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• *BRIEF REPORTS* •

Expression of E-cadherin in gastric carcinoma and its correlation with lymph node micrometastasis

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Abstract

AIM: To examine the expression of E-cadherin in the primary tumor and to evaluate its relationship with lymph node micrometastasis (LNM).

METHODS: The authors studied 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy using reverse transcription polymerase chain reaction (RT-PCR) assay in addition to H&E staining. Cytokeratin-20 (CK-20) gene marker was used in this assay. The level of E-cadherin expression in the primary tumor was examined by immunochemical technique (EliVision™ plus).

RESULTS: LNM was detected in 77 (12.5%) lymph nodes of 14 patients (46.7%) with gastric carcinoma. The incidence of LNM was significantly higher in the diffuse type (12 of 19 cases, 63.2%) than in the intestinal type of gastric carcinoma (2 of 11 cases, 18.2%, $P = 0.026$). The incidence of LNM also increased in accordance with the depth of tumor invasion. The loss of expression of E-cadherin in primary tumors was found in 14 (46.7) of 30 tumors. The absence of E-cadherin expression was significantly associated with the Lauren classification ($P = 0.026$), lymph node metastasis ($P = 0.011$), the grade of differentiation ($P = 0.004$) and the lymphatic invasion ($P = 0.001$). Expression of E-cadherin was negative in 10 (71.4%) of the 14 patients with LNM, and in 4 (25%) of the 16 patients without LNM ($P = 0.026$).

CONCLUSION: The current results indicate that the RT-PCR assay is useful for the detection of LNM and can significantly increase the detection rate of lymph node metastasis in patients with gastric carcinoma. The Lauren classification and depth of tumor invasion are significantly associated with lymph node micrometastases. Our findings also indicate that E-cadherin may play an important role in determining the growth type and differentiation of gastric carcinoma. The loss of E-cadherin expression may contribute to LNM.

Key words: Gastric carcinoma; Lymph node micrometastasis; Cytokeratin-20; E-cadherin; Reverse transcription polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Lymph node metastasis is one of the most important prognostic factors in gastric carcinoma[1-3]. Histopathological examination of resected lymph nodes using H&E staining has been the gold standard for diagnosis of lymph node metastasis; however, the incidence of LNM is often overlooked by the routine histologic method. Recent advances in immunohistochemical and molecular biologic techniques have made it possible to detect LNM not evidenced by routine H&E evaluation. Cytokeratin is a component of the cytoskeleton of epithelial cells that is not present in normal lymph nodes[4]. Several investigators have reported that cytokeratin immunostaining can identify lymph node micrometastases missed by routine H&E staining in patients with gastric carcinoma[5-7]. However, it has been reported that the immunohistochemical technique might still generate false-negative results from overlooking possible micrometastases localized outside the cutting slice or false-positive results due to antibody cross-reactivity with host stromal or inflammatory cells[8]. In the current study, to overcome these problems, we applied RT-PCR assay to detect micrometastasis in the lymph nodes resected from 30 cases of stage I-IV gastric carcinomas, and examined the relationship between LNM and clinicopathologic characteristics. Moreover, the mechanism of LNM is still not completely known presently. E-cadherin is an adhesive molecule of epithelial cells and plays an important role in the formation and maintenance of epithelia architecture. It has been reported that reduced expression of E-cadherin is

closely associated with lymph node metastasis[9,10]. However, the relationship between E-cadherin expression in the primary tumor and LNM has not been extensively discussed. Therefore, the main objective of this study was to explore E-cadherin expression in the primary tumor and its relationship with LNM in patients with gastric carcinoma. The relationship between E-cadherin expression and clinicopathologic features of gastric carcinoma was also investigated.

