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Original article

Clinical significance of disordered beta-catenin expression pattern in human gastric cancers

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Abstract

Background. **Beta-catenin plays two distinct roles, in intercellular adhesion by E-cadherin, and in transcriptional activation via TCF/LEF. Theoretically, the former role is tumor-suppressive, while the latter is oncogenic. We investigated the involvement of beta-catenin in the histogenesis and clinical outcome of gastric cancers.**

Methods. **The expression pattern of beta-catenin was evaluated in stomach and lymph nodes from 82 patients with gastric cancer by immunohistochemistry and Western blot. Its association with E-cadherin expression and clinicopathological factors, including histological type and postoperative survival, was examined.**

Results. **Beta-catenin expression was classified into two patterns, normal (23.2%; 19 patients) and disordered (76.8%; 63 patients), the latter being subclassified as overexpressed (7.3%; 6 patients) and reduced (69.5%; 57 patients). A disordered beta-catenin expression pattern was significantly correlated with diffuse type adenocarcinoma and deep tumor infiltration ($P = 0.0154$), but was not associated with lymph node metastasis ($P = 0.7877$). E-cadherin was always expressed at the cell membrane, and disordered beta-catenin expression was significantly associated with reduced E-cadherin expression ($P < 0.0001$). On univariate analysis, the beta-catenin pattern, as well as depth of invasion and lymph node metastasis, was associated with postoperative prognosis; however, only lymph node metastasis was an independent prognostic factor on multivariate analysis. Interestingly, different disordered patterns of beta-catenin expression, both overexpressed and reduced, were associated with E-cadherin reduction and poorer postoperative survival.**

Conclusion. **Although disordered patterns of beta-catenin expression varied in gastric cancers, they were consistently associated with cancer progression.**

Key words Gastric cancer · Beta-catenin · E-cadherin · Immunohistochemistry · Western blot

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Introduction

Beta-catenin protein has two distinct roles, with different subcellular localizations. In the intercellular adherence junction, beta-catenin binds to both the cytoplasmic domain of cadherin and the amino-terminus of alpha-catenin, and is essential for confirming intercellular adhesion, by connecting cadherins to the cytoskeleton [1]. There is much evidence of E-cadherin-mediated intercellular adhesion displaying inhibitory effects against tumor invasion and metastasis [2,3]. Moreover, it has been revealed that E-cadherin gene mutation is directly involved in gastric carcinogenesis [4]. Therefore, reduced expression of beta-catenin as well as that of E-cadherin, at the cell membrane, is associated with cancer progression [5]. On the other hand, in the cytoplasm, beta-catenin binds to the transcription factor TCF/LEF and is transferred to the nucleus, where it up-regulates transcriptional activity [6]. The accumulation of cytoplasmic beta-catenin is reported to result from genetic mutation of *APC* (adenomatous polyposis coli) and Axin [7] or that of beta-catenin itself [8,9]. Overexpression of beta-catenin in the cytoplasm and/or nucleus has been reported in cancers of various organs, including the large intestine [10], endometrium, ovary [11], esophagus [12], thyroid [13], soft tissue [14], and liver [15].

Gastric cancers display histological variety, being classifiable as differentiated (intestinal) and undifferentiated (diffuse) types, and this complicates the understanding of gastric carcinogenesis. The undifferentiated (diffuse) type tends to be the more aggressive phenotype, with deeper tumor infiltration, higher frequency of peritoneal dissemination and lymph node metastasis, and poorer prognosis [16]. Although various biological markers can be used to differentiate between the two types, one simple morphological characteristic is the better preservation of intercellular adhesion in the differentiated (intestinal) type, and its disruption in

the undifferentiated (diffuse) type. We have reported the downregulation of beta-catenin and E-cadherin in undifferentiated (diffuse) type gastric cancers [17]. With respect to the differentiated type, characteristics similar to those of colorectal cancers have been reported. For example, as in colorectal cancers, E-cadherin is generally well preserved, while beta-catenin is frequently overexpressed in the cytoplasm and/or nucleus due to mutation of the *APC* gene [9] or the beta-catenin gene [6]. Two different beta-catenin expression pattern disorders, i.e., reduction in the cell membrane and overexpression in the cytoplasm, have been demonstrated, but their clinical significance in gastric cancers is not clear. In this study, we classified the expression patterns of beta-catenin by immunohistochemistry, confirmed them by cell fractionation and Western blot, and correlated them with clinicopathological factors, including postoperative survival. The involvement of beta-catenin expression pattern disorders in gastric carcinogenesis is discussed.

