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Original Article

Membranous staining of β-catenin and E-cadherin expression in patients with gastric cancer

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Abstract: Background: β-catenin and E-cadherin are adhesion molecules that promote metastatic potential through epithelial-mesenchymal transition (EMT). Although they have not been extensively studied in gastric cancers, they represent potential testable prognostic markers. Methods: We explored the association between the immunohistochemical expression of these markers and clinicopathologic parameters by retrospectively reviewing 205 cases of gastric cancer from tissue microarrays (TMA). A method was developed to evaluate for membranous staining of β-catenin and E-cadherin using grading criteria that characterized both the intensity of staining and the percentage of cells with loss of staining. Results: Weak membranous expression of E-cadherin and β-catenin were associated with worse overall survival ($p<0.05$). Abnormal expression of E-cadherin and β-catenin were significantly associated with each other ($p<0.01$). Loss of and/or weak membranous staining for both E-cadherin and β-catenin was significantly associated with advanced cancer stage T2-T4 (versus stage T1, $p<0.05$) and tumors that are negative for *H pylori* infection ($p<0.05$). In addition, loss of and/or weak membranous staining for β-catenin was significantly associated with poorly differentiated tumors ($p<0.05$), diffuse Lauren-type gastric tissue ($p=0.02$), and tumors with a significantly higher rate of lymphovascular invasion ($p=0.02$). Conclusion: Loss of/weak membranous expression of both E-cadherin and β-catenin was associated with poorer overall survival rates and clinicopathologic parameters that indicated an aggressive clinical behavior. β-catenin shows significant associations with more clinical parameters, making it a better biomarker than E-cadherin. In our multivariate analysis, weak intensity of staining of β-catenin was an independent prognostic factor for survival and may be a useful immunohistochemical prognostic marker for patients with gastric cancer.

Keywords: β-catenin, E-cadherin, gastric cancer, clinicopathology

Introduction

Gastric cancer is a major health issue, the fourth most common cancer, and the second leading cause of cancer death worldwide [1]. Early gastric cancers are often asymptomatic, and the patients typically have advanced incurable diseases at the time of presentation. The overall five year survival rate of patients with resectable gastric cancer is 32%, which is comparatively low to other malignancies [2]. Most patients die of a metastatic disease or recurrence even after surgical intervention, radiation and chemotherapy. The ability of the tumor to metastasize and to have invasive growth is a major impediment to effective treatment. The epithelial-mesenchymal transition (EMT) is the process in which epithelial cells lose cell polarity from the disappearance of differentiated junctions and instead gain mesenchymal phenotypic traits [3]. This process is normally seen in embryonic cells but is reactivated in cancers, enabling invasive growth and metastatic potential. Once the cells lose their junctions, they are able to disseminate locally or through the bloodstream and lymphatic system [4, 5].

E-cadherin is a transmembrane glycoprotein that is expressed in normal gastric mucosal cells, where it mediates cell-to-cell adhesion

