[25] D. Liu, L. Ge, F. Wang, H. Takahashi, D. Wang, Z. Guo, S.-H. Yoshimura, T. Ward, X. Ding, K. Takeyasu and X. Yao, "Single-Molecule Detection of Phosphorylation-Induced Plasticity Changes during Ezrin Activation," *FEBS Letters*, Vol. 581, No. 18, 2007, pp. 3563-3571. http://dx.doi.org/10.1016/j.febslet.2007.06.071

[26] L. Zhu, R. Zhu, S. Mettler, T. Wu, A. Abbas, J. Delaney and J. G. Forte, "High Turnover Ezrin T567 Phophorylation: Comformation, Activity, and Cellular Function," *American Journal of Physiology Cell Physiology*, Vol. 293, No. 3, 2007, pp. C874-C884. http://dx.doi.org/10.1152/ajpcell.00111.2007

[27] D. Hanzel, H. Reggio, A. Bretscher, J. G. Forte and P. Mangeat, "The Secretion-Stimulated 80K Phophoprotein of Parietal Cells Is Ezrin, and Has Properties of a Membrane Cytoskeletal Linker in the Induced Apical Membrane," *The EMBO Journal*, Vol. 10, No. 9, 1991, pp. 2363-2373.

[28] R. Zhou, X. Cao, C. Watson, Y. Miao, Z. Guo, J. G. Forte and X. Yao, "Characterization of Protein Kinase A-Mediated Phosphorylation of Ezrin in Gastric Parietal Cell Activation," *The Journal of Biological Chemistry*, Vol. 278, No. 37, 2003, pp. 35651-35659. http://dx.doi.org/10.1074/jbc.M303416200

[29] R. Zhou, L. Zhu, A. Kodani, P. Hauser, X. Yao and J. G. Forte, "Phosphorylation of Ezrin on Threonine 567 Produces a Change in Secretory Phenotype and Repolarizes the Gastric Parietal Cell," *Journal of Cell Science*, Vol. 118, No. 19, 2005, pp. 4381-4391. http://dx.doi.org/10.1242/jcs.02559

[30] V. Fykse, E. Solligård, MØ. Bendheim, D. Chen, J. E. Grønbech, A. K. Sandvik and H. L. Waldum, "ECL Cell Histamine Mobilization and Parietal Cell Stimulation in the Rat Stomach by Microdialysis and Electron Microscopy," *Acta Physiologica*, Vol. 186, No. 1, 2006, pp. 37-43. http://dx.doi.org/10.1111/j.1748-1716.2005.01504.x

Original Study

Association of Positive EBAG9 Immunoreactivity With Unfavorable Prognosis in Breast Cancer Patients Treated With Tamoxifen

Nobuhiro Ijichi,[1] Takashi Shigekawa,[1,2,3] Kazuhiro Ikeda,[1] Toshiaki Miyazaki,[1] Kuniko Horie-Inoue,[1] Chikako Shimizu,[4] Shigehira Saji,[3,5] Kenjiro Aogi,[6] Hitoshi Tsuda,[7] Akihiko Osaki,[2] Toshiaki Saeki,[2] Satoshi Inoue[1,8,9]

Abstract

Acquired tamoxifen resistance in breast cancer has not been fully understood. We examined immunohistochemical staining of estrogen receptor-binding fragment associated antigen 9 (EBAG9) in 100 breast cancer specimens excised from patients at surgery before tamoxifen treatment. Positive EBAG9 immunoreactivity (> 50% of the total cells) was significantly associated with decreased disease-free survival. EBAG9 expression will be a prognostic factor in breast cancer patients treated with adjuvant tamoxifen therapy.

