Expression of regulatory factor X1 can predict the prognosis of breast cancer

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Abstract. Breast cancer (BC) is the most common malignancy among women. Identifying novel biomarkers to predict prognosis accurately is important in managing this disease. The regulatory factor X1 (*RFX1*) gene is a member of the regulatory factor X gene family. Its protein reportedly downregulates the proto-oncogene *c-myc*, but its role in BC has been unclear. In this study, expression and methylation status of *RFX1* were determined in BC cell lines. We then evaluated *RFX1* mRNA expression levels with regard to clinicopathological factors including postoperative prognosis in 167 patients with BC. Expression of *RFX1* was heterogeneous among cell lines, and we found no DNA methylation at the *RFX1* promoter region. Patients were categorized into groups with high or low *RFX1* expression, based on ratio of *RFX1* mRNA expression in BC and adjacent non-cancerous tissues. The high *RFX1* group was significantly associated with low T factor (P=0.028), earlier disease stage (P=0.015), positive expression of estrogen receptor (P=0.005) and progesterone receptor (P=0.011), negative expression of human epidermal growth factor receptor 2 (P=0.001). The high *RFX1* group experienced more favorable disease-free survival (P=0.007) and overall survival (P=0.013). In multivariate analysis, *RFX1* expression was an independent prognostic factor for disease-free survival. Our findings indicate that *RFX1* may serve as a prognostic marker for BC.

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer-related death in women worldwide (1). Approximately 1.7 million new cases of BC and 521,900 deaths related to BC are estimated to have occurred worldwide in 2012 (2). Recurrent BC after resection is frequently intractable and recognized as a major challenge in clinical practice. Currently, adjuvant therapies such as chemotherapy, endocrine therapy and anti-human epidermal growth factor 2 (HER2) agent are administered to BC patients with the aim of prevention of disease recurrences (3). However, many patients have suffered from adverse events and other costs caused by such treatment. Risk stratification is necessary for assessing indication for adjuvant therapy; development of biomarkers for this purpose is therefore needed.

Several commercial multigene expression assays (Oncotype Dx®, MammaPrint®, PAM-50 ROR score®, EndoPredict® and Breast Cancer Index®) are currently available to predict patients' prognoses and to evaluate whether adjuvant chemotherapy is appropriate (3). However, such assays are limited to estrogen receptor (ER)-positive, HER2-negative BC. It's still difficult to predict prognosis of all BC subtypes.

The regulatory factor X1 (*RFX1*) gene is a member of the regulatory factor X gene family which codes transcription factors (4). Expression and regulatory mechanisms of *RFX1* transcription have been reported in connection with several malignancies, including brain tumors (5,6) and hepatocellular carcinoma (7). *RFX1* is considered to work as a tumor suppressor by downregulating the proto-oncogene *c-myc* (8). However, the relationship between *RFX1* mRNA expression and patients' clinicopathological factors in BC has not been studied. Here, we focused on *RFX1* and investigated its expression in BC cell lines and patients' tumor samples to determine whether it could be a prognostic marker for BC.

Materials and methods

Sample collection. We obtained 13 BC cell lines (BT-20, BT-474, BT-549, HCC1419, HCC1954, Hs578T, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-468, SK-BR-3 and ZR-75-1) and two non-cancerous mammary gland epithelial cell lines (MCF-10A and MCF-12A). BT-549, HCC1419, HCC1954 and Hs578T were purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), BT-474, MCF-7 and MCF-12A were

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Table I. Primers.

Gene	Experiment	Type	Sequence (5'-3')	Product size	Annealing temperature
RFX1	RT-qPCR	Forward	GCAGACAGAAGTGGGGAGAA	241 bp	60°C
		Reverse	CAGTATACGCCTGTGTTGCC		
	Bisulfite sequencing in promoter region	Forward	TGTTTTTGAGGGTTTAGTTTTTTTT	207 bp	56°C
		Reverse	ACAACTATTACTACCCACCCTAATTAC		
	Bisulfite sequencing in intron 7	Forward	GGTGGAGGTTTGGAGTTT	273 bp	60°C
		Reverse	ACAAAAACAAATATAAAAACAACA		
	MSP	Forward	TTTTTCGTTTTTATTTAATTTCGAC	167 bp	56°C
		Reverse	ATTTCTAACTTCTTACGCTAACGTC		
	U-MSP	Forward	GGAGGTGTTATAGTTATGGTAGTTGT	164 bp	56°C
		Reverse	CTTTTCAAAAATCTCAAAATCAAT		
GAPDH	RT-qPCR	Forward	GAAGGTGAAGGTCGGAGTC	226 bp	60°C
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