Patients and methods

Patients

Eighty-two patients with gastric cancers, evaluated as having pStage IB, II, IIIA, or IIIB tumors by pathological assessment [18], but excluding T4 tumors, underwent partial or total gastrectomy at the Department of Surgery and Clinical Oncology Graduate School of Medicine, Osaka University Medical School, between August 1986 and September 1995. Curative operation with no residual tumor (R0) with D2 lymph node dissection [18] was performed for all patients, with none having received preoperative therapy. The age of the patients (64 men and 18 women) ranged from 26 to 83 years (mean, 61.6 ± 10.9 years).

The resected stomach and lymph nodes were fixed in 10% buffered formaldehyde, and hematoxylin-and-eosin-stained sections were examined under a microscope. The tumors were histologically evaluated according to the *Japanese classification of gastric carcinoma* [19]. Immediately after surgical excision, before fixation, small pieces of tumor and adjacent noncancerous mucosa were snap-frozen and kept in a deep freezer at −80 °C.

Analysis of cellular proteins by Western blot

Fifty-mg pieces of the tumor and normal mucosa were minced and washed three times with phosphate-buffered saline (PBS). They were gently centrifuged and soaked in 500 µl of hypotonic buffer (1 mM $NaHCO_2$) containing 2 mM phenylmethanesulfonyl fluoride (PMSF) and 1 µg/ml of aprotinin for 30 min and centrifuged at 15 000 *g* for 30 min. The supernatant was mixed with half its volume of 3× loading buffer (30% glycerol, 6% sodium-dodecylsulfate [SDS], 62.5 mM Tris-HCl, pH 6.8) (soluble fraction). The pellet was mixed with 750 µl of 1× loading buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) and clarified by centrifugation at 15 000 *g* for 15 min (insoluble fraction). Both the soluble and the insoluble fractions were boiled for 5 min in the presence of 2-mercaptoethanol, and their protein concentrations were measured with a protein assay kit (Bio-Rad, Hercules, CA, USA). Fifty-µg aliquots of protein from the soluble and insoluble fractions were subjected to electrophoresis separately on 7.5% SDS-polyacrylamide gels and transferred to a Protran nitrocellulose transfer membrane (Schleicher and Schuell, Dassel, Germany). After 5% skim-milk blocking, the membranes were first incubated with primary antibodies, then with secondary antibodies coupled with horseradish peroxidase (HRP) (Amersham, Arlington Heights, IL, USA), and finally visualized with an enhanced chemiluminescence (ECL) reagent (Amersham). Bands on ECL-exposed X-OMAT AR film (Eastman Kodak, Rochester, NY, USA) were analyzed by densitometric scanning, using Image Quant (Molecular Dynamics, Sunnyvale, CA, USA). Each membrane contained 25 µg of total cell extract from SW480 cells, which was used to standardize the amount of E-cadherin and beta catenin on a densitometer. Western blots of both E-cadherin and beta-catenin showed some extra bands below the full-length bands. These bands, which have been reported previously and were confirmed to be degraded products by absorption testing, were not included in the densitometric quantification.

