RASEF expression correlates with hormone receptor status in breast cancer

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Abstract. Breast cancer (BC) is the most frequently diagnosed malignant tumor in women worldwide, and the development of new molecules associated with BC is essential for the management of this disease. RAS and EF-hand domain-containing (*RASEF*) encodes the GTPase enzyme that belongs to the Rab family. Although the effects of this gene have been reported in several malignant tumor types, the role of *RASEF* in BC has not been completely elucidated. The aim of the present study was to investigate the importance of *RASEF* expression in BC. *RASEF* mRNA expression levels were evaluated in BC and non-cancerous mammary cell lines. The association between *RASEF* mRNA expression levels and clinicopathological factors in 167 patients with BC were then determined. Among the 13 examined BC cell lines, ER-negative/HER2-negative cell lines expressed lower *RASEF* mRNA levels, when compared with the other examined cell lines (P=0.014). Of the 167 patients examined, patients with negative hormone receptor status exhibited significantly lower *RASEF* mRNA expression levels (P<0.001). In addition low *RASEF* expression in BC tissues was associated with negative estrogen receptor status (P<0.001), negative progesterone receptor status (P<0.001), and triple-negative status (P<0.001). Additionally, although the differences were not statistically significant, patients with low *RASEF* expression levels exhibited poorer disease-free survival (P=0.123) and overall survival (P=0.086) than other patients. The results of the present study indicate that *RASEF* mRNA expression levels are associated with hormone receptor status in BC.

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the major cause of cancer-related deaths in women (1). The establishment of adjuvant therapies including several drugs and radiation has improved the prognosis of patients with BC. Adjuvant drug therapies are selected according to the immunohistochemical detection of relevant target molecules such as estrogen receptor (ER), progesterone receptor (PgR), and anti-human epidermal growth factor 2 (HER2) in surgically resected specimens. Although several multigene expression assays are available to predict patient prognosis and to evaluate the necessity of adjuvant chemotherapy (2), identifying new molecules related to conventional biomarkers could contribute to the selection of more precise treatment strategies.

RAS and EF-hand domain-containing (*RASEF*) is a member of the Rab family of GTPases (3). The regulatory mechanism of *RASEF* and its role in malignancies have been reported for melanoma (4,5), lung cancer (6), esophageal cancer (7), and myeloid leukemia (8,9). These studies demonstrated that *RASEF* has inconsistent roles depending on tumor type: It can function as an oncogene (6,7) or as a tumor-suppressor gene (5,8,9). Rab protein members govern the transportation of substances between cellular compartments to influence various cell functions (10). In malignant tumors, several Rab proteins have been reported as important factors in cancer development and progression (11). In BC, for example, increased RAB25 was associated with lymphatic metastasis and poor prognosis (12,13), and RAB31 is elevated in BC to promote its progression (11). Although various Rab proteins have been studied in BC, there are no reports that describe the roles of *RASEF*.

In the present study, we aimed to investigate the importance of *RASEF* expression in BC by evaluating *RASEF* mRNA expression in BC cell lines and patient specimens.

Materials and methods

Sample collection. Thirteen BC cell lines (BT-20, BT-474, BT-549, HCC1419, HCC1954, Hs578T, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1) and two non-cancerous breast epithelial cell lines (MCF-10A, and MCF-12A) were used in this study. We purchased BT-549, HCC1419, HCC1954, and Hs578T cell lines from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and BT-474, MCF-7 and MCF-12A were kindly gifted from Prof. David Sidransky of Johns Hopkins University (Baltimore, MD, USA). All other cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI 1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum and incubated in an atmosphere with 5% CO_2 at 37˚C (14,15).

BC patients who underwent surgery at Nagoya University Hospital from March 2002 to November 2009 and whose surveillance data for more than five years after surgery were available were selected for this study. We collected primary BC specimens and clinical data from 167 patients in total. The specimens were resected approximately to 1.5 mm in diameter, and frozen immediately at -80˚C. We resected non-cancerous specimens at >3 cm away from the edge of the tumor. The resected BC specimens were diagnosed histologically as BC and classified using the Union for International Cancer Control (UICC) staging system for BC (7th edition). Administration of adjuvant medication therapy was determined by physician discretion considering each patient's general condition, pathological feature and subtype (16,17).