RFX1, regulatory factor X1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MSP, methylation specific PCR; U-MSP, un-methylation specific PCR.

provided to our research team by Johns Hopkins University and Dr. David Sidransky, the director of the Department of Otolaryngology-Head and Neck Surgery of Johns Hopkins University (Baltimore, MD, USA), and others were from the American Type Culture Collection (Manassas, VA, USA). Cells were stored at -80°C using cell preservative solution (Cell Banker; Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and in an atmosphere containing 5% CO_2 at 37°C (9,10). We acquired 167 primary BC tissues from patients who underwent breast surgery at Nagoya University Hospital between March 2002 and November 2009. All tissue samples were diagnosed histologically as BC, frozen immediately after resection, and stored at -80°C. Adjacent non-cancerous specimens were resected >3 cm away from the edge of the tumor. BC specimens were classified histologically using the 7th edition of the Union for International Cancer Control (UICC) staging system for BC. Adjuvant chemotherapy, endocrine therapy and anti-HER2 therapy were administered to selected patients according to the patient's condition, pathological factors, subtype and the physician's discretion.

This study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for use of clinical samples and data was required by the Institutional Review Board at Nagoya University (Nagoya, Japan) and was obtained from all patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Levels of *RFX1* mRNA were determined using RT-qPCR. We extracted RNA from cell lines (8.0x10⁶ cells per each cell line), 167 primary BCs, and adjacent normal tissues using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. cDNA was synthesized from total RNAs (1 μg) by M-MLV Reverse Transcriptase (Invitrogen Life Technologies, Frederick, MD, USA) and Primer 'random' (Sigma-Aldrich). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were quantified to normalize expression levels. RT-qPCR was performed using the SYBR-Green PCR Core Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as follows: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 5 sec, and 60°C for 60 sec. All samples were tested in triplicate, and samples without templates were included in each PCR plate as negative controls. Real-time detection of SYBR Green fluorescence was conducted using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression level of each sample was taken as the value of the *RFX1* amplicon divided by that of *GAPDH* (11,12). Primers specific for *RFX1* and *GAPDH* are listed in Table I.

Surveillance of CpG island around the RFX1 gene. Nucleotide sequence analysis was conducted to determine the presence of CpG island around the promoter region of *RFX1*. CpG island was defined using the following criteria: At least 500-bp region of DNA with a high GC content (>50%) and an observed CpG/expected CpG ratio of ≥0.6 (13). We used the MethPrime (http://www.urogene.org/methprimer/) to determine the location of CpG island (14).

Bisulfite sequence analysis. Genomic DNA samples from 15 cell lines were subjected to bisulfite treatment using the BisulFlash DNA Modification Kit (EPIGENTEK,

Farmingdale, NY, USA); PCR was conducted to detect hypermethylation of the *RFX1* promoter region and intron 7 (5), using specific primers (Table I). The PCR amplification protocols were, for the promoter region: 50 cycles at 94°C for 20 sec, 56°C for 20 sec, and 72°C for 25 sec; and for intron 7: 50 cycles at 94°C for 20 sec, 60°C for 20 sec, and 72°C for 25 sec; both amplifications used an initial denaturation step at 94°C for 2 min. PCR products were purified directly using the MinElute PCR Purification Kit (Qiagen). Sequence analysis was carried out by Eurofins Genomics Co. (Tokyo, Japan) (15).

