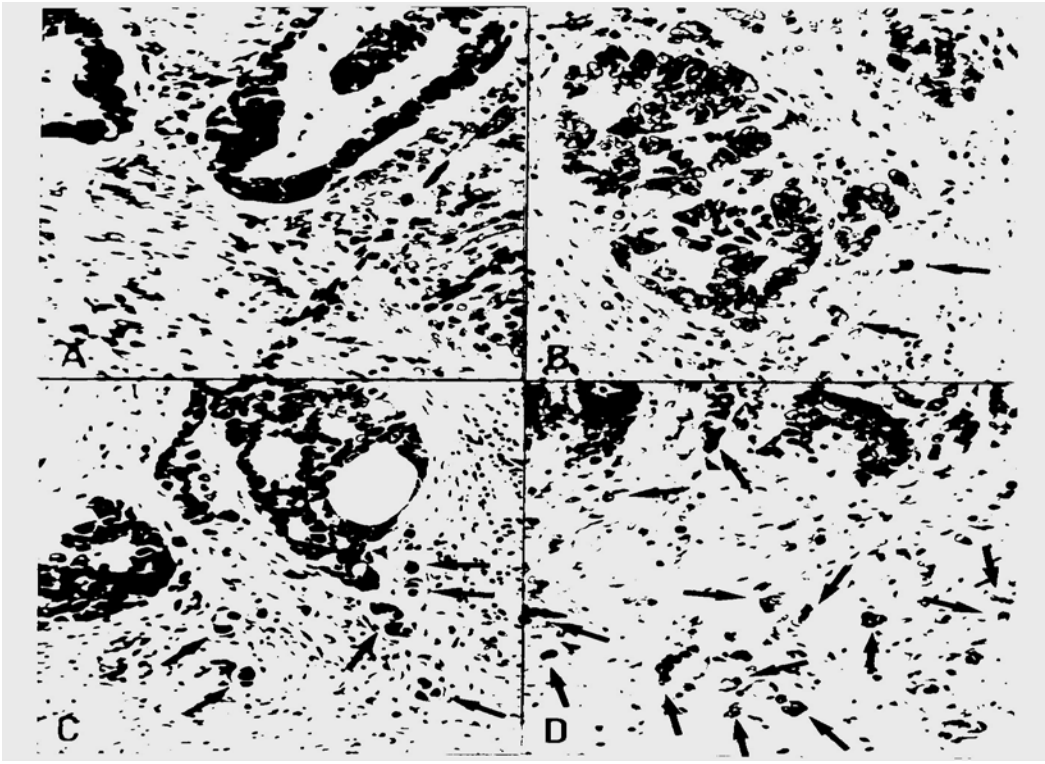

Figure 1. Tumor budding (arrow), showing small clusters of poorly differentiated or undifferentiated cells, ahead of the invasive front of rectal cancer (x50); A. absence of budding (bd-); B. lower grade budding (bd_1); C. moderate grade budding (bd_2); D. higher grade budding (bd_3).

In 2000, Ueno et al. [46] studied 85 consecutive patients with colorectal cancer who had undergone curative resection of primary lesions and metastatic liver diseases. Their goal was to clarify the clinical significance of tumor budding as an indicator of treatment strategy for colorectal liver metastases. They showed that tumor budding was related closely to survival after primary colorectal surgery, as well as to survival after surgery for colorectal liver metastases. Furthermore, multivariate analysis indicated that the aggressiveness of the primary tumor, early liver metastasis, and a large number of liver metastases indicated the only characteristics that could be detected before hepatectomy and that independently indicated a lower chance of survival. They also stressed that among the parameters, the most

powerful prognostic predictor was the aggressiveness of the primary tumor, represented by tumor budding at the invasive front and the extent of nodal involvement.

Regarding a study on tumor budding according to stage, in 2003 Tanaka et al. [16] reported that the presence of moderate or severe budding (Hase's classification) indicated a high risk of liver, local, and peritoneal recurrence and a low cumulative disease-specific survival rate at five years, compared to no or mild budding in patients with Stage II colon cancer after curative surgery. Similarly, in 2003, Okuyama et al. [47] reported that tumor budding is a significant prognostic factor in Stages II and III colon carcinoma after curative resection. That is, patients with budding-positive lesions had a significantly higher incidence of liver metastasis and/or lung metastasis and a worse postoperative survival rate than those with budding-negative lesions. Moreover, they observed no significant difference in the survival curves between patients with budding-positive Stage II lesions and those with Stage III lesions. Finally, multivariate analysis revealed tumor budding as the most significant prognostic cofactor of postoperative survival in Stage II and Stage III colon carcinoma.

Furthermore, in 2002 Okuyama et al. [48] reported the significance of tumor budding in patients with mucinous carcinoma. According these authors, tumor budding was identified exclusively at the invasive margin of concomitant well or moderately differentiated portions of mucinous carcinomas. Both the univariate and multivariate proportional hazard models revealed that the presence of budding was the only significant co-factor of postoperative survival.

A Trial of Preoperative Detection of Tumor Budding by Preoperative Biopsy

Since tumor budding is, as described above, one of the most important prognostic factors, it is useful to evaluate tumor budding by preoperative biopsy specimens in order to select an operative procedure and adjuvant therapy. In 1989, Morodomi et al. [10] were the first to report the significance of tumor budding in relation to lymph node metastasis. By studying preoperative needle biopsy samples of 112 patients with rectal cancer, they found that the most appropriate location for a preoperative biopsy is at the edge or base of the anal side of the tumor. They also found it necessary to fully extract the actively invasive region of the submucosa. They recognized tumor budding in a relatively large portion of the biopsy specimens (52 of 112 specimens, 46%), and found lymph node metastasis in 42 of 52 specimens (79%). They suggested that budding in preoperative biopsy specimens might have predictive value in lymph node metastasis.

The submucosal invasive frontal region of a tumor is part of the tumor's advancing margin, and it may better reflect the true biologic activity of cancer than the deeper invasive frontal region, because associated inflammation and necrosis have less of an influence on the former than on the latter. Ueno et al. [38] examined the presence of tumor budding in the submucosal horizontal invasive front in resected specimens of 437 patients with advanced rectal cancer. They pointed out three unfavorable characteristics in the submucosal horizontal invasive frontal region:

1) tumor budding,

2) poor differentiation, and
3) vascular invasion.

Using multivariate logistic analysis, they found that each of these features affected the number of lymph nodes involved, the development of extranodal tumor deposits, the circumferential surgical margin involvement, and the lateral pelvic lymph node metastases. In addition, they examined these unfavorable findings by preoperative transanal punch biopsy using exploratory excisional forceps in 73 patients. The submucosal biopsy specimens were extracted transanally from the boundary zone between the tumor and normal mucosa, including the submucosal tissue, using exploratory excisional forceps. Among these cases, they found that pelvic recurrence developed in 36% of patients with multiple unfavorable parameters, and that multiple unfavorable parameters were relevant to an increased risk of extensive local spread. They concluded that the histology in the submucosal invasive frontal region reflects the extent of local spread and can be evaluated preoperatively by transanal biopsy, which should become a useful tool for therapy selection for patients with advanced rectal cancer.

Conclusion

Tumor budding appears to indicate colorectal cancer with vigorous biologic activity and aggressiveness of colorectal cancer. Consequently, the presence of tumor budding may be associated with tumor invasion, metastasis, and poor prognosis. Moreover, tumor budding may provide useful information for decision-making on treatment strategies for operative indication, operative procedure, adjuvant therapy, and postoperative follow-up methods. Furthermore, it is easy to evaluate tumor budding by routine pathological examinations. Once tumor budding and an intensity-based grading system are defined clearly, the importance of tumor budding could become better established. Once the necessary definitions are established, we recommend the inclusion of budding in the routine histological examination of colorectal cancer.

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PART 2: BASIC RESEARCH

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Chapter III

MOLECULAR MECHANISMS UNDERLYING COLORECTAL CARCINOGENESIS

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Introduction

It is now generally accepted that cancer results from accumulation of multiple genetic disorders including mutations in the genes controlling cell growth or cell death [1, 2]. Accumulation of evidence from pathological examinations supported the existence of premalignant condition for cancer. In colorectal carcinogenesis, several lines of evidence proved that adenoma is a premalignant lesion of the colon [3]; approximately 30% of patients with invasive colon cancer are accompanied by coexisting adenomas of the colon, the anatomical distribution of adenomas and cancers are similar, and focal cancer is occasionally observed in adenoma. Remnants of adenomatous tissues have been identified in the margin of invasive cancer. In addition, clinical studies of a hereditary disease, familial adenomatous polyposis of the colon (FAP), further corroborated the predisposed condition of adenoma to cancer. Together with premalignant lesions, histologically different stages of tumors led to the proposition of a so-called "adenoma-carcinoma sequence" theory in colorectal carcinogenesis.

Here, I would like to summarize the genetic alterations in sporadic colorectal carcinogenesis, and focus on the possible molecular mechanisms underlying morphological changes in colorectal tumorigenesis.

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Genetic Alterations in Colon Cancer

Loss of Heterozygosity

Earlier allelotype studies of CRCs have disclosed frequent allelic losses (greater than 20%) on chromosomal arms 1p, 4p, 5q, 6p, 6q, 8p, 9q, 17p, 18p, 18q, and 22q [4]. Together with the concept of "adenoma-carcinoma sequence", frequent activating mutations in K-ras oncogene, mutations in p53 on chromosomal band 17p, and losses in the candidate region of FAP on chromosome 5q led Fearon and Vogelstein to propose a model for multistep carcinogenesis of the colon [5] (Figure 1). Subsequent allelotype studies identified additional regions with frequent LOH on 14q [6], 11q [7], and 9p [8]. In addition to *p53* and *APC*, tumor suppressor genes were identified on chromosome 9p (*p16INK4B*) [9] and 18q (*SMAD4/DPC4*) [10] from among the loci with significant allelic loss. Therefore other loci showing frequent LOH may contain tumor suppressor genes involved in colorectal carcinogenesis.

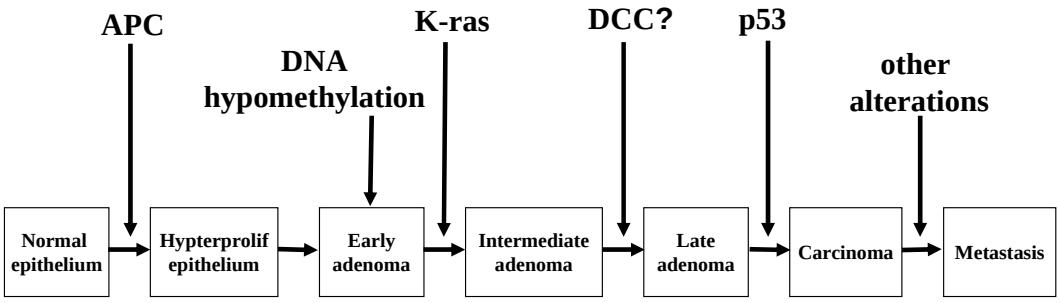

Figure 1. The initial genetic model for colorectal tumorigenesis (by Fearon and Vogelstein, adapted from reference 5).

K-ras

K-ras, one of three of the ras gene family, encodes a 21-kDa protein ($p21^{ras}$) involved in signal transduction critical for cellular proliferation and differentiation [11]. Somatic mutations at codon 12 13, or 61 of *K-ras* have been detected in colon polyps as well as colon cancers [12]. These mutations lead to constitutive activation of $p21^{ras}$ resulting in deregulated signaling, which mimic continuous growth stimulatory response. The activating mutations have been observed in approximately 35-76% of colorectal cancers and at a similar frequency in large adenomas [13, 14, 15]. The frequency of mutation is lower in small adenomas compared to large adenomas [12]. Hence its alteration is considered as a relatively early event in the tumorigenesis of colorectal cancer. Interestingly, mutation of *K-ras* is more frequently observed in elevated or exophytic adenomas (47-67%) compared to flat or superficial adenomas (0-23%), and in polypoid cancers (68%) compared to cancers without polypoid growth (28%)[15, 16, 17, 18], which suggests that aberrant K-ras activation determines macroscopic appearance. Since the primary role of ras oncogenesis transducing signals from receptor tyrosine kinase such as epidermal growth factor receptor (EGFR), its activating mutation may confer growth advantage in tumor cells and lead to exophytic growth and

polypoid morphology of the tumor. Notably *K-ras* mutations were even identified in histologically normal mucosa, and its mutations were equally observed in dysplastic polyps as well as non-dysplastic hyperplastic polyps [19]. Therefore, its activation alone seems to be insufficient for development of tumors or for malignant transformation.

p53

Somatic mutations in *p53* occur in approximately 70-80% of colorectal cancers [12, 20]. Most of the mutations are located within the DNA binding region of the TP53 protein [21], which is necessary for its interaction with the consensus-binding motif in the downstream genes [22, 23]. These target genes include $p21^{WAF1/CIP1}$, *GADD45*, *MDM2*, *BAI1*, *p53AIP*, *p53R2*, *DINP1*, and *NOXA*, implying that p53 controls a variety of cellular functions such as cell cycle, angiogenesis, apoptosis, and DNA repair [22, 23] (Figure 2). Therefore inactivation of both alleles of p53 leads to uncontrolled cell growth, prolonged survival and propagation of impaired integrity of genome to daughter cells. Since mutated p53 protein acquires prolonged half-life by escaping from Mdm2-mediated proteolysis and accumulates within the cells, positive staining by immunohistochemistry reflects mutation of p53. Studies on p53 by mutation screening, LOH on 17p, or immunohistochemical staining revealed that genetic changes of *p53* were observed in only 4-26% of adenomas but in 70-80% of adenocarcinomas, suggesting that inactivation of p53 protein is associated with transition from adenoma to carcinoma or progression of colorectal cancers [12].

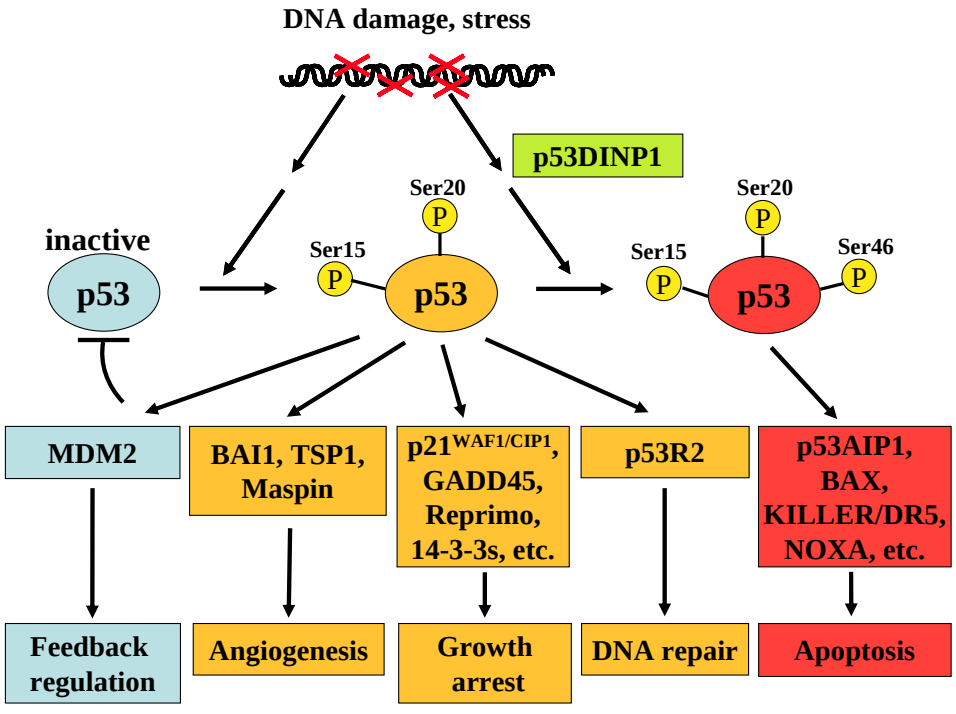

Figure 2. Mechanisms of p53 activation, p53 target genes, and their physiological roles.

APC

Adenomatous polyposis coli (*APC*) gene was isolated as a gene responsible for FAP and germ line mutations of *APC* have been identified in the majority of FAP patients [24]. In addition, somatic mutations of *APC* have been also detected in more than 70% of sporadic colorectal cancers [25], implying that its alteration plays a role in FAP as well as sporadic colorectal tumors. The majority of somatic mutations in sporadic tumors are found in the mutation cluster region (MCR) which is located between codons 1286 and 1513 within exon15, and result in production of a truncated APC protein by frameshift or nonsense mutation [26] (Figure 3). Besides point mutations or small deletions/insertions, most of the colorectal tumors are accompanied by loss of wild type *APC* allele [27, 28], indicating that inactivation of both alleles are important for the development of tumors, which is in good agreement with Knudson's "two-hit theory" [29]. Because FAP is characterized by multiple adenomas, and somatic mutations are detected even in small adenomas, APC exerts a tumor suppressive role in the very early step of colorectal tumorigenesis.

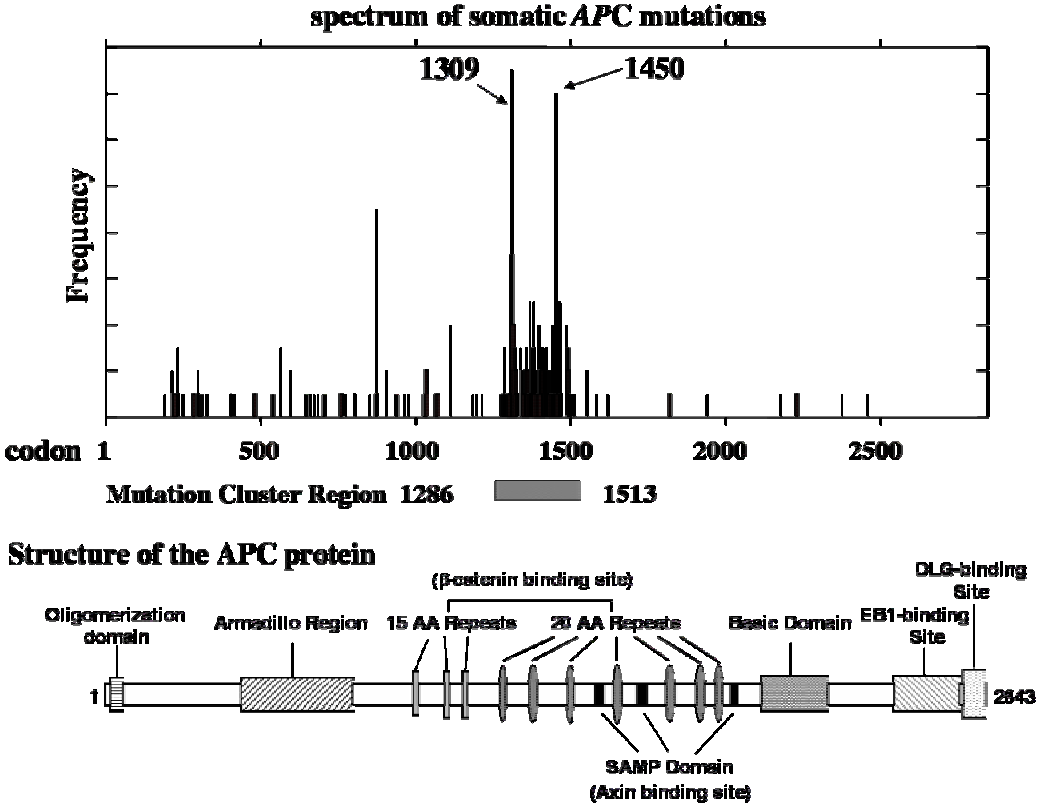

Figure 3. Spectrum of somatic *APC* mutations in sporadic colorectal tumors, and the structure of APC protein.

APC encodes a large 2843-amino acid protein containing various functional domains such as an oligomerization domain, armadillo repeats, 15-amino acid repeats, 20-amino acid repeats, SAMP domains, and a basic domain [30]. Subsequent investigations disclosed that the 20- and 15-amino acid repeats are responsible for its binding with β-catenin [31, 32], the

SAMP domains for binding with Axin/conductin [33, 34], and the carboxyl terminal domain for binding with EB1 [35] (Figure 3). Most *APC* mutations in colorectal tumors produce truncated proteins that lack these domains and are unable to interact with β-catenin, Axin/conductin, or EB1. Since EB1 associates with microtubules and plays a crucial role in mitosis, mutations in *APC* lead to chromosomal instability of the colorectal tumors with APC inactivation [36]. This notion may provide some clues for the reason why the adenoma cells with mutated APC are prone to malignant transformation.

MSI

An important pathway involved in human colorectal carcinogenesis was discovered by the analysis of microsatellite markers that consisted of tandem repeats distributed throughout the genome. de la Chapel and colleagues identified extensive nucleotide changes in tumors from hereditary non-polyposis colon cancer (HNPCC) patients, which was characterized by the altered number of repeats in microsatellite markers [37]. Such alterations, termed as "microsatellite instability (MSI)" or "replication error (RER)", have been observed in a great majority of cancers arising in HNPCC patients, and in approximately 10-15% of sporadic colorectal cancers [38, 39]. Identification of responsible genes for HNPCC, including *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6*, proved that abrogated mismatch repair (MMR) machinery is responsible for the tumorigenesis of HNPCC [40]. The impaired mismatch repair system leads to accumulation of microsatellites within the coding region of some genes, such as *TGF-β type II receptor* and *BAX* [41, 42]. Consequently, accumulated alterations in genes that associate with proliferation and/or cell survival through the impaired MMR, may result in development and progression of colorectal tumors in HNPCC patients. Although mutations in these MMR genes have been less frequently identified in sporadic colorectal cancers, hypermethylation in *MLH1* promoter region leading to reduced expression of MLH1 has been reported [43], suggesting that epigenetic changes may be involved in the carcinogenesis associated with MSI-positive sporadic tumors.

Konishi *et al*. analyzed mutations of *MSH2*, *MLH1*, *APC*, *p53*, *K-ras*, and *TGFβRII*, and MSI in sporadic colorectal tumors as well as those from FAP and HNPCC patients [44]. They observed higher frequency of *K-ras* mutation in tumors with MSI-negative group compared to MSI-positive group, and in tumors from FAP patients compared to HNPCC patients, suggesting that tumors with MSI-negative tumors might be prone to *K-ras* mutation, or that MSI-positive tumors might not need *K-ras* mutation for their progression. This view is in good agreement with the observation that flat adenomas are frequently observed in the colon of HNPCC patients [45], because *K-ras* mutation is associated with flat or non-polypoid morphology. MSI is now analyzed using several microsatellite markers, and MSI-positive tumors are divided into high group (MSI-H) and low group (MSI-L) [46]. Although MSI-H group has been shown to have distinct clinicopathological features such as location in proximal colon and better prognosis compared to MSI-L or microsatellite stable (MSS) tumors [47, 48], the definition of MSI-L tumors and their characteristics remain obscure. Jass and colleagues carried out detailed analysis of genetic alterations in MSI-L tumors and revealed that tumors in this sub-type were associated with higher *K-ras* mutation and promoter methylation of *O-6-methylguanine DNA methyltransferase* (*MGMT*) compared to MSI-H or MSS tumors [49, 50].

CTNNB1 (β-catenin) and AXIN2 (Conductin)

Isolated as a cadherin-associated protein, β-catenin functions as a component adherens junction and plays a crucial role in cell-cell adhesion through its interaction with cytoplasmic domain of E-cadherin [51]. In addition to the identification of β-catenin as an APC-binding protein [31, 32], the view that armadillo, *Drosophila* homologue of β-catenin mediates Wnt/wingless signal transduction pathway in development, shed light on the involvement of this pathway in the development of colorectal tumors.

Interestingly, approximately 1 to 2% of sporadic colon cancers harbor mutations in the β-catenin gene (*CTNNB1*) [52, 53], and its mutations were also observed in 12 to 50% of colorectal cancers without *APC* mutation, in tumors of approximately 40% of HNPCC patients, and in 25% of tumors with MSI [54, 55]. The majority of these mutations are located at or around sequences encoding serine/threonine phosphorylation residues in exon 3, and the mutant β-catenin products become refractory to degradation through the ubiquitin-proteosome pathway and accumulate in the cells. Consistently, clonal expansion of adenomatous epithelia expressing accumulated β-catenin in the cytoplasm was detected by immunohistochemical staining of human adenomas [56, 57], suggesting that elevated β-catenin is involved in development of adenoma. The degree of nuclear staining of β-catenin correlates with tumor size and dysplasia, and a great majority of adenocarcinoma cells reveal augmented β-catenin in the cytoplasm including the nucleus [56]. Notably, high levels of β-catenin have been detected at invasion fronts of cancers [58], which may imply that accumulation of β-catenin is also involved in invasion of colon cancer. It is also worth noting that mutations in *APC* and *CTNNB1* are mutually exclusive [54] indicating that these molecules are in the same signaling pathway and that either mutation in *APC* or *CTNNB1* is sufficient for abrogation of the pathway.

Alterations in *AXIN2*, another molecule involved in Wnt/wingless pathway, have recently been reported in approximately 25% of tumors in HNPCC patients [59]. *AXIN2* encodes a protein that associates with GSK-3β, β-catenin, and APC protein, indicating that the abrogation of the complex or refractory forms of β-catenin is crucial for the development of colorectal tumors.

DPC4

DCC (Deleted in Colorectal Cancer) was identified as a candidate tumor suppressor gene from the commonly deleted region on chromosome 18q that showed frequent LOH in advanced colorectal cancers [60]. However, additional investigations failed to prove a tumor suppressive function for DCC. Located on chromosomal band 18q, *DPC4/SMAD4* was identified as a gene showing frequent losses in pancreatic cancer [10]. Subsequently, somatic mutations in *SDMA4/DPC4* have been identified in human cancers including pancreatic cancer, colorectal cancer, and lung cancer [61, 62].

Since frequency of mutation in *DPC4/SMAD4* increases with the progression of carcinogenesis from 0% in adenomas to 35% in invasive carcinomas with distant metastasis [63], alterations in *DPC4/SMAD4* play a role in the progression of colorectal cancer. SMAD proteins mediate signals of transforming growth factor (TGF) β that negatively regulates the

growth of epithelial cells [64]; consequently the inactivation of SMAD4 should result in uncontrolled growth of cancer cells. Interestingly, reduced SMAD4 expression was more frequently observed by immunohistochemical staining in non-selected colorectal cancers, compared to MSI-positive tumors [65]. Together with frequent mutations in *TGF-β receptor* II in colorectal cancers with MSI [41, 66], TGF-β signaling pathway plays an important role in human colorectal tumorigenesis. Notably germ line mutations of DPC4/SMAD4 have been identified in a hereditary disease, juvenile polyposis syndrome [67].

Gene Amplification

Cytogenetic analysis by means of comparative genomic hybridization (CGH) has facilitated to examine relatively large deletions or amplifications on whole chromosomal regions [68]. The analyses identified 7p, 7q, 8q, 13q, and 20q as the most frequently amplified chromosomal regions in colorectal tumors, suggesting that these regions may harbor oncogenes involved in tumorigenesis [69-71]. Further studies are of necessity to identify the responsible genes and their role in carcinogenesis.

Colorectal Tumorigenesis in Mouse Model

The first mice model of intestinal polyposis was established in C57BL/6J mice treated with ethylnitrosourea, which developed multiple polyps in the small intestine, and was termed as min (multiple intestinal neoplasms) mice [72]. Min mice transmitted intestinal polyposis in an autosomal dominant manner, and mutation screening identified a nonsense mutation at codon 850 in mouse *Apc* gene [73].

A striking finding of the development of very early adenomas in the intestine came out from the study of heterozygous $APC^{\Delta 716}$ knockout mice expressing truncated APC protein at codon 716, which developed a higher number of adenomas [74]. Careful observation of the small intestine in the heterozygous mice identified microadenomas that were covered with a layer of normal villous epithelium. Histological examination of the serial sections of the adenoma disclosed that a contiguous layer of the epithelium extended from the proliferating zone of a single crypt into the adjacent intravillous space (Figure 4). These findings suggest that the *APC* knockout mice develop adenomas from the proliferating zone of the crypt through abnormal cellular migration. The growing adenoma cells appeared to build up a larger polyp with multiple villi in the mucosal layer (Figure 4). Notably, the wild-type *APC* allele was lost in the microadenomas, indicating that both alleles are inactivated in the very early step of colorectal tumor development. They also observed similar intestinal adenomas in mice expressing mutant *β-catenin* gene lacking exon 3 that includes serine and threonine residues phosphorylated by GSK3β [75]. These results underscored the importance of accumulated β-catenin by inactivation of APC or stabilization of β-catenin. However histological features of $APC^{\Delta 716}$ knockout mice are different from those of early human adenomas in the colon, which preserve normal morphology of crypt base but is covered with dysplasic epithelia on the orifices and luminal surface of crypts, depicting top-down morphogenesis of adenoma [76].

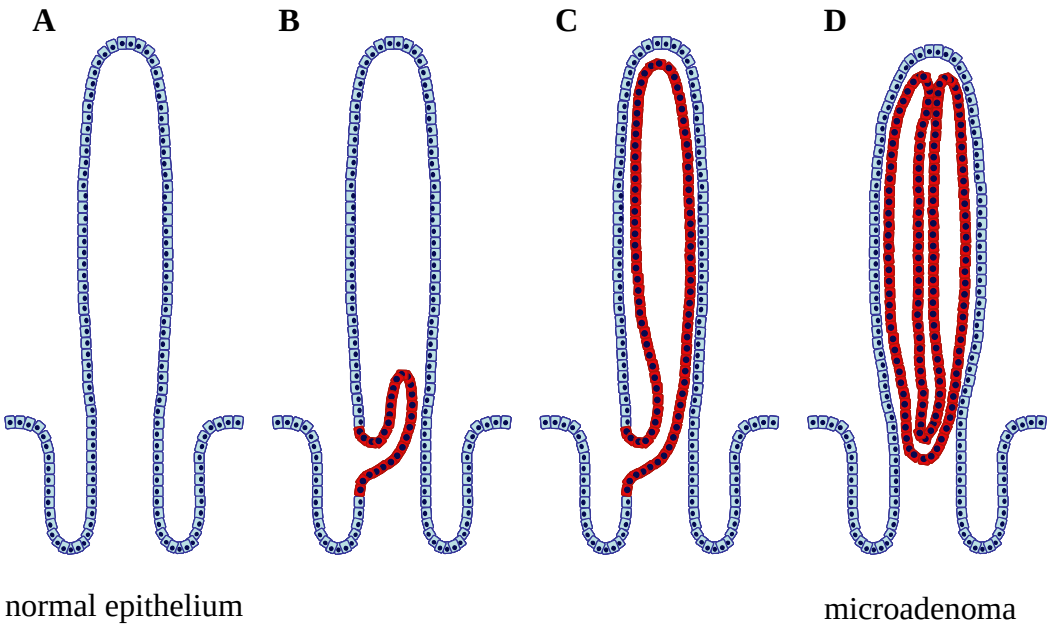

Figure 4. Adenomatious polyp formation in *Apc* mutant mice.

Although normal migration of stem cells from the crypt compartment to the top of villus accompanies cellular differentiation, adenoma cells may escape from apoptosis and migrate out of the crypt into the villus space keeping undifferentiated properties of progenitor cells due to abrogation of cell differentiation, cell growth, cell death, and/or migration.

Abrogated Wnt/wingless Signaling in Colorectal Carcinogenesis

Genes in the Wnt/wingless signaling pathway play critical roles in differentiation and morphogenesis during embryogenesis [77, 78]. In the absence of Wnt signaling, ß-catenin is phosphorylated by a multi-molecular complex composed of ß-catenin, APC, Axin, conductin (AXIN2), and glycogen synthase kinase 3ß (GSK3ß), and is subsequently degraded via the ubiquitin-proteosome system [79] (Figure 5). Upon Wnt signaling, ß-catenin accumulates in the cytoplasm and/or nucleus through its decreased degradation as a result of inhibition of GSK3ß.

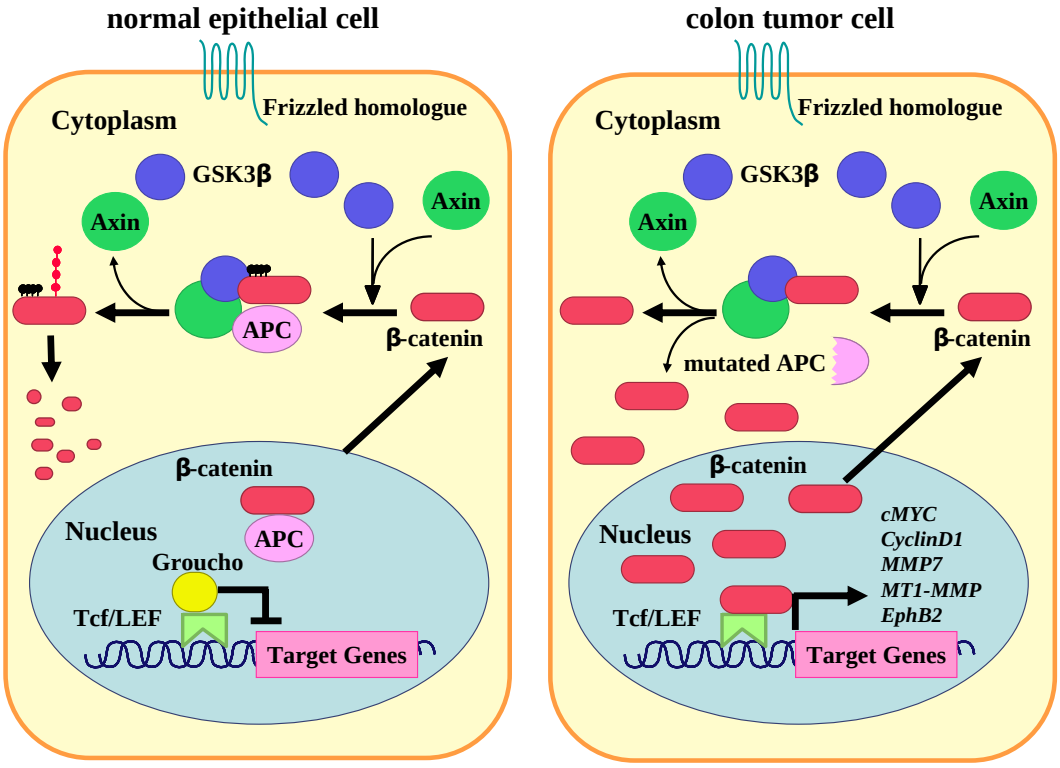

Figure 5. Abrogated Wnt signaling pathway by *APC* mutation in colorectal tumor.

Molecular studies uncovered that impaired regulation of this pathway is a feature of tumors arising in the colon, liver, prostate, stomach, brain, endometrium, or elsewhere [80]. The great majority of colon cancers reveal aberrant accumulation of β-catenin as a consequence of genetic alterations in *APC*, ß-catenin (*CTNNB1*) or *AXIN2* genes [79, 80] (Figure 5). Accumulated β-catenin associates with and activates Tcf/LEF transcription factors, in particular Tcf4, which then upregulates target genes such as *c-myc*, *cyclin D1*, *MMP-7* (*matrilysin*), *urokinase-type plasminogen activator receptor* (*uPAR*), *c-jun*, *fra-1*, *connexin 43*, *CD44*, *PPAR-delta*, *BMP4*, *AF17*, *WISP1, ENC1*, *Laminin-5γ2*, *Id2*, *Claudin-1*, *MT1-MMP*, *APCDD1*, *FGF18*, *EPHB2*, and *EPHB3* (http://www.stanford.edu/~rnusse/pathways/targets.html). These target genes including *Claudin-1*, *CD44*, *ENC1*, and *EPHB2* are not only expressed in adenoma and cancer cells of the colon but also expressed in the progenitor cells in the normal crypt compartment, recapitulating that tumor cells retain properties of crypt progenitor cells [81].

The Function of Target Genes in Tumorigenesis

Among the known downstream targets of the β-catenin/Tcf4 complex, *c-MYC*, *cyclinD1*, and *FGF18* play a role in transformation, regulation of cell cycle, and/or proliferation [82-84]. Since it has been recently reported that elevated c-MYC repressed $p21^{CIP1/WAF1}$ promoter [85], elevated expression of c-MYC and cyclinD1 should result in deregulated cell cycle

progression. FGF18, a secreted protein was shown to enhance the proliferation of cancer cells in an autocrine manner [84].

MMP-7 [86, 87], *CD44* [88], *uPAR* [89], *laminin-5γ2* [90], *claudin-1* [91], and *MT1-MMP* [92] may be involved in cell adhesion and/or migration of colorectal tumors cells. MMP7 and MT1-MMP are matrix metalloproteinases (MMPs) characterized by their ability to degrade various components of the extracellular matrix (ECM). MMP-7 is expressed in more than 90% of colorectal carcinomas and also in colorectal adenomas, where it contributes to tumor formation and progression [93, 94]. In addition, elevated expression of MMP-7 is associated with distant metastasis [95]. MT1-MMP activates MMP-2, which plays a role in degradation of type-IV collagen, a major component of basement membrane [96]. For the activation of MMP-2, MT1-MMP recruits TIMP-2, a process enhanced by claudins -1, -2, -3 and –5 [97]. MT1-MMP also activates proMMP-13 and degrades collagens type I, II, III, V, VII and X, fibronectin, vitronectin, and laminins 1 and 5 [98, 99]. In addition, it plays a role in cell motility mediated by CD44-shedding [100]. It is noteworthy that CD44, laminin-5γ2 and claudin-1 are also up-regulated by the β-catenin/Tcf4 complex [88, 90, 91]. Additionally, in skin epithelial cells, activated β-catenin suppresses expression of E-cadherin [101], an essential molecule for cell-cell adhesion at adherens junction. These data indicate that activation of β-catenin/Tcf4 complex enhance cell migration through activation of molecules important in extracellular matrix or cell-cell adhesion.

EphB2 and -B3 are receptors that control not only cell shape and migration but also cell-to-matrix interaction [102]. EphB3 regulates positioning of Paneth cells into the base of crypt compartment, because in Eph B3 null mice Paneth cells are distributed throughout the crypt villous wall [85]. Interestingly, in the small intestine of newborn mice, EphB2 and B3 are confined to the intravillous pockets of the epithelium, while their ligands, epherin-B1, is highly expressed by epithelial cells excluding the bottom of the pockets [85]. These data suggest that allocation of epithelial cells may be determined by the repulsion between epherin ligands and EphB2/B3 receptors. This notion is in consistent with the observation that Tcf4 knockout mice failed to develop crypt compartment of the intestine [103]. Therefore, deregulated expression of EphB may lead tumor cells to lose their normal compartmentalization along the crypt-villus axis.

In summary, abrogated Wnt/wingless signaling may induce deregulation of cellular survival, cell cycle progression, proliferation, differentiation, adhesion, migration, and/or compartmentalization as a result of altered expression of downstream genes.

Altered Gene Expression in Adenomas and Carcinomas of the Colon

Genetic and epigenetic alterations in tumors lead to deregulated expression of a number of genes. By analyzing expression profiles of thousands of genes simultaneously, microarray has opened the door to a comprehensive understanding of carcinogenesis, detection of biological properties unique to tumor cells, and a molecular classification of tumors according to their gene expression [104-106].

A number of groups have investigated expression profiles in various colon cancer materials from clinical samples, cell lines, and animal models using different microarray slides. Backert *et al.* analyzed expression profiles of six colon cancer cell lines using a cDNA array membrane containing 588 transcripts, and identified 10 genes differently expressed between normal colonic mucosa and cancer cell lines [107]. Alon *et al.* investigated expression profiles of 40 colon cancer tissues and 22 normal mucosae using oligonucleotide arrays representing more than 6500 genes [108]. They carried out hierarchical 2D-cluster analysis, which revealed different expression patterns between cancer and normal tissues. Kitahara *et al.* combined microarray technique with laser capture microdissection, which enabled to detect expression levels of pure population of tumor cells [109]. Because these groups used different techniques and array slides, the number of genes that are differently expressed between cancer tissues and normal mucosa in both studies are limited. As expected, some β-catenin/Tcf4 target genes were included in the list of differentially expressed genes, implying that genetic alterations are also reflected in the expression profiles.

