Epithelial-mesenchymal interconversions and the regulatory function of the ZEB family during the development and progression of ovarian cancer

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Received December 12, 2014; Accepted December 16, 2015

DOI: 10.3892/ol.2016.4092

Abstract. This study assessed the role of epithelial-mesenchymal interconversions and the regulatory functions of the ZEB family during the development and progression of ovarian cancer. E-cadherin, vimentin, ZEB1 and ZEB2 were analyzed using immunohistochemistry in a series of ovarian tissues that included normal tissue, benign tumors, borderline tumors, malignant tumors and metastatic lesions. The correlation between E-cadherin and ZEB was analyzed. We also analyzed the association between the expression of the four factors and clinicopathological features in ovarian cancer. The results revealed that E-cadherin was weakly positive in normal ovarian epithelium. Cytoplasmic E-cadherin was significantly increased in benign tumors ($P<0.01$) and further increased in borderline tumors and ovarian cancers. However, cytoplasmic E-cadherin was markedly reduced in metastatic lesions ($P<0.01$). Membranous E-cadherin was increased in benign tumors, but decreased progressively in borderline, malignant and metastatic tumor tissues ($P<0.05$). The expression profile of vimentin was opposite to that of membranous E-cadherin. Membranous E-cadherin was negatively correlated with ZEB2 expression ($r=-0.514$). Additionally, cytoplasmic E-cadherin, ZEB1 and ZEB2 were associated with the FIGO stage of ovarian cancer. ZEB1 was also correlated with ascitic fluid volume. Our results suggest that epithelial-mesenchymal interconversions are dynamically regulated during the development and progression of ovarian tumors. ZEB2, but not ZEB1, may regulate the expression of membranous E-cadherin during these processes.

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Abbreviations: EOC, epithelial ovarian cancer; EMT, epithelial-mesenchymal transition; OSE, ovarian surface epithelium; MET, mesenchymal-epithelial transition; PBS, phosphate-buffered saline

Key words: ovarian cancer, EMT, MET, ZEB1, ZEB2

Introduction

Epithelial ovarian cancer (EOC) is one of the most common types of cancer among females and the most lethal gynecological malignancy (1). Due to a lack of obvious symptoms to allow for its early detection, EOC is usually diagnosed after the disease has already advanced to a late stage. By this time, treatments are limited or ineffective. Therefore, understanding the mechanisms of EOC initiation and progression is essential for its early diagnosis.

Epithelial-mesenchymal transition (EMT) is a complex and reversible process during which cellular phenotype, function and the expression of a large number of molecules are changed (2,3). EMT is connected not only to tumor invasion and metastasis but also to the early stages of carcinogenesis in epithelial malignancy (4,5). During classical EMT, epithelial markers, including E-cadherin, cytokeratins, ZO-1 and claudins, are downregulated, while mesenchymal markers, including vimentin, N-cadherin, fibronectin and MUC1, are upregulated. Among the epithelial markers, loss of E-cadherin is considered to be a hallmark of EMT. The disruption of E-cadherin-mediated intercellular adhesion is the initiating process in EMT, and this disruption plays a role in malignant transformation and tumor progression in a number of carcinomas (6,7). The expression of E-cadherin is regulated by various transcription factors, including Snail, Slug, Twist, ZEB1 and ZEB2. These transcription factors bind the E-box

sequence in the promoter region of CDH1 and repress E-cadherin expression (8).

Several studies have indicated that epithelial-mesenchymal interconversions are involved in the development and progression of ovarian cancer (9,10). However, the ovarian surface epithelium (OSE) is unique in that it has characteristics of epithelial and mesenchymal cells, and it alters its state of differentiation between epithelial and stromal phenotypes in response to environmental factors (11,12). Mesenchymal-epithelial transition (MET) occurs during the formation of inclusion cysts from OSE. The inclusion cysts then gain epithelial characteristics and may be the origin of EOC (13,14). EMT occurs during the development of ovarian tumors (15). Based on previous studies, we hypothesize that EMT and MET are dynamically regulated during the development and progression of ovarian cancer. The expression of EMT markers, including E-cadherin and vimentin, may also vary during this dynamic regulation. This may help explain inconsistencies in the expression of E-cadherin.

Transcription factors are considered to have a key role in the induction of EMT. The Snail and Twist families of transcription factors have been demonstrated to regulate E-cadherin expression and are associated with tumor progression in ovarian cancer (16-18). Studies into the ZEB family in ovarian cancer are relatively few, and the role of the ZEB family in ovarian cancer is currently unknown (12).

