Forkhead Box I1 in Breast Carcinoma as a Potent Prognostic Factor

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Forkhead box (FOX) proteins are family of transcriptional factors and regulate cell growth and differentiation as well as embryogenesis and longevity. Previous studies have demonstrated that several FOX members regulate growth or metastasis of breast carcinoma, but clinical significance of total FOX members remains unclear. We first examined associations between expression of 40 FOX genes and TNM status of 19 breast carcinoma using microarray data. Subsequently, we immunolocalized FOXI1 in 140 breast carcinomas and evaluated its clinicopathological significance. In the microarray analysis, we newly identified that gene expression of FOXI1 was most pronouncedly linked to metastasis of the breast carcinoma among the FOX members examined. However, clinicopathological significance of FOXI1 has not been examined in the breast carcinoma. FOXI1 immunoreactivity was positive in 44 out of 140 (31%) of breast carcinomas, and it was significantly associated with stage, lymph node metastasis and distant metastasis. The FOXI1 status was significantly associated with worse prognosis of the breast cancer patients, and it turned out to be an independent prognostic factor for both distant disease-free survival and breast cancer-specific survival. These findings suggest that FOXI1 plays important roles in the metastasis of breast carcinoma and immunohistochemical FOXI1 status is a potent prognostic factor.

I. Introduction

Breast cancer is one of the most common malignancies in women worldwide. Invasive breast cancer is generally regarded as a disease that metastasizes in an early phase [10]. Approximately 5% of breast cancer presents metastasis to distant organs, such as bone, lung and liver, at diagnosis [3], and, about 30% will develop metastasis during the evolution of their disease [6]. Since metastasis is the major cause of death of breast cancer patients, and it is important to examine molecular mechanisms of metastasis in breast carcinoma to improve clinical outcome of the patients.

The Forkhead box (FOX) proteins are family of transcriptional factors. FOX regulates cell growth and differentiation as well as embryogenesis and longevity, and they have a conserved FOX domain, which is involved in DNA binding, and extra-FOX protein-protein interaction domains [11]. Human FOX family consists of about 17

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subfamilies and at least 43 members based on sequence similarity [8]. FOX factors are key regulators of various signaling pathways, such as phosphatidilinositol 3-kinese/ protein kinase B (PI3K-AKT), transforming factor β (TGF-β), Wnt/β-catenin, Sonic-Hedgehog and Jagged-Notch [13]. Emerging evidence suggests that several FOX members play important roles in the breast carcinoma [2, 5, 14, 21], but the significance of total FOX members remains unclear.

Therefore, in this study, we first studied the expression profile of FOX genes associated with TNM classification of breast carcinomas by microarray data and newly identified that FOXI1 was the most associated with the metastasis. FOXI1 is required for morphogenesis of mammalian inner ear [19] and plays an important role in early embryogenesis [24], but it has not been examined in the breast carcinoma to the best of our knowledge. Therefore, we subsequently performed immunohistochemistry for FOXI1 in breast carcinoma tissues to clarify its clinicopathological significance.

II.   Materials and Methods

Microarray analysis

The gene expression profile data of estrogen receptor (ER)-positive breast carcinomas (n = 19) were used in the present study, which had been mainly assembled in our previous study [15]. Briefly, total RNA was extracted from 19 snap-frozen specimens using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). A SurePrint G3 Human GE 8 × 60K v2 Microarray Kit (G4851A, ID 028004 (Agilent Technologies, Waldbronn, Germany)) was used, and sample preparation and processing were performed according to the manufacturer's protocol.

