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Expressions of FOXC1 and E-cadherin in gastric cancer tissues and their relationships with pathological characteristics

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Abstract: **To analyze and compare the expressions of two factors, forkhead box C1 (FOXC1) and E-cadherin, between gastric cancer and paracancerous tissues, and to determine the correlations of the two factor expressions with pathological features, thus providing implications for cancer treatment and prognosis prediction. A total of 102 gastric cancer patients who underwent surgery were randomly included into the current study. After excluding 1 case of other organ cancers and 2 cases receiving radiochemotherapy before surgery, 99 patients became the final subjects. The cancer and paracancerous tissues were dissected and processed into paraffin-embedded sections for two-step biotin-free immunohistochemistry (IHC) detection. The expressions of the two factors were detected, and statistical differences analyzed. The relationships between the expression levels and pathological features were further explored. The positive expression rate of FOXC1 in cancer and paracancerous tissues was 56.16% and 11.54%, respectively; and that of E-cadherin in the two tissue types was 24.14% and 94.20%, respectively. These data indicated that the expression levels of FOXC1 and E-cadherin were significantly different in tumor tissue (P<0.05). The expression of FOXC1 was not significantly different between cases of different ages or genders (P>0.05), but was significantly different between cases with distinct TNM classifications, histological subtypes and invasive depths, and presence or absence of lymph node metastasis (P<0.05). Similarly, the expression of E-cadherin was not significantly different between cases divided by ages or genders (P>0.05), but significantly different between cases divided by TNM classifications, histological subtypes, invasive depths, and lymph node metastasis (P<0.05). The two factors, FOXC1 and E-cadherin, may play important roles in the carcinogenesis, development, invasion and metastasis of gastric cancer, and provide implications for the mechanisms underlying gastric oncogenesis. They may also serve as new indicators of prognosis and potential targets for treatment of gastric cancer. Moreover, FOXC1 expression may also be associated with the downregulation of E-cadherin expression. FOXC1 probably regulates E-cadherin expression, thus affecting epithelial-mesenchymal transition (EMT) in gastric cancer.**

Keywords: Gastric cancer; forkhead box C1 (FOXC1); E-cadherin; epithelial-mesenchymal transition (EMT)

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1. Introduction

According to International Guidelines for Treatment of Gastric Cancer, despite decreased morbidity, gastric cancer is still the forth most common malignancy and second leading cause of cancer-related death [1]. The prognosis of gastric cancer patients is generally related to the pathological stage, invasive depth and site, presence of remote metastasis, histological subtype, individual health, genetic factors and treatment choices [2]. Early cancer has a better prognosis than progressive cancer, but the patient is mostly in advanced stages at diagnosis due to unspecific symptoms, leading to bad outcomes. Therefore, to increase the low diagnostic rate and to decrease the high mortality of early gastric cancer [3], it is vitally important to investigate the factors related to gastric cancer. The current study aimed to detect the expressions of forkhead box C1 (FOXC1) and E-cadherin in gastric cancer and paracancerous tissues, and to explore the relationships between the two factors and pathological features, thus providing basis for early diagnosis of gastric cancer.

2. Materials and Methods

2.1. Clinical samples

A total of 102 patients with gastric cancer (diagnosed by pathological examination) admitted into the General Surgery Department, the Affiliated Hospital of Qingdao University from March, 2014 to June, 2016, were primarily included into the current study. After excluding 3 cases not living up to the criteria, we finally included 99 cases into the study. Of the 99 cases, 50 were male and 49 female, aged from 34 to 76 (59.99±5.84) years; 33 cases had moderately to highly differentiated adenocarcinoma while 66 had lowly differentiated adenocarcinoma. The dissected cancer and paracancerous tissues (>6 cm from the edge of tumor) were processed into paraffin-embedded sections. According to the Union for International Cancer Control (UICC) Guidelines for Treatment of Gastric Cancer 2010 [4], 26 cases were classified into I + II stages and 73 into III + IV stages, 22 cases of muscularis mucosa invasion and 77 cases of extraserosal invasion. Of the 99 cases, 67 had lymph node metastasis while 32 did not. All the subjects did

