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Comparison Study of E-cadherin Expression in Primary Breast Cancer and its Corresponding Metastatic Lymph Node

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Abstract

Introduction: E-cadherin is an intercellular adhesion molecule, whose loss of expression may facilitate the process of cancer invasion and metastasis. Although the expression of E-cadherin has been widely studied in primary breast cancers, little is known about its expression at the corresponding metastatic lymph node. Here we comparatively studied their E-cadherin expression patterns and related them to the pathological data of breast cancer patients.

Methods: Only lymph nodes which were pathologically identified as metastases were included in this study to pair up the primary tumors. The quantitative real-time PCR (Q-RT-PCR) technique was used to assess the E-cadherin RNA expression levels in invasive ductal breast cancer subjects. E-cadherin gene copies were normalized using beta-actin gene copies. ER, PR, cerbB2 expressions in the primary tumor were routinely examined by immunohistochemistry method. Tumor characteristics and number of metastatic lymph nodes were gathered from the pathology reports.

Results: We tried to explore the relationship between E-cadherin expression in 21 primary tumors and their corresponding metastatic lymph nodes. However, the Q-RT-PCR data shows that an aberrant expression exists in both primary tumors and the corresponding lymph nodes ($p=0.115$), in which metastatic lymph nodes showed slight higher gene copies comparing to primary sites (77.77 ± 94.74 vs 43.35 ± 40.03, respectively). It is noteworthy that nodal E-cadherin expression was closely but negatively correlated with tumor size ($p<0.01$, $r= -0.775$) and number of meta sized lymph nodes ($p<0.05$, $r=- 0.519$), as tumor size and number of metastasized lymph nodes were already clinically proven to be important prognostic factors. There was no correlation between ER, PR, cerbB2 status in primary tumors and the nodal E-cadherin expression ($p>0.05$).

Conclusions: These study shows that E-cadherin expression is aberrant in invasive ductal cancers and their corresponding metastatic lymph nodes. E-cadherin expression in the metastasized lymph node is closely related to tumor size and number of metastasized lymph nodes.

Keywords: E-cadherin; Primary breast cancer; Metastatic lymph node; Real-time PCR

Introduction

Breast cancer is one of most common malignant tumors in the world and ranked first as a cause of cancer death for females in Shanghai. Tumor invasion with subsequent metastases is one of major cause of morbidity and mortality in patients with breast cancer. The development of metastases is the most important prognostic factor, as almost all patients with distant metastasis succumb to the disease [1].

Detachment of cell-cell adhesion is indispensable for the first step of invasion and metastasis of cancer. This mechanism is frequently associated with the impairment of either E-cadherin expression or function [2]. E-cadherin is one of calcium-dependent transmembrane glycoprotein mediating cell-cell adhesion, specifically involved in epithelial cell-to-cell adhesion [3]. It is mainly localized in adherent junctions and is mediated by extracellular domain cell-cell adhesion through calcium dependent interactions. The E-cadherin gene, located on chromosome 16q22.1, is also an important regulator of morphogenesis [4-6].

In vitro studies, our group found that transfection and expression E-cadherin cDNA inhibited the invasiveness of epithelial tumor cell lines. Other numerous studies have linked aberrant expression of E-cadherin with the development of metastases in breast cancer and other cancer [7]. In cancer, decreased E-cadherin expression is one of the alterations that characterize the invasive phenotype, and the data support its role as a tumor suppressor gene [8,9]. Although in vitro cell lines studies have provided evidence of an association between reduced E-cadherin and invasion, this association has not consistently been shown in vivo [7,10-12]. Few studies have specifically looked at the expression pattern of E-cadherin in breast primary and lymph node or distant metastasis [7,13]. However, only immunocytochemical staining for detection of E-cadherin limited the value of these studies. In the present study, we used the quantitative real-time PCR (Q-RT-PCR) technique [14] to assess the E-cadherin RNA expression levels in invasive ductal breast cancer subjects and their corresponding metastatic lymph nodes. We further studied the relationship among E-

cadherin expression levels and tumor size, ER, PR and numbers of metastatic lymph node.

Materials and Methods

Patient selection and specimens

A total of 21 patients without the history of radiotherapy or chemotherapy who underwent Modified Radical Mastectomy for Breast Cancer between 2003 and 2004 were included in this study. Patients were diagnosed by core-tissue biopsy (CTB) using Bard-Magnum Gun (MG1522, Bard Magnum Biopsy Instrument, CR Bard, Inc.) with 14-gauge 13-cm-long biopsy needles (Bard Magnum Core Tissue Biopsy Needle, CR Bard, Inc.) [15]. The excisional primary tumor tissues and axillary lymph nodes were kept in liquid nitrogen. The axillary lymph nodes were pathologically identified as the metastases. ER, PR, cerbB2 expressions on the primary tumors were routinely examined by immunohistochemistry method [16] by pathologists. Tumor characteristics, ER, PR, cerbB2 expressions and number of metastatic lymph nodes were gathered from the pathology reports.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 15-20 mg liquid-nitrogen-frozen breast cancer tissue and metastatic lymph node tissue using Total RNA Extraction Miniprep System (Cat. No.: GR1001, VIOGENE Inc) [17]. The performance was according to the manufacturer's protocols. Tumor and lymph node sample RNA was diluted in DEPC-treated RNase-free ultra-pure water (DEPC; Sigma-Aldrich, the Netherlands) and stored at -80℃.