Patients and tissues

The specimens of 140 cases of invasive ductal carcinoma, not otherwise specified, of the breast were obtained from Japanese female patients (age range; 27–87 years) who underwent surgical treatment. All the specimens were fixed in 10% formalin and embedded in paraffin wax. Among these, stage IV cases (n = 27) were obtained from 2000 to 2015 from Tohoku University Hospital (Sendai, Japan) and Osaki Citizen Hospital (Osaki, Japan). In contrast, the stage I–III patients (n = 113) were successively treated in Tohoku University Hospital from 2007 to 2008. Among these 113 patients, 56 patients received adjuvant chemotherapy, and 91 patients received adjuvant endocrine therapy after the surgery. The clinical outcome was evaluated by distant metastasis-free survival, which was defined as the time from primary surgery until the first event of distant metastasis [20], and breast cancer-specific survival of the stage I–III patients. The mean follow-up time was 61 months (range; 3–91 months) in this study. The research protocol was approved by the Ethics Committee at the Tohoku University School of Medicine and review board of Osaki Citizen Hospital.

Immunohistochemistry

We purchased monoclonal antibody for FOXI1 (OTI2C1) from Origene technologies, Inc. (Rockville, MD, USA) and mouse monoclonal antibody for Ki-67 (MIB1) from DAKO (Carpinteria, CA, USA). Antigen retrieval was performed by heating the slides in auto clave at 120°C for 20 min in citric acid buffer (pH 6.0) for staining with antibody as described above. Dilutions of primary antibodies for FOXI1 and Ki-67 were 1/400 and 1/50, respectively. We used a Catalysed Signal Amplification II (CSAII) system from DAKO for FOXI1 immunostaining and a Histofine Kit (Nichirei Biosciences, Tokyo, Japan) for Ki67 immunostaining. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution with hematoxylin. Human tissue of the kidney was used as a positive control in this study [18], and as a negative control of the primary antibody, PBS was used instead of that.

Immunohistochemistry for ER (CONFIRM anti-ER (SP1)) and progesterone receptor (PR: CONFIRM anti-PR (1E2); Roche Diagnostics Japan, Tokyo, Japan) was performed with Ventana Benchmark XT (Roche Diagnostics Japan), and that for HER2 was performed by HercepTest (DAKO).

Scoring of immunohistochemistry

FOXI1 was immunolocalized in the nucleus of carcinoma cells, and the cases that had more than 10% positive carcinoma cells were considered positive [27]. ER, PR and Ki-67 were immunolocalized in the nucleus, and the percentage of immunoreactivity (labeling index; LI) was determined. Cases with ER or PR LI of more than 1% were considered ER-positive or PR-positive breast carcinoma according to a previous report [9]. HER2 immunostaining was scored according to the standardized HercepTest scoring system (score 0–3) (DAKO), and the score 3 was considered positive. HER2 gene amplification was also investigated by fluorescence *in situ* hybridization (FISH) in the score 2 cases, and the cases showed positive for FISH were also considered positive for HER2 status. Ki-67 LI was classified into two groups in the uni-and multi-variate analyses using 20% as a cut-off value [17].

Intrinsic subtype of the breast carcinoma was defined according to 2011 St Gallen surrogate definition [7] as follow: luminal A (ER and/or PR positive, HER2 negative, Ki-67 LI < 14%), luminal B (ER and/or PR positive, HER2 negative, Ki-67 LI ≥ 14% (HER2 negative), or ER and/or PR positive, HER2 positive (HER2 positive)), HER2 positive (ER and PR negative, HER2 positive), and triple negative (ER, PR, HER2 negative).

Statical analysis

Association between immunohistochemical status of FOXI1 and clinicopathological factors were evaluated using Student's t test or a cross-table using the χ^2-test. Disease-free and breast cancer-specific survival curves were generated according to the Kaplan-Meier method, and

statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional hazard model (Cox).

P-value < 0.05 was considered significant. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC, USA) in this study.

