ORIGINAL ARTICLE

The Role and Regulatory Mechanism of 14-3-3 Sigma in Human Breast Cancer

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Purpose: 14-3-3 sigma (σ) is considered to be an important tumor suppressor and decreased expression of the same has been reported in many malignant tumors by hypermethylation at its promoter or ubiquitin-mediated proteolysis by estrogen-responsive ring finger protein (Efp). In this study, we investigated the significance of 14-3-3 σ expression in human breast cancer and its regulatory mechanism. **Methods:** Efp was silenced using small interfering RNA (siRNA) in the MCF-7 breast cancer cell line in order to examine its influence on the level of 14-3-3 σ protein. The methylation status of the 14-3-3 σ promoter was also evaluated by methylation-specific polymerase chain reaction (PCR). The expression of Efp and 14-3-3 σ in 220 human breast carcinoma tissues was assessed by immunohistochemistry. Other clinicopathological parameters were also evaluated. **Results:** Silencing Efp in the MCF-7 breast cancer cell line resulted in increased expression of 14-3-3 σ. The Efp-positive human breast cancers were more frequently 14-3-3 σ-negative (60.5% vs. 39.5%). Hypermethylation of 14-3-3 σ was common (64.9%) and had an inverse association with 14-3-3 σ positivity ($p = 0.072$). Positive 14-3-3 σ expression was significantly correlated with poor prognosis: disease-free survival ($p = 0.008$) and disease-specific survival ($p = 0.009$). **Conclusion:** Our data suggests that in human breast cancer, the regulation of 14-3-3 σ may involve two mechanisms: ubiquitin-mediated proteolysis by Efp and downregulation by hypermethylation. However, the inactivation of 14-3-3 σ is probably achieved mainly by hypermethylation. Interestingly, 14-3-3 σ turned out to be a very significant poor prognostic indicator, which is in contrast to its previously known function as a tumor suppressor, suggesting a different role of 14-3-3 σ in breast cancer.

Key Words: *Breast neoplasms, Estrogen-responsive finger protein, Methylation, SFN protein*

INTRODUCTION

Breast cancer is one of the most common cancers in women and a major cancer death in women. The incidence of breast cancer in Korea continues to rise year by year, and its clinical characteristics are becoming closer to those observed in Western countries [1].

Among the many genes that have been known to play important roles in breast carcinogenesis, 14-3-3 sigma (σ) has been directly implicated in the tumorigenesis of breast cancer [2]. 14-3-3 σ is a *p53*-dependent, negative regulator of the cell cycle and contributes to G2/M arrest [2] by inhibiting the formation of the Cdc2-cyclin B1 complex [2,3]. It can also stabilize p53 expression by blocking *MDM2*-mediated *p53* ubiquitination [4]. 14-3-3 σ inactivation has been reported in various types of cancers, such as lung, prostate, and vulvar and oral squamous cell carcinoma [5-7]. This suggests that 14-3-3 σ acts as a tumor suppressor and its inactivation contributes to tumorigenesis by promoting cell cycle progression. Studies have shown that 14-3-3 σ is selectively inactivated by epigenetic silencing [5,6,8]. In fact, loss of 14-3-3 σ by hypermethylation of its promoter is reported to be one of the most consistent molecular alterations discovered in breast cancer so far [9,10]. Recently, a few studies showed that 14-3-3 σ was also downregulated through ubiquitin-mediated proteolysis by estrogen-responsive finger protein (Efp), an estrogen-dependent E3 ubiquitin ligase [11]. Efp is a downstream target of estrogen receptor and mediates estrogen-induced cell growth, which implies possible involvement in the development of

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human breast cancers. However, the exact mechanism of how 14-3-3 σ is inactivated and its association with Efp in breast cancer has not been clearly elucidated. Studies on Efp and 14-3-3 σ in human breast cancer are still very limited, and the biological significance remains unclear.

Therefore, in this study we investigated the regulatory mechanism of 14-3-3 σ in breast cancer. We also evaluated the expression pattern of 14-3-3 σ using clinicopathological features of human breast cancer to deepen our understanding of the role of 14-3-3 σ in breast cancer tumorigenesis and biology. In order to do this, we investigated whether silencing Efp could affect 14-3-3 σ expression *in vitro*. We also examined the expression of Efp and 14-3-3 σ in 220 cases of human breast cancer tissues using immunohistochemistry. To investigate the epigenetic silencing of 14-3-3 σ by methylation, we examined the methylation status of 14-3-3 σ promoter sites in breast cancer tissues by methylation-specific polymerase chain reaction (PCR). We subsequently correlated these findings with various clinicopathological variables, including the clinical outcome of the patients.

METHODS

Patients and tissue specimens

We analyzed the breast cancer registry program of the Department of Surgery, Gangnam Severance Hospital, Yonsei University College of Medicine. Two hundred twenty cases of pure invasive ductal carcinoma ('NOS ductal' according to World Health Organization classification) were obtained from female patients who underwent surgery between 1996 and 2001 in Gangnam Severance Hospital, Yonsei University College of Medicine. Inclusion criteria were cases of pure invasive ductal carcinoma without evidence of remote metastasis. Exclusion criteria were cases of specific histological types (medullary, mucinous, papillary, tubular, lobular, etc.), inflammatory breast cancer, bilateral cases, and those in which tissue samples and clinical data, including the follow-up results, were unavailable. The median follow-up period was 72 months.

Paraffin-embedded tissue sections were selected from the pathological files of the Department of Pathology. As mentioned above, we obtained baseline data and follow-up results, including the clinicopathological characteristics. The histologic grade was determined using a Nottingham combined histologic grade (Elston-Ellis modification of Scarff-Bloom-Richardson grading system) [12]: G1 (well-differentiated), G2 (moderately-differentiated), and G3 (poorly-differentiated). Using a reverse Black method, the nuclear grade was determined to be grade 1 (well-differentiated), grade 2 (moderately-differentiated), or grade 3 (poorly-differentiated). Disease-spe-

cific survival was defined as the time that elapsed from the date of surgery to the date of death due to breast cancer. The follow-up period was defined as the time that elapsed between surgery and death or the last available date of follow-up. In cases in which there was no outpatient record during the most recent 1-year period, we determined the death of the corresponding cases by making an inquiry, based on the Korean social security number, to the Korea National Statistical Office, the district office, and/or the police station.

This study was approved by Institutional Review Board of the Gangnam Severance Hospital, Yonsei University College of Medicine (approval number: 3-2014-0192).

