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Expression of the *ets-1* proto-oncogene in human breast carcinoma: Differential expression with histological grading and growth pattern

S. Katayama[1,*], T. Nakayama[1,*], M. Ito[2], S. Naito[3] and I. Sekine[1]

[1]Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, [2]Department of Pathology, Clinical Research Center, National Nagasaki Medical Center, Omura, Japan and [3]Division of Pathology, Research Laboratory, National Ureshino Medical Center, Saga, Japan

Summary. The proto-oncogene, *ets-1*, is a transcription factor known to control the expression of a number of genes and has been postulated to play a role in cell growth, differentiation and tumour invasion. We examined 137 cases of breast carcinoma by immunohistochemistry and compared the degree of Ets-1 expression among the different histological types of invasive carcinomas. Ets-1 was not expressed in the normal breast epithelium nor in noninvasive carcinomas. Among the 137 breast carcinoma cases, 104 (83.2%) showed positive staining for the Ets-1 protein. Histologically, invasive ductal carcinomas expressed immunopositivity with intense staining for Ets-1 in the tumour cells. Ets-1 expression correlated with Bloom-Richardson grading in invasive ductal carcinoma ($p<0.01$). However, there was no correlation between Ets-1 expression and lymph node metastasis, "t" classification or TNM staging. *In situ* hybridization confirmed the presence of Ets-1 mRNA in breast carcinomas. The expression of Ets-1 mRNA was detected in two of three different kinds of cultured human breast carcinoma cell lines and one of three human breast carcinoma tissues by the reverse transcription polymerase chain reaction method. These findings suggest that ets-1 is overexpressed in ductal cells of the breast that have undergone malignant conversion and that *ets-1* is one of the factors associated with tumour growth and histological differentiation of breast carcinomas.

Key words: Ets-1, Breast, Immunohistochemistry, Cancer

Offprint requests to: Tosiyuki Nakayama, M.D., Ph.D., Department of Molecular Pathology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Fax: 81-95-849-7108. e-mail: toshi-n@net.nagasaki-u.ac.jp

*Sayaka Katayama and Toshiyuki Nakayama contributed equally to this work

Introduction

The prognosis of breast cancer patients is based on the grade of invasion and the presence of lymph node metastasis. These parameters are usually determined by microscopic examination of tissue sections from the primary neoplasm and lymph nodes (Steinberg et al., 1986). However, histopathological examination of primary breast carcinoma specimens cannot always identify the metastatic potential and risk for the patient (Steinberg et al., 1986; Jessup and Gallic, 1992). Recently, the occurrence and progression of cancer is suggested to be related to a series of genetic events affecting the structure and/or the expression of a number of oncogenes, anti-oncogenes and growth factors. However, the mechanism of invasion of breast carcinomas has not been fully elucidated.

Ets-1 was originally characterized as the v-ets retroviral gene, 1 of the 2 oncogenes (*v-myb* and *v-ets*) in the avian leukemia retrovirus, E26 (Leprince et al., 1983). The ets family of genes encode transcription factors for mesodermal cell development during embryonic development (Vandenbunder et al., 1989; Kola et al., 1993). Ets-1 plays a role in the regulation of physiological processes such as cell proliferation and differentiation (Lewin, 1991). Ets-1 is also associated with invasive processes in the stromal tissues of human carcinomas (Vandenbunder et al., 1989). We have reported that Ets-1 is correlated to the progression of the carcinoma cells of stomach, pancreas, thyroid, brain and colon (Nakayama et al., 1996, 1999, 2001; Ito et al., 1998; Kitange et al., 1999). However, the biological function of the *ets-1* proto-oncogene remains unknown. The processes of tumor invasion and metastasis are thought to depend on the increased proteolytic activity of the invading tumor cells (Liotta and Setler-Stevenson, 1990). Matrix metalloproteinases, cathepsins B and D, and plasminogen activator have been proposed to participate in the metastatic cascade (Blasi and Verde,

1990; Kane and Gottesman, 1990; Liotta and Setler-Stevenson, 1990; Matrisian and Bowden, 1990; Rochefort, 1990; Sloane, 1990). Ets-1 protein interacts with the urokinase-type plasminogen activator (u-PA) gene enhancer and with the promoters of the stromelysin-1 and collagenase-1 genes (Nerlov et al., 1992; Vandenbunder et al., 1994). Ets-1, therefore, is thought to regulate increased tumor invasion by activating the expression of u-PA, stromelysin and collagenase.