Methylation-specific polymerase chain reaction (MSP). DNA samples from BC cell lines, clinical BC specimens, and corresponding non-cancerous specimens were subjected to bisulfite treatment. MSP was conducted using specific primers to evaluate the presence of hypermethylation of *RFX1* promoter region (Table I). The PCR amplification protocols for MSP and un-MSP (U-MSP) were as follows: 50 cycles at 94°C for 15 sec, 56°C for 15 sec, and 72°C for 30 sec; both amplifications used an initial denaturation step at 94°C for 2 min. Each PCR product was loaded directly on to 2% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis. Differences in levels of *RFX1* mRNA between BC and adjacent normal tissues were analyzed using the Mann-Whitney U test. The χ^2 test was used to analyze the significance of the association between *RFX1* expression and clinicopathological parameters. Overall survival (OS) and disease-free survival (DFS) rates were calculated using the Kaplan-Meier method; difference in survival curves was analyzed using the log-rank test. We performed multivariate regression analyses using the Cox proportional hazards model to identify prognostic factors, and variables for which $P<0.05$ were entered into the final model. Statistical analyses were performed using JMP 11 software (SAS Institute, Inc., Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RFX1 mRNA expression and DNA methylation status of BC cell lines. *RFX1* mRNA expression levels were heterogeneous among the 13 BC cell lines and two non-cancerous mammary cell lines (Fig. 1). We accepted the reports of each cell line's immunostaining status from previous studies (16,17).

A CpG island was found at upstream of the transcription initiation site of *RFX1* (length 1,519 bases; Fig. 2A). We examined the methylation status of the 15 cell lines using bisulfite sequence analysis, which detected no CpG methylation in the *RFX1* promoter regions of all cell lines, whereas CpG hypermethylation in intron 7 was detected in all cell lines including the non-cancerous mammary cell lines (Fig. 2B). BT-474, MCF-10A, and three representative clinical BC and corresponding non-cancerous tissues were subjected to MSP. We found absence of methylation in the promoter region of *RFX1* gene both in cell lines and clinical samples (Fig. 2C).

Patient characteristics. All 167 patients were women. Their mean age was 54.4±11.6 years (mean ± standard deviation; range, 26-78 years). Their disease stage (by UICC staging
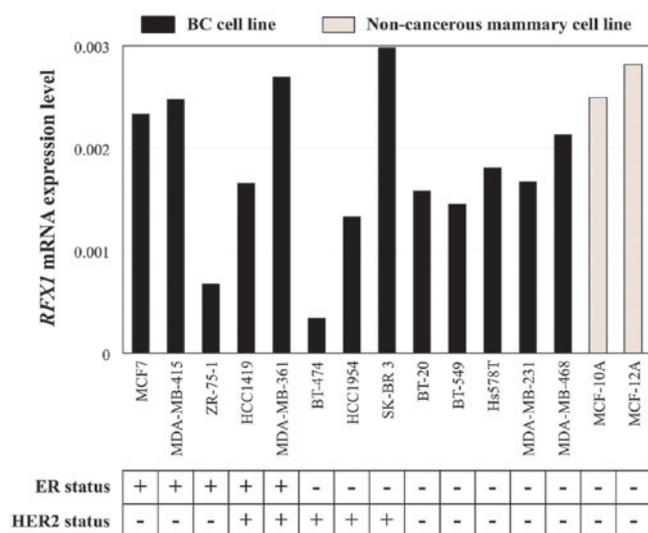

Figure 1. Analysis of *RFX1* mRNA expression. Bar graphs indicate *RFX1* mRNA levels in 13 BC cell lines and 2 non-cancerous mammary cell lines. *RFX1* mRNA expression levels were heterogeneous among cell lines. ER and HER2 status was referred from previous studies.
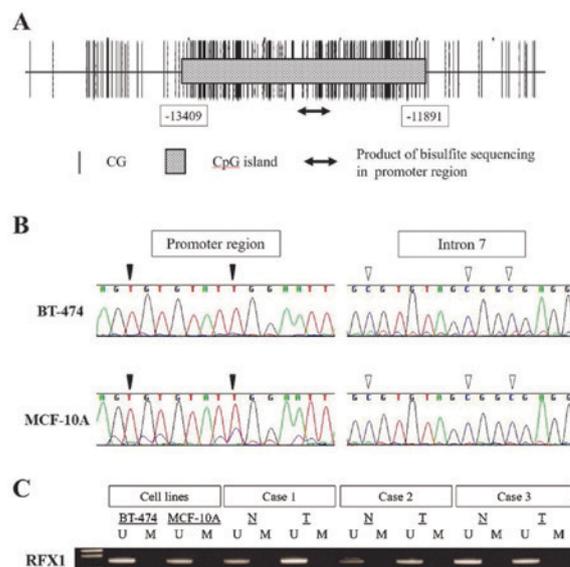

