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# E74-like factor 2 regulates *valosin-containing protein* expression

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Received 26 February 2007
Available online 9 March 2007

**Abstract**

Enhanced expression of valosin-containing protein (VCP) correlates with invasion and metastasis of cancers. To clarify the transcription mechanism of *VCP*, human and mouse genomic sequence was compared, revealing a 260 bp DNA sequence in the 5′-flanking region of *VCP* gene to be highly conserved between the two, in which binding motif of E74-like factor 2/new Ets-related factor (ELF2/NERF) was identified. Chromatin immunoprecipitation assay showed binding of ELF2/NERF to the 5′-flanking region of *VCP* gene. Knock-down of *ELF2/NERF* by siRNA decreased expression level of VCP. Viability of cells under tumor necrosis factor-alpha treatment significantly reduced in *ELF2/NERF*-knock-down breast cancer cell line. Immunohistochemical analysis on clinical breast cancer specimens showed a correlation of nuclear ELF2/NERF expression with VCP expression and proliferative activity of cells shown by Ki-67 immunohistochemistry. These findings indicate that ELF2/NERF promotes VCP transcription and that ELF2/NERF–VCP pathway might be important for cell survival and proliferation under cytokine stress.


*Keywords:* E74-like factor 2/new Ets-related factor; Transcription factor; Valosin-containing protein; Breast cancer; Cell survival

Valosin-containing protein (VCP; also known as p97) belongs to the ATPases associated with various cellular activities (AAA) superfamily, and is essential for a wide variety of cellular functions such as ubiquitin/proteasome-dependent protein degradation, membrane fusion, cell cycle regulation, and endoplasmic reticulum-associated degradation [1–5]. Notably, VCP is involved in activation of nuclear factor (NF) κB, a transcription factor promoting anti-apoptosis, cell proliferation, and invasion [6]. Recently, we showed that VCP is associated with metastatic potential of murine osteosarcoma cell line by using mRNA subtraction technique: the cell lines transfected with *VCP* showed the constant activation of NFκB, decreased apoptosis rates after stimulation with tumor necrosis factor-α (TNFα), and increased metastatic potential [7]. Subsequent studies on the clinical samples showed that the high level of VCP expression in cancer cells correlated with the increase in recurrence rate and poor prognosis of patients with cancer of the liver, stomach, prostate, and esophagus [8–11]. A clear correlation in VCP expression between at the mRNA and protein level was found, indicating that the up-regulation of *VCP* transcription resulted in the enhanced VCP expression in cancers, which is advantageous for cancer cells to survive and metastasize [8,10,11]. This implies that the *VCP* transcription would be a novel target for cancer therapy.

The previous study showed that PBX1 acts as a transcription factor regulating proliferation and differentiation of normal cells and cancer cells [12]. Our very recent study revealed that pre B-cell transcription factor 1 (PBX1) regulates the transcription of *VCP* [13]. The knock-down of *PBX1* by siRNA transfection decreased the expression level of VCP as well as PBX1. *PBX1*-knock-down cells showed a marked susceptibility to TNFα treatment compared to parental cells [13].

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.160

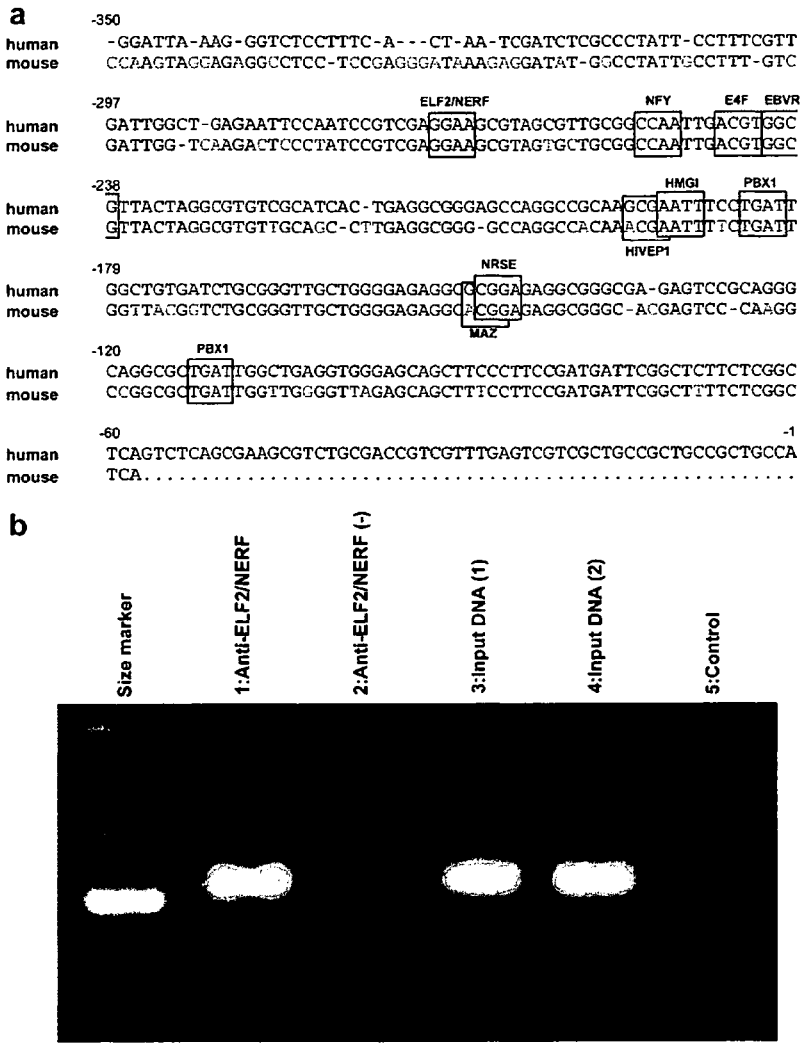


Fig. 1. Comparison of the nucleotide sequence of the 5′-flanking region of human and mouse *VCP* gene. MatInspector software revealed several potential binding sites of transcription factors in this region (a). ChIP assay was performed using MCF7 cells to analyze the binding of ELF2/NERF to the 5′-flanking region of the human *VCP* gene (b). Precipitated DNA fragments with (lane 1) or without (lane 2) anti-ELF2/NERF antibody together with the input DNA samples (lane 3, input sample for lane 1; lane 4, input sample for lane 2) were PCR-amplified with primers specific for the VCP promoter region. Result of PCR without template was shown (lane 5). PCR products were separated on 2.5% agarose gel and stained with ethidium bromide. Positive band in the lane of the sample precipitated with anti-ELF2/NERF antibody (lane 1) was seen at the same size with those of input DNA (lanes 3 and 4).

A 260 bp DNA sequence in the 5′-flanking region of *VCP* gene was identified to be highly conserved through comparison of human and mouse genome. This sequence contains consensus binding sites for several transcription factors. Among them, the binding site of E74-like factor 2/new Ets-related factor (ELF2/NERF), a Ets transcription factor family member, existed (Fig. 1a). In the present study, ELF2/NERF was analyzed for its transcription activity of *VCP* gene, as well as its association with apoptosis and proliferation of cancer cells.

## Materials and methods

*Cell lines and culture.* Human breast cancer cell line, MCF7, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma) in an air incubator with 5% $CO_2$ at 37 °C.

*Analysis of VCP promoter sequence.* The human and mouse genomic sequences of the 5′-flanking region of the *VCP* gene including the transcription start site was obtained from GenBank (Accession Nos. NM007126 and AC005259).

*Bioinformatics resources.* Transcription factor binding site analysis combined with a literature search was performed using MatInspector (Genomatix Software, Munich, Germany).

*ELF2/NERF antibody production and other antibodies.* A peptide sequence of aa 471–487 of ELF2/NERF (KGPEVKSEAVAKKQEHD) was prepared with $NH_2$-terminal cysteines, coupled to keyhole limpet hemocyanin. Freund's complete adjuvant was added to the coupled peptide, which was injected subcutaneously into Japanese white rabbits. Mixture of Freund's incomplete adjuvant and coupled peptide was periodically injected as boosting injections. Anti-ELF2/NERF antiserum was affinity-purified by acetic acid elution from a peptide-conjugated SulfoLink Column (Pierce, Rockford, IL, USA). Specificity of the antibody was confirmed by enzyme-linked immunosorbent assay using the antigen peptide.

Monoclonal antibodies against VCP and Ki-67, and polyclonal antibody against β-actin were purchased from Progen Biotechnik (Heidelberg, Germany), Dako Cytomation (Copenhagen, Denmark), and Sigma, respectively. Anti-mouse Ig G antibody and anti-rabbit Ig G antibody conjugated with horseradish peroxidase purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK), Alexa Fluor 488 goat anti-mouse Ig G antibody and Alexa Fluor 594 goat anti-rabbit Ig G antibody from Invitrogen (Carlsbad, CA), and biotinylated anti-mouse Ig G antibody and anti-rabbit Ig G antibody from Vector Laboratories (Burlingame, CA) were used as secondary antibodies for Western immunoblotting, immunofluorescence, and immunohistochemistry, respectively.

*Chromatin immunoprecipitation assay.* Chromatin immunoprecipitation (ChIP) assay was performed using a kit from Upstate (Lake Placid, NY) according to the manufacturer's protocol. Briefly, MCF7 cells were treated with formaldehyde for protein–DNA crosslinking. Soluble chromatin was incubated with either anti-ELF2/NERF polyclonal antibody or pre-immune Ig G serum. DNA was purified with phenol/chloroform extraction and re-suspended in 20 μl of water. Two microliters of DNA solution was used as a template for 35 cycles of PCR amplification with primers for the 5′-flanking region of the *VCP* gene; 5′-GCTGGCTCCTGATCCGCG AG-3′ and 5′-AGGTTTTGGGAGAAGATGGGTG-3′.

*Small interfering RNAs.* Oligonucleotides to produce plasmid-based small interfering RNA (siRNA) were cloned into pSilencer 4.1-CMV hygro vector (Ambion, Austin, TX) using *Bam*HI/*Hin*dIII restriction sites. siRNA target sequence to *ELF2/NERF* was GAAACCAAGAGA AGGAAAA. Target sequence used as a negative control was ATCCGCGCGATAGTAGTACGTA. The plasmids producing siRNA were transfected into MCF7 cells with Lipofectamine 2000, and clones resistant to hygromycin were selected.