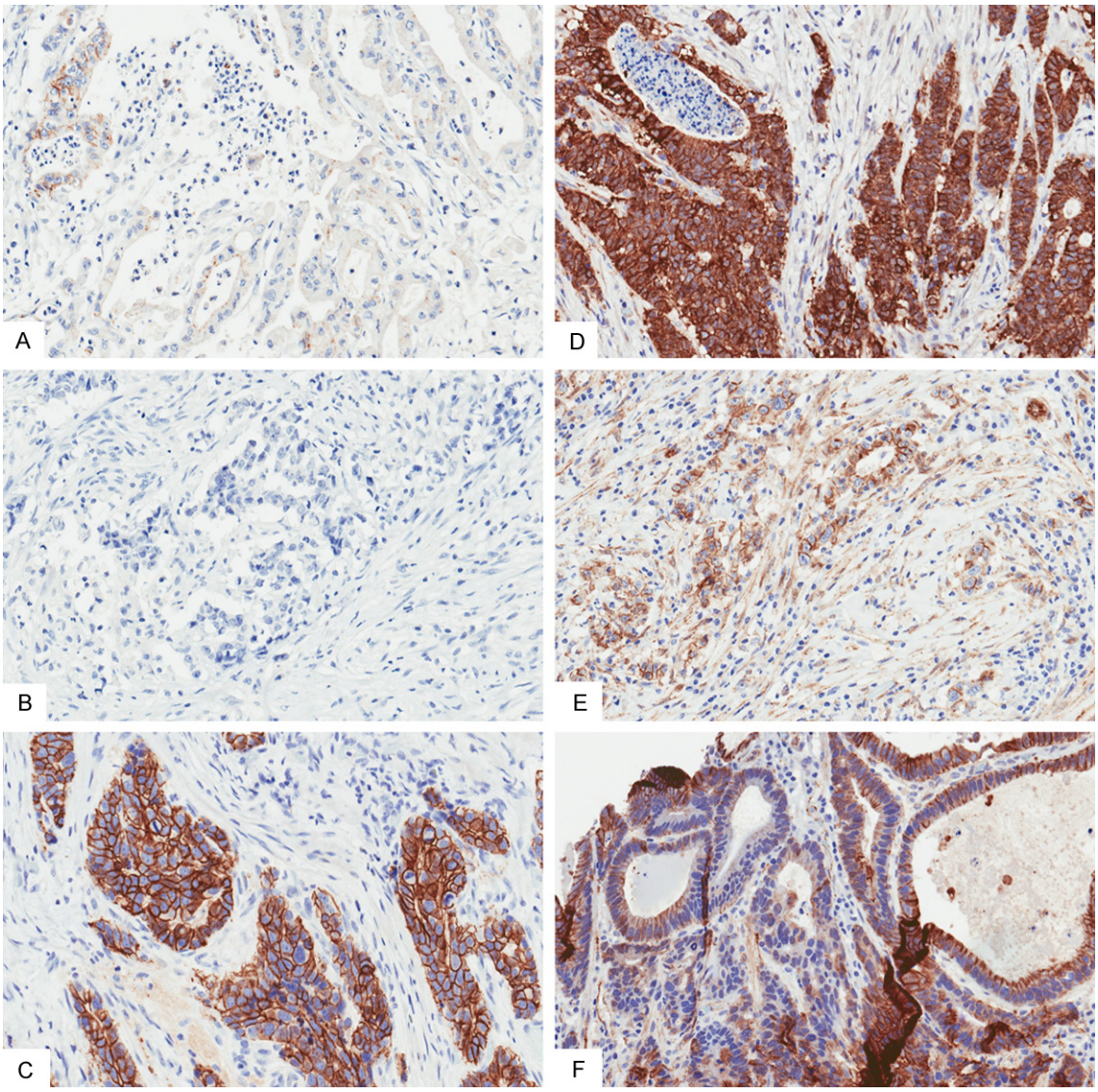


Figure 1. E-cadherin (A-C) and beta-catenin (D-F) staining patterns in a variety of gastric cancers showing staining heterogeneity (20× magnification). (A, B) Examples of tumors with loss of membranous staining in 10% of cells and weak intensity of staining, (C, D) Examples of preserved membranous staining with normal intensity of staining, (E, F) Note the heterogeneity in the staining of some tumors with weak intensity of staining throughout but no areas with loss of staining (E), while other tumors have focal strong intensity of staining but with large areas with loss of membranous staining (F).

and suppresses the cell's ability to invade [4]. However, during carcinogenesis, the factor can be downregulated or inactivated, leading to redistribution of E-cadherin from the cell membrane to the cytoplasm [6]. Loss or downregulation of membranous E-cadherin is a major event during EMT and potentiates the cancer cell to have invasive growth and to metastasize. E-cadherin repression has been analyzed in several carcinomas, including those of colorectum [7], pancreas [8], bladder [9], prostate [10], endometrial, and breast where it has been associated with a more aggressive tumor phenotype.

β-catenin is a cytoplasmic protein that directly interacts with E-cadherin and plays a critical role in the E-cadherin mediated adhesion [11]. Truncation of the β-catenin protein has been reported to cause loss of E-cadherin-dependent intercellular adhesiveness [12]. In vitro, when β-catenin changes its expression pattern from

Table 1. Association between E-cadherin and β-catenin expression intensity in gastric tumors

β-catenin expression intensity	E-cadherin expression intensity		
	Weak	Normal	*p*-value
Weak	99	7	<0.01
Normal	60	39	

Table 2. Association between E-cadherin and β-catenin loss of membranous staining in gastric tumors

β-catenin membranous staining	E-cadherin membranous staining		
	Loss	Preserved	*p*-value
Loss	158	11	<0.01
Preserved	18	18	

membranous to cytoplasmic, a similar change is seen in E-cadherin localization, resulting in cell-cell junctional disorders and potential for metastasis [12]. Futhermore, immunohistochemical analysis of E-cadherin and β-catenin has demonstrated that abnormal E-cadherin and β-catenin expression is closely associated with metastasis of gastric carcinoma.

