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ORIGINAL ARTICLE

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Nuclear accumulation of beta-catenin in intestinal-type gastric carcinoma: correlation with early tumor invasion

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Abstract Mutation of the adenomatous polyposis coli gene, which is known to be an early event in the carcinogenesis of intestinal-type gastric carcinoma, leads to accumulation of beta-catenin. In addition, beta-catenin has been found to activate down stream signaling molecules in the wingless/Wnt pathway. In this study, the clinical significance of nuclear accumulation of beta-catenin was evaluated in gastric carcinoma. Immunohistochemical staining showed nuclear localization in 16 (12%) of 139 (94 intestinal-type and 45 diffuse-type) gastric carcinomas, and all 16 lesions with nuclear staining were intestinal-type adenocarcinomas. Of the 16 cases, 15 were in the early clinical stage. In the remaining case, the lesion had invaded the subserosal layer and showed strong nuclear staining at the invasive front. In 14 of the 16 cases with nuclear localization, there were no abnormal mobility shifts detected using polymerase chain reaction-single strand conformational polymorphism analysis. This was confirmed using direct sequencing analysis, which revealed the wild-type sequence in the 12 cases tested. Nuclear accumulation of beta-catenin did not correlate with lymph node metastasis or 5-year survival. These findings suggest that high intranuclear levels of beta-catenin protein play an important role in early tumor growth and may function in initiation of invasive processes in intestinal-type gastric carcinoma.

Keywords Gastric carcinoma · Beta-catenin · Nuclear accumulation · Mutational analysis · Invasive front

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Introduction

Beta-catenin, a vertebrate homologue of the cytosolic *Drosophila* protein armadillo [20], is involved in at least two different cellular processes. Binding of beta-catenin to the intracellular domain of the adhesion protein cadherin is essential for proper cell adhesion [9, 11], and beta-catenin is an important element in the intracellular wingless/Wnt signal transduction pathway [21]. The role of beta-catenin in signal transduction is not completely understood, but it has been shown to be involved in the wingless/Wnt developmental pathway [5, 8]. When translocated to the nucleus, beta-catenin may act as a transcription factor by binding with the T cell factor-lymphoid enhancer binding factor (Tcf-Lef) family [2] and contribute to acceleration of cell proliferation [25].

Intracellular levels of beta-catenin are mainly regulated by degradation, which is probably initiated by interaction with the adenomatous polyposis coli (APC) protein and glycogen synthetase kinase (GSK)-3 beta [22]. Thus, the amino terminus of the beta-catenin protein, with which APC protein interacts, is an important area in the regulatory mechanism of beta-catenin turnover. Deletion or mutation of this sequence in exon 3 of the beta-catenin gene may result in accumulation of beta-catenin, or phosphorylation of these residues may inhibit degradation [18]. Beta-catenin mutations in exon 3 have been reported to be frequent in various common cancers, in-

cluding colon cancer [6, 7, 13, 15, 23, 27, 28]. However, the correlation between the occurrence of beta-catenin gene mutations and accumulation of its protein is still controversial. In normal human tissue, beta-catenin has also been demonstrated in the nuclei of endometrial glandular cells in the mid- to late proliferative phase, suggesting that it plays a physiological role in the rapid turnover of the cell cycle without mutation [17].

The histologic findings and molecular pathogenesis, including APC gene mutation, of gastric carcinoma of the intestinal type are known to be very similar to those of colon cancer [10], and the beta-catenin mutation was recently reported in exon 3 in intestinal-type gastric carcinoma [19]. However, the relationship between the mutation in the beta-catenin gene and nuclear translocation has not been studied.

We performed an immunohistochemical and mutational analysis of beta-catenin in gastric cancer to determine whether the beta-catenin gene of the cells with protein accumulated in the nucleus carries a mutation, similar to some oncogenes. We also investigated the clinical significance of the nuclear accumulation of beta-catenin.