*Western immunoblot analysis.* MCF7 cells were washed with ice-cold PBS, and lysed with lysis buffer (2% sodium dodecyl sulfate (SDS), 125 mM Tris HCl (pH 6.8)). Protein concentration was measured with the Protein assay kit (Bio-Rad, Hercules, CA). An aliquot of 10 μg from each sample was subjected to SDS polyacrylamide gel electrophoresis, and transblotted onto polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). After blocking the non-specific binding with 5% non-fat milk in PBS 0.1% Tween 20 (PBST), the blotted membranes were reacted with primary antibodies, and then visualized with electrochemiluminescence detection system (Amersham Pharmacia Biotech).

*Immunofluorescence confocal microscopy.* MCF7 cells were cultured in Lab Tek II chamber slides (Nunc Nalgene, Naperville, IL), fixed in methanol at −20 °C for 10 min, washed in PBST, pre-incubated in PBS with 1% bovine serum albumin, then incubated with primary antibodies at a concentration of 200× in PBS. After washing with PBST, the slides were incubated with the secondary antibodies, washed, and examined with Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany).

*Cell viability assay.* MCF7 cells were plated on 96-well plates at $4 \times 10^3$ cells per well, treated with TNFα (Sigma) at a concentration of 5 ng/ml for 30 min, and incubated in DMEM with 0.5% FBS for 6 h. Cell viability was measured with Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI).

*Immunohistochemical assays.* Tumor samples were collected from 27 female patients with breast cancers who underwent surgical resection at the Breast Surgery Division, Osaka University Hospital, Osaka, Japan, during the period from 2000 to 2001. Samples obtained from the breast lesions were fixed in 10% formalin and routinely processed for paraffin-embedding.

Immunohistochemical studies were performed with the avidin biotin peroxidase complex method. Anti-ELF2/NERF polyclonal antibody and anti-VCP and anti-Ki-67 monoclonal antibodies were used as the primary antibodies at a dilution of 1:200, 1:3000, and 1:50, respectively. Counterstaining was performed with methylgreen.

A cell pellet of cultured MCF7 cells was fixed in 10% formalin, paraffin-embedded, cut at 4 μm, and used as a positive control for ELF2/NERF staining. ELF2/NERF expression in the nucleus and cytoplasm in each specimen was independently evaluated: no expression was defined as Level 1, cells with weaker staining compared to MCF7 cells as Level 2, and cells showing strong staining similar to MCF7 cells as Level 3.

Endothelial cells showed positive staining for VCP at a constant intensity [8 11]. The staining intensity in the cytoplasm of the tumor cells was defined in comparison to that of endothelial cells, and categorized as follows: weaker (Level 1 VCP expression), equal to (Level 2), and stronger (Level 3) than that in endothelial cells.

As for Ki-67 immunohistochemistry, cells showing intranuclear staining were judged as Ki-67 positive. The Ki-67 positive cells among 200 tumor cells were counted, and the percentage was shown as the Ki-67 labeling index (LI).

*Statistical analysis.* Data were expressed as means ± standard error. The significance of differences of the mean values was determined by Kruskal–Wallis test. Correlation among clinicopathologic factors was evaluated by $\chi^2$ test. $p$ values of less than 0.05 were considered as statistically significant.

## Results

### *Binding of ELF2/NERF to the 5′-flanking sequence of VCP gene*

In order to confirm the binding of ELF2/NERF to the 5′-flanking sequence of *VCP* gene, ChIP assay was performed. After treatment with formaldehyde to crosslink protein and DNA, soluble chromatin extracted from MCF7 cells was immunoprecipitated with anti-ELF2/NERF antibody. ELF2/NERF binding sequence at the *VCP* promoter region was successfully amplified by PCR. The amplified fragment was confirmed to be the *VCP* promoter region by sequencing. No bands were detected when PCR was performed using DNA precipitated without anti-ELF2/NERF antibody or without template. These findings showed the specific binding of ELF2/NERF to the 5′-flanking sequence of *VCP* gene (Fig. 1b).

### *siRNA interference of ELF2/NERF*

Plasmid expressing *ELF2/NERF* siRNA was stably transfected to MCF7 cells. As a control, plasmid expressing negative siRNA was transfected. Western immunoblotting of the cloned cells showed a marked reduction in ELF2/NERF as well as VCP expression in *ELF2/NERF* siRNA clone compared to negative siRNA clone and original MCF7 cells (Fig. 2a).

Fluorescent immunohistochemical analysis showed that expression of ELF2/NERF and VCP in *ELF2/NERF* siRNA clone was weaker than those in negative siRNA clone and MCF7 cells (Fig. 2b).

Association between *ELF2/NERF* expression and cell viability after TNFα treatment was analyzed. Proportion of viable cells in *ELF2/NERF* siRNA clone was lower than that in MCF7 cells and negative siRNA clone (Fig. 2c; $p < 0.05$).

### *Correlation of ELF2/NERF expression with VCP expression and Ki-67 LI in breast cancer specimens*

Correlation of ELF2/NERF expression with VCP expression and proliferative activity of cells revealed by

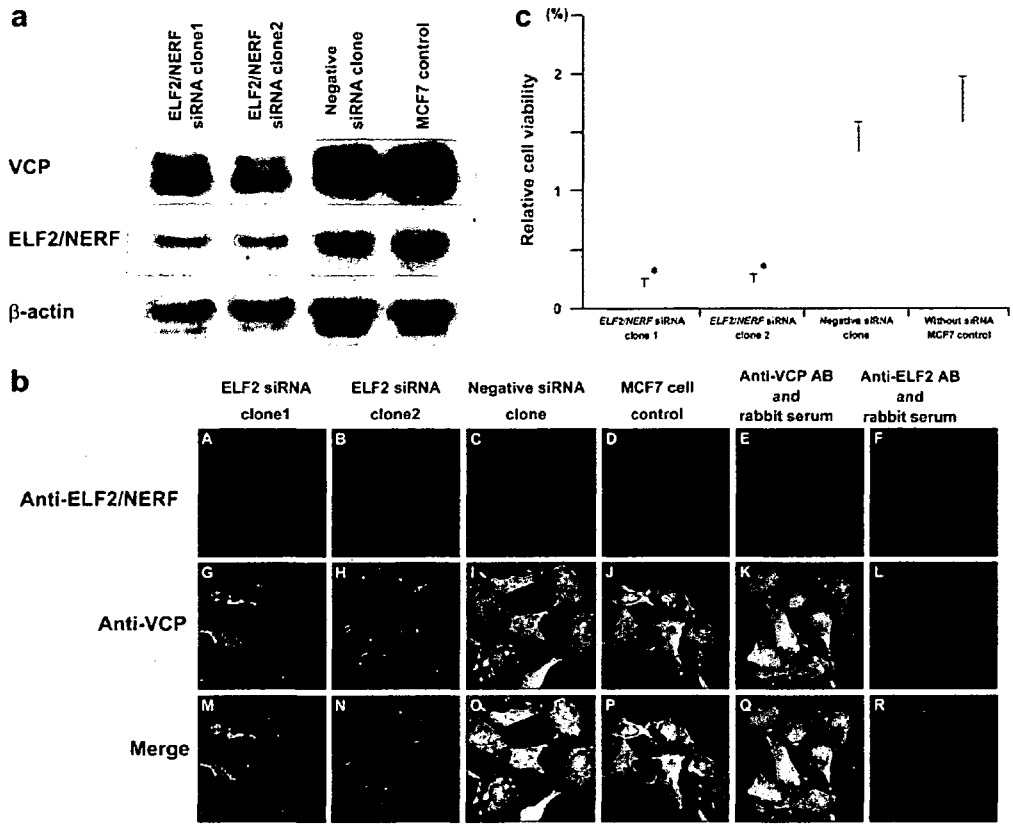


Fig. 2. Western immunoblot analysis of *ELF2/NERF* siRNA transfected MCF7 cells. Equal amounts of whole cell lysate protein were subject to immunoblotting with the indicated antibodies. Expression level of ELF2/NERF and VCP in *ELF2/NERF* siRNA clone was lower than that in the negative siRNA clone and MCF7 cells (a). Fluorescent immunohistochemical analysis of *ELF2/NERF* siRNA transfected MCF7 cells. Immunofluorescence confocal microscopy was conducted for ELF2/NERF (red) and VCP (green). Merged images are shown. ELF2/NERF and VCP expression in *ELF2/NERF* siRNA clones were weaker than those in negative siRNA clone and MCF7 cells. Control analyses with non-immune rabbit or mouse serum as primary antibodies instead of anti-ELF2/NERF and anti-VCP antibodies showed the fluorescence for the specific antibody, but not for the non-immunized serum, indicating the specificity of primary antibodies and absence of bleed-through of the fluorophores. AB, antibody (b). Cell viability assay after TNFα treatment. Cells were treated with TNFα for 30 min at a concentration of 5 ng/ml, incubated in DMEM with 0.5% FBS for 6 h, and cell viability was measured by Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). Relative ratio of viable cells with to without TNFα treatment was calculated. Bars represent means ± standard error of at least three independent experiments. Asterisk denotes significant difference ($p < 0.05$). Proportion of viable cells in *ELF2/NERF* siRNA clones was significantly lower than that in MCF7 cells and negative siRNA clone (c).

Ki-67 immunohistochemistry was analyzed in breast cancer specimens. ELF2/NERF expression in the nucleus and cytoplasm correlated with VCP expression and Ki-67 LI (Table 1A and Fig. 3). Statistically, the VCP expression was more strongly correlated with expression of ELF2/NERF in the nucleus than that of cytoplasm (Table 1B).

