between the 2 investigators, a third investigator (TSh) evaluated the tissue sections, and the mean immunoreactivity results were used. To identify potential correlations between EBAG9 immunoreactivity in the malignant epithelium and clinicopathological characteristics, breast cancer specimens in which > 50% of the cells expressed EBAG9 were regarded as positive, and specimens in which ≤ 50% of the cells expressed EBAG9 were regarded as negative.

Plasmid Construction

Human EBAG9 (hEBAG9) was N-terminally tagged with the Flag epitope and subcloned into the pcDNA3 vector (pcDNA3-Flag-hEBAG9).

Cell Culture and Transfection

Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum was used to maintain 293T cells. Transfection of expression plasmids was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Western Blot Analysis

Whole-cell lysates were prepared using radioimmunoprecipitation assay buffer, resolved using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were probed with anti-EBAG9 antibody and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (GE Healthcare, Buckinghamshire, UK), and bound antibodies were visualized using enhanced chemiluminescence (GE Healthcare).

Statistical Analysis

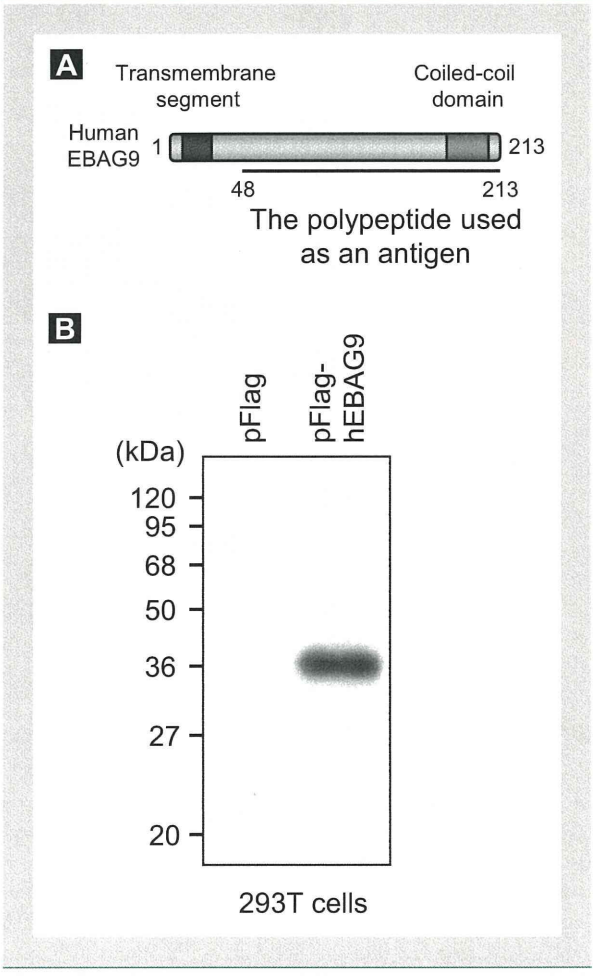
The correlation between the immunoreactivity score and clinicopathological characteristics was evaluated using the χ^2 test. A *P*-value of < .05 was regarded as statistically significant. Differences between the 2 groups were analyzed using 2-sample, 2-tailed Student *t* test. Relapse-free and overall survival curves were obtained using the Kaplan-Meier method and verified using the log-rank (Mantel-Cox) test. Univariate and multivariate analyses were performed using a logistic regression model with JMP 9 software (SAS Institute, Cary, NC).

Results

Characterization of Monoclonal Anti-EBAG9 Antibody Using Western Blot Analysis

We generated a monoclonal antibody against human EBAG9 protein to investigate the expression of EBAG9 in human breast cancer samples. The EBAG9 protein contains 2 functional domains, including an N-terminal transmembrane domain and a C-terminal coiled-coil domain. For antibody production, a 166-amino acid region of EBAG9 (amino acids 48-213) containing the coiled-coil domain was used as the antigen (Fig. 1A). The specificity of the anti-EBAG9 antibody is shown in Figure 1B. Western blotting revealed that the monoclonal antibody reacted with a 32-kDa Flag-tagged human EBAG9 protein in 293T cells transfected with the Flag-hEBAG9 expression plasmid. This result indicated that this antibody specifically reacts with EBAG9.