Antibodies

The following antibodies were used in this study for Western blot and immunohistochemistry: mouse monoclonal antibody (MAb) against beta-catenin (1:2000 dilution; Transduction Laboratories, Lexington, KY, USA), which recognizes amino acid residues from 571 to 781 of beta-catenin, and mouse MAb against human E-cadherin (1:1000 dilution, Takara Shuzo, Shiga, Japan), which recognizes amino acid residues from 129 to 138, the calcium-binding domain of E-cadherin.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor samples were sliced 4-µm-thick on poly-1-lysine coated glass slides, deparaffinized in xylene, rehydrated, boiled in antigen-retrieval buffer [20], and washed with water. They were then treated with 0.3% hydrogen peroxide in

methanol for 30 min to inhibit endogenous peroxidase. After incubation with 3% normal horse serum to block nonspecific binding, the sections were incubated with the primary antibodies against beta-catenin and E-cadherin at 4 °C overnight, and then with biotinylated anti-mouse IgG (Vecstain ABC kit; Vector, Burlingame, CA, USA) for 30 min at room temperature, and then with ABC Elite reagent (Vector) for 30 min at room temperature. Between incubations, sections were washed with PBS. Color was developed with diaminobenzidine tetrahydrochloride, supplemented with 0.04% hydrogen peroxide and counterstained with Mayer's hematoxylin (Chroma, Stuttgart, Germany).

Evaluation of immunohistochemical staining

Beta-catenin was expressed both in the cytoplasm and in the cell membrane. When more than 90% of cells showed both strong membrane staining and weak cytoplasmic staining, this was classified as the "normal" pattern, and samples with other staining patterns were classified as "disordered" pattern. In the disordered pattern, when more than 90% of the cancer cells showed stronger cytoplasmic staining than normal cells, they were classified as "overexpressed", regardless of the membrane expression. The remaining samples were classified as "reduced". In these samples, when membrane staining was observed in 10% to 90% of the cancer cells, the samples were classified as "membrane-preserved", and when the membrane staining was less than 10%, they were classified as "cytoplasmic-preserved". E-cadherin was expressed only in the cell membrane, and its expression pattern was classified as either "preserved" or "reduced", according to the frequency of positively stained cells, as we reported previously. Assesment of the staining was performed independently by two observers (T.U. and Y.D.). Final agreement was obtained using a two-head microscope; cases for which agreement could not be reached were excluded from this study.