Reverse Transcription is carried out with the Super Script First-Strand Synthesis System (Shinegeneinc.) for RT-PCR. The following procedure is based on manufacture's protocol. Prepare the RNA/primer mixture mix including total RNA 5 μg, random primers (50 ng/μl) 3 μl, 10 Mm dNTP 1 μl in each tube. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min. Prepare reaction master mixture. For each reaction: 10x RT buffer 2 μl, 25 mM $MgCl_2$ 4 μl, 0.1 M DTT2 μl, RNAaseOUT1 μl. Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min. Add 1 μl (50 units) of Super Script II RT to each tube, mix and incubate at 25°C for 10 min. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice. Add 1 μl RNase H and incubate at 37°C for 20 min. Store the 1st strand cDNA at -20°C until use for real-time PCR.

Quantitative Real-time RT-PCR

The mRNA level of E-cadherin was measured by quantitive real-time RT-PCR method using a Hot Start Fluorescent PCR Core Reagent Kit for SYBR Green I. Quantitive real-time RT-PCR (FTC2000 Detect System, Funglyn Biotech, Toronto, ON, Canada) was carried out with 5 μl cDNA in a 30μl PCR reaction system. In addition, we used control gene β-actin to normalize the mRNA level of E-cadherin gene. Primer pairs (h e-cadherin forward, 5'-TGCTCACATTTCCCAACTCC-3' reverse, 5'-TTGCCTTCTTTGTCTTTGTT-3') were designed by Oligo 6.0 primer analysis software (Medprobe, Oslo, Norway). Real-time PCR was performed in 50 μl of reaction mixture system, including 25 μl 1x Hotstart Fluo-PCR mix, about 300 nM forward and reverse primers, and containing about 2 μl of tumor sample cDNA as a template. Reaction conditions were as follows: 50℃ for 2 min for UNG activation and 94℃ for 4 min for Taq DNA polymerase activation, followed by 35 cycles of 94℃ for 30 s for denaturation, 55℃ for 30 s for annealing and 72℃ for 1 min for extension. For every transcript measured, serial dilutions (1: 10, 1: 100, 1: 1000, 1: 10 000) of standard-concentration sample were used to generate a standard curve. Data were analyzed with FTC 2000 software (Funglyn Biotech, Toronto, ON, Canada) according to the above standard curve.

Immunohistochemistry

The primary breast cancers tissue and metastatic lymph nodes was collected from each patient, fixed in 10% formalin, embedded in paraffin, and sectioned (3 μm). Immunohistochemistry was performed to detect E-cadherin (mouse anti-human polyclonal antibody, 1:100; Shanghai Changdao Biotech Co, Ltd) according to manufacturer's instructions. Immunohistochemical features were assessed using independent evaluation by a pathologist. For interpretation of the IHC stain results, the IHC tests were categorized as negative (0), "1+," "2+," or "3+" in high-power fields (40× magnification) according to the intensity of cytoplasmic staining in every case.

Statistical Analysis

A two-sample *t* test was performed to compare the difference of Expression of E-cadherin between primary breast cancer and their corresponding lymph node. The correlation between E-cadherin expression and pathological variables were analyzed by correlation analysis using SPSS 14. $p<0.05$ was considered significant.

Results

Clinical and pathological characteristics	
Number of patients	21
Pathological stage at diagnosis [n (%)]	
Stage II (T2N1M0) Stage III (T2N2M0)	12 (57) 9 (43)
Tumor size (range) (cm)	3.4 (2-5)
Histological type	
Ductal	21
Number of invaded lymph node (range)	8.8 (1-18)
Estrogen receptor status [n (%)]	
Negative Positive	11 (52) 10 (48)
Progesterone receptor status [n (%)]	
Negative Positive	8 (38) 13 (62)
CerbB2 receptor status [n (%)]	
Negative Positive	9 (43) 12 (57)

Table 1: Clinical and pathological characteristics of patients

Clinical and pathological features

All patients were females, and all the pathological types were the invasive ductal breast cancers. The tumor characteristics are presented in Table 1. The media age at diagnosis of primary invasive breast cancer was 50.8 years (range, 35-79).