Figure 1 Generation of a Specific EBAG9 Antibody and Immunohistochemical Analysis of EBAG9 in Breast Cancer. (A) Structure of the EBAG9 Protein. The Regions Used for Immunization as the Antigen for Monoclonal Antibody Production are Shown. (B) Specificity of EBAG9 Antibody Determined Using Western Blot Analysis

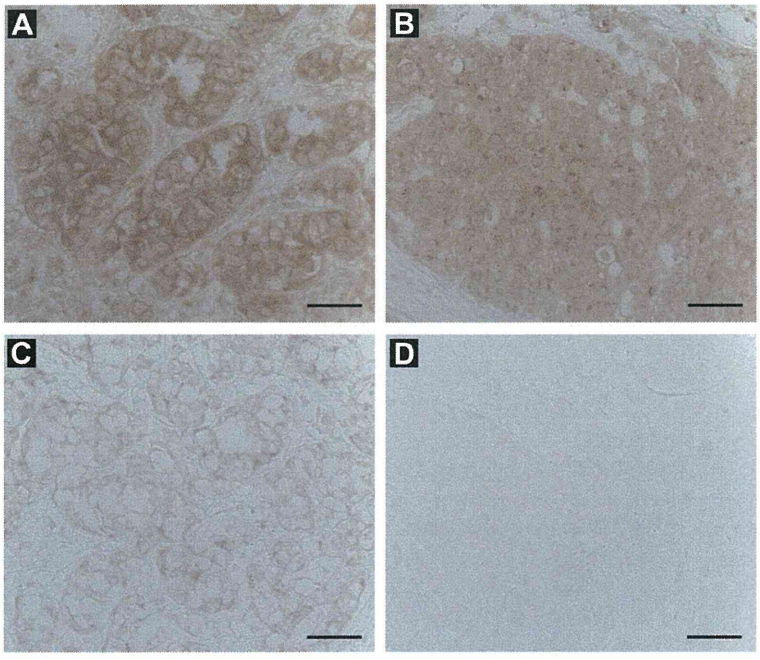


Abbreviations: EBAG9 = estrogen receptor-binding fragment associated antigen 9; hEBAG9 = human EBAG9; pFlag = pcDNA3 plasmid only containing the Flag tag.

Positive EBAG9 Immunoreactivity at Surgery Is Associated With a Poor Prognosis Among Breast Cancer Patients Who Received Postoperative Adjuvant Tamoxifen Treatment

To examine the clinical significance of EBAG9 in tamoxifen treatment of breast cancer, immunohistochemical analysis was performed using 100 breast cancer specimens that were excised from patients at surgery before tamoxifen treatment. The clinicopathological characteristics of the patients are summarized in Table 1. Breast cancer specimens in which more than 50% of the cells expressed EBAG9 were regarded as positive (Fig. 2). EBAG9 immunoreactivity was predominantly observed in the cytoplasm of breast cancer cells, whereas in almost all luminal epithelia, myoepithelia, and stromal cells, EBAG9 immunoreactivity was weak or

Figure 2 Representative Immunohistochemical Staining of Breast Cancer Tissues With Anti-EBAG9 Antibody. Estrogen Receptor-Binding Fragment Associated Antigen 9 Immunoreactivity is Predominantly Observed in the Cytoplasm. (A) and (B) Breast Cancer Specimens in Which More Than 50% of the Cells Expressed EBAG9 Were Regarded as Positive, and (C) Those in Which ≤ 50% of the Cells Expressed EBAG9 Were Regarded as Negative. (D) Mouse Immunoglobulin G was Used in Place of the Primary Antibody as a Negative Control. Scale bar, 100 μm



Abbreviation: EBAG9 = estrogen receptor-binding fragment associated antigen 9.

negative compared with that of cancer cells. In this study, the tamoxifen-treated patients were divided into 2 groups, the relapse and relapse-free groups, on the basis of recurrence within 5 years of surgery. Statistical analysis indicated that the cytoplasmic immunoreactivity of EBAG9 was elevated in the relapse group ($P = .013$; Table 1). Consistent with this observation, the results of Kaplan-Meier survival curve analysis showed that patients with positive EBAG9 immunoreactivity exhibited a shorter relapse-free survival than those with negative EBAG9 staining ($P = .021$ for 5 years and $P = .0024$ for the entire observation period; Fig. 3). These results implied that high EBAG9 expression is correlated with a poor prognosis in patients with tamoxifen-treated breast cancer. The statistical significance of various clinicopathological parameters in this population of breast cancer patients was evaluated using logistic regression analyses (Table 2). In univariate analysis, EBAG9 immunoreactivity ($P = .007$) and lymph node status ($P = .005$) were significantly correlated with a decreased 5-year relapse-free survival. Moreover, in multivariate analysis, EBAG9 immunoreactivity and lymph node status were independent predictors for decreased relapse-free survival (odds ratio, 0.22 and 0.37, respectively; $P = .035$ and .025, respectively). These results suggest that EBAG9 immunoreactivity can independently serve as a biomarker for poor clinical outcomes among breast cancer patients who receive tamoxifen therapy after surgery.

