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Expression of TGF-β1, Snail, E-cadherin and N-cadherin in Gastric Cancer and Its Significance

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OBJECTIVE To investigate the expression of TGF-β1, Snail, E-cadherin and N-cadherin in gastric cancer (GC), and to examine its relationship to malignant features of the tumors.

METHODS The expression of TGF-β1, Snail, E-cadherin and N-cadherin proteins was detected in GC and adjacent tissues by immunohistochemical staining, and compared with the clinico- pathological data .

RESULTS Positive rates of expression for TGF-β1, Snail, E-cadherin and N-cadherin were 63.5%, 83.3%, 37.5% and 44.8% in GC, and 28.8%, 41.3%, 100%, 11.3% in adjacent tissues, respectively. The expression of all four proteins showed a significant difference between the GCs and adjacent tissues (P<0.05). The positive rate of TGF-β1, Snail and N-cadherin, or the negative rate of E-cadherin expression was significantly related to the differentiated degree, histological type, invasion and metastasis of GC. In addition, the expression of N-cadherin was positively related to that of TGF-β1, but negatively related to that of E-cadherin. There was negative correlation between expression of E-cadherin and TGF-β1 and Snail in GC (P<0.05).

CONCLUSION The over-expression of TGF-β1 and Snail and decreased expression of E-cadherin and the abnormal expression of N-cadherin were involved in the process of invasion and metastasis of GC. The data showed that E-cadherin might switch to N-cadherin. TGF-β1 and Snail might play a fundamental role in the process.

KEYWORDS: gastric cancer, TGF-β1 protein, Snail protein, E-cadherin protein, N-cadherin protein, EMT.

INTRODUCTION

Gastric cancer is one of the most common malignancies in the world[1]. Metastasis, a common feature of the natural history of gastric cancer, is a major problem for gastric cancer management. In early invasion and metastasis by carcinoma cells, loss of E-cadherin accompanying the acquisition of a fibroblastic phenotype occurs through a mechanism called epithelial-mesenchymal transition (EMT)[2]. Recently, researchers have found abnormal N-cadherin expression in superficial urothelial tumors, breast carcinomas and pancreatic carcinomas, and that it has a more obvious and direct action than the low-level E-cadherin expression related to cell invasion and metastasis. This change of cadherin from E to N may make a critical contribution to EMT[3-5].

As upstream molecules of E-cadherin, the Snail family members play a central role in regulation of EMT during tumor progression by repressing E-cadherin transcription. Growth factors, especially including transforming growth factor-β (TGF-β), orchestrate the EMT of various epithelial tissues. In the present study, some findings suggest that Snail participates in TGF-β-induced EMT by acting upstream of Akt activation[6-8].

The purpose of this study presented here was to investigate whether a gain in N-cadherin expression in gastric cancer is involved in the process of metastasis via EMT, and whether its expression is affected by growth factors. Correlations among the expressions of N-cadherin, E-cadherin, TGF-β1, and Snail were immunohistochemically examined in gastric cancers and adjacent normal tissues. The mechanism of gastric carcinoma invasion and metastasis, and the possibility of these proteins as an index of the degree of gastric carcinoma malignancy are discussed.

MATERIALS AND METHODS

Materials

Ninety-six gastric cancer tissues were obtained from the Department of Pathology, the First Affiliated Hospital, Anhui Medical University, China, from 1999 to 2005. The designation of stage grouping and the histological classification were made according to the criteria of the Japanese Gastric Cancer Association[9].

Main reagents

Monoclonal mouse immunoglobulin (IgG) antibodies to N- and E-cadherin were purchased from the Zhongshan Corp (Beijing, China). Polyclonal rabbit antibodies against TGF-β1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and antibodies against Snail were purchased from Abcom Biotechnology (Cambridge, UK). The Histostain-SP kit was the product of the Zhongshan Corp.