In this study, we analyzed the expression of E-cadherin and vimentin in various ovarian tissues and assessed the roles of EMT and MET in the development and progression of ovarian tumors. We also examined the regulatory effect of the ZEB family of proteins on E-cadherin, as well as the association of ZEB1, ZEB2, vimentin and E-cadherin with clinical parameters.

Materials and methods

Patients. A total of 72 formalin-fixed paraffin-embedded ovarian tissues and metastatic tissues were obtained from the Department of Pathology at the First and Third Affiliated Hospitals of Harbin Medical University, China, between 2009 and 2011. The samples included 10 normal ovarian samples, 12 benign epithelial ovarian tumors, 8 borderline epithelial ovarian tumors and 31 epithelial ovarian cancers. Additionally, 11 metastatic lesions were obtained from the above 31 cancer cases. The normal ovarian samples were obtained from patients who had received an ovariotomy due to endometrial cancer or cervical carcinoma. The diagnoses for all samples were confirmed by at least two pathologists. None of the patients received any therapy prior to surgery, and all patient samples had complete clinical information. All patients gave their informed consent prior to their inclusion in the study. The study was approved by the ethics committee of Harbin Medical University.

Immunohistochemistry. Immunohistochemistry was performed on 5-μm-thick paraffin-embedded tissue sections for all samples. Briefly, the slides were deparaffinized in xylene and rehydrated with a series of graded ethanol solutions. Endogenous peroxidase was blocked by 3% H_2O_2 at room temperature for 15 min. Antigen retrieval was performed using a microwave treatment at 95˚C for 15 min in citrate buffer (pH 6.0). After washing three times with phosphate-buffered saline (PBS) for 3 min each, the sections were treated with 5% bovine serum albumin for 10 min at room temperature to block nonspecific reactions. The sections were then incubated with primary antibody against E-cadherin (ZSGB Bio, Beijing, China; 1:50), vimentin (ZSGB Bio; 1:50), ZEB1 (Biorbyt, Cambridge, UK; 1:100) or ZEB2 (Sigma-Aldrich, St. Louis, MO, USA; 1:100) overnight at 4˚C. The bound antibodies were detected using a streptavidin-biotin peroxidase kit (ZSGB Bio), and the final staining was completed with DAB (ZSGB Bio). Negative controls were created by replacing the primary antibodies with PBS. The positive controls were samples that had previously been demonstrated to express high levels of the protein being tested.

Evaluation of immunohistochemistry results was carried out by a pathologist who was blinded to the clinical information of the patients. The immunohistochemical expression was scored for intensity and extent. Staining intensity was quantified as follows: negative (0), weak (1), moderate (2) or strong (3). Staining extent was scored according to the percentage of positive cells: none (0), <25% (1), 25-50% (2), 50-75% (3) or >75% (4). The final immunohistochemical score was then calculated as the intensity score multiplied by the extent score.

Statistical analysis. All data are presented as the means ± standard deviation. The two groups were compared using Student's t-test. The Pearson correlation test was performed to determine associations between antibody staining patterns. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of E-cadherin, vimentin, ZEB1 and ZEB2 in ovarian tissues. According to the results of immunohistochemical staining (Fig. 1 and Table I), E-cadherin was almost negative in normal ovarian epithelium and was positive in ovarian neoplastic cells. E-cadherin was localized on the cell membranes and/or in the cytoplasm. Membranous expression of E-cadherin was significant in benign tumors and was reduced in borderline tumors. The majority of ovarian cancer tissues expressed low levels of membranous E-cadherin, and almost all metastatic lesions were negative. Cytoplasmic expression of E-cadherin was gradually increased in benign tumors, borderline tumors and ovarian cancers, although there were no significant differences between them. Notably, cytoplasmic E-cadherin expression was markedly reduced in metastatic lesions.

Normal epithelium tissues were positive for vimentin expression, but almost all benign tumors were negative. The expression level of vimentin was increased in borderline tumors and ovarian cancer tissues and significantly increased in metastatic lesions. Additionally, positive expression of vimentin was mainly localized around the cancer nest in the primary lesion, particularly in the cells which had detached from the cancer nest and migrated into the stroma.

ZEB1 and ZEB2 were expressed mainly in the cytoplasm of the normal epithelium and tumor cells. There was no

Table I. Immunohistochemical scores of E-cadherin, vimentin and ZEB in ovarian tissues.