Discussion

In the present study, increased EBAG9 immunoreactivity was significantly correlated with breast cancer relapse after adjuvant tamoxifen treatment. Furthermore, positive EBAG9 expression was significantly correlated with poor relapse-free patient survival. Multivariate analysis also revealed that EBAG9 expression is an independent predictor of poor prognosis.

Because EBAG9 overexpression has been observed in several carcinomas, this molecule has been considered an independent prognostic marker for disease-specific survival.[5,6] The interpretation of previous clinical data for EBAG9 expression in cancers is rather complicated because some researchers consider that the immunoreactivity recognized by the so-called 22-1-1 immunoglobulin M monoclonal antibody is also identical to EBAG9 immunoreactivity. [13] However, the 22-1-1 epitope has been shown to be distinct from the product encoded by EBAG9 (or RCAS1) cDNA, because the 22-1-1 antibody recognizes tumor-associated *O*-linked glycan antigens.[14] EBAG9 is assumed to modulate adaptive immune responses, particularly those mediated by cytotoxic T lymphocytes. For example, our group demonstrated the reduced intratumoral infiltration of cytotoxic T cells in EBAG9-overexpressing renal cell carcinoma models,[5] and other researchers later reported that EBAG9 negatively regulated the cytolytic capacity of mouse $CD8^+$ T cells.[15]

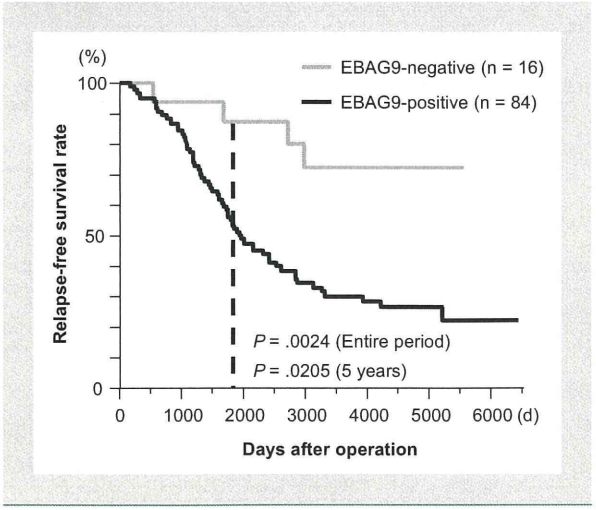
Table 2 Univariate and Multivariate Analyses of Relapse-Free Survival in Breast Cancer Patients Treated With Tamoxifen as Adjuvant Therapy (n = 100)

Variable	Univariate		Multivariate	
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
EBAG9 (Negative vs. Positive)	0.17 (0.03-0.64)	.007[a]	0.22 (0.03-0.91)	.035
Lymph Node (0 vs. ≥1)	0.30 (0.12-0.69)	.005[a]	0.37 (0.15-0.88)	.025
pT (≤30 mm vs. >30 mm)	0.46 (0.20-1.05)	.065	—	—
ERα (Positive vs. Negative)	0.67 (0.15-3.01)	.59	—	—
PgR (Positive vs. Negative)	1.11 (0.40-3.30)	.84	—	—
Age (≤50 vs. >50)	1.43 (0.64-3.20)	.38	—	—

Abbreviations: EBAG9 = estrogen receptor-binding fragment associated antigen 9; ERα = estrogen receptor α; PgR = progesterone receptor; pT = pathological T stage.
[a]Data considered significant in the univariate analyses were examined in the multivariate analyses.