Materials and methods

Blocks of formalin-fixed, paraffin-embedded tissue from 139 patients who underwent gastrectomy for cancer at the Tokyo Medical University Hospital were retrieved from the Diagnostic Pathology Division and used in this study. Clinicopathological data, such as gender, age at the time of diagnosis, tumor size, and stage (depth of invasion), were obtained from the medical records of these patients. Information about lymph node metastasis was available in 116 patients, and 87 patients had been followed for more than 5 years. According to Lauren's classification [14] of gastric cancer, histologically, 94 of the cases were of the intestinal type and 45 were of the diffuse type.

Representative blocks of each tumor were selected for immunohistochemical analysis of beta-catenin. Deparaffinized sections for immunostaining were subjected to heat-induced antigen retrieval with a pressure sterilizer. The sections were heated in 0.01 M sodium citrate buffer (pH 6.0) for 10 min in a conventional pressure sterilizer (ALP KT-2322, Alp Industry, Tokyo, Japan). After cooling, the sections were first incubated with 10% normal swine serum (NSS) in phosphate-buffered saline (PBS) for 10 min and then with an anti-beta-catenin polyclonal antibody (Santa Cruz Biotechnology, Inc., San Francisco, Calif.) diluted to 2 µg/ml overnight. An LSABC kit (Dako Japan, Kyoto, Japan) was used as the detection system. After incubation with the secondary antibody and avidin-biotin peroxidase complex (ABC) reagent, the color reaction was developed in 1%, 3-, 3′-diaminobenzine, and 0.02% H_2O_2 in Tris buffer pH 8.0. Hematoxylin was used for counterstaining. When beta-catenin stained clearly in the nuclei of more than 10% of the tumor cells, expression was judged to be positive for nuclear staining even if it was associated with cytoplasmic and/or membranous staining. When more than 60% of the tumor cells had positive nuclei, the tumor was judged to be having diffuse pattern. When 10–60% of the tumor cells had positive nuclei, the tumor was judged to be showing a heterogeneous pattern.

Genomic DNA was extracted from the same blocks that were determined to be positive for nuclear staining using immunohistochemistry. The tumor area was microdissected from the tissue sections next to those stained by immunohistochemistry. The dissected tissues were deparaffinized by means of microwave heating in digestion buffer [50 mM Tris-HCl pH 8.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.5% Tween 20], and the proteins were digested overnight with proteinase K (10 mg/ml) at 48°C. Samples were then incubated at 100°C for 10 min for protein denaturation.

The tumor DNA was evaluated for mutations in the GSK-3 beta phosphorylation consensus motif of the beta-catenin gene using polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP). The DNA sequence of the third coding exon of the beta-catenin gene was amplified using the forward 5′-primer (GAT TTG ATG GAG TTG GAC ATG G) and the reverse 3′-primer (TGT TCT TGA GTG AAG GAC TGA G). PCR was carried out in a 50-µl reaction mixture containing 0.5 µg genomic DNA, 2.5 µl each primer, 4 µl each dNTP, 10× PCR buffer, and 0.25 µl (1.25U) *Taq* polymerase (TAKARA *Taq*, Takara Biochemicals, Kyoto, Japan). The mixture was heated for 5 min at 95°C for DNA denaturation. This was followed by 40 cycles of denaturation (at 94°C for 30 s), annealing (at 62°C for 30 s), and extension (at 72°C for 7 min) on a Gene Amp PCR System 2400 (Perkin-Elmer Corp, Norwalk, Calif.). The PCR products were electrophoresed in a 3% HTG:GTG agarose gel (3:1) and visualized by staining with bromphenol blue.

For SSCP analysis, 10 µg PCR product was denatured by adding 10 µg stop solution (95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, and 20 mM EDTA) and heated at 95°C for 5 min. The samples were then loaded onto a 12% polyacrylamide gel and run for 4 h at 12 mA at 4°C. After electrophoresis, the DNA was visualized by silver staining (SILVER STAIN II, Daiichi Pharmaceutical, Tokyo, Japan). The bands detected were cut out, and the PCR was repeated using the same primers. The re-amplified PCR products were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, Calif.) and an automated sequencer (model 377, Perkin Elmer).