### Tissue microarray and immunohistochemical evaluation

A tissue microarray was constructed by ISU Abxis Co. (Seoul, Korea) from the paraffin-embedded blocks of 220 breast cancer cases. Briefly, all cases were histologically reviewed and representative tumor areas were marked in the corresponding paraffin blocks. Two selected cylinders (2 mm in diameter) from two different tumor areas were included for each case. Normal control tissues were obtained from surrounding normal breast tissue.

Immunohistochemical staining was done for estrogen receptor (ER), progesterone receptor (PR), c-erbB-2, p53, Efp, and 14-3-3 σ. The tissue microarray slides were deparaffinized by xylene two times and treated with 100%, 95%, and 80% ethanol, and then hydrated with distilled water. These slides were then treated with citric acid buffer (pH 6.0) and boiled in a microwave for 20 minutes for antigen retrieval. Intrinsic peroxidase activity was blocked using a solution containing 3% hydrogen peroxide. Next, the slides were rinsed with tris-buffer solution (TBS) and incubated with the primary antibodies for ER, PR, c-erbB-2, p53, Efp, and 14-3-3 σ overnight at 4°C (Table 1). The slides were rinsed with TBS, and then the Vectastain universal elite ABC kit (Vector Laboratories, Burlingame, USA) was applied. The color development was

Table 1. Primary antibodies for the immunohistochemical staining

Antibody	Manufacturer	Isotype	Dilution
Efp	BD Bioscience (Franklin Lakes, USA)	Mouse monoclonal	1:300
14-3-3 σ	IBL (Takasaki, Japan)	Rabbit polyclonal	1:100
ER	Thermo Fisher Scientific (Fremont, USA)	Mouse monoclonal	1:100
PR	Invitrogen (Carlsbad, USA)	Mouse monoclonal	1:100
c-erbB-2	Thermo Fisher Scientific (Fremont, USA)	Mouse monoclonal	1:100
p53	Invitrogen (Carlsbad, USA)	Mouse monoclonal	1:100

Efp = estrogen-responsive ring finger protein; ER = estrogen receptor; PR = progesterone receptor.
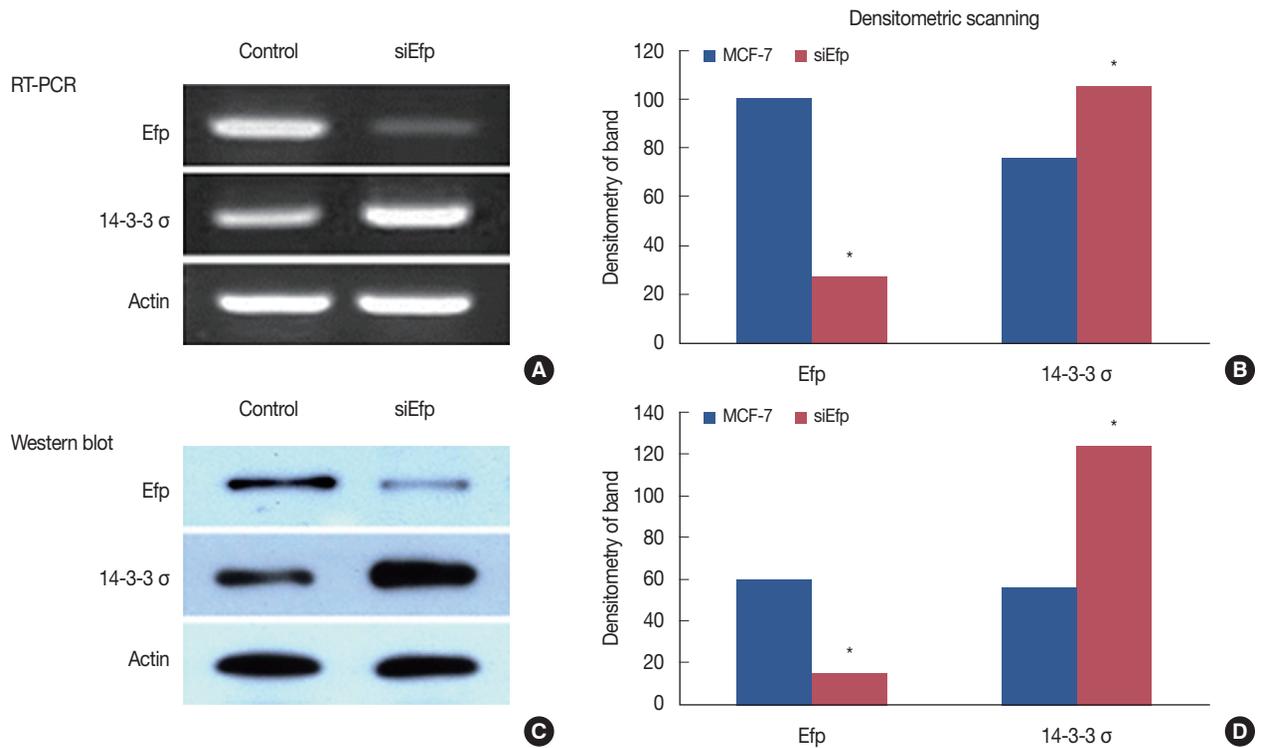
Figure 1. The increased expression of 14-3-3 σ by knocks down of estrogen-responsive ring finger protein (Efp) using small interfering RNA (siRNA). siEfp was transfected in human MCF-7 breast cancer cells. After transfection, total RNA and whole cell lysates were prepared and subjected to reverse transcription polymerase chain reaction (A) and Western blot (C). Relative levels of mRNA and protein expression were determined by densitometric scanning of the bands (B, D). The siEfp transfection resulted in increased levels of 14-3-3 σ mRNA and protein.
*Corresponds to $p < 0.05$.

done with NovaRed (Vector Laboratories), and then the slides were counterstained with hematoxylin. The slides were finally mounted and observed under a microscope.

The immunohistochemical results were interpreted by two experienced pathologists. A positive reaction was determined by the ratio of cancer cells stained by the primary antibody to the total cancer cells in each slide (Figure 1). For ER and PR, a positive diagnosis was determined when intensely stained nuclei were seen in more than 10% of the total cancer cells [13]. For c-erbB-2, a positive reaction was determined when the cell membranes were evenly stained (score 2 or 3 by the four-grade system) [14,15]. For p53 protein, a positive reaction was determined when intensely stained nuclei were seen in more than 5% of the total cancer cells. Efp and 14-3-3 σ expression was graded as 0 (if none of the tumor cells were positive), 1 (if up to 10% of the tumor cells were positive), 2 (if 10% to 50% of the tumor cells were positive), and 3 (if more than 50% of the tumor cells were positive) by the two pathologists independently. The final diagnosis was determined as positive when the sum of the two independent scores was equal or more than five [16,17].