In addition to the potential regulatory function of EBAG9 in antitumor immunity, other mechanisms might also be involved in EBAG9-dependent tumor progression in vivo. One explanation is that altered EBAG9-dependent cell surface glycosylation makes the tumor microenvironment more favorable for tumor growth and cell migration. Interestingly, overexpression of EBAG9 cDNA in cell lines negative for 22-1-1 surface staining led to the generation of the *O*-linked glycan antigens, *N*-acetyl-d-galactosamine (GalNAc; Tn) and Thomsen-Friedenreich (Galβ1-3GalNAc), typical of many carcinomas.[14] In terms of the pathological relevance of *O*-linked glycans, it has been reported that the ectopic expression of sialyl-Tn (GalNAc) in MDA-MB-231 breast cancer cells substantially modifies the *O*-glycosylation pattern and causes decreased adhesion and increased cell migration.[16] Moreover, sialyl-Tn–positive MDA-MB-231 cells exhibit increased tumor growth in severe combined immunodeficiency mice.[16] With respect to tumor-associated glycosylation, EBAG9 is assumed to act as a negative regulator of the endoplasmic reticulum-to-Golgi transport pathway in epithelial cells, which interferes with intracellular membrane trafficking and normal secretion processes.[17]

Figure 3 Kaplan-Meier Survival Analysis According to EBAG9 Immunoreactivity in Breast Cancer Tissues (n = 100). Relapse-Free Survival of Breast Cancer Patients who Received Postoperative Adjuvant Tamoxifen Treatment was Analyzed Using the Log-Rank Test Based on EBAG9 Immunoreactivity



Abbreviation: EBAG9 = estrogen receptor-binding fragment associated antigen 9.

Another possible explanation is that EBAG9 might stimulate angiogenesis by upregulating growth factors or cytokines. Indeed, introduction of the gene encoding EBAG9 in COS-7 cells has been reported to increase the expression of vascular endothelial growth factor and promote the in vivo growth of tumors derived from the COS-7 transfectants.[18] Although tamoxifen has been reported to exert an antiangiogenic effect on breast cancer that opposes the angiogenic effect of estrogen,[19] EBAG9 expression might reverse the effect of tamoxifen from inhibitory to stimulatory in breast cancer in this regard. Overall, we assumed that these functions, namely suppression of antitumor immunity, facilitation of tumor cell migration, and promotion of angiogenesis, would be involved in the acquisition of tamoxifen resistance in EBAG9-overexpressing breast cancer.

Because EBAG9 was originally identified as an estrogen-responsive gene in MCF-7 cells and EBAG9 mRNA levels in this cell line are upregulated by estrogen,[2] ERα appears to be an essential regulator of EBAG9 expression. Our previous clinical data also support this notion because EBAG9 immunoreactivity was significantly correlated with the ERα labeling index in breast cancer.[10] These findings suggest that EBAG9 functions as an estrogen-responsive gene in ER-positive breast cancer cells. However, promoter analysis demonstrated that the 5′-flanking region of the EBAG9 gene contains several transcription factor-binding sites in addition to a prototypic consensus estrogen-responsive element,[20] suggesting that other transcriptional regulatory pathways also regulate EBAG9 expression in breast cancer cells. High-level EBAG9 expression mediated by factors other than ER might result in an unfavorable prognosis in breast cancer patients after tamoxifen treatment. Disruption of tamoxifen-mediated ER regulation might also help maintain the higher level of EBAG9 expression in tamoxifen-resistant breast cancer cells.

The present study shows that EBAG9 immunoreactivity will be a potential biomarker for predicting the prognosis of breast cancer patients treated with adjuvant tamoxifen therapy. This study investigated EBAG9 immunoreactivity in invasive breast cancer treated with tamoxifen, depending on the enzyme immunoassay results of ER or PgR positivity, which was eligible for tamoxifen therapy at the time between 1989 and 1998. Although a category of ER-negative/

PgR-positive tumors is minor, PgR expression has been considered an indication of an intact ER-estrogen response pathway because PgR is primarily a direct ER target gene. Therefore, ER-negative/PgR-positive tumors could be distinct from ER-negative/PgR-negative tumors. Moreover, some of the ER-negative/PgR-positive tumors have been determined falsely using enzyme immunoassay, because the method is less sensitive than immunohistochemisty for ER testing reliability. For these reasons, we assumed that ER-negative/PgR-positive tumors in our study might be also hormone-dependent. Although we evaluated the association between EBAG9 expression and clinical parameters in 8 cases of ER-negative/PgR-positive tumors, no significant correlation was shown in this study. The result might be because of the small number of samples. It is also notable that there is a significant positive correlation between EBAG9 and ER labeling index in clinical specimens from breast cancer patients recruited regardless of ER status.[10] In addition, the research will be further extended by designing new studies in subsets of patient groups, such as cases with tamoxifen or aromatase inhibitor treatment, cases treated with adjuvant chemotherapy alone, cases with ER-negative tumors, or cases with ductal carcinoma in situ. In those studies, it would be interesting if we can collect clinical samples before and after treatment. Future studies will reveal the role of EBAG9 in these subsets of breast cancer.

