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ZEB-1 and E-cadherin expression may predict recurrence-free survival in patients with invasive ductal breast carcinoma

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Abstract

Background: Epithelial-Mesenchymal Transition (EMT) plays an important role in cancer progression and metastasis. We investigated the expression of two EMT-related molecules, ZEB-1 and E-cadherin, in invasive ductal breast carcinoma and evaluated their association with clinicopathological parameters and recurrence-free survival.

Methods: We evaluated the expression of ZEB-1 and E-cadherin in 116 patients of Stage I, II, and III primary invasive ductal breast carcinoma (mean age, 58.5 years, male: female = 0:116) using immunohistochemistry.

Results: Of the 116 patients, 51 (44%) had Stage I, 58 (50%) had Stage II, and 7 (6%) had Stage III tumors. Thirty seven patients (32%) had lymph node metastasis. One hundred and one patients (87%) had ER positive tumors, and 20 patients (17%) had HER2 positive tumors. Positive ZEB-1 expression was observed in the tumors of 66 patients (57%), but it was not significantly associated with clinicopathological parameters. Reduced E-cadherin expression was observed in the tumors of 34 patients (29%). It was significantly associated with negative estrogen receptor expression. Patients with a positive ZEB-1 and / or reduced E-cadherin had a poorer outcome than the other patients in terms of recurrence-free survival.

Conclusion: ZEB-1 and E-cadherin expression may predict recurrence-free survival in patients with invasive ductal breast carcinoma.

Keywords: Breast carcinoma, ZEB-1, E-cadherin, EMT

Introduction

Breast cancer is one of the most common malignant cancer in woman worldwide. Approximately 90 % of breast cancer-related deaths are caused by local invasion and distant metastasis of tumor cells. Metastasis is a complex process divided into a number of steps including detachment of tumor cells from the primary tumor, invasion, migration, intravasation, survival in vasculature, extravasaion, and colonization of the secondary site. The transformation of normal breast cancer epithelial cells to metastatic ones arises during the metastatic cascade[1]. The pioneering work of Hay[2] first described Epithelial-Mesenchymal Transition (EMT). That is a phenomenon that epithelial cells transform to mesenchymal cells. EMT plays crucial role in the organogenesis of embryo. In addition, EMT also allows epithelial tumor cells to lose their attachments to neighboring cells and transit to mesenchymal tissue. EMT contributes to cause

organ fibrosis and promote carcinoma progression through a variety of mechanisms. Recently, it has become clear that EMT also plays an important role in cancer progression, metastasis, and chemoresistance[3, 4]. The involvement of EMT varies between different types of cancers. Although investigations about breast cancer had been performed, much remains to be elucidated [1, 5].

E-cadherin is a transmembrane glycoprotein which is mainly presented in human epithelial cells and mediates cell adhesion between E-cadherin molecules on adjacent cells. It is one of a typical adhesive molecules of epithelium and plays a role of establishment of stable adherent junctions and adhesion of cells. During EMT, expression of E-cadherin is decreased, then epithelial tumor cells lose their adhesion and become migratory and invasive acquiring mesenchymal properties. It is known that tumor malignancy is inversely correlated with its expression. Now it is considered that the most important event of EMT is loss of E-cadherin[3, 4]. Various transcriptional factors regulate expression of E-cadherin. Zinc finger E-box-binding homeobox 1 (ZEB-1) is a member of the zinc-finger E-box-binding homeobox factor family, and can inhibit the expression of E-cadherin by binding to its promoter. ZEB-1 is now considered a one of the typical molecule of EMT by inhibiting E-cadherin expression which induce an epithelial phenotype [6, 7]. EMT molecules induce changes in morphology of mammary epithelial cells with the shift to mesenchymal phenotype, and acquiring malignancy and chemoresistance [8]. Many previous studies have evaluated ZEB-1 expression in several carcinomas such as gastric, pancreatic, hepatocellular, and lung cancers, and reported that the ZEB-1 expressions were associated with high malignancy, development of metastasis and poor prognosis [9-12].

Postoperative recurrence is the key factor affecting the clinical treatment and prognosis of breast cancer patients. We hypothesized that EMT related molecules could be predictive factors of postoperative recurrence of invasive ductal breast cancer. Although ZEB-1 expression in breast cancer has previously been studied using immunohistochemistry [13-16], it is still not clear whether ZEB-1 predicts recurrence-free survival in patients with invasive ductal breast carcinoma. In this study, we investigated the association between the expression status of the EMT related molecule, ZEB-1 and E-cadherin, and postoperative recurrence in invasive ductal breast carcinoma using immunohistochemistry.