Staining methods

Paraffin sections (4 μm) were routinely deparafinized, passed through grades of alcohol, immersed in 3% hydrogen peroxide for 15 min and rinsed 3 times with PBS. The antigens were recovered with heating, and the specimen blocked with 10% goat serum, followed by incubation with first antibodies (N-cadherin was diluted 1:50, TGF-β1 and E-cadherin were diluted 1:100, Snail was diluted 1:250) overnight at 4℃, washed 3 times with PBS, then incubated with biotinylated second antibodies for 30 min at 37℃, washed 3 times with PBS, and incubated with horseradish enzyme labeled streptavidin for 30 min at 37℃. DAB was used as the chromogen followed by counterstaining with hematoxylin, and sealing with neural gum. As a negative control, sections were incubated with PBS instead of the first antibody. The positive control was developed with known positive sections. For immunohistochemistry of N-cadherin, ganglion cells were used as a positive control.

Standards for determination of the results

Prior to inclusion in the study, all cases were reviewed by two pathologists for confirmation of diagnosis, staging and grading. The study was performed blind, so that the patients' clinical characteristics were unknown to investigators performing the immunohistochemical analyses.

According to a classification derived from the work of Nakajima et al.,[5] low TGF-β1 expression is defined when ≤20% of the cancer cells are stained, and high TGF-β1 expression when >20% of the cells are stained. The definition of staining for N-cadherin was the same. Immunostaining of E-cadherin was distinguished as normal and abnormal. If the staining pattern was similar to that of normal epithelial cells (i.e., >90% of the cells with membranous staining), it was evaluated as normal. Abnormal staining was divided into negative staining (<10% of the cells with membranous staining) and heterogeneous staining (between 10% and 90% of the cells with membranous staining). Tumors with abnormal E-cadherin immunostaining, i.e., defined as those giving negative staining or heterogeneous staining, were considered as one group in statistical analysis. Staining was located at the cell membrane and in the cytoplasm close to the membrane in positive cells.

The counting of Snail immunoreactive cells was conducted by scanning and scoring of 10 high-power fields using 400 magnifications. Positive Snail was recognized as intense stain in the tumor cell nucleus. The definition of staining for Snail was also divided into two groups in the same way as that for TGF-β1 staining.

Statistical analysis

SPSS 11.5 software was used. Comparisons of TGF-β1, Snail, N- and E-cadherin positive rates were analyzed by the χ^2 test. A bivariate Spearman test was chosen to assess the correlation between relative expression levels. P values <0.05 were considered statistically significant.

RESULTS

Analysis of the 96 gastric carcinoma samples showed that TGF-β1, Snail, E-cadherin and N-cadherin were positive respectively in 61(63.5%), 80(83.3%), 36(37.5%) and 43(44.8%), of the cases whereas of the 80 adjacent tissues, TGF-β1, Snail, E-cadherin and N-cadherin were positive respectively in 23(28.8%), 33(41.3%), 80(100%) and 9(11.3%)