Based on these findings, we undertook in the present study to determine the role of Ets-1 in the histological types, tumor size and lymph node metastasis of human breast carcinomas.

Materials and methods

Cases and tissues

We studied 24 normal breast tissues and 137 primary human breast carcinomas. The numbers and types of breast carcinomas are listed in Table 1. Tenty three specimens of normal breast tissue, that were taken from patients without breast cancer, were evaluated as the normal control. All tumour specimens were obtained from patients operated on at Nagasaki University Hospital between 2000 and 2003. Each tumor was assigned a histological type according to the World Health Organization classification (Tavassoli and Devilee, 2003), Armed Forces Institute of Pathology grading (Rosen and Oberman, 1993) and according to the TNM classification for tumor size, lymph node metastasis, and invasive grading (Spiessl, 1989). And using the Elston modification of the Bloom-Richardson grading system, patients of invasive ductal carcinoma were divided by histologic grade into 3 groups (Bloom and Richardson, 1957; Elston and Ellis, 1991). Diagnosis was established by two independent pathologists (Katayama and Nakayama), and cases of questionable diagnosis were omitted from the study.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into 4 µm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4 °C with an optimal dilution (0.1 µg/ml) of a primary polyclonal antibody against human *ets-1* (C-20, raised against the C-terminal domain of the *ets-1* protein; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (Hultgardh-Nilsson et al., 1996). The slides were sequentially incubated with an alkaline phosphatase-conjugated horse antirabbit immunoglobulin antibody, and the reaction products were resolved using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersburg, MD, USA). Preabsorption of the primary antibody with excess recombinant *ets-1* peptide (Santa Cruz Biotechnology, Inc.) was used as a negative control. Adrenal gland tissue (Kola et al., 1993) served as the internal positive control for *ets-1* immunostaining. Analysis of the immunohistochemical staining was performed by two investigators (Katayama and Nakayama). Ets-1 expression was classified into three categories depending on the percentage of cells stained and/or the intensity of staining : -, 0 to 10% positive cells; +, 10 to 50% positive tumor cells; and ++, > 50% positive tumor cells.

In situ hybridization

In situ hybridization for the detection of human Ets-1 mRNA was performed using an oligonucleotide probe complementary to a fragment of human *ets-1* mRNA (Dennis et al., 1988). The sequence of the oligonucleotide probe is 5'-GCCCAGCTTCATCACAG AGTCCTATCAGAC-3', and it does not cross-hybridize with other mRNA sequences. The probe was labelled with 5' tailed digoxigenin (DIG, Greiner Japan Inc., Tokyo, Japan) and purified by high performance liquid chromatography (HPLC). Four cases of human breast carcinoma were studied by in situ hybridization. In all of these cases, we were able to obtain relatively fresh (within 6 months) paraffin embedded sections. The presence of cytoplasmic RNA was confirmed by using a methyl green pyronine staining solution (Muto Pure Chemicals, Tokyo, Japan). Prehybridization was carried out as described previously (Nakayama et al., 1996). The sections were treated with 0.2N HCl for 20 minutes and digested with 100 µg/ml of proteinase K (Sigma, St Louis, MO, USA) for 10 minutes at 37 °C. After post-fixation in 4% paraformaldehyde, each section was covered with 20 µl of denatured hybridization mixture containing 4% dextran sulphate, 125 µg/ml sonicated salmon sperm DNA, 9% deionized formamide, 2.5 µg/ml yeast tRNA, 5x Denhardt's medium, 1 mM EDTA (ethylenediamine-tetraacetic acid, pH 7.4), 0.6 M NaCl, 10 mM Tris-HCl, and 1 µg/ml digoxigenin-labelled *ets-1* oligonucleotide probe, and placed in a moist chamber, where it was incubated at 37 °C for 15 hours. After washing, in situ detection was accomplished with a DIG detection kit (BRL). Briefly, the slides were incubated with 100 µl of blocking solution for 15 min at room temperature and incubated with the streptavidin-alkaline phosphatase complex. Ets-1 mRNA expression was evaluated by comparing alkaline phosphatase staining using BCIP/NBT with the results obtained from the positive and negative controls. Each slide was studied in duplicate and negative controls were made using the digoxigenin-labelled sense oligoprobe (5'-GTCTGA TAGGACTCTGTGATGAAGCTGGGC-3'). RNase treatment was carried out prior to hybridization as another negative control. Slides of a human adrenal gland served as a positive control (Kola et al., 1993).