Notterman *et al*. analyzed profiles of 18 adenocarcinomas, four adenomas and their corresponding normal tissues using the Affymetrix Human 6500 GeneChip Set [110]. They identified 19 and 47 transcripts, whose expression levels were augmented or decreased in cancer, respectively, compared to the corresponding normal tissue. Lin *et al.* compared expression profiles of 11 cancer and 9 adenomas with their paired normal mucosae using cDNA microarray containing 23040 genes [111]. They identified 51 and 376 genes that were commonly up-regulated or down-regulated, respectively, in both tumor phenotypes as compared with their paired normal epithelia. The genes with altered expression suggested that activation of oncogenes, augmentation of proliferation signals, attenuation of anti-proliferative signals, avoidance of self-destruction machinery, alteration of cell structure, and adaptation to micro-environmental changes might be the crucial factors for the development and progression of colorectal cancer. They also identified 50 genes differently expressed between cancer cells and adenoma cells, which may represent a signature of malignant transformation. It is of note that both groups succeeded in distinguishing adenocarcinoma cells from adenoma cells by the expression profiles.

Several groups have studied expression profiles of colon cancers at different stages and identified genes associated with disease progression including metastasis [112-116]. Recently, Ramaswamy *et al.* analyzed expression profiles of bulk tissues from solid tumors arising in various organs, with or without metastasis, and identified eight up-regulated genes including *SNRPF, EIF4EL3, HNRPAB, DHPS, PTTG1, COL1A1, COL1A2,* and *LMNB1*, and nine down-regulated genes including *ACTG2, MYLK, MYH11, CNN1, HLA-DPB1, RUNX1, MT3, NR4A1*, and *RBM5*, which appeared to be associated with metastasis [117]. They suggested from these data that ephithelial-mesenchymal interactions play a crucial role for metastasizing activity. Although altered expression of multiple genes may result from the progression of disease and not be the cause of their progression, these information will be useful to understand the mechanisms underlying multistep carcinogenesis in colon tumors.

Metzler and colleagues analyzed expression of tumors with MSI-H, MSI-L and MSS, and found that MSI-H and non-MSI-H tumors were separated by the expression profiles [118]. Additionally they observed a difference between MSI-L and MSS among the non-MSI-H tumors, suggesting that MSH-L may form a distinct tumor subtype that is different from MSI-H or MSS tumors.

Furthermore, microarray has facilitated the identification of genes relevant to prognoses for cancer patients [115], and/or sensitivity to anticancer drugs [119]. Hence, precise analysis of gene expression will allow a better prediction of the biological characters of tumors as well as their response to various treatments, and provide important information vital for personalized medicine.

Conclusion

It is almost 15 years since the proposition of multistep carcinogenesis by Fearon and Vogelstein, and we have acquired a huge amount of information underlying colorectal carcinogenesis from the studies of molecular biology. In this review I have summarized several pathways important for colorectal carcinogenesis, including Wnt/wingless, K-ras, p53, TGF-β pathways, and MMR system. Taking into account of these pathways, I would like to propose an elaborated model of multistep colorectal carcinogenesis, termed "two-way genetic model" (Figure 6). In addition to abrogated signaling by genetic alteration in one or several molecules in these pathways, the deregulated expression of multiple genes is involved. Combining the analysis of these signaling pathways and altered expression of multiple genes in tumors at different stages, or with different phenotypes, will clarify the nature of individual tumors and may provide most effective diagnostic, therapeutic and/or preventive strategies to tumors arising in the colon and rectum.

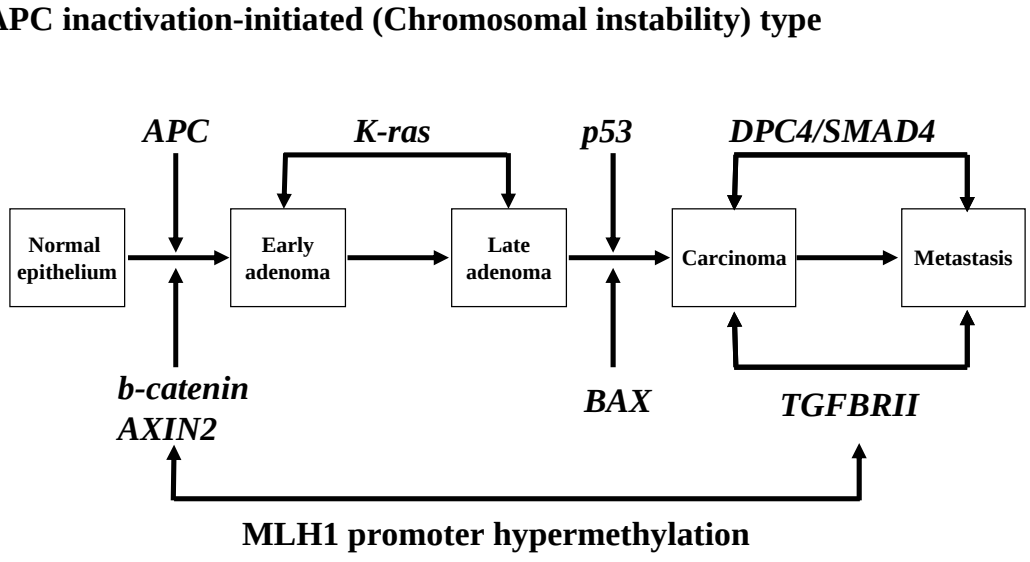

Figure 6. A two-way genetic model for colorectal tumorigenesis

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Chapter IV

BETA-CATENIN AND TUMOR BUDDING

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Introduction

Tumor budding describes a change in the phenotype of epithelial tumor cells and is characterized by certain features: dissociation of epithelial tumor cell structures towards single tumor cells or small tumor cell clusters and loss of epithelial differentiation towards a more mesenchyme-like phenotype. In colorectal carcinomas (CRCs), tumor budding occurs predominantly at the the invasive front of the tumor host interface [1].

Tumor budding in colorectal cancer is of both biological and clinical relevance. Various publications in recent years showed that the extend of tumor budding at the invasive front of CRCs inversely correlates with clinical prognosis, five-year survival, lymph node metastasis, local failure [2-6]. This might be explained by the biological features of budding tumor cells, giving them many advantages for malignant progression: detachment from the primary tumor, better access to blood and lymphatic vessel as single tumor cell or small tumor clusters, ability to actively migrate.

Looking at CRC histopathology, tumor budding seems not to be a clonal effect which could be explained by an additional genetic alteration in a selected tumor cell, because budding can often be found simultanously all over the invasive front and also circular around metastases. Thus tumor budding seems to be a process activated by microenvironmental signals at the tumor host interface. What are these signals and what are the effector molecules involved in tumor budding? Some hints can be found in similar processes, which are normally no more active in adult epithelial tissues, but during embryonic and organ development and in epithelial defect healing. When budding, tumor cell loose epithelial differentiation and gain a mesenchyme-like phenotype a process which is similar to an epithelial-mesenchymal

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transition (EMT). EMT is first activated in early embryonic development during gastrulation, when blastula epithelial cells detach into the lumen and form a primary mesenchyme [7]. Thereby decisive signal pathways, which also play a major role in carcinogenesis, are activated and involved, e.g. the Wnt/wingless pathway, the TGFβ pathway and the notch pathway. In particular the Wnt-pathway is of prime interest, because mutations in the APC tumor suppressor, the initial and most important genetic alteration in colorectal cancer, lead to an aberrant overactivation of this pathway [8,9]. As a consequence of loss of APC functions in CRC, β-catenin, the main effector molecule, is stabilized and accumulates in the tumor cells [10]. β-catenin is now considered as the major oncoprotein in CRC development and progression.

Physiological Functions of β-catenin

β-catenin has two completely diverse cellular functions: 1. Participation in homophilic cell-cell adhesion within E-cadherin/β-catenin complex in the cell membrane. 2. After nuclear translocation, functioning as a transcriptional activator together with LEF/Tcf family members of DNA binding proteins. Thus, the intracellular disribution of β-catenin is decisive and must be striktly regulated. As will be described later, the nuclear compartment is the oncogenic pool of β-catenin.

The E-cadherin/catenin Complex

Cell-cell contacts are made by adherens junctions, desmosomes and tight junctions. Determinants of an epithelial phenotype are homophilic cell adhesions and cellular polarity defining basal and apical orientation. Both are mediated by cell surface expression of the adherens junction molecule E-cadherin. Loss of these epithelial characteristics may be due to loss of E-cadherin function and can indicate a switch towards a dedifferentiated, mesenchyme-like phenotype [11,12]. The E-cadherin/catenin complex is the main molecular complex of adherens junctions (Figure 1). It not only mediates homophilic interaction between epithelial cells, but some components are both active parts of signal transduction pathways and themselves exposed to regulatory signals [13,14]. E-cadherin binds directly to β-catenin, γ-catenin (plakoglobin) and p120catenin, and is indirectly linked to actin and the cytoskeleton via α-catenin. β-catenin participates in the correct positioning and function of E-cadherin [15], thus membranous expression of both proteins determine the epithelial phenotype. E-cadherin's direct interacting partner, β-catenin, also binds to several proteins. β-catenin associates with α-catenin and links cadherin/catenin complexes to the actin cytoskeleton. Its ability to interact with some microtubule-associated proteins such as IQGAP, APC, and the dynein/dynactin complex may link E-cadherin to the microtubule network13.

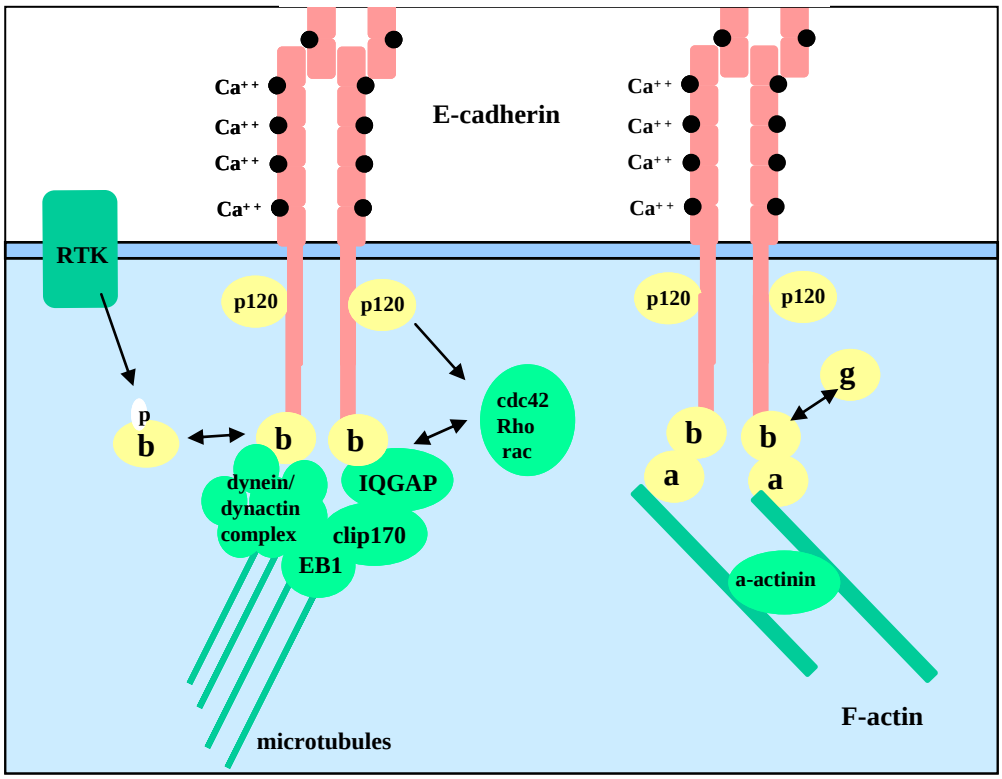


Figure 1. Protein interactions at adherens junctions (AJ).

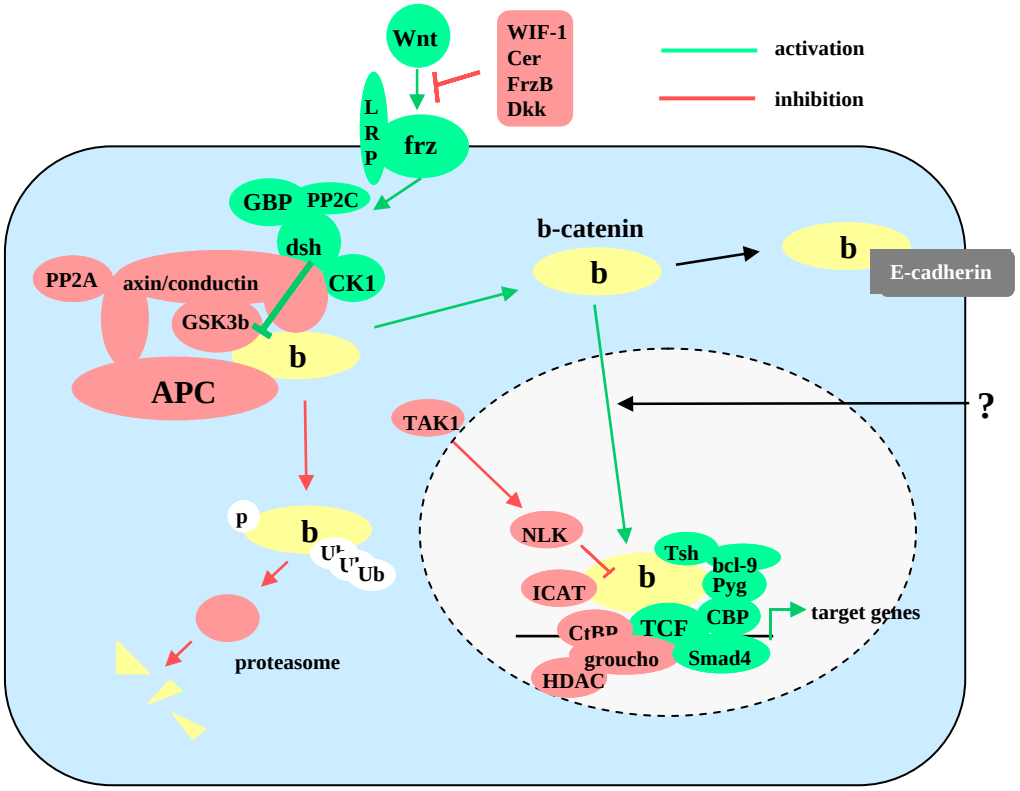
AJs allow homophilic cell-cell adhesion through direct, Ca^{++} dependant interaction of E-cadherin molecules. E-cadherin is indirectly linked to the actin and microtubule cytoskeletons, through associated proteins, which is essential for cell-cell adhesion. E-cadherin´s direct interaction partner β-catenin (β) binds to α-catenin (α) and links cadherin/catenin complexes to the actin cytoskeleton. Thereby α-catenin is the central player in the linkage to F-actin, a process critical for coordinating actin dynamics in the cell. β-catenin binds to the E-cadherin cytoplasmic tail via 12 so-called armadillo repeats. The affinity for this key interaction is increased by phosphorylation of several serine residues in the cadherin tail and reduced by phosphorylation (p) of β-catenin Y654, a known site of action for activated growth factor receptor tyrosine kinases (RTK). Thus, through posttranslational modifications, the strength of the AJ complex can be adapted to the particular needs of the epithelial cell within the context of its tissue. β-catenin and γ-catenin (γ) compete for binding to E-cadherin and α-catenin. β-catenin also interacts with microtubule-associated proteins such as IQGAP and the dynein/dynactin complex. β-catenin and IQGAP also crossreact with the Rho/rac-family of small GTPases. p120catenin (p120) binds independantly to E-cadherin and promotes E-cadherin clustering. p120 has also been suggested to regulate the GTPase Rho.

The Wnt/wingless Pathway

Among the catenins, β-catenin plays a particular role, since it is fundamentally involved in both devopmental processes and pathomechanisms of various diseases, in particular cancer. Besides its function in cell-cell adhesion as a interacting partner of E-cadherin, a second, completely different function of β-catenin was defined: In a nuclear pool, β-catenin interacts

with DNA-binding proteins of the Tcf/LEF (T-cell factor/lymphocyte enhancer factor)-family and acts as a transcriptional activator [16,17] in the so-called canonical Wnt/wingless pathway. This pathway is highly conserved between Drosphila and vertebrates and a potent regulator of early embryonic and organ development [18]. Thus the intracellular distribution of β-catenin is of great importance for the different functions of β-catenin and the subsequent behaviour of differentiating epithelial cells or tumor cells. One of the fundamental results in recent colon cancer research was the demonstration, that the APC tumor suppressor protein interacts with β-catenin (8) and is a negative regulator of the Wnt-pathway (Figure 2) [19].

Nuclear β-catenin is the main downstream effector molecule of the Wnt-pathway. It has no DNA-binding domain but builts up a transcriptional activator complex together with Tcf/LEF familiy members and various cofactors (see reviews by J. Behrens [20], M. Bienz [21], and J. O. Sieber [22]). Tcf/LEF molecules are DNA-binding proteins, which recognize a consensus promoter sequence (TCF-binding sites: WWCAAAG), but do not have a strong intrinsic transcriptional activator function [23]. In the absence of Wnt-signaling Tcfs are bound by transcriptional repressors like groucho, which keeps the target gene promoter inactive [24]. Nuclear accumulation of β-catenin displaces groucho from Tcf-binding. β-catenin/Tcf plays a role in chromatin remodelling and leads to changes in promoter architecture, which makes the promoter accessible for other transcription factors. Thus, TCFs and the corresponding promoter binding sites play a key role in transcriptional regulation by integrating simultaneous signaling by various pathways [25]. In the absence of Wnt-signaling, β-catenin is degraded after phosphorylation by the glycogen synthase kinase3β (GSK3β) in a multiprotein complex. The main components of this complex are the tumor suppressor APC and the scaffolding protein axin/conductin. Only if these molecules are functional, the amount of β-catenin can be efficiently regulated. Loss of function of either APC or axin/conductin leads to a reduced degradation and subsequent overexpression of free cytoplasmic β-catenin, which can exert its nuclear function without efficient control. Since many target genes of β-catenin are itself oncogenes (see 3.2), both APC and axin/conductin function as tumor suppressor. Physiologically Wnt-signaling is induced by binding of the soluble Wnt-factors to their membrane receptors of the frizzled-(frz) family, which leads to an inactivation of GSK3β and subsequent accumulation of β-catenin. However, although active Wnt-signaling or APC mutation in tumor cells lead to accumulation of the cytoplasmic, free pool of β-catenin, it is not fully understood what activates the decisive nuclear translocation of β-catenin. A complex interaction with other pathways might be responsible.



modified from Hülsken and Behrens, J. Cell Sci., 115, 3977, (2002)

Figure 2. The Wnt signaling pathway.

Wnts are secreted glycoproteins that bind to and activate frizzled (frz) seven-transmembrane-span receptors. Low-density lipoprotein receptor-related proteins LRP5/6 act as essential co-receptor. Various secreted factors, like WIF-1, Cerberus (cer), FrzB and Dickkopf (Dkk), antagonise this interaction. Wnt-signalling leads to a stabilisation of cytoplasmic β-catenin, the main effector of the Wnt-pathway. In the absence of Wnts, β-catenin is phosphorylated (p) at the N-terminal serine and threonine residues 33, 37, 41, 45 by glycogen synthase kinase 3β (GSK3β), which triggers ubiquitination and subsequent degradation in proteasomes. This is only possible in a multiprotein complex consisting of APC and the scaffolding protein axin/conductin. In the presence of Wnts, dishevelled (dsh) blocks β-catenin degradation, possibly by recruiting the GSK3β inhibitor GBP. β-catenin degradation is modulated by the casein kinase CK1 and the protein phosphatases PP2A and PP2C. Stabilized β-catenin either is recruted to adherens junctions, or, induced by unknown signals (?), accumulates in the nucleus. There is exerts its oncogenic function as a transcriptional activator after association with DNA binding proteins of the Tcf/LEF-family of transcription factors. Coactivators, such as CBP, pygopus (Pyg), bcl-9, teashirt (Tsh) support the activation of target genes (see table 1). Phosphorylation of Tcfs by NEMO-like kinase (NLK), a target of the MAP kinase kinase kinase TAK1, as well as interaction with ICAT negatively regulate β-catenin transcriptional activity. In the absence of of β-catenin, certain Tcfs suppress target gene transcription by interacting with the corepressors CtBP and histone deacetylase (HDAC) bound to groucho. Interaction with Smad4 might connect the Wnt- and TGFβ-pathways.

Physiological Roles of β-catenin and Wnt-signaling: Embryonic Development and Adult Tissue Homeostasis

Wnt-signaling, characterized by nuclear accumulation and function of β-catenin, regulates fundamental embryonic processes. It was shown to induce epithelial-mesenchymal transitions (EMT) during the gastrulation in sea urchin [26] and also in human cell culture systems [27,28]. Targeted disruption of β-catenin in mice leads to very early embryonic lethality by affecting development at gastrulation [29]. Overactivation of the Wnt-pathway by injection of β-catenin in susceptible cells leads to an axis duplication in xenopus embryos [17], indicating its regulatory role in axis formation. Moreover Wnt-signaling regulates differentiation and development of various organs in fetal development. In skin development β-catenin is involved in hair follicle morphogenesis and control of the epidermal stem cell compartment [30,31]. Also, β-catenin plays an impotant role in neural delevopment: Inactivation of the β-catenin gene in mice by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development [32]. But also the correct development of various other tissues and organs, like angiogenesis [33] and adipogenesis [34], depends on Wnt-signaling and β-catenin.

Of particular interest for tumor formation (see 3.3.1.1) is, that the Wnt-pathway is also essential for intestinal development. Thereby, β-catenin binds an intestine- and mammary epithel- specific Tcf-family member, termed Tcf-4 [35]. Tcf-4 expression characterizes the intestinal stem cell compartment and targeted disruption of Tcf-4 in mice leads to a severe disturbance of gut development [36].

The molecular role of β-catenin in the development of the above mentioned organs and tissues can be deduced by the functions of already its defined target genes. Many of this target genes give the competence to act as a stem cell and in a current model, β-catenin defines the stem-cell compartment [37]. The function in characterizing the stem cell compartment also indicates the role of nuclear β-catenin in the adult organism. Thereby β-catenin maintains tissue homeostasis, in particular in strong-proliferative, self re-newing tissues, like skin, hair and intestine.

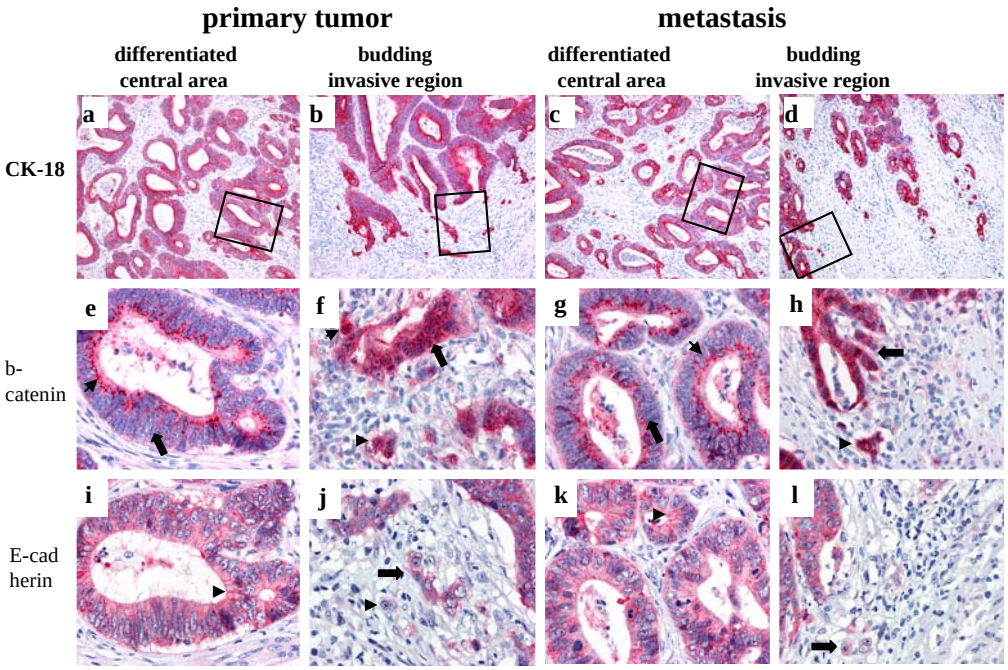
Compiling all data, it is becoming evident that Wnt-signaling and nuclear β-catenin as its main effector do not simply control singular events, but regulate the complex process of morphogenesis, which needs a temporal and spatial coordination of single events like cell-cell-attachment, -migration, -proliferation and -differentiation. The morphogical correlate of these events regulated by β-catenin is often detectable as epithelial cell budding.

Oncogenic Potency of β-catenin

Expression of β-catenin in Colorectal Carcinomas

The decisive genetic alteration, which is found in more than 80% of sporadic colorectal carcinomas and was initially discovered in the familiar adenomatous polyposis (FAP), is the mutation of the APC tumor suppressor gene [38,39]. Its causal nature in colorectal

carcinogenesis was demonstrated in different mouse models (see R. Kucherlapati et al. [40] for review). One of the fundamental results in recent colon cancer research was the demonstration, that the APC tumor suppressor protein interacts with β-catenin [8] and is a negative regulator of the Wnt-pathway. Given the two important, contrary roles of β-catenin in either the E-cadherin dependent determination of the epithelial phenotype (membranous localisation) or as transcriptional regulator and main effector of the Wnt-pathway (nuclear localisation), its function as a oncoprotein in APC-mutant colorectal cancers takes shape. Indeed nuclear β-catenin is detectable in human FAP-associated and sporadic colon adenomas and adenocarcinomas [41]. However the amount of nuclear β-catenin is increasing from early adenomas to adenocarcinomas [42] and its distribution within an individual tumor is very heterogenous: In most well- to moderately-differentiated colon adenocarcinomas nuclear β-catenin is predominantly accumulated in dedifferentiated tumor cells at the invasive front. Strikingly, at the invasive front there is a high coincidence of tumor cell budding and strong nuclear accumulation of β-catenin, which raised the question of a causal connection. These cells also lost membranous expression of E-cadherin. In contrast, in central differentiated areas nuclear accumulation of β-catenin is weak or hardly detectable (Figure 3 e, f) [43,44], but it is often located at the cell membrane together with E-cadherin (Figure 3 i, j). Since all tumor cells in an individual tumor harbour APC-mutations, a nuclear accumulation of β-catenin can not be due to this alteration alone, but its intracellular distribution within different tumor areas has to be explained by additional regulatory events.



(modified from Brabletz et al, Proc Natl Acad Sci, Vol 98, 10356-61, 1999)

Figure 3. Correlation of differentiation and the expression patterns of β-catenin and E-cadherin in colorectal adenocarcinomas.

Central areas (first column) and invasive front (second column) of the primary tumor and central areas (third column) and invasive front (fourth column) of the corresponding metastasis are shown. Stainings are for CK-18 (first row), β-catenin (second row), E-cadherin (third row). Boxes indicate

magnificated regions in stained serial sections. Specific staining is red, nuclear counter-staining is blue, magnifications are x100 (a – d) and x400 (e – l). CK 18 stainings show a differentiated, epithelial growth pattern with tubular structures in the centers of primary tumor (a) and metastasis (c) and loss of tubular growth and tumor cell dissemination in the corresponding invasive fronts (b and d). Tumor cells are clearly polarized in the differentiated central areas in both primary tumor and metastasis, and β-catenin is localized distinctly in the apical cytoplasm and membrane of the tumor cells (arrowheads) (e and g). Note that nuclei are free of β-catenin (arrows). In contrast tubules at the invasive front break up, tumor cells loose their polar orientation and dissociate (arrows) (f and h). This is accompanied by a nuclear accumulation of β-catenin (arrows and arrowheads). Corresponding tumor cells in differentiated areas of the primary tumor express membranous E-cadherin (arrowheads) (i) which is reexpressed in central areas of the metastasis (k). Disseminating tumor cells at the invasive fronts with nuclear β-catenin either completely lost E-cadherin (arrowheads) or show a cytoplasmic expression (arrows) (j and l).

Like the FAP type of hereditary colorectal cancer most sporadic colorectal carcinomas arise from adenomas and have APC mutations as initial genetic alterations. The second major group are the replication error (RER) positive carcinomas, associated with microsatellite instability (MSI). HNPCCs and about 10% of sporadic colorectal carcinomas fall into this group, which is also characterized by a different morphology and clinical prognosis [45]. About half of these carcinomas show normal APC genes [46], however the general importance of a dysregulated Wnt-pathway and its main effector β-catenin in colorectal cancer formation is indicated by the fact that mutations in other components of this pathway, leading to enhanced β-catenin activity, are found in a high percentage of such tumors. In particular, dominant mutations at the target serine residues for GSK3-β in the β-catenin gene itself are found in 27 % of microsatellite instable carcinomas, leading to a stabilisation of the molecule [47]. *Loss of function* mutations in the *conductin/axin-2* gene were demonstrated in 25% of MSI carcinomas, which similar to APC mutations omit degradation of β-catenin [48]. Moreover inactivating frameshift mutations in the TCF-4 gene were found in 39% of human microsatellite instable colon carcinomas [49]. The important role of the dysregulated Wnt-pathway in colon carcinogenesis is also indicated by the fact that also ulcerative colitis associated colon carcinomas show APC gene mutations and nuclear accumulation of β-catenin [50]. Thus a dysregulation of the Wnt-pathway by targeting APC, β-catenin or other components is found in almost all colorectal carcinomas.

Two questions arise from these observations, which may have strong impact on tumor progression: 1. What are the effects of nuclear β-catenin in the dedifferentiating carcinoma cells and is there a direct influence on tumor budding and subsequently on invasion and metastasis? 2. What regulates the heterogenous intracellular distribution of β-catenin and the potential consequences within an individual tumor?

Effects of β-catenin in Carcinoma Cells

Since nuclear β-catenin is a transcriptional activator, the identification of its target genes, characterized by Tcf-binding elements in their promoters, is of particular interest to understand its role in tumor progression (see Table 1). The first identified genes regulated by β-catenin/Tcf in cancers are the well-defined oncogenes *c-myc* [51] and *cyclin D1* [52,53],

linking dysregulated β-catenin activity to dysregulated proliferation. Also the *gastrin* gene, which is discussed to be a trophic factor for intestinal tumor growth is activated by nuclear β-catenin [54]. Overexpression of PPARδ, a member of the nuclear receptor family, which is bound and activated by fatty acids and therefore a potential mediator of dietary effects on colon carcinogenesis, is due to transcriptional activation by β-catenin/TCF [55]. Other target genes of β-catenin are MDR1 [56] and *survivin* [57], which are thought to suppress cell death pathways.

Table 1. β-catenin target genes relevant for cancer

Target Gene	Function
c-myc cyclin D1	proliferation
c-jun ets2 fra-1 ITF-2	oncogenic transcription factors
MMP-7 MMP-26 MT1-MMP uPA-R	protein degradation
VEGF	angiogenesis
BMP-4 ephrinB2/B3	morphogenesis
laminin γ-2 chain fibronectin	migration
CD44	dissemination
Cdx1 Id2 Enc-1	loss of differentiation
gastrin PPARdelta	trophic factors
MDR survivin	cell survival
conductin/axin-2 Tcf-1	negative feedback and tumor suppression

Importantly it was shown that activated β-catenin is involved in loss of epithelial cell differentiation [58,59], indicating a causal role of nuclear β-catenin in the phenotypical switch towards dedifferentiated, budding tumor cells at the invasive front. This view is supported by an increasing number of target genes, which are known to code for regulators of differentiation and effectors supporting invasion and dissemination: Recently *Cdx-1*, encoding a homeobox-factor [60], *Id2* (Inhibitor of differentiation-2) [61]and *ENC1* [62] were identified as β-catenin/TCF target genes. All three proteins inhibit epithelial differentiation and keep cells in a less differentiated, stem cell-like state. An activation of such genes by nuclear β-catenin could explain the dedifferentiated phenotype of nuclear β-catenin expressing, budding colon cancer cells at the invasive front.

Other genes regulated by nuclear β-catenin code for direct effectors of colon cancer progression, like *urokinase-receptor* (*uPAR*) [63], urokinase (Hiendlmayr, Cancer Res, 2004, inpress) MMP-7/*matrilysin* [64,65], *MT1-MMP* [66,67], *MMP-26* [68], *c-jun* [63], *ets2* [69], *VEGF* [70], *fibronectin* [71], *laminin-5 γ2 chain* [72], *bone morphogenetic protein 4* [73], *ITF-2* [74], *CD44* [75]. Both uPAR and MMP-7 are overexpressed by the tumor cells and facilitate extracellular matrix proteolysis, which allows detachment and motility enhancement of the tumor cells. The isolated γ2 chain of laminin-5 is overexpressed selectively in dedifferentiated carcinoma cells at the invasive front and known to be one of the most potent inducer of epithelial cell migration, e.g. in wound healing and embryonic development (see 3.3). The known oncoprotein c-Jun, a component of the transcription factor AP-1, is itself another strong transcriptional activator of invasion factors like uPAR, matrilysin and laminin-5 γ2. A similar role is described for Ets-transcription factors [76]. Another important process in tumor growth and invasion is the generation of surrounding tumor stroma. The stroma and the stromal cells are participating directly in tumor growth and invasion by producing various degrading enzymes like MMPs, by storing cytokines and also supply the tumor with blood vessels [77]. Both VEGF and bFGF, activated by nuclear β-catenin, are cytokines involved in the generation of the tumor stroma and tumor angiogenesis [78]. Splice variants of CD44 (e.g. v6) are known to directly support dissemination of isolated tumor cells and are associated with the presence of metastases and an unfavorable prognosis of colorectal cancer [79]. Finally, a translocation of membraneous β-catenin to the nucleus leads to a loss of E-cadherin function. This further allows detachment of tumor cells from epithelial cell complexes and supports the loss of epithelial features.

Taking together, the strong oncogenic potency of nuclear β-catenin becomes evident: A cluster of genes, associated with a phenotypical switch from differentiated, epithelial towards dedifferentiated, mesenchyme-like tumor cells, are regulated by β-catenin/TCF. Similar phenotypical transitions and subsequent migration are induced by nuclear β-catenin in epithelial cells in the blastula during epithelial to mesenchymal transition (EMT) processes in embryonic gastrulation [80] and it is suggested that strong nuclear accumulation of β-catenin leads to dedifferentiation of epithelial cells [58] and might give the tumor cells a competence similar to embryonic epithelial cells [44]. In analogy, nuclear accumulation of β-catenin in tumor cells at the invasive front might be directly involved in the EMT-like aquisition of the necessary abilities to detach, migrate and disseminate in the body by increasing the morphogenetic competence of the tumor cells. The morphological feature of all these molecular changes, associated with nuclear accumulation of β-catenin, is tumor cell budding.

β-catenin and Tumor Cell Budding

A lot of β-catenin functions are directly involved in tumor cell budding, which can be exemplified by certain β-catenin target genes (Figure 4). β-catenin/Tcf transcriptionally activate the genes of MMP-7, MT1-MMP and the γ2 chain of laminin-5. This set of only three target genes is an extraordinary example of a coordinated interaction of few gene products towards tumor cell budding and a proinvasive phenotype.

MMP-7 was recently shown to not only cleave extracellular molecules, but also E-cadherin on the tumor cells with two important consequences (81): 1. Loss of cell-cell

adhesion allowing detachment. 2. Release of membrane-bound β-catenin, which can now also be translocated to the nucleus and lead to further target gene activation (positive feed-back loop). MT1-MMP can directly cleave the γ2 chain of laminin-5 (MT1-MMP also cleaves and activates MMP-2, which then cleaves the γ2 chain of laminin-5). The γ2 chain of laminin-5 transcription itsself is activated by β-catenin [67] and accumulates in and around budding tumor cells [82-84]. Importantly, cleavage products of the γ2 chain of laminin-5 were shown to be strong inducers of epithelial cell migration [85-88].

Loss of function mutations of the APC gene not only lead to accumulation of cellular β-catenin, but might be involved in tumor cell migration. Kawasaki et al have shown that truncated APC protein strongly interacts with Asef-1, a member of the small family of GTPases, which then induces cellular migration [89,90]. As already mentioned before, one key question is what triggers the whole process of tumor cell budding at the invasive front of the tumor host interface. Strikingly tumor cells in this region express the highest amounts of nuclear β-catenin [91], although all tumor cells, also in differentiated central tumor areas have the same APC gene mutation. Given the potent function of nuclear β-catenin, a very important trigger could be factors from the tumor environment, which induce nuclear translocation of β-catenin at the invasive front. Such factors could be cytokines like TGFβ, TNFα, HGF, EGF, etc.. All these factor may however also be directly involved in tumor budding, independantly of β-catenin action.

One key question of budding tumor cells is how they can survive as single cells or small clusters. Physiologically epithelial cells loosing their epithelial context, characterized by homophilic cell-cell contact and attachment to the basement membrane, very fast die in an apototic process called anoikis. Also in this aspect β-catenin seems to play an important role in budding tumor cells. Recently defined target genes of β-catenin are MDR1 [56] and *survivin* [57], which are thought to suppress cell death pathways, and can prevent anoikis in budding tumor cells detaching from tumor glands.

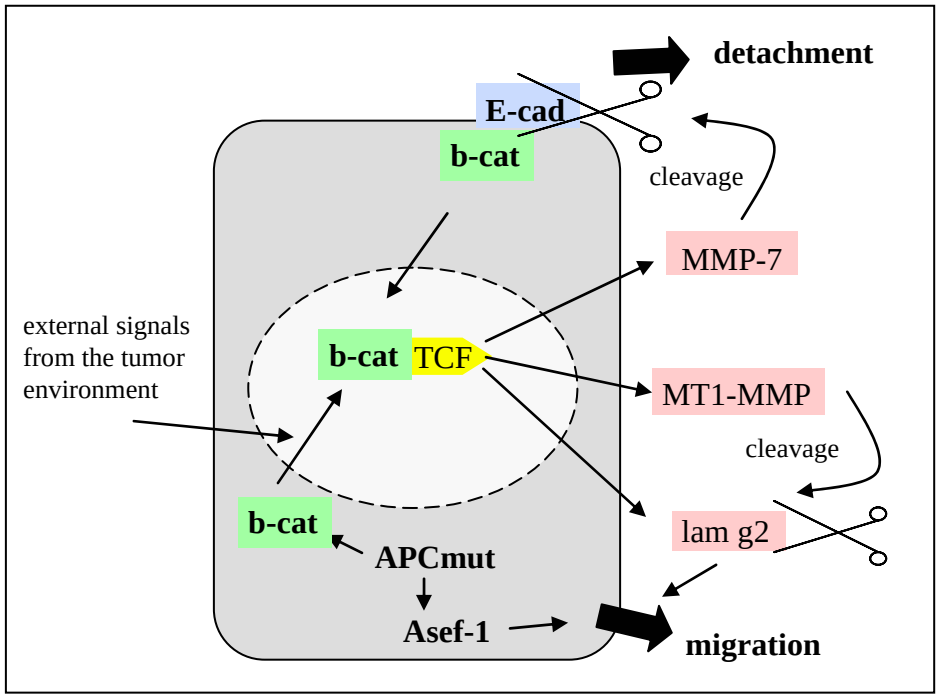


Figure 4. β-catenin and tumor cell budding.

A coordinated interaction of the β-catenin target genes MMP-7, MT1-MMP and the γ2 chain of laminin-5 leads to tumor cell budding by inducing cellular detachment and migration. Migration is further supported by activation of Asef-1 by truncated APC.

Regulators of Intracellular β-catenin Localisation and Tumor Cell Budding and Their Potential Role in Malignant Tumor Progression

In well to moderately differentiated colorectal adenocarcinomas, a strong nuclear expression of β-catenin is found predominantly in budding tumor cells at the invasive front [43,44]. Another feature of colorectal adenocarcinomas is their retained epithelial differentiation in central tumor areas, characterised by polarized tumor cells building up tubular structures. Strikingly the same differentiated phenotype of the primary tumor is found again in corresponding lymph node or distant metastases [72,92-95] (Figure 3 a-d), which means that the dedifferentiated, budding phenotype, allowing the tumor cells to disseminate in the body, can only be transient and thus can not be fixed by genetic alterations in their genomes.