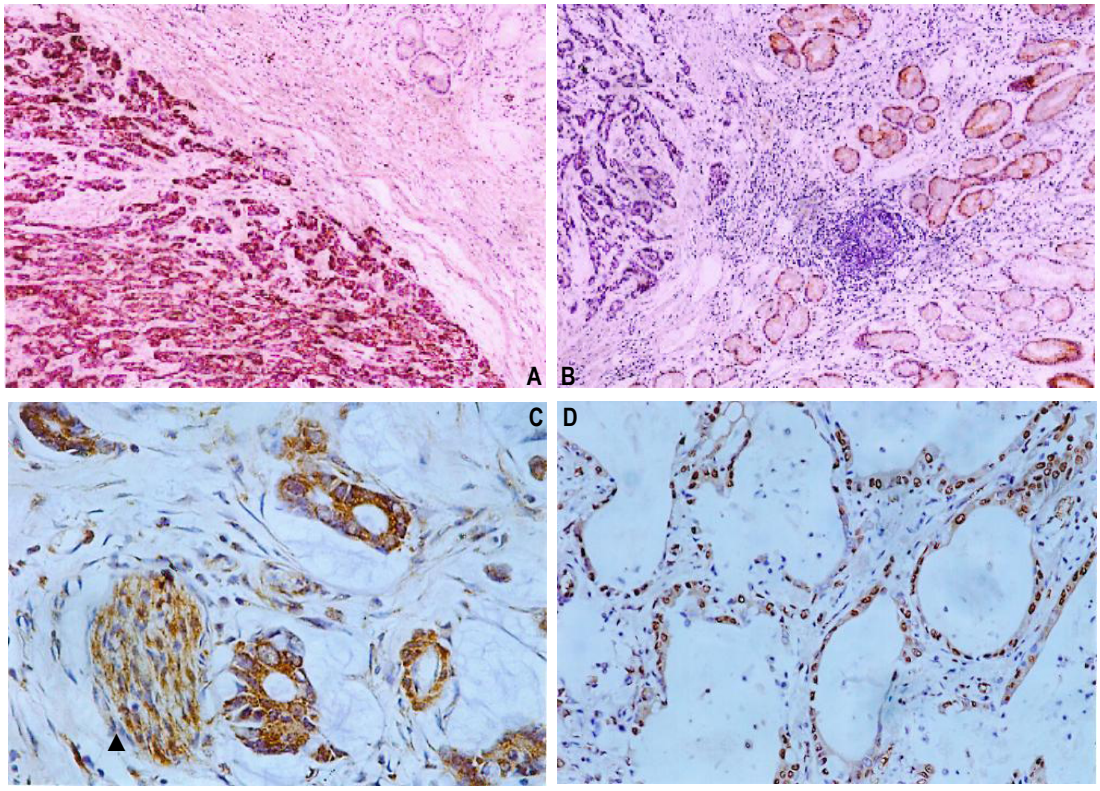

Fig.1. A. Strong positive expression of TGF-β1 in poorly-differentiated adenocarcinoma cancerous tissues, with a negative expression in normal paraneoplastic proper coat (lamina propria) in the glandular organ; B. Positive expression of E-cadherin in poorly-differentiated adenocarcinoma with a positive expression of normal paraneoplastic proper coat (lamina propria) in the glandular organ, and a negative expression in cancerous tissue; C. Positive expression of N-cadherin in poorly-differentiated adenocarcinoma cancerous tissue, ▲ Nervous plexus; D. Strong positive expression Snail in moderately-differentiated adenocarcinoma cancerous tissue, localizing in the cell nucleus and cytoplasm.

Table 1. Relationship between TGF-β1, Snail, N-cadherin and E-cadherin expression and clinicopathologic factors.

Parameters	*n*	TGF-β1		Snail		N-cadherin		E-cadherin	
		Positive	*P*[a]	Positive	*P*[a]	Positive	*P*[a]	Positive	*P*[a]
Differentiation			0.000		0.020		0.016		0.006
High	19	5		12		3		11	
Middle	15	7		12		7		9	
Poor	62	49		56		33		16	
Histological Type[b]			0.000		0.000		0.000		0.000
Pap	10	2		3		1		6	
Tub	24	11		20		12		15	
Por	42	37		38		27		14	
Sig/muc	20	11		19		3		1	
Invasion depth			0.020		0.025		0.017		0.026
Above Serosa	16	6		10		3		10	
Serosa	80	55		70		40		26	
Lymph node metastasis			0.017		0.038		0.019		0.026
No	32	15		23		9		17	
Yes	64	46		57		34		19	
Pathologic stage			0.117		0.068		0.071		0.005
I~II	29	15		21		9		17	
III~IV	67	46		59		34		19	
Distant metastasis			0.045		0.049		0.016		0.038
No	86	52		70		35		35	
Yes	10	9		10		8		1	

[a] *P* was calcutated by χ^2 test.

[b] Japanese Gastric Cancer Association: Pap, papillary adenocarcinoma; Tub, tubular adenocarcinoma; Por, poorly differentiated adenocarcinoma; Sig, signet-ring cell carcinoma; Muc, mucinous adenocarcinoma.

of these cases. The expression of all four proteins showed a significant difference between the gastric cancers and adjacent tissues (P<0.05). Staining of TGF-β1 was detected mainly in the cytoplasm, and only a few tumor cells had staining in the nucleus (Fig.1A). Stains of E-cadherin and N-cadherin were observed at the cell membrane and in the cytoplasm close to the membrane (Fig.1B and C). Stain of Snail was located in the tumor cell nuclei (Fig.1D).