Material and methods

Patients and tumor samples

We evaluated 116 patients of Stage I, II, and III primary invasive ductal breast carcinoma who had undergone curative surgery between 2007 and 2010 at Yamagata University Hospital. All specimens were histologically diagnosed as invasive ductal breast carcinoma on the basis of the General Rules for Clinical and Pathological Recording of Breast Cancer (The Japanese Breast Cancer Society, 2012, 17th edition). Staging was determined according to the TNM Classification of Malignant Tumours (UICC, Seventh Edition, 2009). For bilateral breast carcinomas, we recorded the side of higher stage or malignancy. We used cancer stage recorded at admission if neo adjuvant chemotherapy had been performed.

Estrogen receptor (ER) and progesterone receptor (PgR) were detected by immunohistochemical staining with anti-estrogen receptor rabbit monoclonal antibody (clone SP1, Ventana Japan, Yokohama, Japan) and anti-progesterone receptor rabbit monoclonal antibody (clone 1E2, Ventana Japan, Yokohama, Japan), respectively. They were defined as positive if at least 1% of the tumor cells showed positive immunohistochemical staining. Human epidermal growth factor receptor type 2 (HER2) was detected by immunohistochemical staining using the Hercep Test kit (DAKO JAPAN, Tokyo, Japan). HER2 positivity was defined as immunohistochemical score of 3 or 2 which is fluorescence *in situ* hybridization (FISH) positive.

This study was conducted according to principals of the Declaration of Helsinki, with approval from the ethic committees of Yamagata University Faculty of Medicine. Before tissues were obtained, each patient provided written informed consent to

participate in our study. In our institute during the period of this study, we selected each patients' treatment according to patients'own risk and the clinical practice guidelines used at that time, and any treatments were not performed if they were contraindicated or the patient did not consent to them.

Immunohistochemistry

A mouse anti-human monoclonal antibody to ZEB-1 (Clone CL0151, lot number 02551) was purchased from ATLAS ANTIBODIEDS, Inc. (Stockholm, Sweden), and a mouse anti-human monoclonal antibody to E-cadherin (Clone NCH-38, lot number 10081325) was purchased from DAKO JAPAN (Tokyo, Japan). These were diluted to 1:500 and 1:100, respectively, in phosphate-buffered saline (PBS) according to the manufacturer's instructions.

Each resected specimen was fixed with formalin and embedded in paraffin. Thin serial sections containing representative areas of invasive carcinomas were made with a microtome. Immunohistochemical staining was performed using the labeled streptavidin biotinylated antibody (LSAB) method. After deparaffinization and dehydration, heat-induced antigen retrieval by autoclave pretreatment (120℃ for 20 minutes) in citrate buffer solution (pH 6.0) was performed. Endogenous peroxidase activity was blocked using absolute methanol solution containing 0.3% H_2O_2 for 30 minutes. Unspecific staining was blocked by skimmed milk. Specimens were incubated with diluted anti-ZEB-1 or E-cadherin antibodies at 4℃ overnight. These antibodies were omitted for negative controls. Next day, specimens were incubated with a biotinylated secondary antibody and an avidin-biotin-peroxidase complex, SAB-PO (MULTI) kit purchased from NICHIREI (Tokyo, Japan). Biotin was visualized using diaminobenzidine (DAB). Counterstaining was performed using hematoxylin. We used uterine carcinosarcoma specimens as positive control for ZEB-1 staining, and normal colonic mucosa as positive control for E-cadherin staining. There was no staining in the negative controls.

Evaluation of immunohistochemical staining

Based on a previous study by Soini [15], the immunoreactivity for ZEB-1 of stromal fusiform cells was semi-quantified as follows:

0-5% = negative, 5-25% = weak positivity, 25-50% = moderate positivity, 50-75% = strong positivity, 75-100% = very strong positivity

We considered that staining of weak positivity or over was significant positive, intensity was not evaluated. For epithelial tumor cells, only the presence of nuclear negativity or positivity was assessed. According to Pang's previous report[17], the immunoreactivity of E-cadherin of epithelial tumor cells was semi-quantified as follows:

Intensity of staining was categorized into four grades (0 to 3);

0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.