Therefore, a main driving force inducing the obvious phenotypical dedifferentiation-redifferentiation switches, and thus potentially invasion and metastasis formation, must be the tumor environment acting on the genetically altered tumor cells. Recent observations demonstrated that the expression and localisation of the two decisive molecules E-cadherin and β-catenin are coupled to these changing phenotypes [72,92-95] (Figure 3): Membraneous E-cadherin and β-catenin are found in both differentiated areas of primary colorectal carcinomas and their metastases, whereas the invasive, budding areas of primary tumor and metastases show decreasing E-cadherin expression and nuclear β-catenin. This demonstrates

a stronger difference between distinct morphogenetic areas within a tumor than between primary tumor and corresponding metastases. Since the enormous potential of nuclear β-catenin becomes evident (see above), the search for the driving force of the phenotypical switches may be focused on environmental factors which induce nuclear translocation of β-catenin.

Cell culture experiments revealed a direct or indirect role of environmental factors, including cytokines and extracellular matrix, on the intracellular β-catenin distribution and function (see Table 2): Intestinal trefoil factor (TFF3) [96], insulin-like growth factors (IGF I and IGF II) [97], epidermal growth factor (EGF) [98,99]and hepatocyte growth factor, scatter factor (HGF) [98-100l]ead to a tyrosine phosphorylation of β-catenin with subsequent perturbation of E-cadherin binding, loss of intercellular adhesion and promotion of cell motility. By affecting the function of β-catenin, an overexpression of these cytokines in colon carcinomas is thought to modulate tumor cell adhesion and migration [101,102]. Overexpression of IGF II enhanced colon tumor growth in a mouse model [103] and induced a nuclear translocation of β-catenin coupled with an EMT in bladder and mamma carcinoma cell lines [28]. Very recently it was shown that a combination of TGFβ and TNFα can induce a complete EMT, associated with tumor cell budding in the differentieated colon cancer line LIM1863 [104]. However the in vivo significance of all these cytokines in regulating β-catenin function and tumor cell budding in human colorectal carcinomas has not been demonstrated yet.

Table 2: External factors modulating the intracellular distribution of β-catenin and differentiation.

Factors	Molecular Effects	Consequences
cytokines (TFFs, IGFs, EGF, HGF)	tyrosine phosphorylation of β-catenin	disruption of adherens junctions nuclear accumulation of β-catenin
extracellular matrix	activation of ILK activation of snail	nuclear accumulation of β-catenin inhibition of E-cadherin transcription
MMPs TGFβ, TNFα, HGF	cleavage of e.g. E-cadherin induction of EMT	disruption of adherens junctions loss of epithelial differentiation

Epithelial-mesenchymal interactions are decisive for intestinal development [105]. Thus, mesenchymal factors, in particular components of the surrounding extracellular matrix (EM), could have a potent regulatory effect on tumor cells, which might have a reactive competence similar to embryonic epithelial cells due to APC mutations affecting the Wnt-pathway [44]. In this context the perhaps most significant regulator of intracellular β-catenin distribution is the integrin linked kinase (ILK) [106]. ILK is a serine/threonine kinase which binds to intracellular domains of β1- and β3-integrins. After binding of EM-proteins to their integrin receptors, ILK is activated and exerts various intracellular effects [107,108]. One is the induction of a nuclear translocation of β-catenin and subsequent activation of the β-catenin/TCF transcriptional activator [109]. Moreover it was shown that ILK activation leads to an inhibition of E-cadherin transcription by stimulating the transcriptional repressor snail [117]. Thus ILK may directly be involved in the aquisition of the dedifferentiated phenotype

of nuclear β-catenin expressing tumor cells at the invasive front. However, till now the relevant external stimulators are not known and it will be of interest what particular EM-proteins stimulate ILK activation. Also other pathways known to be altered in colorectal cancers, like the PTEN/Akt [110]- or the TGFβ-pathway [111], are thought to interefere with the Wnt-pathway and possibly the nuclear accumulation of β-catenin and tumor cell budding. However, the relevance of these interactions for colorectal carcinogenesis is still unclear.

Summary

Taking into account its physiological role in embryonic development, dysregulated β-catenin plays a crucial role for the genesis of colorectal carcinomas with both normal and mutated APC. The transcriptional activation of a cluster of target genes necessary for tumor cell budding, invasion and dissemination by nuclear β-catenin underlines its important role for tumor cell budding at the tumor host interface and thus malignant tumor progression.

Moreover, a comparison of central areas of primary colorectal carcinomas and their metastases, showing the same differentiated epithelial growth patterns and a lack of nuclear β-catenin, indicates that the phenotypical transitions and the associated changes in intracellular distribution of β-catenin are dynamic and transient and can not be explained by accumulating genetic alterations alone. In addition a strong regulatory role of the tumor environment for these processes and thus the progression of colorectal tumors must be postulated [112].

Two main conclusions might be drawn: 1. A basic process of tumorigenesis is morphogenesis, which seems to be induced by the variable nuclear accumulation of β-catenin. Thereby modest nuclear accumulation allows short distance movement in epithelial context (adenoma branching) and strong nuclear accumulation is associated with tumor cell budding and dedifferentiation, allowing long range migration and dissemination of stem cell-like isolated carcinoma cells. 2. Reciprocal interactions between the changing tumor environment and the tumor cell regulate the dynamic intracellular β-catenin distribution and E-cadherin expression and thus tumor morphogenesis and progression.

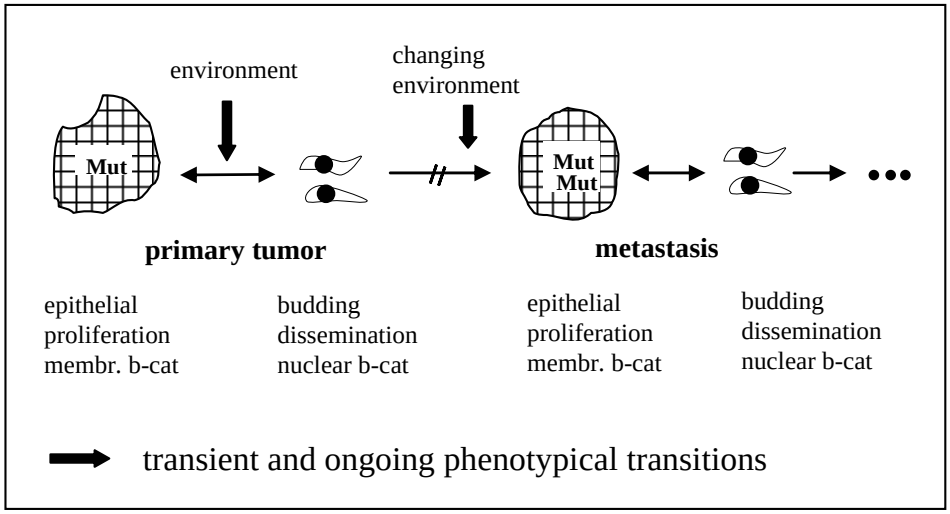


Figure 5. Model for the malignant progression of colorectal carcinomas.

APC gene mutations lead to overexpression of β-catenin, which is located to the cellular membrane in differentiated central areas of the primary tumor. Environmental factors directly or indirectly lead to nuclear translocation of β-catenin and dedifferentiation, allowing tumor cell dissemination. A changing environment at the metastatic site allows epithelial redifferentiation and metastatic growth. The phenotypical switches are transient and ongoing. Additional mutations may be selected upon the ability of the tumor cell to respond to the changing environment.

Subsequently, a model of malignant tumor progression considering both genetic and environmental factors are suggested (Figure 5): In this model two driving forces of tumor progression are integrated: 1. Accumulating genetic alterations in oncogenes and tumor suppressor genes as fixed basis, determining both the cell-autonomous and the reaction competence of the tumor cells. 2. The changing tumor environment, which regulates the adaptation of the genetically altered tumor cells to different conditions. Taking colorectal carcinomas as example, the initial APC mutation, stabilising β-catenin expression, is the decisive genetic alteration, and the tumor environment regulates intracellular β-catenin distribution with strong consequences for the tumor cell behaviour. Thus the dysregulation of the β-catenin activity in colorectal carcinogenesis is an example of how genetic alterations and the tumor environment may exert combined effects on one decisive molecule for tumor progression. Of note, additional genetic alterations supporting tumor progression, like recently described for colorectal carcinomas [113], are not excluded in this model.

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PART 3: PATHOLOGICAL SESSION

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Chapter V

MOLECULAR AND PATHOLOGICAL ASPECTS OF TUMOR BUDDING

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Introduction

The process known as 'tumor budding' or 'de-differentiation' that occurs at the advancing margin of colorectal cancer (Figure 1) and other epithelial malignancies has been shown to be an important prognostic marker in recent years [1-5]. However, the feature has been evident to pathologists for decades. Dukes was aware of the phenomenon and did not regard it as evidence of de-differentiation to a higher grade of malignancy. Bussey, who worked with Dukes for many years on the grading of rectal cancer, likened the process to the extension of a building: 'loose bricks will be found at the site of the extension but in time will be formed into an organized structure' (personal communication). Based on this interpretation, Dukes proposed that rectal cancer be graded on the basis of the overall tumor and not on loss of tissue organization in a small sub-compartment [6]. It is not known if this interpretation related specifically to tumor budding rather than to the bona fide evolution to a more aggressive subclone that clearly exhibits a higher grade of malignancy. In the latter instance, it would be logical to grade a malignancy on the basis of the least differentiated area [7], even if this were not the dominant portion at the time of diagnosis.

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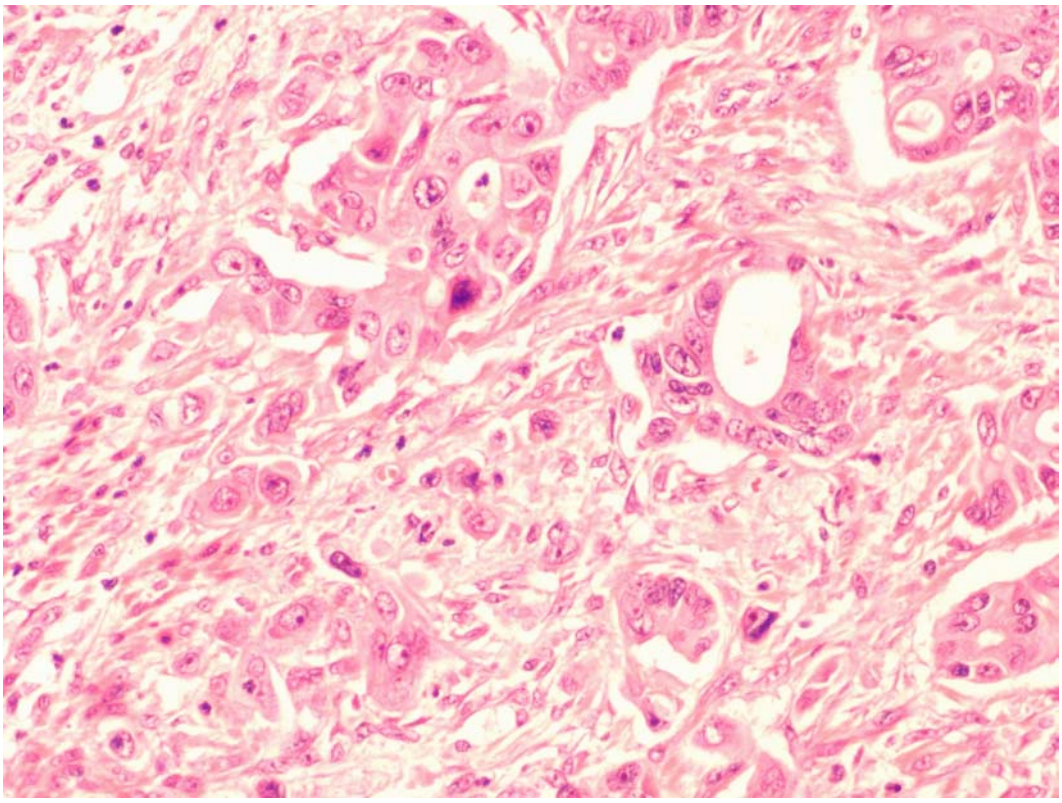

Figure 1. Typical histologic appearance of tumor budding in colorectal cancer (Haematoxylin and eosin).

In retrospect, Dukes was both right and wrong. He was wrong in implying that budding was without prognostic significance. He was correct in viewing budding as a biological process different from transformation to a higher grade of malignancy. The latter must occur at a single point in a tumor following the selection of a new mutation that confers a growth advantage. Budding, by contrast, occurs at the entire invasive front of a cancer. As such it must represent a genetic property present in the tumor as a whole and not a new genetic event. For this reason, the term 'de-differentiation' is somewhat misleading as it generally implies the transformation of a better-differentiated area into a less well-differentiated area by virtue of a specific genetic alteration. While 'budding' may not be an ideal term, it is preferable to the term 'de-differentiation'.

Budding – A Dynamic Process under Genetic Control

Because budding occurs at the tumor-host interface where one frequently observes a heightened inflammatory response characterized by the presence of immature connective tissue (Figure 2), neo-angiogenesis and a mixed inflammatory cell infiltrate, one could interpret the cellular discohesion that is characteristic of the process as a non-specific,

degenerative process. This interpretation is countered by the demonstration of a tightly coordinated repertoire of gene expression within the epithelium at the advancing tumor margin [8]. Beta-catenin was shown to accumulate with a distinct zonal pattern in colorectal cancer, with the most marked nuclear staining occurring at the invasive margin (Figure 3) [9]. The strongest expression of nuclear beta-catenin occurred in 'dedifferentiated' tumor cells at the invasive front of colorectal cancers [9]. By contrast, beta-catenin was mainly expressed along the lateral cell membrane of the more superficial and less actively invasive zones of the tumor, as in normal colorectal mucosa [8, 9]. The aberrant nuclear expression of beta-catenin found in colorectal cancers is usually dependent upon the inactivation of the APC tumor suppressor gene. As the principal effector of the WNT-signaling pathway, nuclear beta-catenin is able to complex with the TCF-family of DNA binding proteins and function as a transcriptional activator [10]. Among the target genes activated by beta-catenin are several known to be critical in the process of invasion and metastasis. These include urokinase-like plasminogen activating receptor (uPAR) [11], matrilysin [12, 13], CD44 [14], and laminin-5 γ 2 [15]. However, beta-catenin and the downstream activated molecules serve a normal physiological role during embryogeneis. Specifically, they regulate the tissue remodeling and migration that occur during gastrulation [9]. For this reason, budding may be regarded as a process that recapitulates the normal patterning observed during embryonic development [9]. The fact that this process will facilitate invasion and metastasis can be viewed as the unwanted by-product of the uncontrolled activation of genes that serve a critical role during embryogensis.

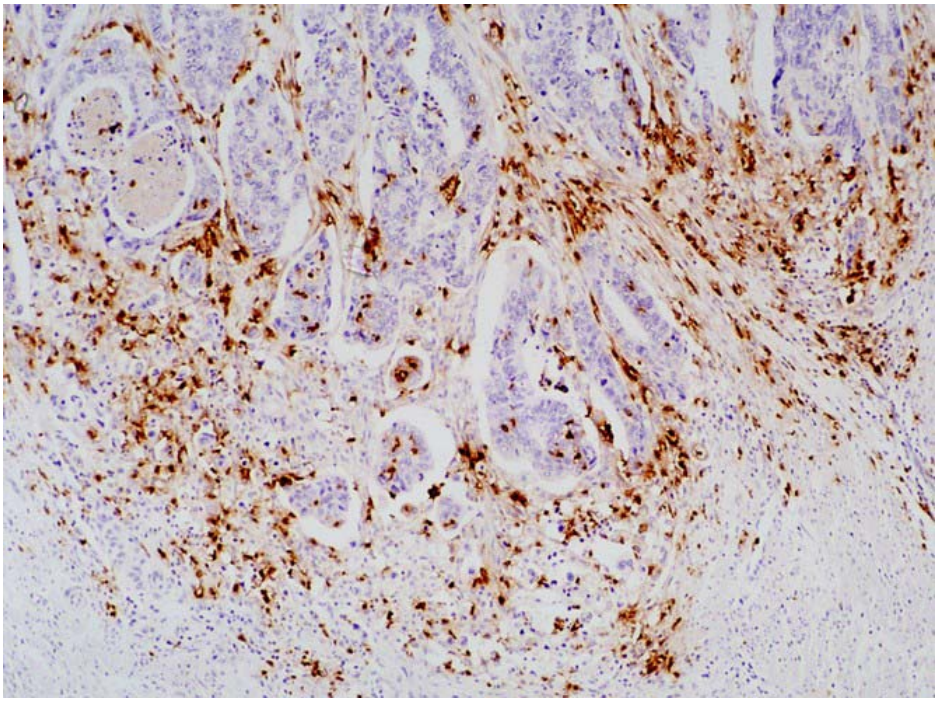

Figure 2. Increased expression of basic fibroblast growth factor by stromal cells in a colorectal cancer that showed tumor budding at the invasive margin (Immunperoxidase).

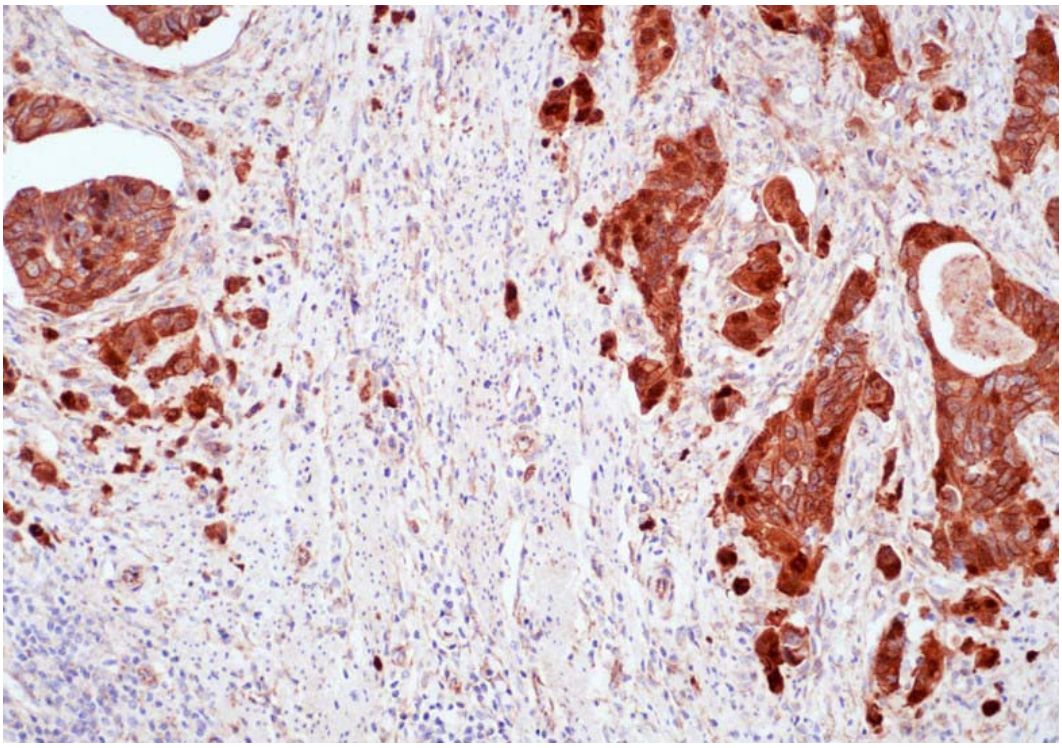

Figure 3. Increased expression of beta-catenin in tumor buds (Immunoperoxidase).

Budding and the WNT Signaling Pathway

Not all colorectal cancers show budding, perhaps around 20-40% [3, 16]. Budding is therefore not a pre-requisite for the development of the malignant phenotype but serves as a biomarker for a more aggressive phenotype. It is important to understand the mechanisms that underlie budding. This knowledge could provide an objective marker for prognostication and assist with the development of novel therapeutic interventions. Beta-catenin and its associated signaling pathway are regarded as crucial in the instigation of colorectal tumorigenesis [17]. Because beta-catenin is regarded as a central component of the mechanisms underlying tumor budding [8, 9], it was hypothesized that the presence of a dysregulated WNT signaling pathway might serve as an absolute pre-requisite for budding [18]. This hypothesis would not be especially attractive if WNT pathway dysregulation were a feature of all colorectal cancers. If this were the case it would be necessary to invoke an additional factor to explain the development of budding in only a subset of colorectal cancer. It is often claimed that *APC* is mutated in the 'vast majority' of colorectal cancers [19]. An exception may be colorectal cancers showing DNA microsatellite instability. However, *beta-catenin* may be mutated and rendered oncogenic in this subset [20] while *APC* could itself be silenced through methylation of its promoter region [21]. These arguments have been used to bolster the somewhat dogmatic view that dysregulation of the WNT signaling is the first step in the initiation of all colorectal adenomas and by implication all colorectal cancers.

In practice, the premise that a single linear model explains all instances of colorectal cancer is unlikely to be true. In fact, *APC* mutation is uncommon in small sporadic adenomas [22] and the overall frequency of *APC* mutation in colorectal cancer is around 60% [23]. The frequency of *APC* mutation is site dependent, being highest in the distal colon and rectum and lowest in the proximal colon [24]. It could be argued that screening for *APC* mutation is imperfect and is less likely to be successful for cancers of the proximal colon. This argument recalls the 'Emperor's new clothes' which had to exist even if they could not be seen. Following immunostaining for aberrantly expressed beta-catenin, it is evident that a subset of colorectal cancers shows a relatively normal distribution of this molecule that is limited to the lateral cell membranes with little or no cytoplasmic or nuclear staining [25, 26]. Studies in both experimental models and humans indicate that colorectal neoplasia may be initiated by oncogenic mutation of either K-*ras* [27] or *BRAF* [28] in addition to *APC*. *BRAF* mutation has recently been associated with the subtype of sporadic colorectal cancer that shows high-level DNA microsatellite instability (MSI-H) [29]. This subset also shows a low frequency of *APC* mutation, a normal pattern of immuno-localization of beta-catenin and occurs mainly in the proximal colon [23]. It is therefore apparent that *APC* mutation is not the exclusive initiating event in colorectal neoplasia and may in fact not occur at any stage in the pathogenesis of some colorectal cancers. While mutation of *beta-catenin* could substitute for mutant *APC*, this alteration appears to be largely restricted to MSI-H cancers from subjects with hereditary non-polyposis colorectal cancer (HNPCC) [30]. Where mutation of *beta-catenin* has been demonstrated in cases of sporadic MSI-H colorectal cancer, the subjects have been young and HNPCC has not been excluded [20].

Budding occurs in approximately 20-40% of colorectal cancer whereas, collectively, mutation of *APC* and *beta-catenin* may affect 70%. Therefore, factors additional to mutation of *APC* and *beta-catenin* must determine tumor budding.

APC Mutation, DNA Microsatellite Instability and Budding

To establish if *APC* mutation might be an important determinant of tumor budding, a series of colorectal cancers was stratified as HNPCC (no. 15), sporadic MSI-H (no. 21), MSI-low (MSI-L) (no. 43) and MS stable (MSS) (no. 16). The frequency of budding and *APC* mutation was determined for each class of colorectal cancer (Table) [18]. Classes of colorectal cancer with the lowest frequency of *APC* mutation (sporadic MSI-H) also had the lowest frequency of budding [18]. While it is reasonable on the basis of the findings to suggest that *APC* mutation may be a key pre-requisite for tumor budding, there was no association between budding and *APC* mutation within the various categories of colorectal cancer [18]. Possible explanations for this lack of concordance could be missed *APC* mutations (not all exons were screened), *beta-catenin* mutations in the case of HNPCC, or the absence of an additional factor in case of cancers with *APC* mutation but no budding. In this study, a subset of cancers was immunostained for beta-catenin and there was a good correlation with *APC* mutation and tumor budding [18].

Table 1. Frequency of *APC* Mutation and Budding in Colorectal Cancers Stratified as MSI-H, MSI-L and MSS

Colorectal cancer (no.)	*APC* mutation no.(%)	Budding no.(%)
MSI-H (21)	1 (4.8%)	0 (0%)
HNPCC (15)	4 (26.7%)	3 (20%)
MSI-L (43)	14 (32.6%)	18 (41.9%)
MSS (16)	6 (37.7%)	9 (56.2%)

E-cadherin, Fibronectin and Budding

In cancer cell lines there is a correlation between the expression of the epithelial adhesion molecule E-cadherin and invasiveness [31]. Cells that express E-cadherin do not infiltrate collagen matrices but show invasive behavior after incubation with monoclonal antibodies to E-cadherin [32]. By contrast, cells that do not express E-cadherin show invasive behavior that is blocked following transfection with complementary DNA (cDNA) that encodes E-cadherin [33]. On the basis of these findings, E-cadherin appears to act as a suppressor of invasion [33]. At the growing margin of colorectal cancers, dissociated single cells (tumor buds) show reduced expression of E-cadherin that may be limited to the cytoplasm [9]. By contrast, the better-differentiated tumor epithelium shows strong membranous expression of E-cadherin (Figure 4). Dissociated cells may also show strong cytoplasmic expression of fibronectin, indicating a mesenchymal phenotype [9]. On basis of these observations it has been suggested that tumor cells with strong membranous E-cadherin expression and no fibronectin expression represent a relatively inert epithelial phenotype, while the finding of dissociated cells with only cytoplasmic E-cadherin and/or fibronectin expression defines a mesenchymal and actively invasive phenotype [9].

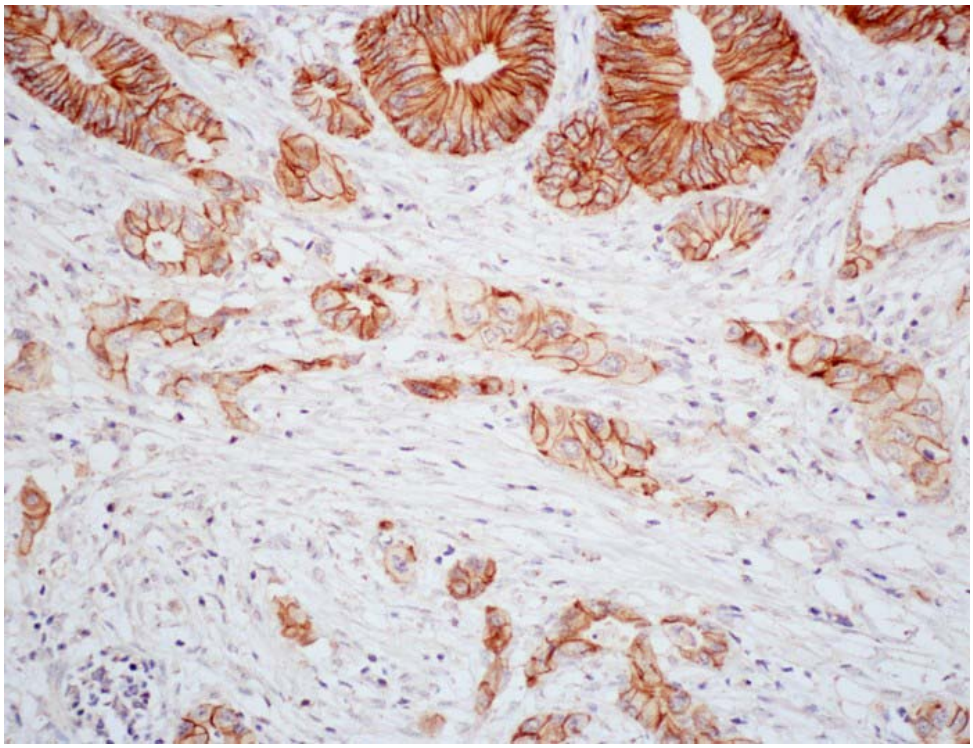

Figure 4. Reduced expression of E-cadherin in tumor buds (Immunoperoxidase).

Proliferation Markers and Budding

It might be assumed that budding at the invasive front of colorectal cancer would be associated with increased cellular proliferation. This might be predicted from the association with invasion, metastasis and prognosis, the fact that tumor buds appear to be de-differentiated, the aberrant nuclear translocation of beta-catenin, and the increased expression of cyclin D1 [8]. Paradoxically, tumor buds show reduced expression of the proliferation marker Ki-67 and concordant increased expression of p16 that normally acts to arrest the cell cycle by directly inhibiting cyclin D1 [8]. These observations have prompted some investigators to question the currently accepted function of cyclin D1 [8]. At any rate, it is clear that in the specific context of budding, beta-catenin is orchestrating a relatively restricted or specialized repertoire of cell responses in which cell proliferation is inhibited. It would appear that trans-differentiation, cellular discohesion and epithelial migration that are occurring in the context of tumor budding are processes that are expressed in a mutually exclusive fashion with respect to proliferation. This raises the interesting question of whether tumor buds may be relatively resistant to both chemotherapy and radiotherapy.

What are the mechanisms for the reduced proliferation within budding cells? $P16^{INK4a}$ is a target gene for beta-catenin and this would account for the co-expression of the two genes at the invasive tumor margin (Figure 5). It has been suggested that cytoplasmic p16 could bind with cdk4 and thereby block its translocation into the nucleus [18]. Cdk4 is required for cyclin D1 activation. In the absence of cdk4, cyclin D1 may complex inappropriately with

cdk2. This is known to give rise to a stable and relatively inert complex [34], a fact that could explain the paradoxically increased levels of cyclin D1 in tumor buds. Moreover, there would be less cdk2 available for cyclins A and E, and this would inhibit the cell cycle. In this model, p16 is conceived as a key molecule in cell cycle arrest and may therefore be critical for the proper development of budding [18]. Loss of expression of p16 may occur through promoter hypermethylation [35]. The latter is observed in colorectal cancers showing the CpG island methylator phenotype (CIMP) [35]. CIMP occurs in around 40% of colorectal cancers, including the subset with methylation of *hMLH1* and MSI-H status. This would provide an additional explanation for the lack of budding in MSI-H colorectal cancers and may also account for the lack of budding in the subset of colorectal cancer with *APC* or *beta-catenin* mutation. Given the predicted lack of budding in colorectal cancer with the CIMP phenotype, it is interesting that this subset of colorectal cancer may show the highest rate of chemo-responsiveness [36].

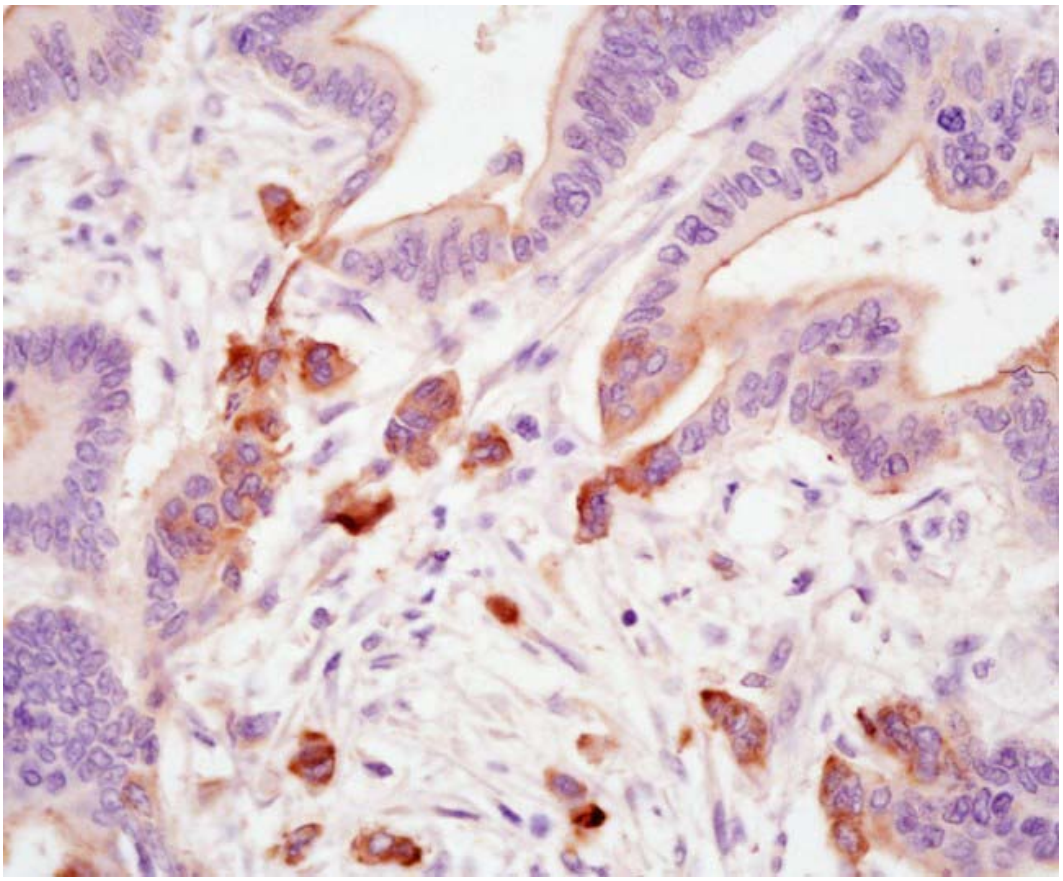

Figure 5. Increased expression of p16 in tumor buds (Immunoperoxidase).

Conclusion

To fully understand the biology of colorectal cancer, it is necessary to dissect out the disordered cellular mechanisms as they occur *in vivo*. There is a distinct patterning of gene expression within different sub-compartments of a colorectal cancer. In particular, investigation of tumor:host interactions at the invasive margin is of critical importance in explaining tumour biology, prognosis and responsiveness to adjuvant therapy. However, it is likely that molecular features applicable to the cancer as a whole will determine whether or not budding can take place. These features will include mutation of *APC* or *beta-catenin* and methylation of $P16^{INK4a}$. The presence or absence of such features will be highly dependent upon the underlying specific pathways of tumorigenesis and the fact that colorectal cancer must now be viewed as a group of diseases rather than a homogeneous disease entity. Therefore, it may not be necessary to diagnose or obtain tissue samples of buds in order to obtain, for example, useful prognostic or predictive information pre-operatively.

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Chapter VI

SIGNIFICANCE OF TUMOR BUDDING IN EARLY COLORECTAL CANCER BASED ON MICROMORPHOLOGIC AND STATISTICAL STUDIES

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Introduction

In early colorectal cancer with submucosal invasion, the choice of treatment varies greatly depending on the status of risk factors for lymph node metastasis. When no risk factors are present, local resection without lymphadenectomy is the treatment of choice. However, a radical operation with lymphadenectomy is needed when a risk factor for lymph node metastasis is present. A number of risk factors have been reported, such as lymphatic permeation [1-3], depth of submucosal invasion [3-4], and histologic grade of malignancy [5-6]. Lymphatic permeation is particularly well known as one of the most important risk factors for lymph node metastasis. But it is not easy for even experienced pathologists to routinely make an accurate diagnosis of lymphatic permeation, as pointed out by Masaki [7]. When cancer cells are detected floating in vessels having lymphatic epithelium, it can be judged that lymphatic permeation has occurred. However, lymphatic permeation consists not only of floating features but may also show embolic features, filling the lymphatic vessels. It is impossible to diagnose such embolic features as lymphatic permeation. Therefore, we paid special attention to tumor budding in the submucosal invasive front as an interesting histologic feature. We micromorphologically studied the features of this budding, and

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statistically analyzed the correlation between the budding and other characteristics such as lymphatic permeation, venous invasion, lymph node metastasis, depth of submucosal invasion and histological grade of malignancy.

However, different researchers have different definitions or concepts of tumor budding. The word “budding” is commonly used as a term related to the germination of yeasts, spores, and plants. “Tumor budding” was first applied as a metaphorical expression by our coworkers [8-10] more than 15 years ago because the biological behavior of the cancer cells resembles the germination of yeast or of a plant. However, other terms have also been used, such as “sprouting” [11,12] or “focal dedifferentiation” [13] and this has caused some confusion regarding the concept or definition of tumor budding. Therefore, we also micromorphologically studied the features of tumor budding in an effort to clarify the relevant concepts and definitions.

Materials and Methods

Materials

One hundred and ninety-six cases of early colorectal cancer were resected between 1980 and 2002. Lymphadenectomy was performed in 161 cases and the presence or absence of lymph node metastasis was reviewed. Local resection without lymphadenectomy was performed in 35 cases and lymph node metastasis was judged as negative if local recurrence did not occur more than one year after operation.

Method of Preparing Specimens

Surgical specimens were initially fixed in 10 % formalin. After 1 week fixation in formalin, the entire tumor mass was sliced into longitudinal sections about 3 mm thick. These sections were dehydrated with alcohol for 1 week and then embedded in paraffin. Next, thin sections of about 3-μm thickness were sliced from paraffin embedded blocks with a microtome. These were mounted on glass slides and stained with hematoxylin-eosin (H.E.) and elastica van Gieson.

Histologic Diagnosis

All of the specimens were reviewed and the histologic findings were recorded in each case. The final diagnosis was checked by the author. The relationships among tumor budding, lymphatic permeation, venous invasion, histologic grade, depth of submucosal invasion and lymph node metastasis were examined. Also, serial thin sections were made in 10 cases to investigate the relationship between budding and lymphatic permeation.

Definition of Lymphatic Permeation

We identified lymphatic vessels in hematoxylin-eosin- and elastica van Gieson-stained specimens and observed the histologic features of the lymphatic vessels, as previously reported [14]. Submucosal lymphatic vessels have few smooth muscle and elastic fibers. Most importantly, a lymphatic vessel appears as an endothelial-lined space, and valvules are often seen within these lymphatic vessels. The diagnosis of lymphatic permeation was made based on the histologic features of normal lymphatic vessels. Lymphatic permeation was defined as positive only when cancer cells were observed floating within an endothelial-lined space. The lumen sometimes contains lymphocytes, and rarely erythrocytes. The differential diagnosis from venous invasion was made on the basis of features such as the presence or absence of smooth muscle and elastic fibers, and valvules. In addition, we looked for pseudo-lymphatic permeation due to tissue shrinkage artifacts created during the process of tissue slide preparation. In pseudo-lymphatic permeation, cancer cells appear to be floating in a space without endothelial cells. However, with careful observation, it can be distinguished clearly from lymphatic permeation. The lymphatic permeation was divided into four degrees, as previously reported [14], with ly0 defined as negative, ly1 slight, ly2 moderate, and ly3 marked lymphatic permeation.

Definition of Venous Invasion

Venous invasion was confirmed by elastica Van Gieson staining. Micromorphologically, venous invasion was classified as embolic type, in which the tumor cells filled the vein and adhered to the vein wall; floating type, in which the tumor cells did not adhere to the vein and floated in the lumen; and intimal type, in which the tumor cells were mainly found in the intima of the vein. Venous invasion was also divided into four degrees, as previously reported [15] by the author, where v0 was defined as negative, v1 slight, v2 moderate, and v3 marked venous invasion.

Morphopathobiological Definition of Tumor Budding

Tumor budding was classified as either intraductal or extraductal. Intraductal budding grows papillarily (defined as Type 0) within the lumen of a tubule, as shown in Figure 1. Extraductal budding grows outside the tubule, and was classified into Type I, II or III according to their micromorphologic features. Type I is an early stage bud (or buds) which is just beginning to protrude out from the parental tubule, as shown in Figure 2. Type II is a growing bud (or buds), which appears as a branch extending from the parental tubule, as shown in Figure 3. Type III is a developing bud (or buds), consisting of an isolated tiny cluster of undifferentiated cells (the number of nuclei is commonly less than 15), without obvious gland formation, including single cells, as shown in Figure 4. Types II and III often coexist, as shown in Figure 5. On this basis, in our system “budding” or “tumor budding” refers to developing buds of morphopathobiological Type III. Tumor budding was judged positive when two or more isolated tiny clusters (developing buds) were present. We further

divided the tumor budding into 4 degrees according to the total number of the clusters in those glass slides in which clusters were most actively and abundantly detected. Intraductal budding (Type 0) was excluded from this study. In our system, bd0 was defined as negative budding, with no or only a single cluster present, as shown in Figure 6; bd1 was defined as slight budding, with fewer than 6 clusters; bd2 was defined as moderate budding, with 6 to 15 clusters; and bd3 was defined as marked budding, with 16 or more clusters.