Conclusion

The current study demonstrated that increased EBAG9 immunoreactivity in breast cancer tissues derived from tamoxifen-treated patients was significantly associated with a poor patient prognosis, suggesting that EBAG9 contributes to tamoxifen resistance in ER-positive breast cancers. EBAG9 expression is a potential marker that can aid in selecting breast cancer treatment options.

Clinical Practice Points

- Acquired tamoxifen resistance is a major clinical challenge in the treatment of breast cancer, however, the factors related to the resistance are not fully characterized.
- Estrogen receptor-binding fragment associated antigen 9 has been implicated in the development and progression of multiple solid tumors including breast cancers, possibly via a mechanism in which EBAG9 hampers antitumor immunity.
- Here we investigated the clinical significance of EBAG9 in breast cancer treated with adjuvant tamoxifen therapy. Immunohistochemical analysis for EBAG9 was performed in 100 breast cancer specimens excised from patients at surgery before tamoxifen treatment.
- Estrogen receptor-binding fragment associated antigen 9 immunoreactivity (> 50% of the total cells) was significantly correlated with relapse events of the patients within 5 years after surgery ($P = .013$).
- In univariate analysis, EBAG9 immunoreactivity ($P = .007$) and lymph node status ($P = .005$) were significantly correlated with decreased 5-year relapse-free survival.
- In multivariate analysis, EBAG9 immunoreactivity and lymph node status were independent predictors for decreased 5-year relapse-free survival (odds ratio, 0.22 and 0.37, respectively; $P = .035$ and .025, respectively).
- Estrogen receptor-binding fragment associated antigen 9 expression would predict the patients' prognosis of tamoxifen treatment after surgery.

Acknowledgments

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Disclosure

The authors have stated that they have no conflicts of interest.

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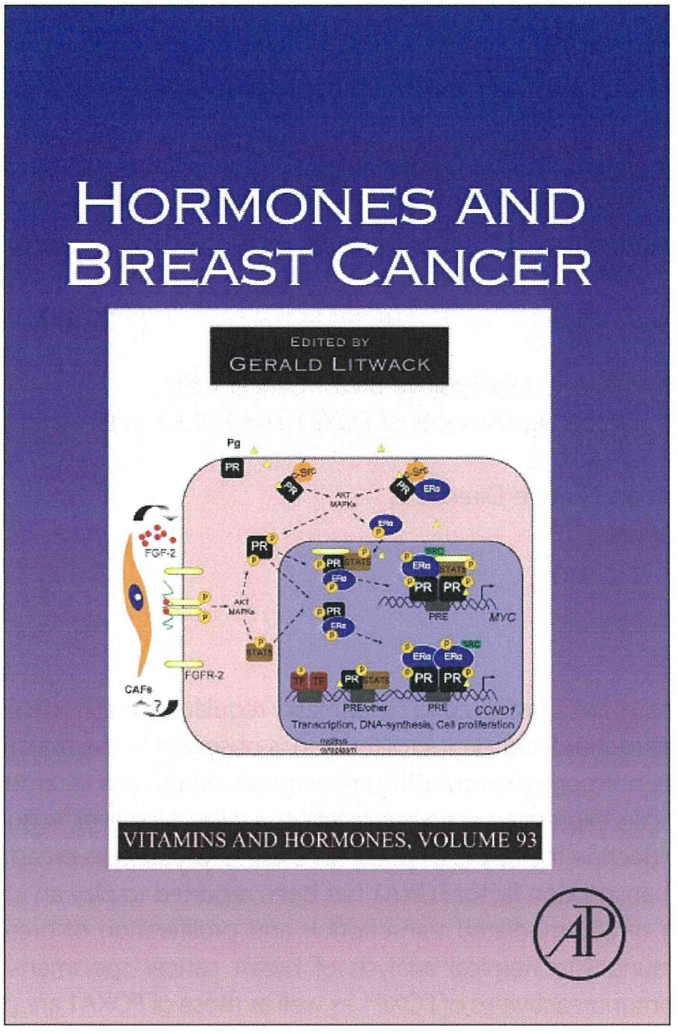