not receive radiochemotherapy before sampling. The specimens were fixed with 10% paraformaldehyde solution and then embedded into paraffin as conventionally described. Paraffin masses containing obvious foci were selected for continuous sectioning, each section of 4-μm thickness. Ready-to-use mouse anti-human monoclonal antibodies to E-cadherin, SP immunohistochemistry (IHC) kit, diaminobenzidine (DAB) staining kit, concentrated rabbit anti-human multiclonal antibodies to FOXC1 (1:50), and other reagents were purchased from Hongda Biotech Group Co., Ltd. (Qingdao, Shandong, China).

2.2. Immunohistochemistry (IHC)

The IHC detection was conducted in strict accordance with the instructions of the IHC Kit. The sections underwent conventional deparaffinization, hydratation and antigen retrieval. The activity of endogeneous peroxidase and non-specific antigens were blocked. Then the specimens were incubated with primary antibodies at room temperature, followed by incubation with enzyme-labeled secondary antibodies. The slides were stained with DAB as the color reagent followed by counterstaining with hematoxylin for nuclei, and then dehydration and sealing. Negative controls were achieved by substituting the primary antibodies with PBS.

2.3. Section scoring

FOXC1-positive cells were mainly located in the nucleus, only a small proportion in cytoplasm or cell membrane, while E-cadherin-positive cells were primarily located in cell membrane and cytoplasm, both factors stained light to dark brown in color (Figure 1 and 2). Each section was scored according to the intensity of stain: 0 (negative), 1 (light brown), 2 (brown), and 3 (dark brown). Each section was also scored according to the percentage of positively stained cells: 0 (0–5% positive cells), 1 (6–25% positive cells), 2 (26–50% positive cells), 3 (51–75% positive cells), and 4 (76–100% positive cells). The scores for intensity and percentage were then added together and recorded as: (-) for 0 and (+) for others. The total score was explained as: low expression (0 < FOXC1/E-cadherin<4) and high expression (5< FOXC1/E-cadherin<7).

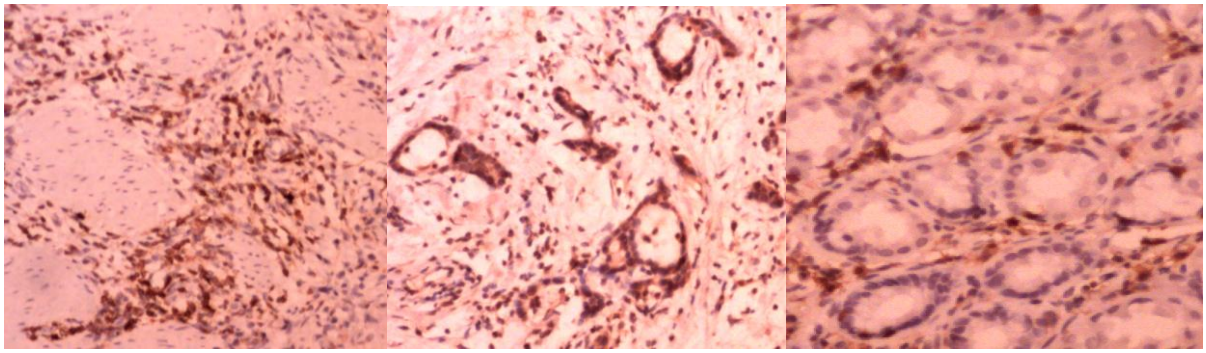

Figure1. A: expression of FOXC1in cancer tissue); B: expression of FOXC1in paracancerous tissues); C: expression of FOXC1 in normal tissue).