III.   Results

Expression profiles of FOX genes associated with TNM classification of breast carcinomas

We first examined associations between expression of FOX genes and TNM status of 19 breast carcinoma using microarray data. We detected 40 FOX genes corresponding 49 probes from the microarray data (Supplementary Table S1), and when the expression ratio of a gene was > 1.50 or < 0.50, we tentatively determined that the expression was predominantly high or low in this study [23]. Among the 40 FOX genes examined, FOXB2 (1.94 fold) and FOXI1 (1.52 fold) were predominantly expressed in higher pT (pT3,4) group compared to the lower pT (pT1,2) group (Fig. 1A). On the other hand, FOXI1 (1.58 fold) and FOXB2 (1.53 fold) were predominantly expressed in cases positive for lymph node metastasis (pN1-3), (Fig. 1B). As shown in Figure 1C, expression level of FOXI1 (3.03 fold) and FOXB2 (1.95 fold) were predominantly high in cases positive for distant metastasis (M1), while that of FOXQ1 (0.47 fold) was predominantly low.

These data suggest that FOXI1 and FOXB2 were associated with advanced TNM staging of breast carcinoma, and especially, FOXI1 showed the highest increase in the metastatic breast carcinoma. Therefore, we selected FOXI1 in this study, and we subsequently performed immunohistochemistry for FOXI1 in the breast carcinoma tissues.

FOXI1 immunolocalization in human breast carcinoma

Immunoreactivity of FOXI1 was detected in the nucleus of breast carcinoma cells (Fig. 2A–D), while it was negative in the non-neoplastic mammary glands or stroma (Fig. 2E). In the positive control, FOXI1 was immunolocalized in the distal tubules of the kidney (Fig. 2F) as reported previously [18]. When we performed immunohistochemistry for FOXI1 in 19 breast carcinomas used in the microarray analysis, the median value of FOXI1 expression level in the FOXI1 immuno-positive cases (n = 5) was 9.8-fold higher than that in the FOXI1-negative cases (n = 14; P = 0.0016 by Mann-Whitney U test). Therefore, FOXI1 immunoreactivity is suggested to reflect its expression level in this study.

Associations between immunohistochemical FOXI1 status and various clinicopathological parameters in the breast carcinoma was summarized in Table 1. The number of FOXI1-positive cases was 44 out of 140 (31%). The immunohistochemical FOXI1 status was positively associated with stage (P = 0.036), lymph node metastasis (P = 0.017) and distant metastasis (P = 0.011), and it was
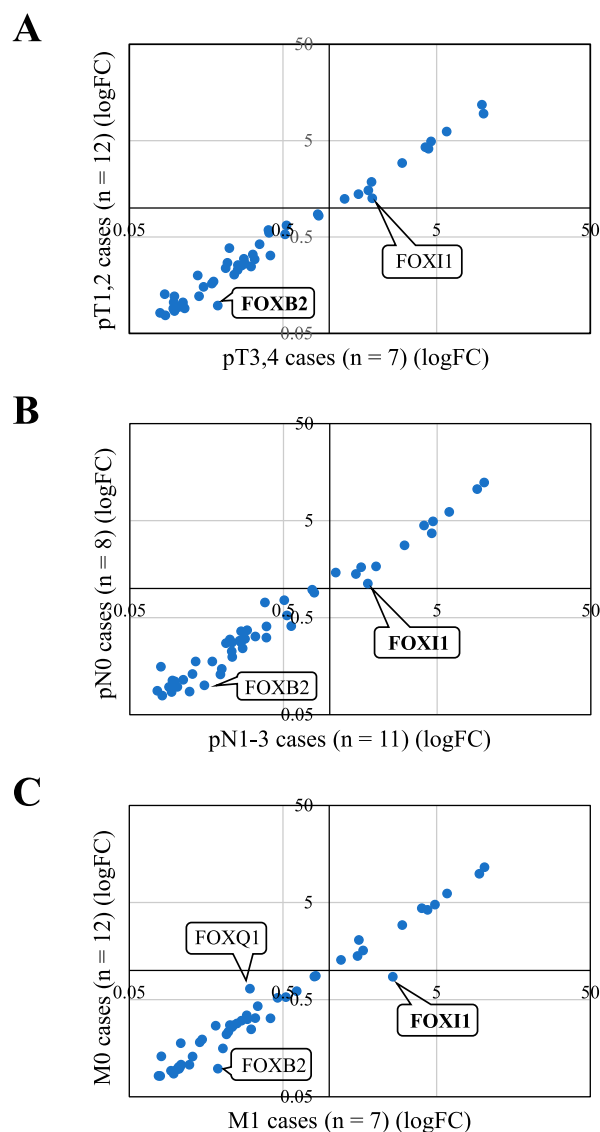