## Discussion

The Ets family proteins consist of a large number of evolutionarily conserved transcription factors which share a conserved winged helix turn helix DNA binding domain (Ets domain) [14,15]. This domain recognizes unique DNA sequences containing GGAA/T named as Ets binding sites [14,15]. Ets factors are supposed to be critical mediators for wide variety of cellular processes, including development, differentiation, growth, and transformation [14 16]. Several dominant negative constructs that inhibit Ets activity have shown that interference of Ets factor function could reverse the transformed phenotype of various types of cells [17]. Some *Ets* genes might be involved in specific chromosomal translocations in different types of cancer [15]. These findings suggest the oncogenic role of most Ets factors. Indeed, overexpression of several members of *Ets* genes was reported in cancers of thyroid, pancreas, liver, colon, lung, and leukemia [14].

ELF2/NERF belongs to Elf subfamily of Ets transcription factors together with ELF1 and myeloid elf-1-like factor (MEF) [15] and regulates a set of genes in B cells and myeloid cells [18]. ELF1 binds and transactivates the *erbB2* promoter [19], and plays a significant role in tumor invasion and vascular development by regulating the gene expressions of the *urokinase-type plasminogen activator* and *Tie2* [20,21]. In fact, overexpression of ELF1 in cancers of breast, prostate, and endometrium has been reported [17,22,23]. ELF2/NERF physically interacts with acute myeloid leukemia 1 (AML1), a frequent target for chromosomal translocations in leukemia [24]. ELF2/NERF

Table 1A
Immunohistochemical analysis of breast cancer specimens

| Case | Age at operation | Histologic diagnosis | ELF2/NERF expression | | VCP expression | Ki-67 labeling index (%) |
|---|---|---|---|---|---|---|
| | | | Nucleus | Cytoplasm | | |
| 1 | 45 | Papillo-tubular carcinoma | Level 1 | Level 1 | Level 1 | 2 |
| 2 | 48 | Lobular carcinoma | Level 1 | Level 1 | Level 1 | 2 |
| 3 | 67 | Papillo-tubular carcinoma | Level 1 | Level 1 | Level 1 | 3 |
| 4 | 69 | Solid-tubular carcinoma | Level 1 | Level 1 | Level 1 | 3 |
| 5 | 87 | Papillo-tubular carcinoma | Level 1 | Level 1 | Level 1 | 4 |
| 6 | 44 | Papillo-tubular carcinoma | Level 1 | Level 1 | Level 1 | 4 |
| 7 | 49 | Scirrhous carcinoma | Level 1 | Level 1 | Level 1 | 4 |
| 8 | 74 | Mucinous carcinoma | Level 1 | Level 1 | Level 1 | 7 |
| 9 | 34 | Solid-tubular carcinoma | Level 1 | Level 1 | Level 1 | 10 |
| 10 | 55 | Papillo-tubular carcinoma | Level 1 | Level 2 | Level 2 | 5 |
| 11 | 53 | Papillo-tubular carcinoma | Level 1 | Level 2 | Level 2 | 30 |
| 12 | 43 | Lobular carcinoma | Level 2 | Level 2 | Level 2 | 4 |
| 13 | 68 | Papillo-tubular carcinoma | Level 2 | Level 2 | Level 2 | 5 |
| 14 | 49 | Solid-tubular carcinoma | Level 2 | Level 2 | Level 2 | 20 |
| 15 | 71 | Papillo-tubular carcinoma | Level 2 | Level 2 | Level 2 | 30 |
| 16 | 37 | Solid-tubular carcinoma | Level 2 | Level 2 | Level 2 | 30 |
| 17 | 45 | Solid-tubular carcinoma | Level 2 | Level 2 | Level 2 | 40 |
| 18 | 57 | Papillo-tubular carcinoma | Level 2 | Level 2 | Level 3 | 30 |
| 19 | 63 | Solid-tubular carcinoma | Level 2 | Level 3 | Level 2 | 20 |
| 20 | 66 | Lobular carcinoma | Level 3 | Level 1 | Level 3 | 10 |
| 21 | 42 | Scirrhous carcinoma | Level 3 | Level 2 | Level 2 | 5 |
| 22 | 58 | Papillo-tubular carcinoma | Level 3 | Level 2 | Level 2 | 5 |
| 23 | 55 | Scirrhous carcinoma | Level 3 | Level 2 | Level 3 | 20 |
| 24 | 42 | Scirrhous carcinoma | Level 3 | Level 2 | Level 3 | 20 |
| 25 | 38 | Papillo-tubular carcinoma | Level 3 | Level 2 | Level 3 | 30 |
| 26 | 61 | Scirrhous carcinoma | Level 3 | Level 3 | Level 3 | 15 |
| 27 | 30 | Papillo-tubular carcinoma | Level 3 | Level 3 | Level 3 | 15 |

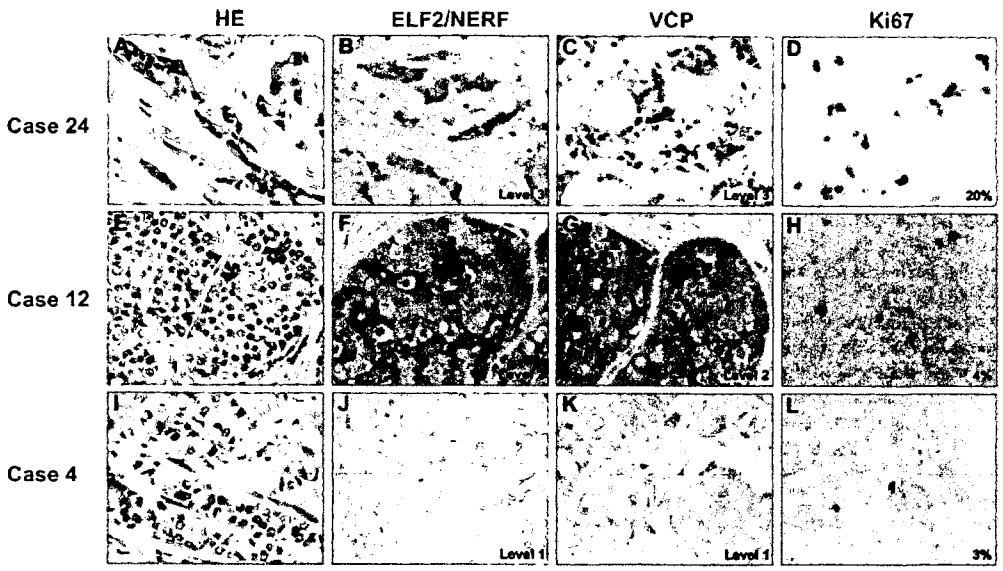


Fig. 3. Immunohistochemical analysis for ELF2/NERF, VCP, and Ki-67 expression in breast cancer specimens. Cases with stronger nuclear ELF2/NERF expression (Case 24) showed stronger VCP expression and higher Ki-67 labeling index compared to those with weaker expression.

expression is increased in endothelial cells in response to hypoxia and angiopoietin-1 [25]. These findings suggest an oncogenic role of ELF2/NERF, but its precise role in tumorigenesis has not been clarified as yet.

In the present study, ELF2/NERF expression was observed in both the nucleus and cytoplasm of breast cancer cells. ELF2/NERF acts as a transcription factor in the nucleus, however, its role in the cytoplasm or its precise transportation mechanism from the cytoplasm to the nucleus has not been clarified as yet. Immunohistochemical analysis on the breast cancer specimens showed that VCP expression was more strongly correlated with expression

Table 1B
Correlation between ELF2/NERF expression and other parameters

| | ELF2/NERF expression | | | |
|---|---|---|---|---|
| | Nucleus | | Cytoplasm | |
| | $\chi^2$ value | $p$ value | $\chi^2$ value | $p$ value |
| Age | 1.49 | 0.47 | 1.36 | 0.51 |
| Histologic subtype | 14.5 | 0.07 | 4.67 | 0.79 |
| VCP expression | 32.9 | 0.0001 | 31.4 | 0.0001 |
| Ki-67 labeling index | 10.2 | 0.006 | 12 | 0.0024 |

*Abbreviations:* ELF2/NERF, E74-like factor 2/new Ets-related factor; VCP, valosin-containing protein.

of ELF2/NERF in the nucleus than in the cytoplasm, indicating the important role of ELF2/NERF as a transcription factor of VCP. Whereas, Ki-67 LI showed a closer correlation with ELF2/NERF expression in the cytoplasm than that in the nucleus. There seems no explanation for this phenomenon at present.

Overexpression of VCP in cancers with higher metastatic potential and poor prognosis has been reported [8 11], which might suggest that ELF2/NERF is also involved in cancer development through *VCP* transcription. Indeed the present study revealed that decreased ELF2/NERF expression induced susceptibility of MCF7 cells to TNFα treatment. These findings further confirm the role of ELF2/NERF–VCP pathway for cell survival under cytokine stress, and indicate a clinical implication of evaluation of ELF2/NERF in cancers.

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Breast Cancer Res Treat (2007) 106:11–17
DOI 10.1007/s10549-006-9465-8

PRECLINICAL STUDY

# Connexin26 expression is associated with lymphatic vessel invasion and poor prognosis in human breast cancer

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Received: 16 November 2006 / Accepted: 25 November 2006 / Published online: 3 January 2007


**Abstract**
*Purpose* Cx26, which is a constituent of the connexin family, has recently been shown to promote metastasis through enhancing the vascular invasion in mouse melanoma cells. In this study, we have investigated whether or not Cx26 expression is associated with vascular invasion and recurrences in human breast cancers.
*Experimental design* Cx26 expression was studied in 152 invasive breast cancers by immunohistochemistry. In order to investigate the blood vessel invasion and lymphatic vessel invasion with precision, immunohistochemical staining of blood vessels and lymphatic vessels was carried out using anti-CD34 and anti-D2-40 antibodies, respectively.
*Results* Cx26 was positive in 51.3% (78/152) of the breast tumors. A statistically significant association was observed between Cx26 expression and large tumor size ($P = 0.013$) or high histological grade ($P = 0.043$). Frequency of blood vessel invasion was higher in Cx26-positive tumors (5.1%, 4/78) than in Cx26-negative tumors (1.4%, 1/74) though not statistically significant ($P = 0.210$). Lymphatic vessel invasion was significantly ($P = 0.001$) more frequent in Cx26-positive tumors (39.7%) than in Cx26-negative tumors (14.9%). Patients with Cx26-positive tumors showed a significantly ($P < 0.001$) poorer prognosis than those with Cx26-negative tumors. Multivariate analysis showed that Cx26 ($P < 0.05$) expression was an independent prognostic factor.
*Conclusions* Cx26 expression is associated with lymphatic vessel invasion, large tumor size, high histological grade, and poor prognosis in human breast cancers. Cx26 seems to enhance the metastasis probably through promoting the lymphatic vessel invasion. Cx26 might be clinically useful as a new prognostic factor.