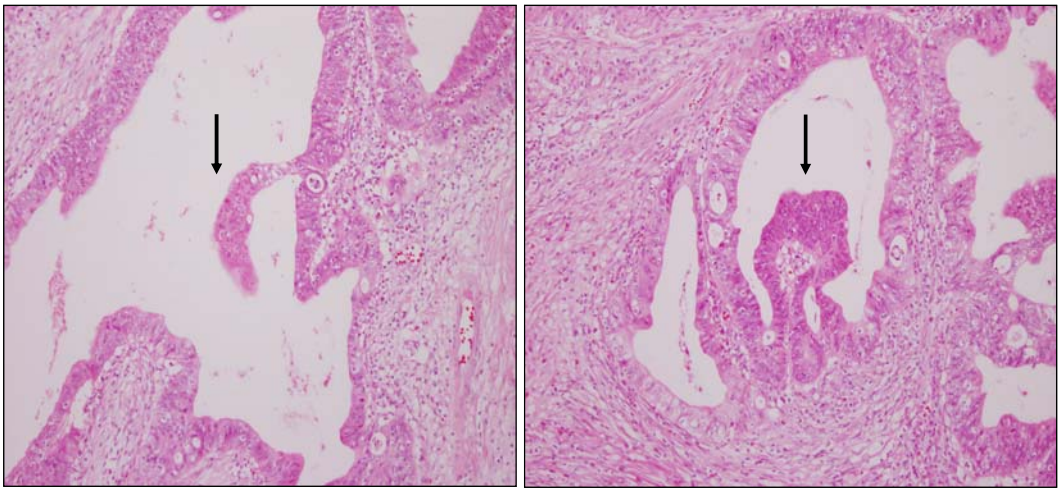

Figure 1. Intraductal budding (Type 0). Tumor protrudes within the lumen of the tubule (arrow). (left : H.E. 5 x 10, right : H.E. 5 x 20). These figures look like a bud. The Isolated undifferentiated cancer cells are not usually observed within the lumen of the tubule.

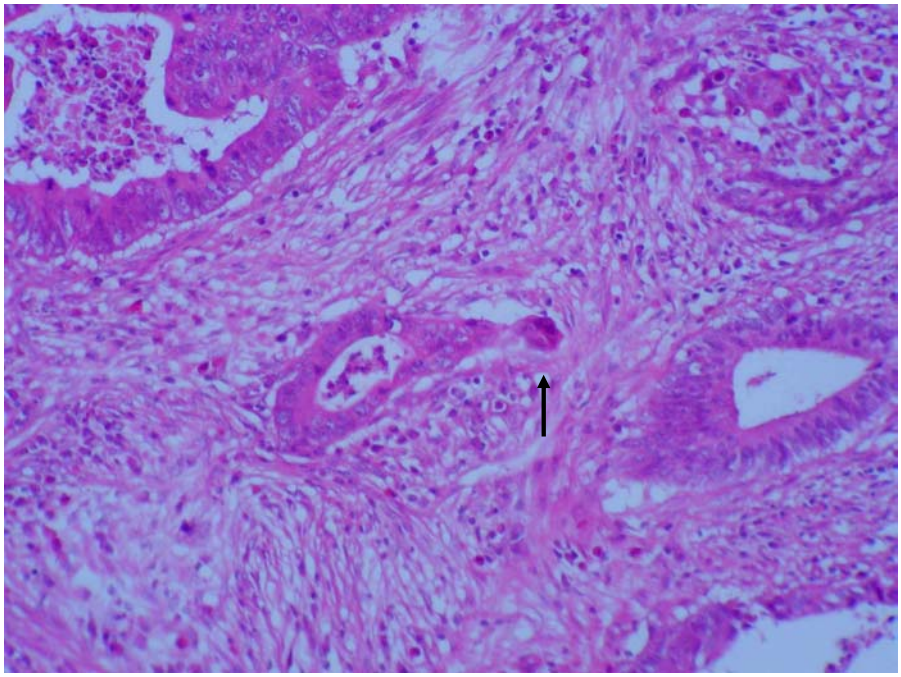

Figure 2. Beginning bud of the Type I budding. A bud begins to just come out from the tubule (arrow). (H.E. 5 x 20)

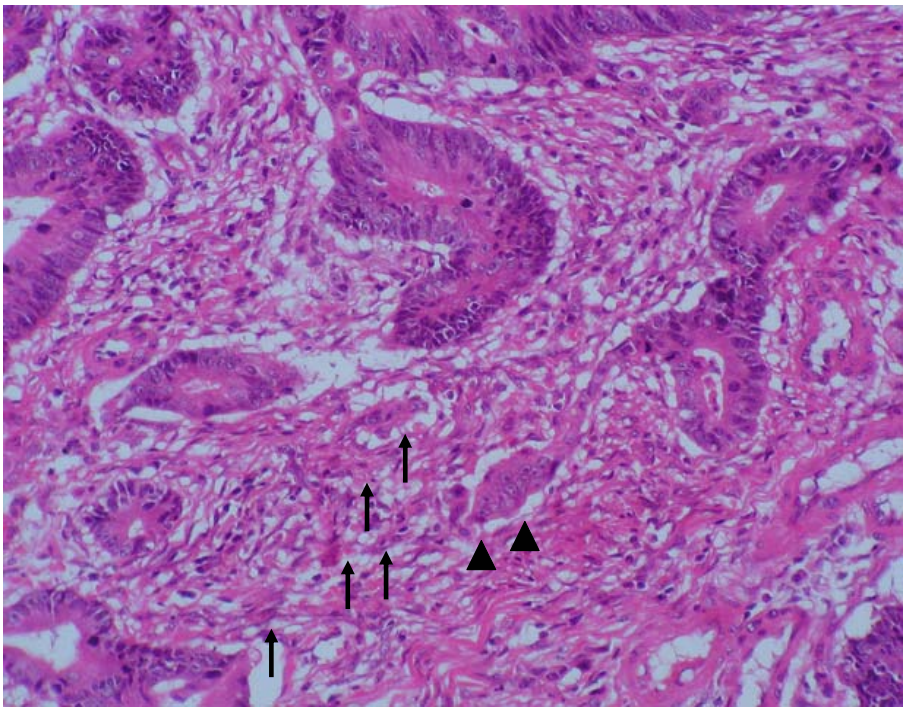

Figure 3. Growing bud of the Type II budding. The growing bud (arrow heads) protrudes into the stroma from the parental tubule. Several clusters of undifferentiated cells (arrows) are observed at the front edge of the growing bud. (H.E. 5 x 20)

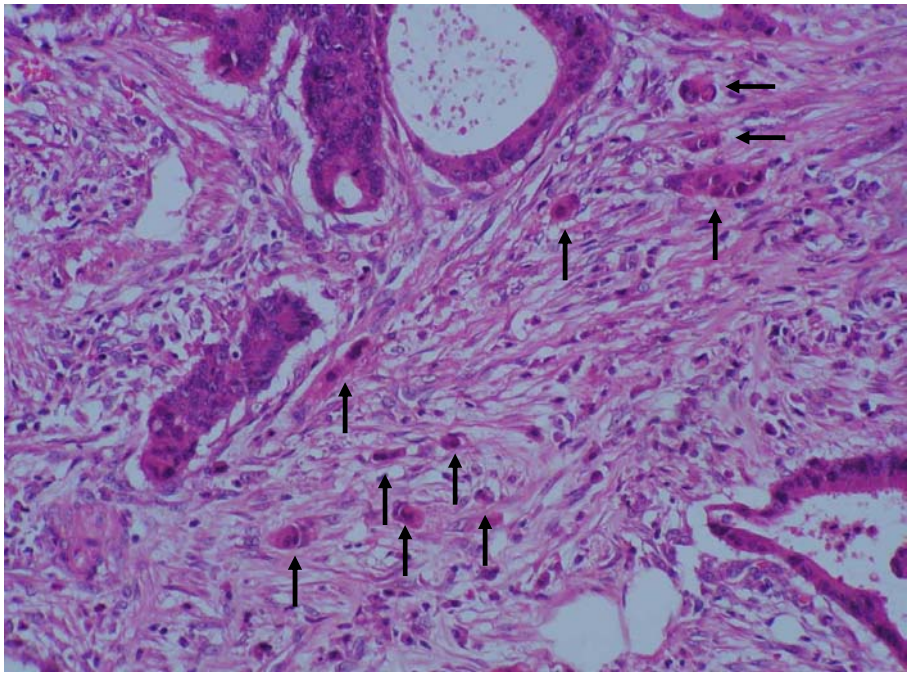

Figure 4. Type III budding. Many tiny clusters of the isolated undifferentiated cells (arrows) are scattering in the stroma. (H.E. 5 x 20)

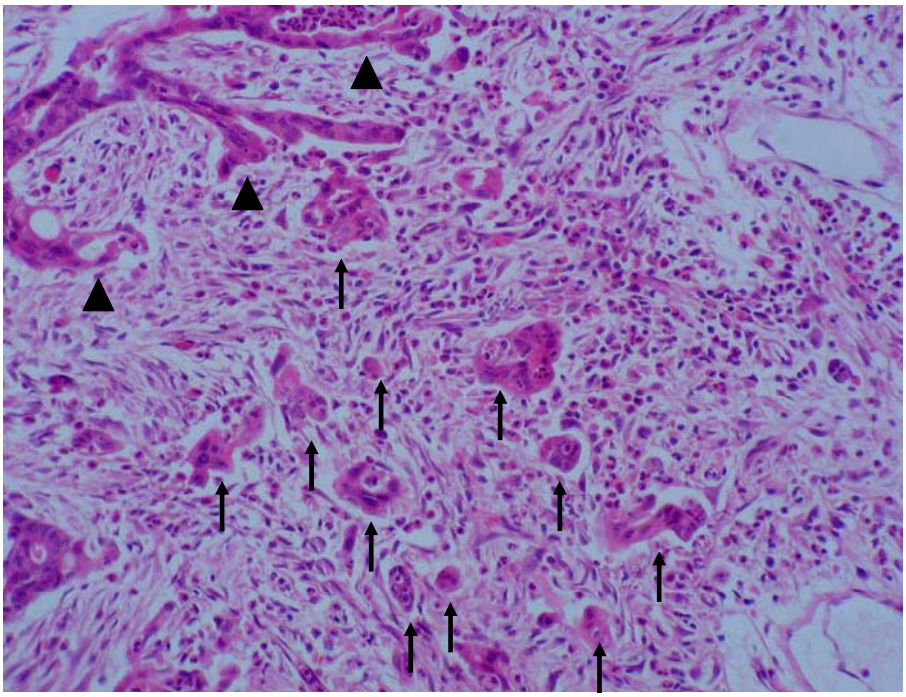

Figure 5. Coexistence of Type II and Type III budding. Many clusters of isolated undifferentiated cells (arrows) are observed at the front edge of the growing buds (arrow heads). (H.E. 5 x 20)

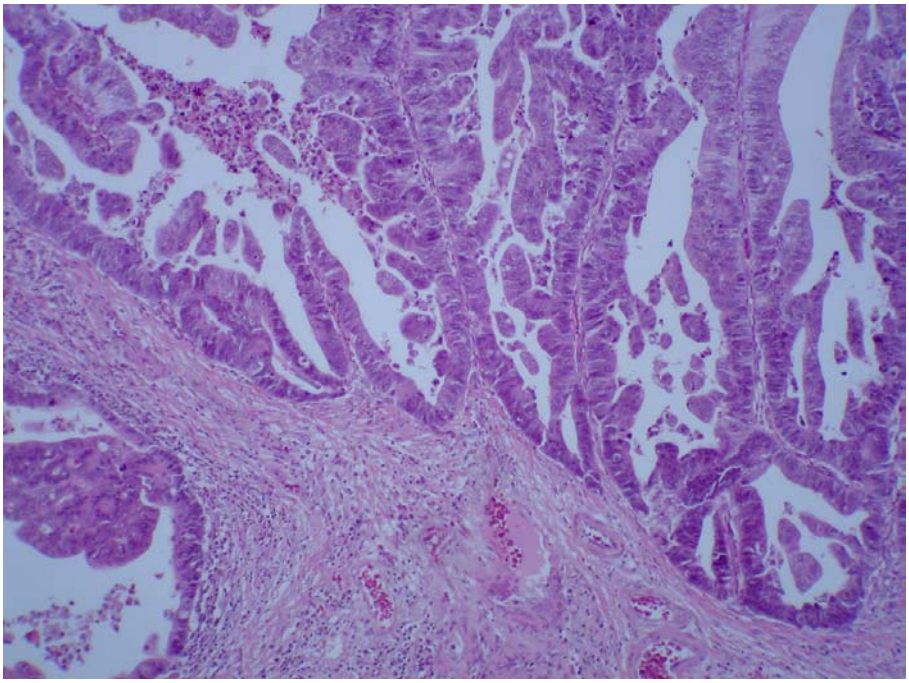

Figure 6. Negative budding. No cluster of undifferentiated cells is seen anywhere. (H.E. 5 x 10)

Definition of the Depth of Submucosal Invasion

Depth of submucosal (sm) invasion was divided into 3 categories, sm1, sm2 and sm3, where sm1 was defined as slight submucosal invasion in which the depth of infiltration is less than 1000 μ m, sm3 was defined as marked invasion into the submucosal layer just over the proper muscle, and sm2 was defined as moderate invasion between sm1 and sm3.

Statistical Analysis

The chi-squared test, Fisher's exact test and multivariate logistic regression analysis were used to determine the statistical significance of any differences. $P< 0.05$ was considered significant.

Results

Chi-squared Test and Multivariate Logistic Regression Analysis Concerning Lymph Node Metastasis

As shown in Table 1, the chi-squared test revealed a significant correlation between lymph node metastasis and lymphatic permeation ($p<0.0001$), budding ($p<0.0002$), and venous invasion ($p=0.0334$). The higher the degree of these three factors, the higher the incidence of lymph node metastasis. Depth of sm invasion ($p=0.1035$) and histologic grade ($p=0.3515$) did not correlate with lymph node metastasis. Multivariate logistic regression analysis was applied to the significant factors to determine the most independent risk factors for lymph node metastasis. As shown in Table 1, lymphatic permeation was the strongest independent risk factor ($p=0.0301$), followed by budding ($p=0.0728$).

Chi-squared Test and Multivariate Logistic Regression Analysis Applied to Budding

As shown in Table 2, the chi-squared test showed that budding was significantly correlated with lymphatic permeation ($p<0.0001$), depth of sm invasion ($p<0.0001$), venous invasion ($p<0.00001$) and histologic grade ($p<0.0004$). The multivariate logistic regression analysis showed that the budding was most strongly associated with lymphatic permeation ($p<0.0001$), followed by depth of sm invasion ($p=0.0019$).

Table 1 Chi-squared and Multivariate Logistic Regression Analysis Concerning Lymph Node Metastasis

		lymph node metastasis			Total	Chi-squared p-value	Logistic p-value
		negative	positive				
lymphatic pemeation							
	ly0	145	6 (4.0)		151	<0.0001	0.0301
	ly1	28	6 (17.6)	(22.2)	34		
	ly2-3	7	4 (36.4)		11		
budding							
	bd0	127	3 (2.3)		130	<0.0002	0.0728
	bd1	35	8 (18.6)	(19.7)	43		
	bd2-3	18	5 (21.7)		23		
venous invasion							
	v0	128	8 (5.9)		136	0.0334	0.5886
	v1	51	7 (12.1)	(13.3)	58		
	v2-3	1	1(50.0)		2		
depth of sm invasion							
	sm1	54	1 (1.8)		55	0.1035	-
	sm2	77	8 (9.4)		85	n.s.	
	sm3	49	7 (12.5)		56		
histologic grade							
	well	167	14 (7.7)		181	0.3515	-
	others	13	2 (13.3)		15	n.s.	

n.s.: not significant.
( ): %.

Table 2. Chi-squared and Multivariate Logistic Regression Analysis Concerning Budding

		bd0	bd1	bd2-3	total	Chi-squared p-value	logistic p-value
lymphatic permeation							
	ly0	119	24	8	151	<0.0001	<0.0001
	ly1	10	14	10	34		
	ly2-3	1	5	5	11		
depth of sm invasion							
	sm1	51	4	0	55	<0.0001	0.0019
	sm2	53	23	9	85		
	sm3	26	16	14	56		
venous invasion							
	v0	101	27	8	136	<0.0001	0.1461
	v1	29	16	13	58		
	v2-3	0	0	2	2		
histilogic grade							
	well	126	38	17	181	<0.0004	0.3567
	others	4	5	6	15		

Lymphatic Permeation

Figure 7a shows a typical lymphatic permeation, in which cancer cells with gland formation are floating within an endothelial-lined space. This type of lymphatic permeation is often observed. Figure 7b shows an embolic type of lymphatic permeation, in which the cancer cells adhere to the endothelium of the lymphatic vessel and are just beginning to embolize the duct.

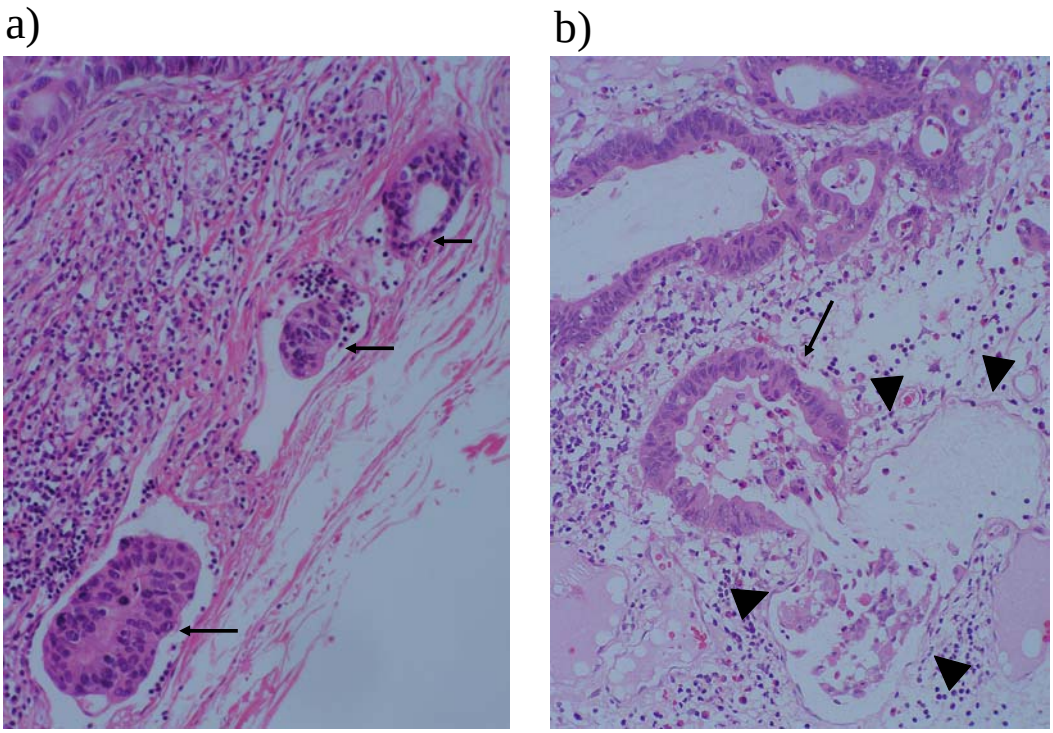

Figure 7. Lymphatic permeation in the submucosal layer. a) Typical lymphatic permeation: cancer cells with glandular differentiation are observed within a endothelial-lined lymphatic vessel (arrow). (H.E. 5 x 20). b) Embolic type of lymphatic permeation: the lumen of the lymphatic vessel (arrow heads) is filled with cancer cells. Tumor replaces the endothelium of the lymphatic vessel with cancer cells. (H.E. 5 x 20)

Lymphatic Permeation and Budding

As shown in Figure 8, lymphatic permeation was often observed near the budding region.

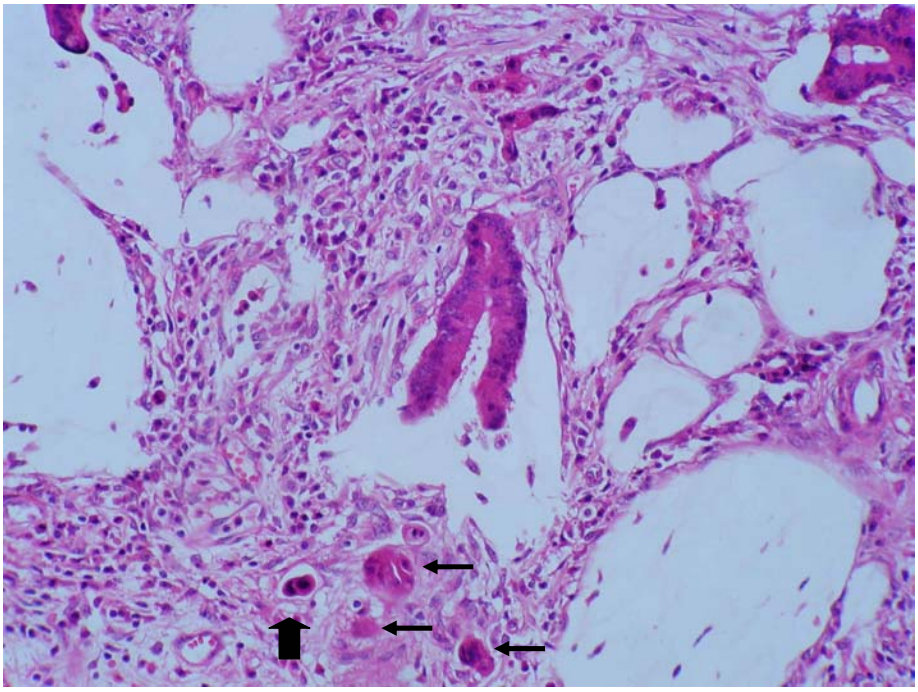

Figure 8. Lymphatic permeation and budding. Lymphatic permeation (large arrow) is observed near the budding region (small arrows). (H.E. 5 x 20)

Transition of the Growing Bud as Seen in the Serial Thin Sections

Figure 9a shows a horizontal slice catching a growing bud (arrow). Figure 9b is an oblique slice catching an interim stage between a growing bud and a developing bud.(arrow). In Figure 9c, one observes a vertical slice showing a developing bud (arrow). These features resemble the germination of a seed.

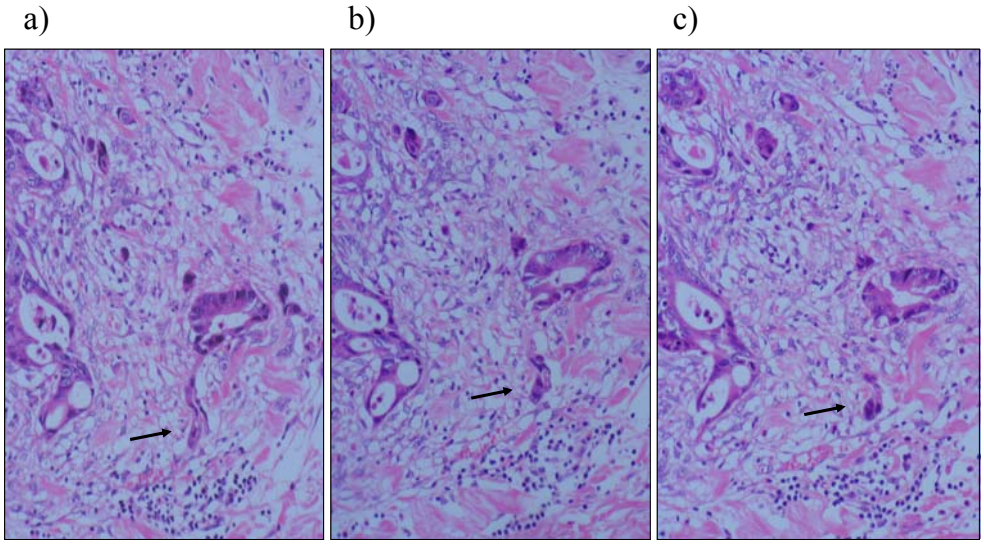

Figure 9. Transition of the growing bud as shown in the serial thin sections. (a) Horizontal slice catches a growing bud (arrow). (b) Oblique slice catches an interim stage between a growing bud and a developing bud (arrow). (c) Vertical slice catches a developing bud (arrow). These features resemble the germination of a seed. (H.E. 5 x 20)

Serial Thin Sections of the Budding Region

Figure 10a shows several clusters of isolated undifferentiated cells gathered closely together (arrows). As shown in Figure 10b, serial thin sections of the budding region reveal new typical lymphatic permeations (ly) appearing to replace the budding in 8 of 10 cases.

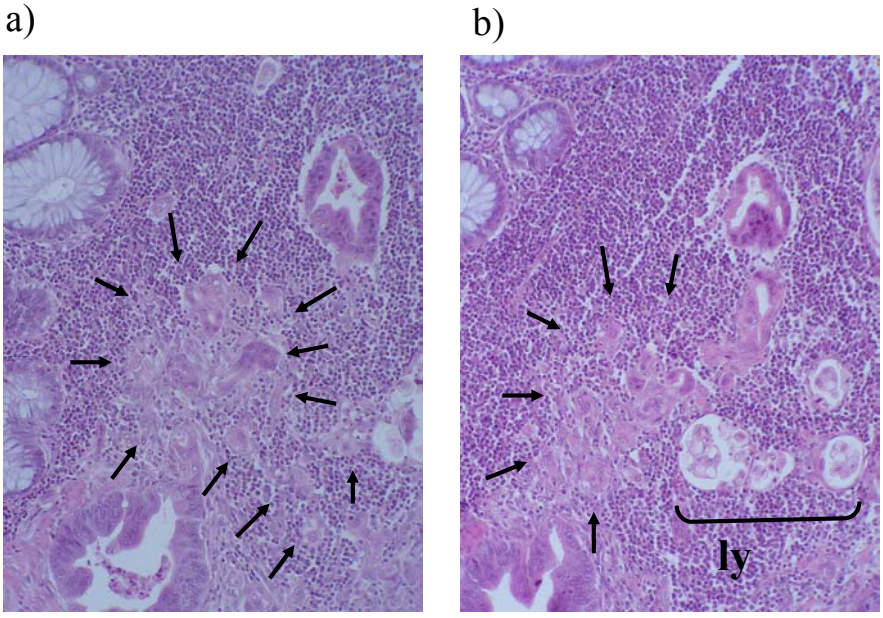

Figure 10. Serial thin sections of the budding region. (a) Some clusters of isolated undifferentiated cells are gathered closely together (arrows). (H.E. 5 x 20). (b) In serial thin sections, new typical lymphatic permeations (ly) appear to replace the budding. (H.E. 5 x 20)

Case Report of Lymph Node Metastasis

Figure 11 shows several clusters of undifferentiated cells (marked budding) in the submucosal layer. The metastatic lymph node appears as a well-differentiated adenocarcinoma.

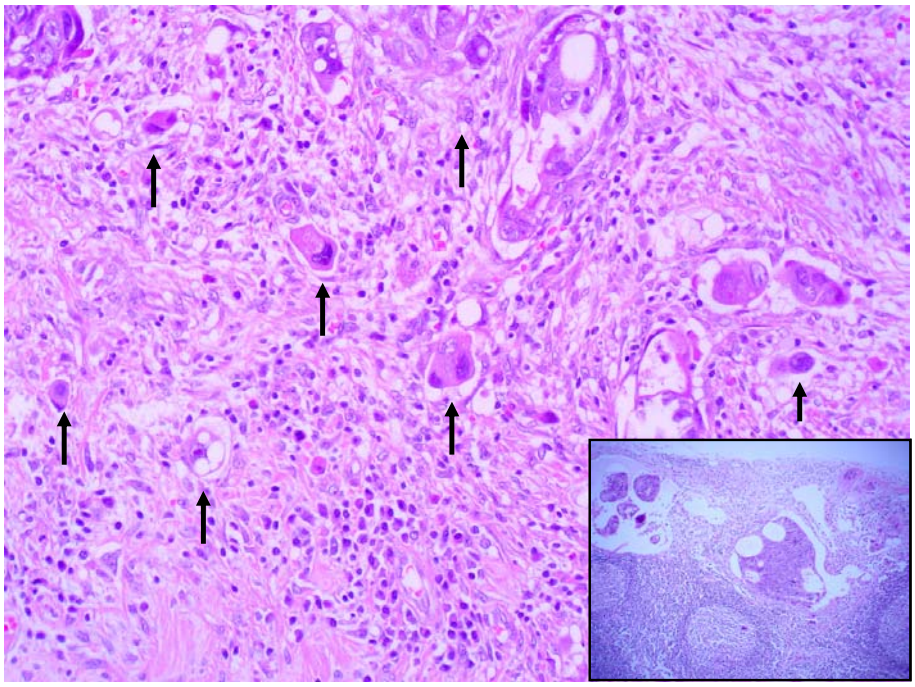

Figure 11. Case of lymph node metastasis. Several clusters of isolated undifferentiated cells (marked budding) are observed. (H.E. 5 x 20). *Inset*: Lymph node metastasis; Cancer cells with glandular differentiation are observed. (H.E. 5 x 10).

Budding as Seen in an Interesting Creature: "Polyandrocarpa Misakiensis"

These pictures were downloaded from Dr. S. Fujiwara's home page (http://www.kochi-u.ac.jp/~tatataa/index2.html), and are shown here with his permission. Figure 12a shows a growing bud, which is an outgrowth of the parental body wall. Figure 12b is a schema of Figure 12a, showing the growing bud and the developing bud. Figure 12c shows the bud separating from the parent and starting cell differentiation and morphogenesis to reconstruct a new individual.

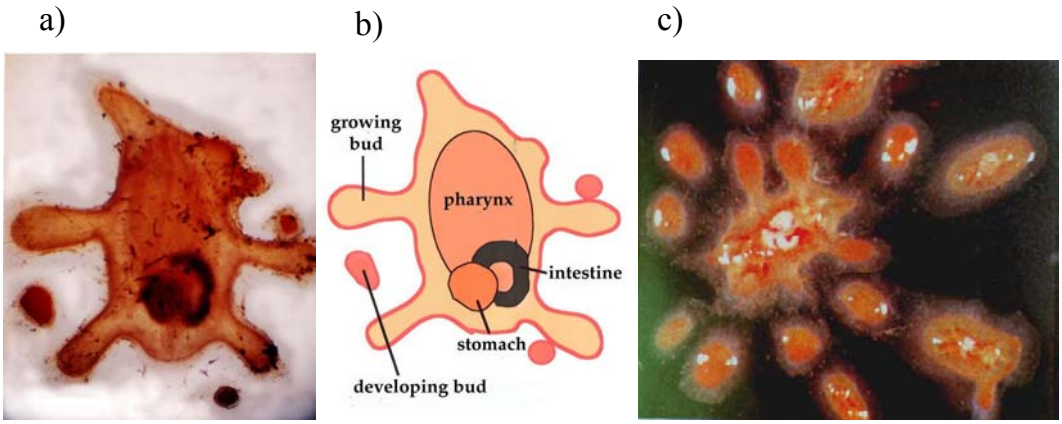

Figure 12. Polyandrocarpa Misakiensis. (a) The growing bud is an outgrowth of the parental body wall. (b) Schema of the Figure 12a shows the growing bud and the developing bud. (c) The bud separates from the parent and starts cell differentiation and morphogenesis to reconstruct a new individual. These pictures were downloaded from Dr. S. Fujiwara's home page (*http://www.kochi-u.ac.jp/~tatataa/index2.html*).

Discussion

In resected specimens of colorectal cancer, we usually observe that the tumor proliferates with glandular differentiation. In the stromal part of the active invasive front, however, protruding or branching morphogenesis of the glands (Type I or Type II budding) or tiny isolated undifferentiated cells without obvious gland differentiation (Type III budding) often appears. The former two types are connected with parental cancer cells and seem to be relatively differentiated cancer cells. On the other hand, the tiny, isolated undifferentiated cells of Type III budding seem to have been derived from the parental cancer cells. However, their biological behavior appears to be different from that of the differentiated parental cancer cells, and their malignant potential seems to be higher. Therefore, we focused our attention on these tiny isolated undifferentiated cells and attempted to investigate their clinical significance. They usually consist of several undifferentiated cells and sometimes form into clusters. They seem to be able to easily invade the lymphatic ducts and blood vessels, perhaps because of their extremely small size. Also, they are probably able to form tubules after acquiring the ability to differentiate as they develop in metastatic lesions. We often observe glandular formation mixed with the undifferentiated cells in lymph vessels (Figure 7) or in the metastatic lymph node (Figure 11), so these undifferentiated cells seem to have the capacity for multipotential differentiation.

Broders [16] previously reported that the degree of development of the undifferentiated cells in epithelioma of the lip was associated with the prognosis. Imai [11] applied this concept to evaluate the prognostic value of these undifferentiated cells in gastric cancer. He morphopathologically defined the undifferentiated cells as "Zokushutsu" in Japanese. In 1960, he translated the term as "sprouting" in his English report [12], because the growth pattern was morphologically similar to that of a sprouting plant. When the sprouting is visible in the low power view field (less than x 100), it seems to be an indicator of the growth pattern of the tumor as a whole [12]. This probably represents a clinically advanced stage of carcinoma.

However, to our knowledge, these kinds of reports disappeared from the literature after that time. In 1987, our coworker Hayashida [8] used the status of undifferentiated cells to evaluate the risk of lymph node metastasis in colorectal early cancer (submucosal cancer) for the first time. He used the term “budding” for the undifferentiated cells because their appearance resembled that of a germinating seed (Figure 9). Since 1987, our coworkers [9,10,17,18] have reported the significance of budding in colorectal early cancer. The term “budding” is only a metaphorical expression. Jass [19] described that the buds appeared to “drip” down from the main mass of a more differentiated tumour. This is also an interesting metaphorical expression. It does not matter at this point what terminology we use. Researchers are free to choose the term that they feel most descriptive. Eventually, the most appropriate term will, no doubt, remain while less suitable ones will disappear. The important thing that we have to remember is that cancer cells are malignant creatures even observed on the glass slide. We should imagine the migration of these cancer cells as we look at the two-dimensional glass slide images, and try to understand the budding in three-dimensional terms.

The tiny isolated undifferentiated cancer cells morphopathologically and biologically (in other words *morphopathobiologically*) appear extremely malignant. Our *morphopathobiological* definition of budding is philosophically different from the *morphopathological* definition even if the morphologic findings are similar. The process by which we came to our conclusions differs from that of others. We used the term "budding" to emphasize the importance of the biologic behavior. In 1977, Si-chun [20] proposed a new *pathobiological* classification of gastric carcinoma that emphasized its biologic behavior. His concept influenced our study. Recently, the term "budding" has been commonly accepted in descriptions of colorectal cancer and has spread worldwide. Combination studies of budding and other prognostic factors have stimulated new research in adhesion molecules, genetic abnormalities and many other areas [21-34]. However, the term “focal dedifferentiation” reported by Ono [13] seems to be a definition that emphasizes the cancer cell dissociation mechanism but is not suitable as a morphological definition. The same may be said of the term “cohort-migration” [35].

The proliferation features of those isolated undifferentiated cells are quite similar to those of the growing buds of spores, plants and some kinds of cell division. So, the term “bud or budding” is widely accepted not only in pathology but in other fields as well [36].

In the biological field, Fujiwara [37] and his coworkers [38-40] have studied the mechanism of bud development in the extremely interesting creature “Polyandrocarpa misakiensis”. They used the terms “growing bud” and “developing bud” to describe the developmental mechanisms of this creature. The term “growing bud” was metaphorically used to describe parts projecting from the parental body, and “developing bud”, describe parts isolated from them (Figure 12). The developing buds have multipotential ability and enter into the cell division cycle after dedifferentiation from some part of the parental body. They then redifferentiate in the stomach and intestine.

The growing buds look like the branching morphogenesis of cancer cells, and the developing buds look like the undifferentiated cells. Tumor cells are also creatures having a cell division cycle. A similar phenomenon seems to occur in the process of cancer cell initiation, progression and development. Therefore, we micromorphologically classified the tumor budding into four types. The pathological and clinical significance of Type 0 budding is uncertain. The Type II budding is detected as a horizontal slice in the plane image of the microscopic glass slide. On the other hand, when Type II budding is detected in a vertical

slice, it may sometimes appear as clusters of undifferentiated cells in the plane image (Figure 9). In other words, Type III budding cells may also coexist with Type II budding. Although we can sometimes observe Type II budding alone (Figure 3), Types II and III occur together in many cases (Figure 5). Also, Type II budding seems to be relatively well differentiated because it is connected to the parental cancer cells. That is why tumor budding was judged negative when only Type II budding was detected in the glass slide. However, Type III budding appears *morphopathobiologically* to be extremely malignant. Therefore only Type III budding was defined as positive in our system. In addition, we quantitatively classified the Type III budding into 4 degrees and studied the relationships between these degrees of development and other clinicopathologic characteristics.

It is the relationship with lymph node metastasis that has the greatest clinical significance. Neither histologic grade nor depth of submucosal invasion was associated with lymph node metastasis (Table 1). Predominant histologic grade is not always useful for estimating not only the risk of lymph node metastasis but also the characteristics of the entire tumor. Especially when well-differentiated adenocarcinoma is predominant, the partial histologic features of the moderately or poorly differentiated region are easily overlooked. This is probably why, in the case of ordinary colorectal cancer predominantly showing well-differentiated adenocarcinoma, histologic grade is not correlated with lymph node metastasis. With careful observation, however, the poorly or undifferentiated components of the carcinoma may well be considered important features, as reported by some authors [5,11,12,16].

Depth of submucosal invasion is also an important risk factor for lymph node metastasis. Some authors [3,4] reported that sm3 invasion (marked submucosal invasion) was a significant risk factor. Our definition of the degree of submucosal invasion was slightly different from the Haggitt [41] classification. Our definition was based on the classification as reported by Nascimbeni [3], because of its extreme simplicity and convenience. We were able to classify the depth of submucosal invasion in 98 % of all submucosal colorectal cancers, similar to the rate reported by Nascimbeni [3], although our results did not agree with his. On the other hand, in 1987 our coworker Hayashida [8] first reported the relationship between the interesting parameter of “budding” and lymph node metastasis in submucosal colorectal cancer. Also, in 1989 our coworker Morodomi [9,10] first reported the relationship between budding in preoperative biopsy specimens and lymph node metastasis in rectal cancer. Ueno [22] also reported that tumor budding in biopsy specimens was useful to evaluate the extent of local spread. In our present study, budding was significantly associated with lymphatic permeation and lymph node metastasis, as previously reported [17,18]. Multivariate analysis showed it to be an important risk factor, along with lymphatic permeation. Masaki [7] reported that lymphatic permeation alone was associated significantly with tumor budding using a statistical analysis. Other authors [1-3] also reported that lymphatic permeation was a risk factor for lymph node metastasis. In addition, lymphatic permeation often appeared around the budding regions in serial thin sections (Figure 10). Those findings signify that Type III budding is a feature that appears prior to lymphatic permeation, as previously reported by Morodomi [10]. Hase [21] also supported his concept. Otherwise, tumor budding may signify tumor emboli due to lymphatic permeation (Figure 7b). Okuyama [42] also reported that budding was a risk factor for lymph node metastasis in pT1 or pT2 well-differentiated colorectal cancer. Hase [21] reported that the tumor budding was associated with the prognosis of colorectal cancer.

Some statistical studies demonstrated that budding was an important factor related to other malignant parameters in colorectal cancer [7,21,23]. Our data was very similar to their results. For this reason, budding might be an index of the malignant potential of colorectal cancer. However, one must ask why the isolated undifferentiated cells of Type III budding are so malignant. According to the studies of Makino [27] and Guzinska-Ustymowicz [34], tumor budding was significantly associated with the p53 or Bcl-2 protein. The biologic behavior of the budding cells seems to be extremely malignant. As for the mechanism of the invasion into lymphatic vessels, tiny cancer cells first need to separate from the parental tumor cell, and then infiltrate into the lymphatic vessels through the epithelium. Ruoslahti [43] also described that cancer cells must become detached from the tumor mass. Gabbert [44] reported that the first and essential step in tumor invasion is tumor dedifferentiation and dissociation at the invasive front in colonic carcinoma of the rat. He also described that the interstitial edema caused the stromal space to open widely and allowed tumor cells to mobilize out of the main tumor. These dissociated tumor cells may be absorbed into the lymphatic vessels together with stromal liquid. In any case, cancer cells need to be very tiny in order to enter the lymphatic vessels. Type III budding cells can easily enter the lymphatic vessels because they are extremely tiny undifferentiated cells without glandular differentiation.

Moreover, cell-cell or cell-matrix adhesion is probably reduced in budding cells, as reported by Frenette [45]. Other authors [25,26,28-32] also reported abnormality of the adhesion molecules in budding cells. On the other hand, if the cancer cells form tubules, they cannot easily enter into the lymphatic vessels because the cell-cell adhesion is extremely strong.