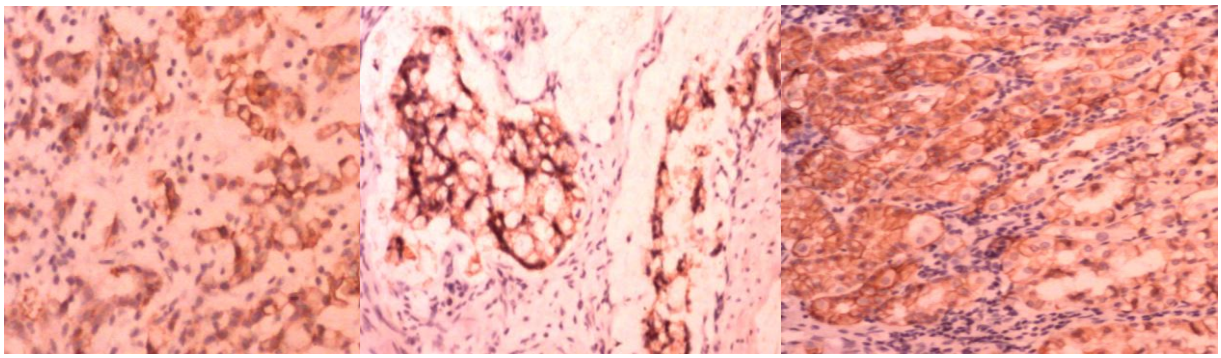

Figure2. D: expression of E-Cadherin in cancer tissue); E: expression of E-Cadherin in paracancerous tissues); F: expression of E-Cadherin in normal tissue).

2.4 . Statistical analysis

All statistical analyses were performed using SPSS 23.0 software package. The expressions of FOXC1 and E-cadherin in gastric cancer and paracancerous tissues were assessed by paired chi-square test, and the correlations of FOXC1 and E-cadherin with pathological features were analyzed using R×C contingency table chi-square test. All comparisons with a $P<0.05$ were considered statistically significant.

3. Results

3.1. Expressions of FOXC1 and E-cadherin in

gastric cancer and paracancerous tissues

The positive expression rate of FOXC1 in cancer and paracancerous tissues was 56.16% and 11.54%, respectively. Thus, FOXC1 expression was significantly higher in cancer tissue than in paracancerous tissue (P<0.05). In contrast, the positive expression rate of E-cadherin in cancer and paracancerous tissues was 24.14% and 94.20%, respectively; E-cadherin expression was significantly lower in cancer tissue than in paracancerous tissue (P<0.05) (Table 1, 2). In cancer tissue, the expression of FOXC1 had a significantly negative correlation with that of E-cadherin (P<0.05), the positive rate of FOXC1 and E-cadherin being 75.80% and 24.20%, respectively. Similarly in paracancerous tissue, the expression of FOXC1 had a significantly negative correlation with that of E-cadherin (P<0.05), the positive rate of FOXC1 and E-cadherin being 41.4% and 75.8%, respectively (Table 3, 4).

Table 1 Expression of FOXC1 gene in gastric cancer tissue and adjacent mucosa

FOXC1 gene	Adjacent mucosa		X^2	P	Positive rate%	
	High	Low			Cancer tissue	Adjacent mucosa
Cancer tissue	41	32	15.46	P<0.05	56.16%	11.54%
	3	23				

Table description: the difference was statistically significant, the positive rate of FOXC1 gene in cancer tissues was not equal with the adjacent tissues, the positive rate in cancer tissues was 56.16%, and the positive rate in the adjacent tissues was 11.54%, P < 0.05.

Table 2 Expression of E-Cadherin gene in gastric cancer tissue and adjacent mucosa

E-Cadherin gene	Adjacent mucosa		X^2	P	Positive rate%	
	High	Low			Cancer tissue	Adjacent mucosa
Cancer tissue	7	22	51.42	P<0.05	24.12%	94.20%
	65	4				

Table description: the difference was statistically significant, the positive rate of E-Cadherin gene in cancer tissues was not equal with the adjacent tissues, the positive rate in cancer tissues was 24.12%, and the positive rate in the adjacent tissues was 94.20%.