Fig. 1. Scatter plot analysis of microarray data for 49 probes containing 40 FOX genes in 19 breast carcinoma tissues. **A:** comparison between pT3,4 and pT1,2 cases, **B:** comparison between pN1-3 (cases positive for lymph node metastasis) and pN0 (cases negative for lymph node metastasis), **C:** comparison between M1 (cases positive for distant metastasis) and M0 (cases negative for distant metastasis). FOX genes with the relative expression ratio > 1.5 or < 0.5 were summarized in each figure, and the gene showed the highest or lowest ratio was described in bold.

marginally significant with pathological T factor (pT) (P = 0.059).

Association between FOXI1 and clinical outcome of breast cancer patients

As demonstrated in Figure 3A, FOXI1 status was significantly associated with an increased incidence of recurrence in stage I–III patients (n = 113) (P = 0.0007 using the log-rank test). Association between FOXI1 status and breast cancer-specific survival was summarized in Figure
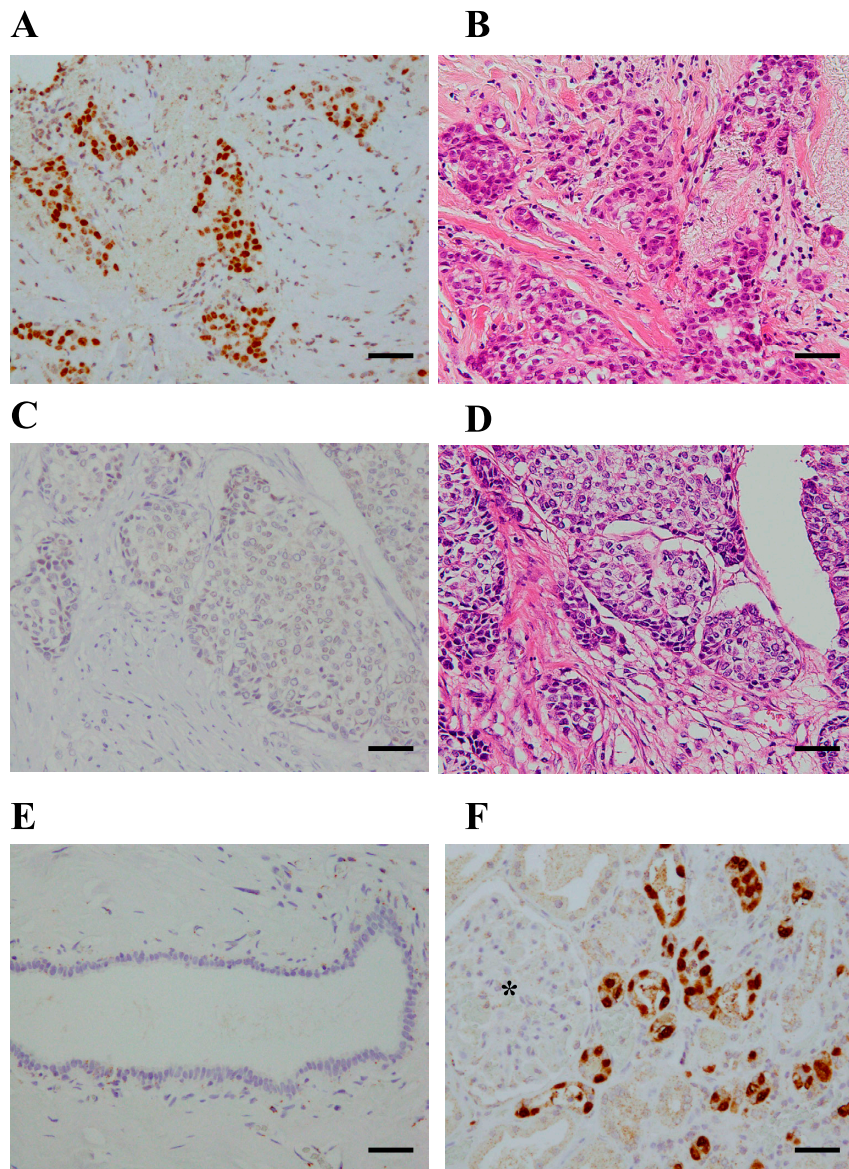
Fig. 2.　Immunohistochemistry for FOXI1 in invasive breast carcinoma. **A:** FOXI1 was immunolocalized in the nucleus of breast carcinoma cells. **B:** HE staining of the same area as **A**. **C:** FOXI1-negative case. **D:** HE staining of the same area as **C**. **E:** FOXI1 immoreactivity was negative in the normal breast tissue. **F:** As a positive control, FOXI1 immunoreactivity was detected in distal tubules of the kidney, but not in the glomerulus (*). Bar = 50 μm, respectively.