Methylation-specific PCR

We analyzed the methylation status of 14-3-3 σ in randomly selected samples from 111 cases. The methylation status of the samples was investigated by methylation-specific PCR, as described previously [9,18]. Briefly, genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, USA) from the formalin-fixed, paraffin-embedded tumor tissue after manual microdissection under a microscope to obtain a sample that consisted of more than 70% tumor cells. One microgram of genomic DNA was then treated with sodium bisulfite using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, USA) and was analyzed by methylation-specific PCR (MSP) using a primer set that covered CG dinucleotide numbers 3, 4, 8, and 9. Primers specific for methylated DNA (59-TGGTAGTTTTTATGAAAGGCGTC-39 [sense] and 59-CCTCTAACCGCCCACCACG-39 [antisense]), and primers specific for unmethylated DNA (59-ATGGTAGTTTT-TATGAAAGGTGTT-39 [sense] and 59-CCCTCTAAC-CACCCACCACA-39 [antisense]) yielded a 105 to 107-bp PCR product. The PCR conditions were as follows: 1 cycle of 95°C for 5 minutes; 31 cycles of 95°C for 45 seconds, 56°C for 30 seconds for the unmethylated primer, 60°C for 30 seconds

for the methylated primer, and 72°C for 30 seconds; and 1 cycle of 72°C for 4 minutes.

The MCF-7 cell line, previously reported to be unmethylated at the 14-3-3 σ promoter [9], was used as the unmethylated control. Commercially available methylated DNA (Universal Methylated Human DNA Standard; Zymo Research) was used as the methylated control. Methylated bands on MSP which were more dense than the corresponding unmethylated bands or the ones as dense as the universally methylated DNA control were determined as hypermethylated.

Small interfering RNA silencing of Efp

For the transfection procedure, MCF-7 cells were grown to 60% confluence, and Efp small interfering RNAs (siRNAs) (5'-GGTGGAGCAGCTACAACAATT-3') were transfected using the RNAiMax reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, the RNAiMax reagent was incubated with serum-free medium for 10 minutes. Subsequently, a mixture of the siRNA was added. After incubation for 15 minutes at room temperature, the mixture was diluted with medium and added to each well. The final concentration of siRNAs in each well was 100 nM. After culturing for 40 hours, cells were washed, resuspended in new culture media for reverse transcription (RT)-PCR and Western blot. Total RNA and whole cell lysates was prepared and subjected to RT-RCR and Western blot. Relative levels of mRNA and protein expression were determined by densitometry of the bands.

RT-PCR analysis and Western blot

Confluent MCF-7 cell monolayers were washed with PBS. Total RNA (12 μg) was isolated using TRIzol reagent. One milliliter of TRIzol reagent (Invitrogen) per well was added to cells in 6-well plates, and total RNA was extracted using the manufacturer's protocol (Invitrogen). RT was performed using oligo (dT) primers and superscript reagent (Invitrogen). Five micrograms of RNA were used for first strand cDNA synthesis using oligo (dT) in a final volume of 20 μL. Seven microliters of the cDNA mixture was used to amplify mRNA for *Efp*, *14-3-3 σ*, and 18s ribosomal RNA (*18s rRNA*) as a loading control by PCR. *Efp*, *14-3-3 σ*, and *18s rRNA* were amplified using the following primer sets: *Efp* (forward 5'-AACATCTCTCAAGGC-CAAGGT-3' and reverse 5'-AGATGCCTACCCCACAGAA-GT-3'), *14-3-3 σ* (forward 5'-GTGTGTCCCCAGAGCATGG-3' and reverse 5'-ACCTTCTCCCGGTACTCACG-3'), and 1*8s rRNA* (forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'-GCTGGAATTACCGCGGCT-3'). The RT product (7 μL) was amplified in a 20-μL volume containing 10 pmol of primers and 2.5 units of Taq DNA polymerase. Reaction conditions were as follows: 95°C for 1 minute, 60°C for 1 minute,

72°C for 1 minute for 33 cycles, and then 72°C for 7 minutes. The PCR products were resolved on a 1.2% agarose gel and identified by ethidium bromide staining. Normalization of *Efp* and *14-3-3 σ* expression was achieved by comparing the expression of *18s rRNA* for the corresponding sample.

For Western blot analysis, proteins were isolated from MCF-7 and siEfp-treated MCF-7 cells and lysed in solubilizing buffer (1×PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors, PMSF, aprotinin, and sodium orthovandate). Equal amounts of protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, USA). The membrane was blocked with 5% nonfat milk in TBS containing 0.05% Tween, and then incubated with antibody mouse anti-Efp (1:5,000; BD Bioscience, Franklin Lakes, USA), rabbit anti-14-3-3 σ (1:1,000; IBL Co., Ltd., Takasaki, Japan), and mouse antiactin (1:5,000; Santa Cruz Biotechnology, Santa Cruz, USA). The membrane was then incubated with the secondary antibody and thoroughly washed. Immunoreactive bands were visualized with a Super-Signal (Thermo-Fisher Scientific, Waltham, USA).

Statistical analyses

All statistical analyses were done using SPSS version 13.0 for Windows (SPSS Inc., Chicago, USA). To evaluate correlations between Efp, and 14-3-3 σ expression and clinicopathologic parameters, data were cross-tabulation (chi-square test/2×2 table; Pearson or Fisher exact test) was done. Univariate analysis with patient survival was evaluated using life tables constructed from survival data with Kaplan-Meier plots. Comparisons of the different groups were done with the log-rank test. The end point in the present study was disease-specific survival ranging from the date of surgery until the date of breast cancer-related death or, if no information was documented, until the date of last follow up information (=censored). Multivariate survival analysis was carried out on samples where all clinical parameters were available using the Cox proportional hazard model to evaluate the independent power of each variable. Statistical significance was set at *p*-value <0.05 (95% level of confidence).

