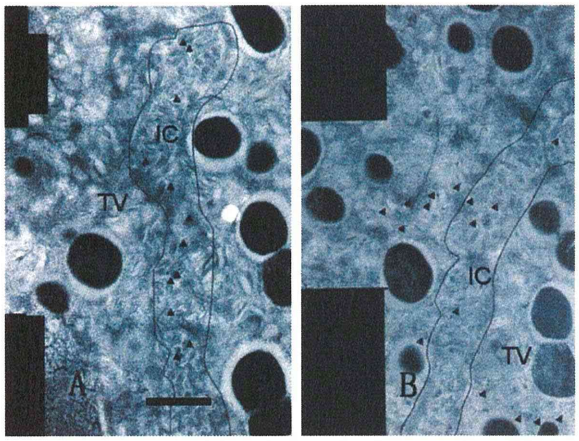


**Figure 3. Adjacent serial sections immunostaining. Each photograph is composed of several distinct pictures (montaged pictures) and serially sectioned. Labeled gold particles are shown with arrowheads. The anti-phosphoryl ezrin antibody bound only to the IC microvilli (A), while the anti-CD44 antibody labeled both the IC and TV (B). Scale bar = 1 μm.**

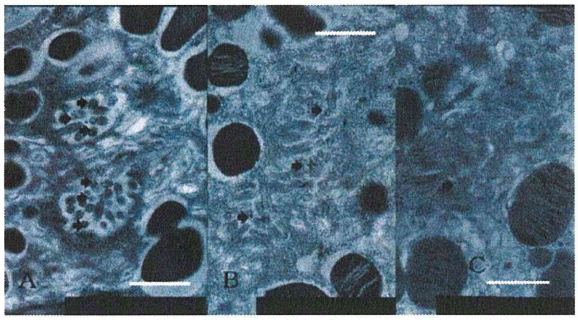


**Figure 4. Labeling differences in each level of gland of parietal cell stained with immunogold using anti-phosphoryl ezrin antibody. (A) Neck region, adjoining mucous neck cell in active animals; (B) Lower region of base, neighboring chief cell; (C) Neck region from starved rats. Anti-phosphoryl ezrin antibody labeling is strong on IC membrane of parietal cell from fed (active secreting) rats (A), and moderate to weak in lower half of base (B) and through gland from starved (inactive resting or inactive secreting) rats (C). Scale bar = 1 μm.**

anti-phosphoryl ezrin antibody in each cell between the gland segments. Parietal cells adjacent to mucous neck cells or chief cells were deemed to be in the neck or base region, respectively. The gold particle numbers were compared between the neck and the base region of the glands, and between active and inactive resting glands (**Figure 4**). The number of labeled gold particles was divided by the IC area to give the labeling density (per $\mu m^2$). Labeling density zonation was clear from the neck to the base, with a significantly higher density in parietal cells located in the isthmus to neck region (mean ± SE; 25.501 ± 3.736 $\mu m^2$) compared with the glandular lower base (17.082 ± 7.275 $\mu m^2$) or from inactive starved rats (1.926 ± 0.465 $\mu m^2$) (**Figures 5(A)** and **(B)**).

Statistical analysis using IMAGE-J revealed that phosphoryl ezrin expression in the neck and upper base was significantly higher than that in lower base (25.501 ± 3.736 vs 17.082 ± 7.275, $p < 0.05$) and than that in starved gland (25.501 ± 3.736 vs 1.926 ± 0.465, $p < 0.01$). These findings suggest that the phosphoryl ezrin assemble in the membrane of active parietal microvilli at neck to upper base.

## 4. Discussion

The component cells of gastric glands include pit mucous cells, progenitor cells, parietal cells, mucous neck cells, chief cells and endocrine cells and have previously been studied in rodents. These cells undergo mitosis in the isthmus, from where they migrate and differentiate along the longitudinal axis of the gland in an upward or downward direction [19-21]. The parietal cells migrate upwards and downwards then mature, while chief cells derive from the mucous neck cell through an intermediate cell type to the mature chief cell in a downwards migration [17]. Parietal cells adjoining the mucous neck cell are considered to be in the neck region and those next to the chief cell are in the base region of the gland.

Gastric juice is very acidic, with a pH of around 1.5. The average pH value measured in this study was pH 2.0 in fed rats and pH 6.4 in starved animals, indicating that the parietal cells of fed rats correspond to cells in the active secretory-phase, while those of starved animals correspond to cells in the inactive resting phase. The pa-