The χ^2 test was used to test correlations between nuclear accumulation of beta-catenin and clinicopathologic factors, histologic type, depth of invasion, lymph node metastasis, and 5-year survival. A P value less than 0.05 was considered significant. The Fisher test was used for correlations between beta-catenin and depth of invasion, and a P value of less than 0.05 was considered to indicate a significant tendency.

Results

In the normal gastric epithelial cells, beta-catenin staining was seen along the cell membrane, but it was never seen in the nuclei (Fig. 1A). Nuclear staining for beta-catenin was observed in 16 (12%) of the 139 cases (Fig. 1B). However, the beta-catenin staining in the tumors whose nuclei were positive was focally retained along the cell membrane where beta-catenin was usually localized in the normal gastric epithelial cells. All 16 tumors that showed nuclear accumulation of beta-catenin were well-differentiated adenocarcinomas of the intestinal type.

Localization of beta-catenin in the other cases was as follows: strong cytoplasmic staining was seen in more than 10% of the tumor cells in 10 (7%) of the 139 cases (Fig. 1C), positivity with continuous distribution along the membrane was seen in 28 (20%) of the 139 cases (Fig. 1D), and discontinuous distribution was seen in 85 (61%) of the 139 cases (Fig. 1E, F). There were 39 (87%) diffuse-type carcinomas with discontinuous membranous distribution (Table 1). None of the diffuse-type carcinomas showed nuclear or cytoplasmic staining. The localization of beta-catenin was significantly correlated with tumor type ($P<0.05$).

Of the 16 cases with nuclear accumulation, nuclear staining within the tumor was heterogeneous in six cases. In all six cases, nuclear expression was found at