E-cadherin mRNA in primary breast cancers and metastatic lymph nodes

The quantitative real-time PCR (Q-RT-PCR) was used to assess the mRNA level of E-cadherin in primary breast cancers and their corresponding metastatic lymph nodes. The data was shown in Figure 1.

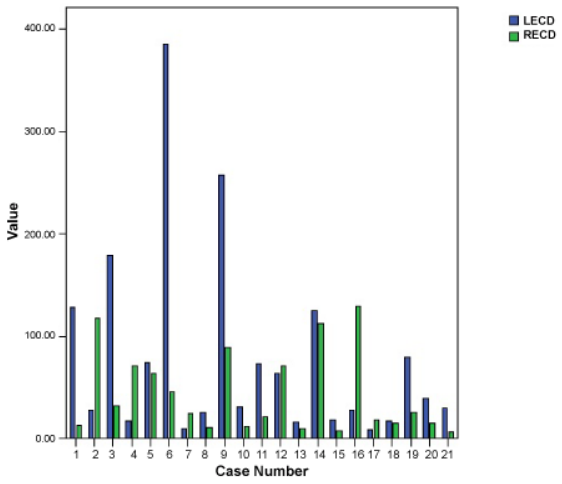

Figure 1: E-cadherin gene expression levels in paired samples. Blue bars represent metastasis samples, and green bars represent primary tumors. Vertical axes show normalized densities for the indicated genes

An aberrant expression exists in both primary tumors and the corresponding lymph nodes, in which metastatic lymph nodes showed slight higher gene copies comparing to primary sites (77.77 ± 94.74 vs 43.35 ± 40.03, respectively). But the statistical analysis showed that there was no significant difference (P >0.05) (Table 2). The relationship between the target gene expression and clinicopathologic parameters was shown in Table 3.

	Gene copy (X ± SD)	t-Test (t)	P Correlation (r)		P
Primary breast cancer	43.349 ± 4.028	1.694	>0.05	0.306	>0.05
Metastatic lymph node	77.767 ± 9.472				

Table 2: E-cadherin expression in primary breast cancer and corresponding metastatic lymph node

Immunohistochemical expression of E-cadherin in primary breast cancers tissue and metastatic lymph nodes

Immunohistochemistry was performed to detect E-cadherin in the primary breast cancer tissue and metastatic lymph nodes. Representative images were taken from the results of Immunohistochemistry (Figure 2). Statistically, E-cadherin was expressed in 85.7% (18/21) of metastatic lymph nodes tissue, which was higher than the 66.7% (14/21) in Primary breast cancers tissue, while the difference was not significant (P >0.05) (Table 4).

	T E-cadherin (r)	P	L E-cadherin(r)	P
Tumor Size	0.177	0.443	-0.775	0.0001
N lymph node	0.029	0.902	-0.519	0.016
ER	0.100	0.665	0.009	0.97
PR	0.188	0.415	-0.08	0.729
CerbB2	0.278	0.223	-0.085	0.715

Table 3: The relationships between the target gene expression and clinico pathologic parameters

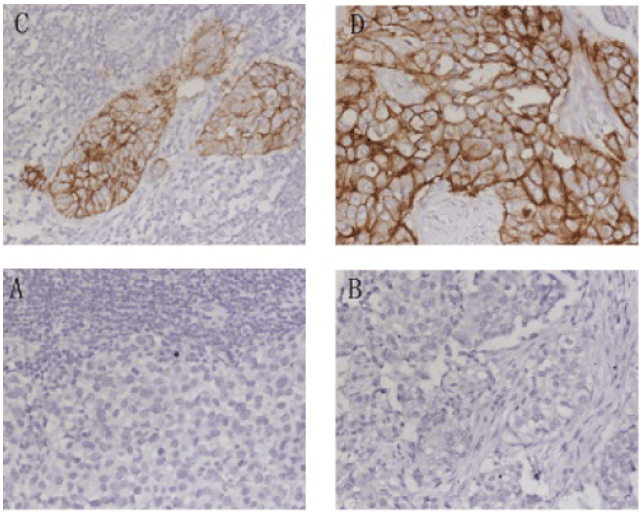

Figure 2: Immunohistochemical analysis of E-cadherin. E-cadherin expression was evaluated at high-power field (× 40 magnifications) by an experienced pathologist. A Negative for E-cadherin in Primary Breast cancers tissue. B Negative for E-cadherin in metastatic lymph nodes. C Three positive for E-cadherin in Primary Breast cancers tissue .D Three positive for E-cadherin in metastatic lymph nodes

The relationship between expression of E-cadherin and tumor characteristics

The relationships between the E-cadherin expression and clinicopathologic parameters were summarized in Table 3. It is noteworthy that nodal E-cadherin expression was closely but negatively correlated with tumor size ($p<0.01$, $r= -0.775$) and number of metastasized lymph nodes ($p<0.05$, $r= - 0.519$), as tumor size and number of metastasized lymph nodes were already clinically proven to

be important prognostic factors. There was no correlation between ER, PR, cerbB2 status in primary tumors and the nodal E-cadherin expression ($p>0.05$).