Statistical analysis

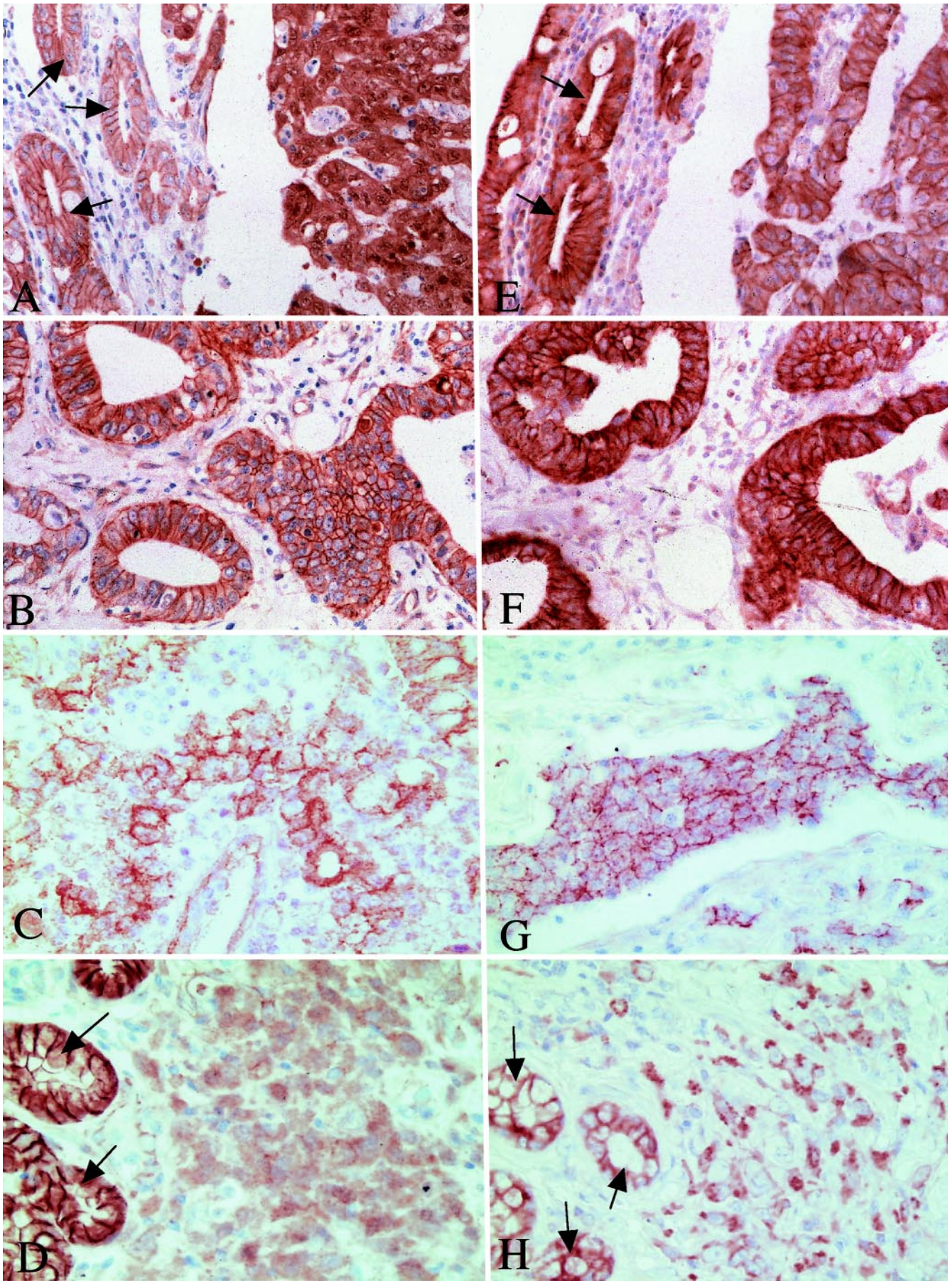
Associations between two parameters were analyzed by the Mann-Whitney *U*-test or Spearman's rank correlation test. For continuous parameters, differences between two groups were analyzed by Student's *t*-test. Postoperative survival was analyzed by the Kaplan-Meier method and statistically assessed by the log-rank test. Multivariate analysis for survival curves was performed with the Cox proportional hazard model. In all analyses, *P* values of less than 0.05 were considered statistically significant. All statistical analyses were performed using the software package Stat View ver. 5.0 (Abacus Concepts, Berkeley, CA, USA).

Results

Various expression patterns of beta-catenin in gastric cancer tissue

In the noncancerous epithelium of gastric mucosa, beta-catenin was expressed strongly at the cell-cell adhesion site and was also observed faintly in the cytoplasm, as reported for epithelium of the large intestine [21]. Chronic gastritis, whether associated with *Helicobactor pylori* or not, did not affect the expression pattern of beta-catenin (data not shown). Infection with *H. pylori* was checked by microscopy and urease test. As for the cancer tissues, in 19 cases (23.2%), beta-catenin was strongly expressed at the intercellular adhesion site, as in normal mucosa, and was classified as being of the normal pattern, while the remaining 63 cases (76.8%) were classified as being of the disordered pattern. Of these 63 cases, 6 exhibited strong beta-catenin expression in the cytoplasm, sometimes with nuclear staining, and were classified as the overexpressed pattern. The remaining 57 cases were classified as being of the reduced pattern. Weak expression of beta-catenin was observed in the cell membrane in 30 of these 57 cases (reduced-membrane-preserved), while diffuse cytoplasmic staining was apparent in the other 27 cases (reduced-cytoplasmic-preserved) (Fig. 1).