Variable	Normal	Benign	Borderline	Malignant	Metastatic lesions
E-cadherin-C	0.90±0.99	4.42±1.93[b]	6.63±3.25	8.29±3.37	1.91±0.83[b]
E-cadherin-M	0.80±0.79	7.58±2.97[b]	5.00±1.85[a]	1.61±1.17[b]	0.27±0.47[b]
Vimentin	3.20±1.87	1.33±1.97[a]	2.88±1.64	4.74±2.31[a]	8.00±2.10[b]
ZEB1	8.60±2.07	8.67±2.77	7.75±2.43	7.48±2.67	7.73±2.53
ZEB2	8.10±1.85	4.25±1.48[b]	6.64±1.92[a]	8.29±2.44	9.09±2.70

[a]Compared with the previous group, P<0.05; [b]Compared with the previous group, P<0.01. E-cadherin-C, staining of E-cadherin in the cytoplasm; E-cadherin-M, staining of E-cadherin in the cell membrane.

Table II. Correlation of E-cadherin, vimentin and ZEB with clinical pathological parameters in ovarian cancer.

Variable	E-cadherin-C	E-cadherin-M	Vimentin	ZEB1	ZEB2
Age					
≤50 years	8.59±3.02	1.65±1.22	4.24±2.05	7.41±2.87	8.47±2.87
>50 years	7.93±3.83	1.57±1.16	5.36±2.53	7.57±2.50	8.07±1.86
CA125					
≤35 U/ml	8.0±3.74	1.25±0.96	5.50±1.00	5.50±3.00	7.50±1.73
>35 U/ml	8.33±3.39	1.67±1.21	4.63±2.44	7.78±2.55	8.41±2.53
Ascitic fluid volume					
≤100 ml	7.18±3.22	1.73±1.19	4.27±2.49	6.18±2.75[a]	8.09±2.84
>100 ml	8.90±3.37	1.55±1.19	5.00±2.22	8.20±2.40	8.40±2.26
Residual tumor					
≤2 cm	8.30±3.43	1.80±0.63	4.90±2.51	7.20±1.03	8.80±2.66
>2 cm	8.29±3.42	1.52±1.36	4.67±2.27	7.62±3.19	8.05±2.36
FIGO stage					
I-II	6.53±2.65[b]	1.71±1.26	5.00±2.42	5.88±1.93[b]	7.41±2.29[a]
III-IV	10.43±2.93	1.50±1.09	4.43±2.21	9.43±2.10	9.36±2.24
Tumor grade					
High or moderate	7.60±3.74	1.80±1.21	4.53±2.88	7.67±2.87	8.73±2.87
Low	8.94±2.95	1.44±1.15	4.94±1.69	7.31±2.55	7.88±1.96
Histological type					
Serous	8.77±3.52	1.64±0.90	4.73±2.19	7.68±2.68	8.68±2.40
Mucinous	7.11±2.80	1.56±1.74	4.78±2.73	7.00±2.74	7.33±2.40

[a]P<0.05, [b]P<0.01. E-cadherin-C, staining of E-cadherin in the cytoplasm; E-cadherin-M, staining of E-cadherin in the cell membrane.

difference in the expression of ZEB1 among the various types of ovarian tissues, although the expression of ZEB2 was higher in normal ovarian tissues, reduced in benign tumors and increased progressively in borderline tumors, ovarian cancer and metastatic lesions.

Correlation between E-cadherin and ZEB2 in ovarian tissues. Membranous E-cadherin expression was significantly negatively correlated with that of ZEB2 during the progression of ovarian cancer, with a correlation coefficient of -0.514. However, the expression of cytoplasmic E-cadherin was not associated with that of ZEB2. There was no correlation between E-cadherin and ZEB1.

Correlation of E-cadherin, vimentin and ZEB with clinical pathological parameters in ovarian cancer. The correlation of E-cadherin, vimentin, ZEB1 and ZEB2 with clinical pathological parameters was analyzed in 31 patients with ovarian cancer. As shown in Table II, the expression of cytoplasmic E-cadherin was higher in patients with FIGO stage III/IV ovarian cancer than in those with FIGO stage I/II ovarian cancer (P<0.01). ZEB1 and ZEB2 were also more highly expressed in patients with FIGO stage III/IV cancer (P<0.01 and P<0.05, respectively). Additionally, the expression of ZEB1 was associated with ascitic fluid volume, such that patients with more ascitic fluid had increased expression of ZEB1 (P<0.05).