MATERIALS AND METHODS

Patients and specimens

A total of 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy at the Department of Gastrointestinal Pancreatic Surgery at Sun Yat-Sen University of Medical Sciences were studied. There were 17 men and 13 women, ranging in age from 26 to 82 years, with a mean age of 56.8 years. None of these patients had received preoperative chemotherapy or radiotherapy. Total gastrectomy was performed in 16 patients, distal subtotal gastrectomy in 13 patients, and proximal subtotal gastrectomy in one patient. One patient underwent D1 lymphadenectomy, 22 patients underwent D2 lymphadenectomy, four patients underwent D3 lymphadenectomy, and three patients underwent palliative resection. According to the Lauren' criteria[11], 19 tumors were classified as diffusive type carcinomas, and 11 tumors were classified as intestinal type carcinomas. Depth of tumor invasion and extent of lymph node metastasis were classified according to UICC TNM classification published in 1997.

Half of each resected lymph node was fixed in 40 g/L formaldehyde and embedded in paraffin for routine histopathological examination. The other half was stored in 1 mL RNA later (Sigma, USA) at 4 ℃ overnight, then transferred to a clean freezing tube and stored at 70 ℃ for RNA extraction. The resected primary tumors were also fixed in 40 g/L formaldehyde and embedded in paraffin. Two consecutive sections, each 4 μm-thick, were examined by ordinary H&E staining and immunostaining with anti-E-cadherin antibody, respectively.

RNA extraction

Lymph node samples were homogenized in 1 mL of TRIzol Reagent (Invitrogen) per 50-100 mg of tissue using power homogenizer. RNA extraction was carried out according to the protocol recommended by the manufacturer. Total RNA was dissolved in diethylpyrocarbonate-treated water and the volume and quality of the RNA then assessed by the ultraviolet spectrophotometer.

The Access RT-PCR

Complementary DNA (cDNA) was synthesized and amplified from total RNA using the Access RT-PCR system (Promega). The primer sequences used for CK-20 detection were 5'-ggtcgcgactacagtgcatattaca-3' (sense) and 5'-cctcagcagccagtttagcattatc-3' (anti-sense)[12]. cDNA synthesis was monitored by beta-actin RT-PCR using the following primers: 5'-caaatgcttctaggcggact-3' (sense) and 5'-atgctatc-acctcccctgtg-3' (anti-sense). The RT-PCR was performed in a 25-μL reaction mixture containing nuclease-free water 11 μL, 5×Reacton Buffer 5 μL, dNTP (10 mmol/L) 0.5 μL, each of beta-actin primers (20 μmol/L) 0.5 μL, each of CK-20 primers (10 μmol/L) 1.25 μL, $MgSO_4$ (25 mmol/L) 1 μL, AMV reverse transcriptase (5 U/μL) 0.5 μL, Tfl DNA polymerase (5 U/μL) 0.5 μL, and RNA sample 3 μL. The Access RT-PCR condition was set up as follows: 1 cycle at 48 ℃ for 45 min (reverse transcription), 1 cycle at 94 ℃ for 2 min (AMV RT inactivation), followed by 40 cycles at 94 ℃ for 30 s (denaturation) and at 62 ℃ for 1 min (annealing) and at 68 ℃ for 1.5 min (extension), followed by a final extension at 68 ℃ for 7 min. The resultant cDNA products of CK-20 and beta-actin were 121 and 381 base pairs, respectively. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. In this report, "micrometastasis" in the regional lymph nodes was defined as metastasis that was detected only by the RT-PCR assay but not by ordinary H&E staining (Figure 1).

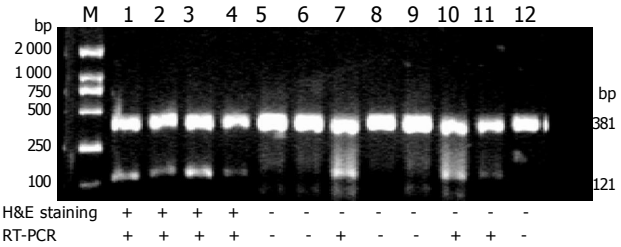

Figure 1 Comparison of the results of RT-PCR and H&E staining. M: DL2000Marker; lanes 1-12: dissected lymph nodes. The estimated size of the amplified products in base pairs (bp) appears on the right. Lymph nodes of lanes 7, 10, and 11 were diagnosed LNM.