Introduction: Breast cancer is primarily a hormone-dependent tumor that is regulated by the status of the estrogen and progesterone receptors. We previously identified EBAG9 as an estrogen-responsive gene in MCF-7 human breast carcinoma cells. Upregulation of EBAG9 expression has been observed in several malignant tumors such as advanced breast cancers, indicating that EBAG9 might contribute to tumor progression. **Patients and Methods:** In the present study, we generated a monoclonal antibody against EBAG9, and then performed immunohistochemical analysis of EBAG9 expression in specimens obtained from breast cancer patients treated with tamoxifen as an adjuvant therapy. **Results:** EBAG9 immunoreactivity was detected in the cytoplasm of breast cancer cells and was significantly elevated in breast cancer samples from patients who relapsed during or after adjuvant tamoxifen treatment. Positive EBAG9 immunoreactivity was significantly correlated with poor patient prognosis. **Conclusion:** These results suggest that EBAG9 expression in tumor regions is associated with an unfavorable prognosis in breast cancer patients treated with tamoxifen.

Clinical Breast Cancer, Vol. 13, No. 6, 465-70
Keywords: EBAG9, Endocrine therapy, Immunohistochemistry, Prognostic factor, Tamoxifen resistance

Introduction

Estrogen signaling pathways regulate various cellular events, including cell growth and apoptosis, through activation of the estrogen receptor (ER).[1] The ER functions as a transcription factor that activates the expression of target genes. ERα expression is clinically recognized as the defining feature of the luminal subtype of breast cancer, which is a predominant subtype of breast cancer characterized by a specific mRNA expression profile. The luminal subtype is generally sensitive to endocrine therapies, including the first-generation selective ER modulator, tamoxifen, which antagonizes the function of the ER in breast cancer cells. In general, patients with ER-positive luminal breast cancers are considered to

[1]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan
[2]Department of Breast Oncology, International Medical Center, Saitama Medical University, Saitama, Japan
[3]Division of Clinical Trials and Research and Department of Surgery, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, Tokyo, Japan
[4]Department of Breast and Medical Oncology, National Cancer Center Hospital, Tokyo, Japan
[5]Department of Target Therapy Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[6]Department of Breast Oncology, National Shikoku Cancer Center, Ehime, Japan
[7]Department of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan
[8]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[9]Department of Anti-Aging Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Submitted: Dec 28, 2012; Revised: Jun 5, 2013; Accepted: Aug 26, 2013; Epub: Oct 8, 2013

Address for correspondence: Satoshi Inoue, MD, PhD, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Fax: +81-42-984-4541; e-mail contact: INOUE-GER@h.u-tokyo.ac.jp

1526-8209/$ - see frontmatter
http://dx.doi.org/10.1016/j.clbc.2013.08.015

have a better prognosis than patients with ER-negative breast cancers. Nonetheless, patients with the luminal subtype often acquire resistance to endocrine therapy during the course of breast cancer management and experience recurrences. Thus, identification of the factors involved in endocrine resistance and recurrence of breast cancer will be useful for determining molecular targets for the diagnosis and treatment of the disease.

We previously identified ER-binding fragment associated antigen 9 (EBAG9) as a primary estrogen-responsive gene using the genomic binding-site cloning technique.[2] EBAG9 is an approximately 32-kDa single-pass transmembrane protein with a C-terminal coiled-coil domain.[3] The physiological function of EBAG9 has not been well defined. However, this protein has been implicated in cancer development and progression because tumor-associated aspects of EBAG9 expression have been noted in multiple malignant tumors, including prostate,[4] renal cell,[5] bladder,[6] testicular,[7] hepatocellular,[8] ovarian,[9] and breast cancers.[10] The results of our previous studies suggest that EBAG9 hampers antitumor immunity because overexpression of this molecule reduces the number of tumor-infiltrating lymphocytes.[5,10] Thus, it is assumed that EBAG9 contributes to the pathophysiology of various cancers by modulating endocrine-immune interactions in the tumor microenvironment.

In the present study, we investigated the clinical relevance of EBAG9 expression in breast cancer tissues from patients treated with tamoxifen as an adjuvant therapy. Positive cytoplasmic EBAG9 immunoreactivity was correlated with breast cancer relapse in tamoxifen-treated patients. Our findings suggest that EBAG9 is a potential predictive marker for the therapeutic effect of tamoxifen on breast cancers.