In addition, we compared the peripheral part of the tumor, such as infiltrating fatty tissue, with the central part of the tumor. We considered E-cadherin expression to be reduced if whole tumor cells were weak staining, or if the peripheral part of the tumor were stained less than the central part of the tumor. We verified that normal ductal tissue showed moderate or greater staining as an internal positive control.

Statistical analysis

We used the JMP 11.0.0 software for Windows (SAS Japan, Tokyo, Japan) for all statistical analyses. Associations between ZEB-1 or E-cadherin expression status and clinicopathological parameters were evaluated using the Chi squared test. Recurrence-free survival rates were evaluated by Kaplan-Meier method and differences were evaluated using the log-rank test. The time to recurrence was defined as the duration from the original operation to the date on which patients were diagnosed with recurrence based on the radiological findings. Prognostic factors were analyzed using univariate and multivariate analyses (Cox proportional hazard model). A P-value of less than 0.05 was considered statistically significant.

Results

Characteristics of patients

Characteristics of patients are summarized in Table1. There were no male patients in the period of this study. All of the patients were female, and their mean age was 58.5 years (range 32-88). Fifty one patients (44%) had Stage I disease, 58 patients (50%) had Stage II disease, and 7 patients (6%) had Stage III disease. Thirty seven patients (32%) had lymph node metastasis. One hundred and one patients (87%) had ER positive tumors, and 20 patients (17%) had HER2 positive tumors, and 9 patients (8%) had triple negative tumors. None of the patients died in the perioperative period. During the median follow-up period of 59.5 months (range 8-89) from surgery to recurrence, 12 patients experienced postoperative recurrence (7 in the lymph nodes, 4 in the bone, 3 in the lung, 1 in the liver; and in some cases the disease recurred simultaneously at several sites). Of the 12 recurrent patients, the median time from surgery to recurrence was 20.5 months (range 12-43). During the median follow-up period of 61 months (range 8-89) from surgery to the most recent follow-up, 5 patients died from the disease. Of the 5 died patients, the median time from recurrence to death was 24 months (19-68).

Table 1. Characteristics of patients

Patients	n = 116
Sex, n (%)	
Male/Femal	0 (0%) / 116 (100%)
Age	58.5 ± 13.3 (32 - 88)
T classification	
T1/T2/T3/T4	62 (54%)/ 45 (39%)/ 4 (3%)/ 5(4%)
Lymph node metastasis	
Positive	37 (32%)
Negative	79 (68%)
TNM Stage	
I/II/III	51 (44%) / 58 (50%)/7 (6%)
ER	
Potitive	101 (87%)
Negative	15 (13%)
PgR	
Potitive	86 (74%)
Negative	30 (26%)
HER2	
Potitive	20 (17%)
Negative	96 (83%)
Triple negative	9 (8%)
Menoposal status	
Pre	34 (29%)
Post	82 (71%)
Operation	
Mastectomy	64 (45%)
Conservative surgery	52 (55%)
Axillar disection	
Axillar	105 (91%)
Sentinel node biopsy	7 (6%)
None	4 (3%)
Systemic therapy	
Chemothrapy	45 (39%)
Trastuzumab	9 (8%)
Hormonal therapy	100 (86%)
None	5 (4%)
Radiation	36 (31%)
Recurrence	
Present	12 (10%)
Died	5(4%)

ZEB-1 expression

In the positive control, ZEB-1 staining was detected in the nuclei of sarcoma lesion of uterine carcinosarcoma (Fig. 1 a). ZEB-1 expression was detected only in the nuclei of stromal fusiform cells surrounding the invasive ductal breast carcinomas (Fig. 1 b). Only a few nuclei of stromal fusiform cells surrounding non-invasive carcinomas were stained. No epithelial tumor