Correlation between TGF-β1, Snail, N-cadherin and E-cadherin expression and clinicopathologic features

The relationship between these protein expressions and the clinicopathologic factors of gastric carcinoma is summarized in Table 1. The positive rate of TGF-β1, Snail and N-cadherin expression increased with an increase in carcinoma invasion depth and a decrease in tumor differentiation (P<0.05), which implied that there was a positive correlation between the expression of these proteins and the ability for tumor invasion, and malignancy; while E-cadherin expression decreased with the increase in carcinoma invasion depth and the decrease of tumor differentiation (P<0.05). These findings implied that there was a negative correlation between E-cadherin expression and tumor invasion ability and the degree of malignancy. With regard to lymph node metastasis and distant metastasis shown in Table1, N-cadherin positive rates were 53.1% (34/64) and 80% (8/10, P<0.05) and E-cadherin negative rates were 70.3% (45/64) and 90% (9/10, P<0.05), which implied the presence of a positive correlation between N-cadherin and tumor metastasis, but a negative correlation between E-cadherin and tumor metastasis.

Table 2. Correlation between N-cadherin and E-cadherin according to Spearman analysis.

	N-cadherin		*r*	*P*
	Positive	Negative		
E-cadherin			-0.222	0.030
Positive	11	25		
Negative	32	28		

Table 3. Correlations among TGF-β1, Snail and N-, E-cadherin according to Spearman analysis.

	r	*P*
TGF-β1 and Snail	0.242	0.018
TGF-β1 and E-cadherin	-0.218	0.033
TGF-β1 and N-cadherin	0.378	0.000
Snail and E-cadherin	-0.234	0.029
Snail and N-cadherin	0.009	0.928

Correlations among TGF-β1, Snail, N-cadherin and E-cadherin expression in gastric cancer tissue

The relationships between the expression of N-cadherin staining and those of other molecules were analyzed on the basis of expressions only in cancer cells. From Table 2 we can see that the expression of N-cadherin negatively related to that of E-cadherin. There was a negative correlation between expression of E-cadherin and TGF-β1 and Snail. No correlation could be established between abnormal expression of N-cadherin and over-expression of Snail. Statistical results are shown in Table 3.

DISCUSSION

Relationship between EMT and cadherin

Epithelial-mesenchymal transition (EMT) is characterized by the loss of proteins associated with a polarized epithelial phenotype and by the de novo synthesis of proteins associated with mesenchymal, migratory morphology of transitioning cells. Greenburg and Hay were the first to describe that epithelial cells in culture may acquire mesenchymal features verifying the principles for the EMT process[10]. Early discoveries of EMT resulted largely from studies of embryonic development. It was then implicated in the progression of primary tumors towards metastases. Various clinical studies of tumor tissues and tumor cell lines demonstrated that reduced expression of E-cadherin represent a key step in the EMT process .

The cadherin family is composed of a series of structural and functionally similar single stranded transmembrane, glycoproteins. The classical cadherins are classified as E (epithelial), N (neural), P (placental) with the same 120 kDa molecular weight. They mainly participate in mediating Ca^{2+} dependent adhesion between homotype cells. E-cadherin is predominantly expressed in epithelia, and it binds to cytoplasmic adhesion proteins to form an E-cadherin/catenin complex as an anchorage site for actin-based cytoskeleton. The E-cadherin/catenin/cytoskeleton complex plays an important role in the maintenance of structural and functional stability of epithelial tissues. Research has shown that the loss of E-cadherin expression is a major characteristic of highly invasive and metastatic cancer. Our results showed expression of E-cadherin is at a low-level or there was no expression in gastric carcinomas, in accordance with previous reports. Staining was predominantly located at the cytoplasm in many positive cells while the cell membrane was not obvious, which may decrease the ability of cell-cell contacts.