Cell culture

Three human breast carcinoma cell lines: MCF-7 , SK-BR-3 , MDA-MB231 (Improta-Brears et al., 1999; Roetger et al., 1998; Palyi et al., 1999), were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cultures of all three cell lines were maintained in Dubecco's modified Eagle's medium (DMEM, GIBCO/BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Improta-Brears et al., 1999; Roetger et al., 1998; Palyi et al. 1999). Plastic culture dishes were purchased from Becton Dickinson (Oxnard, CA, USA). All of these cells were provided by Health Science Research Resources Bank (Osaka, Japan).

Reverse transcription - polymerase chain reaction (RT-PCR)

Total RNA was prepared from normal human breast and carcinoma tissue, and the human breast carcinoma cell lines MCF-7, SK-BR-3 and MDA-MB231 (Importa et al., 1999; Roetger et al., 1998; Palyi et al., 1999) using the acid guanidine phenol method (Van Dekken et al., 1990). Cellular RNA (1 µg) was incubated at 37 °C for 1 hour in 50 µl of reverse transcriptase buffer containing 20 units of RNAs in (Promega Corp., Madison, WI, USA), 100 pmol of random hexamer primers (Boehringer Mannheim, Mannheim, Germany), and 400 units of Moloney murine leukemic virus reverse transcriptase (GIBCO/BRL). Reverse transcription was terminated by heating at 95 °C for 10 minutes, and 20% of the resulting cDNA was removed for PCR. PCR samples were incubated with 50 pmol of each primer and 2.5 units of Taq DNA polymerase. The human *ets-1* PCR primers were 5'-GGGTGACGACTTCTTGTTTG-3' (sense) and 5'-GTTAATGGAGTCAACCCAGC-3' (antisense). The human ß-actin PCR primers were 5'-TCCTCCCTGGAGAAGACTA-3' (sense) and 5'-AGTACTTGCGCTCAGGAGGA-3' (antisense). The *ets-1* and ß-actin primers are designed to amplify 274 and 313 bp DNA fragments, respectively. Both primer pairs were chosen to span introns of their respective human genes. Samples were subjected to 28 cycles of PCR amplification using a thermocycler. Each cycle included denaturation at 94 °C for 1 minute, annealing at 57 °C for 1 minute, and primer extension at 72°C for 1.5 minutes. An aliquot of each amplification mixture was subjected to electrophoresis on a 2.0% agarose gel, and DNA was visualized by ethidium bromide staining. The sequence of the product of RT-PCR were analyzed by DNA analyzer (ABI PRISMTM 310NT, Applied Biosystems, Inc., CA).

Statistical analysis

The Stat View II program (Abacus Concepts Inc., New York, NY, USA) was used for statistical analyses. Analyses comparing the intensities of Ets-1 expression were performed by the Mann-Whitney's U test, Chi-square test and Spearman's test.

Results

Ets-1 immunohistochemical staining is shown in some types of invasive ductal carcinomas in Figures 1A-F. Carcinoma cells expressed strong intensity in the cytoplasm and/or nucleus. The results from immuno-

Table 1. Immunohistochemical Staining and Histological Types of Breast Carcinomas (137 cases).