RESULTS

Demographic data of the subjects

Basic clinical and demographic data are summarized in Table 2. Mean patient age was 47.1±9.9 years old. 66.3% (146/220) are positive to at least one of the ER, PR, and HER2 (receptor-positive group). ER-positive cases, 19.5% (43/220) were HER2-positive cases, and all receptor groups were negative in 26.4% (58/220), i.e., the triple-negative group. The triple-nega-

Table 2. Baseline clinicopathologic characteristics of breast cancer patients examined

| Characteristic | Subgroup* | | | p-value§ |
	Receptor (+)† (n=129) No. (%)	Triple (-)‡ (n=58) No. (%)	HER2 only (+) (n=17) No. (%)	
Age (yr)‖	46.9±10.1	47.0±10.1	49.1±8.1	0.939¶
Follow-up (mo)**	72 (1–144)	73 (5–135)	83 (7–135)	0.648¶
Tumor size (cm)				
≤2	54 (74.0)	18 (24.7)	1 (1.4)	0.010
>2	75 (57.3)	40 (30.5)	16 (12.2)	
Axillary node				
Negative	59 (58.4)	33 (32.7)	9 (8.9)	0.353
Positive	70 (68.0)	25 (24.3)	8 (7.8)	
Stage				
I	33 (71.7)	13 (28.3)	0	0.178
II	70 (61.4)	33 (28.9)	11 (9.6)	
III	26 (59.1)	12 (27.3)	6 (13.6)	
Histologic grade				
I	36 (87.8)	3 (7.3)	2 (4.9)	0.001
II & III	93 (57.4)	54 (33.3)	15 (9.3)	
Nuclear grade				
1 & 2	84 (79.2)	17 (16.0)	5 (4.7)	<0.001
3	44 (45.8)	40 (41.7)	12 (12.5)	
ER				
Negative	43 (36.4)	58 (49.2)	17 (14.4)	-
Positive	86 (100.0)	0	0	
PR				
Negative	18 (19.4)	58 (62.4)	17 (18.3)	-
Positive	111 (100.0)	0	0	
HER2				
Negative	103 (64.0)	58 (36.0)	0	-
Positive	26 (60.5)	0	17 (39.5)	
p53				
Negative	98 (75.4)	26 (20.0)	6 (4.6)	<0.001
Positive	27 (39.1)	31 (44.9)	11 (15.9)	

ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2.
*Excluding unknown cases; †Receptor-positive group: positive to at least one of the ER, PR, and HER2; ‡Triple-negative group: negative to all of the ER, PR, and HER2; §p-value from chi-square test (2×2 Pearson or Fisher exact test; except TNM stage); ‖Mean±SD; ¶p-value of age and follow-up duration: from independent sample t-test; **Median (range).

tive group frequently showed high nuclear grade, high histologic grade, and p53 positive cells.

Efp silencing upregulates 14-3-3 σ expression *in vitro*

Efp silencing was performed by transfection of siEfp in the human breast cancer cell line, MCF-7. Transfection of siRNA resulted in the downregulation of *Efp* mRNA levels by RT-PCR and increased the expression of 14-3-3 σ protein levels by Western blot (Figure 1). All three independent experiments gave similar results. This clearly shows that Efp is associated with decreased expression of 14-3-3 σ, and silencing of Efp can

Table 3. Correlation between Efp and 14-3-3 σ expression

| | No. | Efp | | p-value* |
		Negative No. (%)	Positive No. (%)	
Total 14-3-3 σ				<0.001
Negative	172	146 (84.9)	26 (15.1)	
Positive	45	28 (62.2)	17 (37.8)	
Total†	217	174	43	
Hormonal receptor-positive 14-3-3 σ				<0.001
Negative	108	87 (80.6)	21 (19.4)	
Positive	20	8 (40.0)	12 (60.0)	
Total†	128	95	33	
Triple-negative 14-3-3 σ				0.067
Negative	38	36 (94.7)	2 (5.3)	
Positive	19	15 (78.9)	4 (21.2)	
Total†	57	51	6	

Efp=estrogen-responsive ring finger protein.
*p-value from chi-square test (2×2 Pearson); †Total number of the group (excluding unknown cases).

increase the expression of 14-3-3 σ.

Expression of Efp and 14-3-3 σ in subtypes of breast cancer

Both Efp and 14-3-3 σ showed cytoplasmic staining patterns. 14-3-3 σ was strongly expressed in the myoepithelial cells. In the normal breast epithelial cells, Efp was only weakly positive. About two-thirds of the total tumors (146/217 cases, 67.3%) were negative for both Efp and 14-3-3 σ. About one-third of the total cases (71/217 cases, 32.7%) were positive for at least one of the two genes. Fifty-four of 217 cases (24.9%) were positive for only one of the two genes, while only 17 of 217 cases (7.8%) showed positive staining for both. For the cases when at least one gene was positive, the majority (54/71 cases, 76%) demonstrated a mutually exclusive pattern of positivity, i.e., positive to only one of the two proteins (Table 3, Figure 2). Only 17 cases (23.9%) of the positive 71 cases were positive for both Efp and 14-3-3 σ. The Efp-positive cases were more frequently negative (26/43 cases, 60.5%) for 14-3-3 σ than positive (17/43 cases, 39.5%).

The majority (36/43 cases, 85.7%) of the Efp-positive tumors were receptor-positive (p=0.019). Triple-negative tumors were more frequently positive for 14-3-3 σ (19/58 cases) than receptor-positive tumors (26/145 cases) (p=0.006) (Table 4).