Table 1 Clinicopathological Findings in Adjuvant Tamoxifen-Treated Invasive Breast Cancer Patients Followed-Up For 5 Years After Surgery (n = 100)

Clinical Findings	Relapse (n = 41)	Relapse-Free (n = 59)	*P*
Age (Mean ± SD)	52.4 ± 10.0	54.3 ± 12.2	.424
Age			.383
≤50	21 (21.0)	25 (25.0)	
>50	20 (20.0)	34 (34.0)	
pT			.064
≤30 mm	21 (21.0)	41 (41.0)	
>30 mm	20 (20.0)	18 (18.0)	
Lymph Node			.005
Positive (n ≥ 1)	30 (30.3)	26 (26.3)	
Negative (n = 0)	11 (11.1)	32 (32.3)	
ERα			.869
Positive	37 (37.0)	55 (55.0)	
Negative	4 (4.0)	4 (4.0)	
PgR			.949
Positive	34 (34.0)	48 (48.0)	
Negative	7 (7.0)	11 (11.0)	
EBAG9			.013
Positive	39 (39.0)	45 (45.0)	
Negative	2 (2.0)	14 (14.0)	

Data are presented as n (%) except where otherwise noted.
Abbreviations: EBAG9 = estrogen receptor-binding fragment associated antigen 9; ERα = estrogen receptor α; PgR = progesterone receptor; pT = pathological T stage.

Patients and Methods

Tissue Selection and Patient Characteristics

For a nested-control study of the therapeutic effect of tamoxifen on recurrent breast cancer, 100 patients were recruited from 3 institutions (National Hospital Organization Shikoku Cancer Center, Matsuyama, Japan; National Cancer Center Hospital, Tokyo, Japan; and Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan). These patients had been diagnosed with breast cancer between 1989 and 1998, and developed distant metastases during or after adjuvant tamoxifen therapy. The hormone receptor status of the patients was determined using enzyme immunoassays of ER and progesterone receptor (PgR) proteins. They did not receive any other systemic therapy. Patients experiencing relapse were defined as those with distant metastases within 5 years of surgery followed by tamoxifen treatment, and patients without distant metastases were considered relapse-free. Formalin-fixed paraffin-embedded sections of tissues obtained during biopsy or surgery were used. These studies were approved by the institutional review board of participating hospitals and Saitama Medical University, and informed consent was obtained from all patients. The clinicopathological characteristics of the patients are presented in Table 1.

Antibodies

Mouse monoclonal anti-EBAG9 antibody (#C57-8) was generated using a recombinant protein containing amino acids 48-213 of the human EBAG9 protein fused to glutathione S-transferase as described elsewhere.[11] The antibody was affinity purified using the antigen.

Immunohistochemistry

Immunohistochemical analysis of EBAG9 expression was performed using an EnVision+ visualization kit (Dako, Carpinteria, CA) as previously described.[12] The tissue sections (6 μm) were deparaffinized, rehydrated through a graded ethanol series, and rinsed in Tris-buffered saline containing 0.05% Tween-20 (TBST). For antigen retrieval, the sections were heated in an autoclave at 121°C for 5 minutes in a 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with endogenous peroxidase (0.3% H_2O_2) and incubated in 10% fetal bovine serum for 30 minutes. The primary antibody, a monoclonal antibody against EBAG9 (1:200 dilution), was applied, and the samples were incubated overnight at 4°C. The sections were rinsed in TBST and incubated with EnVision+ horseradish peroxidase-labeled polymer for 1 hour at room temperature. The antigen-antibody complex was visualized using a 3,3′-diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Mouse immunoglobulin G was used in place of the primary antibody as a negative control.

Immunohistochemical Assessment

The slides were evaluated for the proportion of positively stained cells. Two investigators (HT and AO) evaluated the tissue sections independently. If the immunoreactivity score differed