N-cadherin is expressed in ganglial cells, myocardium and so on. Normal epithelia do not express this adhesion molecule, but recent studies have demonstrated that N-cadherin is associated with an increased invasive cancer potential. In vitro experiments also indicate that negative N-cadherin expression in breast cancer cells show enhanced invasiveness after being transfected with N-cadherin[11]. Our results demonstrated the aberrant expression of N-cadherin was significantly related to the differentiated degree, histological type, invasion and metastasis of gastric cancer. Furthermore, we found endothelial cells and other mesenchymal cells also express N-cadherin. So it is possible that the N-cadherin expressed on the surface of tumor cells might promote homophile interactions with the endothelium and matrix components. In our study the positive rate of N-cadherin was only 44.8% (43/96), which may be related to low-level expression of this adhesion molecule. Rosivatz et al.[12] also found expression of N-cadherin could not be detected at the protein level by antibody immunohistochemical staining, although in contrast mRNA expression was seen by TaqMan analysis in many cases[12]. Nakajima[5] utilized a Catalyzed Signal Amplification System in their study, as this technique is up to 1000 times more sensitive than the usual immunoenzymatic detection systems[5].

N-cadherin and E-cadherin are both classic cadherins which are involved with the same mechanism in mediating intercellular adhesion. While their actions in tumors are contrary to each other, the mechanism is unclear. A new concept entitled the "cadherin switch" has been proposed that involves the change of cadherin from E to N making the tumor cells turn from a benign phenotype into an invasive malignant phenotype[13]. In our results, after gastric cells undergo a carcinomatous change, E-cadherin turns from a high expression to a low-level or non-expression, while N-cadherin changes from non-expression to high expression. This results in tumor cells easily separating from the gland tissue and infiltrating toward the stroma, which may be the cause of gastric carcinoma invasion and metastasis. Our results are in accordance with recent experiments with cultured human gastric carcinoma cells[14].

Relationship between TGF-β1, Snail and cadherin

TGF-β has a dual role during tumor progression: it represses tumor growth during the early phases of tumor genesis by inducing cell-cycle arrest and programmed cell death (apoptosis), but during the late phases of carcinogenesis, it promotes EMT, tumor invasion and metastatic dissemination of tumor cells. Some scholars have suggested that TGF-β acts as a "double-edged sword"[15]. Consistent with its tumor-suppressor functions, several components of the TGF-β-mediated signaling pathways are impaired in gastric cancer, which has been verified by reports from China and abroad. The mechanism of TGF-β-induced EMT is not yet known. Studies of pancreatic carcinoma indicated that TGF-β1 can enhance the expression of mesenchymal molecules, such as N-cadherin and vimentin[5].Our results showed that the expression of TGF-β1 displayed a significant difference between gastric cancer and adjacent tissues. The positive rate of TGF-β1 was significantly related with the differentiated degree, histological type, invasion and metastasis of gastric carcinoma. In addition, the expression of TGF-β1 was positively related to that of N-cadherin, but negatively related to that of E-cadherin. As a result, TGF-β1 can repress E-cadherin expression to weaken cell-cell contacts accompanying the acquisition of a fibroblastic phenotype, occurring through N-cadherin expression, which enhances tumor-cell motility and migration.

A further protein known to trigger EMT is Snail, which is possibly involved in E-cadherin and N-cadherin conversion during EMT. Snail was first described in *Drosophila melanogaster*, where it was shown to be essential for the formation of the mesoderm[16]. In the 20 or so years since its isolation, the Snail family members have been shown to be involved in regulation of EMT during embryonic development and tumor progression. The Snail zinc-finger protein functions as repressor of E-cadherin transcription by recognizing E-box elements in the E-cadherin promoter. Snail activity is regulated by various signaling pathways at multiple levels. For example, Snail is required for transforming growth factor-β-induced EMT by activating the PI3 kinase/Akt signal pathway[8]. So Snail plays a central role in regulation of EMT. Our results showed that the positive rate of Snail in gastric cancer was much higher than that in adjacent tissues, and it was significantly related with some clinicopathologic factors. According to the Spearman test, the expression of Snail was positively related to that of TGF-β1, but negatively related to that of E-cadherin. These results support the view points stated above.

Research is currently under way to define the role of TGF-β1, Snail and N- and E-cadherin in gastric neoplastic progression, and to identify the cellular-signaling pathway used by these molecules. Extensive studies are now in progress aiming to validate the use of adhesion molecules and upstream markers as molecular tools for the diagnosis and assessment of gastric cancer.

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