Correlation between Efp, 14-3-3 σ expression, and clinicopathological variables

Correlation between Efp, 14-3-3 σ expression status, and other clinicopathological parameters of patients are summarized in Table 5. Notably, Efp was negatively correlated with axillary lymph node metastasis (p=0.021) or positive p53 ex-
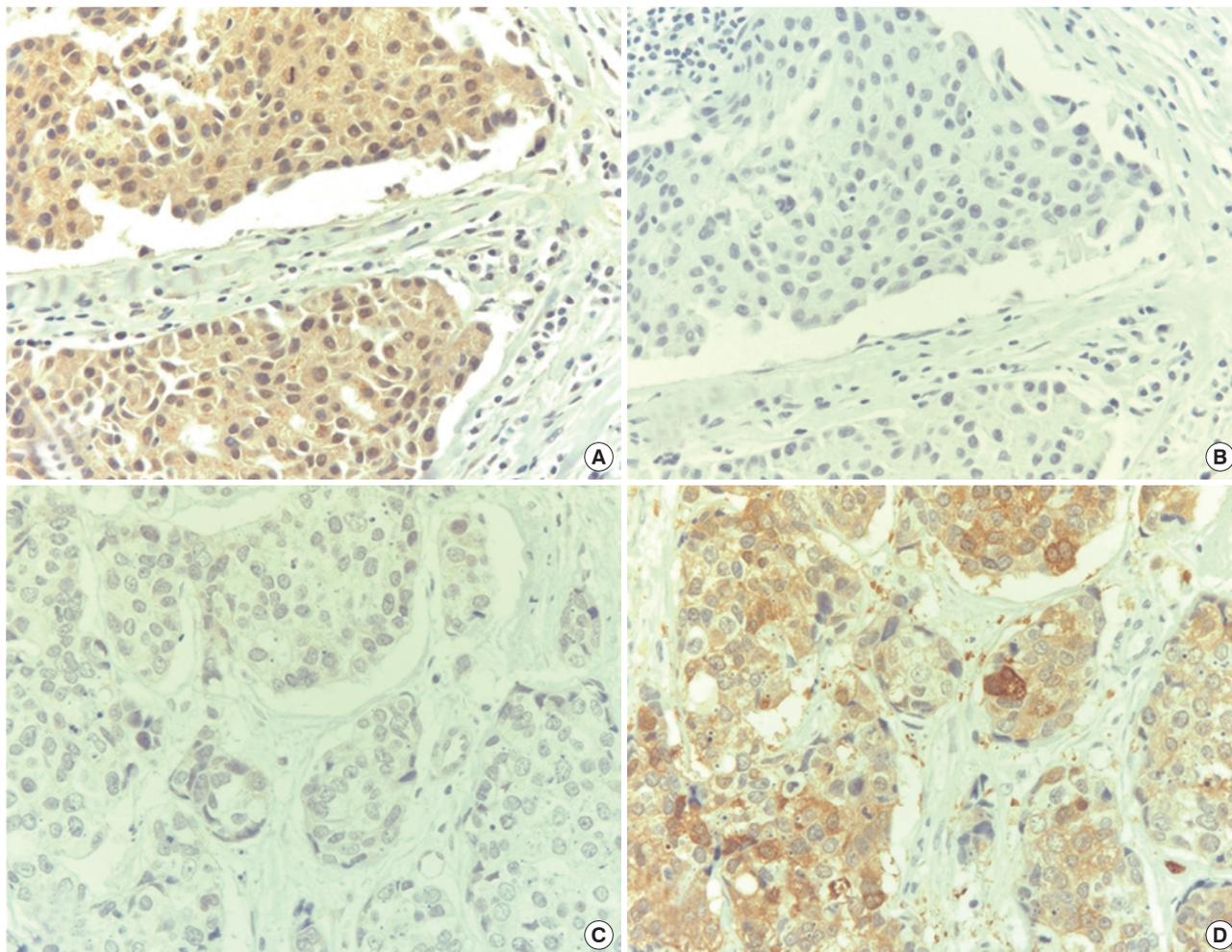
Figure 2. Expression of estrogen-responsive ring finger protein (Efp) and 14-3-3 σ in breast cancer. An estrogen receptor-positive breast cancer was positive to Efp (A) and negative to 14-3-3 σ (B) while a triple-negative breast cancer was negative to Efp (C) and positive to 14-3-3 σ (D) (NovaRed with Hematoxylin counterstain, ×400).

Table 4. Expression of Efp and 14-3-3 σ according to the subtype of breast cancer by receptor expression

	Receptor-positive (n=128)* No. (%)	Triple-negative (n=57)* No. (%)	p-value†
Efp			0.019
Negative	95 (74.2)	51 (89.5)	
Positive	33 (25.8)	6 (10.5)	
14-3-3 σ			0.006
Negative	108 (84.4)	38 (66.7)	
Positive	20 (15.6)	19 (33.3)	

Efp = estrogen-responsive ring finger protein.
*Excluding unknown cases; †p-value from chi-square test.

pression ($p = 0.009$). There was an increase in the proportion of Efp-negative cases as the histologic grade and nuclear grade increased, but it was not statistically significant. No other clin-icopathological variables, such as tumor size, stage, histologic grade, nuclear grade, ER, or c-erbB-2 appeared to be significantly related to Efp expression.

Positive 14-3-3 σ expression was significantly correlated with high histologic grade, high nuclear grade, and positive p53 ($p = 0.012$, $p = 0.033$, and $p = 0.001$, respectively). There was a statistically significant inverse correlation between 14-3-3 σ expression and both ER-positivity ($p = 0.020$) and PR-positivity ($p = 0.032$), which is in accordance with our previous finding that the triple-negative group was more frequently 14-3-3 σ-positive than the receptor-positive group.

MSP for the 14-3-3 σ promoter

Methylation status of the 14-3-3 σ promoter was evaluated with MSP, as shown in Figure 3. Of the 111 cases examined, 72 cases (64.9%) were hypermethylated by MSP (Table 6). The

Table 5. The correlations between the Efp, 14-3-3 σ, and other clinicopathological parameters

Clinicopathologic parameters	No.*	Efp			14-3-3 σ		
		Negative No. (%)	Positive No. (%)	p-value[†]	Negative No. (%)	Positive No. (%)	p-value[†]
Tumor size (cm)				0.236			0.630
≤2	79	60 (75.9)	19 (24.1)		64 (81.0)	15 (19.0)	
>2	138	114 (82.6)	24 (17.4)		108 (78.3)	30 (21.7)	
Axillary node				0.021			0.346
Negative	107	79 (73.8)	28 (26.2)		82 (76.6)	25 (23.4)	
Positive	110	95 (86.4)	15 (13.6)		90 (81.8)	20 (18.2)	
Stage				0.196			0.692
I	48	36 (75.0)	12 (25.0)		39 (81.3)	9 (18.8)	
II	123	97 (78.9)	26 (21.1)		95 (77.2)	28 (22.8)	
III	46	41 (89.1)	5 (10.9)		38 (82.6)	8 (17.4)	
Histologic grade				0.061			0.012
I	43	30 (69.8)	13 (30.2)		40 (93.0)	3 (7.0)	
II & III	172	142 (82.6)	30 (17.4)		130 (75.6)	42 (24.4)	
Nuclear grade				0.052			0.033
1 & 2	111	83 (74.8)	28 (25.2)		94 (84.7)	17 (15.3)	
3	103	88 (85.4)	15 (14.6)		75 (72.8)	28 (27.2)	
ER				0.086			0.020
Negative	126	106 (84.1)	20 (15.9)		93 (73.8)	33 (26.2)	
Positive	91	68 (74.7)	23 (25.3)		79 (86.8)	12 (13.2)	
PR				0.022			0.032
Negative	99	86 (86.9)	13 (13.1)		72 (72.7)	27 (27.3)	
Positive	117	87 (74.4)	30 (25.6)		99 (84.6)	18 (15.4)	
HER2				0.965			0.544
Negative	160	127 (79.4)	33 (20.6)		126 (78.8)	34 (21.3)	
Positive	43	34 (79.1)	9 (20.9)		32 (74.4)	11 (25.6)	
p53				0.009			0.001
Negative	130	97 (74.6)	33 (25.4)		110 (84.6)	20 (15.4)	
Positive	70	63 (90.0)	7 (10.0)		45 (64.3)	25 (35.7)	