3B, and a significant association was detected between FOXI1 status and an adverse clinical outcome of patients (P = 0.0013). Similar tendencies were detected in both ER-positive cases (P = 0.016 for disease free survival (Fig. 2C) and P = 0.028 for breast cancer-specific survival) and ER-negative cases (P = 0.014 for disease free survival (Fig. 2D) and P = 0.012 for breast cancer-specific survival). Significant association between FOXI1 status and a worse prognosis was also observed in cases positive for lymph node metastasis (P = 0.0085 for disease free survival (Fig. 2E) and P = 0.013 for breast cancer-specific survival) or cases received chemotherapy (P = 0.0024 for disease free survival (Fig. 2E) and P value was not evaluated for breast

cancer-specific survival because no patient died in FOXI1-negative group).

　　Results of univariate analysis of distant disease-free survival using Cox (Table 2), pT, lymph node metastasis, Ki-67 status, FOXI1 status, histological grade and ER status were demonstrated to be significant prognostic factors. Following multivariate analysis revealed that FOXI1 (P = 0.0015) and Ki67 status (P = 0.036) were turned out independent worse prognostic factors for disease-free survival. On the other hand, as shown in Table 3, univariate analysis for breast cancer-specific survival revealed FOXI1 status, lymph node metastasis, histological grade, pT and ER status as significant prognostic variables, in

Table 1. *Association between immunohistochemical FOXI1 status and clinicopathological factors in 140 breast carcinomas*

	FOXI1 status		*P* value
	+ (n = 44)	− (n = 96)	
Age† (years)	56.2 ± 1.8	56.4 ± 1.3	0.91
Menopausal status			
Premenopausal	15	36	
Postmenopausal	29	60	0.70
Stage			
I	15	51	
II–IV	29	45	**< 0.05**
Pathological T factor (pT)			
pT1	20	60	
pT2–4	24	36	*0.06*
Lymph node metastasis			
Positive	25	34	
Negative	19	62	**< 0.05**
Distant metastasis			
Positive	14	13	
Negative	30	83	**< 0.01**
Histological grade			
1–2	34	75	
3	10	21	0.91
ER status			
Positive	34	79	
Negative	10	17	0.48
PR status			
Positive	29	65	
Negative	15	31	0.83
HER2 status			
Positive	6	17	
Negative	38	79	0.55
Ki-67 LI† (%)	18.8 ± 2.8	15.1 ± 1.4	0.20
Intrinsic subtype			
Luminal A	23	50	
Luminal B	12	30	
HER2 positive	3	7	
Triple negative	6	9	0.88

†; Data are presented as mean ± SEM. All other values represent the number of cases.
P-value < 0.05 and 0.05 ≤ *P*-value < 0.10 were significant (in bold) and borderline significant (in italics).

addition to Ki-67 status as a marginally significant variable. Subsequent multivariate analysis demonstrated that only FOXI1 was an independent worse prognostic marker (*P* = 0.035).