**Keywords** Connexin26 · Breast cancer · Lymphatic vessel invasion · Prognosis

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## Introduction

Gap junctions form channels between adjacent cells, which permit the passage of small molecules of approximately 1 kDa between neighboring cells. Gap junctional intercellular communication (GJIC) plays an important role in a variety of cellular processes including homeostasis, morphogenesis, cell differentiation and growth control [1, 2]. Gap junction channels are composed of two hemi-channels (connexons) and each connexon is formed by six connexins. Until now, 21 members of the connexin transmembrane protein family have been identified [3, 4]. It has been shown that, in general, loss or reduced function of GJIC is implicated in the progression of a variety of tumors including hepatocellular cancer, gastric cancer, lung cancer, glioma, prostate cancer, malignant melanoma, and breast cancer [5–11]. Loss or reduced function of GJIC is usually induced by down-regulation of connexins (e.g., loss of Cx32 in gastric cancer [7], loss of Cx43 in prostate cancer [10] and glioma [9], reduced expression of Cx32 and Cx43 in lung cancer [8], and

reduced expression of Cx43 in breast cancer [11]). In addition, restoration of function in these connexins has been shown to result in the retardation of cell growth and induction of more normal phenotype [12, 13]. Thus, connexins are generally considered as a tumor suppressor gene.

Cx26 is a constituent of the connexin family and was initially isolated as a gene of which expression is down-regulated in breast cancer cell lines as compared with normal human breast epithelial cell lines [14]. Furthermore, transfection of Cx26 into various tumor cell lines including breast cancer cell lines has been shown to confer growth suppression [15–17]. Thus, in analogy to other connexins, Cx26 has also been considered to serve as a tumor suppressor gene. However, recent studies have disclosed a very unique feature of Cx26 in tumor progression. Ito et al. [18] have shown that Cx26 up-regulation is strongly associated with a metastatic potential in B16 mouse melanoma cells, because BL6 cells could transfer dye into vascular endothelial cells and transfection with wild-type Cx26 rendered F10 cells competent for coupling with endothelial cells and as spontaneously metastatic as BL6 cells. They have also suggested that formation of heterologous gap junctions between Cx26 and Cx43, which are expressed in melanoma cells and vascular endothelial cells, respectively, might facilitate the invasion of tumor cells into the blood vessels, being based on the observation that melanoma cells could transfer dye into vascular endothelial cells [18]. It is reported that, although Cx26 is not expressed in human melanoma cells in the epidermis, it is up-regulated in the areas invading into the dermis. Moreover, very recently, Ito et al have demonstrated the up-regulation of Cx26 in human lung squamous cell cancer as well as its association with poor prognosis [19]. These observations taken together, it is speculated that Cx26, unlike other connexins, might have a potential to stimulate the tumor invasion and metastasis.

It has been reported that Cx26 is over-expressed in a significant proportion of breast cancers although Cx26 is rarely expressed in the normal breast epithelial cells [20], and that breast cancers with Cx26 expression are associated with high histological grade [20], suggesting an association of Cx26 up-regulation with a biologically aggressive phenotype and, possibly, poor prognosis in human breast cancer. Since a prognostic significance of Cx26 expression in breast cancer has yet to be reported, we have investigated, in this study, the relationship between Cx26 expression and relapses in breast cancer patients as well as the relationship between Cx26 expression and the various clinicopathological parameters of breast tumors including blood vessel and lymphatic vessel invasion.

## Materials and methods

### Patients and tumor tissues

Tumor tissue samples were obtained from 152 female patients who underwent breast conserving surgery or mastectomy due to invasive breast cancer from 1998 to 2001 at Osaka University Hospital. Tumor tissues were fixed in 10% buffered-formalin and embedded in paraffin. Informed consent to the study had been obtained from each patient in advance.

As adjuvant therapy, 84 patients were treated with hormonal therapy, 12 with chemotherapy, and 34 with chemo-hormonal therapy. Hormonal therapy included tamoxifen 5 years or tamoxifen 5 years plus goserelin 2 years, chemotherapy included CMF (cyclophosphamide 100 mg/day p.o. days 1–14 + methotrexate 40 mg/m$^2$ i.v. days 1, 8 + 5-FU 600 mg/m$^2$ i.v. days 1, 8) × 6 cycles or EC (epirubicin 60 mg/m$^2$ i.v. day 1 + cyclophosphamide 600 mg/m$^2$ i.v. day 1) × 4 cycles, and chemo-hormonotherapy included a combination of these therapies.

Indication for adjuvant treatment was decided essentially according to St. Gallen's recommendation [21, 22]. Physical examinations were performed every 3 months for 2 years postoperatively and every 6 months thereafter, combined with blood tests and chest X-ray examinations every 6 months postoperatively. The median follow-up period of these 152 patients was 77 months, ranging from 52 to 98 months. Thirty-one patients developed recurrences (i.e., 8 developed bone metastases, 4 developed liver metastases, 4 developed lung metastases, 2 developed brain metastases and 13 developed soft tissue metastases). Ipsilateral breast recurrences after breast conserving surgery were not counted as recurrences.

### Immunohistochemistry

Paraffin sections (4 μm) of tumor tissues were subjected to immunohistochemcial staining of Cx26 protein with the avidin–biotin–peroxidase method. In brief, endogeneous peroxidases were quenched by incubating the sections for 20 min in 3% $H_2O_2$. Antigen retrieval was performed by heating the samples in 10 mmol/l citrate buffer (pH 6.0) at 95°C for 40 min. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan)

for 30 min at room temperature, the sections were incubated with a mouse monoclonal anti-Cx26 antibody (working dilution, 1:500) purchased from ZYMED LABORATORIES, USA) at 4°C overnight. The avidin–biotin–peroxidase complex system (Elite ABC kit, VECTASTAIN, USA) was used for color development.

Immunostaining of Cx26 was evaluated essentially according to the method previously described by Ito et al. Entire tumor lesion was observed with a special attention to the periphery of tumors since tumor cells in the periphery were reported to be more likely to be Cx26-positive than those in the center in other tumors. One thousand tumor cells were counted and, when tumor cells clearly positive for Cx26 staining were seen in more than 1%, the tumor was considered as Cx26-positive.

### Estrogen receptor (ER) and progesterone receptor (PR) assay

ER and PR contents of breast cancer tissues were measured by means of enzyme immunoassay using a kit provided by Abbott Research Laboratories (Chicago, IL).

### Statistics

The relationship between Cx26 expression and clinicopathological parameters of breast tumors was analyzed by chi-square test or Mann-Whitney's $U$ test. The relapse-free survival (RFS) curves were calculated by the Kaplan–Meier method, and the log-rank test was used to determine differences in RFS rates. The Cox proportional hazards model was used to calculate the hazard ratio for each parameter in univariate analysis and multivariate analysis. Statistical significance was defined as $P < 0.05$.

## Results

### Immunohistochemical examination of Cx26 expression in breast cancer

Representative results of immunohistochemical examination of Cx26 expression in breast cancer tissues are shown in Fig. 1. Figure 1A shows a tumor strongly positive for Cx26 immunostaining where cytoplasmic staining was observed in almost all tumor cells. Figure 1B shows a tumor in which about 10% of

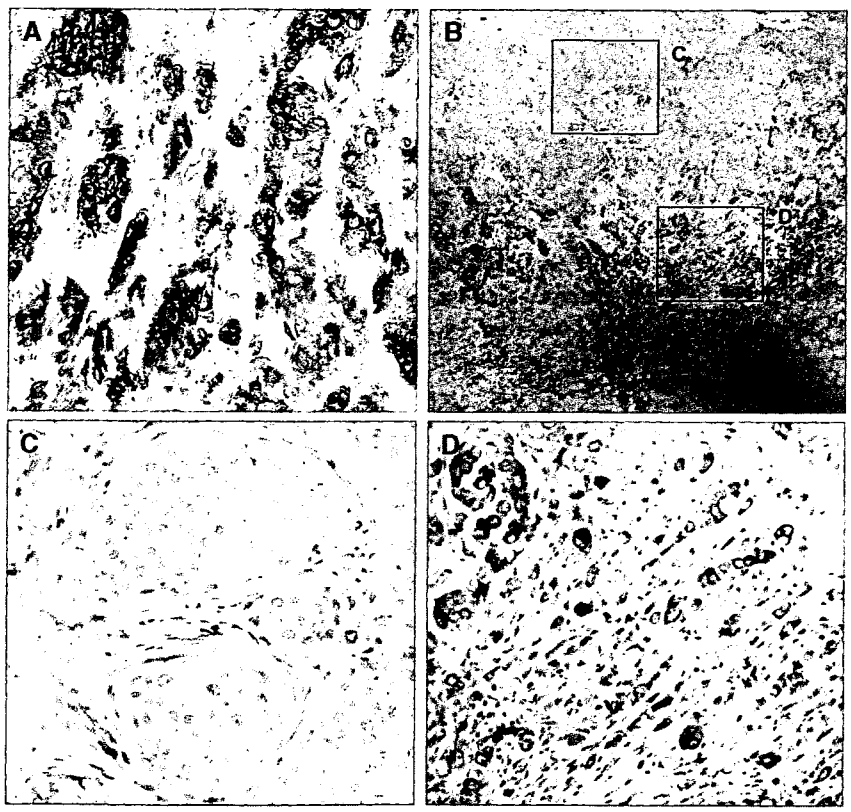


**Fig. 1** Representative results of immunohistochemical staining of Cx26 in breast cancer tissues. (**A**) A tumor strongly positive for Cx26 immunostaining (×400). (**B**) When a small percentage of tumor cells (about 10% in this tumor) was positive for Cx26 immunostaining, positive tumor cells were mostly observed in the periphery (×40). (**C**) Tumor cells in the center were negative and (**D**) those in the periphery were positive for Cx26 immunostaining (×200)

tumor cells were positive for Cx26 staining. In such a weakly positive tumor, Cx26-positive cells were mostly observed in the periphery (Fig. 1D), but not the center (Fig. 1C) of the tumor.