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CHAPTER SEVEN

FOXP1 and Estrogen Signaling in Breast Cancer

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Contents

Abstract

Breast cancers are considered to be primarily regulated by estrogen signaling pathways because estrogen-dependent proliferation is observed in the majority of breast cancer cases. Thus, hormone therapy using antiestrogen drugs such as tamoxifen is effective for breast cancers expressing estrogen receptor α (ERα). However, acquired resistance during the endocrine therapy is a critical unresolved problem in breast cancer. Recently, a forkhead transcription factor FOXA1 has been reported to play an important role in the regulation of ERα-mediated transcription and proliferation of breast cancer. Interestingly, immunohistochemical analysis of breast cancer specimens has revealed that nuclear immunoreactivities of FOXP1 as well as those of FOXA1 are positively correlated with hormone receptor status, including ERα and progesterone receptor. In particular, the double-positive immunoreactivities of FOXP1 and FOXA1 are significantly associated with a favorable prognosis for survival of breast cancer patients receiving adjuvant tamoxifen therapy. The functions of FOXP1 and FOXA1 have been characterized in cultured cells; further, similar to FOXA1, FOXP1 is assumed to be a critical transcription factor for ERα signaling, and both forkhead transcription factors can serve as predictive factors for acquired endocrine resistance in breast cancer.

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1. INTRODUCTION

Estrogen is a sex steroid hormone that regulates various cellular events through its cognate estrogen receptor α (ERα), which functions as a transcription factor that activates the transcription of its target genes (Platet, Cathiard, Gleizes, & Garcia, 2004). Clinically, ERα is noted as the defining feature of luminal breast cancer, which accounts for a large portion of breast cancers. Luminal breast cancer is generally treated with endocrine therapy using classical antiestrogen agents such as tamoxifen, which acts as an antagonist for ERα in breast cancer cells. Because of the sensitivity to endocrine therapy, ERα-positive luminal breast cancer is considered to have better prognosis than ERα-negative breast cancer. However, resistance to antiestrogen therapies is often acquired in a substantial fraction of recurrent breast cancers. Identification of the factors involved in the mechanisms underlying endocrine resistance, recurrence, or poor prognosis of breast cancer will be useful for understanding the exact pathophysiology of the disease and for developing alternative diagnostic methods and treatment specific to the disease.

The transcriptional activity of ERα is regulated by a number of regulatory cofactors, including chromatin-remodeling complexes, coactivators, and corepressors (Hall & McDonnell, 2005). Moreover, several transcription factors, including those belonging to the forkhead box (FOX) family, modulate the transcriptional activity of ERα. In particular, as described in detail below, FOXA1 plays a crucial role in the ERα-mediated transcription in breast cancer cells (Carroll et al., 2005; Hurtado, Holmes, Ross-Innes, Schmidt, & Carroll, 2011; Lupien et al., 2008). In addition, recent clinicopathological and *in vitro* studies have shown that another member belonging to the FOX family, FOXP1, is closely related to the biology of breast cancer (Ijichi et al., 2012; Shigekawa et al., 2011). This chapter focuses on the potential role of FOXP1 compared to that of FOXA1 in the pathophysiology of breast cancer and discusses the clinical relevance of these forkhead factors in the disease, particularly in association with hormone therapy.

2. FOXP1 AND FOXA1 IN ERα-POSITIVE BREAST CANCER CELLS

FOXP1 and FOXA1 are transcription factors, which belong to the FOX family that includes a conserved forkhead DNA-binding domain