	N	Ets-1 EXPRESSION		
		-	+	++
Total carcinomas	137	23 (16.8%)	31 (22.6%)	83 (60.6%)
Non-invasive ductal carcinoma*	3	3 (100.0%)	0 (0.0%)	0 (0.0%)
Invasive ductal carcinoma with a predominant intraductal component	4	1 (25.0%)	2 (50.0%)	1 (25.0%)
Invasive ductal carcinoma	115	16 (13.9%)	26 (22.6%)	73 (63.5%)
Invasive lobular carcinoma	4	1 (25.0%)	1 (25.0%)	2 (50.0%)
Medullary carcinoma	4	1 (25.0%)	1 (25.0%)	2 (50.0%)
Mucinous carcinoma	2	0 (0.0%)	0 (0.0%)	2 (100.0%)
Adenoid cystic carcinoma	1	0 (0.0%)	0 (0.0%)	1 (100.0%)
Apocrine carcinoma	3	0 (0.0%)	1 (33.3%)	2 (66.6%)
Carcinoma with metaplasia, Spindle-cell type	1	1 (100.0%)	0 (0.0%)	0 (0.0%)
*Benign breast lesions***				
Intraductal papilloma	3	3 (100.0%)	0 (0.0%)	0 (0.0%)
Fibroadenoma	10	10 (100.0%)	0 (0.0%)	0 (0.0%)
Adenosis	10	10 (100.0%)	0 (0.0%)	0 (0.0%)
*Normal breast tissue***	23	23 (100.0%)	0 (0.0%)	0 (0.0%)
Lactating breast	1	1 (100.0%)	0 (0.0%)	0 (0.0%)

*: $p<0.05$, **: $p<0.001$

histochemical staining are summarized in Table 1. Ets-1 antigen was expressed heterogeneously in carcinomas. Strong staining of Ets-1 was observed in 83.2% of all carcinomas (114 of 137). Ets-1 immunoreactivity in relation to histological type is shown in Table 1. However, all three cases of noninvasive ductal carcinoma and all of the twenty four cases of benign tumours and normal breast tissues were negative for Ets-1. The immunopositivity of Ets-1 in the stromal fibroblast around carcinoma cell was not intensly.

There was no correlation between the intensity of Ets-1 expression and histological types. However, in histological growth patterns of invasive carcinoma, the intensities of Ets-1 staining were variable, as shown in Table 2. Positivity for Ets-1 expression was distributed to varying degrees among the eight different growth patterns. The solid carcinoma and the carcinoma with fibrous stroma patterns recorded the highest expressions, while the clinging and low papillary patterns showed the lowest.

Ets-1 staining intensity was compared with the presence of lymph node metastasis, 't' classification and TNM staging in the primary tumors (Tables 3-5). The intensity of Ets-1 expression was not correlated with the presence of lymph node metastasis, 't' classification and TNM staging. And, in Table 6, we summarized the results of the correlation between cellular atypism and Ets-1 expression. Bloom-Richardson grading is significantly correlated with Ets-1 expression ($p<0.01$).

As with the antigenic intensity shown by immunostaining, Ets-1 mRNA expression demonstrated by in situ hybridization was positive in both the cytoplasm and the nucleus of breast carcinomas (Fig. 1F). No specific hybridization was observed with the sense labelled probe. RNase treatment of the sections hybridized with the *ets-1* oligonucleotide probe yielded no positive signals.

The results from RT-PCR of Ets-1 mRNA in human breast carcinoma cell lines and human breast carcinoma tissues are shown in Figure 2. Two of three human breast