Jass [19] pointed out that budding was a dynamic process under genetic control and not merely the result of architectural disruption caused by a host immune reaction at the tumor margin. Type I budding seems to be initiated by some kinds of abnormal genetic activity. In Types II and III the budding seems to have progressed and acquired the ability to easily invade the extra-cellular matrix. Budding is also related to histologic grade. The cell-cell adhesion is very strong in most cases of well-differentiated adenocarcinoma, so the budding may be slight. When the tumor cells show a tendency toward poorly differentiated adenocarcinoma, detection of budding increases remarkably in most cases through the appearance of many tiny, isolated undifferentiated cells. Sordat [25] also reported results similar to ours.

As for the depth of invasion, budding rarely appears in cases of slight submucosal invasion, because cancer cells are just beginning to invade the submucosal layer. The deeper the invasion of cancer cells into the submucosal layer, the more budding appears at the active invasive front.

As I have described above, tumor budding has been studied from a number of various viewpoints. Budding was found useful for evaluating malignant potential and tumor prognosis. However, further studies are required to clarify the clinical or biological significance of this budding from a variety of viewpoints.

We concluded that tumor budding was one of the most important risk factors for lymph node metastasis and that it had a close relationship with lymphatic permeation. The budding cells were considered to easily enter lymphatic vessels and to display extremely malignant behavior.

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Chapter VII

AGGRESSIVE AND NON-AGGRESSIVE TUMOR BUDDING IN COLORECTAL CANCERS

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Introduction

Colorectal cancer is one of the most common types of cancer in Western countries including Poland. The mortality of colorectal cancer is ranked as the second in Western countries, and the third in Poland, amongst all cancers. Recent studies concerning the prognostic factors in colorectal cancer have paid attention to tumor budding as a potential prognostic factor [1-7]. Morodomi et al. defined tumor budding as either bundles of five or more cancer cells occurring in a well-differentiated region (mainly the actively invasive area), showing tubular structures, which were classified as microtubular cancer nests, or isolated cancer cells without a distinct structure, which were classified as undifferentiated cells. Since both of these findings appeared to be due to budding from the main mass of the tumor, they called them "budding" [1]. Several published studies have indicated that tumor budding is associated with metastasis in colorectal cancers [7-9]. These results strongly suggest that tumor budding could play a crucial role as a prognostic factor in colorectal cancers. The previous work of Masaki et al. [11], in which the budding process was examined in relation to other factors such as alterations in adhesion molecules or proteolytic enzymes, is worth consideration. They observed that up-regulation of CD44 variant 6 through nuclear β-catenin activation may contribute to the formation of tumor budding, and immunostaining of these two adhesion molecules may be useful in identifying those at a high-risk for locoregional failure in patients with T1 colorectal carcinomas [10]. The same authors reported that tumor

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budding at the invasive margin and matrilysin expression are more useful in identifying the high-risk group for adverse outcome in patients with T1 colorectal carcinomas [11].

Unfortunately, no attention has been paid to the association between tumor budding and p53 or c-erbB-2/HER-2/neu protein expression in buds. Both the tumor suppressor gene p53 product and the proto-oncogene c-erbB-2 have been found to be expressed in a number of human adenocarcinomas. These proteins are encoded by two genes located in the same chromosome.

p53 is a 53-kDa nuclear phosphoprotein, which is encoded by a tumor suppressor gene located on the short arm of chromosome 17. Vogelstein and Kinzler [12] suggested that p53 is a cell cycle protein and plays a regulatory role in the control of cell proliferation. p53 is inactivated by mutation, deletion, or binding to other proteins. Such inactivation eliminates its growth suppressive function and can result in overexpression of the protein [13,14]. Amongst others, mutation of p53 is the commonest change and may significantly influence tumor progression [15,16]. It has been shown that p53 could be an indicator of rapid proliferation, low differentiation, advanced stage or poor prognosis [16]. However, the importance of p53 as a prognostic indicator is controversial. Some authors reported that p53 expression in colorectal cancers is an indicator of shortened patient survival [17,18], while others reported that it has limited value in predicting clinical outcome [19,20]. The value of p53 in predicting lymph node metastasis has also been questioned [21].

The c-erbB-2 gene was first isolated due to its homology with v-erbB and human epidermal growth factor receptor probes [22]. This gene was mapped on chromosome 17 at q21 [23]. The c-erbB-2 gene product is a 185,000 molecular weight transmembrane protein with characteristics of a tyrosine kinase growth factor receptor [24,25]. This protein is involved in mediating a number of normal cellular processes including proliferation [26]. Thus far, previous studies on the prognostic utility of c-erbB-2 protein expression in human cancers have obtained conflicting results. Recent studies have shown that the c-erbB-2 proto-oncogene is amplified in 25-33% of human mammary carcinomas, and there is a significant association between c-erbB-2 amplification and lymph node metastasis and prognosis [27]. c-erbB2 amplification and/or expression are also associated with poor prognosis in a number of tumors, such as gastric [28,29] and ovarian cancers [30]. The correlations of p53 and c-erbB-2 protein expression with nodal status of breast, gastric and colorectal cancers have been reported in several studies [31-33]. However, McKay et al. showed that c-erbB-2 protein expression was not associated with lymph node metastasis in colorectal cancers [34]. These conflicting results encouraged me to investigate their expression in tumor buds with reference to lymph node metastasis and vascular invasion in colorectal cancers.

Patients and Methods

Fifty-five patients with colorectal cancer treated by surgery from 2000 to 2003 at the Department of Surgery, The J. Śniadecki Memorial Hospital (Białystok, Poland) were studied. The median age was 69 years with a range of 43 to 89 years. Twenty-one patients were men and 34 were women. Tissue specimens were collected immediately after tumor removal, fixed in 10% buffered formaldehyde solution and embedded in paraffin. Hematoxylin-eosin-stained sections were examined according to the TNM classification.

The present study paid special attention to the following histologic features at the invasive front:

1. Vascular invasion - lymphatic and venous invasion were examined, and assessed together as vascular invasion.
2. Tumor budding according to the criteria of Morodomi et al. [1]
3. Lymphocytic infiltration according to the criteria of Jass JR et al. [35]

Only cases with pT3 stage and G2 grade of histological differentiation were included in this study.

Immunohistochemical Study

Slides of 4-μm-thick serial sections of the primary tumor and regional lymph nodes were prepared from each patient. A standard avidin-biotin immunoperoxidase method (Novostain Super ABC Kit Universal) was used for the detection of c-erbB-2 expression. Immunostaining of p53 protein was performed using the labeled streptavidin biotin (LSAB) method protocol described by DAKO (DAKO, LSAB Kit, Dako, Poland). In brief, the slides were dewaxed using xylene and transferred to alcohol. They were then placed in citric acid buffer (10 mM) and heated in a microwave oven (700W) for 15 minutes to expose antigens. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 minutes. The slides were then washed three times in phosphate-buffered saline (PBS) and incubated in 10% normal horse serum for 15 minutes to reduce nonspecific antibody binding. After washing with PBS, the slides were incubated overnight at 4°C with primary monoclonal antibodies. Anti-human c-erbB-2 protein monoclonal antibody (Novocastra/c-erbB-2, No NCL-c-erbB-2-316, dilution 1:50, Biokom, Poland) was used for one slide, and mouse anti-human p53 monoclonal antibody (clone DO-7; M7001; dilution 1:100; Dako, Poland) was used for another slide. Nonspecific mouse IgG was used as negative control. The reaction products were visualized with diaminobenzidine DAB (DAKO S3000, Dako, Poland). Appropriate positive and negative controls were used. Nuclear immunostaining was observed for p53, and cytoplasmic immunostaining for c-erbB-2. p53 and c-erbB-2 expression were semi-quantitatively assessed in neoplastic cells of the primary tumor and lymph node metastases, and defined as follows: p53 and c-erbB-2-negative (lack of staining, or staining reaction present in less then 10% of tumor cells), and p53 and c-erbB-2-positive (staining reaction present in more then 10% of tumor cells). The percentages of p53 and c-erbB-2-positive cells were calculated in at least 500 neoplastic cells per each sample using a light microscope (x400).

Method of Determining Budding (Microtubular Cancer Clusters or Undifferentiated Cancer Cells)

Budding was evaluated according to the criteria of Morodomi et al. [1]. The invasive front was observed in a 500 μm x 2500 μm square visual fields at four locations in each slide,

and tumor budding was classified as negative when no bud was observed, and positive when at least one bud was observed. Similar conditions were used for evaluating p53 and e-crbB-2 protein expression in tumor budding; i.e. negative when no reaction was observed and positive when protein expression was present in the buds.

Statistical Analysis

Fisher's exact test was used for statistical analysis, and p-values less than 0.05 were considered statistically significant.

Results

Tumor Budding, Clinicopathological Parameters, and p53 and c-erbB-2 (HER-2/neu) Protein Expression (Table 1)

Statistical analysis revealed no significant correlation between tumor budding and sex, tumor location and lymphocytic infiltration. However, significant correlations were found between tumor budding and vascular invasion (p=0.0005) and lymph node involvement (p=0.002). Immunostaining of p53 showed apparent nuclear localization (Figure 1), and c-erbB-2-positive reaction was observed mostly in the cytoplasm of cancer cells in both the main mass of the tumor and the buds (Figure 2). p53 and c-erbB-2 protein expression in the main mass of the tumor were associated with tumor budding (p=0.04, p=0.0009, respectively). Expression of these proteins in the metastatic lymph nodes was significantly correlated with tumor budding (p=0.005, p=0.002, respectively).

Table 1. Tumor Budding, Clinicopathologic Parameters and Immunostaining

Variable	TB(-)	TB(+)	Significance
Sex			p>0.05
Male	6 (28.57%)	15 (71.43%)	
Female	6 (17.65%)	28 (82.35%)	
Tumor location			p>0.05
Colon	5 (15.63%)	27 (84.38%)	
Rectum	7 (30.43%)	16 (69.57%)	
Vascular invasion			p=0.0005
Absent	11 (44.00%)	14 (56.00%)	
Present	1 (3.33%)	29 (96.67%)	
Lymphocytic infiltration			p>0.05
Absent	9 (26.47%)	25 (73.53%)	
Present	3 (14.28%)	18 (85.72%)	
Lymph node metastasis			p=0.002
N0	11 (39.29%)	17 (60.71%)	
N1+2+3	1 (3.70%)	26 (96.30%)	

Table 1. Tumor Budding, Clinicopathologic Parameters and Immunostaining (cont.)

Variable	TB(-)	TB(+)	Significance
p53[1] exp. (tumor)			p=0.04
Negative	8 (38.10%)	13 (61.90%)	
Positive	4 (11.76%)	30 (88.24%)	
p53[2] exp. (l.n.m.)			p=0.005
Negative	12 (32.43%)	25 (67.57%)	
Positive	0 (0%)	18 (100%)	
HER-2[1] exp. (tumor)			p=0.0009
Negative	9 (50.0%)	9 (50.0%)	
Positive	3 (8.11%)	34 (91.89%)	
HER-2[2] exp. (l.n.m.)			p=0.002
Negative	11 (37.93%)	18 (62.07%)	
Positive	1 (3.85%)	25 (96.15%)	

N 1+ 2 +3 = total number of tumors with lymph node metastasis.
[1] c-erbB-2/HER-2, p53 exp. (tumor) = c-erbB-2/HER-2, p53 expression in primary tumor.
[2] c-erbB-2/HER-2, p53 exp. (l.n.m.) = c-erbB-2/HER-2, p53 expression in lymph node metastases.

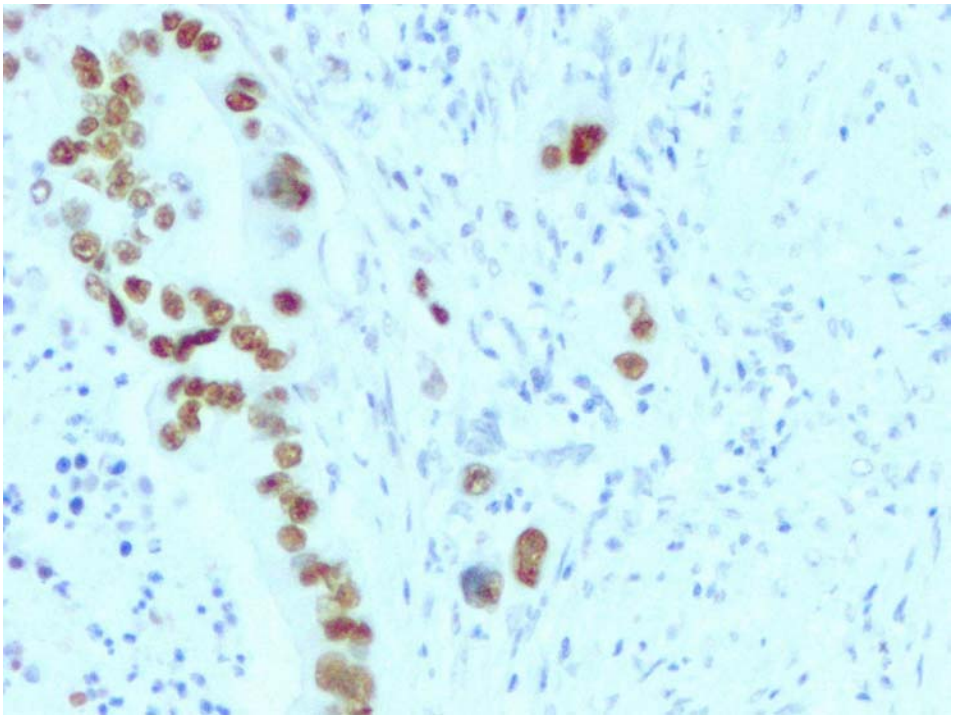

Figure 1. Immunostaining of p53 shows intense nuclear staining in the main tumor mass and buds (x100).

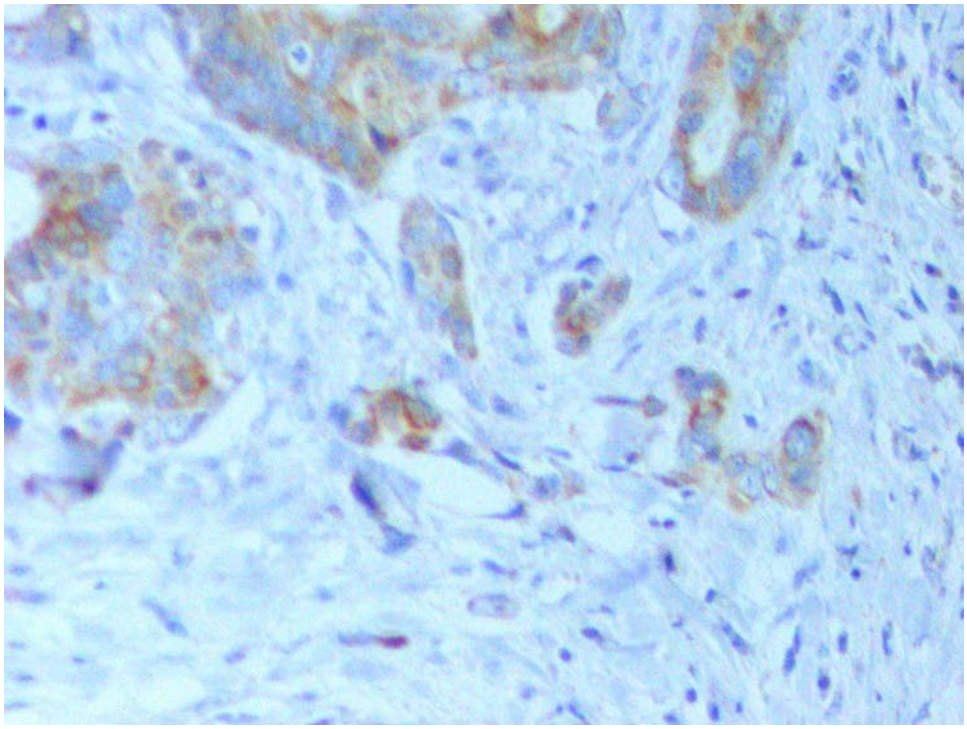

Figure 2. Immunostaining of c-erbB-2 shows positive cytoplasmic staining in the main tumor mass and buds (x100).

p53 and c-erbB-2 (HER-2/neu) Protein Expression and Clinicopathologic Parameters (Table 2)

As shown in Table 2, p53 and c-erbB-2 protein expression in the main mass of the tumor were significantly associated with vascular invasion and lymph node metastasis.

Table 2. p53 and c-erbB-2/HER-2 Protein Expression in Main Tumor Mass and Clinicopathologic Parameters

Variables	p53 negative	p53 positive	Significance	HER-2 negative	HER-2 positive	Significance
Lymph node metastasis						
N0	17 (60.71%)	11 (39.29%)	p=0.0007	18 (64.29%)	10 (35.71%)	p=0.00001
N1+2+3	4 (14.81%)	23 (85.19%)		0 (0%)	27 (100%)	
Vascular invasion						
Absent	14 (56%)	11 (44%)	p=0.02	15 (60%)	10 (40%)	p=0.0001
Present	7 (23.33%)	23 (76.67%)		3 (10%)	27 (90%)	

N 1+ 2 +3 = total number of tumors with lymph node metastasis.

p53 and c-erbB-2 (HER-2/neu) Protein Expression in Tumor Buds and Clinicopathologic Parameters (Table 3)

Statistically significant correlations were noted between p53 protein expression in the buds and lymph node metastasis (p=0.00634) and vascular invasion (p=0.00415). It was also noted that expression of p53 protein in the buds was correlated with p53 expression in the primary tumor (p=0.000001) and lymph node metastasis (p=0.00001). Expression of p53 protein in the buds was not correlated with the expression of c-erbB-2 in lymph node metastases, but was significantly associated with the expression of c-erbB-2 in the main tumor mass (p=0.01237).

Expression of c-erbB-2 in the buds was significantly correlated with the presence of lymph node metastasis (p=0.00011) and vascular invasion (p=0.01115). Statistically significant correlation was found between expression of c-erbB-2 in the buds and expression of p53 in lymph node metastases (p=0.00270), and expression of c-erbB-2 in both the main tumor mass (p=0.000001) and lymph node metastases (p=0.000001). However, there was no correlation between expression of c-erbB-2 in the buds and p53 expression in the main tumor mass (p>0.05).

Table 3. p53 and c-erbB-2/HER-2 Protein Expression in Tumor Buds and Clinicopathologic Parameters

Variables	p53(-)*	p53(+)†	Significance	HER-2(-)*	**HER-2(+)	Significance
Lymph node						
N0	11	6 (35.29%)	p=0.00634	11	6 (35.29%)	p=0.00011
N1+2+3	6 (23.08%)	20		2 (7.69%)	24 (92.31%)	
Vascular invasion						
Absent	10	4 (28.57%)	p=0.00415	8 (57.14%)	6 (42.86%)	p=0.01115
Present	7 (24.14%)	22		5 (17.24%)	24 (82.76%)	
p53[1] exp. (tumor)						
Negative	13 (100%)	0 (0%)	p=0.000001	6 (46.15%)	7 (53.85%)	p>0.05
Positive	4 (13.33%)	26		7 (23.33%)	23 (76.67%)	
p53[2] exp. (l.n.m.)						
Negative	17 (68.0%)	8 (32.0%)	p=0.00001	12 (48.0%)	13 (52.0%)	p=0.00270
Positive	0 (0%)	18 (100%)		1 (5.55%)	17 (94.45%)	
HER-2[1] exp. (tumor)						
Negative	7 (71.43%)	2 (28.57%)	p=0.01237	9 (100%)	0 (0%)	p=0.000001
Positive	10	24		4 (11.76%)	30 (88.24%)	
HER-2[2] exp. (l.n.m.)						
Negative	10	8 (44.45%)	p=0.06827	13	5 (27.78%)	p=0.000001
Positive	7 (28.0%)	18 (72.0%)		0 (0%)	25 (100%)	

N 1+ 2 +3 = total number of tumors with lymph node metastasis.
* p53, c-erbB-2/HER-2 (-) = negative p53, HER-2 expression in tumor buds.
† p53, c-erbB-2/HER-2 (+) = positive p53, HER-2 expression in tumor buds.
[1] c-erbB-2/HER-2, p53 exp. (tumor) = c-erbB-2/HER-2, p53 expression in primary tumor.
[2] c-erbB-2/HER-2, p53 exp. (l.n.m.) = c-erbB-2/HER-2, p53 expression in lymph node metastases.

Discussion

Tumor budding refers to tumor cells dissociating from the main tumor. In the literature, Hase et al. [2] first used the term “tumor budding”. However, the same phenomenon was originally described by Imai [36], who reported that tumors with “sprouting” tumor cells had higher malignant potential. The significance of tumor budding as a prognostic indicator has been reported by many authors [1-3,6]. Tumor budding is associated with lymph node metastasis and a poor prognosis in patients with colorectal cancer [1,2,7,9,37]. According to these authors, if the degree of differentiation of colorectal adenocarcinoma is moderate (G2), lymph node involvement is highly probable. However, if tumor budding is absent, lymph node metastasis is less likely, even in moderately differentiated tumors. In well differentiated adenocarcinomas (G1), tumor budding and lymphatic invasion are usually not observed; however, if tumor budding is observed in these cases, lymph node metastasis is more likely. Using a dimethylhydrazine-induced murine colonic carcinoma model, Gabbert showed that the first and essential step in tumor invasion is tumor dedifferentiation and tumor cell dissociation at the invasive front [38]. This finding in experimental colon cancers corresponds well to the budding in human colorectal cancers. Ueno et al. suggested that tumor budding may be a good index to estimate the aggressiveness of rectal cancers [6]. They divided 638 rectal cancers into two groups by the degree of tumor budding: rectal cancer with high-grade ‘budding’ (≥10 foci per x250 field), and low grade ‘budding’ (<10 foci per field). They found a statistically significant correlation between the degree of tumor budding and the number of involved nodes.

Of interest in their study, the number of patients with lymph node involvement was nearly the same in tumors with a high degree of tumor budding and those with a low degree (131/383 vs. 122/253). We also showed in the present study that lymph node metastasis was not seen in 17 out of 43 cases with positive tumor budding. These findings suggest that, although the degree of budding at the invasive front may be associated with tumor aggressiveness, other factors should be investigated to determine the high risk group for lymph node metastasis more precisely.

Makino et al. investigated p53 protein expression and tumor budding in early invasive colorectal cancers, and found that lymph node metastasis and tumor budding were more frequent in tumors with p53 protein expression than without [4]. The current study also showed that expression of p53 protein in the primary tumor was significantly correlated with the presence of tumor budding and lymph node metastasis. However, Yamaguchi et al. could not find any significant correlation between p53 protein expression and lymphatic invasion, venous invasion or lymph node metastasis [17]. Other researchers also argued that the association between p53 protein expression and metastasis in colorectal cancers is still controversial [39-44].

It is known that colorectal carcinoma is a heterogeneous neoplasm made up of different cell clones with diverse growth rates and metastatic potential [45]. This is one of the reasons for the diverse biological aggressiveness among tumors classified as the same pathologic or clinical stage. The current study suggests that the presence of tumor budding at the invasive front may give additional information about the biological behavior of colorectal cancers with the same stage.

Until now, the behavior of colorectal cancers has been believed to be dependent on multiple genetic events throughout the genome. Although mutations or other changes in the p53 gene are the most frequent genetic alterations found in human malignancies, the role of c-erbB-2 protein in colorectal carcinogenesis is controversial. Several studies suggested that patients with tumors negative or weakly positive for c-erbB-2 protein had significantly longer survival than patients with moderately or strongly positive tumors [27,28,46-50]. Saeki et al. showed that c-erbB-2 protein expression may be one of the most reliable indicators of metastasis and prognosis in human colon cancers [50]. However, contradictory results, showing no association between c-erbB-2 protein expression and prognosis, were reported by others [51,52].

Unfortunately, none of the previous studies examined the association between c-erbB-2 protein expression in the main tumor mass and tumor budding at the invasive front in colorectal cancers. According to my observations, c-erbB-2 protein expression in the primary tumor was significantly correlated with the presence of tumor budding and lymph node metastasis.

The present results revealed that the correlations between c-erbB-2 or p53 protein expression in the primary tumor and lymph node metastasis or vascular invasion were weaker than those between the expression of these proteins in the buds and lymph node metastasis or vascular invasion. Expression of p53 and c-erbB-2 protein in lymph node metastases was detected only when the buds were positive for these proteins. Simultaneously, p53 and c-erbB-2 proteins were found in the buds only when they were detected in the main tumor mass. These findings suggest that budding tumor cells may play a role as carriers of the investigated proteins to lymph node metastases. However, among 43 cases with tumor budding, only 26 cases had lymph node metastasis, and the remaining 17 cases did not have lymph node metastasis, suggesting that there are different kinds of buds oncologically. As shown in Table 3, among 30 cases with c-erb B-2 expression in the buds, 24 cases had lymph node metastasis, while among 13 cases without c-erb B-2 expression in the buds, 11 cases had no lymph node metastasis. Furthermore, among 26 cases with p53 expression in the buds, 20 cases had lymph node metastasis, while among 17 cases without p53 expression in the buds, 11 cases had no lymph node metastasis. Taking these results together, if the buds show p53 and/or c-erbB-2 protein expression, lymph node metastasis is highly probable, but if not, lymph node metastasis is not likely. This is the reason I classified the buds into aggressive buds associated with lymph node involvement and non-aggressive buds without. The possible diversity of buds may explain differences in the prognostic significance of tumor budding in colorectal cancers.

Because there was no previous report focusing on p53 and c-erbB-2 protein expression in the buds, it was not possible to compare my results with others. However, the present results suggest the usefulness of tumor budding and evaluation of p53 and c-erbB-2 protein expression in the buds in predicting lymph node metastasis in colorectal cancers.

In conclusion, two types of buds - aggressive and non-aggressive - occur at the invasive front.

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Chapter VIII

COHORT MIGRATION AND IQGAP1

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Introduction

One of the reasons that carcinoma is thought to be a malignant and mortal disease is that carcinoma cells metastasize. In addition to the treatments that minimize the growth of primary tumors, those that limit their spread to other sites have been long waited for being established. In the metastatic cascade, cell migration plays an important role because it appears repeatedly in the cascade. Understanding this process hopefully leads to a new therapeutic breakthrough.

Microscopial observation of tissues is a very basic way of research. However, it often gives us a very important clue(s) that leads to a disease mechanism or a prognostic factor because it shows us what really occurs in a patient. Concepts of cohort migration and tumor budding are both obtained through the histopathologic observation. Starting from the observation, molecular mechanisms have been explored until now. In this chapter, we will discuss about the types of cell migration, in special reference to cohort type migration and IQGAP1, a molecule involved in regulation of cell-cell adhesion and extension of leading edges during epithelial cell movement.

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Cohort Migration

In pathologic sections, carcinoma cells, which are malignant counterparts of epithelial cells, proliferate and invade as coherent cell nests, maintaining cell-cell contact with one another, and this mode of growth is also seen even at the invasion front. These findings let us assume that there might be a way by which carcinoma cells move together as coherent cell clusters, and we have called this type of movement "cohort migration." It has been actually shown in vitro that carcinoma cells can move *en mass* keeping cell-cell contact each other, including our models [1, 2]. Now it is considered that there are two types of tumor cell migration: single-cell migration and cohort migration [3, 4]. Single-cell migration is observed as a scattered type migration during invasion by the hematopoietic system neoplasm, such as lymphoma and leukemia, as well as sarcoma and poorly differentiated carcinoma. On the contrary, cohort migration, also called collective migration [4], is frequently observed during invasion by well to moderately differentiated carcinoma cells. Although the basic mechanisms involved in cell migration, which have predominantly been investigated using *in vitro* single cell migration models [5], are common between the two types of migration, there are two additional characteristics that are very unique to cohort type migration. One is compartmentalized release from cell-cell adhesion: cells extend leading lamellae to move via this release whilst keeping cell-cell contact in other portions [6, 7]. The other is the regulation of expression and localization of matrix metalloproteinases (MMPs) via cell-cell contact within migrating cell sheets. In our model, membrane type (MT)1-MMP and gelatinase A (Gel A, MMP-2) are expressed and localized specifically at the front pathfinder cells of the migrating cell sheets, and reorganization of gelatin matrix by these MMPs is essential for this type of migration [8]. We will discuss these two characteristics more in detail later.

Cohort Migration and Tumor Budding

A concept of cohort migration is not new and has been referred by pathologists. Imai subdivided the histological growth pattern of gastric and uterine cervical carcinoma into nonsprouting, sprouting and lymphatic-permeating types [9, 10]. The nonsprouting type was defined as carcinoma cells which spread by forming large cell nests or glandular structures and the sprouting type as carcinoma cells which propagate as more or less anaplastic thin cell cords or as individual cells. Leighton et al. also described occurrence of increasing numbers of aggregates of carcinoma cells as the tumor grew and involved larger volumes of tissue [11]. They speculated that aggregates of cells function as integrated units in the propagation of similar groups, and also referred to the possibility that there were migrations of multicellular portions of aggregates. To examine how often cohort type migration occurs in vivo, we classified the invasion front (IF) of colon carcinoma into three types on histopathology sections: (i) IF consisting of compact tumor glands (type Ia cohort migration); (ii) IF consisting of partially resolved tumor glands (type Ib cohort migration); and (iii) IF consisting of more markedly resolved tumor glands with small clusters of carcinoma cells lying ahead (type II cohort migration) [3]. The former two types are associated predominantly with an expanding pattern of growth [12], whereas type II cohort migration shows the features called "tumor budding" and is often associated with worse prognosis [13]. A half of our cases (total

74 cases) showed type I cohort migration and the others type II. Type Ia invasion front was observed in carcinomas which were confined to the mucosa or submucosa, whereas type Ib and II were observed in carcinomas showing further invasion. Single cell invasion was noted only in the cases with type II cohort migration, especially in the cases which extended into the subserosa. Thus, in cases with tumor budding, both cohort migration and single cell migration may be involved in their invasion, while cohort migration seems to be the predominant mechanism in cases with compact or partially resolved tumor glands. Therefore, cohort migration can be involved in more than half of the cases in colon carcinomas.

Tumor budding (type II cohort migration) is different from type I cohort migration in the extent of cell dissociation and the size of invading carcinoma cell nests, and is a useful marker in predicting lymph node metastasis, hematogenous metastasis or locoregional failure in colorectal carcinomas [13-16]. The micropapillary pattern, which has been reported in breast, ovary, urinary bladder and lung carcinomas in association with frequent lymph node metastasis and worse prognosis, may be similar to tumor budding in colorectal carcinomas or at least an extreme case of tumor budding since it is defined as a component of neoplasm exhibiting clusters of crowded, discrete small groups of neoplastic cells arranged in solid nests or in tubules within clear empty spaces in breast carcinomas [17-21].

In vitro Cohort Migration Model

To elucidate the mechanisms involved in cohort type migration we developed a two-dimensional simple Lab-Tek chamber motility assay, which meets the requirements for morphological observation and biochemical and molecular analysis [1, 6, 7]. In this assay, a highly metastatic variant L-10 of the human colon adenocarcinoma cell line RCM-1 [22] formed interlinked and piled-up cell islands on the tissue culture glass substrate of the Lab-Tek chamber slide. In response to a naturally occurring motogenic factor, hepatocyte growth factor/scatter factor (HGF/SF), the L-10 cells and several other human colon carcinoma cells migrated as localized coherent cell sheets at intervals along the margin of the cell islands [23]. (Figure 1A) The cells at the edges of the migrating cell sheets showed motile cell features such as fan-shaped leading lamellae, while the following cells had cell contact with one another. Involvement of passive cell movement due to cell growth was examined, but there was no contribution of cell proliferation to this L-10 cell translocation. Ultrastructural study of the migrating-cell sheets clearly demonstrated that the cells were in a state of active locomotion whilst maintaining cell-cell contact. This was performed via localized modulation of cell-cell adhesion such as the occurrence of wide intercellular gaps in the lower portion of the cells to allow the cells to extend tapering cytoplasmic processes and leading lamellae forward while cell-cell contacts remained close in the upper portion of the cells with desmosomes [6, 23]. (Figure 1B) Additionally, since our RCM-1 cells also invaded the gel as organized clusters with tubular structure when cultured on reconstituted collagen gels [24], the manner of locomotion in the two-dimensional model described above appears to reflect the locomotion in a three-dimensional system.

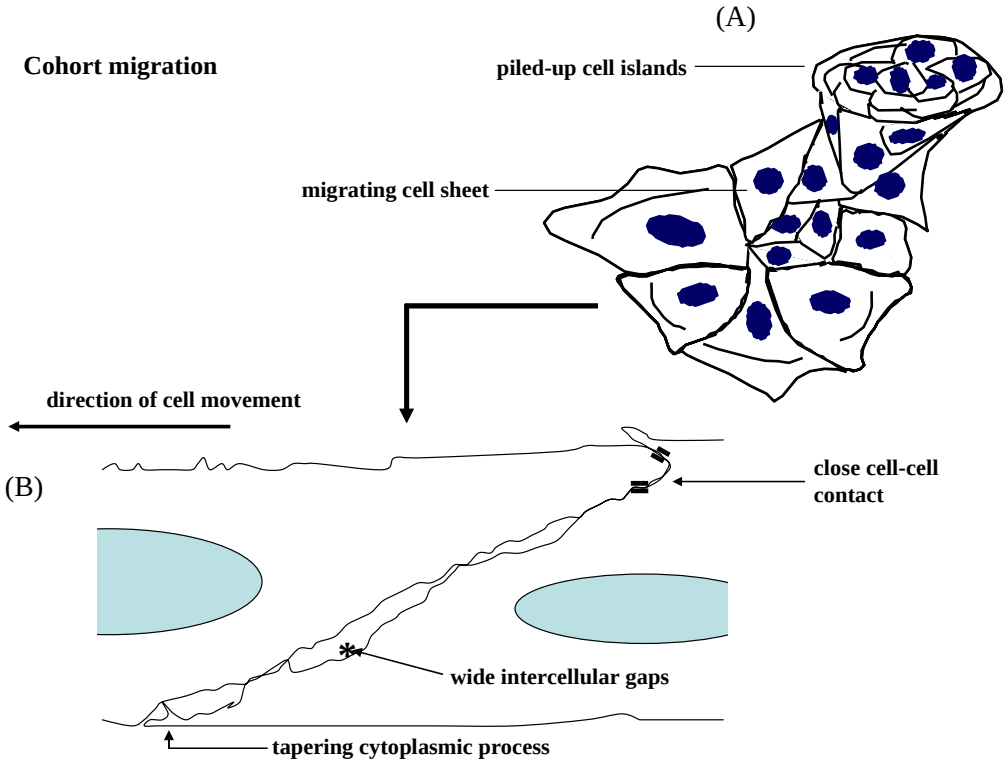

Figure 1. Schema of in vitro cohort migration model. (A) Two dimensional schema of migrating cell sheets. (B) A vertical view of migrating cells.

Mechanisms Involved in the Localized Release from the Cell-cell Adhesion during Cohort Migration

The E-cadherin/catenin System

In a cohort migration model, as mentioned above, localized disruption of cell-cell adhesion is most characteristic: migrating cells maintain close cell-cell adhesion with one another in the upper portion of cells with tight junctions and desmosomes, whereas wide-open intercellular spaces are formed in the lower portions, which enables cells to extend leading lamella forward to move. The cell-cell adhesion of epithelial cells is mediated predominantly by a transmembrane glycoprotein, E-cadherin and a set of cytoplasmic cadherin-associated molecules collectively called the catenins [including α-, β-, γ- (plakoglobin) and p120cas catenins [25, 26]. Catenins form a complex with E-cadherin and link it to the actin cytoskeleton, which is essential for E-cadherin to express its full adhesive function. This cadherin/catenin complex-based cell-cell adhesion, which corresponds to the adherens junction structurally, is possibly regulated via genetic and epigenetic alterations. Although mutations and deletions in E-cadherin, α- and β-catenin genes have been reported [27-30], in

the gastrointestinal tract those abnormalities are confined predominantly to poorly differentiated adenocarcinomas, most of which show scattered histological phenotypes. In contrast, only few mutations of the E-cadherin gene have been found in well to moderately differentiated adenocarcinomas [28], which show characteristic epithelial morphology both in vivo and in vitro. In these better differentiated cases, temporal and spatial regulation of the cadherin/catenin complex seems to be more important for their movement [26, 31]. β-catenin is a regulatory molecule involved in both cell-cell adhesion and signal transduction. As a signal transducer molecule, it translocates to the nucleus to activate transcription, and abnormal nuclear accumulation of β-catenin has been reported in carcinomas [32, 33]. As a cell adhesion regulatory molecule, β-catenin suppresses cadherin-mediated cell-cell adhesion via its tyrosine phosphorylation [26, 31]. When cohort migration was induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment, a localized release from cell-cell adhesion at the lower portion of cells was associated with increased tyrosine phosphorylation of the E-cadherin/catenin complex (Ecc), including β-catenin [7]. In HGF/SF-induced cohort migration, however, the tyrosine phosphorylation level was not altered, and instead the level of α-catenin that formed complex with E-cadherin was reduced [23]. Since α-catenin is a key molecule that links the E-cadherin/β-catenin complex to the actin cytoskeleton, we looked for the mechanism responsible for this dissociation of α-catenin from the complex.

IQGAP1

IQGAP1, a recently identified target molecule of Cdc42 and Rac 1 small GTPases, has been shown to negatively regulate the Ecc-based cell-cell adhesion by dissociating α-catenin from Ecc [34-39] (Figure 2). The binding site on β-catenin for IQGAP1 overlaps with that for α-catenin; thus, IQGAP1 dissociates α-catenin from the β-catenin/α-catenin complex in a dose-dependent manner *in vitro*. Consistent with this, overexpression of IQGAP1 in mouse L fibroblasts stably expressing E-cadherin has resulted in a reduction of Ecc-based cell-cell adhesion associated with dissociation of α-catenin from Ecc. In contrast, inhibiting interactions between IQGAP1 and β-catenin increases interactions between β-catenin and α-catenin, which in tern results in the strong association of the cadherin/catenin complex with the cytoskeleton and promotes adhesive interactions between cells. 38, 39].

IQGAP1 is also an actin binding protein [40, 41], and overexpression of IQGAP1 in mammalian cells enhances cell migration in a Cdc42- and Rac1-dependent manner [42]. Moreover, cell motility is significantly decreased both by knock down of endogenous IQGAP1 using small interfering RNA and by transfection of a dominant negative IQGAP1 construct. Invasion mediated by constitutively active Cdc42 has been attenuated by the dominant negative IQGAP1 construct.

Rho family GTPases, [together with phosphatidylinositol 3-kinase (PI3K)], play a central role in establishing a polarized migratory cell morphology in fibroblasts [43]. In polarized motile cells, Cdc42 and Rac1 localize to the leading edge of the cells and are actually activated there [44]. Rac1 activity is the highest immediately behind the leading edge, whereas Cdc42 activity is the highest at the tip of the leading edge [45]. Rho GTPases also capture and stabilize microtubules (MT) through IQGAP1 near the cell cortex, leading to polarized cell morphology and directional cell migration. When the cells are polarized, the

plus ends of MTs are targeted, captured and stabilized near the leading edge, where MTs play critical roles in directed transport of specific proteins and vesicles for cell polarization. IQGAP1 directly interacts with CLIP-170 (a cytoplasmic linker protein), and Rac1 and Cdc42 form a tripartite complex with IQGAP1/CLIP-170 [46]. It is proposed that activated Rac1 and Cdc42 could provide docking sites for MT plus ends near the cortex through IQGAP1 and CLIP-170 and could reinforce cell polarization by establishing a polarized MT arrays.

IQGAP1 in Cohort Migration

Using our cohort migration model, we have demonstrated possible association of IQGAP1 with localized disruption of cell-cell adhesion that is essential for cohort migration of carcinoma cells [47]. In our study, the amount of IQGAP1 bound to Ecc increased in migrating cells in association with a decrease in the α-catenin level in Ecc. In accordance with this, IQGAP1 showed a shift from the cytosol to the membrane fraction. Moreover, immunofluorescent study with a confocal laser microscope demonstrated the localization of IQGAP1 at the membranes of the lower portion of migrating cells, the site of compartmentalized disruption of cell-cell adhesion during cohort migration. IQGAP1 has been shown to negatively regulate the Ecc-based cell-cell adhesion by dissociating α-catenin, a key molecule that links the E-cadherin/β-catenin complex to actin cytoskeleton, from Ecc as described above [36, 37]. Taken together, these lines of evidence indicate close association of an IQGAP1-related regulation mechanism with compartmentalized modulation of cell-cell adhesion during cohort type migration. (Figure 3) Correlation between the levels of IQGAP1 bound to Ecc and the extents of migration that was modulated by enhancing or blocking cell-ECM interactions also supported the above association [47]. Furthermore, transfection of dominant active mutant of Rac1, which inhibits IQGAP1-induced cell dissociation, into carcinoma cells suppressed HGF/SF-induced cohort migration, while transfection of dominant negative mutant of Rac1, which stimulates IQGAP1-mediated cell dissociation, enhanced the cohort migration (K. Nabeshima, et al., unpublished results). These findings also support involvement of IQGAP1-mediated mechanisms in cohort migration.