The goal of our study was to explore the association between the immunohistochemical expression patterns of these EMT markers and clinicopathologic parameters, including survival in patients with gastric cancer.

Methods

Patient selection

We retrospectively reviewed 205 cases of gastric cancer patients who were previously diagnosed at The University of Texas MD Anderson Cancer Center. There were 84 female patients and 121 male patients, with an overall mean age of 61 years (range 26 to 89 years) at the time of diagnosis. Of the 205 patients, 87 had moderately differentiated tumors and 118 had poorly differentiated tumors; 43 had early gastric cancer (T1), and 162 had advanced gastric cancer (T2-T4). Complete demographic and clinical data were collected. This study was approved by the Institutional Review Board of the University of Texas M.D. Anderson Cancer Center.

Tissue microarray construction

Tissue microarrays were constructed using formalin-fixed, paraffin-embedded archival tissue blocks with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) [13]. Their matching hematoxylin and eosin-stained (H&E) slides were retrieved, reviewed, and screened for representative tumor regions. For each patient, three cores of tumor were sampled from representative areas using a 1.0-mm punch.

Immunohistochemical analysis

Immunohistochemical stains were performed on 4-μm unstained sections from the tissue microarray blocks using primary mouse monoclonal antibodies as follows: anti-E-cadherin (1:7000 dilution, HECD-1, Invitrogen, Carlsbad, CA, USA) and anti-β-catenin (1:1500 dilution, 14, BD Biosciences, San Jose, CA, USA). Antigen retrieval was performed on the tissue sections at 100°C in a steamer containing either Tris-EDTA buffer for 20 minutes (E-cadherin) or Citrate buffer for 10 min (β-catenin) after deparaffinization. The sections were then incubated with the primary antibody at 35°C for 15 min. Subsequently, they were immersed in 3.0% hydrogen peroxidase at 35°C for 5 min to block the endogenous peroxidase activity. A primary enhancer solution was then applied to the slides and incubated at 35°C for 8 minutes. The sections were then incubated with secondary anti-mouse immunoglobulin at 35°C for 8 min. Diaminobenzidine (DAB) was used as a chromogen and hematoxylin was used for counterstaining. The immunohistochemically stained slides of tissue microarrays were examined using standard light microscopy (Olympus BX40, Tokyo, Japan) with the appropriate positive and negative controls.

Scoring of immunohistochemical stains

The staining results were scored semiquantitatively by a pathologist (M.X.), who was blinded to the clinicopathologic data. The immunohistochemical results showed a heterogeneous pattern of staining in some tumors where there was only very focal strong staining or moderate but diffuse staining as shown in **Figure 1**. Therefore, slides were evaluated for membra-

Table 3. Association between multiple clinicopathological factors and expression of both E-cadherin intensity of staining as well as loss of membranous staining in gastric tumors