IV. Discussion

Since FOX transcription factors regulate a variety of cellular functions, several studies have been reported regarding their biological roles or clinical significance in the breast cancer. For instances, FOXC1 [21] and FOXK1 [5] were shown as the worse prognostic factors, and FOXC2 [2] and FOXP4 [14] promoted invasion property of the breast carcinoma cells. On the other hand, FOXM1 inhibited metastasis of breast carcinoma cells [12], and FOXN2 decreased the proliferation and invasion [30].

Table 2. *Univariate and multivariate analyses of distant disease-free survival in 113 stage I–III breast cancer patients*

Variable	Univariate	Multivariate	
	P value	*P* value	Relative risk (95% CI)
pT			
(pT2–4/pT1)	**< 0.001†**	*0.06*	5.61 (0.91–34.52)
Lymph node metastasis			
(positive/negative)	**< 0.001†**	0.27	2.43 (0.50–11.89)
Ki-67 status			
(≥ 20%/< 20%)	**< 0.01†**	**0.04**	5.31 (1.11–25.28)
FOXI1 status			
(positive/negative)	**< 0.01†**	**< 0.01**	5.53 (1.93–15.87)
Histological grade			
(3/1,2)	**< 0.01†**	0.32	2.04 (0.50–8.36)
ER status			
(positive/negative)	**0.02†**	0.29	0.53 (0.16–1.72)
HER2 status			
(positive/negative)	0.63		

Statistical analysis was evaluated by a proportional hazard model (Cox).
P value < 0.05 and 0.05 ≤ *P* value < 0.10 were considered significant and borderline significant, and were listed in bold and italic, respectively.
†; Significant (*P* < 0.05) and borderline-significant (0.05 ≤ *P* < 0.10) values were examined in the multivariate analyses in this study.
95% CI, 95% confidence interval.

Table 3. *Univariate and multivariate analyses of breast cancer-specific survival in 113 stage I–III breast cancer patients*

Variable	Univariate	Multivariate	
	P value	*P* value	Relative risk (95% CI)
FOXI1 status			
(positive/negative)	**< 0.01†**	**< 0.01**	1034 (1.90–56.38)
Lymph node metastasis			
(positive/negative)	**0.01†**	*0.09*	14.96 (0.64–350.26)
Histological grade			
(3/1,2)	**0.02†**	0.94	1.10 (0.12–10.25)
pT			
(pT2–4/pT1)	**0.02†**	0.90	1.17 (0.11–12.83)
ER status			
(positive/negative)	**< 0.05†**	0.11	0.21 (0.11–20.23)
Ki-67 status			
(≥ 20%/< 20%)	*0.06†*	0.75	1.52 (0.11–20.23)
HER2 status			
(positive/negative)	0.76		

Statistical analysis was evaluated by a proportional hazard model (Cox).
P value < 0.05 and 0.05 ≤ *P* value < 0.10 were considered significant and borderline significant, and were listed in bold and italic, respectively.
†; Significant (*P* < 0.05) and borderline-significant (0.05 ≤ *P* < 0.10) values were examined in the multivariate analyses in this study.
95% CI, 95% confidence interval.

FOXOs are generally known as tumor suppressors, but they also promote metastasis of subsets of breast cancer [1]. Emerging evidence suggests importance of FOX family in the breast carcinoma, but significance of total FOX members remains unclear in the breast carcinoma.