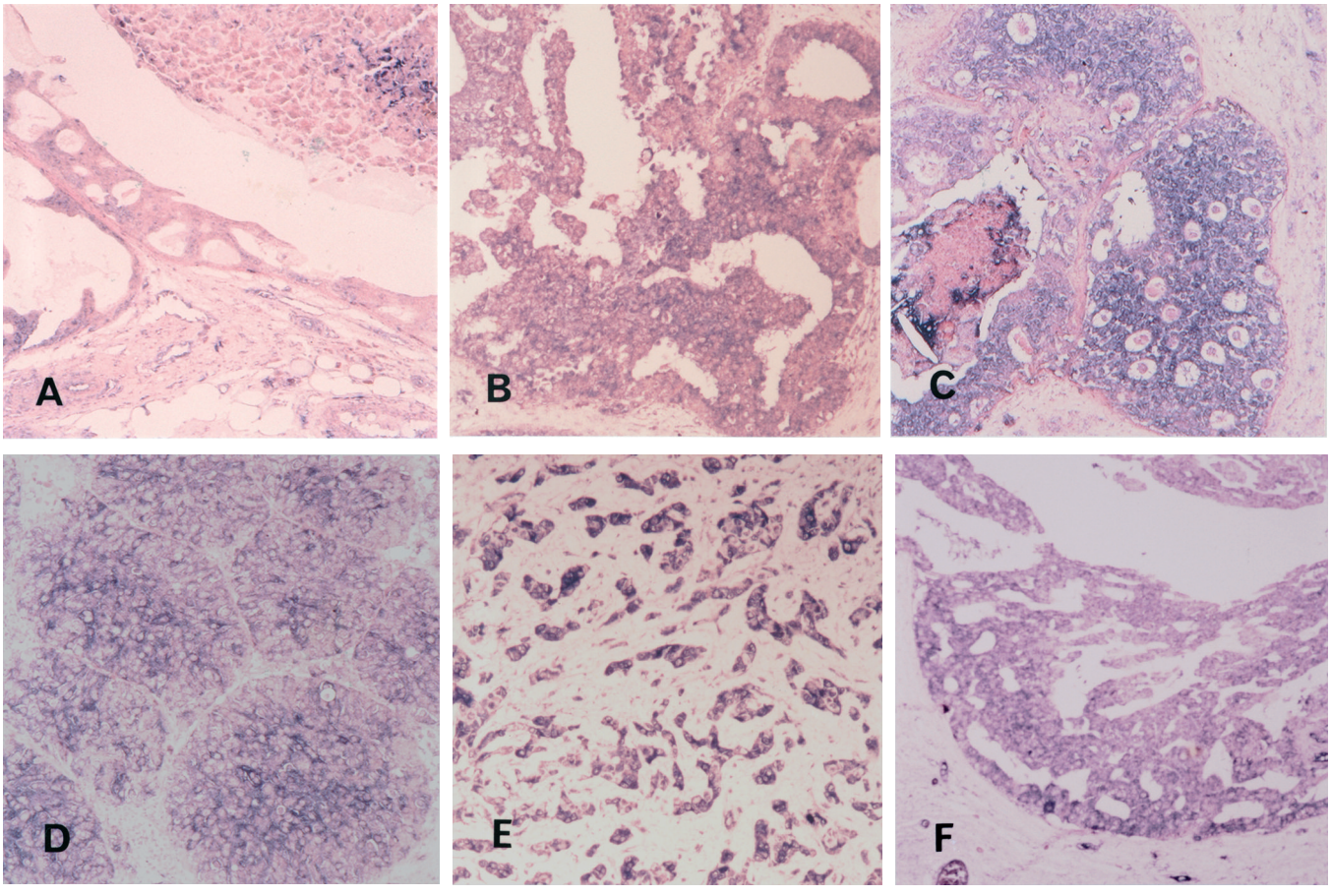

Fig. 1. Immunohistochemical studies of Ets-1: (Note: changed to colon instead of semicolon) Breast carcinoma of low papillary type (**A**, staining category; -, x 150) did not express Ets-1 protein. Positive staining for Ets-1 is shown in cytoplasm of carcinoma cells of high papillary type (**B**; +, x150), Cribriform type (**C**; ++, x 150), solid type (**D**; +, x 150) and carcinoma with fibrous stroma type (**E**; ++, x 150) growth patterns. Using the in situ hybridization method, the expression of *ets-1* mRNA in the cytoplasm of carcinoma cells is demonstrated (**F**, x 150).

carcinoma tissues and one (MDA-MB231) of three human carcinoma cell lines expressed Ets-1 mRNA. ß-actin mRNA, a control to demonstrate the equivalent amounts of tissue RNA, was used for cDNA synthesis and was detected in all of the samples. And the sequence of RT-PCR products were completely homologous to the sequences of *ets-1* gene.