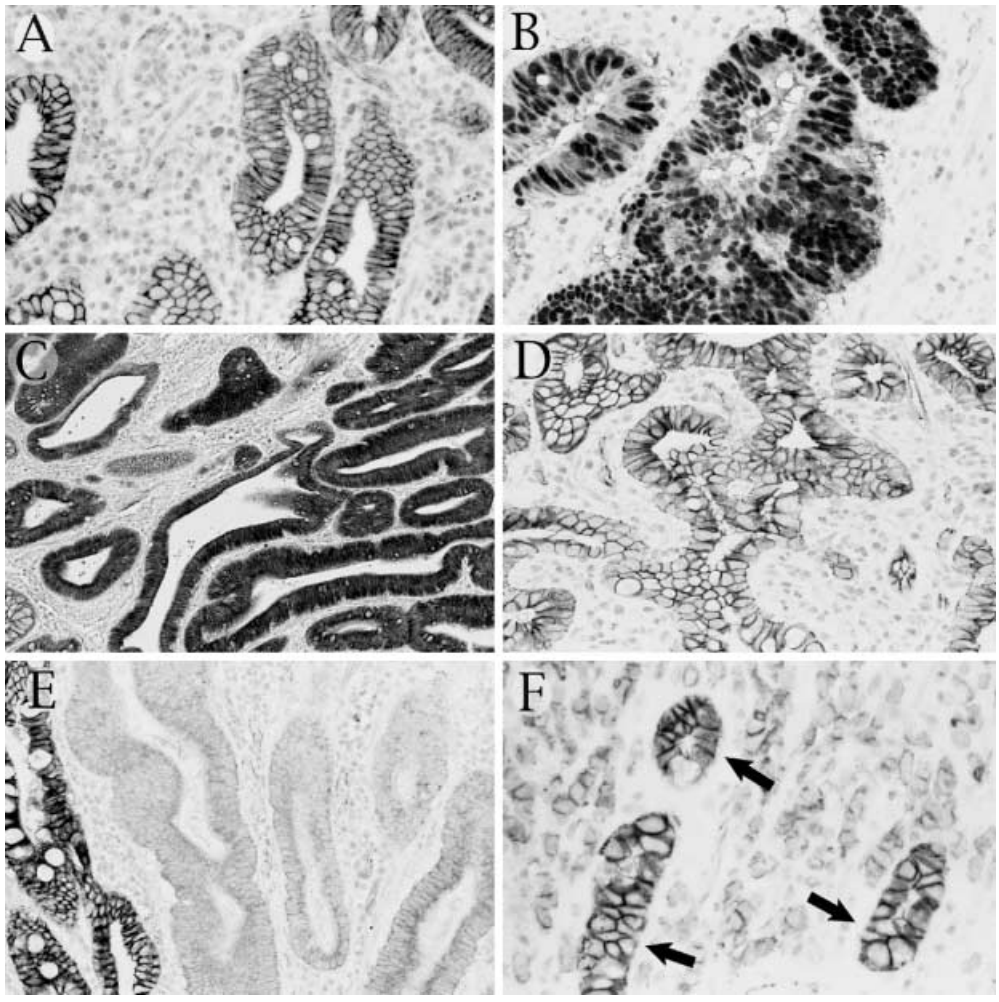

Fig. 1 Localization of beta-catenin in normal cells and cancer cells of the stomach. **A** Normal mucosa with continuous membranous distribution of beta-catenin (×40). **B** Intestinal-type adenocarcinoma with nuclear localization of beta-catenin (×400). **C** Intestinal-type adenocarcinoma with strong cytoplasmic staining (×100). **D** Intestinal-type adenocarcinoma maintaining membranous staining similar to normal mucosa (×200). **E** Intestinal-type adenocarcinoma showing uneven membranous distribution (×100). **F** Diffuse type carcinoma cells with discontinuous membranous staining invaded around the normal gastric mucosa (×100)

Table 1 Relationship between localization of beta-catenin and tumor type

Tumor type	Nucleus	Cytoplasm	Membrane	Discontinuous membrane	*P* value
Intestinal	16 (17%)	10 (11%)	22 (23%)	46 (49%)	<0.05
Diffuse	0 (0%)	0 (0%)	6 (13%)	39 (87%)	
Total	16 (12%)	10 (7%)	28 (20%)	85 (61%)	139 (100%)

the invasive front, where 80–100% of the tumor cells were positive. Other areas showed very weak nuclear expression or retained membranous staining without nuclear positivity. The heterogeneous distribution of nuclear positivity is illustrated in Fig. 2A. In the remaining ten cases, the nuclei of more than 60% of the cancer cells were positive throughout the tumors (Fig. 2B).

Table 2 lists the localization of beta-catenin, depth of invasion, and tumor size in the16 cases with nuclear staining. Only one was advanced carcinoma invading to the subserosal layer. The staining in the advanced case was heterogeneous, and nuclear accumulation was seen at the invasive front. The other 15 cases were early carcinomas, 10 of which were intramucosal carcinomas, while the other 5 had invaded the submucosa. Diffuse staining was detected in seven (70%) of the ten intramucosal carcinoma cases. Two of the five submucosal carcinomas had invaded the deeper half of the submucosa, which showed focal positivity at the invasive front. The other three showed minimal invasion and diffuse nuclear staining. The average tumor size of the six heterogeneous tumors was 4.5 cm, whereas the ten tumors with diffusely distributed nuclear positivity averaged 2.1 cm in size.

Nuclear accumulation of beta-catenin showed a significant correlation with depth of invasion (mucosal/submucosal vs muscularis propria/subserosa; $P<0.05$), but no correlation was seen with 5-year survival or lymph node metastasis (Table 3, Table 4, and Table 5). SSCP analysis was performed in 14 of the16 cases with nuclear staining, but the area of positive nuclear staining in the other two cases was too small to extract DNA for SSCP analysis. No abnormal mobility shifts were detected in the 12 cases confirmed by means of nucleotide sequencing (Fig. 3).