Clinicopathologic factor	Intensity of E-cadherin staining [cases (%)]		*p* value	Membranous E-cadherin staining [cases (%)]		*p* value
	Weak	Normal		Loss	Preserved	
Total	159	46		176	29	
Age						
≤60	62 (39.0)	20 (43.5)	0.61	74 (42.1)	8 (27.6)	0.16
>60	97 (61.0)	26 (56.5)		102 (58.0)	21 (72.4)	
Gender						
Male	97 (61.0)	24 (52.2)	0.31	105 (59.7)	16 (55.2)	0.69
Female	62 (39.0)	22 (47.8)		71 (40.3)	13 (44.8)	
Tumor Depth						
Early cancer (T1)	28 (17.6)	15 (32.6)	0.04	32 (18.2)	11 (38.0)	0.02
Advanced cancer (T2-T4)	131 (82.4)	31 (67.4)		144 (81.8)	18 (62.1)	
Histologic Type						
Well to moderate	67 (42.1)	20 (43.5)	0.86	72 (41.0)	15 (51.7)	0.31
Poor and others	92 (57.9)	26 (56.5)		104 (59.1)	14 (48.3)	
Lauren Type						
Intestinal	55 (34.6)	17 (37.0)	0.86	59 (33.5)	13 (44.8)	0.49
Mixed	14 (8.8)	3 (6.5)		15 (8.5)	2 (6.9)	
Diffuse	90 (56.6)	26 (56.5)		102 (58.0)	14 (48.3)	
H pylori infection						
Negative	155 (97.5)	40 (87.0)	0.01	168 (95.5)	27 (93.1)	0.64
Positive	4 (2.5)	6 (13.0)		8 (4.6)	2 (6.9)	
Lymphovascular Invasion						
Negative	44 (27.7)	10 (21.7)	0.45	49 (27.8)	5 (17.2)	0.26
Positive	115 (72.3)	36 (78.3)		127 (72.2)	24 (82.8)	
Lymph Node Metastasis						
Negative	40 (25.2)	14 (30.4)	0.57	44 (25.0)	10 (34.5)	0.36
Positive	119 (74.8)	32 (69.6)		132 (75.0)	19 (65.5)	
Distal Metastasis						
Negative	129 (81.1)	40 (87.0)	0.51	144 (81.8)	25 (86.2)	0.8
Positive	30 (18.9)	6 (13.0)		32 (18.2)	4 (13.8)	

nous staining using two methods to account for the heterogeneity of the tumors and to more precisely characterize the staining patterns. The first method was a system similar to a HercepTest, and the second method was a test based on the percentage of cells with loss of membranous staining.

When grading the intensity of staining, we used a system similar to the HercepTest modified for gastric cancer [14, 15]. Normal intensity of staining was considered as strong, complete membranous staining or basolateral staining similar to a HercepTest result of 3+. Weak intensity of staining was considered as none, faint or moderate, incomplete membranous or incomplete basolateral staining similar a HercepTest result of 0-2+. Cases with strong but incomplete membranous or basolateral staining were considered to have weak intensity of staining.

The slides were then reevaluated based on the percentage of cells with loss of membranous staining. Cases considered to have no preserved membranous staining had >90% cells with evidence of at least trace membranous staining (this includes both complete and incomplete membranous and basolateral staining). Cases that were considered to have loss of membranous staining had ≥10% cells with

Table 4. Association between multiple clinicopathological factors and expression of both β-catenin staining intensity and loss of staining in gastric tumors

Clinicopathologic factor	Intensity of β-catenin staining [cases (%)]		*p*-value	Membranous β-catenin staining [cases (%)]		*p*-value
	Weak	Normal		Loss	Preserved	
Total	106	99		169	36	
Age						
≤60	48 (45.3)	34 (34.3)	0.12	73 (43.2)	9 (25.0)	0.06
>60	58 (54.7)	65 (65.7)		96 (56.8)	27 (75.0)	
Gender						
Male	68 (64.2)	53 (53.5)	0.16	100 (59.2)	21 (58.3)	1
Female	38 (35.9)	46 (46.5)		69 (40.8)	15 (41.7)	
Tumor Depth						
Early cancer (T1)	13 (12.3)	30 (30.3)	0.001	29 (17.2)	14 (38.9)	0.006
Advanced cancer (T2-T4)	93 (87.7)	69 (69.7)		140 (82.8)	22 (61.1)	
Histologic Type						
Well to moderate	37 (35.0)	50 (50.5)	0.03	64 (37.9)	23 (63.9)	0.005
Poor and others	69 (65.1)	49 (49.5)		105 (62.1)	13 (36.1)	
Lauren Type						
Intestinal	31 (29.3)	41 (41.4)	0.13	53 (31.4)	19 (52.8)	0.02
Mixed	8 (7.6)	9 (9.1)		13 (7.7)	4 (11.1)	
Diffuse	67 (63.2)	49 (49.5)		103 (61.0)	13 (36.1)	
H pylori infection						
Negative	105 (99.0)	90 (90.9)	0.008	161 (95.3)	34 (94.4)	0.69
Positive	1 (0.9)	9 (9.1)		8 (4.7)	2 (5.6)	
Lymphovascular Invasion						
Negative	20 (18.9)	34 (34.3)	0.02	42 (24.9)	12 (33.3)	0.3
Positive	86 (81.1)	65 (65.7)		127 (75.2)	24 (66.7)	
Lymph Node Metastasis						
Negative	22 (20.8)	32 (32.3)	0.08	42 (24.9)	12 (33.3)	0.3
Positive	84 (79.3)	67 (67.7)		127 (75.2)	24 (66.7)	
Distal Metastasis						
Negative	84 (79.3)	85 (85.9)	0.27	137 (81.0)	32 (88.9)	0.34
Positive	22 (20.8)	14 (14.1)		32 (19.0)	4 (11.1)	