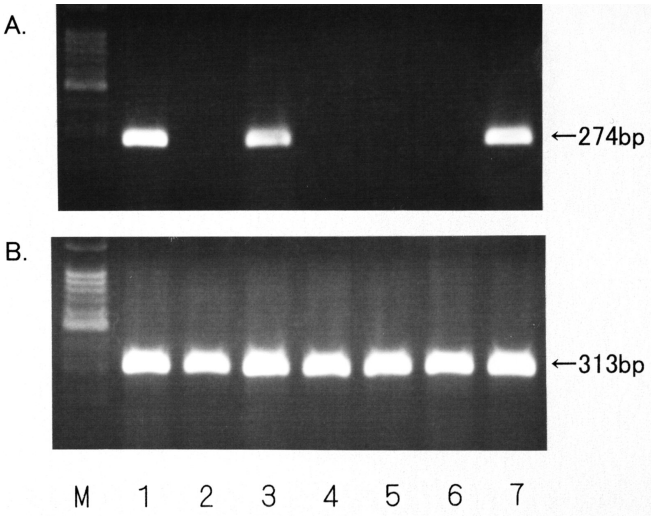

Fig. 2. RT-PCR analysis of *ets-1* mRNA expression in human breast tissue using the specific primer pairs predicted to amplify fragment size on the right. **A:** *ets-1* (274bp). **B:** ß-actin as internal control (313bp). Total RNA was prepared from human breast carcinoma tissue (lanes 1-3), one normal breast tissue specimen (lane 4) and three human breast carcinoma cell lines; MCF-7 (lane 5), SK-BR-3 (lane 6) and MDA-MB231 (lanes 7). Size marker (lane M) is 100 bp ladder markers.

Discussion

The *ets* family of genes is expressed during embryonic life, particularly in the intestine, and this expression is dramatically reduced in later fetal age (Vandenbunder et al., 1989; Kola et al., 1993). In our study, Ets-1 was not expressed in the normal breast epithelium nor in breast adenomas. However, 83.2% (114 of 137) of carcinomas showed overexpression of the Ets-1 protein. In non-invasive carcinomas, Ets-1 expression was not expressed, but was enhanced in

Table 2. Growth pattern of invasive ductal carcinoma and immunohistochemical Ets-1 staining (246 areas/115 cases).

GROWTH PATTERN	N	Ets-1 EXPRESSION		
		-	+	++
Clinging	18	16 (88.9%)	2 (11.1%)	0 (0.0%)*
Low papillary	24	18 (75.0%)	5 (20.8%)	1 (4.2%)*
High papillary	15	3 (20.0%)	5 (33.3%)	7 (46.7%)
Cribriform	8	2 (25.0%)	3 (37.5%)	3 (37.5%)
Comedo	15	1 (6.7%)	3 (20.0%)	11 (73.3%)
Solid	40	5 (12.5%)	4 (10.0%)	31 (77.5%)**
Tubular	49	6 (12.2%)	19 (38.8%)	24 (49.0%)
Carcinoma with fibrous stroma	77	13 (16.9%)	7 (9.1%)	57 (74.0%)**
Total area	246	64 (26.0%)	48 (19.5%)	134 (54.5%)

*; p<0.0001, **; p<0.01

Table 3. Expression of Ets-1 in primary breast carcinoma with or without lymph node Metastasis (64 cases).

LYMPH NODE METASTASIS	N	Ets-1 EXPRESSION		
		-	+	++
Present	23	2 (8.7%)	3 (13.0%)	18 (78.3%)
Absent	42	7 (16.7%)	9 (21.4%)	26 (66.9%)

n.s.

Table 4. Relationship between Ets-1 expression and "t" classification in breast carcinoma (57 cases).

	N	Ets-1 EXPRESSION		
		-	+	++
t 1	24	8 (33.3%)	6 (25.0%)	10 (41.7%)
t 2	22	7 (31.8%)	3 (13.6%)	12 (54.5%)
t 3	10	0 (0.0%)	2 (20.0%)	8 (80.0%)
t 4	1	0 (0.0%)	0 (0.0%)	1 (100.0%)

n.s.

Table 5. Relationship between Ets-1 expression and TNM stage in breast carcinomas (37 cases).

TNM STAGE	N	Ets-1 EEXPRESSION		
		-	+	++
I	21	5 (23.8%)	4 (19.0%)	12 (57.1%)
II	6	1 (16.7%)	0 (0.0%)	5 (83.3%)
III	4	1 (25.0%)	2 (50.0%)	1 (25.0%)
IV	6	1 (16.7%)	0 (0.0%)	5 (83.3%)

n.s.

Table 6. Correlation between Ets-1 expression and cellular atypism in invasive ductal carcinoma (n=134).