Efp = estrogen-responsive ring finger protein; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2.
*Total number of the group (excluding unknown cases); [†]p-value from chi-square test (2×2 Pearson; except TNM stage).
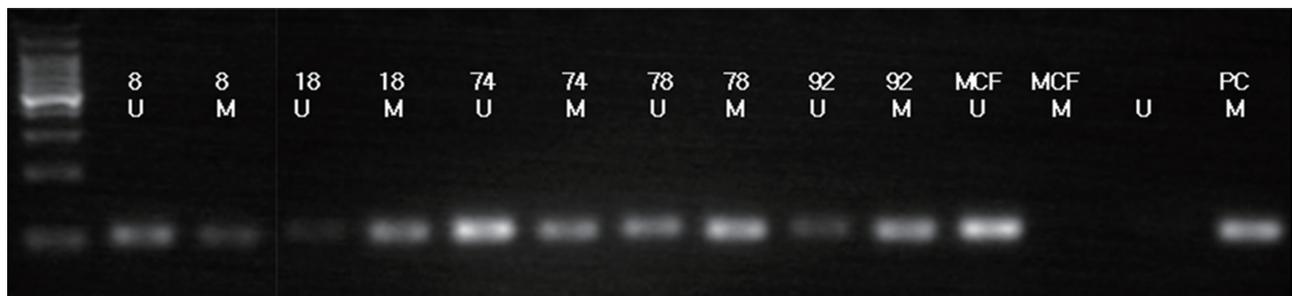
Figure 3. Methylation-specific polymerase chain reaction (MSP) in breast cancer. Methylated and unmethylated bands on MSP. Methylated bands which are brighter than the corresponding unmethylated band or the ones as dense as the universally methylated DNA control were determined as hyper-methylated.
8, 18, 74, 78, and 92 = case numbers; MCF = MCF-7 cell line; PC = universally methylated DNA; U = unmethylated band; M = methylated band.

hormone receptor-positive group was more frequently hyper-methylated (45/71 cases, 63.4%) than the triple-negative group (17/28 cases, 60.7%), but the difference was not statistically significant (Table 6). The 14-3-3 σ positive results by immunohistochemistry were negatively associated with the hypermethylation of the 14-3-3 σ promoter, but only showed marginal significance ($p = 0.072$) (Table 6).

Correlation between Efp, 14-3-3 σ expression, and clinical outcome

Univariate analysis of various clinicopathological factors that may be correlated with the expression of Efp and 14-3-3

Table 6. 14-3-3 σ expression and its methylation status in breast cancer

| Methylation status | No.* | 14-3-3 σ | | p-value† |
		Negative No. (%)	Positive No. (%)	
Overall case				0.072
Methylation				
Unmethylated	38	26 (68.4)	12 (31.6)	
Methylated	72	60 (83.3)	12 (16.7)	
Receptor-positive group				0.182
Methylation				
Unmethylated	25	19 (76.0)	6 (24.0)	
Methylated	43	38 (88.4)	5 (11.6)	
Triple-negative group				0.094
Methylation				
Unmethylated	11	5 (45.5)	6 (54.5)	
Methylated	17	13 (76.5)	4 (23.5)	

*Total number of the group (excluding unknown cases); †p-value from chi-square test (2×2 Pearson).

σ showed that 14-3-3 σ expression was significantly associated with an increased risk of recurrence and disease-related death ($p = 0.011$ and $p = 0.022$), while Efp was not. Other traditionally well-known prognostic factors, such as tumor size, axillary lymph node status, TNM stage, and histologic grade, were also significant prognostic variables for survival (data not shown).

Multivariate analysis revealed that the axillary lymph node status ($p = 0.028$) and 14-3-3 σ expression ($p = 0.008$) were the only two independent prognostic factors for recurrence with relative risks over 1.0, whereas tumor size, TNM stage, and histologic grade were not significant (Table 7). Only TNM stage ($p = 0.015$) and 14-3-3 σ expression ($p = 0.009$) were independent prognostic factors in cancer-related deaths, but other factors including Efp were not significant prognostic indicators (Table 7).

In both the univariate and multivariate analysis, 14-3-3 σ appeared to be the single most powerful prognostic indicator. Survival curves based on 14-3-3 σ expression also demonstrated a significant correlation between positive 14-3-3 σ expression and adverse clinical outcome of the patients (Figure 4A and 4B). This correlation appeared to be more pronounced in the triple-negative group (Figure 4E and 4F) than in the receptor-positive group (Figure 4C and 4D). In the receptor-positive group, the difference between the 14-3-3 σ positive group and negative group was not statistically significant.

DISCUSSION

The role of the 14-3-3 σ as a tumor suppressor has been shown by its known function as a negative cell cycle regulator, a G2/M checkpoint by inhibiting the formation of the Cdc2-

Table 7. Multivariate analysis of recurrence and cancer-related death

|	Coefficient	SE	RR (95% CI)	p-value*
For recurrence				
Tumor size (cm)				
≤2	-	-	1.0	
>2	0.854	0.493	2.350 (0.894–6.172)	0.083
Axillary node				
Negative	-	-	1.0	
Positive	1.071	0.487	2.917 (1.123–7.576)	0.028
TNM stage				0.077
I	-	-	1.0	
II	-0.066	0.952	0.936 (0.145–6.051)	0.945
III	0.772	1.012	2.165 (0.298–15.743)	0.445
Histologic grade				
I	-	-	1.0	
II & III	0.929	0.746	2.531 (0.586–10.926)	0.213
14-3-3 σ				
Negative	-	-	1.0	
Positive	0.937	0.351	2.551 (1.283–5.075)	0.008
For cancer-related death				
Tumor size (cm)				
≤2	-	-	1.0	
>2	-0.912	0.634	2.489 (0.719–8.620)	0.150
Axillary node				
Negative	-	-	1.0	
Positive	0.449	0.586	1.567 (0.497–4.948)	0.443
TNM stage				0.015
I	-	-	1.0	
II	-0.279	1.046	0.756 (0.097–5.877)	0.789
III	1.144	1.133	3.139 (0.341–28.890)	0.313
Histologic grade				
I	-	-	1.0	
II & III	1.052	1.042	2.864 (0.372–22.071)	0.312
14-3-3 σ				
Negative	-	-	1.0	
Positive	1.116	0.428	3.502 (1.319–7.063)	0.009

Data were considered significant in the univariate analyses and were examined in the multivariate analyses. Relative risk (RR) less than 1.00 represent a decreased risk of death, whereas RR greater than 1.00 represent an increased risk of death.
SE = standard error; CI = confidence interval.
*p-values determined by a Cox proportional hazard model.

cyclin B1 complex [2,3], or by stabilizing p53 through blocking *MDM2*-mediated *p53* ubiquitination [4]. This has been supported by several observations of decreased expression of 14-3-3 σ in various malignant tumors, such as cancer of the stomach, prostate, lung, and oral cavity [5-8]. In breast cancer, 14-3-3 σ expression has also been shown to be downregulated [9,10,19]. The mechanism by which 14-3-3 σ is inactivated has not been completely elucidated yet, but epigenetic silencing by hypermethylation of the 14-3-3 σ gene is one of the commonly proposed mechanisms [5,8-10].