The present study complies with the Declaration of Helsinki and was approved by our institutional review board (approval no: 2016-0224). Participants granted written informed consent for use of clinical samples and data.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). We evaluated *RASEF* mRNA expression levels by RT-qPCR. RNA was extracted from cell lines ($8.0x10^6$ cells per cell line), and BC and non-cancerous specimens from 167 patients. cDNA was synthesized as previously described (16,17). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were evaluated for normalizing *RASEF* mRNA expression levels. *RASEF*-specific primers were: Forward 5'-ATCAGACTTCAAAGCACAGAA ATGG-3' and reverse 5'-TTCCTCTTCCAACTCACTCAA CTG-3', which generated a 96-bp product. *GAPDH*-specific primers were: Forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3', which generated a 226-bp product. We used a SYBR Green PCR core reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for RT-qPCR with these cycling conditions: One cycle at 95˚C for 10 min, followed by 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec, using an ABI StepOnePlus real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). All samples were assayed in triplicate. The mRNA expression level of *RASEF* in each sample was obtained from the *RASEF* value divided by *GAPDH* value (18,19).

Statistical analysis. Differences in the levels of *RASEF* mRNA between two groups were evaluated with the Mann-Whitney test. When they were compared between multiple groups, ANOVA with Tukey's post-hoc test was performed. We analyzed the association between *RASEF* mRNA expression levels and patient clinicopathological factors using the χ^2 test. We utilized the Kaplan-Meier method for evaluating disease-free survival (DFS) and overall survival (OS) rates; the survival curves were compared using the log-rank test. Patients' *RASEF* expression levels were divided into quartiles with low *RASEF* levels being taken as the lowest quartile. JMP 12 (SAS Institute, Inc., Cary, NC, USA) was exploited for the statistical analysis, and P<0.05 was considered to indicate a statistically significant difference.

Results

RASEF mRNA expression levels in BC cell lines. We evaluated the levels of *RASEF* mRNA expression in 13 BC cell lines and two non-cancerous cell lines of mammary gland (Fig. 1). The ER, PgR, and HER2 statuses of the cell lines have been evaluated in previous studies (20,21). All ER-positive BC cell lines expressed higher levels of *RASEF* mRNA than non-BC cell lines. Although *RASEF* mRNA expression levels did not significantly differ among ER-positive and -negative (P=0.083), PgR-positive and -negative (P=0.833), or HER2-positive and -negative (P=0.053) cells, the expression levels in ER-negative/HER2-negative BC cell lines were lower than in other cell lines (P=0.014).

Patient characteristics. A total of 167 BC patients were enrolled in the present study and all were women. The mean age (± standard deviation) was 54.4±11.6 years (range, 26-78 years). The UICC stage distribution was as follows: Stage 0, seven patients; stage I, 47 patients; stage II, 78 patients; stage III, 34 patients; and stage IV, one patient. The median follow-up duration was 100.0 months (range, 8-155 months) or until death. The conventional biomarkers status determined from immunohistochemistry tests in primary tumors was as follows: ER-positive, n=127; ER-negative, n=40; PgR-positive, n=115; PgR-negative, n=52; HER2-positive, n=39; HER2-negative, n=119 (HER2 data missing for nine patients); triple-negative, n=18; and non-triple-negative, n=148 (data missing for one patient). The patients who expressed at least one molecule among ER, PgR, and HER2 were defined as 'non-triple-negative'. Because eight patients among nine whose HER2 statuses were unknown showed ER-positivity, they were categorized as non-triple-negative.