Figure 2. (A) The CpG island (indicated by dotted zone) was found upstream of the *RFX1* transcription initiation site. (B) Representative results of bisulfite sequence analyses of CpG regions in *RFX1* promoter region and intron 7. All CpG sites in the *RFX1* promoter region of BT-474 and MCF-10A cells were converted to TG, whereas CpG sites in intron 7 of the same cell lines were CG. Black arrows: Un-methylated CpG. White arrows: methylated CpG. (C) MSP was conducted in BT-474, MCF-10A, three representative clinical BC and corresponding non-cancerous tissues. No methylation was found at the promoter region of *RFX1* gene.

system) were stage 0, 7; stage I, 47; stage II, 78; stage III, 34; and stage IV, 1. The median duration of patient follow-up was 100.0 months (range, 8-155 months) or until death. The immunostaining status of primary tumors were ER positive, n=127; negative, n=40; progesterone receptor (PgR) positive, n=115; negative, n=52; and HER2 positive, n=39; negative, n=119.

Mean expression levels of *RFX1* mRNA in BC tissues was significantly lower than those in corresponding non-cancerous tissues (P<0.001; Fig. 3A). In 43 (26%) of the 167 patients,

Table II. Associations between expression of *RFX1* mRNA and clinicopathological parameters in 167 patients with breast cancer.

Clinicopathological parameter	The low *RFX1* group (n=124)	The high *RFX1* group (n=43)	P-value
Age			
≤60 year	78	30	
>60 year	46	13	0.413
Histology			
DCIS	4	3	
IDC	110	38	
ILC	5	2	
Others	5	0	0.265
UICC T factor			
Tis/T1	51	26	
T2/T3/T4	73	17	0.028[a]
Node status			
Negative	62	23	
Positive	62	20	0.693
UICC pathological stage			
0/I/II	92	39	
III/IV	32	4	0.015[a]
ER status			
Positive	88	39	
Negative	36	4	0.005[a]
PgR status			
Positive	79	36	
Negative	45	7	0.011[a]
HER2 status			
Positive	37	2	
Negative	82	37	
Unknown	5	4	0.001[a]
Triple-negative			
True	15	3	
False	108	40	
Unknown	1	0	0.323
Adjuvant therapy			
Endocrine therapy alone	35	22	
Chemotherapy alone	27	3	
Endocrine and chemotherapy	48	16	
None	14	2	0.012[a]

χ^2 test. [a]Statistically significant (P<0.05). DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; UICC, union for international cancer control; Tis, carcinoma *in situ*; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.
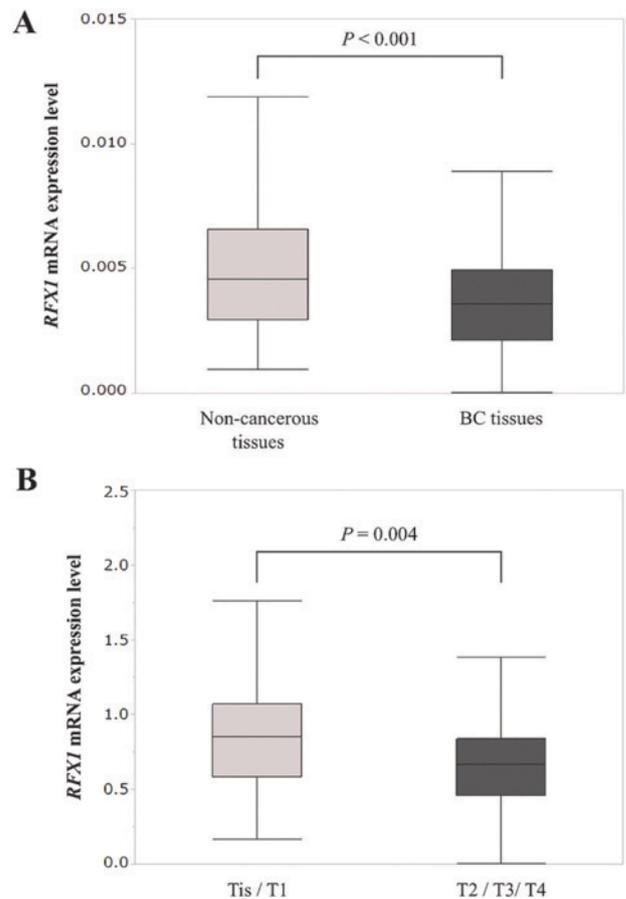

Figure 3. (A) Mean expression level of *RFX1* mRNA in BC tissues was significantly lower than in corresponding non-cancerous tissues. (B) Expression of *RFX1* in BC tissues. *RFX1* mRNA in T2/T3/T4 specimens was expressed at significantly lower levels than in the Tis/T1 group. Tis, carcinoma *in situ*.