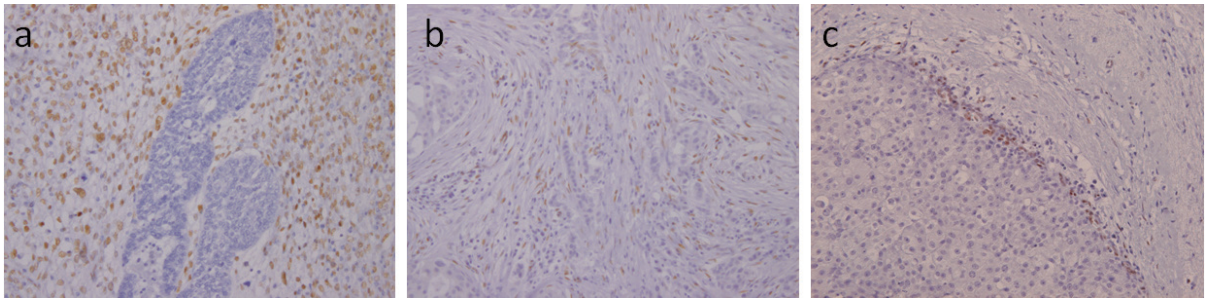

Fig. 1 Representative images of ZEB-1 staining. Original magnification, × 20
a Uterine carcinosarcoma as a positive control; Tumor cells in the sarcoma lesion were stained.
b Stromal fusiform cells in invasive ductal breast carcinoma were stained.
c ZEB-1 expression in the marginal lesion of tumor.

Table 2. Clinicopathological parameters of stromal ZEB-1 and tumor E-cadherin expression in breast invasive ductal carcinoma.

Variable	ZEB-1				E-cadherin		
	Total no. (n = 116)	positive (n = 66)	negative (n = 50)	p-value	preserved (n = 82)	reduced (n = 34)	p-value
Stage, n (%)							
I		25 (38%)	26 (52%)	0.28	38 (46%)	13 (38%)	0.705
II		36 (54%)	22 (44%)		39 (48%)	19 (56%)	
III		5 (8%)	2 (4%)		5 (10%)	2 (6%)	
Lymph node							
Positive		25 (38%)	12 (24%)	0.109	23 (28%)	14 (41%)	0.172
Negative		41 (62%)	38 (76%)		59 (72%)	20 (59%)	
ER							
Positive		56 (85%)	45 (90%)	0.408	76 (93%)	25 (77%)	0.008
Negative		10 (15%)	5 (10%)		6 (7%)	9 (23%)	
PgR							
Positive		46 (70%)	40 (80%)	0.201	63 (77%)	23 (67%)	0.31
Negative		20 (30%)	10 (20%)		19 (23%)	11 (33%)	
HER2							
Positive		15 (23%)	5 (10%)	0.065	14 (17%)	6 (18%)	0.941
Negative		51 (77%)	45 (90%)		68 (83%)	28 (82%)	
Triple negative							
Yes		5 (8%)	4 (8%)	0.932	2 (2%)	7 (21%)	0.002
Other		61 (92%)	46 (92%)		80 (98%)	27 (79%)	
Recurrence							
Absent		56 (85%)	48 (96%)	0.04	78 (95%)	26 (76%)	0.004
Present		10 (15%)	2 (4%)		4 (5%)	8 (24%)	
ZEB-1							
Positive					42 (51%)	24 (71%)	0.052
Negative					40 (49%)	10 (29%)	

cell was stained. Therefore, we only evaluated expression in the stromal fusiform cells. Table 2 shows the associations between ZEB-1 expression and clinicopathological parameters. Positive expression of ZEB-1 in stromal fusiform cells was observed in 66 patients (57%). It was significantly associated with postoperative recurrence ($p = 0.04$). There was no significant association between ZEB-1 expression and other parameters including stage, lymph node metastasis and biomarkers. ZEB-1 expression in the marginal lesion of a tumor as if it was starting invasion was observed in 7 patients (Fig. 1c). But this was not significantly associated with clinicopathological parameters or postoperative recurrence (date not shown).

E-cadherin expression

E-cadherin staining was detected in the cellular membrane of epithelial tumor tissues, and intensities of staining were shown in Fig. 2. We also show an example that the peripheral part of tumor such as tumor infiltration to fatty tissue