A previous study demonstrated that 14-3-3 σ expression was gradually decreased as a lesion progressed from benign to ma-
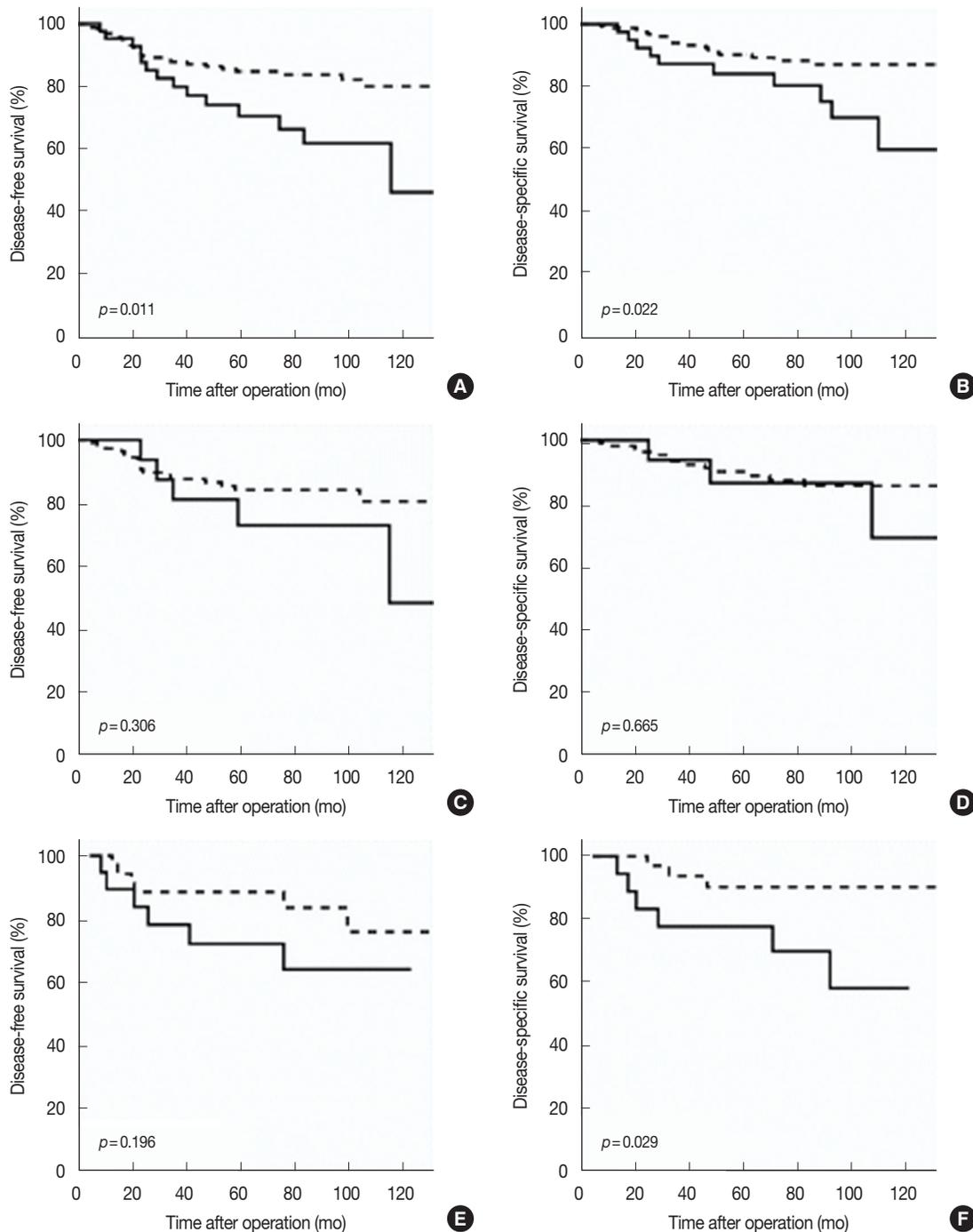
Figure 4. Disease-free survival and disease-specific survival curves according to 14-3-3 σ expression in each subgroup. (A, C, E) Disease-free survival; (B, D, F) Disease-specific survival. (A, B) Total cases: 14-3-3 σ expression was significantly associated with an increased risk of recurrence and disease-related death. (C, D) Receptor-positive group: 14-3-3 σ expression was associated with an increased risk of recurrence and disease-related death, but statistically insignificant. (E, F) Triple-negative group: 14-3-3 σ expression was not associated with an increased risk of recurrence. But 14-3-3 σ expression was significantly associated with an increased risk of disease-related death. Dashed line, 14-3-3 σ negative; linear line, 14-3-3 σ positive.

lignant with the loss of 14-3-3 σ expression in 8%, 35%, and 77% of usual ductal hyperplasia, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) lesions, respec-

tively, which indicates the role of 14-3-3 σ as a tumor suppressor in breast carcinogenesis [20]. Another recent study showed that the hypermethylation of 14-3-3 σ occurred at rates of zero

in hyperplasia without atypia, 38% in atypical hyperplasia, 83% in DCIS, and 96% in IDC [10]. These studies showing a gradual decrease in 14-3-3 σ expression and a corresponding increase of hypermethylation with progression of the breast lesions, and the fact that hypermethylation of the 14-3-3 σ gene was also found in some benign, and sometimes even apparently normal epithelia adjacent to cancer, suggest and support the idea that 14-3-3 σ inactivation by hypermethylation may be an early event in breast carcinogenesis [10,19].

On the other hand, it has also been reported that 14-3-3 σ is a primary target for proteolysis by Efp, and expression of the 14-3-3 σ protein is regulated by ubiquitin-mediated proteolysis by Efp *in vitro* [11,21]. Efp is a RING finger-dependent protein and functions as a ubiquitin-ligase (E3) that can ubiquitinate 14-3-3 σ [11,21]. Our experimental data suggested that the Efp may promote unlimited proliferation of breast cancer cells by accelerated destruction of 14-3-3 σ, a postulated cell cycle inhibitor. This is supported by another recent study in human breast cancer tissue which showed that immunoreactivity of 14-3-3 σ was inversely associated with Efp immunoreactivity [22].