RFX1 mRNA expression levels in BC tissues were higher than in adjacent normal tissues. We calculated the ratio of *RFX1* mRNA expression levels between BC and adjacent non-cancerous tissues. Patients whose *RFX1* mRNA levels were lower in BC tissue than in adjacent tissues were designated as 'the low *RFX1* group' (n=124), and patients with higher *RFX1* expression were designated as 'the high *RFX1* group' (n=43) in the following analyses.

Clinical significance of RFX1 mRNA levels. The high *RFX1* group was significantly associated with less advanced UICC T factor (P=0.028) and lower pathological stage (P=0.015). The high *RFX1* group correlated significantly with ER positive (P=0.005), PgR positive (P=0.011), HER2 negative status (P=0.001; Table II).

Mean *RFX1* mRNA expression in T2/T3/T4 specimens (n=90) was significantly lower than in the Tis (carcinoma *in situ*)/T1 group specimens (n=77; P=0.004; Fig. 3B). *RFX1* mRNA levels did not significantly differ between patients with negative and positive lymph node metastases (n=85 and 82 respectively, P=0.181), or between UICC stage 0/I/II specimens (n=131) and stage III/IV (n=36; P=0.069).

With regard to relevance to the conventional biomarkers (Fig. 4), *RFX1* mRNA levels in ER positive specimens (n=127) were expressed significantly higher than in ER negative specimens (n=40; P=0.017); PgR positive specimens'

Table III. Prognostic factors for disease-free survival in 167 patients with breast cancer.

Variables	n	Univariate Hazard ratio	95% CI	P	Multivariate Hazard ratio	95% CI	P
Age (>60)	59	1.06	0.49-2.17	0.879			
UICC T factor (T2-4)	90	3.96	1.73-10.7	<0.001[a]	1.85	0.67-5.65	0.239
Lymph node metastasis (positive)	82	4.98	2.18-13.4	<0.001[a]	2.84	0.79-10.0	0.107
UICC pathological stage (III/IV)	36	7.73	3.78-16.4	<0.001[a]	4.80	1.80-14.9	0.001[a]
ER status (negative)	40	2.02	0.93-4.14	0.073			
PgR status (negative)	52	1.81	0.87-3.67	0.112			
HER2 status (positive)	39	2.31	1.09-4.76	0.031[a]	0.94	0.42-2.08	0.883
Triple-negative (yes)	18	2.37	0.88-5.40	0.084			
Adjuvant chemotherapy (yes)	94	2.37	1.10-5.65	0.026[a]	0.32	0.09-1.07	0.064
The low *RFX1* group	124	5.69	1.72-35.2	0.002[a]	4.77	1.32-30.9	0.014[a]

[a]Statistically significant in multivariate analysis (P<0.05). CI, confidence interval; UICC, Union for International Cancer Control; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model.
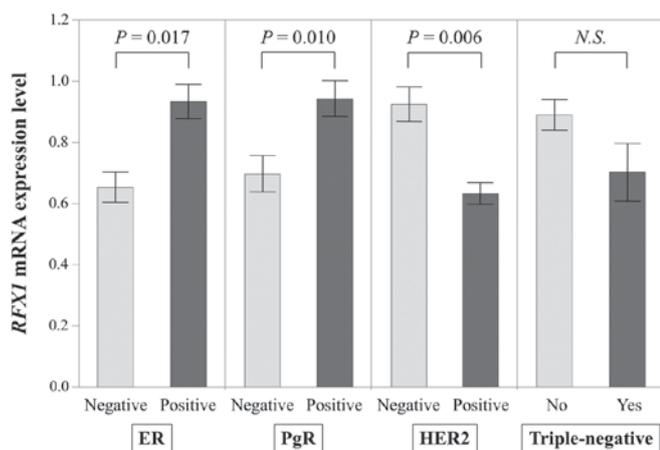