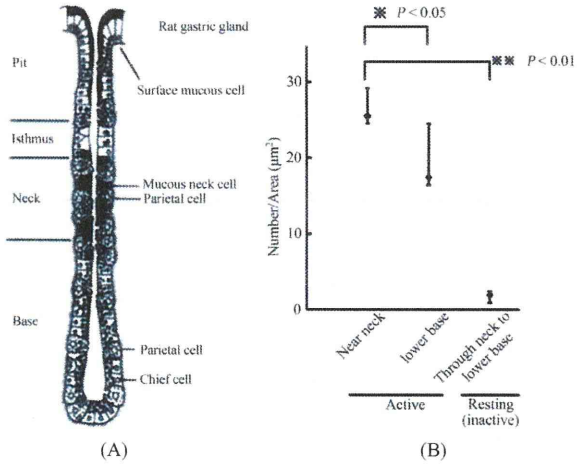


**Figure 5. (A) Schematic drawing of rat gastric gland. After proliferation in the glandular isthmus, the parietal cell migrates and differentiate through neck to base of gland; (B) Statistical analysis of phosphoryl-ezrin plotting. The labeling of the cell in the near neck site was significantly higher than that in the lower base, or whole site of the gland in the resting (the inactive).**

rietal cells are distributed broadly through the gland from the isthmus to the base. The cells are large and plump from the isthmus to the upper base, although they become smaller and more slender in the lower base as they migrate downwards [19-22].

Routine light microscopy revealed parietal cells of an acidophilic nature with numerous mitochondria in the cytoplasm. The cells were stained with immunolabeling using an anti-$H^+$, $K^+$-ATPase antibody, and the reaction products had a thread-like appearance, especially in the isthmus to the neck region [5,18]. The expression pattern followed the contour line of the stained apical-cell membrane. In previous studies, we used high pressure-frozen, freeze-substituted and resin-embedded tissue samples to demonstrate the intracellular localization of $H^+$, $K^+$-ATPase [3,23]. This antibody clearly stained the IC containing many microvilli in the active phase, while TV was stained in the inactive resting phase. Diffuse cytoplasmic staining was observed in cells scattered widely from the isthmus to the glandular base. This aspect is thought to derive from TV membrane staining at the light microscopy level.

Yao *et al.* [15] revealed that most actin in the gastric gland is present in parietal cells, which are largely globular during the inactive resting secretion phase. However, during the active phase, actin forms a filamentous structure. Jöns *et al.* [24] reported that the binding of actin to the plasma membrane is dependent on phosphoryl ezrin molecules, and the initiation of ultrastructural changes in parietal cells is thought to be induced by the polymerization of cytoskeletal actin [2,7,15] followed by binding of the actin molecule to the C-terminal of phosphoryl ezrin and intramembranous CD44 to the N-terminus [8-10, 16,25,26]. Non-phosphoryl ezrin molecules are distributed throughout the cytoplasm without binding actin in the inactive resting phase [16,29-29], while localization of the ezrin-actin linkage in the cytoplasm occurs in the active phase during the formation of phosphorylated ezrin molecules and filamentous actin-molecule binding. This indicates that phosphoryl ezrin and actin are related to the cell surface distribution of the $H^+$, $K^+$-ATPase proton pump.

In the present study, immunostaining with the phosphoryl ezrin antibody resulted in heavy labeling in the neck to the upper base region (**Figures 3** and **4**), revealing that the labeled cells are in the active phase [5]. A statistical morphometric data comparison of neck and base cells showed that phosphoryl ezrin and $H^+$, $K^+$-ATPase were present in similar sites within the same cell [18]. The apical membrane of IC containing microvilli in the active phase was labeled by immunogold staining with the anti-phosphoryl ezrin antibody. On the other hand, labeling of these structures was weak in the inactive resting phase (**Figures 4** and **5**).

These findings suggest that the parietal cells in the neck and upper base are more active than those in the lower base. Karam *et al.* [20] described the intracellular mitochondrial distribution of parietal cells at various levels of the mouse gastric gland and revealed a high number in the isthmus and neck. Moreover, the integral $H^+$, $K^+$-ATPase is exposed on the luminal surface when IC is enlarged, and the enzyme is activated by a successive $K^+$ ion supply. On the other hand, the increasing TV volume results in a decreased surface area of the canaliculi membrane as the IC and TV derive from similar membrane systems [12]. The TV is transformed into the apical plasma membrane during acid secretion [2]. $H^+$, $K^+$-ATPase then becomes an intravesicular membrane protein and acid secretion is halted because of an interruption in the $K^+$ ion supply. This transformation is limited at sites with small amounts of phosphoryl ezrin, such as the middle to the bottom region of the gland [18,22] where parietal cells are small and slender. Here, it is thought that the acid secretory capacity is limited and unresponsive to physiological or feeding conditions.

The purpose of this work was to decide at which level parietal cells are most active. Acid secretion activity in the gland appears to be higher in the segment from the isthmus to the upper glandular base, which agrees with the findings of Fykse *et al.* [30] who reported that parietal cells in the second part of four segments in the gland were activated by histamine treatment. Jiang *et al.* [5] reported that the acid-secreting potency of individual parietal cells was higher in the upper third of the gland (containing the superficial part). Further experiments are required to clarify the secretion system of gastric acid in parietal cells.

## 5. Acknowledgements

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

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## 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.