IQGAP1-mediated regulation of cell-cell adhesion was initially demonstrated in mouse L fibroblasts stably expressing human E-cadherin by means of overexpressing IQGAP1 [36, 37]. However, the physiological situations in which this IQGAP1 regulatory system operates, especially in humans, have yet to be clarified [37]. Our work suggests its possible involvement in human carcinoma cell movement.

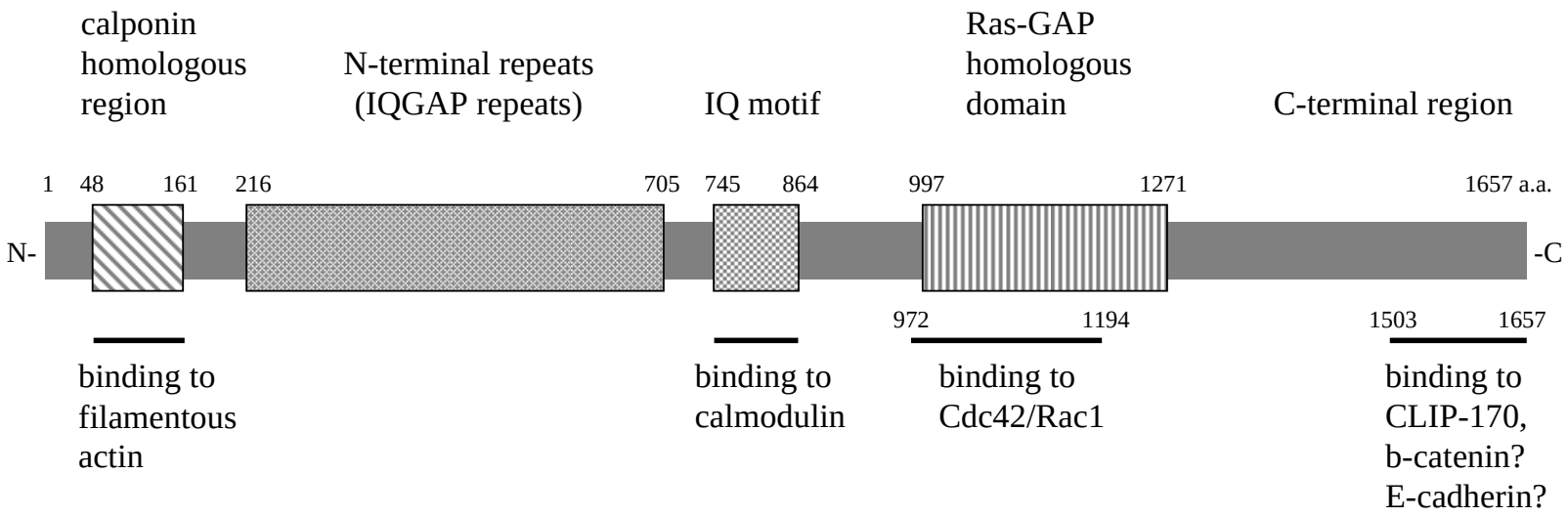

Figure 2. IQGAP1 molecule.

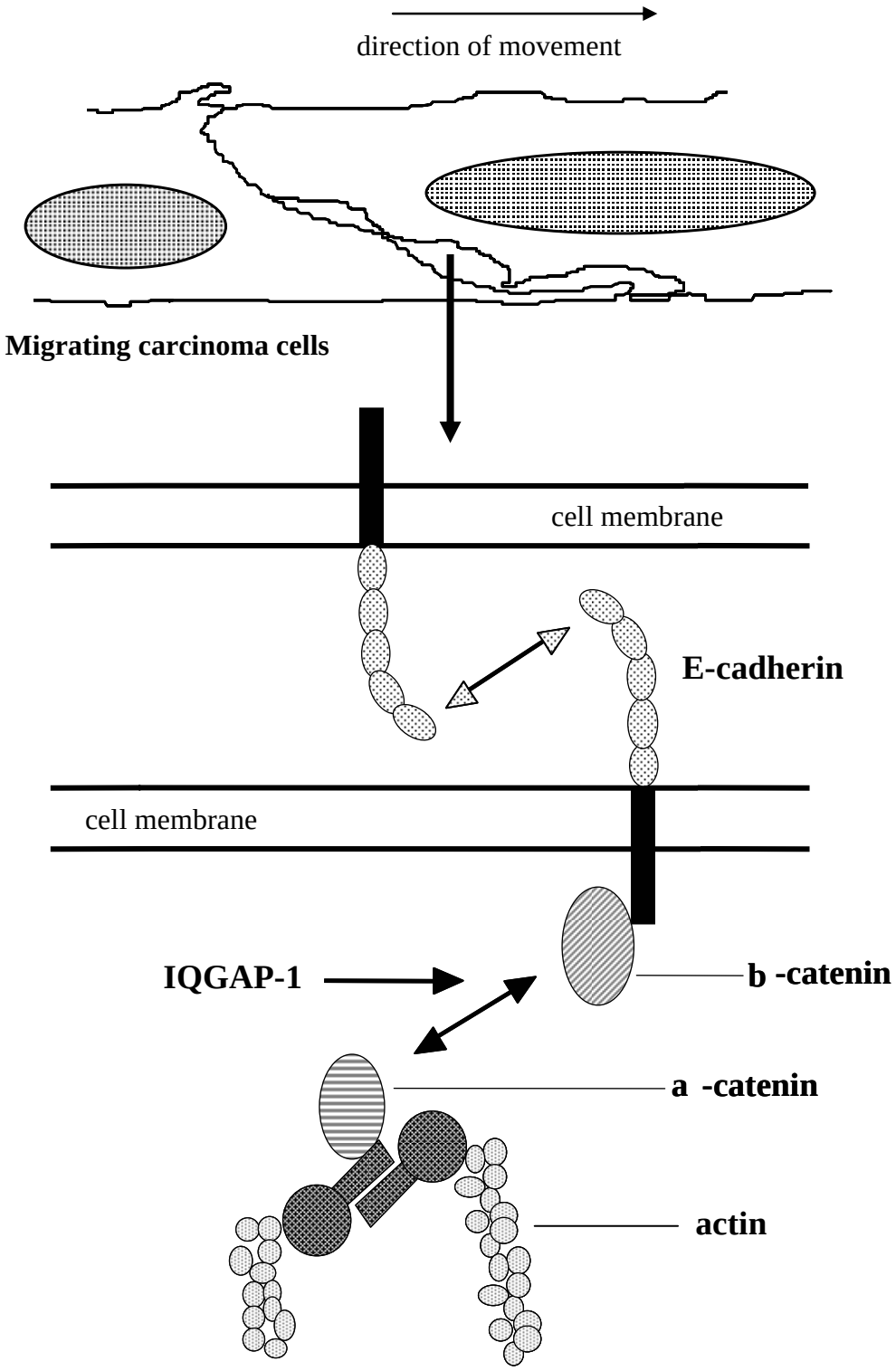

Figure 3. Compartmentalized release from cell-cell adhesion during cohort migration.

Involvement of IQGAP1 in Cross-talk between Signals Induced by Motogenic Factors and Cell-extracellular Matrix (ECM) Interactions

Using the in vitro cohort migration model, we have also shown that there is cross-talk between signals induced by HGF/SF and cell-ECM interactions during cohort migration, and that this cross-talk involves IQGAP1 [47]. HGF/SF-induced cohort migration was enhanced by fibronectin (FN) or type I collagen-coated substratum and almost completely inhibited by blocking cell-ECM interactions with Arg-Gly-Asp (RGD)-containing peptides. During the enhancement of HGF/SF-induced migration by ECM proteins, the level of IQGAP1 in Ecc increased more than that caused by HGF/SF alone. On the contrary, when migration was inhibited with RGD peptides, the level of IQGAP1 in Ecc did not increase as in non-stimulated cells despite the presence of HGF/SF stimulation. These results may indicate possible involvement of IQGAP1-mediated regulation of cell-cell adhesion in modulation of migration by cross-talk between signals from HGF/SF stimulation and cell-ECM interactions. (Figure 4) The cross-talk mechanism for coordinate regulation of cell-cell and cell-ECM adhesions during epithelial cell movement has been an interesting topic. It was reported in KYN-2 human hepatocellular carcinoma cells that integrin-mediated cell-substratum adhesion inhibited E-cadherin-mediated cell-cell adhesion, possibly through activation of c-Src bound to E-cadherin [48]. In migrating neural crest cells, treatment of cells with RGD peptides or antibodies to FN or β1 and β3 integrins blocked cell migration and at the same time caused rapid accumulation of N-cadherin molecules into adherens junctions in tight association with the cytoskeleton [49]. Moreover, coordinate expressions of cell-cell and cell-ECM adhesion molecules have recently been described. In general, expression levels of FN, integrins and integrin-binding proteins are inversely correlated to those of cell-cell adhesion molecules, such as E-cadherin. For example, overexpression of integrin-linked kinase (ILK), a serine/threonine kinase that binds to the integrin β1 cytoplasmic domain, stimulated FN matrix assembly in epithelial cells, and this was accompanied by a reduction in the E-cadherin expression [50]. Xenopus XTC cells stably transfected with E-cadherin showed downregulation of FN and α3β1 integrin expression, associated with impaired adhesion to FN and laminin [51]. Similarly, in mouse mammary epithelial cells, transition from epithelial to fibroblastic phenotype by transforming growth factor (TGF)-β1 correlated with decreased expression of E-cadherin, ZO-1 (a tight junction molecule) and desmoplakin I and II (desmosomal molecules) and increased expression of FN [52].

It is now considered that whether an epithelial cell migrates or not represents an integration of migration-inhibitory cell-cell interactions and migration-promoting cell-substrate interactions [53]. IQGAP1 may play a key role in this integration mechanism.

Further investigation of the signals involved in these coordinate expressions of cell-cell and cell-ECM adhesive molecules and modulation of their functions might provide a new approach to prevent invasion and metastasis of carcinoma cells.

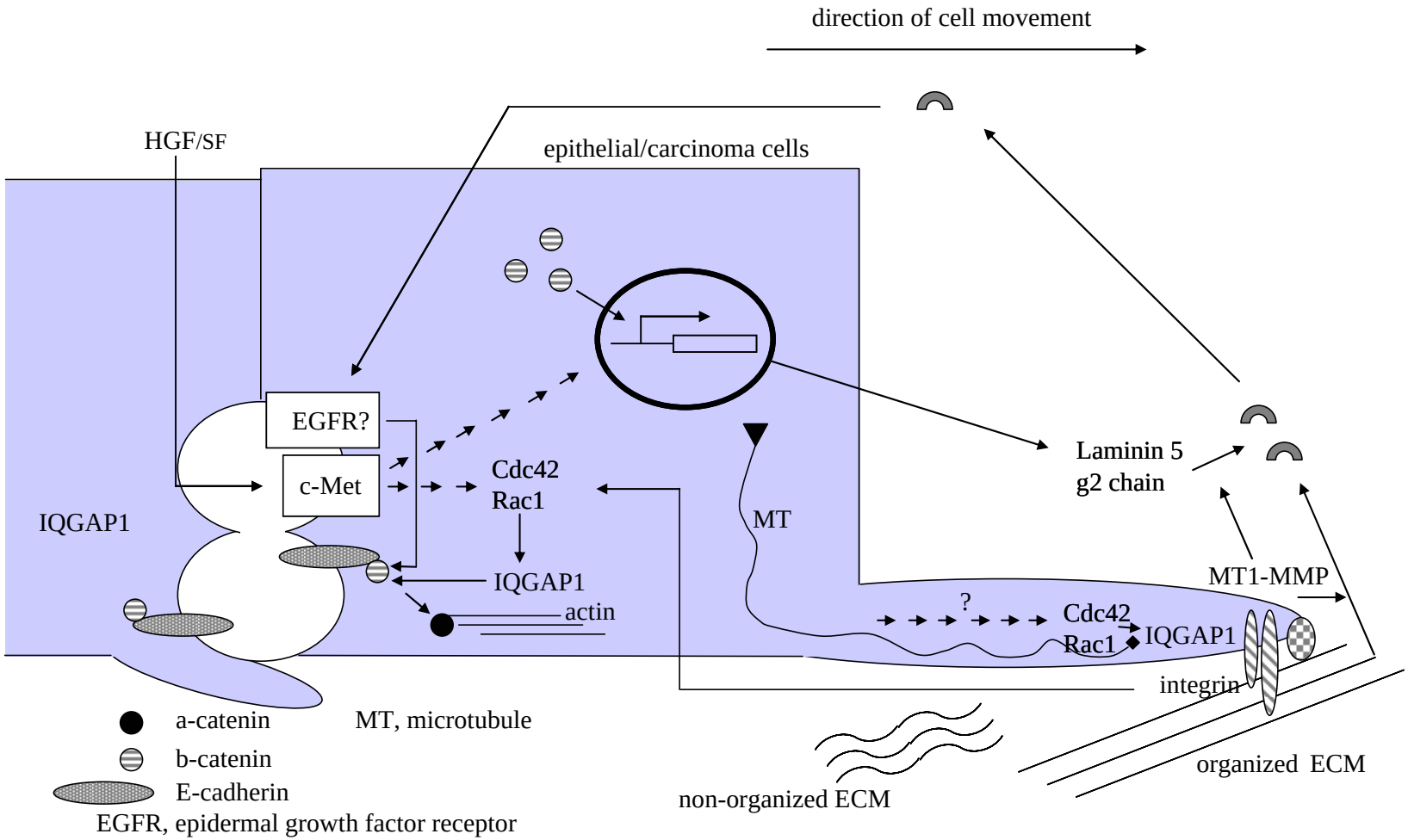

Figure 4. Coordinate links in between cell-cell adhesion, extension of leading edges and limited proteolysis on the cell surface.

Expression of IQGAP1 in Human Colorectal Carcinomas

We examined expression of IQGAP1 immunohistochemically in human colorectal tissues and found that IQGAP1 was overexpressed in carcinoma tissues compared with normal counterparts [54]. Within the carcinoma tissue, IQGAP1 was strongly demonstrated along the cell membrane at the invasion front, and higher levels of expression were observed in deeper two-thirds of carcinoma tissues than in the superficial one-third. This expression pattern showing stronger signals in deeper portions was most apparent in advanced carcinomas that invaded into the subserosa. Moreover, in our recent study in pT2-3 colorectal carcinomas, the cases with more pronounced IQGAP1 expression in deeper one-third than in superficial one-third showed the significantly higher nodal metastasis rate (Nabeshima et al., unpublished data). The cases with diffuse (>75% in both superficial and deep portions) and strong expression of IQGAP1 showed significantly worse prognosis in terms of a 5-year survival rate. Demonstration of IQGAP1 preferentially at the invasion front may support a role of IQGAP1 in cohort-type carcinoma cell migration during colorectal carcinoma invasion in vivo. Considering that IQGAP1 negatively regulates the Ecc-based cell-cell adhesion, IQGAP1 may facilitate dissociation of cell-cell adhesion at the invasion front.

In normal intestinal crypts, epithelial cells show migration-associated differentiation from the bottom to the surface [55]. Our study showed IQGAP1 expression predominantly in the zone of maturation that consists of the surface epithelium and the most superficial portion of crypts. The cells of this zone come off regularly at the end of maturation pathways, followed by replacement by next migrating cells. They are also involved in epithelial restitution in mucosal healing via cell migration. These findings suggest that cell dissociation mechanisms frequently operate there, like at the tumor invasion front. Considering the fact that IQGAP1 immunoreactivity was not usually demonstrated in the migrating cells of the zones of proliferation and simple transit within a crypt, the IQGAP1-dependent mechanism might be additional and involved preferentially in enhanced and frequent cell dissociation in normal epithelium.

IQGAP1 Expression and Carcinomas

IQGAP1-knockout mice showed no obvious defects during development, suggesting absence of essential non-redundant roles of IQGAP1 during tissue remodeling [56]. Loss of IQGAP1 also did not affect tumorigenesis, but mutant mice exhibited a significant increase in late-onset gastric hyperplasia. Although gastric carcinoma development was not seen, many hyperplastic lesions included areas of dysplasia [56]. These lines of evidence did not deny the possibility of partial involvement of IQGAP1 in gastric carcinogenesis. In fact, in cell lines established from a diffuse type of gastric cancer that shows invasive and scattered growth, IQGAP1 was upregulated by gene amplification, accompanied by corresponding increases in mRNA and protein expression [57]. Also, in human endometrial [58] and gastric [59] carcinomas, IQGAP1 expression was demonstrated immunohistochemically. In poorly differentiated endometrial carcinomas, abnormal complexes containing E-cadherin and IQGAP1 were found at cell adhesive sites, where α–catenin was not demonstrated [58]. In gastric carcinomas, IQGAP1 was frequently observed diffusely in the cytoplasm in intestinal-

type carcinomas (mostly well to moderately differentiated adenocarcinomas), but was expressed at the cell membrane in diffuse-type tumors (mostly poorly differentiated adenocarcinomas) [59]. In these gastric carcinomas, membranous expression of IQGAP1 was inversely correlated with that of E-cadherin or α-catenin. These lines of evidence suggest that IQGAP1 might be bound to the E-cadherin-β-catenin complex and interfere with intercellular adhesion in poorly differentiated adenocarcinomas that show scattered-type migration. Our study as mentioned above, however, suggested that this IQGAP1-mediated negative regulation of Ecc-based cell-cell adhesion may be involved also in cohort type migration of carcinoma cells observed in well to moderately differentiated adenocarcinomas. It may induce transient release from cell-cell adhesion, which occurs focally along the cell borders and enables cells to extend leading edges and move as cell clusters or tubules.

Similarly, overexpression of other regulators of E-cadherin-mediated cell-cell adhesion has also been demonstrated at the cancer invasion front. For example, overexpression of c-erbB-2/epidermal growth factor receptor (EGFR) and tyrosine phosphorylation of β-catenin, which is known to disrupt E-cadherin-based cell-cell adhesion, have often been observed in focal dedifferentiated cells at the invasion front of colorectal cancers [60, 61]. Phosphorylated EGFR is shown to colocalize with the E-cadherin-catenin complex and induces tyrosine phosphorylation of β-catenin, leading to dissociation of homophilic binding of E-cadherins [62, 63].

Tumor Cell Migration and MMP

Hereafter, we discuss about another characteristic very unique to cohort migration: the regulation of MMP expression via cell-cell contact within migrating cell sheets. Degradation of ECM by MMPs is assumed to be a prerequisite for the cells to migrate into native or provisional tissue matrix based on studies in many systems [64]: cell migration can be enhanced by overexpression of MMPs [65], while the overexpression of tissue inhibitor of metalloproteinases (TIMPs) or use of MMP inhibitors results in reduced migration [66]. Moreover, analysis in knockout mice for MMPs has shown alterations in migration-related processes such as pathologic inflammatory reactions, reduced angiogenesis, or delayed tumor progression, supporting a function of individual MMPs in cell motility [67]. Regulation of MMPs, including their expression and localization, is different according to the type of cell migration; single cell migration and cohort/collective migration [68].

Front Cell-specific Expression of MT1-MMP and Gel A during Cohort Migration

In two-dimensional cohort migration assays, when migration of colon carcinoma cells was induced with HGF/SF, MT1-MMP and Gel A were expressed only at the front cells of migrating cell sheets, with the following migrating cells being negative [8]. In contrast, when cell scattering (a dispersion of cells as single cells) was induced by stimulating cell migration in the presence of anti-E-cadherin antibody, the front cell-specific pattern of expression

observed during cohort migration was lost: individual scattering cells expressed both MT1-MMP and Gel A in their leading edges and cytoplasm. The same pattern, expression in front cells during cohort migration and in individual cells during scattering, was also shown at the mRNA level by *in situ* hybridization [68], suggesting the regulation at the gene expression level. When cohort migration was induced on gelatin-coated substratum, these MMPs expressed in the front cells degraded the gelatin matrix at the leading edges. Inhibition of this matrix lysis with BB94, a synthetic inhibitor specific to MMPs, TIMP-1 and -2, and the hemopexin domain (PEX) of Gel A suppressed migration on gelatin matrix, indicating that the ECM lysis by front cell MMPs is essential for cohort migration. 8].

Although the precise mechanisms involved in the front cell-specific localization of these MMPs are currently unknown, gene expression of MT1-MMP in the following cells of migrating cell sheets appears to be downregulated via cell-cell contact among migrating cell sheets when considering its different expression patterns in cohort migration and scattering. Similarly, gene expression of MT1-MMP is reported to decrease in confluent cultures of mouse mammary gland epithelial cells compared with their sparse cultures [69]. The cellular binding of Gel A also reduces in confluent cultures compared with that in sparse cultures of breast carcinoma cells [70]. On the other hand, expression of MT1-MMP and Gel A in the front pathfinder cells may be related to abundant interaction with ECM there. FN is one of the candidate ECM components, since FN is preferentially produced and deposited extracellularly by migrating cells during cohort migration [71-73] and that culturing of colon carcinoma cells on FN substratum stimulates production and activation of Gel A (K. Nabeshima, unpublished results). Taken together, the presence of abundant cell-cell contact within migrating cell sheets as in confluent cultures seems to suppress MT1-MMP expression and Gel A binding, whereas specialized cell-ECM contact may facilitate expression of these enzymes in the front pathfinder cells.

MMP-Dependent Reorganization of ECM and Exposure of Putative Cryptic Promigratory Sites within ECM Proteins, including Laminin-5 γ2 Chain

We have shown that MMPs expressed preferentially by front pathfinder cells are essential for cohort migration as mentioned above. However, this does not necessarily mean that MMPs serve only as a path clearing mechanism by removing a kind of barrier ECM. Since ECM can provide cells with good substrate to move on, proteolytic degradation of ECM is also a way of reorganizing the matrix to facilitate cellular interactions. In cohort migration, removal of gelatin matrix at the leading edges of the front cells of migrating cell sheets was not random or complete [8]. Instead, it was performed in a very coordinate and organized manner, leaving radially arrayed gelatin matrix at the frontmost part. This limited and organized clearing of gelatin matrix was necessary for cell migration because MMP inhibitors efficiently inhibited migration. Collectively, these findings suggest that a role of MMP is not just to remove ECM but to rearrange it to suit cell migration. This is the same for single cell migration. In three-dimensional matrix-based models, fibroblastic type tumor cells are reported to cause initial fiber traction at the leading edge followed by radial fiber alignment

towards the cell, which then favors persistent migration in direction of maximal traction [74]. Moreover, it is suggested that binding of integrins to this kind of pre-stressed ECM fibers would strengthen the linkage between those receptors and the force-generating cytoskeleton at that side of cells, thereby causing the cell to migrate along the direction of the rigid substrate [75].

Exposure of migration-permissive epitopes by enzymatic modification of ECM has also been demonstrated. The neurite-promoting activity of endoneurial laminin is inhibited by a Schwann cell-derived chondroitin sulfate proteoglycans (CSPGs) in non-injured peripheral nerves. Treatment of peripheral nerve sections with Gel A resulted in the removal of CSPGs and exposure of epitopes of laminin permissive for neurite extension [76]. Similarly, in breast epithelial cells, exogenous addition of activated Gel A stimulated migration onto laminin-5, which is a trimer of laminin α3, β3 and γ2 chains and found in many epithelial basement membranes, via cleavage of the γ2 chain [77]. Plasma membrane bound MT1-MMP also directly cleaves laminin-5, with a pattern similar to that of Gel A, leading to migration over laminin-5 in human carcinoma cells including those from the colon [78]. Cleavage of β3-chain of laminin-5 by MT1-MMP is also reported, and this cleavage product enhanced migration of prostate cancer cells [79]. Laminin-5 may be a static adhesive substrate in quiescent tissues, whereas it may deliver migratory stimuli during wound healing responses or tumor invasion [78].

More interestingly, laminin-5 γ2 chain is observed exclusively in the cytoplasm of budding tumor cells at the invasion front [80]. This altered expression pattern of laminin-5 γ2 chain has already been reported in colorectal carcinomas, together with a suggestion that laminin-5 γ2 chain might represent a valuable marker for the invasive potential [80-82]. However, little is known about the regulation of laminin-5 γ2 chain localization in budding tumor cells. Assembly of laminin-5 has been proposed to proceed from a γ2β3 heterodimer to an α3γ2β3 heterotrimer and secretion of whole laminin-5 requires assembly of subunits into a heterotrimer [81, 83]. In invading colon carcinoma cells, the under-expression of the α3 subunit, together with intracellular retention of the γ2 and β3 subunits, may link budding activity to alterations in laminin-5 assembly, secretion and deposition [81]. Recently β-catenin is reported to activate the laminin-5 γ2 gene through two T-cell factor-binding elements in a synergistic manner together with HGF/SF in intestinal epithelial cells, indicating that laminin-5 γ2 is another target gene of nuclear β-catenin during tumor progression [84]. The stimulatory effect of HGF/SF is mediated through activation of the transcription factor junD binding to two AP-1 sites in the laminin-5 γ2 gene promoter [84, 85]. Moreover, the production of the laminin-5 by carcinoma cells may be regulated at least partially by the surrounding cells and stroma. Tumor budding activity as well as laminin-5 expression in the cytoplasm of carcinoma cells was suppressed when these tumors grew at ectopic sites (subcutis) in nude mice, whereas the laminin-5-associated budding was restored when the cells were implanted orthotopically [81].

Once the laminin-5 γ2 chain is secreted extracellularly, both enzymatically active MT1-MMP and MMP-2 may promote its cleavage into the pro-migratory γ2' and γ2x fragments [86]. The deposition of these fragments into the tumor extracellular milieu is suggested to result in increased migration, invasion, and vasculogenesis mimicry by tumor cells in highly aggressive melanomas. Epidermal growth factor (EGF)-like sequence repeats, which are

contained in laminin-5 and released on MMP-mediated cleavages, could be chemotactic for tumor cells [87].

Expression of laminin-5 γ2 and β3 chains in budding tumor cells has been reported to be associated with focal under-expression of the E-cadherin-β-catenin complex in colorectal carcinomas [81], suggesting a link between γ2 chain-mediated budding activity and decreased cell-cell adhesion. Involvement of IQGAP1 in this link remains to be clarified. However, expressions of intracellular laminin-5 γ2 chain and IQGAP1 are both preferentially observed at the invasion front in colorectal carcinomas, and both γ2 chain expression and cohort migration can be induced in vitro by HGF/SF. Thus, in cohort migration, to examine a possibility that there may be a linkage between HGF/SF and γ2 chain-induced cell motilities via IQGAP1-mediated mechanisms is one of our interests.

Perspectives

Carcinoma cell migration involves a number of steps which include: partial or complete loss of cell-cell adhesion, extension of leading edges or pseudopods in the direction of movement, proper adhesion to ECM, contraction of the actin cytoskeleton in order to move the cell body, and remodeling of ECM by limited proteolysis. Digested ECM provides additional migratory stimuli. As we see in the text, all these steps proceed in a quite well coordinated manner, linking their controlling signals with one another. (Figure 4) Theoretically, disconnection of any of the link will lead to defective cell migration. Future therapeutic targeting of the carcinoma invasion cascade will require taking these coordinate regulatory pathways, especially between cell-cell and cell-ECM interactions, into consideration. To further determine similarities and differences in cohort type versus single cell migration will help to clarify the coordinate system. Furthermore, the cellular or/and stromal factor that regulates the invasion front-specific expression of invasion-related molecules, such as IQGAP1 and laminin 5 γ2 chain, needs to be clarified.

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PART 4: CLINICAL RESEARCH

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Chapter IX

NEW PROGNOSTIC MARKERS IN SUBMUCOSALLY INVASIVE COLORECTAL CARCINOMAS: CLINICAL IMPLICATION OF TUMOR BUDDING

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Introduction

Early colorectal carcinomas are divided into two categories: carcinoma in situ and submucosally invasive carcinoma. Carcinoma in situ is a lesion that contains abnormal glands lined by cytologically malignant cells, where all cells are confined within the epithelial layer and the basement membrane is not involved. As lymphatics within the colorectal mucosa are relatively sparse [1], carcinoma in situ probably has no potential for lymph node metastasis [2-6], and endoscopic polypectomy alone, when it is technically feasible, is generally accepted as adequate treatment [5]. However, abundant lymphatic and venous channels exsist in the submucosal layer of the large bowel; therefore, submucosally invasive carcinomas or malignant polyps have some risk of lymph node metastasis and hematogeneous metastasis. In the literature, the term "malignant polyp" was often used to designate a colorectal polyp that contains carcinoma invading through the muscularis mucosae into the submucosal layer [2, 7-14]. It is still controversial whether endoscopic polypectomy alone is satisfactory or additional bowel resection with lymph node dissection is mandatory for submucosally

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invasive carcinomas. This article discusses this controversial issue with special attention to the clinical implication of tumor budding in submucosally invasive colorectal carcinomas.

Malignant Potential of Submucosally Invasive Carcinomas

In previous studies, the prevalence of lymph node metastasis in patients with submucosally invasive carcinomas undergoing colectomy after endoscopic polypectomy varied from 0 to 29%, 15% on average [15]. Some investigators have asserted that all patients with submucosally invasive carcinoma should undergo bowel resection with lymph node dissection [16]. However, the majority of patients with submucosally invasive carcinoma undergoing colectomy after endoscopic polypectomy do not have lymph node metastases; therefore, several other researchers have proposed various histopathologic criteria to identify patients with high risk for lymph node metastases so as to treat them properly. In the literature, the following histological parameters in polypectomized specimens have been paid special attention with respect to risk factors for lymph node metastasis [17-21]; poorly or undifferentiated histology, massive invasion in the submucosal layer or positive margin, and lymphovascular permeation.

We surveyed and examined the utility and the limitations of these clinicopathologic parameters in the treatment of patients with submucosally invasive carcinomas [22]. Although poorly differentiated histology is easily identified, it is an extremely rare pathological finding, the average prevalence being about only 9%. The diagnosis of lymphatic invasion is often subjective and uncertain because of interobserver variation, sampling error, or artifact. Tissue retraction around tumor foci may mimic lymphatic invasion. Muller et al. studied the validity of immunohistochemical staining, using specific antibodies for the accurate detection of lymphatic invasion [12]; however, lymphatic invasion was not confirmed by this method. Venous invasion has been a rare finding in malignant polyps, the prevalence being from 2% to 6% except for an unusually high rate reported by Geraghty et al. [11] (37%). Except for Muller et al. [12] and Cooper et al. [9], other investigators reported that its positive predictive value was less than 5%, suggesting that venous invasion was not an independent risk factor and should not be used as the only criterion for further surgery [15].

There has been much interobserver and interinstitutional variation in the evaluation and interpretation of massive invasion in the submucosal layer. To evaluate the depth of submucosal invasion objectively, Haggitt et al. [3] proposed a new classification in 1985. They stratified the depth of invasion of early colorectal carcinoma into five levels, and examined the associations between the presence or absence of adverse outcome and various clinicopathologic parameters with special reference to the level of invasion in 129 early colorectal carcinomas. In their series, none of 65 patients with level 0 invasion (carcinoma in situ), 18 patients with level 1 invasion, and 8 patients with level 2 invasion had an adverse outcome. However, 1 of 10 patients with level 3 invasion, and 4 of 28 patients with level 4 invasion had an adverse outcome. They concluded that level 4 invasion ($p<0.001$) and rectal location ($p=0.025$) were the only statistically significant adverse prognostic factors. Nivatvongs et al. [23] examined 158 colorectal polyps with invasive adenocarcinoma treated

by resection using Haggitt et al.'s classification, and found that only level 4 invasion was significantly associated with lymph node metastasis.

In Haggitt et al's classification, submucosally invasive carcinomas were classified into two types only, pedunculated and sessile. However, about 40% of polyps with invasive carcinoma in our series had intermediate morphology, having a broad-based shape. Those polyps had no stalk, however, the muscularis mucosae was always lifted up above the level of the adjacent muscularis mucosae histologically, which was not categorized in Haggitt et al.'s classification. In these polyps, Level 2 and 3 of their classification could not be determined. To overcome this shortcoming, we adopted a modified relative criterion (Figure 1). Submucosal invasion was graded into three levels, as reported previously [22].

(1) In the case of pedunculated or broad-based morphology, the height of the submucosal layer above the adjacent muscularis mucosae was equally divided into two levels. Submucosal invasion limited to the upper half was defined as Level 1, and that extending into the lower half was defined as Level 2. If cancer cells invaded below the level of the adjacent muscularis mucosae, invasion was defined as Level 3 (Figure 1, left / right, upper). If the adjacent mucosa was not included in the polypectomized specimen, accurate differentiation of Level 1 and 2 on the slides was theoretically impossible. In such cases, the size of the head and stalk were obtained from the endoscopic report and the level of submucosal invasion was determined approximately.
(2) In the case of sessile or ulcerated morphology, submucosal invasion was always below the level of the adjacent muscularis mucosae, and consequently, the invasion level was defined as Level 3 (Figure 1, right, lower). Our system is assumed to be applicable to all types of polyps with invasive carcinoma.

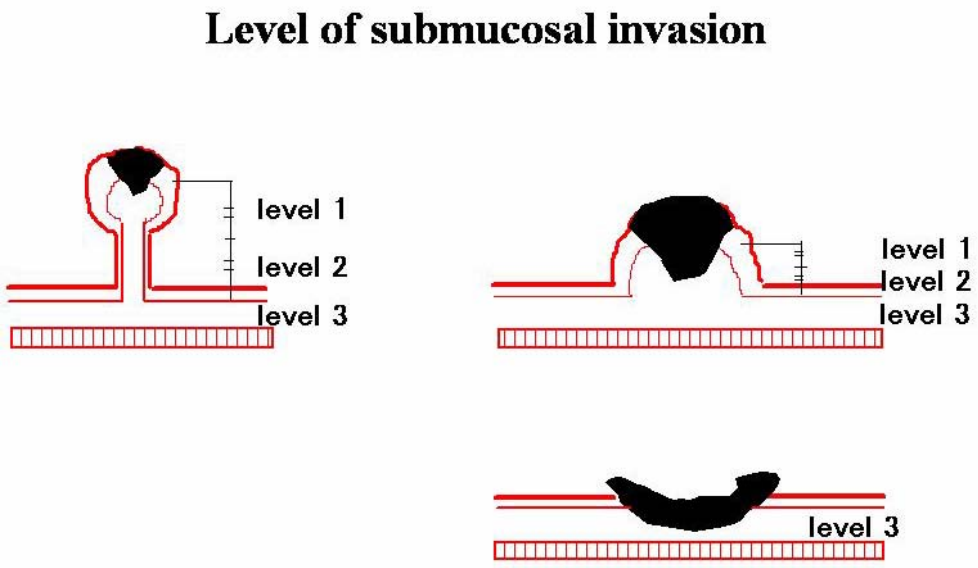

Figure 1. Level of invasion into submucosal layer. Left and right (upper); In a pedunculated and broad-based lesion, the submucosal layer above the level of the muscularis mucosae of the surrounding normal mucosa is divided equally into two, denoting. level 1 and level 2. Level 3 denotes the level beneath the muscularis mucosae of the surrounding normal mucosa. Right (lower); In a sessile lesion, the submucosal layer of the lesion is located beneath the level of the muscularis mucosae of the surrounding normal mucosa, which implies level 3 invasion.

On the other hand, some Japanese researchers have advocated an absolute grading criterion for determining malignant potential of submucosally invasive colorectal carcinomas [24-26]. As an absolute grading criterion, the depth and width of submucosal invasion were measured using a micrometer under light microscopy, and graded into three categories as follows: sm1, the depth of invasion less than or equal to 0.5mm; sm2, the depth of invasion greater than 0.5mm and less than 1.0mm; sm3, the depth of invasion greater than or equal to 1.0mm; sma, the width of invasion less than or equal to 1.0mm; smb, the width of invasion greater than 1.0mm and less than 3.0mm; smc, the width of invasion greater than or equal to 3.0mm [27].

Recently, we examined the clinical utility of relative and absolute grading criteria for submucosal invasion in predicting locoregional failure in 51 submucosally invasive colorectal carcinomas, and found that massive invasion in the submucosal layer *per se,* either relative or absolute, was not a significant factor [28]. This result is consistent with the official recommendation by the American College of Gastroenterology in 2000, suggesting that invasion of the stalk of a pedunculated malignant polyp, by itself, is not an unfavorable prognostic finding, as long as the cancer does not extend to the margin of stalk resection [29].

Tumor Budding at the Invasive Margin

Many researchers have already reported that dedifferentiated histology at the invasive margin (tumor budding) is significantly associated with tumor aggressiveness in many types of cancers including lip [30], tongue [31], lung [32], and colorectum [22, 33-38]. An isolated single cancer cell or a small nest of cancer cells with poorly differentiated histology at the invasive margin were defined as tumor budding (Figure 2). This histological finding can be easily and objectively diagnosed in routine pathological practice. Morodomi et al firstly reported that the degree of tumor budding can be of great help in predicting lymph node metastasis in advanced rectal cancers [33].

As for submucosally invasive colorectal carcinomas, Coverlizza et al first reported that 2 of 14 patients (14%) who underwent subsequent surgery had this histologic feature in polypectomy specimens, and in both of them, the surgically resected specimens contained lymph node metastasis [18]. Subsequently, many researchers reported similar findings [22, 34, 39, 40]. The positive predictive value of tumor budding for predicting lymph node metastasis or regional failure ranged from 21% to 100%. By multivariate analysis, we demonstrated that tumor budding is more useful than widely accepted criteria such as massive submucosal invasion, vascular invasion, or poorly differentiated histology in predicting locoregional failure (lymph node metastasis or local recurrence) in submucosally invasive colorectal carcinomas [22].

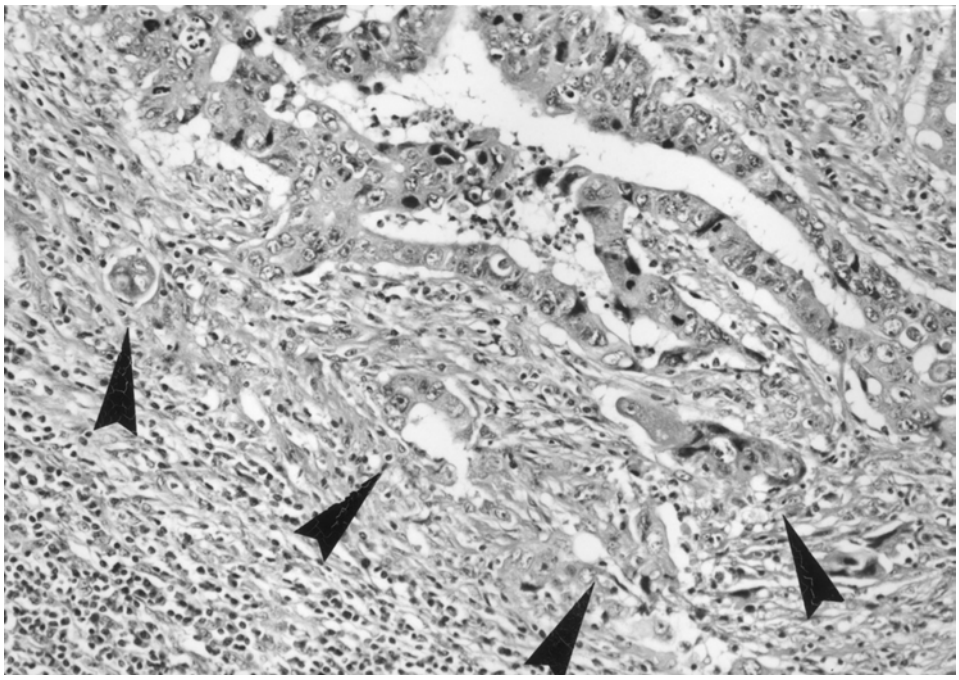

Figure 2. Representative photograph of tumor budding at the invasive margin, showing small nests of cancer cells with poorly differentiated histology (arrowheads). H&E, x50.