Tissue samples from representative cases were separately extracted into soluble and insoluble fractions, using hypotonic buffer without any detergent. As we have reported, the soluble fraction contained most of the cytosolic protein, while the insoluble fraction had membrane proteins, such as E-cadherin [12] (Fig. 2). In gastric mucosa and cancerous tissue, beta-catenin was distributed in both the soluble and insoluble fractions. In noncancerous mucosa, as well as in normal pattern gastric cancers, more beta-catenin was observed in the insoluble fraction than in the soluble fraction. In the overexpressed pattern cancers, the total amount of beta-catenin was much higher than that in noncancerous mucosa (more than twofold: 1738.9 ± 579.9 vs 4123.1 ± 1779.8 arbitrary units). In the reduced pattern samples, the amount of beta-catenin in the insoluble fraction was lower than that in the noncancerous mucosa (962 ± 320 vs 479 ± 219 arbitrary units). Reduced pattern (cytoplasmic-preserved) samples showed a relatively large amount of beta-catenin in the soluble fraction (1085 ± 614 arbitrary units), while reduced pattern (membrane-preserved) samples did not express much beta-catenin in the soluble fraction (395 ± 87 arbitrary units). The amount of E-cadherin was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correlated with that of beta-catenin in the insoluble fraction, except in the overexpressed pattern samples, in which there was more insoluble beta-catenin, but less E-cadherin than in noncancerous mucosa.

Association of beta-catenin expression and clinicopathological factors

Table 1 summarizes the relationship between the beta-catenin expression pattern and the histological type of gastric cancer, classified as differentiated type (including well and moderately differentiated adenocarcinoma) and undifferentiated type (including poorly differentiated adenocarcinoma [solid and diffuse growth], signet ring cell carcinoma, and mucinous carcinoma). Some associations were noted between the beta-catenin expression pattern and histological type. The normal pattern was more frequently observed in the differentiated type tumors (16/41 patients; 39%) than in the undifferentiated type (3/41 patients; 7%), and, likewise, four of six of the overexpressed pattern were found among the differentiated types. In contrast, the reduced pattern was more frequent in the undifferentiated type (36/41; 88%) than in the differentiated type (21/41; 51%). As for subcellular localization of beta-catenin in the reduced pattern cases, the reduced pattern (membrane-preserved) was more frequent among the differentiated type (16/21; 76%), while the reduced pattern (cytoplasmic-preserved) was dominant in the undifferentiated type (22/36; 61%), especially in poorly differentiated adenocarcinoma with diffuse growth (17/21; 85%).

This study included pStage IB, II, and III patients but not patients with T4 cancer in order to reveal the

Table 1. Relationship between beta-catenin expression pattern and histological type of gastric cancer

	Differentiated type		Undifferentiated type				
	Well	Moderately	Poorly (solid)	Poorly (diffuse)	Signet ring	Mucinous	Total
Normal[a]	8	8	1	0	1	1	19
Overexpressed[a]	4	0	1	0	0	1	6
Reduced[a]	4	17	5	20	11	0	57
Membrane-preserved[a]	(4)	(12)	(4)	(3)	(7)	(0)	(30)
Cytoplasmic-preserved[a]	(0)	(5)	(1)	(17)	(4)	(0)	(27)
Total	16	25	7	20	12	2	82