Table 3 Expression of FOXC1 gene and E-Cadherin gene in cancer tissue

Cancer tissue		E-Cadherin gene		X^2	P	Positive rate%	
		High	Low			FOXC1%	E-Cadherin%
FOXC1 gene	High	3	72	69.03	P<0.05	75.80%	24.20%
	Low	21	3				

Table description: In cancer tissues, the expression of FOXC1 was correlated with the expression of E-Cadherin factor, the positive rate of FOXC1 was 75.80%, the positive rate of E-Cadherin was 24.20%, P<0.05.

Table 4 Expression of FOXC1 gene and E-Cadherin gene in adjacent mucosa

Adjacent mucosa		E-Cadherin gene		X^2	P	Positive rate%	
		High	Low			FOXC1%	E-Cadherin%
FOXC1 gene	High	40	35	18.11	P<0.05	41.40%	75.80%
	Low	35	23				

Table description ：The expression of FOXC1 factor and E-Cadherin factor were related to the adjacent tissues, the positive rate of FOXC1 was 41.4%, and the positive rate of E-Cadherin was 75.8%, P<0.05.

Table 5 Relationship between FOXC1gene and clinicopathologic features

Clinicopathologic features	Cases(n)	FOXC1 gene level Positive	Negative	X^2	P	F-positive%
Male	50	40	10	0.99	P>0.05	80.00%
Famale	49	35	14			71.40%
Age（years）						
≤60	46	35	11	0.005	P>0.05	76.10%
>60	53	40	13			75.50%
TNMstage						
Ⅰ－Ⅱ	34	19	15	11.14	P<0.05	55.90%
Ⅲ－Ⅳ	65	56	9			86.20%
Local invasion						
T_{1-2}	75	10	65	9.52	P<0.05	84.42%
T_{3-4}	24	12	12			54.17%
Lymph node matastasis						
Yes	78	65	13	9.52	P<0.05	84.42%
No	23	12	11			54.17%
Tumour differentiation						
poor	66	61	5	29.95	P<0.05	94.42%
Well+moderate	33	14	19			42.42%

Table description: There was no difference between FOXC1 gene and age, sex in cancer tissues, FOXC1gene were significant differences in TNM stage, tumor type, depth of invasion and lymphatic metastasis. The positive rate of FOXC1 factor increased with the increase of malignant degree.

Table 6 Relationship between E-Cadherin gene and clinicopathologic features

Clinicopathologic features	Cases (n)	E-Cadheringene level Positive	Negative	X^2	P	E-positive%
Male	75	34	41	2.14	P>0.05	82.00%
Famale	24	15	9			69.39%
Age（years）						
≤60	51	12	39	0.41	P>0.05	76.47%
>60	48	14	34			70.83%
TNMstage						
Ⅰ－Ⅱ	78	13	65	6.57	P<0.05	83.33%
Ⅲ－Ⅳ	21	9	12			57.14%
Local invasion						
T_{1-2}	25	15	10	19.66	P<0.05	60.00%
T_{3-4}	74	11	63			14.86%
Lymph node matastasis						
Yes	69	8	61	19.83	P<0.05	11.59%
No	30	14	16			53.33%
Tumour differentiation						
poor	67	9	58	13.50	P<0.05	13.24%
Well+moderate	32	15	17			46.88%

Table description： There was no difference between E-Cadherin gene and age 、sex in cancer tissues, E-Cadherin gene were significant differences in TNM stage, tumor type, depth of invasion and lymphatic metastasis .The positive rate of E-Cadherin factor reduce is with the increase of malignant degree.

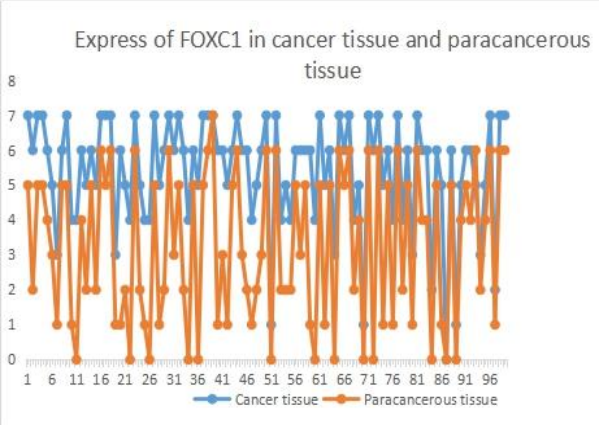

Figure 3. FOXC1of expression was significantly higher in the cancer tissue (5.34±1.60) than paracancerous tissues (3.21±2.18)