Figure 4. Expression of *RFX1* mRNA levels in tumor tissues about the relevance to the conventional biomarkers. Mean *RFX1* mRNA levels in the ER positive BC subgroup were significantly higher than in the ER negative subgroup. The PgR positive patients' mean *RFX1* mRNA levels was also significantly higher than that of PgR negative patients. The HER2 negative subgroup had a significantly higher mean *RFX1* mRNA level than the HER2 positive subgroup. The triple-negative and non-triple-negative subgroups did not significantly differ. NS, not significant.
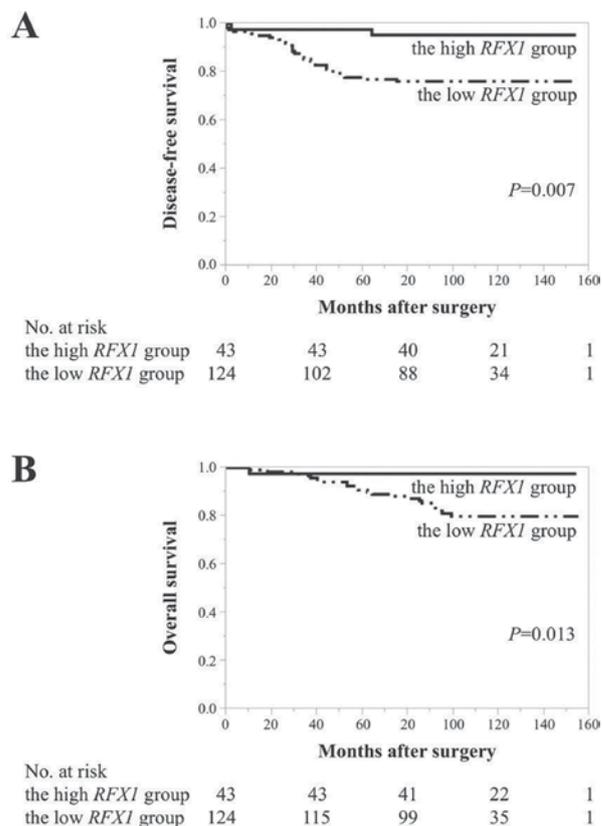

Figure 5. *RFX1* as a prognostic marker. (A) The high *RFX1* group experienced significantly longer disease-free survival than the other patients. (B) Overall survival was also significantly longer for the high *RFX1* group.

RFX1 mRNA level (n=115) was also significantly higher than that of PgR negative specimens (n=52; P=0.010); HER2 negative specimens (n=119) expressed significantly more *RFX1* mRNA than HER2 positive specimens (n=39; P=0.006; missing HER2 data for 1 patient). *RFX1* mRNA levels did not significantly differ between triple-negative (n=18) and other BC subtypes (n=148; P=0.363; missing data for 1 patient).

Prognostic impact of RFX1 mRNA expression. The high *RFX1* group experienced significantly longer DFS than the low *RFX1* group (5-year DFS rates, high *RFX1* group: 95%; low *RFX1* group: 77%; P=0.007; Fig. 5A). Among 43 patients of the high *RFX1* group, only two suffered recurrences. The OS in the high *RFX1* group was also significantly longer than in the low *RFX1* group (5-year OS rates, high *RFX1* group: 98%; low *RFX1* group: 90%; P=0.013; Fig. 5B). Multivariate analysis of DFS identified UICC pathological stage III/IV [hazard ratio (HR): 4.80; 95% confidence interval (CI): 1.80-14.9, P=0.001] and the low *RFX1* group (HR, 4.77; 95% CI: 1.32-30.9, P=0.014) as independent prognostic factors (Table III).

Discussion

Our data showed that higher *RFX1* expression was associated with less advanced T factor and lower disease stage, and patients with higher *RFX1* expression had excellent prognoses.