[a] See text for explanation of terms

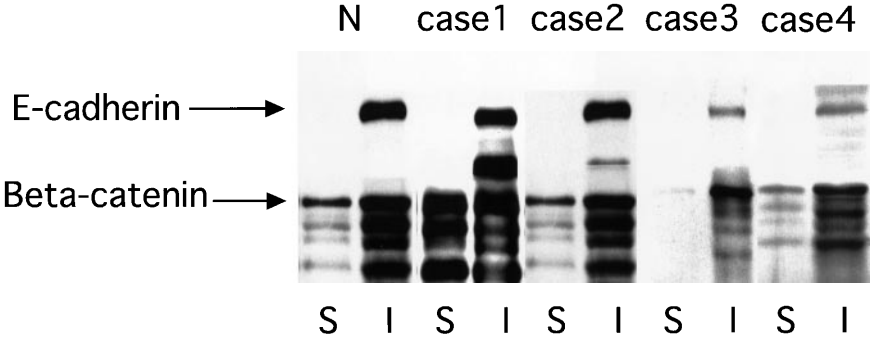

Fig. 2. Western blotting of E-cadherin and beta-catenin in representative gastric cancers and noncancerous mucosa. Tissue samples were separately extracted from soluble (*S*) and insoluble (*I*) fractions and subjected to immunoblotting for E-cadherin (120kD) and beta-catenin (88kD). Representative cases are shown from noncancerous mucosa (*N*) and cancers with different beta-catenin expression patterns, i.e., case 1 (overexpressed), case 2 (normal), case 3 (reduced-membrane-preserved), and case 4 (reduced-cytoplasmic-preserved)

Fig. 1A–H. Representative immunohistochemical expression of beta-catenin (**A**,**B**,**C**, and **D**) and E-cadherin expression (**E**,**F**,**G** and **H**) in cancer tissues from gastric cancer patients. The expression patterns of beta-catenin were classified as overexpressed (**A**), normal (**B**), reduced (membrane-preserved) (**C**), and reduced (cytoplasmic-preserved) (**D**). Expression of E-cadherin was classified as preserved (**F**) and reduced (**E**,**G**, and **H**). *Arrows* indicate adjacent noncancerous epithelium in the cancer tissue. ×200

biological malignant potential of disordered beta-catenin expression pattern. The relationship between the beta-catenin expression pattern and pathological factors is shown in Table 2. The normal pattern was observed in 44% (4/9) of pT1 cases and in 5.2% (1/19) of pT3 cases, with the correlation being significant ($P = 0.0426$). More than half of the patients in this series (54/82; 66%) exhibited lymph node metastasis; however, there was no significant correlation between beta-catenin status and lymph node metastasis. Advanced tumor stage was less frequently found with normal pattern beta-catenin expression, although the relationship was not significant ($P = 0.0750$).

Association with E-cadherin expression

Because beta-catenin is strongly bound to the cytoplasmic domain of E-cadherin at the intercellular adherence junction, we examined E-cadherin expression along with beta-catenin expression by immunohistochemistry. As we reported previously, E-cadherin is always expressed at the cell membrane, and cancer cells frequently show weaker staining for it, never stronger staining, than noncancerous epithelium [22,23]. As shown in Table 3, there was a strong association between reduced E-cadherin expression and disordered beta-catenin expression pattern ($P < 0.0001$). Interestingly, the disordered beta-catenin pattern, whether overexpressed, reduced (membrane-preserved) or reduced (cytoplasmic-preserved), did not affect the E-cadherin expression pattern.

Table 2. Relationship between beta-catenin expression pattern and pathological factors

	Beta-catenin expression			
	Normal	Disordered	Total	
Depth of invasion				
pT1	4	5	9	
pT2	14	40	54	
pT3	1	18	19	$P = 0.0426$
Lymph node metastasis				
pN0	6	22	28	
pN1	10	20	30	
pN2	3	21	24	$P = 0.826$
pStage				
IB	10	21	31	
II	6	18	24	
IIIA	2	15	17	
IIIB	1	9	10	$P = 0.0750$
Total	19	63	82	

Postoperative survival

Postoperative survival curves, by the Kaplan-Meier method (Fig. 3), were calculated according to beta-catenin expression. Lymph node metastasis ($P = 0.0045$; data not shown) and disordered beta-catenin expression status ($P = 0.0405$) were significantly associated with poor postoperative prognosis in patients from pStage IB to pStage IIIB. Although tumors with deeper infiltration showed poorer prognosis, the trend was not significant ($P = 0.0742$). The subclassification of the beta-catenin disordered expression pattern into overexpressed, reduced pattern (membrane-preserved), and reduced pattern (cytoplasmic-preserved) did not reflect postoperative survival. Multivariate analysis showed only lymph node metastasis (hazard ratio, 5.265; $P = 0.0026$) as a significant prognostic factor, with beta-catenin expression (hazard ratio, 3.061; $P = 0.0739$) and depth of invasion (T2 [hazard ratio, 5.547; $P = 0.1$] and T3 [hazard ratio, 5.961; $P = 0.0944$]) not being significant.