### Relationship between Cx26 expression and clinicopathological features of breast cancers

Expression of Cx26 was studied in 152 breast tumors by immunohistochemistry and its relationship with the clinicopathological features was evaluated. A statistically significant association was observed between Cx26 expression and large tumor size ($P = 0.013$), high histological grade ($P = 0.043$) or PR negativity ($P = 0.008$) but other parameters such as menopausal status, histological type, lymph node status, and ER status did not show a statistically significant association with Cx26 expression (Table 1).

**Table 1** Relationship between Cx26 expression and clinicopathological parameters in breast cancers

| | Cx26 expression | | *P* |
|---|---|---|---|
| | Negative | Positive | |
| *Menopausal status* | | | |
| Premenopausal | 39 | 42 | |
| Postmenopausal | 35 | 36 | 0.761 |
| *Histological type* | | | |
| Invasive ductal carcinoma | 68 | 70 | |
| Invasive lobular carcinoma | 3 | 6 | |
| Mucinous carcinoma | 2 | 2 | |
| Apocrine carcinoma | 1 | 0 | 0.722 |
| *Tumor size* | | | |
| ≦2 cm | 40 | 26 | |
| >2, ≦5 cm | 34 | 46 | |
| >5 cm | 0 | 6 | 0.013 |
| *Histological grade* | | | |
| I + II | 68 | 62 | |
| III | 6 | 16 | 0.043 |
| *Lymph node metastasis* | | | |
| Positive | 27 | 28 | |
| Negative | 47 | 50 | 0.862 |
| *Estrogen receptor* | | | |
| Positive | 54 | 55 | |
| Negative | 20 | 23 | 0.906 |
| *Progesterone receptor* | | | |
| Positive | 56 | 45 | |
| Negative | 18 | 33 | 0.008 |
| *Adjuvant therapy* | | | |
| None | 11 | 11 | |
| Hormonotherapy | 43 | 41 | |
| Chemotherpay | 3 | 9 | |
| Chemohormonotherapy | 17 | 17 | 0.241 |

### Relationship between Cx26 expression and blood or lymphatic vessel invasion

Tumor cell invasion into the blood vessel and lymphatic vessel was evaluated by immunohistochemical visualization of the blood vessels and lymphatic vessels using anti-CD34 and anti-D2-40 antibodies, respectively. Representative results of blood vessel invasion and lymphatic vessel invasion are shown in Fig. 2A and B, respectively. Frequency of blood vessel invasion was higher in Cx26-positive tumors (5.1%, 4/78) than in Cx26-negative tumors (1.4%, 1/74), though there was no statistical significance (Table 2). On the other hand, lymphatic vessel invasion was significantly more frequently observed in Cx26-positive tumors (39.7%, 31/78) than in Cx26-negative tumors (14.9%, 11/74). Figure 2C shows that Cx26-positive tumor cells are seen in the lymphatic vessel.

### Prognosis of breast cancer patients according to Cx26 expression

There was a statistically significant ($P < 0.001$) difference in RFS rates between patients with Cx26-positive and negative tumors as shown in Fig. 3. Proportions of patients treated with adjuvant hormonal therapy, chemotherapy, or chemohormonotherapy were similar between patients with Cx26-positive and negative tumors (Table 1). Univariate analysis showed that tumor size, lymph node status, histological grade, PR status, and Cx26 expression were significant prognostic factors (Table 3). Then, multivariate analysis was conducted to see whether or not Cx26 expression would be an independent prognostic factor. The multivariate analysis showed that PR status and Cx26 expression were mutually independent prognostic factors.

## Discussion

Cx26 expression was observed in 51.3% (78/152) of breast tumors. As previously reported, we were able to show a significant association between Cx26 expression and high histological grade and, in addition, between Cx26 expression and a high recurrence rate, suggesting that Cx26 expression might indicate a biologically aggressive phenotype. Our present observation is consistent with the recent report on human squamous cell lung cancer that lung tumors expressing Cx26 are associated with a high recurrence rate [19] and with that on mouse melanoma that Cx26 expressing BL6 mouse melanoma cells increase metastatic properties [18].

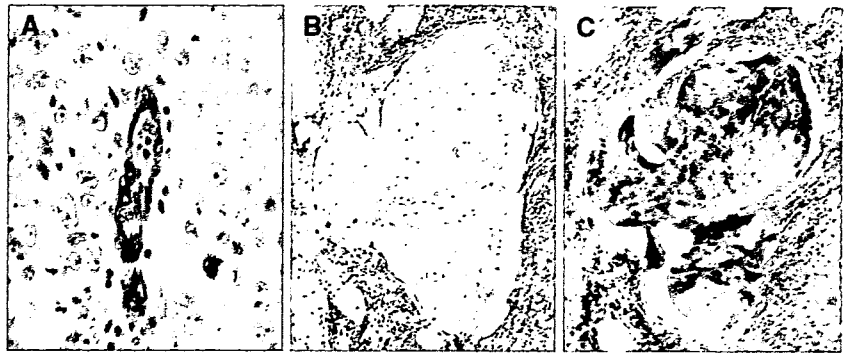


**Fig. 2** Representative results of blood vessel invasion and lymphatic vessel invasion detected by immunohistochemistry. (**A**) Blood vessel invasion detected by immunostaining with an anti-CD34 antibody (×400). (**B**) Lymphatic vessel invasion detected by immunostaining with an anti- D2-40 antibody (×200). (**C**) In the next section to (**B**). Cx26-positive cancer cells were seen in the lymphatic vessels (×200)

**Table 2** Correlation between Cx26 expression and lymphatic vessel or blood vessel invasion in breast cancers

| | Cx26 expression | | *P* |
|---|---|---|---|
| | Negative | Positive | |
| *Lymphatic vessel invasion* | | | |
| Positive | 11 | 31 | |
| Negative | 63 | 47 | 0.001 |
| *Blood vessel invasion* | | | |
| Positive | 1 | 4 | |
| Negative | 73 | 74 | 0.214 |

Such an increased metastatic potential of tumor cells with a high expression of Cx26 is speculated to be attributable to the formation of a heterologous (Cx26–Cx43) gap junction between the CX26-expressing tumor cells and Cx43-expressing vascular endothelial cells [23, 24], resulting in the increased invasion of tumor cells into the blood vessels as has been demonstrated in BL6 mouse melanoma cells [18, 25]. Therefore, in this study, we have investigated the relationship between Cx26 expression and blood vessel invasion. For the purpose of differentiating blood vessel invasion from lymphatic vessel invasion with precision, we carried out immunohistochemical staining of blood and lymphatic vessels using anti-CD34 [26] and anti-D2-40 [27] antibodies, respectively. As anticipated, incidence of blood vessel invasion was higher in Cx26-positive tumors (5.1%, 4/78) than in Cx26-negative tumors (1.4%, 1/74), though no significant difference was observed probably due to such a small number ($n$ = 5) of tumors with blood vessel invasion. Since lymphoepithelial cells are also reported to express Cx43 [24], it is expected that Cx26-positive tumors might be associated with lymphatic vessel invasion. Thus, we have also investigated the relationship between Cx26 expression and lymphatic vessel invasion. We were able to show a highly significant association between Cx26 expression and lymphatic vessel invasion. These observations seem to indicate that Cx26 expression might play a significant role in the formation of metastases through the increased invasion into lymphatic vessel and, probably, into the blood vessel in human breast cancers. It might be of interest to study whether Cx26 expressing tumor cells can form heterologous gap junction with lymphoendothelial cells as has been demonstrated between Cx26 expressing tumor cells and vascular endothelial cells. Momiyama et al. [17] have recently reported that Cx26-transfected MCF-7 cells show a decreased tumor growth as compared with parent MCF-7 cells in nude mice but, interestingly, have reported that one nude mouse with an inoculation of Cx-26 transfected MCF-7 cells developed lung metastases which are a very unusual

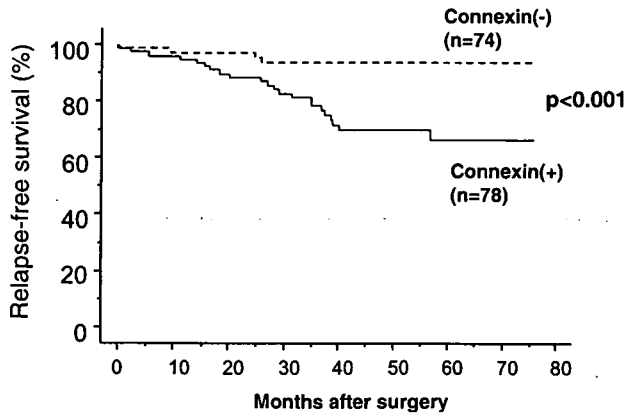


**Fig. 3** Relapse-free survival curves of breast cancer patients according to the expression levels of Cx26

**Table 3** Univariate and multivariate analysis of various prognostic factors

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| | HR* (95% CI) | *P* value | HR* (95% CI) | *P* value |
| Menopausal stasus | 1.02 (0.59–1.77) | 0.941 | – | |
| Tumor size | 3.21 (1.20–8.57) | 0.019 | 2.13 (0.77–5.91) | 0.151 |
| Histological grade | 1.74 (1.10–2.76) | 0.017 | 1.03 (0.54–1.95) | 0.932 |
| Lymph node status | 2.46 (1.11–5.41) | 0.026 | 2.14 (0.93–4.88) | 0.072 |
| ER status | 0.49 (0.22–1.10) | 0.083 | – | |
| PR status | 0.24 (0.11–0.54) | <0.001 | 0.35 (0.15–0.84) | 0.018 |
| Cx26 expression | 5.35 (1.83–15.4) | 0.002 | 3.08 (1.01–9.35) | 0.048 |

* Hazard ratio of premenopausal against postmenopausal, large tumor size (>2.0 cm) against small tumor size, histologic grade III against grade I + II, lymph node positive against lymph node negative, ER-positive against ER-negative, PR-positive against PR-negative, and Cx26 expression high against low levels

Abbreviations: HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor

phenomenon. It is tempting to speculate that Cx26 does not stimulate (or somewhat reduce) cell proliferation but increases metastatic potential by enhancing the blood vessel and lymphatic vessel invasions through the heterologous gap junction formation.