Association between RASEF mRNA expression level and patient clinicopathological factors. In 78 (47%) of the 167 patients, BC specimens expressed lower *RASEF* mRNA levels than non-cancerous specimens. *RASEF* mRNA expression levels did not differ between Tis (carcinoma *in situ*)/T1 (n=77) and T2/T3/T4 (n=90; P=0.337), lymph node metastasis-positive (n=82) and -negative (n=85; P=0.326), or stage 0/I (n=54) and stage II/III/IV (n=113; P=0.075) disease.
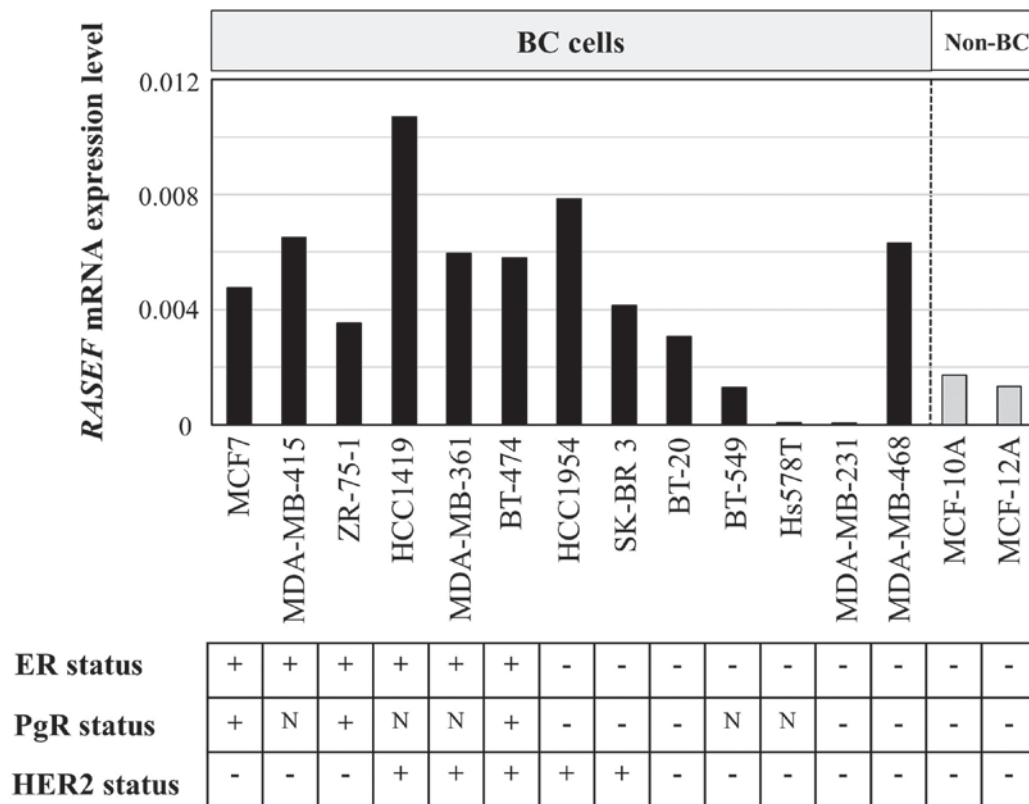
Figure 1. Analysis of *RASEF* mRNA expression levels in cell lines. Bar graphs are showing *RASEF* mRNA levels in 13 BC cell lines and two non-cancerous breast cell lines. *RASEF* mRNA levels in ER-negative/HER2-negative BC cell lines were significantly lower than those in non-triple-negative BC cell lines. *RASEF,* RAS and EF-hand domain-containing; BC, breast cancer; non-BC, non-cancerous cell lines; N, no previous data available; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.

When we investigated conventional biomarkers, we found that ER-negative specimens (n=40) exhibited significantly lower *RASEF* mRNA expression levels than ER-positive specimens (n=127; P<0.001; Fig. 2A). PgR-negative specimens (n=52) also exhibited lower *RASEF* mRNA expression levels than PgR-positive specimens (n=115; P<0.001). Additionally, *RASEF* mRNA expression levels were significantly lower in triple-negative specimens (n=18) than in non-triple-negative specimens (n=148; P<0.001; data missing for one patient). The expression levels between HER2-positive (n=39) and -negative specimens (n=119) did not differ significantly (P=0.180; data missing for nine patients). When we focused on *RASEF* mRNA levels among ER-positive/PgR-positive (n=115), ER-positive/PgR-negative (n=12), and ER-negative/PgR-negative specimens (n=40), we found that ER-negative/PgR-negative specimens exhibited significantly lower *RASEF* mRNA expression levels than ER-positive/PgR-positive specimens (P<0.001; Fig. 2B). ER-positive/PgR-negative specimens tended to have lower *RASEF* mRNA expression levels than ER-positive/PgR-positive specimens, although there was no significant difference (P=0.086).

Patients with *RASEF* expression levels in the lowest quartile were distributed into a 'low *RASEF* group' (n=41), and the remaining patients were designated as 'others' (n=126). The low *RASEF* group was associated with more advanced UICC T factor (P=0.031; Table I), and ER-negative (P<0.001), PgR-negative (P<0.001), and triple-negative status (P<0.001).

Although the differences were not statistically significant, the low *RASEF* group tended to have poorer DFS (5-year DFS rates, low *RASEF* group: 72.6%; others: 85.6%; P=0.123; Fig. 3A) and OS (5-year OS rates, low *RASEF* group: 90.1%; others: 93.5%; P=0.086; Fig. 3B).

Discussion

In this study, we demonstrated that low *RASEF* mRNA expression levels were associated with negative hormone receptor status.