The *RFX1* gene is cytogenetically located at 19p13.1, and belongs to the *RFX* family genes which share the conserved DNA-binding domain named 'winged helix' (4,18). The *RFX* family codes transcription factors that contain a highly conserved 76-amino acid DNA binding domain (4). RFX1 forms a homodimer or heterodimers with the RFX2 or RFX3 proteins through a dimerization domain (19,20). RFX1-4 can bind methylated DNA sequences preferentially within a sequence-specific, 14-bp consensus sequence (21). As RFX1 downregulates the proto-oncogene *c-myc* (8), it is considered to be a tumor suppressor. In hepatocellular carcinoma (HCC), RFX1 regulates SC-2001-mediated *SHP1* transcription in HCC cell lines, and SC-2001 inhibits tumor growth (7). *RFX1* downregulation in tumors has been also verified in glioma (5), neuroblastoma (6), and esophageal adenocarcinoma (22).

We investigated the mRNA expression level and methylation status of *RFX1* using BC cell lines and non-cancerous mammary gland epithelial cell lines. The *RFX1* mRNA expression levels were heterogeneous across cell lines. There were no reports on hypermethylation of the promoter region of *RFX1* gene in non-neoplastic tissues (5). In this study, we found no CpG methylation within the promoter region in cell lines and some clinical tissues. Reportedly, CpGs in intron 7 were hypermethylated in glioma cell lines and tissue samples, although CpG methylation in the promoter region was not seen (5). BC cells were also reported to have hypermethylated CpGs in intron 7 (23). CpGs in gene bodies are sometimes methylated in a tissue-specific manner. Gene body methylation has been shown to increase gene expression contrary to methylation in the promoter region (24). In the current study, we found CpG hypermethylation in intron 7 of all BC cell lines and the non-cancerous mammary cell lines. These results suggest that methylation status is not associated with downregulation of *RFX1* mRNA in BC cells.

This study demonstrated that *RFX1* mRNA expression has a significant relationship with patients' clinicopathological factors and prognosis. The most striking finding was that the high *RFX1* group experienced excellent prognosis (5-year DFS rates, high RFX1 group: 95%; low *RFX1* group: 77%). Notably, only two of 43 patients suffered recurrences in the high *RFX1* group. Multivariate analysis of DFS identified UICC pathological stage and the low *RFX1* group as independent prognostic factors. These results emphasize the usefulness of *RFX1* as a prognostic marker. *RFX1* mRNA level was indicated as a potent prognostic factor which was independent of ER, PgR or HER2 status. Considering these findings, if a patient's BC specimen's *RFX1* mRNA expression is high, adjuvant chemotherapy could be tailored, thus reducing the adverse events and expense associated with chemotherapy.

Breast cancers are commonly treated according to their subtypes (3), which are determined by investigating their immunostaining status for ER, PgR and HER2. The subtype reflects each tumor's character, prognosis and sensitivity to medication. In this study, the high *RFX1* group correlated significantly with ER positive, PgR positive and HER2 negative. This result corresponds to our understanding that BC with such phenotypes are less aggressive. Although triple-negative BC is thought to be a more malignant phenotype with a poorer prognosis, our results showed no significant differences between triple-negative BC and other types. This might be because of the small sample size of the triple-negative group.

This is the first study to show the relationship between *RFX1* mRNA expression and BC prognosis; and our findings may have several possible clinical applications. For example, *RFX1* levels in resected samples might help evaluate each patient's prognosis and need for adjuvant chemotherapy. Multigene expression assays might be refined by adding *RFX1* expression. In addition, *RFX1* expression can be analyzed by needle biopsy samples before operation, thus aiding in treatment decisions for neoadjuvant therapy.

This study has some limitations. First, the function of RFX1 in BC cells is unknown. Basic functional analyses in which *RFX1* expression is inhibited are needed. Second, the mechanism of *RFX1* downregulation is still unclear. The methylation status of two regions (the promoter and intron 7) in the *RFX1* gene was determined in the present study. Therefore, it remains formally possible that additional intragenic or intergenic regions may have undergone methylation in breast cancer tissues. Mechanisms other than methylation (such as histone modification and micro RNAs) should also be investigated for this gene and its product. Further, this study was conducted retrospectively. Therefore, therapeutic intervention might affect significance of *RFX1* mRNA level. Thus study for larger series of patients or prospective study is warranted.

In conclusion, our data indicate that higher expression of *RFX1* mRNA level in BC tissue than adjacent normal site predicts better prognosis. *RFX1* could become a useful prognostic marker for BC.

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