In our present microarray analysis, gene expression level of a great majority of FOX members did not markedly change according to the TNM status of breast carcinoma.
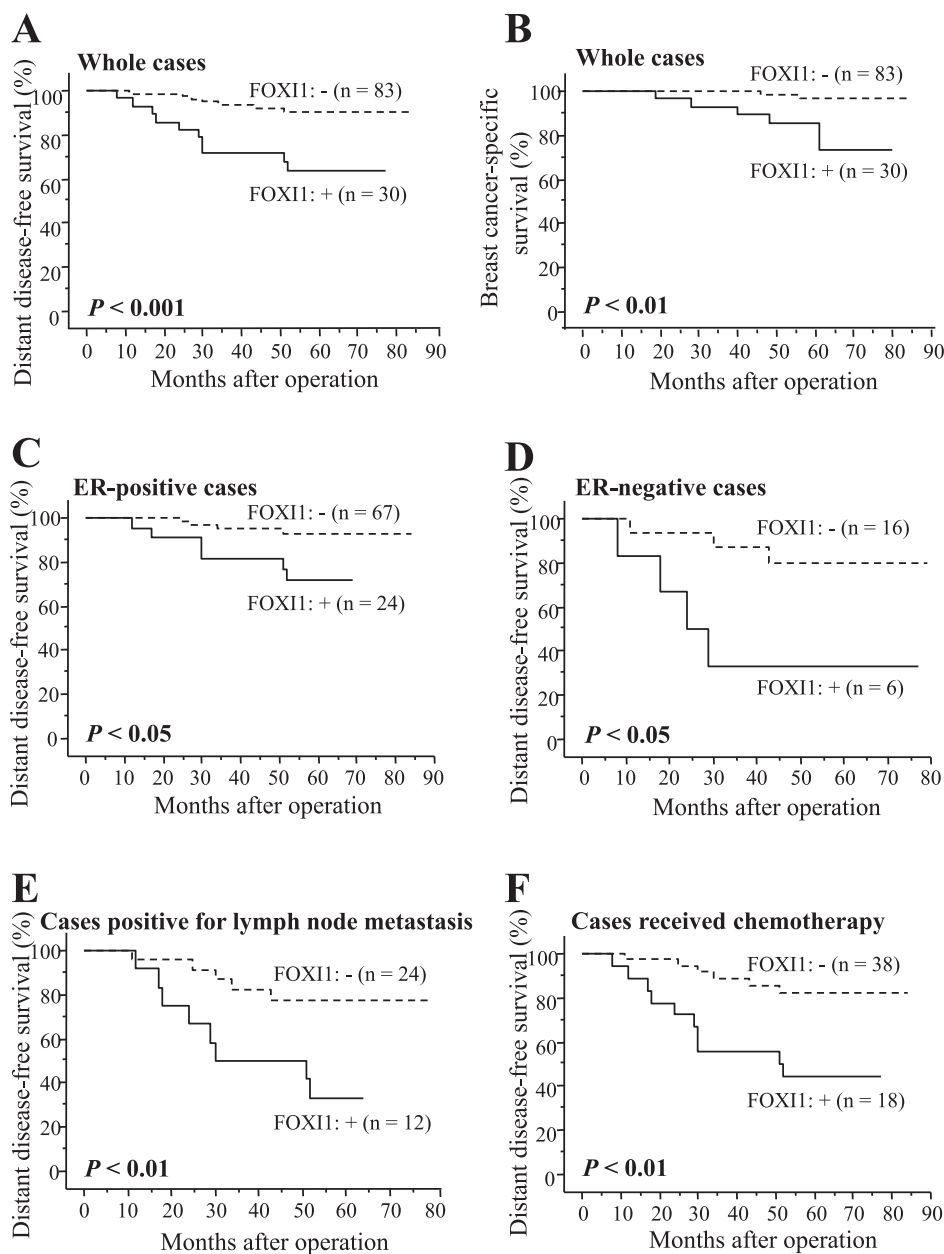
Fig. 3. Distant disease-free (**A, C–F**) and breast cancer-specific survival (**B**) of stage I–III breast cancer patients according to FOXI1 status. **A, B:** FOXI1 status in whole cases (n = 113), **C:** ER-positive cases (n = 91), **D:** ER-negative cases (n = 22), **E:** cases positive for lymph node metastasis (n = 36) and **F:** cases received chemotherapy (n = 56). The solid line shows FOXI1-positive group, and the dashed line shows FOXI1-negative group. *P*-values < 0.05 were considered significant and shown in bold.