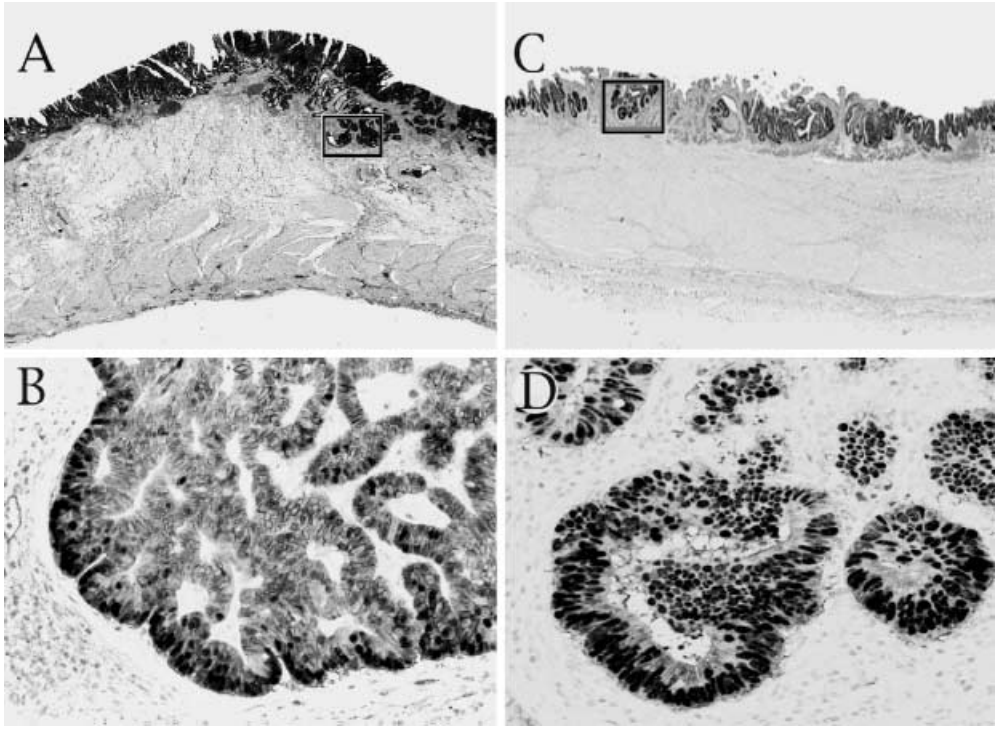

Fig. 2 Heterogeneous and diffuse distribution of nuclear positivity in gastric carcinomas. **A** Submucosal carcinoma that has invaded the deep layer shows heterogeneous distribution of nuclear positivity at the invasive front. **B** High-power view of **A**. Nuclei in the periphery of the tumor nests are positive. **C** Intramucosal carcinoma with diffuse nuclear positivity throughout the tumor. **D** High-power view of **C**. All nuclei are positive

Table 2 Immunohistochemical staining of beta-catenin, depth of invasion, and tumor size in 16 cases with nuclear positivity

Tumor samples	Immunohistochemistry			Depth[d]	Size (mm)	Average size (mm)
	Nucleus[a]	Cytoplasm[b]	Membrane[c]			
1	++	+	+	m	12	
2	++	+	++	m	15	
3	++	+	++	m	16	
4	++	+	++	m	20	
5	++	+	++	m	24	
6	++	+	++	m	32	
7	++	+	+	sm	17	
8	++	+	++	sm	20	
9	++	+	–	sm	39	21
10	+	–	–	m	10	
11	+	+	–	m	15	
12	+	–	–	m	28	
13	+	+	++	m	37	
14	+	+	++	sm	38	
15	+	–	–	sm	125	
16	+	+	+	ss	60	45