complete negative membranous staining. The same grading criterion was used for both E-cadherin and β-catenin.

Statistical analysis

The patients' follow-up information was extracted from the medical records. We associated the abnormal expression of E-cadherin and β-catenin with age (≤60 vs >60), sex, tumor depth (T1 vs T2-T4), histologic type (well to moderate vs. poor), Lauren type gastric tissue (intestinal vs. diffuse vs. mixed), presence of *H pylori* infection, lymphovascular invasion, lymph node metastasis, and distal metastasis. We also obtained information on the patients' overall survival rates. The recurrence information was updated whenever patients came to the clinic for a follow-up visit.

Chi-square analysis or Fisher's exact tests were used to compare categorical data. The survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. Overall survival (OS) was calculated as the time from the date of diagnosis to patient death or last follow-up. The prognostic significance of clinicopathologic characteristics was determined using multivariate Cox proportional hazards regression analysis. All statistical analyses were performed using Statistical Analysis

Table 5. Summary of significant associations between abnormal E-cadherin and β-catenin membranous expression and clinicopathologic parameters

Staining Patterns	Clinicopathologic Factors
Weak β-catenin intensity	Advanced Cancer (T2-T4) > Early Cancer (T1)
	Poorly Differentiated > Well or Moderate Differentiated
	Negative *Helicobacter pylori* Infection
	Lymphovascular Invasion
Loss of β-catenin staining	Advanced Cancer (T2-T4) > Early Cancer (T1)
	Poorly Differentiated > Well or Moderate Differentiated
	Diffuse Lauren Type > Intestinal or Mixed
Weak E-cadherin intensity	Advanced Cancer (T2-T4) > Early Cancer (T1)
	Negative *Helicobacter pylori* Infection
Loss of E-cadherin staining	Advanced Cancer (T2-T4) > Early Cancer (T1)

System 9.1 software (for Windows, SAS Institute, Cary, North Carolina). We used a two-sided significance level of 0.05 for all statistical analyses.

Results

Abnormal expression of E-cadherin and β-catenin in gastric cancer

Weak membranous expression of E-cadherin and β-catenin was seen in 159 (77.6%) and 106 (51.7%) patients, respectively. In addition, when separately looking at tumors where ≥10% of cells had loss of membranous staining, 176 (85.9%) and 169 (82.4%) patients had loss of staining with E-cadherin and β-catenin respectively. When abnormal expression of E-cadherin was associated with abnormal expression of β-catenin, the results were significant both in terms of intensity and loss of membranous staining as shown in **Tables 1** and **2** ($p<0.01$).