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

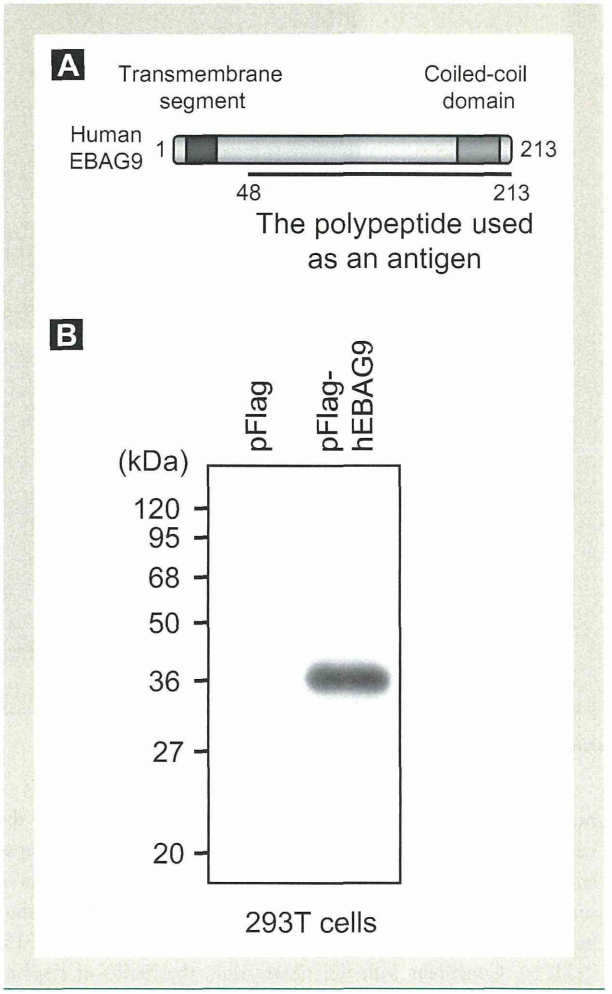
The correlation between the immunoreactivity score and clinicopathological characteristics was evaluated using the χ^2 test. A *P*-value of < .05 was regarded as statistically significant. Differences between the 2 groups were analyzed using 2-sample, 2-tailed Student *t* test. Relapse-free and overall 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

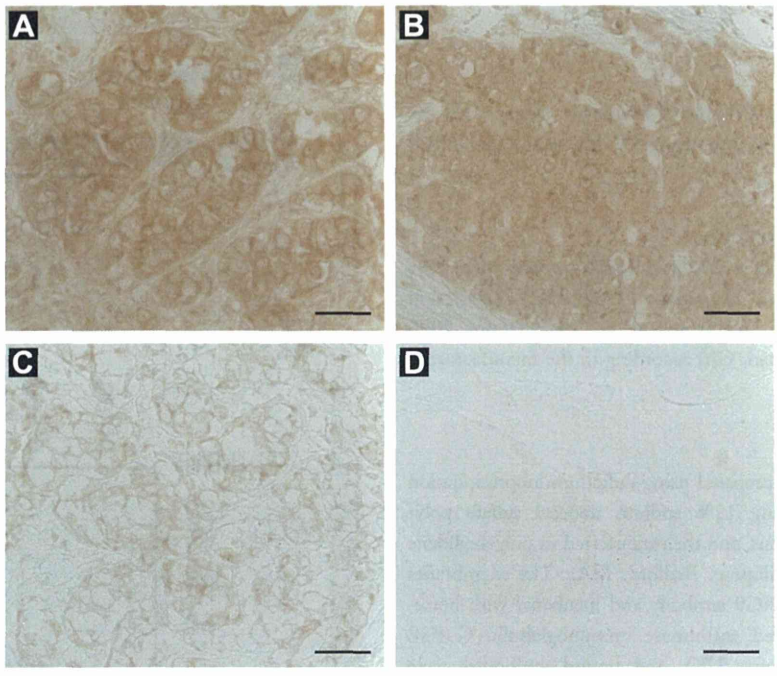


Abbreviations: EBAG9 = estrogen receptor-binding fragment associated antigen 9; hEBAG9 = human EBAG9; pFlag = pcDNA3 plasmid only containing the Flag tag.