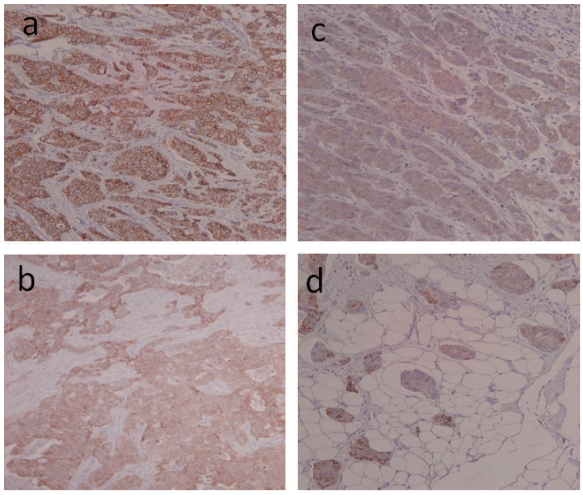

Fig. 2
Immunohisitochemical analysis of E-cadherin expression.
Original magnification, × 20. Examples of positive staining of membranes in breast cancer tissue: a strong, b moderate, c weak; d weak staining in peripheral part.

was stained less than the central part of tumor (Fig. 3). Table 2 also shows the associations between E-cadherin expression and clinicopathological parameters. Reduced E-cadherin expression was observed in the tumor of 34 patients (29%). It was significantly associated with ER negative (p = 0.008), triple negative (p = 0.002) and postoperative recurrence (p = 0.004). As with ZEB-1, no significant association was observed with other factors including stage, lymph node metastasis. Positive ZEB-1 expression occurred more frequently in tumors with reduced E-cadherin expression, but this was not significant (p =0.052).

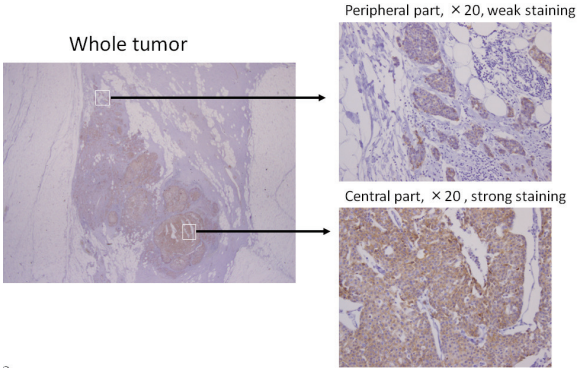

Fig. 3
An example comparing the peripheral part of tumor with the central part of tumor of E-cadherin expression.

Recurrence-free survival

During the median follow-up period of 59.5 months (range 8-89), 12 patients experienced disease recurrences, of whom 10 patients had tumors with positive for ZEB-1 expression, and 8 had tumors of reduced E-cadherin expression. Figure 4 shows recurrence-free survival rate after resection by Kaplan-Meier curve according to ZEB-1, E-cadherin expression or matching them. The patients with positive ZEB-1 expression group had a poorer outcome in terms of recurrence-free survival than the negative ZEB-1 expression group (5-year 83.2% vs. 95.8%, p = 0.036) (Fig. 4a). Similarly, the reduced E-cadherin expression group had a poorer outcome than the preserved E-cadherin expression group (5-year 74.5% vs. 94.7%, p = 0.002) (Fig. 4b). Among patients with positive ZEB-1 expression, reduced E-cadherin group had a poorer outcome than the