Another important finding in this study is that a significant association between Cx26 and relapses was seen when a very low cut-off value of 1% was employed. We used the various cut-off values when the association between Cx26 expression and prognosis was evaluated, and we have found that a cut-off value of as low as 1% is optimal in the separation of patients with poor prognosis from those with good prognosis. Interestingly, although there was a significant difference in the positivity of lymphatic vessel invasion between Cx26-positive and negative tumors; its positivity was similar between tumors with high Cx26 expression (>10% positive tumor cells), 40.9% (9/22), and those with low Cx26 expression (1–10% positive tumor cells), 39.3% (22/56). In tumors with low Cx26 expression, Cx26-positive tumor cells are mostly localized in the periphery (invasion front) of a tumor, where blood and lymphatic vessels are also most frequently seen. Thus, it is speculated that Cx26-positive tumor cells localizing in the invasion front, even if the number of such tumor cells is small, seem to have a good chance of invading the adjacently existing lymphatic vessels. It is also speculated that Cx26 expression in tumor cells is under the control of various cytokines locally produced from tumor cells, stromal cells, and infiltrating inflammatory cells at the invasion front, and that such local cytokines milieu might affect the potential of invasion into the blood and lymphatic vessels, and, finally, the metastatic potential of tumor cells.

In conclusion, we have been able to show that Cx26 is up-regulated in 50.1% of breast cancers, and such up-regulation is associated with lymphatic vessel invasion, high histological grade, and poor prognosis. Our results seem to suggest that Cx26 is unlikely to serve as a tumor suppressor in human breast cancers and, rather, Cx26 enhances the metastases probably through promoting blood and lymphatic vessel invasion. Cx26 might be clinically useful as a new prognostic factor, but our preliminary results need to be confirmed by a future study including a larger number of patients.

**Acknowledgement** The authors are deeply grateful to Professor Hiroshi Nojima (Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University) for his valuable and helpful advice on the planning of this study. *Grant support*: Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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J Cancer Res Clin Oncol (2007) 133:501–509
DOI 10.1007/s00432-007-0194-0

ORIGINAL PAPER

# Low *LATS2* mRNA level can predict favorable response to epirubicin plus cyclophosphamide, but not to docetaxel, in breast cancers

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Received: 5 June 2006 / Accepted: 21 December 2006 / Published online: 13 February 2007


**Abstract**
*Purpose* Putative tumor suppressor genes LATS1 and LATS2 are implicated in the regulation of the cell cycle at the G2/M and G1/S phase, respectively. This study investigated possible correlations of intra-tumoral *LATS1* and *LATS2* mRNA levels with response to epirubicin plus cyclophosphamide (EC) or docetaxel (DOC) treatment.
*Methods* mRNA expression levels of *LATS1* and *LATS2* were determined by means of real-time PCR assay in 56 locally advanced breast cancers and 15 recurrent breast cancers treated with EC ($n = 32$) or DOC ($n = 39$).
*Results* Among the patients treated with EC, *LATS2* mRNA levels of responders ($0.72 \pm 0.11$, mean $\pm$ SE) were significantly ($P < 0.05$) lower than those of non-responders ($1.62 \pm 0.44$), and responders showed a tendency ($P = 0.05$) towards reduced *LATS1* mRNA levels. Patients with low *LATS2* mRNA levels ($n = 16$) showed a significantly ($P < 0.05$) higher response rate (75%) to EC treatment than those with high *LATS2* mRNA levels ($n = 16$; response rate = 31%). Positive predictive value, negative predictive value, and diagnostic accuracy of *LATS2* mRNA levels for prediction of response to EC were 75, 69, and 72%, respectively. On the other hand, neither *LATS1* nor *LATS2* mRNA levels were associated with response to DOC treatment.
*Conclusion* These results suggest the possibility that intra-tumoral *LATS2* mRNA levels may be clinically useful for the prediction of response to EC treatment by breast cancer patients. We speculate that disruption of the checkpoint function at the G1/S phase induced by down-regulation of LATS2 plays some part in the favorable response to EC.

**Keywords** LATS1 · LATS2 · Chemosensitivity · Breast cancer

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## Introduction

Anthracycline and taxanes are the most active chemotherapy components for breast cancers, and are often used for relieving symptoms and prolonging survival under metastatic conditions as well as improving survival in the adjuvant setting. These chemotherapies, however, are not necessarily effective for all patients. In fact, the response rates of metastatic breast cancers are 50–60% to anthracycline-containing regimens (A-regimens) (French Epirubicin Study Group 1988; Italian Multicentre Breast Study with Epirubicin 1988) and 50–60% to taxanes (Seidman et al. 1993; Adachi et al. 1996). The annual risk reduction rates improved by 30–40 and 40–50%, respectively, as a result of administering adjuvant A-containing regimens alone and A-containing regimens plus taxanes (Henderson et al. 2003; Mamounas et al. 2005). On the other hand, various side effects are observed essentially in all patients treated with these chemotherapies though the type, frequency, and grade of these side effects differ among patients. Thus, it is of vital importance to identify the factors, which can predict response to each of

the various chemotherapies. Such predictive factors would be useful for the selection of the most effective chemotherapy as well as for the elimination of ineffective chemotherapies on an individual patient basis. Although various biological parameters have been postulated as candidates for predictive factors of response to anthracyclines (Faneyte et al. 2001; Geisler et al. 2001; Egawa et al. 2003) or taxanes (Egawa et al.2001; Miyoshi et al.2002; Hasegawa et al.2003), their clinical value remains controversial, so that, at present, clinically useful predictive factors for these chemotherapies have yet to be established.

The *LATS1* and *LATS2* genes are human homologues of the Drosophila *lats* gene, which encodes a putative serine/threonine kinase (Tao et al. 1999; Yabuta et al. 2000; Hori et al. 2000). *LATS1* is thought to be a tumor suppressor gene since *LATS1*-deficient mice develop soft tissue sarcomas or ovarian stromal cell tumors (St John et al. 1999) and since frequent loss of heterozygosity (LOH) of this gene has been reported in human ovarian, cervical, and breast cancers (Cooke et al. 1996; Lee et al. 1990; Mazurenko et al. 1999; Fujii et al. 1996; Theile et al. 1996; Noviello et al. 1996). In addition, in vitro over expression of *LATS1* was found to cause G2/M arrest through the inhibition of CDK1 activity in a breast cancer cell line (Yang et al. 2001). *LATS2* (also known as KPM) is also considered a tumor suppressor gene since frequent LOH of this gene has been reported in various human cancers including breast, ovary, and liver (Lee et al. 1988; Sato et al. 1991; Wang and Roger 1988) and in vitro over expression of *LATS2* was seen to cause G1/S arrest through the inhibition of CDK2 activity (Li et al. 2003). Furthermore, we recently found that a reduced expression of *LATS1* mRNA or *LATS2* mRNA is associated with a biologically aggressive phenotype of breast cancer (Takahashi et al. 2005), indicating that the reduced function of these tumor suppressor genes leads to accelerated cell proliferation, resulting in a high incidence of distant metastases.

Several clinical studies have reported that tumors with increased cell proliferation detected by Ki-67 (or Mib-1) immunostaining and flowcytometry show an enhanced response to chemotherapy (Chang et al. 2000; Pohl et al. 2003), although contradictory results have also been reported (Chang et al. 1999; Bottini et al. 2001). These findings seem to suggest that factors involved in the regulation of the cell cycle may be useful for predicting response to chemotherapy. In fact, several cell cycle regulators such as p53, p21, p27, cyclin E, and BRCA1 are reportedly associated with response to chemotherapies including those using anthracylines or taxanes (Egawa et al. 2003; Colleoni et al. 1999; Taguchi et al. 2004). As mentioned earlier, LATS1 and LATS2 are tumor suppressor genes, which are implicated in the regulation of the cell cycle. Thus, it is speculated that the expression levels of *LATS1* mRNA and *LATS2* mRNA may be associated with chemosensitivity. In the present study, we therefore investigated possible correlations between *LATS1* mRNA and *LATS2* mRNA levels in breast cancer tissues determined with a real-time PCR assay with the response to epirubicin plus cyclophosphamide (EC) or docetaxel (DOC) monotherapy.

## Materials and methods

### Tumor specimens and patient treatments

For this study, 71 females breast cancer patients (56 locally advanced primary breast cancer patients and 15 locally recurrent breast cancer patients) were recruited. These patients were treated with either EC ($n = 32$) or DOC ($n = 39$). Tumor samples were obtained from primary breast tumors or locally recurrent lesions by means of incisional biopsy or vacuum-assisted core needle biopsy prior to chemotherapy. Part of each tumor sample was subjected to pathological diagnosis, and the rest was snap frozen in liquid nitrogen and kept at −80°C until use for RNA extraction and for estrogen receptor (ER) and progesterone receptor (PR) assay. An additional 41 primary breast tumor samples from patients without prior treatment were obtained interoperatively and also used for immunohistochemical analyses. Informed consent for these studies was obtained from all patients.

### Chemotherapy and evaluation of response

Four cycles of EC (epirubicin 60 mg/m$^2$ i.v. day 1 + cyclophosphamide 600 mg/m$^2$ i.v. day 1, q3w) or DOC (60 mg/m$^2$ i.v. day 1, q3w) were administered to the patients with locally advanced breast tumors before surgery or to patients with recurrent tumors until disease progression was observed. Chemotherapeutic response was evaluated according to the WHO clinical criteria: complete response (CR), disappearance of all known disease; partial response (PR), 50% or more decrease in tumor size; no change (NC), less than 50% decrease or less than 25% increase in tumor size; and progressive disease (PD), 25% or more increase in tumor size or appearance of new lesions (Miller et al. 1981). Patients showing CR or PR were considered responders, and those showing NC or PD non-responders.