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]

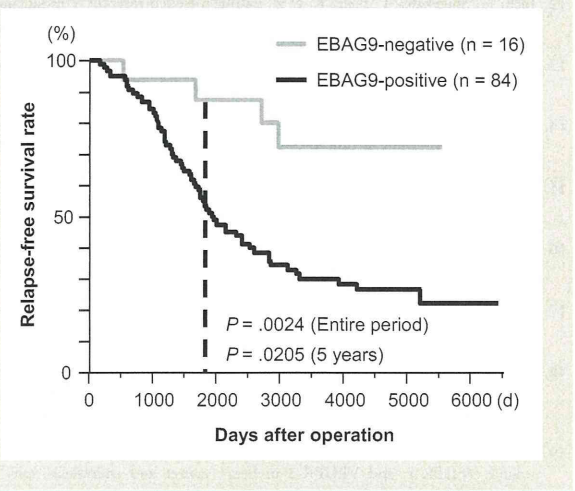
Table 2 Univariate and Multivariate Analyses of Relapse-Free Survival in Breast Cancer Patients Treated With Tamoxifen as Adjuvant Therapy (n = 100)

	Univariate		Multivariate	
Variable	**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

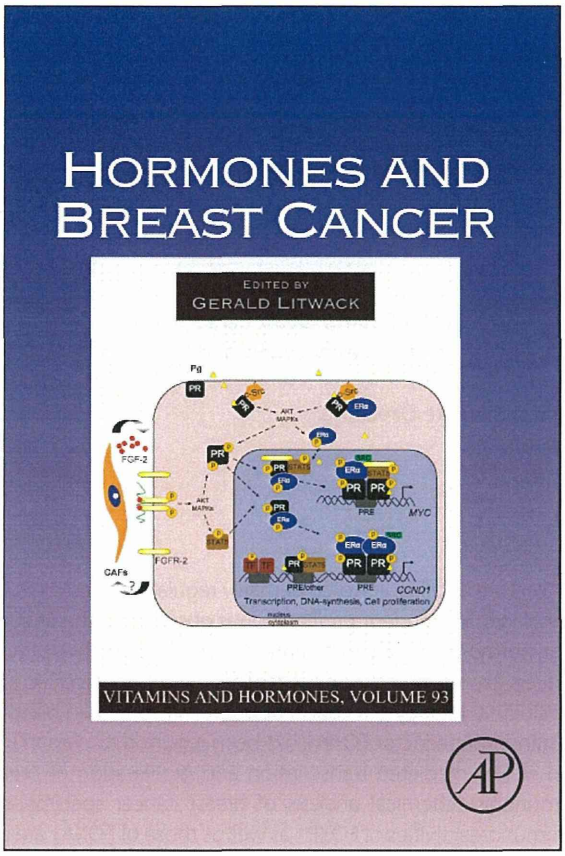
The authors thank Mr W. Sato, Ms S. Miyoshi, and Ms K. Kanegae for their technical assistance, and Dr S. Horiguchi, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, for preparation of specimens. This work was supported in part by Grants-in-Aid for Cancer Research (21-4) from the Ministry of Health, Labor and Welfare; the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation; grants from Japan Society for the Promotion of Science, grants of the Cell Innovation Program and the Support Project of Strategic Research Center in Private Universities from the Ministry of Education, Culture, Sports, Science and Technology.