Clinicopathologic association of E-cadherin and β-catenin in gastric cancer

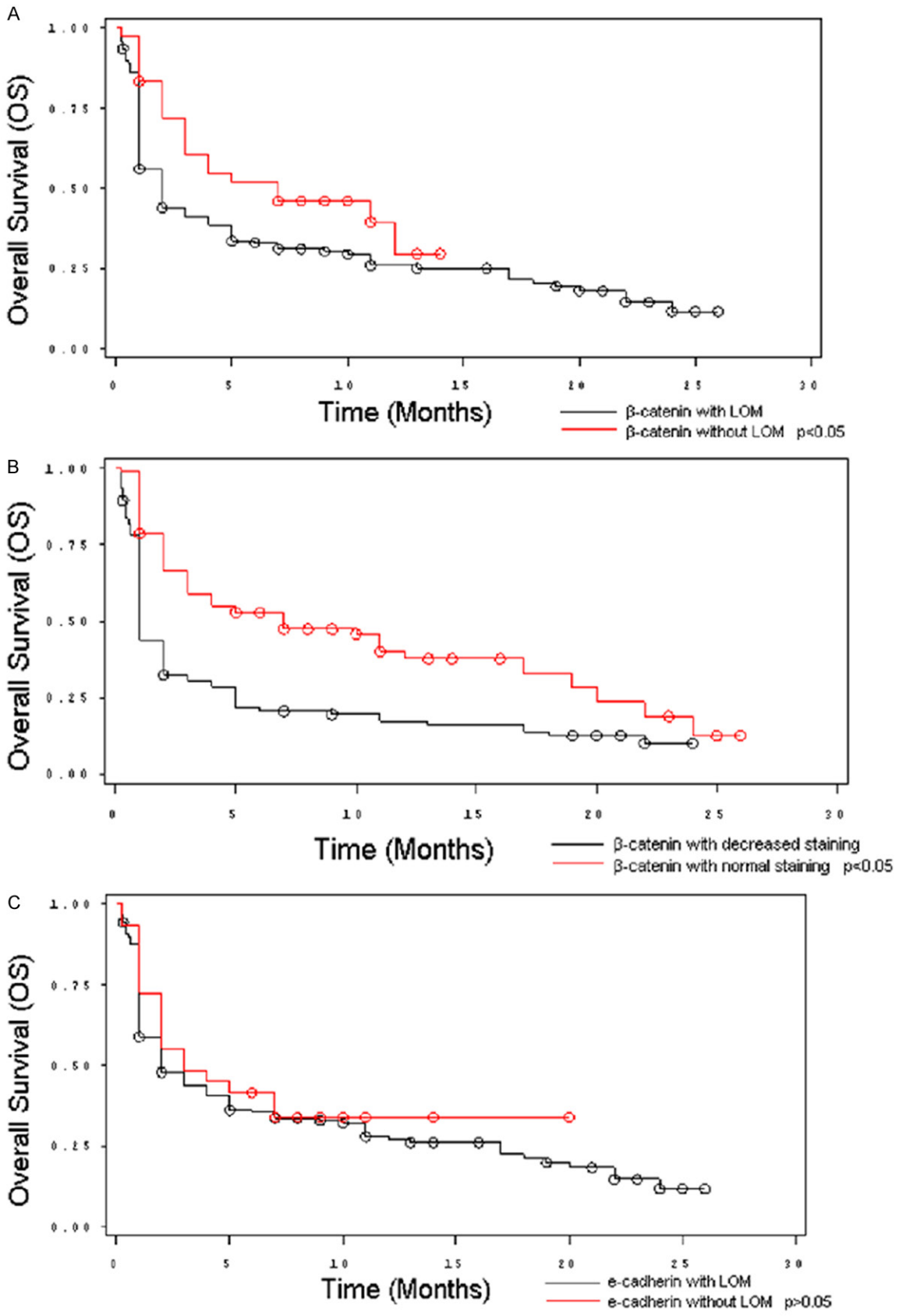
Loss of and/or weak membranous staining for both E-cadherin and β-catenin was significantly associated with advanced cancer stage T2-T4 (versus stage T1, $p<0.05$) and tumors that are negative for *H pylori* infection ($p<0.05$). In addition, loss of and/or weak membranous staining for β-catenin was significantly associated with poorly differentiated tumors ($p<0.05$), diffuse Lauren-type gastric tissue ($p=0.02$), and tumors with a significant higher rate of lymphovascular invasion ($p=0.02$). We found no additional association of the abnormal expression of E-cadherin and β-catenin with any other clinicopathologic parameters including age, sex, lymph node and distal metastasis ($p>0.05$). This information is presented in **Tables 3** and **4** with significant findings summarized in **Table 5**.

Survival analysis of E-cadherin and β-catenin in gastric cancer

Patients with tumors that had either weak membranous expression of E-cadherin or weak membranous expression of β-catenin had worse overall survival ($p<0.05$). In addition, loss of membranous staining of β-catenin was also associated with poorer overall survival ($p<0.05$). There was a trend towards poorer overall survival in patients with loss of membranous staining of E-cadherin; however, this was not statistically significant ($p>0.05$). The Kaplan-Meier curves for overall survival comparing patients with normal expression with those with abnormal expression of E-cadherin and β-catenin are shown in **Figure 2**. We then performed a multivariate analysis as shown in **Table 6**. The results showed that weak β-catenin expression was an independent predictor of short survival ($p<0.05$).