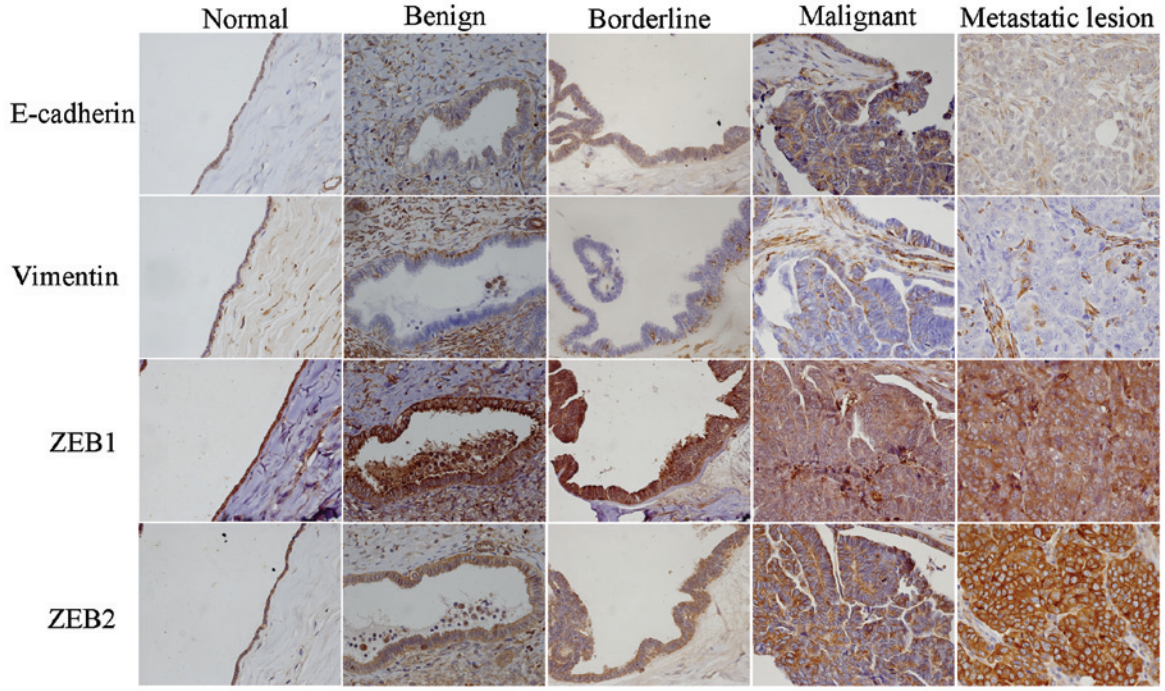

Figure 1. Immunohistochemistry staining in ovarian tissues. E-cadherin, vimentin, ZEB1 and ZEB2 were analyzed in continuous sections of a series of ovarian tissues including normal tissue, benign tumors, borderline tumors, malignant ovarian tumors and metastatic lesions.

Discussion

Epithelial-mesenchymal interconversions are involved in the carcinogenesis and progression of ovarian cancer. However, the expression of EMT markers in the normal and neoplastic ovary are complex and do not fully follow the typical EMT model. Normal OSE expresses little or no E-cadherin, while OSE cells that line the wall of inclusion cysts are typically positive for E-cadherin (14). E-cadherin expression in OSE was also reported to vary with different locations within the ovary and with cell shape (19). The literature describing E-cadherin expression in ovarian cancer is inconsistent. Certain studies indicate that E-cadherin expression is reduced in primary ovarian cancer and is re-expressed in ovarian cancer effusions at a higher level (20). Other studies indicate that primary ovarian cancer expresses E-cadherin, and its expression is reduced in advanced tumors (21). Further studies revealed that E-cadherin expression is increased in metastatic ovarian lesions compared with the primary lesion (22).