[a] ++ Diffuse pattern: more than 60% of the tumor cells have positive nuclei; + heterogeneous pattern: 10–60% of the cancer cells have positive nuclei
[b] + Cytoplasmic staining is observed in more than 10% of the cancer cells; – weak or no cytoplasmic staining
[c] ++ Membranous staining is retained in more than 80% of the cancer cells; + membranous staining is retained in 10–80% of the cancer cells; – weak or no membranous staining
[d] *m* mucosal layer; *sm* submucosal layer; *ss* subserosal layer

Table 3 Relationship between nuclear accumulation of beta-catenin and depth of invasion (early or advanced cancer). *m* mucosal layer, *sm* submucosal layer, *mp* proper muscle layer, *ss* subserosal layer

Depth of invasion	Nucleus	Membrane and cytoplasm	*P* value
m, sm (early)	15 (17%)	72 (83%)	<0.05
mp, ss (advanced)	1 (2%)	43 (98%)	
Total	16 (12%)	115 (88%)	

Table 4 Relationship between nuclear accumulation of beta-catenin and 5-year survival

5-Year survival	Nucleus	Membrane and cytoplasm	*P* value
Alive	11 (14%)	68 (86%)	0.98
Dead	1 (13%)	7 (87%)	
Total	12 (14%)	75 (86%)	

Table 5 Relationship between nuclear accumulation of beta-catenin and lymph node metastasis

Lymph node metastasis	Nucleus	Others	*P* value
+	2 (17%)	10 (83%)	0.98
–	14 (14%)	90 (86%)	
Total	16 (14%)	100 (86%)	

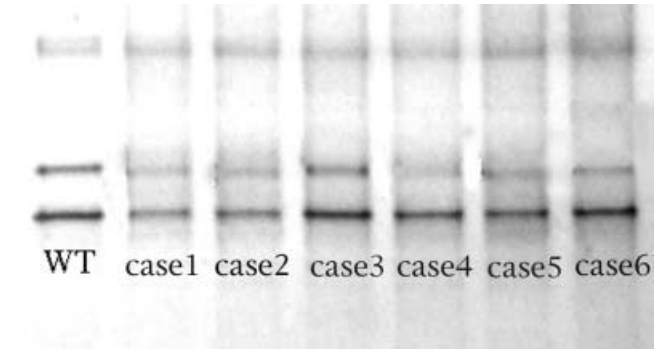

Fig. 3 Single strand conformation polymorphism (SSCP) analysis of the polymerase chain reaction (PCR) amplification of beta-catenin exon 3. *Lane WT* shows the wild-type band pattern. The next *six lanes* (six tumor samples of 12 intestinal-type gastric adenocarcinomas) also show wild-type band pattern. No mobility shifts are seen

Discussion

We analyzed the clinical significance of nuclear accumulation of beta catenin in gastric cancer, and our findings indicate that nuclear accumulation of beta-catenin dose not occur in the diffuse type of gastric adenocarcinoma but is frequent in the intestinal type, especially in the early clinical stage. Strong beta-catenin staining of the nuclei was also demonstrated at the invasive front, suggesting that the abnormal accumulation of beta-catenin plays an important role in the acquisition of invasive potential by intestinal-type gastric adenocarcinoma.

Gene alterations in intestinal-type gastric carcinoma have been shown to be very similar to those of colorectal carcinoma [16, 19], and APC gene mutations are frequent in its early phase of carcinogenesis [24]. Our finding that all gastric cancers that showed nuclear accumulation were intestinal-type adenocarcinomas is consistent with many reports that degradation of beta-catenin is closely linked to the APC gene [1, 22] and that the APC gene mutation is necessary for nuclear accumulation of beta-catenin. Thus, the APC gene needs to be directly examined in a future study to clarify the mechanism of nuclear accumulation of beta-catenin in intestinal-type gastric cancer.