Discussion

The clinical significance of EMT has been reported in many diverse human tumors, where it enables invasive growth and increases metastatic potential [12]. Several studies have evaluated EMT phenotype in metastatic sites where EMT-derived migratory cells that established colonies at distant metastatic sites were shown to lose the mesenchymal phenotype via mesenchymal-epithelial transition (MET) during


A
Overall Survival (OS)
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
Time (Months)
β-catenin with LOM
β-catenin without LOM p<0.05
B
Overall Survival (OS)
Time (Months)
β-catenin with decreased staining
β-catenin with normal staining p<0.05
C
Overall Survival (OS)
Time (Months)
e-cadherin with LOM
e-cadherin without LOM p>0.05

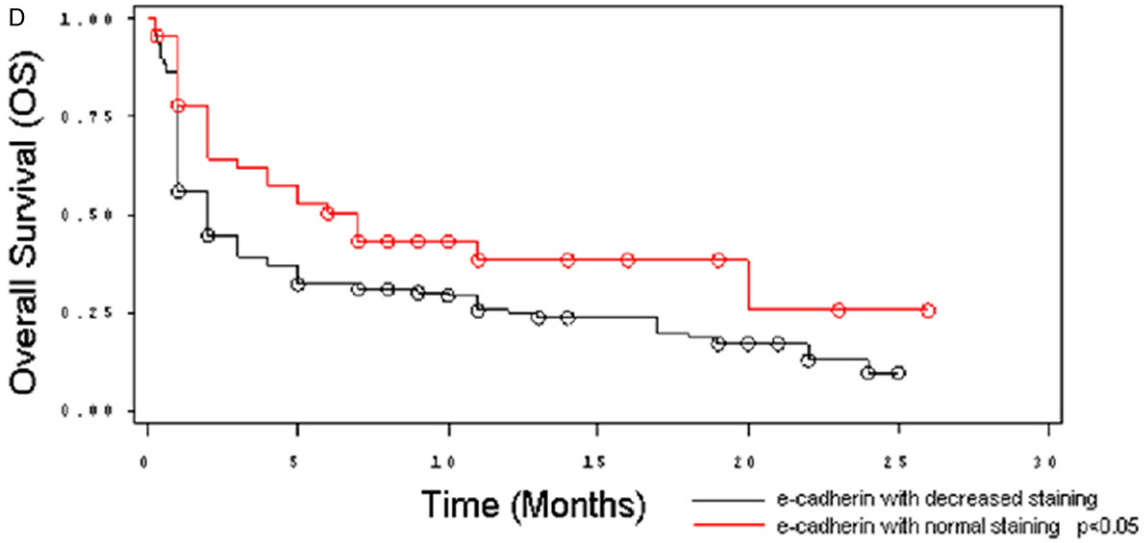


Figure 2. Kaplan-Meier Curves showing the overall survival of 205 gastric cancer patients based on E-cadherin and β-catenin expression patterns. A. Curve shows a significant association between loss of β-catenin staining and worse overall survival. B. Curve shows a significant association between weak β-catenin and worse overall survival. C. Curve shows a trend towards worse overall survival with loss of E-cadherin staining. D. Curve shows a significant association between weak E-cadherin staining and worse overall survival.

Table 6. Multivariate analysis with respect to overall survival (OS) in gastric cancer

Parameter	Hazard Ratio	*p*-value
β-catenin intensity		
Normal	0.706	0.017
Weak	1.000	
Gender		
Female	0.745	0.048
Male	1.000	
Tumor Depth		
Early cancer (T1)	1.000	0.013
Advanced cancer (T2-T4)	1.612	
Lymphovascular Invasion		
Negative	1.000	0.006
Positive	1.617	
Distal Metastasis		
Negative	1.000	0.046
Positive	1.468	

β-catenin intensity is an independent prognostic factor for these patients.

secondary tumor formation [5, 16, 17]. In addition, EMT has been linked to cancer progression and poorer patient survival through mechanisms such as evasion of apoptosis, resistance to chemotherapy, and acquisition of stem cell-like properties [18-21].