Group	Case	Expression of E-cadherin				χ^2	P value
		+	++	+++	−		
Primary breast cancer tissue	21	7	6	4	4	2.108	0.55
Lymph node	21	3	8	5	5		

Table 4: Immunohistochemical expression of E-cadherin in Primary breast cancers tissue and metastatic lymph nodes

Discussion

The expression of E-cadherin in breast cancer metastases is largely unknown and, to our knowledge, few studies specifically investigate the expression of E-cadherin in primary breast cancer in relationship to their corresponding metastatic lymph nodes [13,18]. We performed the Real-time RT-PCR method to study the relationship between E-cadherin expression in node-positive patients, with matched primary tumors and metastatic lymph nodes. We found that aberrant E-cadherin expression is a common event in primary invasive ductal breast cancer [7].

We also compared E-cadherin expression in primary invasive ductal tumors and their corresponding metastatic lymph nodes, and found no significant difference in mean expression. However, in metastatic lymph nodes, E-cadherin expression is slight higher gene copies comparing to primary sites (77.77 ± 94.74 vs 43.35 ± 40.03, respectively). According to these findings, one of the possible explanations is that cancer cells may re-expression E-cadherin protein once they reach distant sites [12]. Previous studies in breast cancer also have shown that loss of E-cadherin in primary tumors may be a transient phenomenon, enabling cells to break away and to be subsequently re-expressed in metastatic sites, possibly by facilitating lymphatic tumor emboli [7,19]. Bukholm et al. [12] also found that 19 of 20 lymph node metastases strongly expressed E-cadherin protein. Kowalski et al. [7] specifically evaluated paired primary breast tumors and matched distant metastases, and found that in a subset of patients the metastases had stronger E-cadherin expression than primary specimens. Park et al. [20] also found that abnormal expression of the adhesion molecules in the primary tumors with re-expression in corresponding nodal metastases is a common event in breast ductal carcinomas and may play a central role in establishing metastasis. However, the mechanism and biologic role of E-cadherin re-expression at the metastatic site has not been elucidated, although it appears that translational regulation and post-translational events are probable mechanisms of E-cadherin re-expression [21].

Interestingly, a lot of evidence showed that decrease or loss of E-cadherin expression can enhance the invasive ability of cancer cells. Some researchers found that chemotherapy can enhance cancer invasion/metastasis, and both *in vitro* and *in vivo* studies have shown that metastases can be more invasive than primary tumors [22-26]. The mechanism that is still unknown and need further researches. Our data showed that E-cadherin expression is higher in metastatic lymph nodes than primary tumors, the metastases were proved to be more invasive. This finding indicated that E-cadherin may not play an important role in the metastases' invasiveness. However, Ando et al. [27] believe that it is reasonable to suggest that the tumor suppressor E-cadherin may serve as a tumor enhancer when exposed to leptin and estradiol, that its ability to help cells aggregate then enhances the transformation of normal cells to cancerous cones, stimulating the growth of tumor mass. When the researchers used an E-cadherin antibody or a calcium-chelating agent to block E-cadherin function in the present of estradiol, this enhanced cell growth stopped [27].

In breast cancer, a relationship between E-cadherin expression and ER expression has been noted previously [10]. ER-positive tumors have been demonstrated to express normal amounts of E-cadherin protein, and loss of E-cadherin and ER genes has been linked to disease progression in invasive carcinomas of the breast. Nass et al. [28] found an association between coincident methylation of E-cadherin and the ER gene during breast cancer progression, probably not attributable to coincidence of methylation for two genes. In our study, however, we did not find an association between E-cadherin expression and the ER, PR, HER-2/neu status. We further studied the relationship between E-cadherin expression and tumor size. It is noteworthy that nodal E-cadherin expression was closely but negatively correlated with tumor size ($p<0.01$, $r= -0.775$) and number of metastasized lymph nodes ($p<0.05$, $r=-0.519$), as tumor size and number of metastasized lymph nodes were already clinically proven to be important prognostic factors.

In summary, the present study provides evidence that aberrant E-cadherin expression is a common event in primary invasive ductal breast cancer. E-cadherin is expression or re-expression at metastatic lymph nodes of invasive ductal breast cancer, supporting the hypothesis that re-expression of E-cadherin may play a role in the establishment of the metastatic cells at distant sites. Because of the difficulty in collecting fresh tumor tissues and matched invasive lymph nodes, we only collected 21 pairs of specimens, which may be too small to give a definite conclusion. But it still provides some evidence in E-cadherin expression in metastatic lymph nodes and E-cadherin may play a role in metastatic sites.

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