RASEF is a member of the Rab GTPase protein family and contains a Rab GTPase domain in its C-terminal region. Uniquely, RASEF has 2 EF-hand domains in the N-terminal region, which are important for binding to calcium ions, and an internal coiled-coil motif (3). The Rab protein family consists of 70 Rab proteins, and they govern the transportation of various molecules among cellular compartments (10). Recently, several Rab proteins have been revealed to contribute to cancer development and progression, and some have been focused on as novel therapeutic targets (11). In BC, RAB25 was shown to promote epithelial-mesenchymal transition (22), and its expression was associated with more aggressive stage and poor prognosis (12,13). In addition, FIP1C, an effector of RAB11, promoted lysosomal degradation of HER2 to suppress tumor progression. Despite these studies of Rab proteins, there are no reports that describe RASEF in BC. Several previous reports have described the roles of *RASEF*
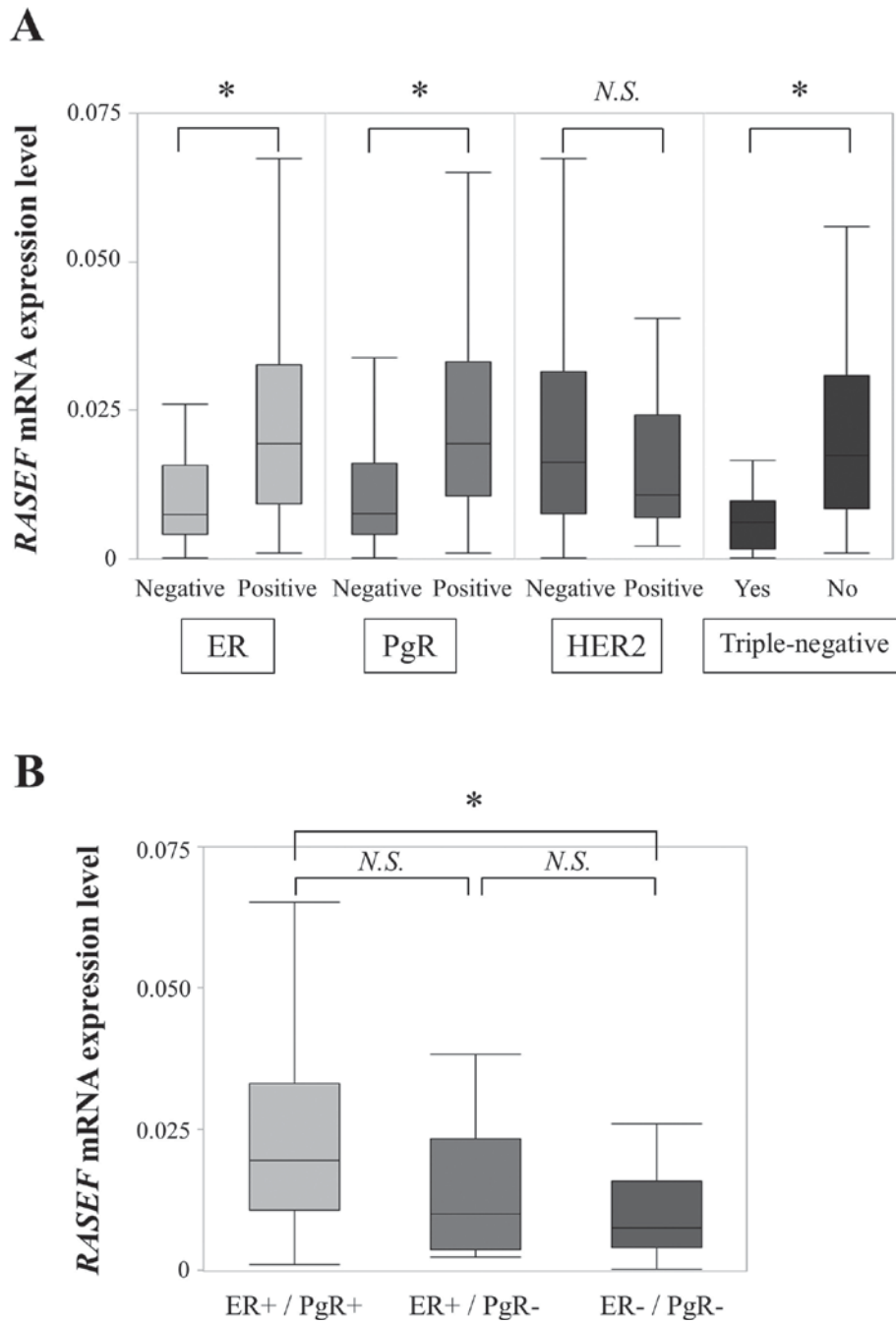
A