### RNA extraction, reverse transcription, and real-time PCR assay of *LATS1* and *LATS2* mRNA levels

Total RNA was extracted from the frozen tumor specimens using TRIZOL reagent according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). About 3 μg of total RNA was reverse-transcribed for single strand cDNA, using the oligo-$(dT)_{15}$ primer and Superscript II (Life Technologies Inc., Gaithersburg, MD, USA) at 42°C for 90 min, followed by heating at 70°C for 10 min. The ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) was used for real-time PCR reactions of *LATS1* and *LATS2*. The sequence of the primers and probes for *LATS1* and *LATS2* as well as the reaction conditions were described previously (Takahashi et al. 2005). *β-glucoronidase* transcripts for quantitative control were used to normalize the transcript content of the sample. The primer and probe mixture for *β-glucoronidase* was purchased from Perkin-Elmer Applied Biosystems and used according to the manufacturer's protocol. The standard curves for *LATS1*, *LATS2* and *β-glucoronidase* mRNA were generated using serially diluted solutions of plasmid clones inserted with *LATS1*, *LATS2* or *β-glucoronidase* cDNA as templates. The amount of target gene expression was then calculated from these standard curves with $10^{-9}$ μg of the PCR product for *LATS1* and *LATS2*, and $10^{-8}$ μg of the PCR product for *β-glucoronidase*, which was defined as 1. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the relative expression levels. The final expression levels of *LATS1* and *LATS2* mRNA were expressed as ratios to those of *β-glucoronidase*.

### Immunohistochemical staining of Mib-1, LATS1, LATS2, and Geminin

From the 71 tumors, 53 samples were available for immunohistochemical analysis for Mib-1 detection with the avidin–biotin–peroxidase method using a rabbit anti-human Mib-1 polyclonal antibody (MIB-1; Immunotech, Cedex, France) following a previously described method (Takamura et al. 2002). Another set of 60 formalin fixed paraffin embedded breast tumor tissues obtained interoperatively from patients without prior treatment were used for immunohistochemical study of LATS1, LATS2, and Geminin. Their expression was detected by using the Histofine Simple Stain system, Nichirei, Tokyo, Japan, for LATS1 and Geminin, or the CSA system (DAKO, Kyoto, Japan) for LATS2. Polyclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, for LATS1 and Geminin, and from Abgent, San Diego, CA, USA, for LATS2. Antigen retrieval was performed by incubating the sections in Histo VT One (Nakarai, Kyoto, Japan) for LATS1, or in a target retrieval solution (DAKO, Kyoto, Japan) for LATS2 and Geminin, in a hot water bath at 98°C for 40 min. After quenching endogenous peroxidase with 3% $H_2O_2$ in methanol for 20 min, non-specific binding was blocked by incubating the slides with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min. The slides were then incubated with primary antibody (1 μg/ml for LATS1 and Geminin, 5 μg/ml for LATS2) at 4°C overnight followed by incubation for 60 min with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for LATS1 and Geminin, or with peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for LATS2. Next, the antibody complex was visualized with the 3, 3′-diaminobenzidine tetrahydrochloride (Merck KGaA, Darmstadt, Germany). The sections were counter-stained with hematoxylin, the lesions showing the most active staining selected microscopically and 1,000 tumor cells examined for calculation of the percentage of stained cells. Nuclear positive staining was determined for Mib-1 and Geminin, and 10% or more nuclear and/or cytoplasmic staining was judged to indicate positivity for LATS1 and LATS2. All the slides were examined by skilled observers blinded to the clinical data.

### Estrogen receptor (ER) and progesterone receptor (PR) assay

An enzyme immunoassay was used to measure ER and PR protein levels in breast cancers with the kits provided by Abbott Research Laboratories, Chicago, IL, USA, according to the manufacturer's instructions. The cut-off value for ER and PR was set at 5 fmol/mg protein.

### Statistical methods

Student's *t*-test was used for comparison of *LATS1* and *LATS2* mRNA expression levels as well as Mib-1 expression levels among various groups. The relationship between *LATS1* or *LATS2* mRNA high or low expression with response to EC or DOC, and the relationship between LATS1 or LATS2 expression and Geminin expression were analyzed with the chi-square test. Statistical significance was assumed for $P < 0.05$.

## Results

### Patient characteristics and *LATS1* and *LATS2* mRNA levels in breast cancer tissues

Characteristics of patients treated with EC ($n = 32$) or DOC ($n = 39$) were similar as shown in Table 1. *LATS1* and *LATS2* mRNA levels in the EC group ($1.91 \pm 0.28$ and $1.14 \pm 0.23$, respectively, mean ± SE) were not significantly different from those in the DOC group ($2.54 \pm 0.51$ and $0.98 \pm 0.14$, respectively) (Table 2). There were no significant associations between *LATS1* and *LATS2* mRNA levels and any clinical parameters including menopausal status, disease site, stage, ER, and PR status (Table 2).

### Relationship between Mib-1, *LATS1 mRNA* or *LATS2* mRNA levels and response to EC or DOC

In the EC group, percentages of cancer cells positive for Mib-1 immunohistochemistry were similar ($P = 0.96$) for responders ($30.25 \pm 4.25\%$, mean ± SE) and non-responders ($30.63 \pm 6.67\%$) (Table 3; Fig. 1). On the other hand, *LATS2* mRNA levels of responders ($0.72 \pm 0.11$) were significantly ($P < 0.05$) lower than those of non-responders ($1.62 \pm 0.44$), while *LATS1* mRNA levels of responders ($1.42 \pm 0.20$) were marginally significantly ($P = 0.05$) lower than those of non-responders ($2.47 \pm 0.51$) (Table 3; Fig. 1). The DOC group showed no significant differences between responders and non-responders in Mib-1 positivity, *LATS1* mRNA levels, or *LATS2* mRNA levels (Fig. 1).

Patients were divided into high and low expression groups for Mib-1, *LATS1* mRNA, or *LATS2* mRNA levels by using the median value as the cut-off value. Response rates to EC or DOC showed no significant differences between the Mib-1 high and low groups and between the *LATS1* mRNA high and low groups (Table 3). Patients with low *LATS2* mRNA levels showed a significantly ($P < 0.05$) higher response rate

**Table 1** Clinicopathological characteristics of patients treated with EC or DOC

| | EC[a] ($n = 32$) | DOC[b] ($n = 39$) |
|---|---|---|
| Age (years) [average (range)] | 50.1 (30–74) | 51.7 (34–67) |
| Menopausal status | | |
| Premenopausal | 17 (53)[c] | 16 (41) |
| Postmenopausal | 15 (47) | 23 (59) |
| Disease site | | |
| Locally advanced breast tumors | | |
| Stage II | 13 (41) | 11 (28) |
| Stage III | 7 (22) | 16 (41) |
| Stage IV | 5 (15) | 4 (10) |
| Locally recurrent tumors | 7 (22) | 8(21) |

[a] Epirubicin plus cyclophosphamide

[b] Docetaxel

[c] Percent (%)

**Table 2** *LATS1* and *LATS2* mRNA levels in breast cancer tissues

| | *n* | *LATS1* mRNA levels | *LATS2* mRNA levels |
|---|---|---|---|
| Chemotherapy | | | |
| EC[a] | 32 | 1.91 ± 0.28[c] | 1.14 ± 0.23 |
| DOC[b] | 39 | 2.54 ± 0.51 | 0.98 ± 0.14 |
| Menopausal status | | | |
| Premenopausal | 33 | 2.33 ± 0.59 | 1.01 ± 0.18 |
| Postmenopausal | 38 | 2.19 ± 0.28 | 1.08 ± 0.18 |
| Disease site | | | |
| Locally advanced breast tumors | | | |
| Stage II | 24 | 2.42 ± 0.80 | 1.02 ± 0.16 |
| Stage III | 23 | 2.04 ± 0.36 | 1.14 ± 0.31 |
| Stage IV | 9 | 2.10 ± 0.55 | 0.70 ± 0.14 |
| Recurrent tumors | 15 | 2.42 ± 0.41 | 1.18 ± 0.25 |
| ER status | | | |
| Positive | 27 | 2.90 ± 0.73 | 0.94 ± 0.16 |
| Negative | 39 | 1.86 ± 0.23 | 1.03 ± 0.17 |
| Unknown | 5 | 1.89 ± 0.54 | 1.87 ± 0.89 |
| PR status | | | |
| Positive | 20 | 2.56 ± 0.95 | 0.93 ± 0.18 |
| Negative | 46 | 2.17 ± 0.25 | 1.02 ± 0.15 |
| Unknown | 5 | 1.89 ± 0.54 | 1.87 ± 0.89 |

[a] Eepirubicin plus cyclophosphamide

[b] Docetaxel

[c] Mean ± SE

**Table 3** Relationship between Mib-1, *LATS1*, or *LATS2* expression levels and response to EC or DOC

| mRNA level | Responders (*n*) | Non-responders (*n*) | Response rate (%) | *P*-value |
|---|---|---|---|---|
| Mib-1 expression | | | | |
| EC[a] | | | | |
| High | 3 | 5 | 38 | 0.31 |
| Low | 5 | 3 | 63 | |
| DOC[b] | | | | |
| High | 11 | 8 | 58 | 0.41 |
| Low | 8 | 10 | 44 | |
| *LATS1* expression | | | | |
| EC | | | | |
| High | 8 | 8 | 50 | 0.72 |
| Low | 9 | 7 | 56 | |
| DOC | | | | |
| High | 13 | 7 | 65 | 0.15 |
| Low | 8 | 11 | 42 | |
| *LATS2* expression | | | | |
| EC | | | | |
| High | 5 | 11 | 31 | <0.05 |
| Low | 12 | 4 | 75 | |
| DOC | | | | |
| High | 10 | 9 | 53 | 0.88 |
| Low | 11 | 9 | 55 | |