Disclosure

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

References

1. Platet N, Cathiard AM, Gleizes M, et al. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev Oncol Hematol* 2004; 51:55-67.
2. Watanabe T, Inoue S, Hiroi H, et al. Isolation of estrogen-responsive genes with a CpG island library. *Mol Cell Biol* 1998; 18:442-9.
3. Tsuchiya F, Ikeda K, Tsutsumi O, et al. Molecular cloning and characterization of mouse EBAG9, homolog of a human cancer associated surface antigen: expression and regulation by estrogen. *Biochem Biophys Res Commun* 2001; 284:2-10.
4. Takahashi S, Urano T, Tsuchiya F, et al. EBAG9/RCAS1 expression and its prognostic significance in prostatic cancer. *Int J Cancer* 2003; 106:310-5.
5. Ogushi T, Takahashi S, Takeuchi T, et al. Estrogen receptor-binding fragment-associated antigen 9 is a tumor-promoting and prognostic factor for renal cell carcinoma. *Cancer Res* 2005; 65:3700-6.
6. Kumagai J, Urano T, Ogushi T, et al. EBAG9 is a tumor-promoting and prognostic factor for bladder cancer. *Int J Cancer* 2009; 124:799-805.
7. Fujimura T, Takahashi S, Urano T, et al. Estrogen receptor-binding fragment-associated gene 9 expression and its clinical significance in human testicular cancer. *Int J Urol* 2009; 16:329-32.
8. Aoki T, Inoue S, Imamura H, et al. EBAG9/RCAS1 expression in hepatocellular carcinoma: correlation with tumour dedifferentiation and proliferation. *Eur J Cancer* 2003; 39:1552-61.
9. Akahira JI, Aoki M, Suzuki T, et al. Expression of EBAG9/RCAS1 is associated with advanced disease in human epithelial ovarian cancer. *Br J Cancer* 2004; 90: 2197-202.
10. Suzuki T, Inoue S, Kawabata W, et al. EBAG9/RCAS1 in human breast carcinoma: a possible factor in endocrine-immune interactions. *Br J Cancer* 2001; 85: 1731-7.
11. Kuhara M, Yoshino T, Shiokawa M, et al. Magnetic separation of human podocalyxin-like protein 1 (hPCLP1)-positive cells from peripheral blood and umbilical cord blood using anti-hPCLP1 monoclonal antibody and protein A expressed on bacterial magnetic particles. *Cell Struct Funct* 2009; 34:23-30.
12. Ijichi N, Shigekawa T, Ikeda K, et al. Estrogen-related receptor γ modulates cell proliferation and estrogen signaling in breast cancer. *J Steroid Biochem Mol Biol* 2011; 123:1-7.
13. Nakashima M, Sonoda K, Watanabe T. Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1. *Nat Med* 1999; 5:938-42.
14. Engelsberg A, Hermosilla R, Karsten U, et al. The Golgi protein RCAS1 controls cell surface expression of tumor-associated *O*-linked glycan antigens. *J Biol Chem* 2003; 278:22998-3007.
15. Rüder C, Höpken UE, Wolf J, et al. The tumor-associated antigen EBAG9 negatively regulates the cytolytic capacity of mouse $CD8^+$ T cells. *J Clin Invest* 2009; 119:2184-203.
16. Julien S, Adriaenssens E, Ottenberg K, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. *Glycobiology* 2006; 16:54-64.
17. Wolf J, Reimer TA, Schuck S, et al. Role of EBAG9 protein in coat protein complex I-dependent glycoprotein maturation and secretion processes in tumor cells. *FASEB J* 2010; 24:4000-19.
18. Sonoda K, Miyamoto S, Yamazaki A, et al. Biologic significance of receptor-binding cancer antigen expressed on SiSo cells (RCAS1) as a pivotal regulator of tumor growth through angiogenesis in human uterine cancer. *Cancer* 2007; 110: 1979-90.
19. Garvin S, Nilsson UW, Dabrosin C. Effects of oestradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells. *Br J Cancer* 2005; 93:1005-10.
20. Ikeda K, Sato M, Tsutsumi O, et al. Promoter analysis and chromosomal mapping of human EBAG9 gene. *Biochem Biophys Res Commun* 2000; 273:654-60.



From Nobuhiro Ijichi, Kazuhiro Ikeda, Kuniko Horie-Inoue, Satoshi Inoue, FOXP1 and Estrogen Signaling in Breast Cancer. In Gerald Litwack, editor: *Vitamins and Hormones,* Vol. 93, Burlington: Academic Press, 2013, pp. 203-212.
ISBN: 978-0-12-416673-8

CHAPTER SEVEN

FOXP1 and Estrogen Signaling in Breast Cancer

Nobuhiro Ijichi*, Kazuhiro Ikeda*, Kuniko Horie-Inoue*, Satoshi Inoue*,†,‡,1

*Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan
†Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
‡Department of Anti-Aging Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
1Corresponding author: e-mail address: Inoue-Ger@h.u-tokyo.ac.jp

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.

Vitamins and Hormones, Volume 93
ISSN 0083-6729
http://dx.doi.org/10.1016/B978-0-12-416673-8.00006-X

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