In our study, we demonstrated that silencing Efp can result in increased expression of 14-3-3 σ in the MCF-7 breast cancer cell line. In human breast tissue, we found that the Efp-positive cases were more frequently 14-3-3 σ negative. This *in vitro* and *in vivo* counter-correlation between Efp and 14-3-3 σ possibly supports the Efp-mediated ubiquitination of 14-3-3 σ in breast cancer. However, the MCF-7 cell line we used in Efp-silencing is known to be luminal ER-positive in phenotype. We did not test basal-like cell lines. The ER-negative, basal-like cell lines might have low baseline Efp levels, which might compromise the silencing effect. The difference in the 14-3-3 σ responsiveness to Efp in different cell lines requires further investigation.

Efp positivity was not common in our cohort with since only about one-fourth of the total cases were positive for Efp whereas 14-3-3 σ was negative in about two-thirds of the total cases. In contrast, hypermethylation of 14-3-3 σ was common, occurring in more than 60% of the breast cancer samples. This may suggest that even if Efp can ubiquitinate and accelerate the degradation of 14-3-3 σ *in vitro* and even *in vivo*, it may not be a dominant mechanism in the inactivation of human breast cancer. Instead, hypermethylation may play a major role in the inactivation of 14-3-3 σ in human breast cancer.

The Efp is also a downstream target of estrogen receptor α (ERα) [23-25]. In a previous study, Efp immunoreactivity was significantly associated with ERα status in breast carcinoma tissues [24]. The Efp gene has an estrogen-responsive element at the 3'-untranslated region [23]. Therefore, it has been suggested that Efp is mainly produced in carcinoma cells through ERα as a result of estrogenic action in breast carcinoma. However, in our study there was no significant correlation between Efp and ER. In our study, the frequency of Efp positive cancers was low, only 36 of 145 cases (24.8%) in the receptor-positive group. It was even lower in the triple-negative group (positive in 6/57 cases, 10.5%). Suzuki et al. [22] found higher Efp immunoreactivity in ERα-negative breast carcinomas (52.4%). This difference in the frequency of Efp positivity in ER-negative breast cancers may be partially due to the difference in the criteria by which immunohistochemical positivity was determined. In our study, we used strict criteria, considering only diffuse strong expression (grade 3) as positive. Another possible explanation for the lack of correlation between Efp and ERα status is the escape of breast cancer cells from estrogenic control in tumorigenesis, as suggested in another recent study [25]. Ikeda et al. [26] suggested another possibility by analyzing the 5'-flanking region of the human Efp gene, and reporting the regulation of the Efp promoter by multiple elements and/or interacting factors. Therefore, factors other than ERα may be also be involved in the expression of Efp in some breast carcinomas. Suzuki et al. [22] reported that Efp immunoreactivity was significantly associated with an increased risk of recurrence or worse prognosis for both recurrence and overall survival in breast carcinomas, and that the effect is similar to that of the lymph node status, a well-established prognostic factor. However, in our study Efp expression was inversely correlated with axillary lymph node status ($p = 0.021$) although it was not significantly associated with patient survival.

Interestingly, in contrast to previous reports showing decreased expression or inactivation of 14-3-3 σ in various malignant tumors supporting its function as a tumor suppressor, the current results clearly show that 14-3-3 σ expression was not lost, but strongly expressed in at least a small subset of breast cancer cases. Although 14-3-3 σ expression in breast cancer was not compared to that in normal breast epithelia or benign proliferative lesions, another result of our study revealed the significant correlation of 14-3-3 σ positivity with high grade breast cancers and poor prognosis also clearly shows that 14-3-3 σ may play a more complicated role than just a tumor suppressor in breast cancer. In our study, the expression of 14-3-3 σ was inversely correlated with ERα, PR, and even Efp, which partially explains the more frequent positivity in triple-negative tumors than in receptor-positive tumors. In addition, 14-3-3 σ is correlated with high histologic grade, high nuclear grade, and positive p53 expression, all of which are characteristics of triple-negative breast cancers. The significance of 14-3-3 σ as a poor prognostic indicator was also more pronounced in triple-negative tumors than in receptor-positive ones. This association of 14-3-3 σ with poor

prognosis was in contrast to the previously known function of 14-3-3 σ as a tumor suppressor. However, the role of 14-3-3 σ as a poor prognostic factor has also been reported by a few recent studies in other types of cancer. Perathoner et al. [27] reported that 14-3-3 σ was significantly correlated with tumor differentiation and stage in colorectal carcinoma, and clearly demonstrated that 14-3-3 σ was an independent poor prognostic marker. Nakayama et al. [28] examined the expression of 14-3-3 σ, ERα, and Efp in endometrial carcinomas and showed that high 14-3-3 σ expression was significantly correlated with myometrial invasion and lymph node metastasis. The expression of 14-3-3 σ in normal and hyperplastic endometrium was also evaluated, and demonstrated that interestingly 14-3-3 σ expression gradually decreased from normal to hyperplastic to malignant tumors, which is a similar result to the previous studies [5-8,20]. These conflicting findings on the role of 14-3-3 σ in various types of cancer suggest that 14-3-3 σ is not just a tumor suppressor. Instead, it may have a more complex and complicated role in the process of carcinogenesis. It might also be possible that it plays different roles in early and late carcinogenesis. In early carcinogenesis, when malignant transformation occurs from the normal epithelium, 14-3-3 σ may function as a tumor suppressor, but once a malignant tumor is established, it may have a role in promoting tumor growth and propagation. Another hypothesis is that the deregulation or dysfunction of the ubiquitin-proteosome pathway may result in accumulation of 14-3-3 σ despite of the high level of Efp. Deregulation or dysfunction of the ubiquitin-proteosome pathway has been reported in various diseases, mainly neurodegenerative or metabolic diseases [29]. There is also evidence that the ubiquitin-proteosome system plays a role in tumor development, such as in colon cancer or hepatocellular carcinoma [30], in which the system is not exactly dysregulated, but activated degrading the tumor suppressor molecules. The exact role of 14-3-3 σ in breast and other cancers needs to be further elucidated. However, the significant correlation of 14-3-3 σ and poor prognosis suggests its potential usefulness as a therapeutic target in breast cancer.

Our data demonstrated that 14-3-3 σ was negatively correlated with Efp *in vitro* and less frequently *in vivo*. 14-3-3 σ was negatively correlated with hypermethylation in human breast cancer tissue, which was more common. This suggests that in human breast cancers the regulation of 14-3-3 σ may involve both mechanisms, i.e., ubiquitin-mediated proteolysis by Efp and downregulation by hypermethylation. However, the inactivation of 14-3-3 σ is probably achieved mainly by hypermethylation. Interestingly, 14-3-3 σ turned out to be a very significant prognostic indicator in breast cancer, being correlated with poor prognosis, which is the opposite of its previ-

ously known function as a tumor suppressor, suggesting a different role of the 14-3-3 σ in breast cancer.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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