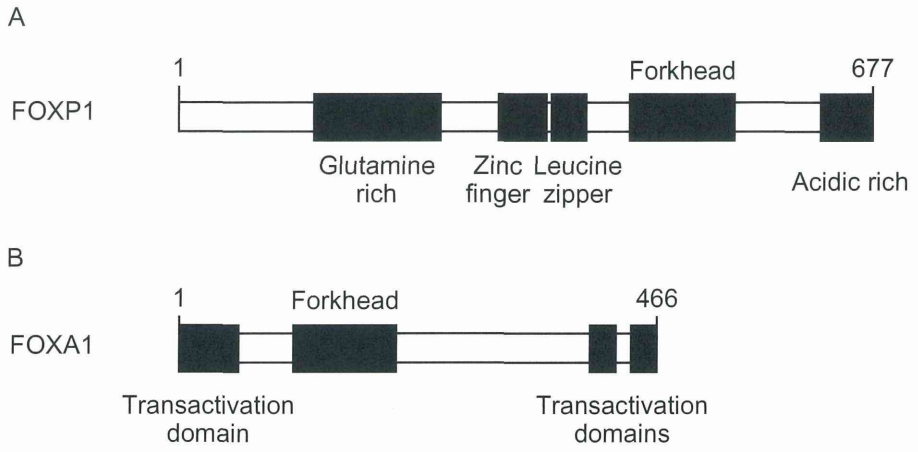

Figure 7.1 Schematic representation of the domain structure of forkhead box transcription factor FOXA1 and FOXP1 proteins. (A) FOXP1 structure. Forkhead domain is located in the C-terminal region of the FOXP1 protein. Zinc finger and leucine zipper domains, responsible for dimerization of FOXP1, are located in the central region. (B) FOXA1 structure. Forkhead domain is located in N-terminal region of FOXA1 protein. Three transactivation domains are located in both terminal regions.

(Fig. 7.1; Li, Weidenfeld, & Morrisey, 2004; Wang, Lin, Li, & Tucker, 2003). Recently, genome-wide studies with an aim of identifying ERα- and androgen receptor (AR)-binding sites have shown that FOXA1 plays a role in regulation of the nuclear receptor-mediated gene networks (Carroll et al., 2005, 2006; Lupien et al., 2008). FOXA1 is recognized as a pioneer transcription factor because binding of FOXA1 to chromatin DNA facilitates subsequent recruitment of ERα and AR to the genome (Grange, Roux, Rigaud, & Pictet, 1991; Lupien et al., 2008). Genome-wide mapping of ERα-, AR-, and FOXA1-binding events in breast and prostate cancer cells using high-throughput sequencing has further uncovered the involvement of several collaborative factors, including TLE1 and activator protein 2γ (AP-2γ), in the nuclear receptor-mediated transcription (Holmes et al., 2012; Tan et al., 2011). In breast cancer cells, several FOX family transcription factors may contribute to the ERα-mediated transcription by directly interacting with the ERα protein, as exemplified by FOXA1 and FKHR/FOXO1 (Carroll & Brown, 2006; Schuur et al., 2001).

Recent studies have shown that FOXP1 and FOXA1 play critical roles in estrogen signaling and in the biology of ERα-positive breast cancer (Ijichi et al., 2012; Shigekawa et al., 2011). These studies showed an upregulation in the expressions of *FOXP1* and *FOXA1* mRNAs induced by 17β-estradiol (E2) stimulation in ERα-positive MCF-7 cells. The

upregulation of both genes was observed in the early phase (3 h) after E2 stimulation, which suggests that both FOXP1 and FOXA1 are transcriptionally regulated by the estrogen in breast cancer cells. In addition, Giguère and his colleagues reported that estrogen upregulates the levels of FOXA1 protein 4–8 h after E2 stimulation (Laganière et al., 2005). Consistent with these findings, the findings of genome-wide chromatin immunoprecipitation (ChIP) analysis based on microarrays (ChIP-chip) showed three and two functional estrogen receptor-binding sites (ERBSs) within the *FOXP1* and *FOXA1* gene loci, respectively, in the genome of MCF-7 cells (Carroll et al., 2005). Conventional ChIP assay showed more than twofold enrichments of estrogen-dependent recruitment of ERα in these ERBSs, which suggested that the recruitment of ERα in the *FOXP1* and *FOXA1* loci contributes to the estrogen-dependent transcription of both *FOX* genes.

Further, FOXP1 and FOXA1 have been shown to serve as transcription factors that directly regulate the ERα-mediated transcription. Luciferase reporter analysis using a vector containing an estrogen-responsive element (ERE, ERE-tk-*luc*) showed that overexpression of either FOXP1 or FOXA1 significantly stimulated the ERα-mediated transactivation in MCF-7 cells in response to estrogen. siRNA-mediated knockdown of FOXA1 reduced ERα-mediated transactivation in the presence or absence of estrogen in MCF-7 cells. Consistent with these observations, the results of other studies showed upregulation of known estrogen-responsive genes, including *SHP* (Lai, Harnish, & Evans, 2003) and *LRH-1* (Annicotte et al., 2005), in FOXP1-overexpressing MCF-7 cells treated with estrogen. Similarly, the contribution of FOXA1 to ERα-mediated transcription was further confirmed by the FOXA1 siRNA-dependent reduction in estrogen-induced expressions of prototypic ERα target genes, progesterone receptor (*PgR*), and growth regulation by estrogen in breast cancer 1 (*GREB1*) (Ghosh, Thompson, & Weigel, 2000; Kastner et al., 1990). These observations suggest that both FOXP1 and FOXA1 stimulate ERα transcription activity in response to estrogen.