No mutations in exon 3 of the beta-catenin gene were found in the cases that showed nuclear localization in our study, and the possible participation of other genes should be investigated. For example, GSK-3 beta and axin interacted with beta-catenin, resulting in the phosphorylation and/or dephosphorylation and stabilization of beta-catenin, which translocates to the nucleus. When beta-catenin was injected into the cytoplasm of living cells, it was shown to possess the ability to migrate rapidly and constitutively into the nucleus, suggesting that nuclear translocation does not always require gene alterations of beta-catenin or its binding proteins [29]. This is consistent with our observation of heterogeneous expression of nuclear accumulation of beta-catenin without gene mutation.

Strong nuclear accumulation was demonstrated at the invasive front, and it became weaker toward the center of the tumor. The 10 carcinomas that showed diffuse nuclear positivity among the 16 cases with nuclear staining were small, having an average size of 2.1 cm. The other six cases, which showed heterogeneous staining, were large (average 4.5 cm), and strong nuclear positivity was detected at the invasive front. If it is assumed that small intramucosal carcinomas are very early cancers, nuclear localization of beta-catenin must be an essential phenotype in the progression to acquire invasive growth. When beta-catenin, with its diverse functions, is translocated to the nuclei, cell-to-cell adhesion becomes loose and cancer cell increases G1 cell-cycle regulatory proteins. Indeed, we observed that cyclin D1 was strongly expressed in the nuclei of the cancer cells that accumulated beta-catenin (data not shown). At the same time, expression of MMP-7 may degrade basement membranes and the extracellular matrix to create a microenvironment that supports the initiation of invasive growth. Recently defined genes activated by beta-catenin/TCF include the cyclin D1 gene and the MMP-7 gene. Our immunohistochemical findings of beta-catenin in gastric carcinoma were similar to those of colon carcinoma in which cyclin D1 and MMP-7 were frequently overexpressed [3, 4, 26].

We have performed an immunohistochemical study of Ki-67 antigen using Mib-1 antibody in 20 cases that consisted of 10 tumors positive for nuclear staining of beta-catenin and 10 tumors negative for nuclear staining. There was no significant correlation between nuclear beta-catenin accumulation and the proliferative activity of the tumor cells estimated by Ki-67 labeling index including the invasive front (data not shown). The acceleration of the cell cycle may not always be necessary for invasive growth. We believe that high intranuclear levels of beta-catenin are an important step in progression from the autonomous growth phase to the invasive phase in gastric carcinoma of the intestinal type. Our observations suggest that after invasions, beta-catenin seems to translocate to the membrane again to construct the structure of cancer tissue. Because there were no mutations of the beta-catenin gene in cases with nuclear staining, and because peak accumulation of nuclear positivity seems to be temporary, part of the developmental or physiological signaling pathway may participate in the mechanism of invasive growth in intestinal-type gastric adenocarcinoma.

In our study, nuclear accumulation of beta-catenin did not correlate with lymph node metastasis (P=0.98) or with 5-year survival (P=0.98). These findings conflict with a recent study reporting that loss of membranous staining of beta-catenin correlated with poor survival [12]. This discrepancy is probably due to the fact that our material contained a small number of diffuse-type carcinomas that showed frequent loss of membranous distribution. With regard to the clinical significance of beta-catenin, we believe that the immunohistochemical detection of nuclear beta-catenin is a marker of early invasion in intestinal-type adenocarcinoma. If nuclear staining is detected throughout the cancerous tubules in biopsy material of suspected early cancer, then endoscopic mucosal resection should include the submucosal layer in order to totally remove early invasive lesions. Further clinicopathological studies should be carried out to clarify the significance and differences between nuclear accumulation of beta-catenin in adenoma and intramucosal carcinoma. In addition, the mechanism for the nuclear accumulation of beta-catenin for initiation of invasive growth should be clarified in small tumors, which may use a different mechanism of invasion from that of large tumors.

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