Immunohistochemistry

The dissected primary tumors were fixed in 40 g/L formaldehyde and embedded in paraffin. Specimens from the paraffin embedded block were cut into 4 μm sections and then placed on slides. E-cadherin immunohistochemical staining was performed using EliVision™ plus IHC Kit (Maixin Biological, Fuzhou, China). After the slides were deparaffinized with xylene and dehydrated with ethanol, they were placed in 10 mmol/L citrate buffer and heated in a microwave at 700 W for 5 min for the retrieval of antigens in the specimens. Endogenous peroxidase activity was blocked by incubation of the slides in 3% hydrogen peroxide in absolute methanol at room temperature for 10 min. The slides were then incubated sequentially with 50 μL of mouse monoclonal antibody against human E-cadherin (Maixin Biological, Fuzhou, China) overnight at 4 ℃, with 50 μL of polymer enhancer for 20 min, and 50 μL of polymerized HRP-anti mouse IgG for 30 min. The reaction products were visualized with diaminobenzidine (DAB Kit, Maixin Biological, Fuzhou, China), and slides were counterstained with hematoxylin, dehydrated, and evaluated by light microscopy. Tris-buffered saline (TBS) solution was used instead of the primary antibody for negative controls.

For the purpose of data analysis, the expression level

of E-cadherin in the primary tumor was graded according to the proportion of positive tumor cells. If more than 25% of the tumor cells were positively stained for E-cadherin, the tumor was classified as having preserved E-cadherin expression. In contrast, if 25% or less of the tumor cells were positively stained, the tumor was classified as having the loss of E-cadherin expression. The stained slides were observed independently by two pathologists who had no knowledge of the clinicopathological data. All the staining results for E-cadherin were examined in relation to the clinicopathological parameters of the tumors.

Statistical analysis

Statistical analysis was performed by Fisher's exact test to examine the association of LNM and the expression of E-cadherin in the primary tumor with the clinicopathologic characteristics of the primary tumor, and examine the relationship between the expression of E-cadherin in the primary tumor and LNM. Statistical significance was defined as $P<0.05$.

RESULTS

Correlation between LNM and clinicopathologic characteristics

Routine examination by H&E staining confirmed metastasis in 233 lymph nodes from 20 patients. All these 233 lymph nodes were cytokeratin-20 positive; moreover, LNM was detected in an additional 67 lymph nodes in 12 cases of these 20 patients. LNM was also detected in 10 lymph nodes from two cases of 10 patients who had no obvious metastases identified by H&E staining. Totally, LNM was identified only by the RT-PCR assay in 77 (12.5%) lymph nodes of 14 patients (46.7%) with gastric carcinoma. From Table 1, we also found that the incidence of LNM was significantly higher in the diffuse type (12 of 19 cases, 63.2%) than in the intestinal type of gastric carcinoma (2 of 11 cases, 18.2%, $P = 0.026$). Micrometastases increased in accordance with the depth of tumor invasion. Seven of 21 cases that had lesions within submucosa layer were detected to have micrometastases in the lymph nodes; in contrast, seven of nine cases with invasion reaching the muscularis propria and deeper invasion had micrometastases ($P = 0.046$). Other clinicopathologic findings including gender, age, location, diameter, histologic differentiation, lymphatic invasion and vascular invasion had no statistically significant correlation with the incidence of LNM ($P>0.05$).

Table 1 Correlation between LNM and clinicopathologic characteristics

Variable	Patients (*n*)	LNM		
		Negative (%)	Positive (%)	*P*
Gender				
Male	17	9 (52.9)	8 (47.1)	
Female	13	7 (53.8)	6 (46.2)	$P = 1.000$
Age				
<50 yr	10	4 (40)	6 (60)	
≥R50 yr	20	12 (60)	8 (40)	$P = 0.442$
Superficial diameter				
<5 cm	17	9 (52.9)	8 (47.1)	
≥R5 cm	13	7 (53.8)	6 (46.2)	$P = 1.000$
Tumor location				
Upper/middle third	17	8 (47.1)	9 (52.9)	
Lower third	13	8 (61.5)	5 (38.5)	$P = 0.484$
Histologic type				
Intestinal	11	9 (81.8)	2 (18.2)	
Diffuse	19	7 (36.8)	12 (63.2)	$P = 0.026$
Depth of invasion				
T1/T2	21	14 (66.7)	7 (33.3)	
T3/T4	9	2 (22.2)	7 (77.8)	$P = 0.046$
Histologic differentiation				
Well/moderate	13	9 (69.2)	4 (30.8)	
Poor	17	7 (58.8)	10 (41.2)	$P = 0.159$
Lymphatic invasion				
Positive	18	7 (38.9)	11 (61.1)	
Negative	12	9 (75)	3 (25)	$P = 0.072$
Vascular invasion				
Positive	5	3 (60)	2 (40)	
Negative	25	13 (52)	12 (48)	$P = 1.000$