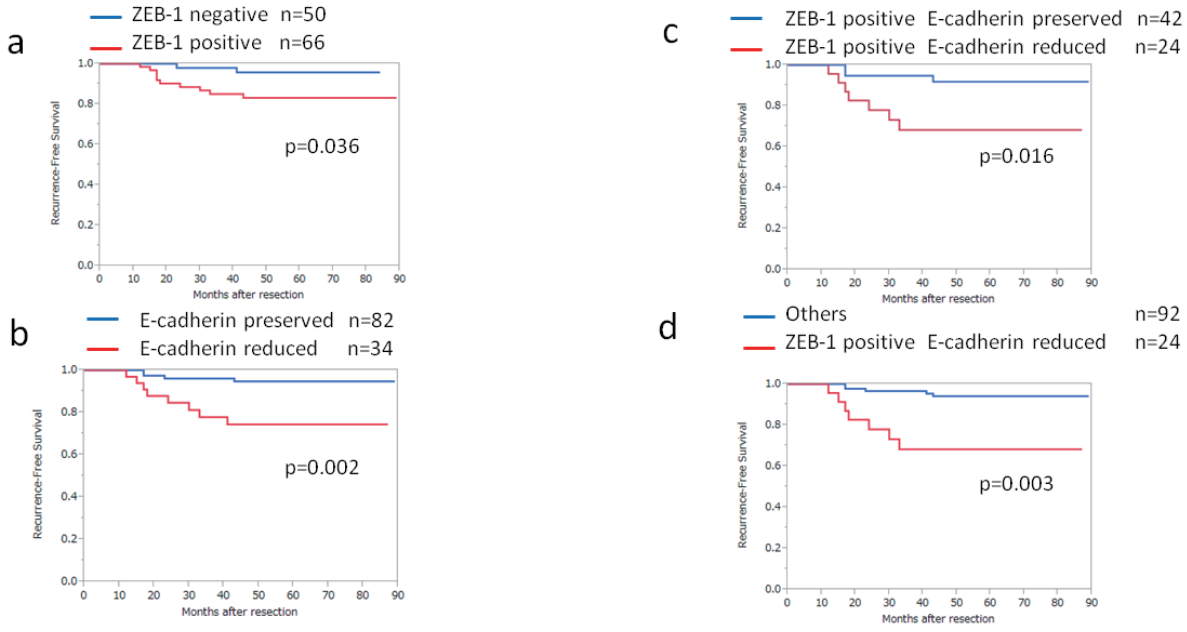

Fig. 4
Kaplan-Meier curves of recurrence-free survival for each immunohistochemical staining group;
a.the ZEB-1 positive group vs. the ZEB-1 negative group (5-year 83.2% vs. 95.8%, p = 0.036). b. the E-cadherin negative group vs. the E-cadherin positive group (5-year 74.5% vs. 94.7%, p = 0.002). c. among ZEB-1 positive groups, the E-cadherin reduced group vs. the E-cadherin preserved group (5-year 68.2% vs. 91.8%, p = 0.016). d. the ZEB-1 positive/ E-cadherin reduced group vs. the other group (5-year 68.2% vs. 94.1%, p = 0.003).

other group (5-year 68.2% vs. 91.8%, p = 0.016) (Fig. 4c). The positive ZEB-1 and reduced E-cadherin group had a poorer outcome than the other group (5-year 68.2% vs. 94.1%, p = 0.003) (Fig. 4d).

Univariate and multivariate analyses

The prognostic factors relating to recurrence-free survival were evaluated using univariate and multivariate analyses (Table 3). Univariate analyses showed that tumor size > 2.0 cm (p =0.0003), HER2 positive (p = 0.027), lymph node metastasis (p = 0.009), positive ZEB-1 expression (p = 0.027), and reduced E-cadherin expression (p = 0.004) were significantly related to recurrence-free survival. Multivariate analyses showed that tumor size > 2.0 cm (p = 0.011) and reduced E-cadherin expression (p = 0.045) were independent factors for recurrence-free survival. Lymph node metastasis had borderline significance (p = 0.052).

Discussion

We investigated the relationship between the expression of the EMT related molecules ZEB-1 and E-cadherin, and postoperative recurrence-free survival of invasive ductal breast carcinoma patients. Recurrence-free survival rates were significantly lower in the group of positive ZEB-1 and reduced E-cadherin.

ZEB-1

Montserrat et al. [14] previously reported that 19 % of specimen of invasive breast carcinoma had positive ZEB-1 expressions in epithelial tumor cells using immunohistochemistry, but its expression was not related with prognosis. Surprisingly down-regulation of ZEB-1 gene expression was related with poor overall survival and disease-free survival. Soini et al. [15] reported that 75 % of specimens of breast carcinomas had positive ZEB-1 expression in stromal fusiform cells using immunohistochemistry, but no epithelial tumor cells had positive expression. They also reported that ZEB-1 expression in stromal fusiform cells did not related with prognosis. In our study, positive ZEB-1 expression using immunohistochemistry was detected in the nuclei of fusiform cells surrounding the invasive ductal breast carcinomas and we observed positive ZEB-1 expressions in only a few nuclei of stromal fusiform cells surrounding non-invasive carcinoma lesions contained in the specimen of this study (data not shown). We used uterine carcinosarcoma as a positive control, and in this sample, ZEB-1 expression was observed only in the sarcoma lesion, and no expression in the carcinoma lesion. We also observed ZEB-1 expressions in some colonic carcinomas, no epithelial tumor cells showed positive staining (data not shown). Recent studies have shown that cells experience drastic shape changes from epithelial cells to a spindle-like shape of mesenchymal cells, and a portion of myofibroblasts within fibrotic lesion arise from epithelium during EMT[18]. The fusiform cells which we detected positive ZEB-1 expression might be derived from epithelial tumor cells and associated with tumor malignancy. We also observed ZEB-1 expression in the marginal lesion of tumors in 7 of the 116 patients. We named it Kimura.W-Shibata.K type infiltration. These were presumed to be undergoing EMT and infiltration, but these were not significantly associated with clinicopathological parameters or postoperative recurrence. Further studies are needed to verify the associations.