Discussion

Based on immunohistochemistry and immunoblot findings, we classified beta-catenin expression in gastric cancers into four patterns: normal, overexpressed, reduced pattern (membrane-preserved), and reduced pattern (cytoplasm-preserved). This variety in expression pattern is characteristic of beta-catenin, probably because it has two distinct functions, a relatively static one as a structure protein involved in cell adhesion and a dynamic one as a signal transducer

Table 3. Relationship between beta-catenin expression pattern and E-cadherin expression pattern in gastric cancer

	Beta-catenin expression					
	Normal	Disordered	Overexpressed	Membrane-preserved	Cytoplasmic-preserved	Total
E-cadherin expression						
Preserved	13	8	(1)	(4)	(3)	21
Reduced	6	55	(5)	(26)	(24)	61
Total	19	63	(6)	(30)	(27)	82
						$P < 0.0001$

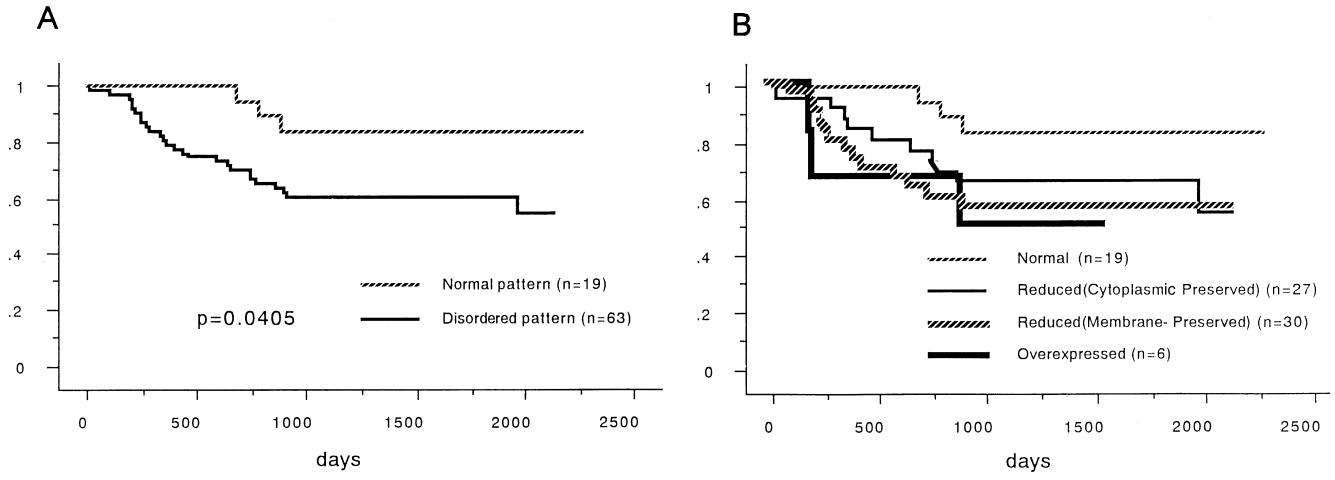

Fig. 3A,B. Postoperative survival curves (Kaplan-Meier method) of gastric cancer patients according to beta-catenin expression. **A** Postoperative survival curves according to beta-catenin expression classified as normal or disordered. **B** Postoperative survival curves according to disordered expression patterns of beta-catenin, subclassified as overexpressed, reduced (membrane-preserved), and reduced (cytoplasmic-preserved)

involved in oncogene transcription. These two aspects of beta-catenin function may, in part, explain the following expression patterns.