[a] Epirubicin plus cyclophosphamide

[b] Docetaxel

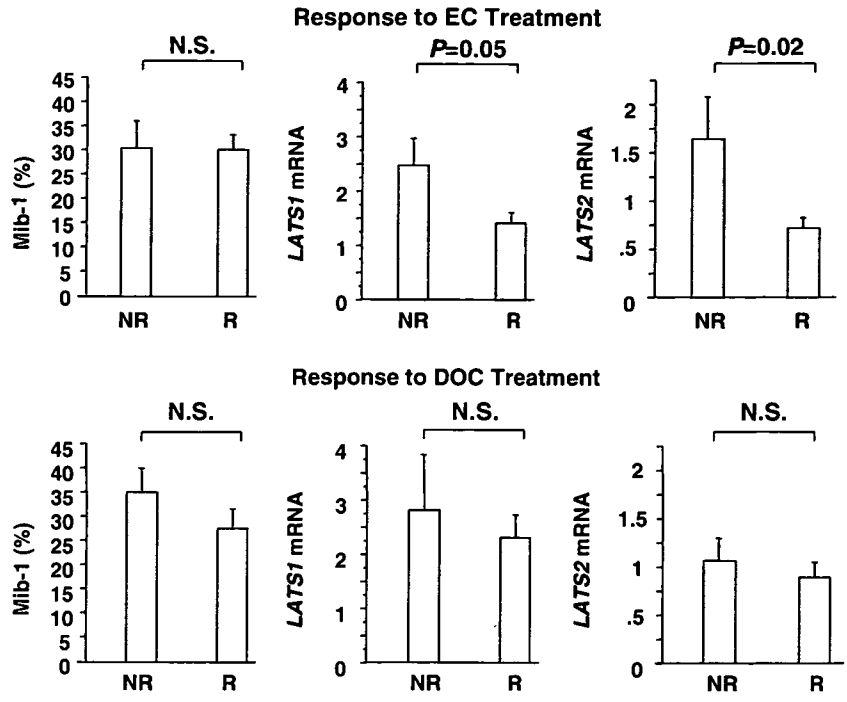


**Fig. 1** Mib-1 positivity (%) and *LATS1* or *LATS2* mRNA levels in non-responders (*NR*) and responders (*R*) to epirubicin plus cyclophosphamide (*EC*) treatment (*upper panel*) and docetaxel (*DOC*) treatment (*lower panel*)

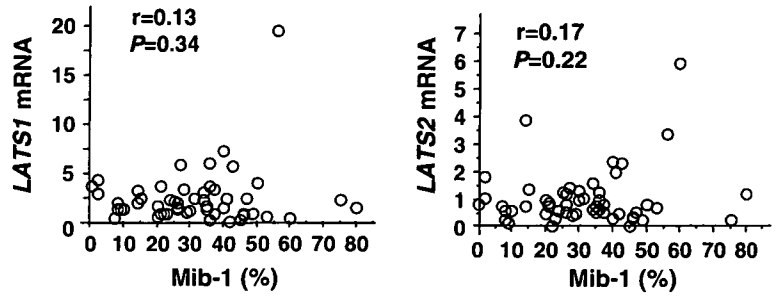


**Fig. 2** Correlation of Mib-1 positivity (%) with *LATS1* or *LATS2* mRNA levels in breast cancers

(75%) to EC than did those with high *LATS2* mRNA levels (31%) (Table 3), while, no significant association was found between *LATS2* mRNA levels and the response rate to DOC ($P = 0.88$). Positive predictive value, negative predictive value, and diagnostic accuracy of *LATS2* mRNA levels for the prediction of response to EC were 75, 69, and 72%, respectively.

### Relationship between Mib-1 expression levels and *LATS1* mRNA or *LATS2* mRNA levels in breast cancer tissues

There was no significant association between percentages of Mib-1 positive cells and *LATS1* mRNA ($r = 0.13$ and $P = 0.34$) or *LATS2* mRNA ($r = 0.17$ and $P = 0.22$) levels (Fig. 2).

### Immunohistochemical analyses of LATS1, LATS2, and Geminin in breast cancer tissues

In order to confirm the LATS1 and LATS2 expression in breast cancer cells, a different set of 41 tumor samples was subjected to immunohistochemical staining for further investigation. As shown in Fig. 3, nuclear and/or cytoplasmic staining of LATS1 and LATS2 were recognized in breast cancer cells in 29 (71%) and 24 (59%) tumors, respectively. Next, the expression of Geminin in cancer cells was examined and the relationship between Geminin expression and LATS1 or LATS2 expression is shown in Table 4. The proportion of Geminin-positive tumor cells tended to be higher ($P = 0.05$) in LATS1-negative than in LATS1-positiv tumors, while it was significantly ($P < 0.01$) higher in LATS2-negative than in LATS2-positive tumors.

## Discussion

In the study presented here, we were able to show that tumors with low *LATS2* mRNA expression are significantly associated with a high response rate to EC. The anti-neoplastic activity of EC is thought to be mostly attributable to epirubicin because the response rate of metastatic breast cancers to EC is similar to that to

**Table 4** Relationship between LATS1, or LATS2 expression and Geminin expression

| | Positive *n* (%) | Negative *n* (%) | *P*-value |
|---|---|---|---|
| LATS1 expression | | | |
| Geminin-positive cells (%) | | | |
| <10 | 24 (83) | 6 (50) | 0.05 |
| 10≤, <20 | 4 (14) | 3 (25) | |
| 20≤ | 1 (3) | 3 (25) | |
| LATS2 expression | | | |
| Geminin-positive cells (%) | | | |
| <10 | 22 (92) | 8 (47) | 0.006 |
| 10≤, <20 | 1 (4) | 6 (35) | |
| 20≤ | 1 (4) | 3 (18) | |

epirubicin monotherapy (Ormrod et al. 1999) and because the action of cyclophosphamide monotherapy is reported to be generally very low (Carter 1972). Anthracyclines including epirubicin exert their anti-neoplastic effect through formation of a complex with DNA by intercalating the DNA strands and inhibiting the function of topoisomerase II-alpha, which leads to the induction of DNA damage and, finally, the inhibition of DNA replication (Capranico et al. 1990). Since topoisomerase II-alpha is most strongly expressed in the S and G2/M phases (Boege et al. 1995), it is speculated that the cells in these phases are the most sensitive to anthracycline. This speculation is supported by Hill et al., who showed that cancer cells in the S-phase were most sensitive to anthracyclines in vitro (Hill and Whelan 1982). Since LATS2 negatively regulates CDK2 activity (Li et al. 2003), low *LATS2* mRNA expression is thought to lead to high levels of CDK2 activity, which then drives the transition of cancer cells from the G1 to the S phase, resulting in high sensitivity to anthracyclines.

Low expression of *LATS1* mRNA in our study tended ($P = 0.05$) to be associated with a high response rate to EC. Since LATS1 inhibits CDK1 activity (Yang et al. 2001), low *LATS1* mRNA expression is thought to lead to acceleration of the transition from the G2 to the M phase. Although the S-phase is the most sensitive to anthracylcines, the cancer cells in the early M-phase also reportedly show a relatively high sensitivity to anthracyclines (Hill and Whelan 1982). We therefore speculate that tumors with low *LATS1* mRNA expression are more sensitive to EC than those with high expression because the proportion of cancer cells in the M-phase is higher in tumors with low than in those with high *LATS1* mRNA levels. Another explanation is that the association between low *LATS1* mRNA expression and a high response rate to EC is not a causal relationship but simply an indirect association since *LATS1* mRNA expression significantly correlates with *LATS2* mRNA expression (data not shown). If this expression is low, it would then increase the population of cancer cells in the S phase when the cancer cells are most sensitive to anthracyclines.

The association of Mib-1 with sensitivity to anthracyclines has been studied by several investigators but not with consistent results. Some investigators reported a significant relationship between high Mib-1 expression and a favorable response to anthracyclines (Chang et al. 2000; Pohl et al. 2003) but others did not (Linn et al. 1997). We could not find a significant association either between Mib-1 expression and response to EC. Tumors with a high percentage of Mib-1 positive cells are generally considered to be characterized by high proliferation but the percentage of Mib-1 positive cells does not necessarily correlate with the percentage of cells in the S-phase since Mib-1 is expressed in all cells except those in the G0-phase (Gerdes et al. 1984). Thus, even if the percentage of Mib-1 positive cells is the same, the percentage of cells in the S phase

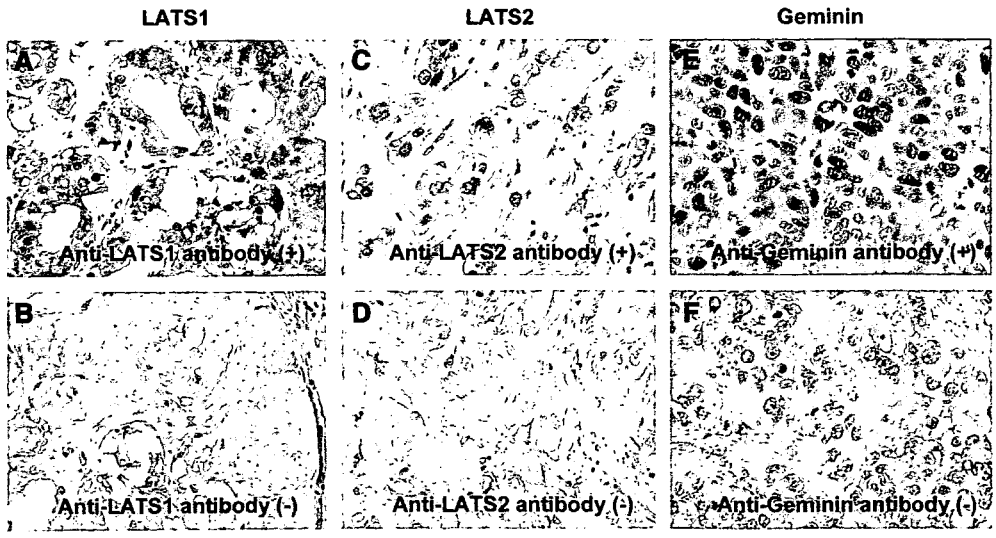


**Fig. 3** Representative results of immunohistochemical staining of LATS1, LATS2, and Geminin in cancer cells with (**a**, **c**, **e**) and without (**b**, **d**, **f**) antibodies (X400). Nuclear and/or cytoplasmic staining of LATS1 (**a**) and LATS2 (**c**), and nuclear staining of Geminin (**e**) were detected