BLOOM-RICHARDSON GRADING*	N	Ets-1 EXPRESSION		
		-	+	++
I	59	16 (27.1%)	14 (23.7%)	29 (49.2%)
II	49	3 (6.1%)	11 (5.8%)	35 (71.4%)
III	26	1 (3.8%)	6 (23.1%)	19 (73.1%)

*: p<0.01

invasive carcinomas (Table 1). Therefore, the degree of Ets-1 expression was correlated to the extent of breast carcinoma invasion. And the atypism of carcinoma was significantly correlated with Ets-1 expression in Table 6. Recently, Ets-1 has been reported as an independent prognostic marker for relapse-free survival in breast cancer (Span et al., 2002). The concept of multi-step carcinogenesis has been proposed for the development of breast carcinoma, and has been generally accepted (Farber, 1984; Fearon and Vogelstein, 1990). These results suggest that *ets-1* plays some role in the carcinogenesis of breast carcinoma in one of the multi-steps in carcinogenesis and in the development of invasive characteristics.

The product of *ets-1* is localized in the nucleus and binds to DNA (Pognonec et al., 1989). However, in other reports, the Ets-1 protein was detected in the cytoplasm (Fujiwara et al., 1988) or both in the cytoplasm and in the nucleus in human colorectal carcinoma cell lines (Koizumi et al., 1990). In this study, we showed immunohistochemically that Ets-1 protein was widely expressed in the cytoplasm of carcinoma cells as well as in the nucleus. We believe that Ets-1 protein is overproduced in the cytoplasm and bound to DNA in the nucleus of colorectal carcinoma cells. It was shown that Ets-1 protein regulates the gene expression of some cytokines and peptides such as Fos and Jun (Wasylyk et al., 1990), integrin (Rosen et al., 1994), stromelysin (Higashino et al., 1995), parathyroid hormone-related protein (Dittmer et al., 1994; Lindemann et al.,2003), u-PA and collagenase-1 (Vandenbunder et al., 1989; Nerlov et al., 1992). The expression of these substances has also been observed in colorectal carcinoma cells (Nomura et al., 1986; McDonnal et al., 1991; Fujita et al., 1992; Abdeen et al., 1995), and they may play important roles in tumor growth and progression.

It was necessary to determine whether Ets-1 is produced by mesenchymal cells or carcinoma cells. Ets-1 is thought to be involved not only in tumor invasion but in connective tissue remodelling (Liotta et al., 1991). We observed increased Ets-1 expression during vascular smooth muscle cell migration and/or proliferation induced by serum stimulation in vitro and by balloon injury *in vivo* (Hultgardh-Nilsson et al., 1996). Ets-1 is known to be expressed in stromal fibroblasts around carcinoma cells (Wernart et al., 1994; Bolon et al., 1995, Behrens et al., 2001). The expression of the Ets-1 gene has also been demonstrated in vascular sarcomas (Wernert et al., 1992) and astrocytomas (Amouyel et al., 1988). In this study, however, *ets-1* mRNA was detected in the breast carcinoma cells themselves, but not intensely in stromal fibroblasts, as shown by immunohistochemistry and in situ hybridization. There was no correlation between Ets-1 expression in stromal cell and clinicopathological factors. RT-PCR of the *ets-1* mRNA from one of three human carcinoma cell lines confirmed that it is produced in the carcinoma cells themselves, as we previously reported in the carcinoma cells of the stomach, pancreas, thyroid, brain and colon (Nakayama et al., 1996, 1999, 2001; Ito et al., 1998; Kitange et al., 19991). And some papers supported our results of RT-PCR (Barrett et al.,2002; Lindemann et al., 2003). These findings suggest that Ets-1 is produced by carcinoma cells and may play a critical role in the progression of breast carcinoma cells.

Non-invasive ductal carcinoma may have a variety of growth patterns including micropapillary, papillary, solid, comedo, or cribriform in pure form or in combination (Rosen and Oberman, 1993). Almost all cases of invasive carcinomas have an intraductal lesion.of carcinoma. In this study, we showed the differential expression of Ets-1 protein in accordance with the histological growth patterns in intraductal lesions. Our results supported the previous report that Ets-1 protein plays an important role in the regulation of cell differentiation (Lewin, 1991).

In conclusion, our results suggest that Ets-1 plays an important role in carcinogenesis and/or tumour growth of breast carcinoma. The overexpression of the *ets-1* proto-oncogene product may be one of the steps in the multi-step carcinogenesis of human breast carcinomas.

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