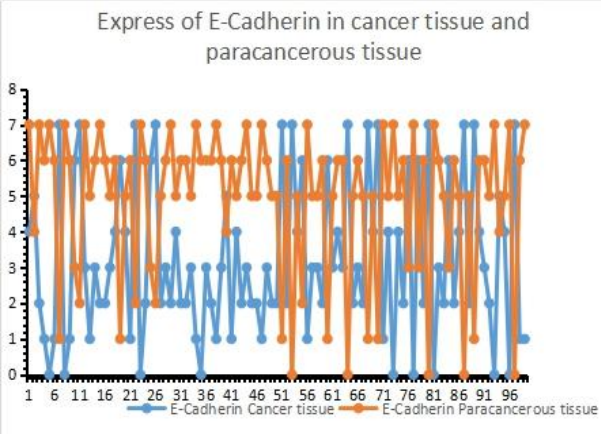

Figure 4. E-Cadherin of expression was signficantly lower in the cancer tissue (3.16±2.17) than paracancerous tissue (4.87±2.03).

3.2. Relationships between FOXC1 and E-cadherin expressions and pathological features of gastric cancer

The expression of FOXC1 was not significantly different between cases of distinct ages or genders no matter in cancer or paracancerous tissues (P>0.05), but was significantly different between cases of distinct TNM stages, histological subtypes, lymphatic metastasis and invasive depths (P<0.05). Similarly, E-cadherin expression was not significantly different between cases of distinct ages or genders in cancer or paracancerous tissues (P>0.05), but was significantly different between cases of distinct TNM stages, histological subtypes, lymphatic metastasis and invasive depths (P<0.05) (Table 5, 6).

4. Discussion

The carcinogenesis and development of gastric cancer is a process involving many factors. Exploring oncogenes and tumor suppressor genes and further identifying new targets for individualized treatment have become hotspots for research on gastric cancer. Transcription factors are a class of proteins that affect a series of biological processes including the growth, development, differentiation and metabolism of cells, tissues and organs by regulating the expressions of target genes. Forkhead box (FOX) transcription factors are a family of important transcription factors conservatively existing from lower organisms such as yeasts to higher eukaryotes such as humans. At present, FOX transcription factor superfamily can be divided into 19 sub-families from A to S according to different homologies of the DNA-binding domain, composed of over 100 family members. The DNA-binding domain of FOX transcription factors is designated as "forkhead domain", consisting of 100 amino acids, in which the core part constitutes 3 α-helices with a loop structure linked by a β-chain at each of the two sides. Due to these conformational features, FOX transcription factors are also known as winged-helix proteins [5]. As a member of FOX transcription factor superfamily, FOXC1 plays essential roles. It has been reported that mutations of FOXC1 is closely related to abnormal development of fetuses. Recent findings also show that FOXC1 is possibly involved in the carcinogenesis and development of tumors, including breast, prostate and ovarian cancers [6]. A study on breast cancer demonstrated that the epigenetic changes of FOXC1 may contribute to the carcinogenesis and development of the tumor , suggesting that FOXC1 may affect cell proliferation via regulating cell cycles, specifically, partially regulating the expression of cyclin D1 [7]. In addition, Salcedo and coworkers [8] revealed that both VEGF signaling and FOXC1 could induce the expression of CXCR4, thus promoting tumor angiogenesis. However, the role and the functional mechanisms of FOXC1 in tumors remain unclear and to be elucidated. Despite increasing reports about the involvement of FOXC1 in various tumors, the role of FOXC1 in gastric cancer remains rarely studied [9]. In the current study, we found that FOXC1 was expressed in both gastric cancer and paracancerous tissues, with a positive rate of 56.16% and 11.54%, respectively. The positive rate was significantly higher in cancer tissue than in paracancerous tissue, suggesting that FOXC1 may be correlated with the carcinogenesis and development of gastric cancer. As shown in Figure1 (A, B and C), FOXC1 was stained dark brown, brown and light brown or no color in cancer tissue, paracancerous tissue and normal tissue, respectively; the decreasing stain intensity and percentage of positive cells indicated that FOXC1 was highly expressed in gastric cancer tissue while lowly expressed in paracancerous and normal tissues. Specifically, the expression of FOXC1 was significantly higher in cancer tissue than in paracancerous tissue as shown in the line chart (Figure 3), indicating that FOXC1 is correlated with carcinogenesis and tumor development. Analysis of