The overexpressed pattern showed strong beta-catenin staining in the cytoplasm as well as in the nucleus, and abundant beta-catenin protein especially in the soluble fraction. The overexpressed pattern is frequently observed in colorectal adenomas and cancers [24], because approximately 70% of them show mutation of the *APC* gene [25], and some of the remaining cases show mutation of exon 3 of the beta-catenin gene [26]. Mutations of these genes are also detected in differentiated (intestinal) type gastric cancers, but not in the undifferentiated (diffuse) type [27,28]. However, the overexpressed pattern was not found frequently in differentiated type gastric cancers (4/41 patients). Thus, the overexpressed pattern seems to be associated with, but not to be necessary for the differentiated (intestinal) phenotype.

The reduced pattern (membrane-preserved) was frequently observed in moderately differentiated adenocarcinoma and signet ring cell carcinoma. These cancers frequently showed reduced E-cadherin expression. Mutation of the E-cadherin gene and methylation of its promoter region are reported to be involved in the mechanism of reduced E-cadherin expression [29]. In addition, we and others recently have shown the involvement of proteolysis of extracellular and cytoplasmic domains of E-cadherin in reduced E-cadherin expression [30,31]. Recently, the transcriptional factor Snail has been reported to repress E-cadherin gene transcription. When E-cadherin disappears from the membrane, beta-catenin loses its ability to bind to the cell membrane, is released into the cytosol, and immediately degraded by the ubiquitin degradation system [32].

The reduced pattern (cytoplasmic-preserved) may result from translocation of beta-catenin from the membrane to the cytoplasm. We have shown tyrosine phosphorylation of beta-catenin in colorectal cancers in vivo [10] and in esophageal cancer cell lines upon epidermal growth factor (EGF) stimulation [33], and others have demonstrated it by hepatocyte growth factor (HGF) treatment or v-src transfection [34,35]. This tyrosine phosphorylation of beta-catenin results in the disruption of E-cadherin-mediated adhesion, cytoplasmic localization of beta-catenin, and cancer cell invasion [36–39]. In the present study, the reduced pattern (cytoplasm-preserved) was commonly observed in undifferentiated type cancers, especially in poorly differentiated diffuse adenocarcinomas. In agreement with this, Akimoto et al. [40] have recently reported tyrosine phosphorylation of beta-catenin, and its cytoplasmic localization in undifferentiated type gastric cancers. They also found concomitant overexpression of K-sam a receptor type tyrosine kinase, with these beta-catenin alterations. Another candidate involved in the disordered pattern of beta-catenin expression is integrin-linked kinase, which is reported to recruit beta-catenin from the cell adhesion site to the cytoplasm without affecting the total amount of beta-catenin [41,42].

The effect of cytoplasmic/nuclear beta-catenin in cancer progression is not clear. Theoretically, it should facilitate cancer progression, because various oncogenes, including c-*myc* [43], cyclin D1 [44], matrix metalloproteinase-3 (*MMP-3*) [45], and fibronectin [46],

have been reported to be target molecules of the TCF/LFF transcription factor and beta-catenin. Supporting this idea is the association of cytoplasmic beta-catenin with poor prognosis in breast cancers. However, other studies have demonstrated that nuclear beta-catenin is not associated with a poor clinical outcome [47]. It was recently reported that cytoplasmic beta-catenin had no prognostic significance in gastric cancers [39]. Another possibility is that cytoplasmic/nuclear beta-catenin may not be involved in cancer progression, but may be involved in the early stage of carcinogenesis, as seen in colorectal polyps [36].

Stomach and lymph specimens from patients with gastric cancers from pStage IB to pStage III, but not T4, were examined in this study, because standard D2 lymph node dissections were performed for these patients. For the remaining patients at either pStage I or IV, postoperative survival depended on the anatomical spread of cancer; this is, the clinical stage, rather than the biological malignant potential of the cancer, such as that shown by disordered beta-catenin expression pattern. Multivariate analysis showed that beta-catenin expression was not an independent prognostic factor; therefore, the clinical application of beta-catenin evaluation to gastric cancer treatment remains questionable. However, as mentioned above, the disordered patterns of beta-catenin expression varied. Further study with a large number of patients is needed to reveal the significance of each disordered expression pattern.

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