Expression of E-cadherin

The loss of expression of E-cadherin in primary tumors was found in 14 (46.7) of 30 tumors. From Table 2, we found that the absence of E-cadherin expression was significantly associated with The Lauren classification ($P = 0.026$), lymph node metastasis ($P = 0.011$), the grade of differentiation ($P = 0.004$) and the lymphatic invasion ($P = 0.001$), but not correlated with age, gender, location, diameter and depth of tumor invasion ($P>0.05$).

Table 2 Correlation between the expression of E-cadherin in primary tumors and clinicopathologic characteristics

Variable	Patients (*n*)	Expression of E-cadherin		
		Negative (%)	Positive (%)	*P*
Gender				
Male	17	7 (41.2)	10 (58.8)	
Female	13	7 (53.8)	6 (46.2)	$P = 0.713$
Age				
<50 yr	10	3 (30)	7 (70)	
≥R50 yr	20	11 (55)	9 (45)	$P = 0.260$
Superficial diameter				
<5 cm	17	7 (41.2)	10 (58.8)	
≥R5 cm	13	7 (53.8)	6 (46.2)	$P = 0.713$
Tumor location				
Upper/middle third	17	8 (47.1)	9 (52.9)	
Lower third	13	6 (46.2)	7 (53.8)	$P = 1.000$
Histologic type				
Intestinal	11	2 (18.2)	9 (81.8)	
Diffuse	19	12 (63.2)	7 (36.8)	$P = 0.026$
Depth of invasion				
T1/T2	21	8 (38.1)	13 (61.9)	
T3/T4	9	6 (66.7)	3 (33.3)	$P = 0.236$
Histologic differentiation				
Well/moderate	13	2 (15.4)	11 (84.6)	
Poor	17	12 (70.6)	5 (29.4)	$P = 0.004$
Lymph node metastasis				
Positive	18	12 (66.7)	6 (33.3)	
Negative	12	2 (16.7)	10 (83.3)	$P = 0.011$
Lymphatic invasion				
Positive	18	13 (72.2)	5 (27.8)	
Negative	12	1 (8.3)	11 (91.7)	$P = 0.001$

Correlation between LNM and the expression of E-cadherin

From Table 3, we found that expression of E-cadherin was negative in 10 (71.4%) of the 14 patients with LNM, and in 4 (25%) of the 16 patients without LNM. The difference between these two groups was statistically significant ($P = 0.026$).

Table 3 Correlation between LNM and the expression of E-cadherin

LNM	Patients (*n*)	Expression of E-cadherin		
		Negative (%)	Positive (%)	*P*
Positive	14	10 (71.4)	4 (28.6)	
Negative	16	4 (25)	12 (75)	*P* = 0.026