pathological features demonstrated that FOXC1 expression was not associated with age or gender but related to TNM staging, histological subtypes, invasive depths, and presence or absence of lymphatic metastasis. With the decreasing degree of tumor differentiation, the tumor was ranked in a higher stage, the lymphatic metastasis increased in area and number, and the positive expression rate of FOXC1 increased in cancer tissue; whereas in paracancerous tissue the reverse changing trend was observed, indicating that FOXC1 may contribute to the invasion and metastasis of gastric cancer.

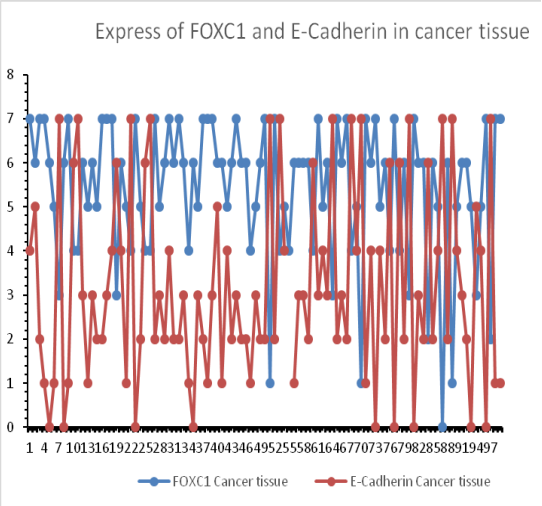

Figure 5. FOXC1of expression (5.34±1.60) was significantly higher than expression of E-Cadherin (3.16±2.17) in the cancer.

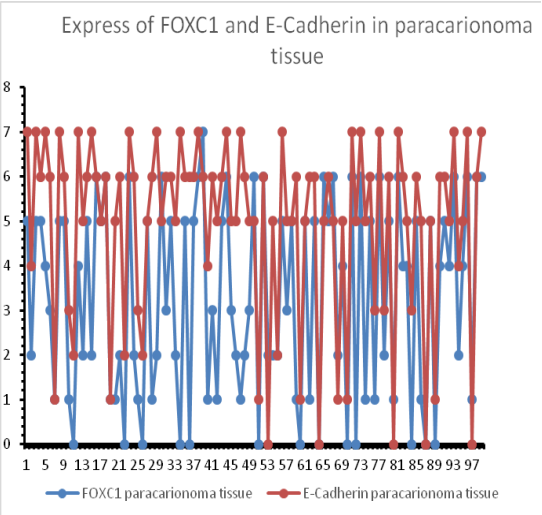

Figure 6. FOXC1of expression (3.21±2.18) was signficantly lower than expression of E-Cadherin (4.87±2.03) in paracancerous tissue.