However, FOXI1 and FOXB2 predominantly expressed in higher TNM status of the breast carcinomas, while FOXQ1 was predominantly expressed in the M0 cases, suggesting particular importance of these FOX members in the breast carcinoma. Among these, Elian *et al.* (2021) very recently reported that low expression of FOXQ1 mRNA was indicative of poor prognosis in patients with breast cancer [4], which is consistent with our present results. FOXB2 activated Wnt signaling and neuroendocrine differentiation of prostate carcinoma cells [16], but it has not been reported in the breast carcinoma. FOXI1 was most pronouncedly linked to metastasis in the breast carcinoma in this study, but its clinicopathological significance has not been examined in the breast carcinoma to the best of our knowledge.

This is the first study that immunolocalized FOXI1 in the breast carcinoma. In this study, FOXI1 immunoreactivity was detected in 31% of breast carcinomas, whereas it was negative in morphologically normal mammary glands. FOXI1 plays a key role in differentiation and functional maintenance for the renal intercalated cells, and FOXI1 immunoreactivity was detected in 91% of chromophobe renal cell carcinoma and 72% of renal oncocytoma, but

it was negative in renal neoplasms derived from non-intercalated cells [28]. No information is currently available about the FOXI1 expression in other carcinoma tissues. Considering that FOXI1 is required for morphogenesis of mammalian inner ear [19] and plays an important role in early embryogenesis [24], FOXI1 may be a differentiation regulator or a lineage selector factor, and aberrant expression of FOXI1 may cause dedifferentiation and high-grade malignancy in the breast carcinoma.

Biological function of FOXI1 remains largely unknown in carcinoma. Only Sun *et al.* (2017) reported that FOXI1 prohibited cell proliferation of gastric cancer [25]. They also shown that FOXI1 regulated expression of various protein-coding genes (118 genes upregulated and 72 genes downregulated after FOXI1 overexpression) and non-coding RNAs in the gastric cancer cells [26]. Among these FOXI1-regulated genes reported, for instance, ATF3 enhanced breast cancer metastasis and had predictive value for the clinical outcomes [29], while LINC00052 was reported as a suppressor of breast cancer cell migration [22]. Considering that FOX members regulate a variety of biological process and play pleiotropic roles as described in the Introduction section, it is suggested that FOXI1 directly or indirectly regulates various gene expression in the breast carcinoma and aberrant activation of FOXI1-associated-signaling cascades leads to metastasis of the breast carcinoma. Further examinations are required to clarify molecular functions and possible therapeutic potential of FOXI1 in human breast carcinoma.

In summary, we examined gene expression profile of FOX family according to the TNM status of breast carcinoma by microarray analysis and newly identified that FOXI1 was most associated with the metastasis. A subsequent immunohistochemical analysis demonstrated that FOXI1 immunoreactivity was positive in 31% of breast carcinomas, and it was significantly associated with stage, lymph node metastasis and distant metastasis. Moreover, multivariate analysis turned out that the FOXI1 status was an independent worse prognostic factor in breast cancer patients. These findings suggest that FOXI1 plays important roles in the metastasis of breast carcinoma and immunohistochemical FOXI1 status is the potent prognostic factor.

V. Conflicts of Interest

The authors have no conflict of interest to declare, in this study.

VI. Acknowledgments

VII. References

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