The mutual transcriptional regulations of *ERα* and *FOX* genes indicate that both FOXA1 and FOXP1 have the potential to promote estrogen-dependent proliferation of breast cancer cells. Moreover, FOXA1 also upregulates the migration of MCF-7 cells. These findings suggest that FOXP1 and FOXA1 regulate ERα in a positive feedback manner and play crucial roles in the estrogen-dependent cellular responses of ERα-positive breast cancer cells (Fig. 7.2).

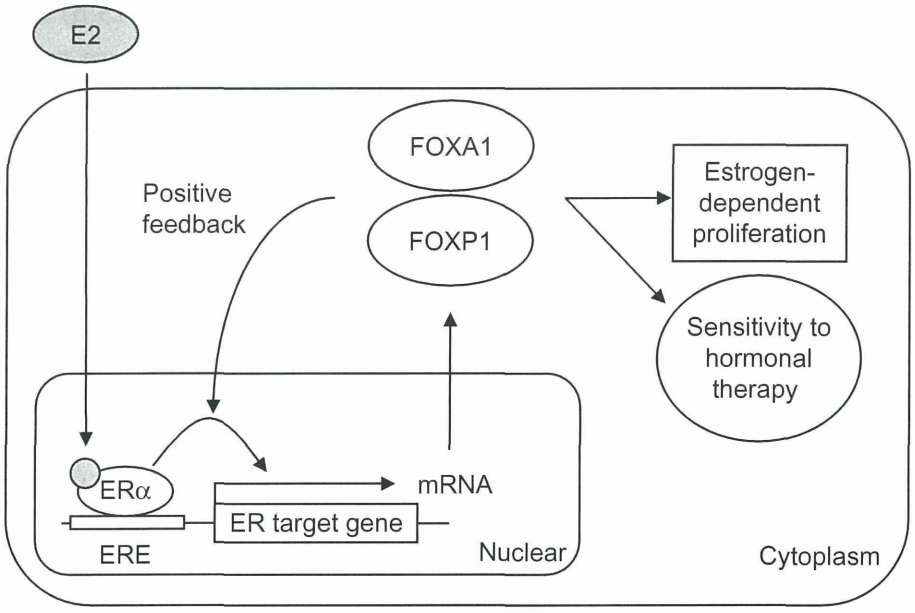

Figure 7.2 Model for cellular functions of FOXP1 and FOXA1 in estrogen signaling. *FOXP1* and *FOXA1* are primary target genes for estrogen receptor α (ERα) and regulate the ERα-mediated transcription in a positive feedback manner. FOXP1 and FOXA1 promote estrogen-dependent proliferation of breast cancer cells and contribute to the sensitivity to hormone therapy.

3. CLINICOPATHOLOGICAL SIGNIFICANCES OF FOXP1 AND FOXA1 IN ER-POSITIVE BREAST CANCER

Recent global gene expression studies on breast cancer have shown that high FOXA1 expression was positively correlated with the status of hormone receptors ERα and PgR and negatively correlated with histological grade and proliferation markers (Badve et al., 2007; Habashy et al., 2008; Thorat et al., 2008). In addition, FOXA1 expression was associated with better prognosis of cancer-specific survival, which indicated that FOXA1 can serve as a predictor for good prognosis of breast cancer (Badve et al., 2007; Habashy et al., 2008; Thorat et al., 2008; Wolf et al., 2007). On the basis of gene expression profiling studies, researchers have classified breast cancers into the following five intrinsic subtypes with unique molecular characteristics and prognostic significance (Perou et al., 2000; Sørlie et al., 2001): luminal A and B, HER2+/ERα−, basal-like, and normal-like subtypes. Luminal subtypes A and B are ERα-positive breast cancers, distinguishing subtype A from B by its higher levels of ERα and better prognosis of the patients (Sørlie et al., 2001). Among these subtypes, FOXA1