Evaluation of Tumor Budding

The evaluation of tumor budding has not yet been standardized. Some researchers categorized only the presence or absence of this histological finding [41], whereas others classified its grade subjectively into, none, mild, moderate and severe [34]. Others counted the actual number of budding tumor cells at the invasive margin. Ono et al counted the number of dedifferentiation units along the entire invasive front, and classified its grade into 4 levels; none(0 unit), mild(1-20 units), moderate(21-50 units), and severe(>50 units) [35]. Goldstein and Hart also counted the number of tumor budding foci along the entire invasive front, and categorized its grade into 3 levels; absent to focally present (<3 isolated foci), moderately present (3-10 foci or <3 mm of continuous pattern), or extensively present (>10 foci or >3 mm of continuous patter) [38]. On the other hand, Morodomi et al counted the budding in a visual field 500 μm x 2500 μm square at four locations in each slide, and the average budding count was classified into 3 levels; negative (0-4 per square), mildly positive(5-14 per square), and strongly positive(15 or more per square) [33]. Ueno et al counted the number of budding foci in a field measuring 0.385 mm^2 using a x25 objective lens, in which the intensity was considered maximal, and graded the count into 4 levels; <5, 5-9, 10-19, and $\geqq$20 [42]. It has not been determined yet which classification is most useful clinically. We have recently attempted to construct a formula for predicting the risk of lymph node metastasis in submucosally invasive colorectal carcinomas using the actual number of budding tumor cells *per se*, and to determine the indication for additional surgery after

endoscopic mucosal resection of submucosally invasive colorectal carcinoma using decision analysis (data under submission).

Seventy-six submucosally invasive colorectal carcinomas resected surgically from 75 patients were examined. Twenty-one lesions were initially resected locally by endoscopic mucosal resection, or trans-anal or trans-sacral resection, and additional bowel resection with lymph node dissection was performed. The remaining 55 lesions were initially resected by radical surgery. Tumors were evaluated for predominant grade, lymphatic vessel invasion, blood vessel invasion, regional lymph node involvement, degree of submucosal invasion according to relative and absolute grading criteria, surgical margin status, presence or absence of adenomatous component, and presence or absence of tumor budding. For objective evaluation of tumor budding, the number of dedifferentiation units along the entire invasive margin was counted under light microscopy according to a previous publication [35]. The associations between lymph node metastasis and clinicopathological parameters were examined statistically. Five (6.6%) out of 76 tumors had lymph node metastasis in the resected specimens. Univariate analysis showed that histology and tumor budding were significantly associated with the presence of lymph node metastasis (p=0.024, 0.006). Neither vascular invasion nor depth of invasion was significant. Logistic regression analysis using these two factors as independent variables showed that tumor budding alone was significantly associated with lymph node metastasis, consistent with our previous publication [22].

The probability of lymph node metastasis was calculated as follows:

$Z=0.062 \text{ X (budding counts)}-3.638$

$\text{Probability}= 1/1+e^{-Z}$

Table 1 shows the associations between actual budding count and probability of lymph node metastasis, showing that the higher the budding count, the higher the probability of lymph node metastasis. Interestingly, this formula was able to predict the presence or absence of lymph node metastasis in the 5 successive submucosally invasive colorectal cancers subsequently operated on at our institution. In one of these cases, the number of budding cells was 94, and the probability of lymph node metastasis was calculated as 0.89, and this patient was found to have pericolic lymph node metastasis.

Table 1. Tumor budding and Probability of Lymph Node Metastasis

Budding counts	0	10	20	30	40	50	60
Probability	0.026	0.047	0.083	0.145	0.239	0.369	0.52

Decision Analysis

Decision analysis is a logical method for the improvement of decision-making in the economic, military and medical fields. It utilizes decision trees to model each uncertain issue in a structured and efficient manner. A chronological sequence of choices and possible events

can be shown clearly. Decision trees after endoscopic mucosal resection were constructed using two parameters, the risk for lymph node metastasis and operative mortality. Outcomes of each arm were determined as predicted survival time. Information was obtained from annual reports on the life table of Japanese people, the multi-institutional registry of the Japanese Society for Cancer of the Colon and Rectum [43], and previous articles. Two-way sensitivity analysis was performed using computer software "Precision Tree"(Palisade Corporation, New York, USA).

Figure 3 shows a representative decision tree in a case of a 75 year-old male undergoing endoscopic mucosal resection for a polypoid colonic submucosally invasive carcinoma. Two-way sensitivity analysis is shown in Figure 4. If the budding count was zero, the risk of lymph node metastasis was about 2%. If the patient had medical complications, and the operative risk was high (about 2.0%), a policy of observation was justified. On the other hand, if the budding count was about 20, the risk of lymph node metastasis was about 8%. In this case, additional surgery was justified.

We could show that the malignant potential of submucosally invasive colorectal carcinomas can be determined objectively by a mathematical formula using the actual number of tumor budding foci. This finding is clinically useful, because the probability of lymph node metastasis can be applicable for decision analysis when determining the indication for additional surgery after endoscopic treatment for submucosally invasive colorectal carcinomas. Using 2-Way sensitivity analysis, we could determine the indication for additional surgery more objectively than before. These refinements will be useful not only in helping patients to understand their medical situations, but also in obtaining informed consent from them. However, this study is still preliminary, and multi-institutional, prospective controlled study is now in planning.

In conclusion, multivariate analysis showed that the actual number of tumor budding foci alone was significantly associated with the presence or absence of lymph node metastasis in submucosally invasive colorectal carcinomas. The actual number of tumor budding foci may be useful for decision-making in patient-oriented treatment of submucosally invasive colorectal carcinomas.

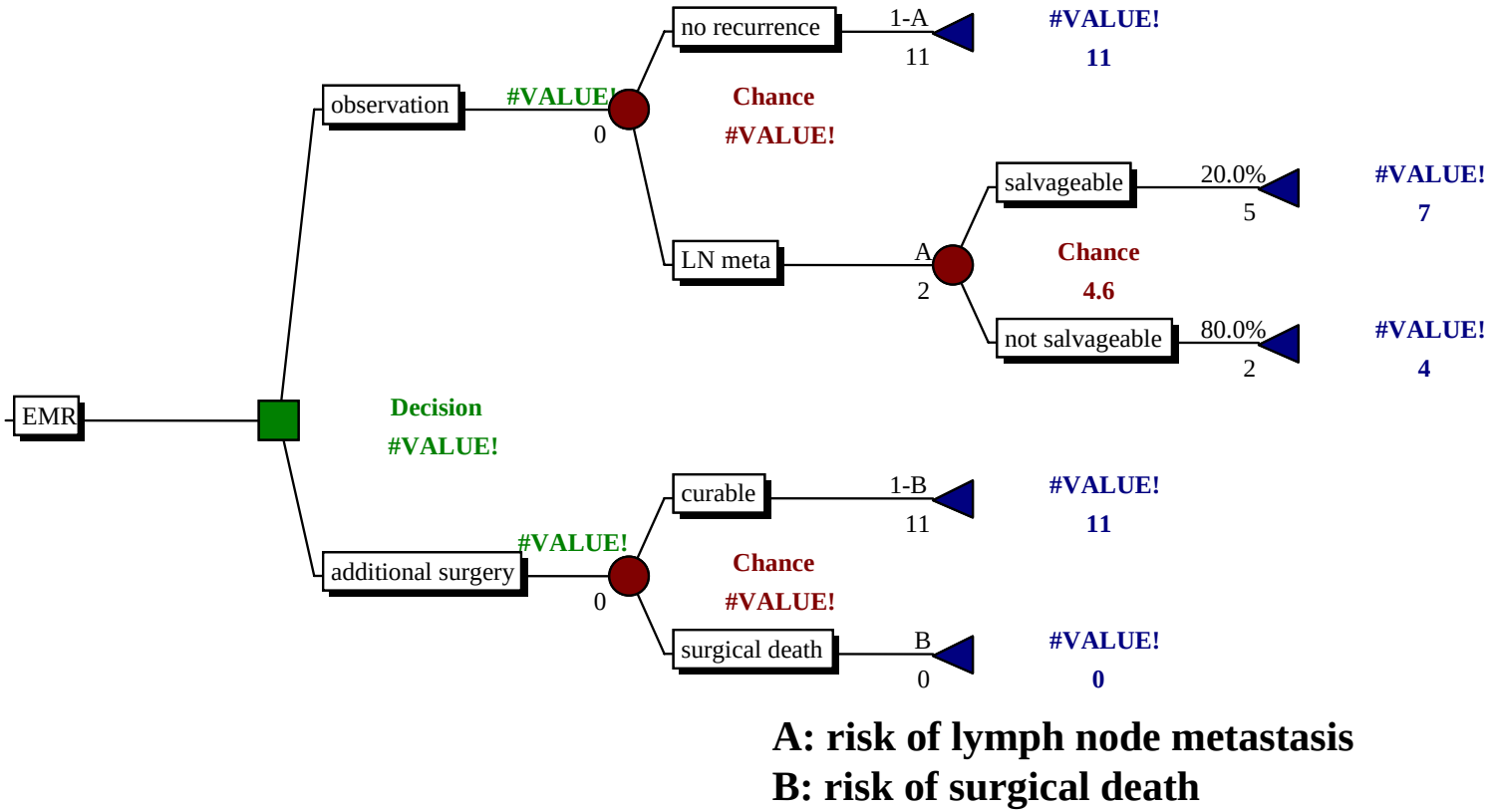

Figure 3. Representative decision tree in 75-year-old man undergoing endoscopic mucosal resection for a polypoid colonic submucosally invasive carcinoma. The probability of lymph node metastasis ("A") and the risk of surgical death ("B") are variables. On the assumption that salvageability with lymph node metastasis is 20.0%, the expected years of survival of each arm can be calculated.

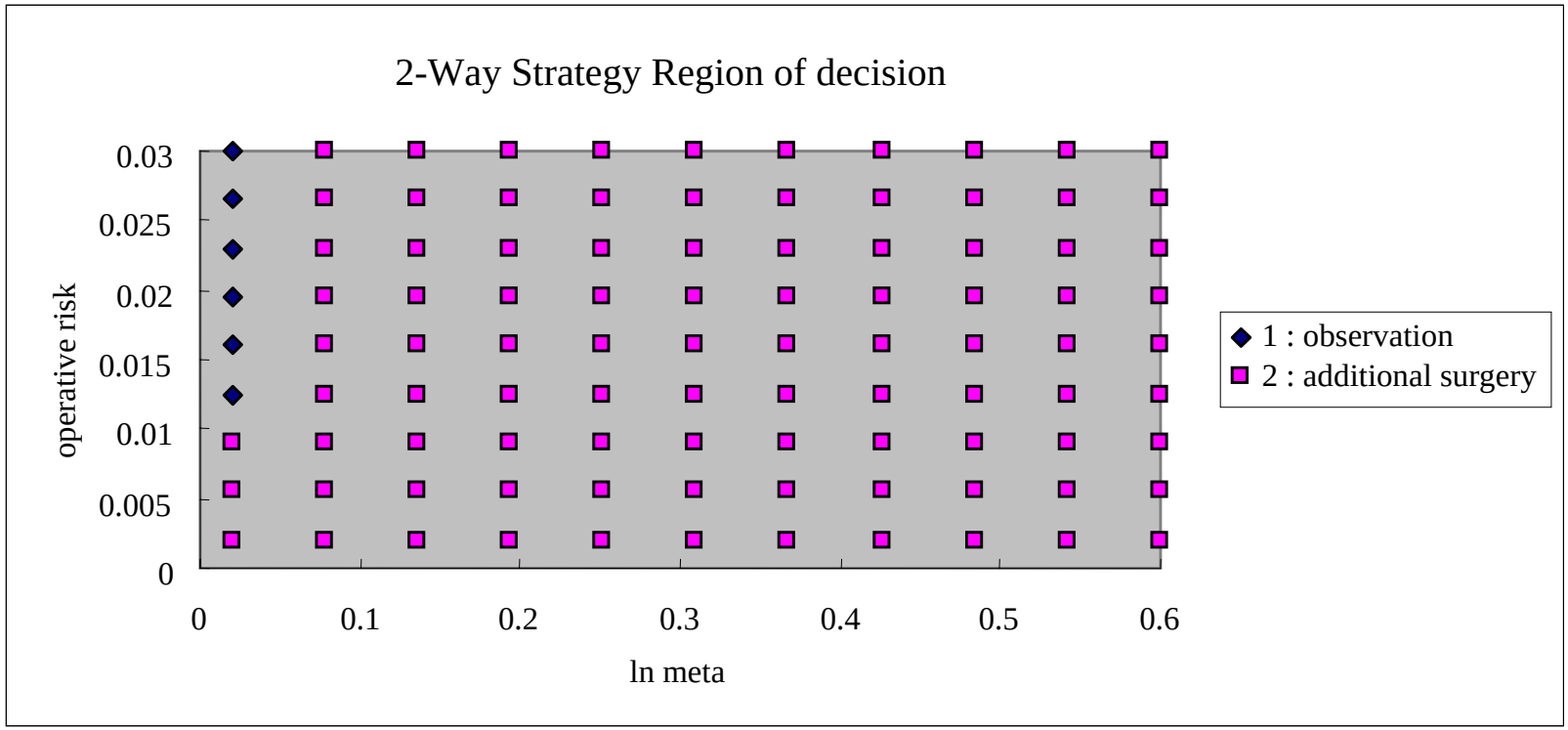

Figure 4. Two-way sensitivity analysis of decision. This figure shows the best decision depending on the probability of lymph node metastasis and the risk of surgical death. If the former value is 0.02 and the latter is 0.018, a policy of observation is justified. If the former value is 0.08 and the latter is 0.018, a policy of additional surgery is justified.

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Chapter X

TUMOR BUDDING IN ADVANCED RECTAL CARCINOMA

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Introduction

The first step in invasion and metastasis of a solid tumor is loss of differentiation (de-differentiation) and dissociation at the invasive front, followed by tumor cells mobilizing out of the main tumor [1]. Tumor budding, an isolated single cancer cell or a small cancer cluster observed in an actively invasive region (Figure 1), is a histological expression of this event [2]. With regard to molecular background of tumor budding, it has recently been demonstrated that this tumor characteristic is relevant to cell-to-cell or cell-to-extracellular matrix interactions through regulation of Laminin-5 [3,4], CD44 [5], and syndecan-1 protein [6], and has also been demonstrated to be associated with carbonic anhydrase, a marker of mucosal differentiation [7].

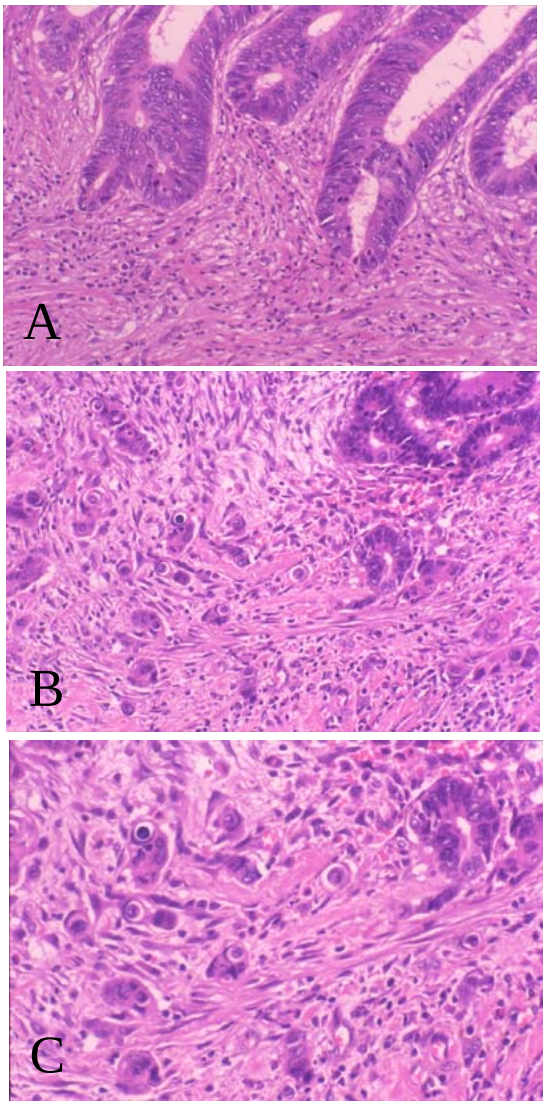


Figure 1. Invasive frontal region of rectal cancer. A) Negative for tumor budding. No small cancer clusters are observed. (original magnification X66) B,C) Positive for tumor budding. An isolated single cancer cell or a cancer cluster composed of fewer than five cancer cells is defined as tumor budding focus. The field where the tumor was fragmented because of the aggregation of inflammatory cells or because of a technical artifact excluded from consideration. (original magnification B:X66, C:X100).

An understanding of the mechanisms underlying tumor cell invasion requires an analysis of the complex interactions between the neoplastic cells and the surrounding matrix [8]. Collagen-I protein in the centre of the tumor, produced through the desmoplastic response, was reported to inhibit the de-differentiation process of the tumor [9]. In differentiated colorectal cancers, the process of de-differentiation and dissociation of neoplastic cells is always strictly confined to the tumor invasive front. Inductive signals from the host microenvironment are thought to be involved in initiating and maintaining this rapid and even reversible phenotypic shift through activating or repressing the preformed genetic programme of the tumor cells [1].

Morphologically, the tumor center is usually composed of fine and mature fibres stratified into multiple-layers ("mature stroma"), whereas fibrotic stroma including keloid-like collagen ("intermediate stroma") and myxoid stroma ("immature stroma"), observed in particular differentiated tumors, are practically confined to the tumor invasive front (**Figure 2**). Keloid-like collagen in rectal cancer has morphological similarity to the collagen observed in keloids, which is characterized by an overabundant deposition of collagen. [10] The presence of keloid-like collagen suggests that fibroblasts, which play an important role in tumor growth [11], morphogenesis [12], differentiation [13], and cell migration [14], may be aberrantly activated by some kinds of modulators. [10] Myofibroblasts are distributed more pervasively in the immature fibrotic stroma, compared to mature stroma or intermediate stroma [15]. The myofibroblast has recently been spotlighted because of its production of enzymes able to degrade the basement membrane surrounding tumor glands, and its synthesis of extracellular matrix components in the tumor stroma, which could subsequently alter the adhesive and migratory properties of the epithelial cancer cells [16]. It has actually been observed that maturation of fibrotic cancer stroma has a significant negative correlation with the intensity of tumor budding (Figure 3) (data in St Mark's Hospital) [15]. Based on these findings, intermediate and immature fibrotic stroma seem to be a transitory phenotypes which are associated with the de-differentiation of cancer cells, whereas mature stroma is a later and more stable phenotype which is associated with reduced invasive activity of neoplastic cells, whether they are located in the tumor center or the invasive front.

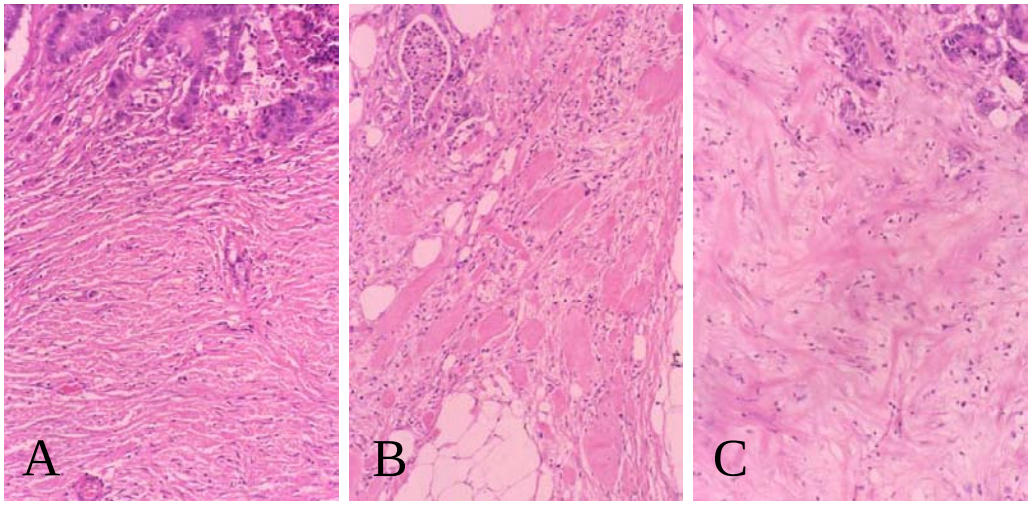


Figure 2. Type of fibrotic cancer stroma in invasive frontal region. A) "Mature" stroma. B) Fibrotic stroma containing keloid-like collagen ("intermediate" maturation). C) Myxoid stroma ("immature" stroma). (original magnification X50)

The phenotypic features which may conceptually overlap with tumor budding are differentiation and growth pattern (expanding / infiltrating). Although tumor budding has a significant relationship with these two features, as well as other tumor behavior-related parameters such as extramural spread, nodal involvement, and venous invasion (**Table 1**), for practical reasons, these should be distinguished from tumor budding. Tumor budding is usually observed in only a confined region of the invasive front. A carcinoma should only be classified as poorly differentiated when lack of cell cohesiveness and deficient gland

formation is found widely elsewhere in the tumor. The difference between tumor budding and growth pattern is the field size estimated. The growth pattern is a parameter that is based on the comprehensive estimation of the nature of the invasive margin, namely, it includes the naked eye inspection of pathological specimens as well as low power microscopic examination [17]. On the other hand, tumor budding is identified and graded with the use of microscopy alone at a higher magnification. Tumor budding has been demonstrated to correlate with postoperative prognosis independent of tumor differentiation or growth pattern [18].

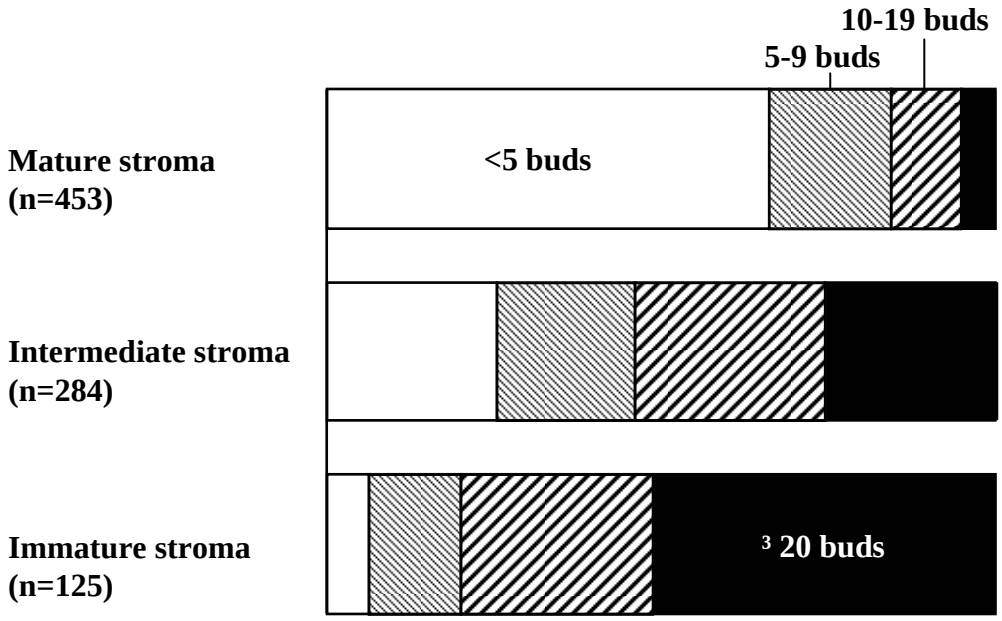


Figure 3. Negative correlation between the intensity of tumor budding and maturation of fibrotic cancer stroma. Intensity of budding: the number of budding foci in a microscopic field of X250 where budding was the most intensive. P<0.0001 (Spearman's test)

Table 1. Tumor Budding and Other Clinicopathologic Characteristics

Variable	Categories	Intensity of tumor budding*		p-value
		0-9 (n=446)	10+ (n=192)	
Differentiation	Well	134	13 (9%)	<0.0001
	Moderate	289	116 (29%)	
	Poor	23	63 (73%)	
Growth pattern	Expanding	409	88 (18%)	<0.0001
	Infiltrating	37	104 (74%)	
Extramural spread	Absent	148	22 (13%)	<0.0001
	Present	298	170 (36%)	

Table 1. Tumor Budding and Other Clinicopathologic Characteristics (cont.)

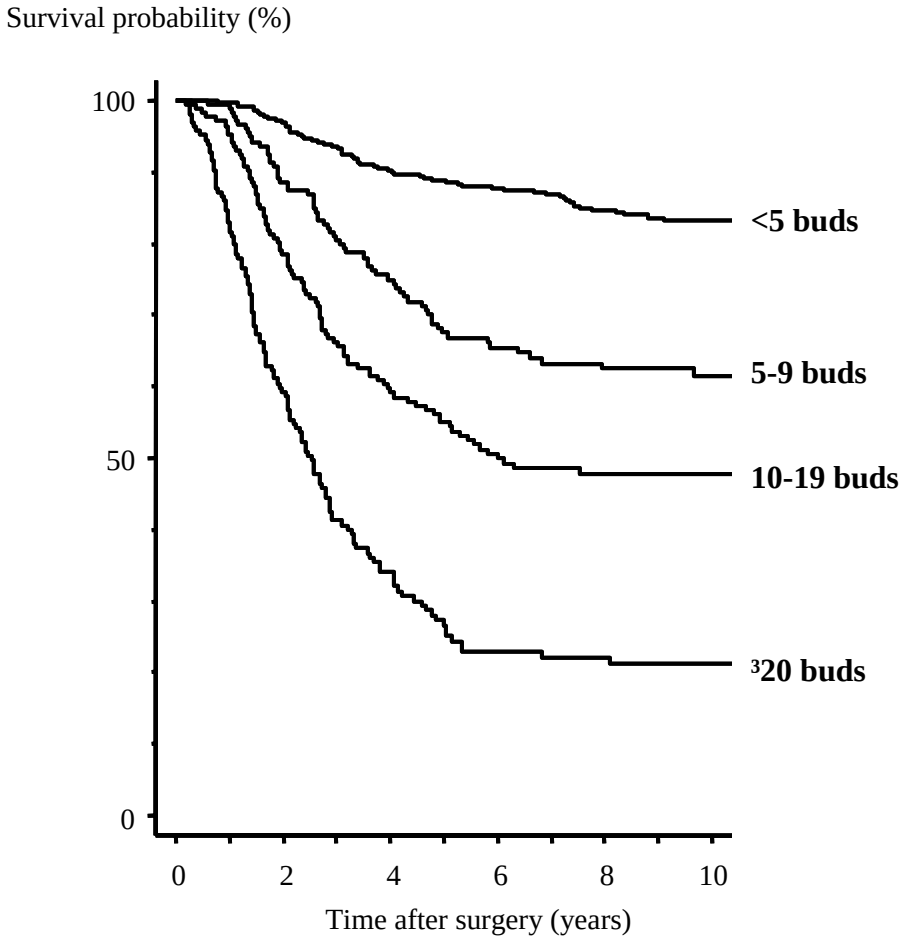
Variable	Categories	Intensity of tumor budding*		p-value
		0-9 (n=446)	10+ (n=192)	
Number of nodes involved	0	324	61 (16%)	
	1-3	102	90 (47%)	<0.0001
	4 or more	20	41 (67%)	
	Present	8	20 (71%)	
Lymphocyte infiltrate	Conspicuous	169	15 (8%)	<0.0001
	Other	277	177 (39%)	
Extramural venous invasion	Absent	364	135 (27%)	0.0015
	Present	82	57 (41%)	

Number of budding foci in a microscopic field of X250 where budding was the most intensive.

Value of Tumor Budding as a Prognosticator

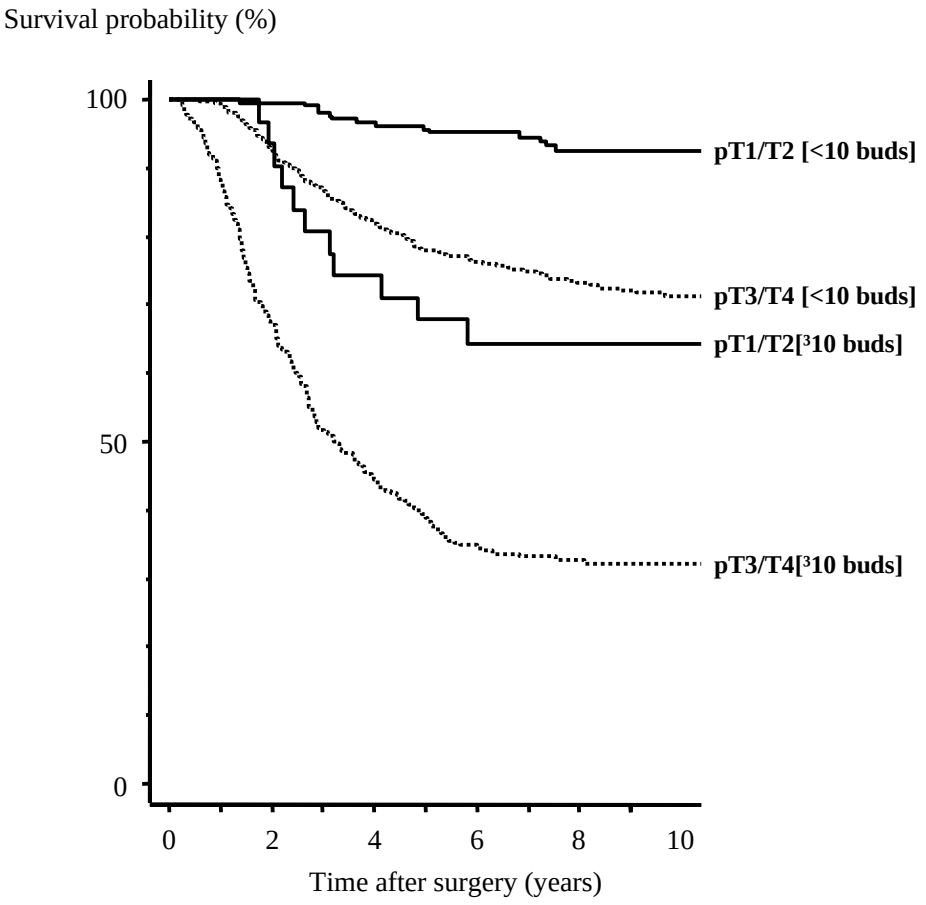
Dukes's original classification was proposed more than 70 years ago [19,20]. Since then, this classification for rectal cancer, composed of the two parameters, depth of tumor penetration and nodal involvement, has been the most widely employed prognostic classification for colorectal cancer, even after some modifications such as the Astler-Coller's classification [21], the UICC classification [22], and the Japanese classification [23]. Although it is generally accepted that these two parameters are the best standard prognostic indicators, pathologists have been striving for other pathological indicators in order to predict the prognostic outcome with more accuracy [2,24,25,26].

Figure 4 shows prognostic utility of a grading system based on tumor budding that could stratify patients undergoing surgery for rectal cancer into different postoperative outcome groups. One flaw of the prognosis-predicting system using tumor depth and nodal involvement is that the Dukes B group, or the UICC stage II group, has proved to be a perplexingly broad category with respect to survival outcome, primarily because it is difficult for current routine techniques to exclude patients with occult lymph node metastases from this category. Tumor budding is independent from tumor depth (**Figure 5**) or nodal involvement (**Figure 6**) in terms of prognostic impact. Its use as a marker for prognosis minimizes the erroneous staging of advanced disease as early-stage disease, possibly due to occult metastasis; i.e., 9% of UICC stage I patients and 22% of stage II patients were demonstrated to be upstaged by estimating tumor budding (**Figure 7**).



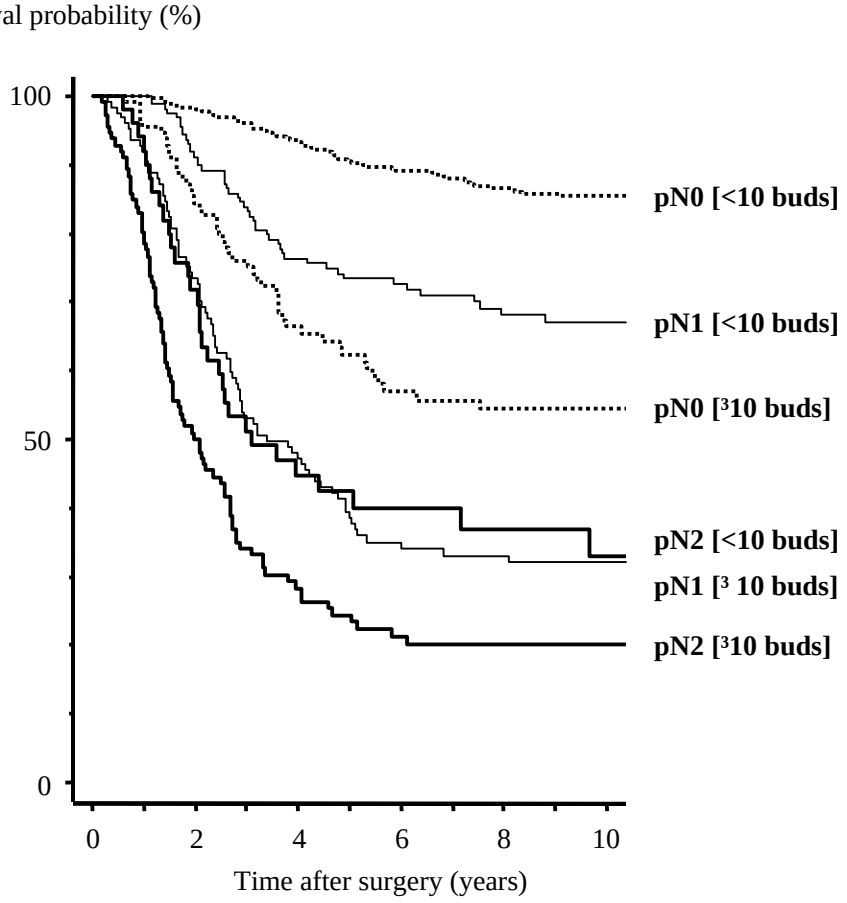
Number of budding foci			Number at risk			
<5 buds	563	513	445	374	262	191
5-9 buds	192	156	122	91	76	54
10-19 buds	193	139	100	76	57	40
³20 buds	166	94	52	30	24	19

Figure 4. Kaplan-Meier survival curves by the intensity of tumor budding. Intensity: the number of budding foci in a microscopic field of X250 where budding was the most intensive.
<5 buds vs 5-9 buds, P<0.0001; 5-9 buds vs 10-19 buds, P=0.0032; 10-19 buds vs ³20 buds, P<0.0001



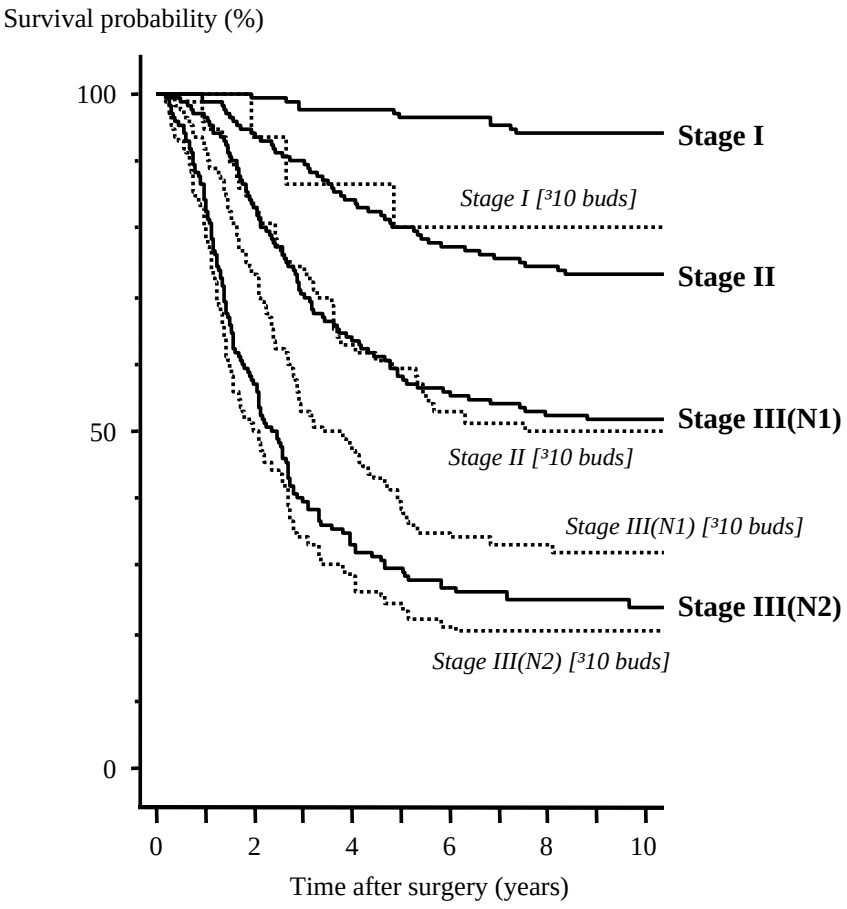
Number of budding foci			Number at risk			
T1/T2 [<10 buds]	227	212	197	168	121	86
T1/T2[³10 buds]	36	29	23	19	17	12
T3/T4 [<10 buds]	528	457	370	297	217	159
T3/T4[³10 buds]	323	204	129	87	64	47

Figure 5. Correlation between tumor budding and pT in terms of their influence on postoperative survival. Intensity: the number of budding foci in a microscopic field of X250 where budding was the most intensive.
<10 buds vs ³10 buds: pT1/T2, P<0.0001; pT3/T4, P<0.0001



Number of budding foci	Number at risk					
N0 [<10 buds]	536	493	440	359	260	184
N0 [³10 buds]	118	91	67	49	38	29
N1 [<10 buds]	169	141	107	91	66	54
N1 [³10 buds]	129	88	56	38	30	19
N2 [<10 buds]	50	35	20	15	12	7
N2 [³10 buds]	112	54	29	19	13	11

Figure 6. Correlation between tumor budding and pN in terms of their influence on postoperative survival. Intensity: the number of budding foci in a microscopic field of X250 where budding was the most intensive.
<10 buds vs ³10 buds: pN0, P<0.0001; pN1, P<0.0001; pN2, P=0.016



UICC (TNM) Stage *["budding"]*			Number at risk			
I	213	196	186	156	114	79
II	441	388	321	252	184	134
III(N1)	298	229	163	129	96	73
III(N2)	162	89	49	34	25	18
I [³10 buds]	19	14	13	11	10	8
II [³10 buds]	99	77	54	38	28	21
III(N1) [³10 buds]	129	88	56	38	30	19
III(N2) [³10 buds]	112	54	29	19	13	11

Figure 7. Survival impact of high-grade budding on the UICC (TNM) classification. No significant survival difference was observed between UICC stage II patients (5-year survival rate, 80%) and stage I patients with high-grade budding (³10 buds) (80%). High-grade budding reduced the survival probability of UICC stage II patients (5-year survival rate, 59%) to the same level as that of UICC stage III (N1) patients (58%).

Jass et al. have established a prognostic staging system utilizing four pathological parameters: tumor depth, nodal involvement, peritumoral lymphocytic infiltration, and tumor growth pattern [24].Compared to the Dukes's classification, Jass's staging system is superior in that it can place twice as many patients into the most favorable and the worst prognostic categories [24]. However, it has been pointed out that the reproducibility of the observation of lymphocytic infiltration and growth patterns can be variable in routine practice [17,26,27].

Because tumor budding is a semi-quantifiable parameter, similar to the mitotic index, it offers a less subjective basis than current golden schemes and is a worthy candidate to supplement the two acknowledged two gold-standard prognostic parameters (i.e. depth of penetration and nodal involvement) in routine practice. Using a grading system with three parameters will provide a wider spectrum of 5-year survival rates compared with conventional systems, enabling patient selection to be performed efficiently both for postoperative adjuvant therapy and intensive follow-up examination.