EMT plays a significant role during late invasion and metastasis (15). We detected the two classic EMT markers, E-cadherin and vimentin, in normal and ovarian tumors. Our results revealed that membranous and cytoplasmic E-cadherin were expressed at low levels in normal OSE. Additionally, membranous E-cadherin expression was higher in benign ovarian tumors, decreased in borderline and malignant tumors, and almost non-existent in metastatic lesions. The expression profile of vimentin was opposite to that of membranous E-cadherin. These findings indicate that epithelial-mesenchymal interconversions are dynamic during the development and progression of ovarian tumors. Furthermore, as OSE is the site of frequent metaplastic and dysplastic changes and is considered to be the origin of tumor formation, a theory which is supported by certain authors, MET may in fact occur here first (14,19). Traditionally, E-cadherin is regarded as a significant component of cell-to-cell adherens junctions, and most investigators evaluate the expression of E-cadherin on the membranes of different tumor cells. However, E-cadherin is also observed in the cytoplasm and nucleus of tumor cells. Voutilainen *et al* (23) reported that strong cytoplasmic expression of E-cadherin was present in 9% of EOC cases. In our study, we noted that cytoplasmic E-cadherin was progressively increased in benign, borderline and malignant ovarian tumors and significantly decreased in metastatic lesions. It has been reported that E-cadherin in the cytoplasm or nucleus may participate in specific signaling networks to promote tumor progression (24). It may also act as a regulator of gene transcription by modulating the activity of several signaling pathways (25,26). Our results also support the emerging correlation between cytoplasmic E-cadherin and tumor progression. However, the exact function of cytoplasmic E-cadherin remains unclear. One intriguing question is why cytoplasmic E-cadherin is significantly decreased in metastatic lesions.

A number of studies have indicated that E-cadherin, the hallmark of EMT, is mainly regulated by Snail, Twist, ZEB2/SIP1 and other transcription factors (16,27). Yoshida *et al* (16) observed that the expression of Snail, Slug, ZEB2/SIP1 and Twist increased progressively in benign, borderline and malignant tumors. Among these molecules, the expression of Snail was significantly negatively correlated with E-cadherin expression. Nuclear Snail expression was demonstrated to be correlated with tumor progression, but was not associated with clinicopathological factors or prognosis (18). In recent years, Twist has been recognized as having a central role in EMT (28), and data from clinical studies suggest a prognostic role for Twist (17). The ZEB family includes ZEB1 and ZEB2/SIP1. The clinical role of ZEB1 and ZEB2 in ovarian carcinoma is currently not well established. Our results revealed

that ZEB1 and ZEB2 are expressed at high levels in ovarian tissue. ZEB1 expression did not change among the various types of ovarian tissues, but ZEB2 expression was higher in OSE, decreased in benign ovarian tumors, and increased progressively in borderline tumors, malignant tumors and metastatic lesions. In contrast to our data, ZEB1 mRNA levels were previously reported to be significantly higher in metastases compared with primary carcinomas and effusions (29). A similar expression profile for ZEB2 was observed in another study (16). However, ZEB2 mRNA expression levels were significantly higher in effusions compared with primary tumors and solid metastases, and ZEB2 was revealed to be a main regulator of E-cadherin in effusions (26). Additionally, our results revealed that ZEB1 and ZEB2 were mainly located in the cytoplasm, which contradicts the results of most other studies. In order to confirm the specificity of antibodies, we analyzed ZEB1 and ZEB2 in brain tissues according to the manual, and the staining was nuclear. ZEB1 and ZEB2 are expressed in brain tissues and may, therefore, be used as a positive control. Therefore, our results are credible. Gamba *et al* (30) also reported nuclear and cytoplasmic staining for ZEB2 in invasive micropapillary carcinoma, and suggested that cytoplasmic ZEB2 might be a significant factor in the early stages of malignancy and predicts a poor overall survival rate. Li *et al* (31) also demonstrated cytoplasmic staining of ZEB1 and ZEB2 in HCC cells and adjacent non-tumoral liver cells.

A correlation analysis revealed that membranous E-cadherin was significantly negatively correlated with ZEB2 expression during the progression of ovarian cancer. Our results suggest that ZEB2 is involved in the regulation of epithelial-mesenchymal interconversions in ovarian cells.

In summary, the expression profiles of membranous E-cadherin and vimentin indicated that dynamic epithelial-mesenchymal interconversions occur during the development and progression of ovarian cancer. ZEB2, but not ZEB1, participated in the regulation of E-cadherin expression during this process. These results provide a molecular basis for studying the pathogenesis of ovarian cancer and exploring these potentially valuable therapeutic targets further.

Acknowledgements

This study was supported by the Heilongjiang Provincial Health Bureau (grant no. 2012-518), the National Natural Science Foundation of China (grant nos. 81302061 and 81241092) and the 973 Program for an earlier research project (grant no. 2012CB526705).

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