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**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

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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*

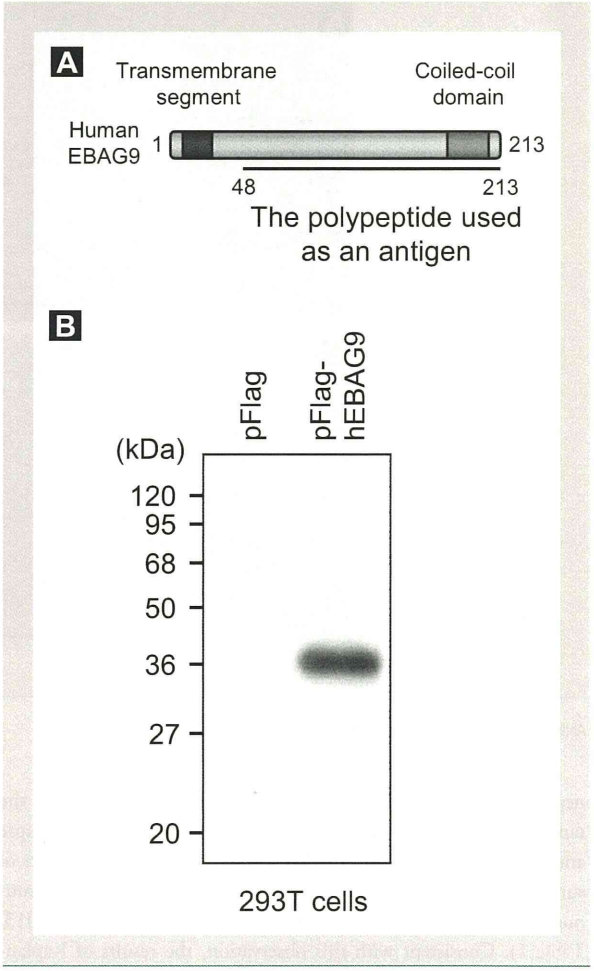
The correlation between the immunoreactivity score and clinicopathological characteristics was evaluated using the $\chi^2$ test. A *P*-value of < .05 was regarded as statistically significant. Differences between the 2 groups were analyzed using 2-sample, 2-tailed Student *t* test. Relapse-free and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

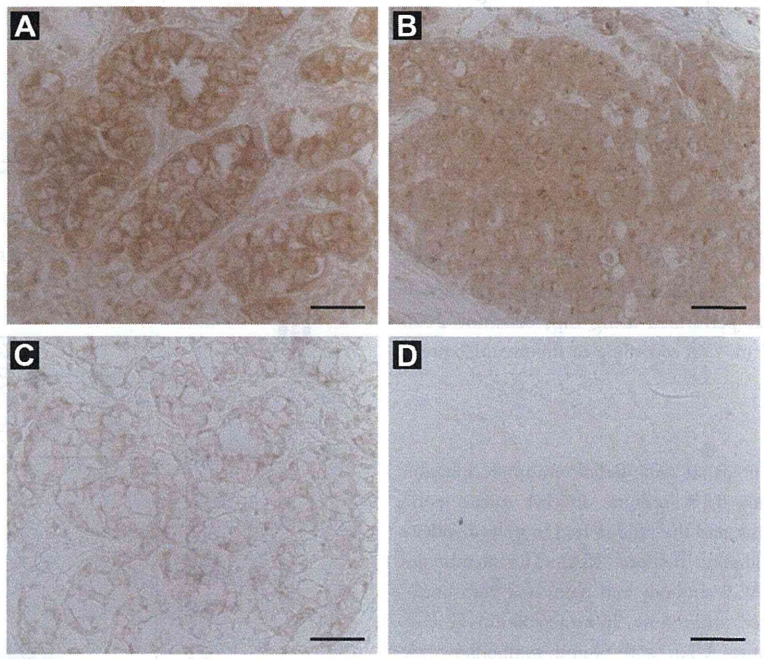



Abbreviations: EBAG9 = estrogen receptor-binding fragment associated antigen 9; hEBAG9 = human EBAG9; pFlag = pcDNA3 plasmid only containing the Flag tag.

### *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]

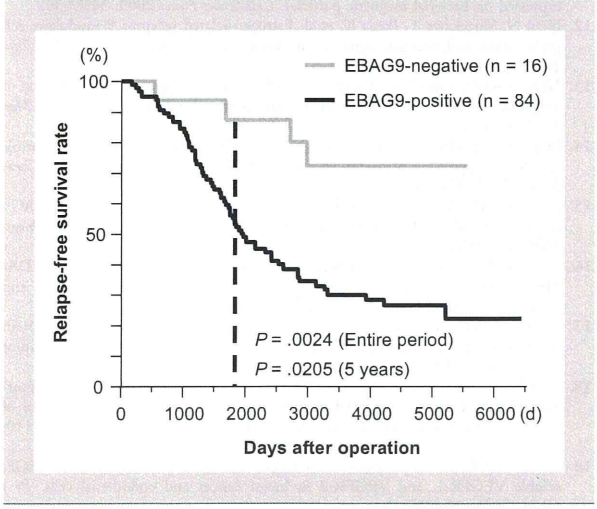
**Table 2** Univariate and Multivariate Analyses of Relapse-Free Survival in Breast Cancer Patients Treated With Tamoxifen as Adjuvant Therapy (n = 100)

| 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.

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## Disclosure

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

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