E-cadherin and β-catenin are important markers of EMT. In our study, we attempted to characterize the expression of these markers in gastric cancers. Initially, the grading was based on a combined grading system incorporating both the intensity of staining and loss of staining, with 10% cell staining representing the cut-off between 0 staining and 1-3+ staining. However, we felt that due to the large amount of heterogeneity in the tumor samples, separating the grading for intensity from the percentage of cells with staining was important. We therefore devised two separate grading systems.

In our study, 77.6% and 51.7% of patients had weak expression of E-cadherin and β-catenin respectively. In addition, 85.9% and 82.4% of patients had loss of membranous staining of E-cadherin and β-catenin respectively. This number may appear to be elevated, which is likely due to the fact that MD Anderson is a secondary referral center for patients who tend to have more aggressive tumors. Nevertheless, we found that a significant proportion of patients had abnormal expression of these markers.

There is strong evidence that abnormal expression of both E-cadherin and β-catenin are significantly correlated with EMT and malignant

phenotype in gastric cancer cell lines in vitro [22-25]. However, to the best of our knowledge, there are only two published studies on the association between survival and the immunohistochemical expression pattern of these markers in fixed tissue, with conflicting results [26, 27]. In the study from Jawhari et al. [26], 89 gastric cancer cases were evaluated, and the retention of membranous expression of β-catenin was significantly associated with a survival advantage ($p<0.05$). However, although the E-cadherin staining pattern showed a trend toward worse survival, it was not statistically significant ($p>0.05$). In the study from Yoon et al. [27], 251 cases were examined, and no association was found between survival and the expression of β-catenin and E-cadherin.

The results of our study are similar to the results from Jawhari et al. [26], as we found a significant association between weak intensity of staining of β-catenin and E-cadherin with poorer overall survival. In addition, loss of membranous staining of β-catenin was significantly associated with poorer overall survival ($p<0.05$), and a trend towards poorer overall survival was observed when evaluating for loss of membranous staining of E-cadherin ($p>0.05$). Our study showed that weak membranous expression of β-catenin appeared to be the best marker of survival compared to E-cadherin as our multivariate analysis indicated that it was an independent prognostic factor. Although there was a strong association between abnormal expression levels of E-cadherin and β-catenin, ($p<0.05$), our data suggests that β-catenin may be important in the process of EMT through other mechanisms other E-cadherin mediated adhesion.

Association of abnormal membranous staining of E-cadherin and β-catenin with other clinicopathologic parameters is also poorly understood with a limited number of published articles [26-32]. We found a significant association of abnormal expression of either β-catenin and/or E-cadherin with advanced stage cancers, poorly differentiated tumors, diffuse Lauren-type gastric tissue, lymphovascular invasion, and tumors with negative *H pylori* infection status. An important observation was that abnormal expression of E-cadherin and β-catenin was more frequently seen in tumors with a diffuse morphology. This further supports the theory that the E-cadherin-β-catenin complex is essential in mediating cell adhesion. It is reasonable to postulate that with abnormal membranous expression of these proteins, the gastric epithelium becomes disorganized and loses its normal glandular architecture, and that EMT might be a key process involved in the development of diffuse gastric adenocarcinomas. Since *H pylori* infection is mostly associated with intestinal type adenocarcinomas, this can present as a confounding variable in our examination of the *H pylori* infection status in our patients. Nevertheless, given our results, it is likely that tumorigenesis by *H pylori* (chronic atrophic gastritis leading to the formation of intestinal type adenocarcinomas) does not depend on changes in expression of E-cadherin-β-catenin complex.

Conclusion

In summary, loss of and/or weak membranous expression of both E-cadherin and β-catenin may be associated with poorer overall survival and clinicopathologic parameters that indicate a more aggressive clinical behavior. β-catenin appears to be a better biomarker than E-cadherin, because it shows a significant association with more clinical parameters. In our multivariate analysis, the intensity of staining of β-catenin was an independent prognostic factor for survival. It may be a useful immunohistochemical prognostic marker for patients with gastric cancer.

Disclosure of conflict of interest

None.

Authors' contribution

DFT and MX participated in the conception of the study, research design, study management, review of the pathology slides, data interpretation and manuscript writing. YX was involved in the data collection, analysis and data interpretation. LZ, SR, CT, JL, GL, DZ were involved in the provision of study materials. All authors contributed to the drafting of the manuscript and all authors have read and approved the final manuscript.

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