Clinical Implication of Tumor Budding in Biopsy Specimens

Local recurrence of rectal cancer after potentially curative surgery is one of the most common recurrent forms and influences survival [28]. Several mechanisms have been postulated as the cause of local recurrence, including insufficient resection of the mesorectum [29,30] and extramesorectal region such as iliac nodes [31,32]. To prevent local recurrence, surgical treatment options, such as wide local surgical clearance [33] and pelvic lymphadenectomy (iliac nodal dissection) [34,35] have been proposed, as well as preoperative radiotherapy [36,37,38]. Currently, reliance on ascertainment of extent of local spread alone provides few selection gates to either extended lymphadenectomy or preoperative radiotherapy. The optimal treatment varies on a case-specific basis, and thus the appropriate therapy selection requires an accurate evaluation of each tumor.

Insufficient distal clearance for patients with low advanced rectal cancer is another important cause of local recurrence. There are no generally accepted guidelines as to which low rectal cancer patients should undergo abdominoperineal resection with colostomy, rather than conventional sphincter preserving surgery (i.e., low anterior resection) or more aggressive sphincter-preserving surgery such as intersphincteric resection. Although we have the “two-centimeter rule” concerning the distal clearance, this has recently been shown to sometimes mislead us into selecting excessive surgery [39,40,41].

We herewith describe the value of defining a tumor’s aggressiveness by examination of the submucosal invasive frontal region, including tumor budding, as the clinical yardstick to evaluate the extent of extramural spread and the possibility of intramural distal spread (**Figure 8**). This study was based on data of patients undergoing curative surgery with no preoperative adjuvant therapy for advanced rectal cancer in the National Defense Medical College.

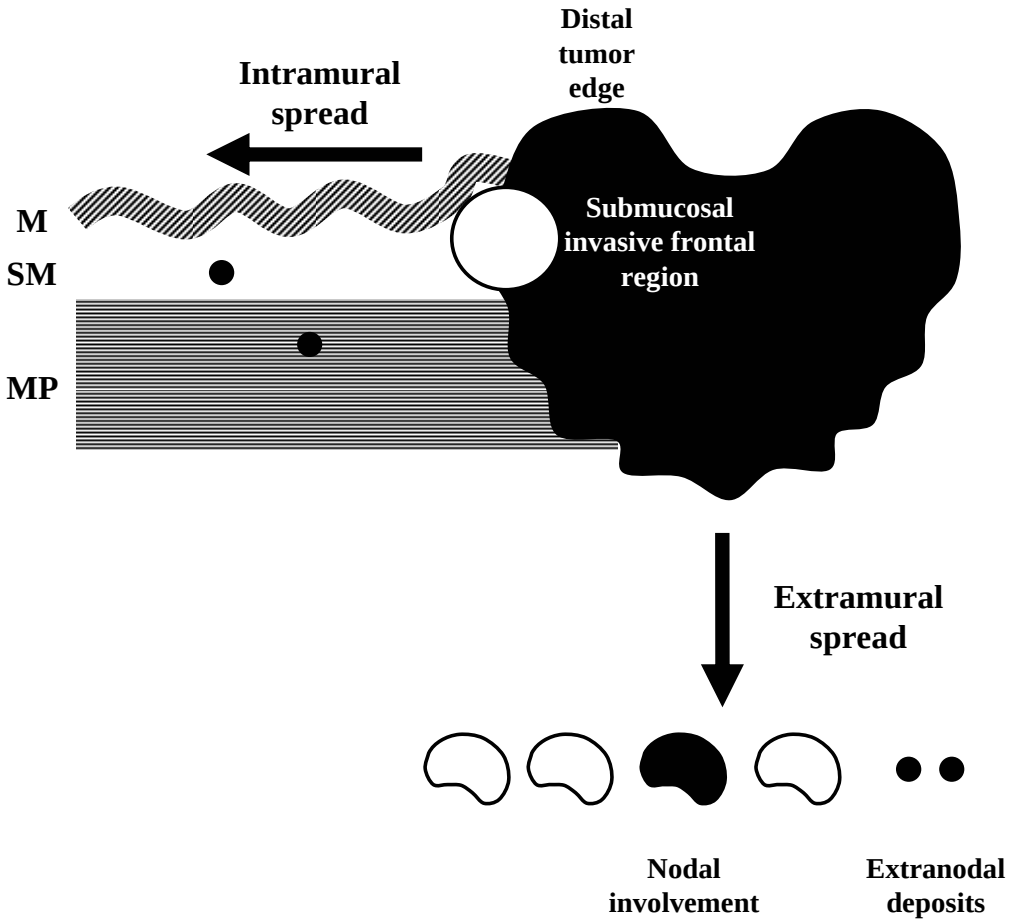


Figure 8. Submucosal invasive frontal region and extent of extramural- and intramural cancer spread. Nodal involvement, including iliac nodal involvement, extranodal deposits, and circumferential margin involvement were evaluated as the extent of extramural cancer spread. Intramural spread length was measured between the beginning of macroscopic elevation on histologic slides and the most distant microscopic cancer deposits or the most advanced point of direct spread.
M: mucosal layer, **SM**: submucosal layer, **MP**: muscularis propria.

Assessment of the Extent of Extramural Spread

Grading of rectal cancer by biopsy histology was first reported in 1929 by Stewart and Spies [42]. They stated that biopsy specimens were sufficient for the histological grading proposed by Ratkin and Broders [43] and were of value in estimating the probable course of the disease. Since that report, however, biopsy histology has not received much attention as a tool for deciding among treatment modalities. This is because the biopsied superficial parts of most colorectal cancers are not representative of the deeper parts of the tumor, resected tumors often showing more unfavorable histology than the original biopsy [44]. For instance, the incidences of tumor budding, poor differentiation and vascular invasion observed in the endoscopic biopsy specimens were no more than 10% (4%, 10% and 6%, respectively) and it was only 14% of patients who were classified as showing one or more unfavorable features

[45].The difficulty of measuring these parameters preoperatively has rendered them effective solely as postoperative indicators.

Various histological features that reflect aggressiveness of cancer are observed at the actively invasive regions in the submucosal layer, which can be estimated by transanal biopsy using exploratory excisional forceps [45]. In surgical specimens of 437 patients with advanced rectal cancers, 26% of patients were judged as positive for tumor budding in the submucosal invasive region, when five or more budding foci in a field of X200 (diameter of the field, 1000μm), where budding was most intensive, was regarded as positive. Tumor differentiation in the submucosal invasive region was classified as poor in 14% and vascular invasion was observed in 26% of patients. Patients who had none of these unfavorable features numbered 239; 124 patients had one unfavorable parameter, and 74 patients had multiple unfavorable parameters.

In **Table 2**, significant correlation between three parameters of tumor aggressiveness (tumor budding, differentiation and vascular invasion) and nodal involvement is demonstrated. The risk of lymph node metastases increased in proportion to the number of such unfavorable histological parameters. These parameters have significant impact on not only nodal status, but also other features relevant to extent of local spread, such as development of extranodal tumor deposits, which have been demonstrated to be an unfavorable prognosticator [46,47], or circumferential surgical margin involvement, which has been shown to influence local recurrence [48,49]. With respect to those patients undergoing systematic pelvic lymphadenectomy, the incidence of iliac nodal involvement was affected by three unfavorable parameters, and was only 4 % in patients with no unfavorable parameter, whereas it was 22% in patients with a single unfavorable parameter and 39% in patients with multiple unfavorable parameters (**Table 2**).

Table 2. Pathological Findings at Submucosal Invasive froNt in Surgical Specimens and the Extent of Extramural Cancer Spread

		Lymph node metastases			Lateral pelvic node metastasis		
Parameter	Categories	Absent	Present	P value	Absent	Present	P value
Tumor budding*	Negative	190	132(41%)	<0.0001	127	14(10%)	0.0002
	Positive	23	92(80%)		36	17(32%)	
Differentiation	Well-moderate	201	176(47%)	<0.0001	147	17(10%)	<0.0001
	Poor	12	48(80%)		16	14(47%)	
Vascular invasion	Absent	185	139(43%)	<0.0001	121	16(12%)	0.0113
	Present	28	85(75%)		42	15(26%)	
Number of risk factors†	0	161	78(33%)	<0.0001	96	4(4%)	<0.0001
	1	42	82(66%)		43	12(22%)	
	2-3	10	64(87%)		24	15(39%)	

* Positive: At least five budding foci had to be present in a filed of X200.

† Tumor budding (+), poor differentiation, vascular invasion (+).

The tight correlation between histology in the submucosal invasive frontal region and nodal status is well reproduced when we evaluate the submucosal invasive front in the submucosal biopsy specimens from the boundary zone between the tumor and the normal mucosa, extracted transanally (**Figure 9**). Unfavorable features can be evaluated more satisfactorily than in endoscopically excised specimens. Tumor budding was observed in 19%

of patients and 15% of patients were classified as having a poorly differentiated tumor. Vascular invasion was found in 33% of patients. The number of unfavorable histological characteristics in the submucosal layer was positively correlated with the risk of lymph node metastases (**Table 3**). Furthermore, lateral pelvic node metastasis was found in 20% of patients who had one unfavorable submucosal-layer characteristic, in 46% of patients who had multiple unfavorable characteristics, and in only 7% of patients who had no unfavorable characteristics.

Table 3. Pathological Findings at Submucosal Invasive Front in Transanal Submucosal Biopsy Specimens and the Extent of Extramural Cancer Spread

		Lymph node metastases			Lateral pelvic node metastasis		
Parameter	**Categories**	**Absent**	**Present**	**P value**	**Absent**	**Present**	**P value**
Tumor budding*	Negative	37	22(37%)	<0.0001	38	7(16%)	0.2435
	Positive	0	14(100%)		9	4(31%)	
Differentiation	Well-moderate	36	26(42%)	0.0031	42	5(11%)	0.0034
	Poor	1	10(91%)		5	6(55%)	
Vascular invasion	Absent	34	15(31%)	<0.0001	32	3(9%)	0.0182
	Present	3	21(88%)		15	8(35%)	
Number of risk factors*	0	33	11(25%)	<0.0001	28	2(7%)	0.0067
	1	4	11(73%)		12	3(20%)	
	2-3	0	14(100%)		7	6(46%)	

* Positive: At least five budding foci had to be present in a filed of X200.
† Tumor budding (+), poor differentiation, vascular invasion (+).

Morodomi et al., who reported the significance of tumor budding in needle biopsy samples in relation to lymph node metastasis, stated that the most appropriate location for a preoperative biopsy is at the edge or the base of the anal side of the tumor and that it is necessary to fully extract the actively invasive region of the submucosa [50]. As shown above, important histological features in the submucosal invasive frontal region, i.e., tumor budding, tumor differentiation and vascular invasion, reflect the extent of local cancer extension and can be estimated by preoperative transanal punch biopsy. Used together with imaging modalities such as CT and MRI, biopsy specimens taken from the submucosal invasive front could make an important contribution to decision-making in selecting the appropriate treatment strategy for patients with advanced rectal cancer.

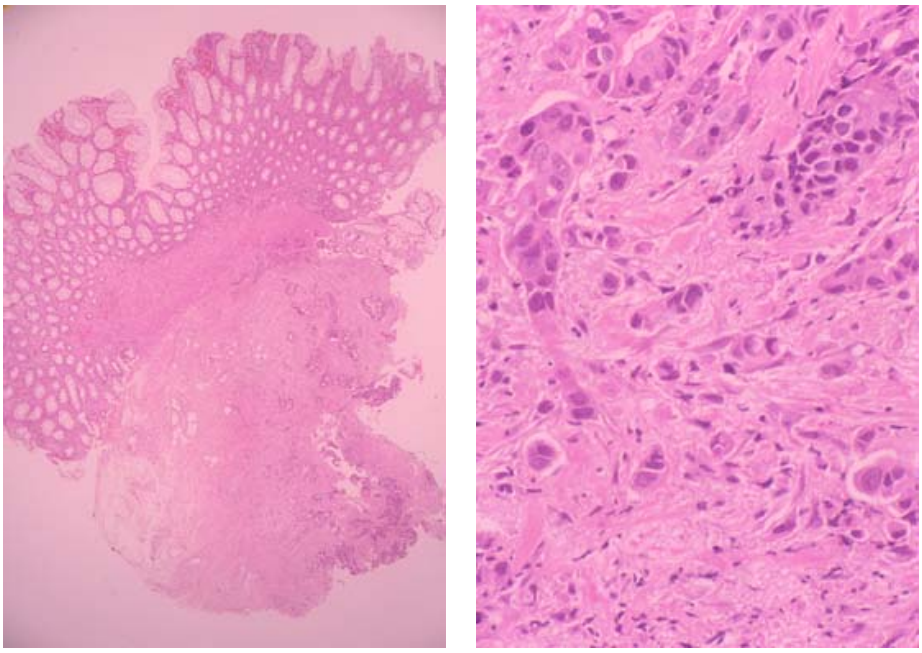


Figure 9. Transanal submucosal biopsy. A) Biopsy specimen (original magnification X6.5). Specimens were extracted transanally from the boundary zone between the tumor and the normal tissue, including the submucosal tissue, using excisional forceps (TKZ-M5538-3, Takasago, Tokyo, Japan). The average diameter of the specimens extracted was 4.4mm. B) Tumor budding observed in the submucosal biopsy specimen. (original magnification X100)

Assessment of the Possibility of Intramural Distal Spread (IM)

Intramural distal spread (IM) is a well-known mode of cancer growth that has been recognized for the last 10 decades [51]. It has been shown to occur in 4% to 24% of patients who have undergone curative surgery [41,52,53]. Although the direct-type of intramural spread has been detected using transrectal ultrasonography [54], little is known regarding the kind of preoperative parameters we can use to predict distal spread including discontinuous microscopic spread, which has long been known as the dominant mode of IM [41,52]. Although surgery has been restricted by the "two-centimeter rule" of distal clearance margin, it has often been recently argued that a distal margin of resection of 1 cm is sufficient for most cancers of the rectum, because of the rarity of extensive intramural tumor spread [41,55] and the lack of survival difference between patients with and without 1 cm of resection margin. [39,40], During the last 10 years in particular, in order to avoid colostomy, there has been pressure to find reliable markers of distal cancer spread, for cases in which sufficient distal margin (i.e. 2 cm) cannot be obtained.

Tumor differentiation has been the most frequently recommended IM risk factor [53,56], and Schiessel et al. argued that poorly differentiated tumors should be excluded from intersphincteric resection [57]. However, as endoscopic biopsies taken from a tumor include only very limited and superficial portions and do not necessarily represent the histology of the tumor as a whole, tumor differentiation is not considered effective as a guide for determining

an adequate margin [52], Similarly, although vascular invasion is strongly correlated with IM, it is not evident in endoscopic biopsies.

Table 4 demonstrates the potential risk parameters of IM evaluated on the surgical specimens and actual incidence of IM. Three histological features in the actively invasive submucosal field at the tumor's distal edge (i.e., tumor budding, differentiation, and vascular invasion) were significantly correlated with IM. Based on multivariate analysis, tumor budding and vascular invasion were the significant, independent parameters which had impact on IM, while tumor differentiation was a parameter which was almost independent of these two parameters (p=0.08). The annularity and gross appearance of tumor have been reported to show a significant correlation with IM [52,58], and the combination of annularity, gross appearance of tumor, and the histology of the submucosal region can be expected to predict IM, because of the independent association of each of these factors with IM [59]. By estimating intramural spread based on the three risk factors, i.e., presence of unfavorable histology in submucosal region, 3/4 or more annularity, and type 3 gross appearance (ulcerated type with infiltration) [23], patients who had little risk of IM could be selected; among patients showing no risk factors, only 3% of patients showed IM (**Table 5**). This group included only one patient with an intramural spread of 5 mm or more.

Table 4. Cancer Histology in Submucosal Layer at Distal Tumor Edge in Surgical Specimens and Distal Intramural Spread (IM)

Parameters	Categories(number)	IM(+) cases		Multivariate analysis‡	
		Number [IM≧10mm (%)]	P-value	OR [95%CI]	P- value
Tumor budding*	Negative (473)	36 (8%)	<0.0001	1	
	Positive (83)	23 (28%)		3.15 [1.63-6.10]	0.0007
Differentiation	Well / moderate (492)	43 (9%)	<0.0001	1	
	Poor (64)	16 (25%)		1.93 [0.92-4.04]	0.0817
Vascular invasion	Absent (491)	41 (8%)	<0.0001	1	
	Present (65)	18 (28%)		3.17 [1.62-6.18]	0.0007
Number of unfavorable histology †	0 (400)	21 (5%)	<0.0001		
	1 (110)	22 (20%)			
	2,3 (46)	16 (35%)			

* Positive: At least five budding foci had to be present in a filed of X200.

† positive tumor budding, poor differentiation, and presence of vascular invasion.

‡ logistic analysis, OR: odd's ratio CI:confidence interval.

Table 5. Risk Factors of the Distal Intramural Spread (IM) and Actual incidEnce of IM

Risk factors	Number of risk factors	Number of IM(+) tumor	Distance of IM (mm) 1-4	5-9	10-19	20-
a) Presence of unfavorable histology in submucosal region at tumor edge in surgical specimens*	0 (n=211)	7 (3%)	6	0	1	0
b) 3/4 or more annularity	1 (n=242)	22 (9%)	14	4	3	1
c) Type 3 gross appearance†	Multiple (n=103)	30 (29%)	17	5	7	1

* positive tumor budding, poor differentiation, and presence of vascular invasion.
† gross appearance: Type 1, protuberant type; Type 2, ulcerated type with clear margin; Type 3, ulcerated type with infiltration; Type 4, diffusely infiltrating type; Type 5, unclassified type.

Using the same method as the study of resected surgical specimens, the incidence and length of IM was examined based on three risk factors assessed preoperatively (i.e., presence of unfavorable histology, 3/4 or more annularity, and type 3 gross appearance). IM was observed (length: 2 mm) in only one (4%) of 27 tumors which showed no-risk factors (**Table 6**). In contrast, IM was seen with 19% tumors with one-risk factor and with 22% of tumors with multiple-risk factors. The two groups with risk factors include patients with tumors accompanied by IM at lengths of 5 mm or more. Based on such precise estimation of the required distal margin for individual tumors, which is essential to increase the sphincter-sparing procedure rate, we can prudently select cases of very low rectal cancer patients to whom the "one-centimeter rule" can be applied, and consequently, fewer colostomies in combination with intersphincteric resection may be performed.

Table 6. Number of Preoperative Risk Factors for Distal Intramural Cancer Spread (IM) and Actual Incidence of IM

Risk factors	Number of risk factors	Number of IM(+) tumor *	Distance of IM (mm) 1-4	5-9	10-19	20-
Presence of unfavorable histology in submucosal biopsy specimens*	0 (n=27)	1 (4%)	1	0	0	0
3/4 or more of annularity (diagnosed endoscopically)	1 (n=36)	7 (19%)	6	1	0	0
Type 3 gross appearance (diagnosed endoscopically)	multiple (n=27)	6 (22%)	4	1	0	1

* positive tumor budding, poor differentiation, and presence of vascular invasion.
† gross appearance: Type 1, protuberant type; Type 2, ulcerated type with clear margin; Type 3, ulcerated type with infiltration; Type 4, diffusely infiltrating type; Type 5, unclassified type.

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Chapter XI

MOLECULAR PATHOLOGIC APPLICATION AS A PREDICTOR OF LYMPH NODE METASTASIS IN SUBMUCOSAL COLORECTAL CARCINOMA: IMPLICATION OF IMMUNOHISTOCHEMICAL ALTERATIONS AT THE DEEPEST INVASIVE MARGIN

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Keywords: molecular pathological marker, submucosal colorectal carcinoma, lymph node metastasis, endoscopic treatment, MUC1, cathepsin D, MMP-7, CD34, Ki-67

Introduction

With the development and popularization of endoscopic mucosal resection (EMR), it has become easy to treat large broad-base lesions and superficial depressed lesions endoscopically [1]. Moreover, the adaptive expansion of radical EMR in cases of submucosal (sm) carcinoma of the colorectum has been studied by detailed analysis of a large number of

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accumulated cases, and the results suggest that some sm deeply infiltrating carcinomas (the so-called sm2 carcinomas [1]) can be radically cured by EMR alone [2]. In addition, with the development of various precise preliminary diagnostic tools, EMR has established an important position not only as a therapeutic tool for cancer but also as a diagnostic tool, used for so-called total biopsy. Nevertheless, there are still many cases of surgery for non-metastatic sm carcinoma of the colorectum, and we have been pursuing the development of more effective metastasis predictors.

With reference to the adaptive expansion of EMR in the treatment of sm carcinoma of the colorectum, the conditions of lymph node non-metastatic carcinomas were studied using molecular pathological markers, in addition to the conventional histopathological findings, and the possibility of their clinical application is discussed.

Criteria for EMR Radical Cure of Colorectal Sm Carcinoma Based on Conventional Histopathologic Findings in HE Sections

The conditions for local radical cure of sm carcinoma of the colorectum with EMR are (1) that no metastasis of the lesion is found, and (2) that complete local radical cure is achievable. Generally, lymph node metastasis is found in about 10% of sm carcinomas of the colorectum, and radical cure cannot be achieved by local excision alone [2]. In considering the criteria for EMR radical cure of sm carcinoma of the colorectum, the depth of sm invasion and the histologic differentiation at the leading edge of the infiltrate, determined using HE sections of resected specimens, are important [2]. With regard to the depth of sm invasion, EMR specimens do not contain muscle tissue proper and the relative classification, obtained by dividing the submucosa into three equal layers [1], cannot be used. Due to this, classification using the distance of sm infiltration measured has become an important trend. Based on detailed analysis of the results from a large number of patients, we reported that, with several limited histological differentiation degrees of the leading edge of the infiltrate, sm carcinoma does not have metastases if the measured distance of infiltration is under 1,500 μm [2]. In concrete terms, for malignancy of carcinoma of the colorectum, the most important factor is the histological degree of the differentiation of the leading edge of the infiltrate [2], rather than the predominant histologic type, on which "the General Rules of the Japanese Research Society for Cancer of the Colon and Rectum [3]" are based. We focused specifically on the histological variety of moderately differentiated adenocarcinomas, and subclassified them into “moderately” to “moderately-well (Mw)”, which is close to “well-differentiated (W)”, and “moderately-poor (Mp)”, which is close to "poorly-differentiated (Por)" (i.e., tumors in which carcinoma-sprouting were found) [2]. Based on these results, the criteria for endoscopic radical cure of sm carcinoma of the colorectum were set as histopathologic findings in resected specimens of (1) histologic differentiation at the leading edge of the infiltrate between W and Mw, and (2) sm infiltrating carcinoma with a measured distance of infiltration under 1,500 μm and no vessel invasion. Since an Mp to Por lesion has lymph node metastasis even with minimal infiltration, it is unsuitable for radical cure by EMR alone, regardless of the depth of the sm invasion [2].

Vascular invasion is an important process in lymph node metastasis and distant metastasis, and patients with positive ly and v factors do not meet the criteria for endoscopic radical cure. It should be noted that in the evaluation of HE sections, identification of the lymphatic vessel endodermis is often difficult, and therefore false negatives are not uncommon with lymphatic vessel invasion.

Molecular Organization in Colorectal Carcinoma Infiltration and Metastasis

The proliferation, infiltration and metastasis of cancers involve several steps, each of which is associated with many molecular pathologic factors (Figure 1). Adhesive molecules and anti-adhesive molecules such as E-cadherins and MUC1 [4-7], and various proteases, such as metalloproteases (MMPs) which are extracellular matrix catabolic enzymes released by interstitial cells, all participate in the process of cancer proliferation, destruction of the basal membrane and interstitial infiltration and proliferation. MMP-2, -7, -9 and -11 are specifically associated with metastasis of colorectal carcinoma. [8, 9] Moreover, VEGF, FGF and PD-ECGF are involved in the induction of angiogenesis, one of the most important factors in metastasis. Microvascular density (MVD) has also been reported to be a prognostic factor for carcinoma of the colorectum [10-12].

Studying such molecular pathological markers involves examining formalin-fixed paraffin-embedded sections of standard EMR specimens treated by relatively simple methods such as immunostaining and in situ mRNA hybridization [13-17], and the stable evaluation they provide makes these methods clinically applicable.

Moreover, the expressions of various molecular pathologic markers in sm carcinoma of the colorectum are often intratumoral site-specific, and evaluation of the leading edge of the infiltrate is essential for the evaluation of true malignancy. Preliminary evaluation of various molecular pathologic factors in the surface tissue of a lesion by means of a biopsy is therefore insufficient, and only EMR specimens, obtained by deep stump-free complete bulk excision, can be considered useful for these various molecular pathologic examinations.

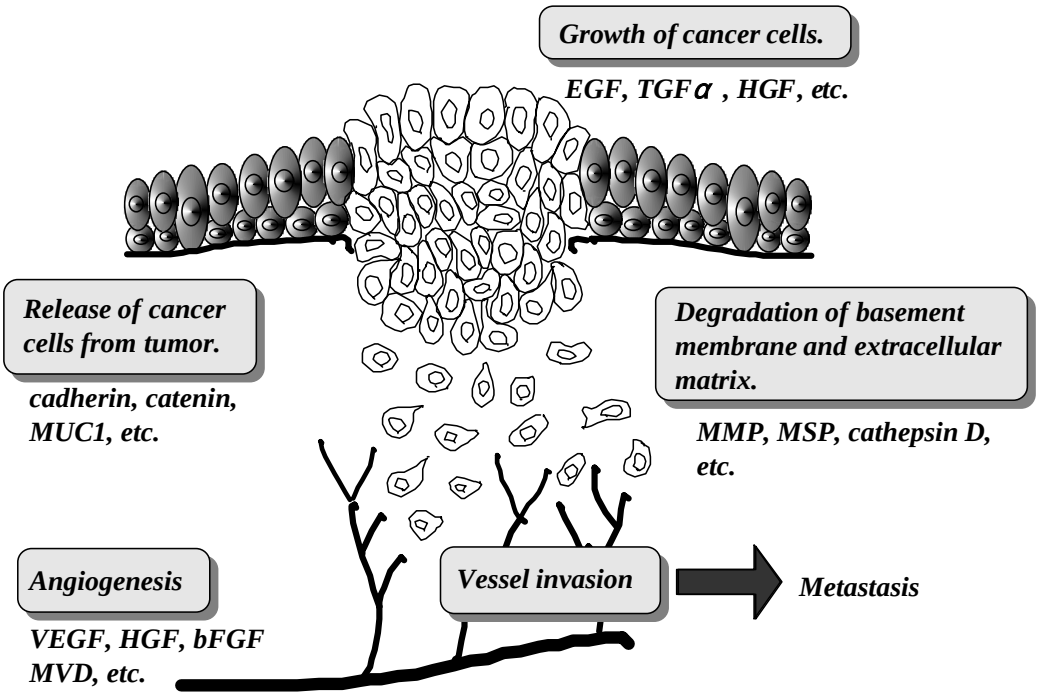

Figure 1. Molecular Organization in Colorectal Carcinoma Infiltration and Metastasis.

Prediction of Lymph Node Metastasis by Means of Molecular Pathologic Markers

As molecular pathologic markers indicative of lymph node metastasis, we studied the proliferative activity using the Ki-67 labeling index (LI), the expression of the anti-adhesive molecule MUC1 (Figure 2a), the vascular density (microvessel density: MVD) through CD34 labeling, the expression of the protease MMP-7 (Figure 2b), and the expression of cathepsin D in the cancer cells (Figure 3) and interstitial cells (macrophages).

With reference to the lymph node metastasis rate, MVD was significantly lower in the low-rate group than in the high-rate group (3.2% vs. 18.1%), and the Ki-67 LI was significantly lower in the low-rate group than in the high-rate group (6.8% vs. 14.4%). Lymph node metastasis was significantly lower in the MMP-7-negative cases than in the MMP-7-positive cases (3.4% vs. 19.4%). Lymph node metastasis was significantly lower in the MUC1-negative cases than in the MUC1-positive cases (4.0% vs. 21.9%). Lymph node metastasis was significantly lower in the cathepsin D-negative cases than in the cathepsin D-positive cases in carcinoma cells (3.4% vs. 19.9%). Nevertheless, on their own, none of these markers fully included all the non-metastatic cases, and therefore none of these can be used as the sole indicator of lymph node non-metastatic carcinomas.

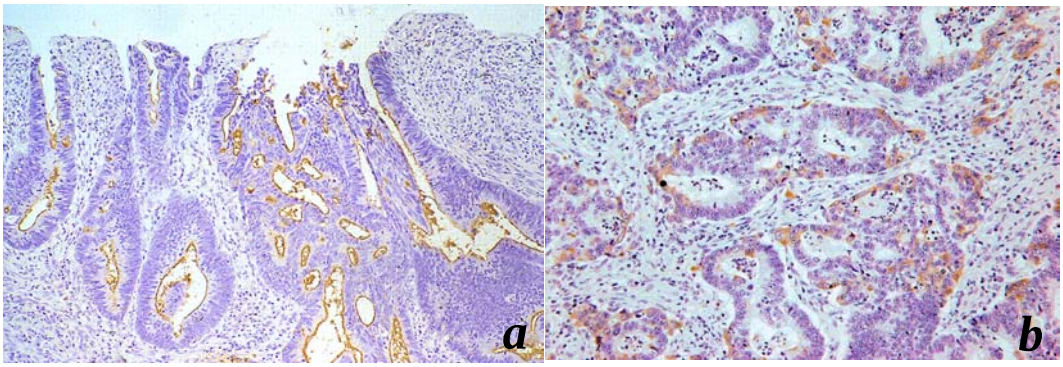

Figure 2. MUC1 and MMP-7 expression. a) MUC1 immunostaining. Diffuse MUC1 expression is seen in the basal membrane of the carcinoma. If MUC1 appeared in 10% or more of the carcinoma cells the case was determined positive. b) MMP-7 immunostaining. MMP-7 expression can be seen in the carcinoma cytoplasm. If MMP-7 appeared in 10% or more of the cancer cells the case was determined positive

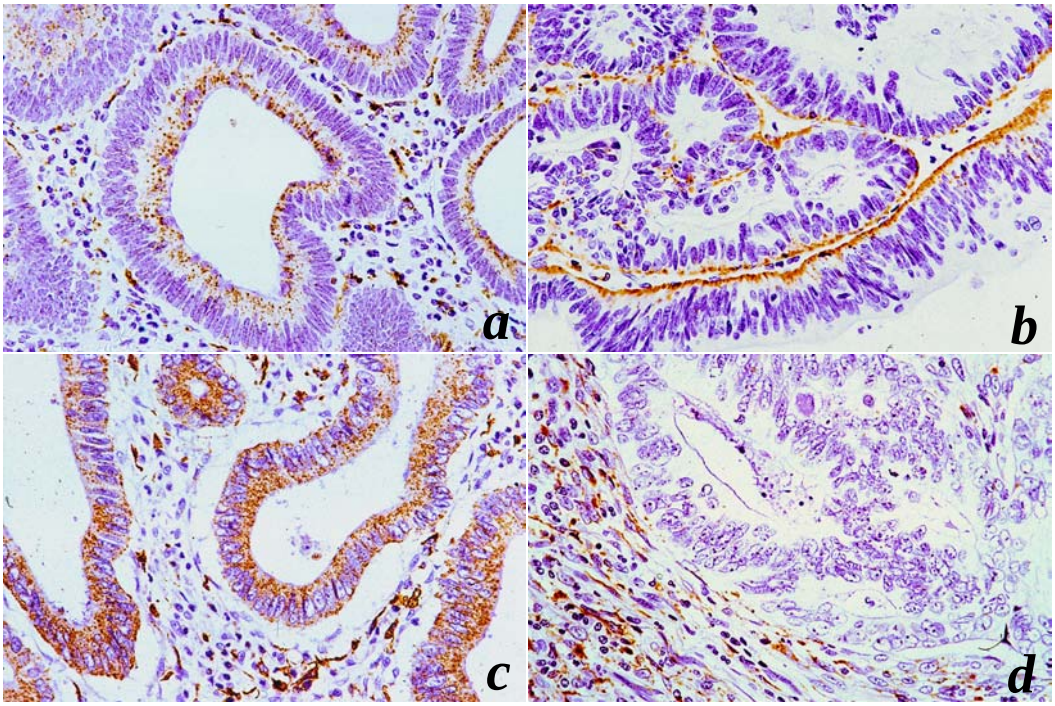

Figure 3. Cathepsin D expression. Cathepsin D immunostaining in carcinoma cells shows various patterns. a) Polarity positive-apical type (PA): Expression found in the cytoplasm apical side. b) Polarity positive-basal type (PB): Expression found in the basal side. c) Polarity negative (PN): Expression found in the cytoplasm but no polarity was found. d) No expression (NE): No expression found in the cytoplasm. Both a) and b) were determined positive. Others were determined negative.

Table 1. Incidence of Lymph Node Metastasis in Submucosal Colorectal Carcinoma in Relation to Each Molecular Pathologic Marker at the Deepest Invastive Margin

Molecular patologic markers expression		No. of cases	Cases with lymph node metastasis (%)	P-value
MVD	low	158	5 (3.2)	<0.0001†
	high	171	31 (18.1)	
Ki-67 LI	low	148	10 (6.8)	0.0279*
	high	181	26 (14.4)	
MMP-7	(-)	174	6 (3.4)	<0.0001
	(+)	155	30 (19.4)	
MUC1	(-)	201	8 (4.0)	<0.0001
	(+)	128	28 (21.9)	
Cathepsin D	(-)	118	4 (3.4)	0.0001
	(+)	136	27 (19.9)	

MVD: micro vessel density. LI: labeling index. * P<0.05. † P<0.01.

Prediction of Lymph Node Metastasis by Combining Several Molecular Pathologic Markers

(1) Prediction of lymph node metastasis based on a combination of MUC1 expression and Ki-67 LI (Table 2): No positive cases of lymph node metastasis were found among the 87 cases in the low Ki-67 LI group in which the MUC1 expression was negative. (2) Prediction of lymph nose metastasis based on a combination of interstitial cell and cancer cell cathepsin D expression, MUC1 expression and MVD (Table 3): No cases of lymph node metastasis were found among cases that were negative for interstitial cell (macrophage) cathepsin D expression and positive for cancer cell cathepsin D expression, among cases that were negative for interstitial cell cathepsin D expression and negative for MUC1 expression, and among cases that were negative for interstitial cell cathepsin D expression and in the low MVD group. The combination of all of these factors identified a group without lymph node metastasis. (3) Prediction of lymph node metastasis based on a combination of MMP-7 appearance and Ki-67 LI (Table 4): No cases of lymph node metastasis were found among the 82 cases with a low Ki-67 LI that were negative for MMP-7 expression.

The above results suggest that, although evaluation of each of these factors alone cannot be used clinically as a predictor of lymph node metastasis, by combining several factors, it may be possible to identify cases with no lymph node metastasis, without having to take into account the histopathologic factors used to date, such as the depth of sm invasion, histologic differentiation at the leading edge of the infiltrate, and vessel involvement.

Table 2. Incidence of Lymph Node Metastasis in Submucosal Colorectal Carcinoma in Relation to MUC1 Expression and Ki-67 at the Deepest Invasive Margin

MUC1 expression	Ki-67 LI	
	low	high
negative	0/87 (0 %)[a]	2/35 (6 %)[c]
positive	10/61 (16 %)[b]	24/67 (36 %)[d]

LI: labeling index. [a] vs [d]: P<0.01. [a] vs [b, c] vs [d]: P<0.05.

Table 3. Incidence of Lymph Node Metastasis in Submucosal Colorectal Carcinoma in Relation to Cathepsin D, MUC1 expression and MVD at the Deepest Invasive Margin

Molecular pathologic markers	Cathepsin D expression in interstitial cells	
	negative	positive
Cathepsin D expression in cancer cells		
PA	0/22 (0 %)	1/20 (5.0 %)
PB	0/37 (0 %)	5/39 (12.8 %)
PN	1/10 (10.0 %)	12/45 (26.7 %)
MUC1 expression		
negative	0/62 (0 %)	5/64 (7.8 %)
positive	4/37 (10.8 %)	26/91 (28.6 %)
MVD		
low	0/63 (0 %)	11/98 (11.2 %)
high	4/36 (11.1 %)	20/57 (35.1 %)

PA: polarity positive, apical type.
PB: polarity positive, basal type.
PN: polarity negative.
NE: no expression.
MVD: microvessel density.

Table 4. Incidence of Lymph Node Metastasis in Submucosal Colorectal Carcinoma in Relation to MMP-7 Expression and Ki-67 LI at the Deepest Invsive Margin

MMP-7 expression	Ki-67 LI	
	low	high
negative	0/82 (0 %)[a]	6/92 (1 %)[c]
positive	10/66 (15 %)[b]	20/89 (22 %)[d]

LI: labeling index. [a] vs [d]: P<0.01. [a] vs [b, c] vs [d]: P<0.05.

Guidelines for Treatment of Colorectal Sm Carcinoma of the Colon Using Molecular Pathologic Markers

Our concept for guidelines for the treatment of colorectal sm carcinoma can be explained based on the above results (Figure 4). Although we previously reported that sm infiltrating carcinomas with a measured distance of infiltration of under 1,500 μm are candidates for local radical cure by EMR [2], lesions in which the distance of infiltration is determined, in preliminary precise examinations, to be under 1,500 μm, are currently fully excised by EMR as a total biopsy for histologic-diagnostic purposes. Lesions whose resected specimens are found by histopathologic evaluation to meet the criteria for EMR radical cure of colorectal sm carcinoma are followed. In cases with lesions that do not meet the criteria for radical cure, the expression of the molecular pathologic markers related to lymph node metastasis are examined at the leading edge of the infiltrate and the treatment strategy is decided based on the examination results. In this manner, we are able to select, from among the non-metastatic cases that are currently still subjected to additional surgical excision, those cases that can be followed up.

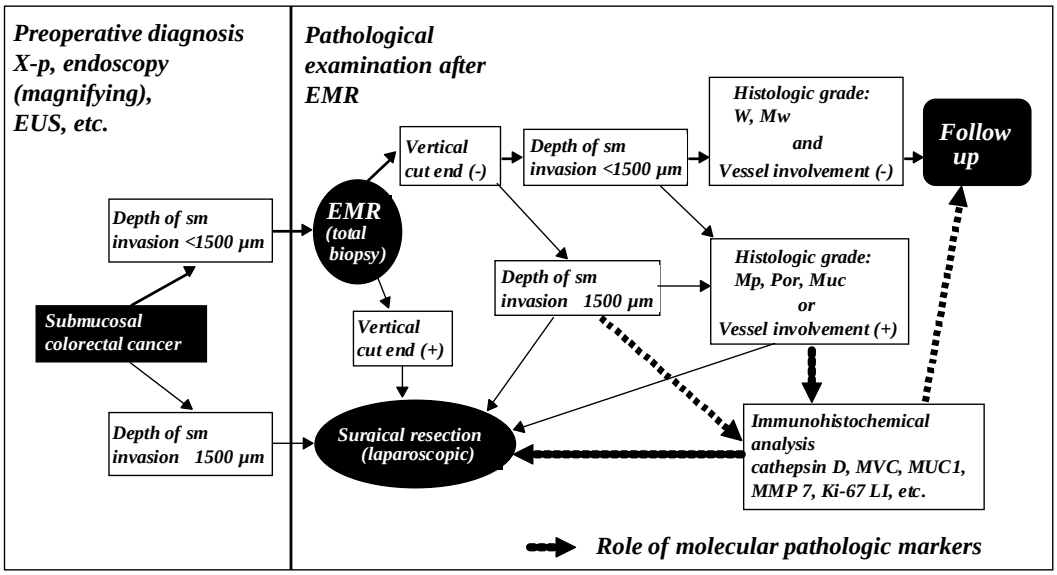

Figure 4. Management algorithm for patients with submucosal colorectal cancer.

Conclusion

We discussed the adaptive expansion of EMR to colorectal sm carcinoma using molecular pathologic markers. While EMR is a therapeutic tool, it simultaneously serves as an important diagnostic tool for total biopsy. In cases of sm carcinomas in particular, evaluation of the leading edge of the infiltrate and consideration of the histologic

differentiation are important, and EMR specimens are essential for evaluation of the true malignancy of lesions. We expect that, by investigating the expression of each molecular pathologic marker at the leading edge of the infiltrate, we will be able to predict lymph node metastasis with greater certainty, and further expand the number of colorectal sm carcinomas that can be radically cured by EMR alone. Accumulation of a worldwide database of cases in which these molecular pathologic markers were clinically used, and verification of their effectiveness will be necessary in the future.

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