Table 3. Univariate and multivariate analyses of recurrence-free survival using Cox proportional hazard model.

	Univariate			Multivariate		
Variables	Hazard ratio	95%CI	p-value	Hazard ratio	95%CI	p-value
Tumor size > 2cm	14.75	2.87-269.53	0.0003	8.24	1.50-153.88	0.011
HER2 positive	4.03	1.19-12.66	0.027	2.17	0.65-8.45	0.213
Lymph node metastasis	4.66	1.47-17.46	0.009	3.58	0.99-12.56	0.052
positive ZEB-1	4.42	1.16-28.72	0.027	2.32	0.53-16.08	0.280
reduced E-cadherin	5.37	1.69-20.15	0.004	3.62	1.03-15.05	0.045

CI, confidence interval

E-cadherin

There are many studies about E-cadherin expression of breast cancers using immunohistochemistry and the meta-analysis has been performed. However many of these studies reported that loss of E-cadherin expression might be an independent negative prognostic indicator for infiltrating ductal breast carcinoma, immunohistochemical techniques and interpretation algorithms have not been standardized [19]. Recently, some studies evaluated E-cadherin expression in whole tumor cells[17, 20]. If the staining of whole tumor cells is uniformly weak, it is possible that the results are influenced by preservation of the specimens or immunohistochemical techniques. We presumed that loss of E-cadherin initially occurs in the peripheral part of tumor, so we evaluated not only whole tumor cells, but also the peripheral part of tumors comparing with the central part of tumors. We also verified that normal breast tissue in the same specimens were stained properly and loss of E-cadherin was observed in only tumor tissues. Although these evaluations performed as accurately as possible due to differences in each specimen, we could improve the precision of evaluation avoiding influences of preservation and immunohistochemical techniques.

Recurrence-free survival

In this study, although the patients with positive ZEB-1 expression and the reduced E-cadherin had a lower recurrence-free survival rate, they are not sufficiently useful predictive factors alone. We evaluated the recurrence-free survival rate by matching them. The positive ZEB-1 and reduced E-cadherin group had a lower recurrence-free survival rate than the other group (5-year 68.2 vs. 94.1%, $p = 0.0003$). We propose that the combination of ZEB-1 and E-cadherin expression could be a more useful predictive factor in invasive ductal breast carcinoma.

Multivariate analysis

We evaluated prognostic factors by multivariate analyses. Only tumor size > 2.0 cm and reduced E-cadherin expression were significant factors. Positive ZEB-1 expression was not significant in multivariate analyses. We also evaluated E-cadherin expression with the new criterion comparing expression of the peripheral part of tumors with the central part of tumors. We could show that reduced E-cadherin expression was a significant and independent prognostic factor of postoperative recurrence of invasive ductal breast carcinoma based on this criterion. We concluded that reduced E-cadherin expression was a better predictor of postoperative recurrence than positive ZEB-1 expression.

As far as we know, this is the first study of invasive ductal breast carcinoma which identified the combination of ZEB-1 and E-cadherin expression as a predictive factor in terms of recurrence-free survival rate using immunohistochemistry. We should consider that the patients of positive ZEB-1 and reduced E-cadherin expression are at higher risk of recurrence and require more aggressive adjuvant therapies. Although many studies have reported the malignant significance and prognostic value of ZEB-1 and E-cadherin, no drugs or treatment procedures have been established which are targeted to them. Innovations are required to establish new therapeutic strategies. A limitation of this study is that neither the expression of ZEB-1 nor E-cadherin were significantly associated with overall survival rate. Of the 116 patients, only 5 patients died during our follow up period. Further study is needed with larger number of patients and longer follow up period.

Conclusions

Positive ZEB-1 expression in stromal fusiform cells and reduced E-cadherin expression in epithelial tumor cells are associated with a poorer outcome in terms of recurrence-free survival in invasive ductal breast carcinoma patients.

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Conflict of interest

The authors declare that they have no conflict of interest.

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