DISCUSSION

It is well known that lymph node metastasis is the most important prognostic factor for patients with gastric carcinoma. However, even after undergoing radical resection of primary tumors and lymph nodes, about 20% of patients with gastric carcinoma reportedly die of recurrence[13], and about 3% of patients with early-stage gastric carcinoma also reportedly die of recurrence[14]. These findings suggest the existence of LNM that cannot be identified by routine H&E staining. Several investigators have demonstrated the usefulness of immunohistochemical technique for detection of micrometastases in lymph nodes of gastric carcinoma patients[5-7,15,16]. In the present study, RT-PCR assay was applied to detect micrometastsis in the lymph nodes resected from 30 cases of stage I-IV gastric carcinomas. Totally, LNM was detected by the RT-PCR assay in 77 (12.5%) lymph nodes of 14 patients (46.7%) with gastric carcinoma. The incidence of LNM was significantly higher in the diffuse type (12 of 19 cases, 63.2%) than in the intestinal type of gastric carcinoma (2 of 11 cases, 18.2%, $P = 0.026$). Similar to our results, Ishida *et al*, studied 2 446 lymph nodes removed during surgery for 109 cases of gastric carcinoma, including Stages I-IV. Metastases were confirmed in 230 lymph nodes (9.4%) stained with H&E, and an additional 201 lymph nodes (17.6%) had micrometastases identified only by immunostaining. They also demonstrated that the diffuse type had more micrometastases than the intestinal type[5].

In addition, we also found that there was a significant correlation between LNM and depth of tumor invasion. Seven of 21 cases that had lesions within submucosa layer were detected to have micrometastases in the lymph nodes; in contrast, seven of nine cases with invasion reaching the muscularis propria and deeper invasion had micrometastases ($P = 0.046$). Micrometastases increased in accordance with the depth of tumor invasion. Tsujitani *et al*, obtained almost the same results as ours. They reported that micrometastases in the lymph nodes were found in 18% of mucosal cancer, 25% of submucosal cancer, and 65% of T3 (serosal) cancer specimens, with cancer-free nodes examined by H&E staining[17].

These results indicate that RT-PCR assay is clearly more sensitive than routine histopathological examination for detection of micrometastases in lymph nodes of gastric carcinoma patients. Lymph node micrometastases are significantly associated with the Lauren classification and depth of tumor invasion.

E-cadherin is a 120-ku transmembrane glycoprotein that is responsible for calcium-dependent intercellular adhesion by homotypic interactions. E-cadherin plays an important role in maintaining cell polarity and tissue morphology[18]. Previous studies showed that 43.5-72% of gastric carcinoma had reduction or loss of E-cadherin expression[19,20]. In our study, the loss of E-cadherin expression was observed in 46.7% (14 of 30 cases) of gastric carcinoma. We also found the absence of E-cadherin expression was significantly related with the histologic type and the grade of tumor differentiation. In 19 gastric carcinomas with diffuse type of growth 12 showed negative E-cadherin expression, while in 11 gastric carcinomas with intestinal type of growth only two showed negative E-cadherin expression. The difference was statistically significant ($P = 0.026$). In 17 poorly-differentiated gastric carcinomas 12 showed negative E-cadherin expression, while in 13 well- or moderately-differentiated gastric carcinomas only two showed negative E-cadherin expression. The difference was also statistically significant ($P = 0.004$). Recently, several scholars also reported that the reduction or absence of E-cadherin expression occurred more frequently in diffuse than intestinal type of gastric carcinoma, and correlated with poor differentiation[21,22]. These findings indicate that E-cadherin may play an important role in determining the growth type and differentiation of gastric carcinoma.

The E-cadherin gene has generally been recognized as an invasion-suppressor gene[23,24]. Chen *et al*[22], reported that the loss of E-cadherin expression was significantly associated with tumor invasion. Yonemura *et al*[20], also reported that reduced E-cadherin expression showed a strong relationship with positive serosal involvement and infiltrating type. In the present study, the absence of E-cadherin expression was more frequent in T3 or T4 tumors (six of nine tumors, 66.7%), compared with T1 or T2 tumors (8 of 21 tumors, 38.1%), but this difference was not statistically significant ($P = 0.236$). This may be explained by the fact that the cases in our study were comparatively few. To draw a further conclusion, larger sample investigations on gastric carcinoma are needed.

It has been reported that reduced expression of E-cadherin plays an important role in the development of lymph node metastases in patients with gastric carcinoma. However, the relationship between E-cadherin expression in the primary tumor and LNM has not been extensively discussed. In our study, we found that expression of E-cadherin was negative in 10 (71.4%) of the 14 patients with LNM, and in 4 (25%) of the 16 patients without LNM. The difference between these two groups was statistically significant ($P = 0.026$). The result indicates the loss of E-cadherin expression may contribute to LNM.

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