E-cadherin is a member of the adhesion molecule family and receives attention due to its role in carcinogenesis and tumor development. It is a calcium-dependent cell adhesion molecule located on cell surface, which is involved in forming cell junctions, maintaining the integrity of epithelial tissue, and transducing intercellular signals due to its transmembrane structure [10]. E-cadherin is an alternative biomarker used to predict the prognosis of gastric cancer patients [11]. The current study found that E-cadherin expression was significantly associated with TNM staging, histological subtypes, invasive depths, and presence or absence of lymphatic metastasis. With the decreasing degree of tumor differentiation, the tumor was ranked in a higher stage, the invasive depth increased, and the lymphatic metastasis increased in area and number, but the positive expression rate of E-cadherin decreased; and the decrease became greater when the malignancy increased. The expression of E-cadherin was significantly lower in cancer tissue than in paracancerous tissue (94.20% vs 24.14%), suggesting that E-cadherin indeed plays a role in carcinogenesis and tumor development. As shown in Figure2 (D, E and F), E-cadherin was stained light brown or no color, brown and dark brown in cancer, paracancerous and normal tissues, respectively. The percentage of positively stained cells progressively increased from the cancer to normal tissue while the expression level of E-cadherin continuously decreased. The expression of E-cadherin was significantly lower in cancer tissue than in paracancerous tissue as shown in the line chart (Figure 4). All the results in Chart 2, Table (2,4)and Figure 2 indicate that E-cadherin is correlated with carcinogenesis, development, invasion and metastasis of gastric cancer.

Greenburg and coworker [12] first proposed the concept of epithelial-mesenchymal transition (EMT) in 1982. EMT was primarily considered a characteristic of embryogenesis, as it was closely related to the development of fetal tissues and organs, specifically contributing to the formation of gastrula, neural crest, cardiac valves and myocytes [13]. Since the report by Theiry [14] that malignant tumors acquired the invasive and metastatic abilities through EMT, EMT has become an important explanation for tumor metastasis. EMT is a dynamic process composed of multiple phases. The first phase includes desmosome rupture, cell dispersing, and separation of cell boundaries; the second phase involves inhibition of cytokeratin expression, activation of vimentin expression, destruction of basement membrane, and rearrangement of cytoskeleton; and the third phase is characterized by migration of cells into interstitial substance and phenotypic transformation from epithelial cells into mesenchymal cells. During the whole process of EMT, cells take on three phenotypes, including epithelial phenotype (E state), partial EMT phenotype (P state), and mesenchymal phenotype (M state). Some malignant tumors derived from epithelial cells undergo EMT during their oncogenesis and development, thus promoting invasion and metastasis [15]. Downregulation or loss of E-cadherin expression is a basic feature that characterizes EMT, and a large

number of transcription factors can inhibit the expression of E-cadherin. Numerous studies have revealed that downregulated expression of E-cadherin is related to tumor invasion and metastasis [6]. For gastric cancer, one study on E-cadherin reported that tumors lacking cell adhesion were likely to undergo lymphatic metastasis and to have poor prognosis. For other malignant tumors, studies showed that dysfunction of E-cadherin impaired the adhesive ability of tumor cells, leading to metastasis. The current study analyzed the relationships between FOXC1 and E-cadherin expressions in gastric cancer and paracancerous tissues and discovered that the positive expression rate of FOXC1 was remarkably higher than that of E-cadherin (75.8% vs 24.2%) in cancer tissue. In the presence of high FOXC1 expression, 72 cases had downregulated E-cadherin expression and 3 cases normal level (downregulation rate 96.0%); whereas in the presence of low FOXC1 expression, 3 cases had downregulated E-cadherin expression and 21 cases normal level (downregulation rate 12.5%). Thus, the downregulation rate of E-cadherin was significantly higher in FOXC1-high cases than in FOXC1-low cases. Comparison of the results in Figures (1, 2) and Figure (5, 6) revealed that FOXC1 had a higher expression than E-cadherin in cancer tissue while a lower expression than E-cadherin in paracancerous tissue. Therefore, we speculate that FOXC1 may promote EMT in gastric cancer cells via downregulating E-cadherin expressions, thus facilitating tumor invasion and metastasis.

In summary, FOXC1 and E-cadherin may play roles in the carcinogenesis, development, invasion and metastasis of gastric cancer. FOXC1 probably promotes tumor invasion and metastasis via regulating the expression of E-cadherin and further regulating the process of EMT. However, the specific relations and mechanisms remain to be elucidated by future research. FOXC1 can hopefully become a new biomarker to facilitate diagnosis and to predict prognosis, and provide a potential target for gastric cancer treatment.

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