B

Figure 2. (A) Correlation between expression of *RASEF* mRNA and conventional biomarkers. *RASEF* mRNA levels were significantly lower in ER-negative, PgR-negative, and triple-negative specimens than in ER-positive, PgR-positive, and non-triple-negative specimens. (B) Comparison of *RASEF* mRNA levels among ER-positive/PgR-positive, ER-positive/PgR-negative, and ER-negative/PgR-negative groups. ER-negative/PgR-negative specimens exhibited significantly lower *RASEF* mRNA expression than ER-positive/PgR-positive specimens. *P<0.001. *RASEF*, RAS and EF-hand domain-containing; N.S, not significant; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.

in malignant tumors. Interestingly, *RASEF* has shown inconsistent behavior in different studies: It has been reported to function as an oncogene and as a tumor suppressor gene. Oshita *et al* (6), showed that RASEF protein expression was positively associated with poor prognosis in non-small cell lung cancer. They demonstrated that RASEF interacted with extracellular signal-regulated kinase (ERK) 1/2 and enhanced ERK 1/2 signaling. Another study used cDNA microarray to demonstrate that *RASEF* was overexpressed in esophageal squamous carcinoma compared with non-cancerous tissues (7). Conversely, Maat *et al* (5) identified *RASEF* as

a tumor suppressor regulated by epigenetic mechanisms in uveal melanoma. They revealed that missense mutation and methylation of the *RASEF* gene is related to poor survival. In the present study, we aimed to clarify the significance of *RASEF* expression in BC patients.

Regarding *RASEF* mRNA expression levels in BC and non-cancerous mammary cell lines, ER-positive BC cell lines expressed higher *RASEF* mRNA levels than non-BC cell lines, and ER-negative/HER2-negative BC cell lines expressed low *RASEF* mRNA levels. Because of the small sample size, there were no significant differences between ER-positive and

Table I. Associations between *RASEF* mRNA expression and clinicopathological characteristics of 167 patients with BC.

Clinicopathological parameters	Low RASEF group (n=41)	Others (n=126)	P-value
Age (years)			0.847
≤60	26	82	
>60	15	44	
Histology			0.231
DCIS	1	6	
IDC	39	109	
ILC	0	6	
Others	1	5	
UICC T factor			0.031[a]
Tis/T1	13	64	
T2/T3/T4	28	62	
Node status			0.164
Negative	17	68	
Positive	24	58	
UICC pathological stage			0.413
0/I/II	34	97	
III/IV	7	29	
ER status			<0.001[a]
Positive	21	106	
Negative	20	20	
PgR status			<0.001[a]
Positive	17	98	
Negative	24	28	
HER2 status			0.873
Positive	10	29	
Negative	29	90	
Unknown	2	7	
Triple-negative			<0.001[a]
Yes	13	5	
No	27	121	
Unknown	1	0	
Adjuvant therapy			0.005[a]
Endocrine therapy alone	7	50	
Chemotherapy alone	14	16	
Endocrine and chemotherapy	15	49	
None	5	11	

[a]χ^2 test. *RASEF*, RAS and EF-hand domain-containingp; BC, breast cancer; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; UICC, Union for International Cancer Control; Tis, carcinoma *in situ*; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2.

ER-negative BC cell lines or PgR-positive and PgR-negative BC cell lines.

When patient data was analyzed, although there were no significant differences, patients with *RASEF* expression levels in the lowest quartile (designated the 'low *RASEF* group') tended to experience poorer DFS and OS. In this study, adjuvant therapy was administered to most patients, which might have abated the impact of *RASEF* mRNA expression. As a possible explanation for poor prognosis, we found that low *RASEF* expression correlated with ER-negative, PgR-negative and triple-negative status. These cancers are known to be more aggressive and to result in poorer survival than ER-positive, PgR-positive, and non-triple-negative cancers (23-26). Nakamura *et al* (8), suggested that RASEF overexpression induced caspases-3 and -9, and increased p38 phosphorylation levels, which induced apoptosis and inhibited proliferation of chronic myeloid leukemia progenitor cells. Among these molecules, caspase-9 is the apoptotic initiator protease of the apoptotic pathway (27).

A

No. at risk					
Low *RASEF* group	41	33	30	17	1
Others	126	111	97	38	1

B

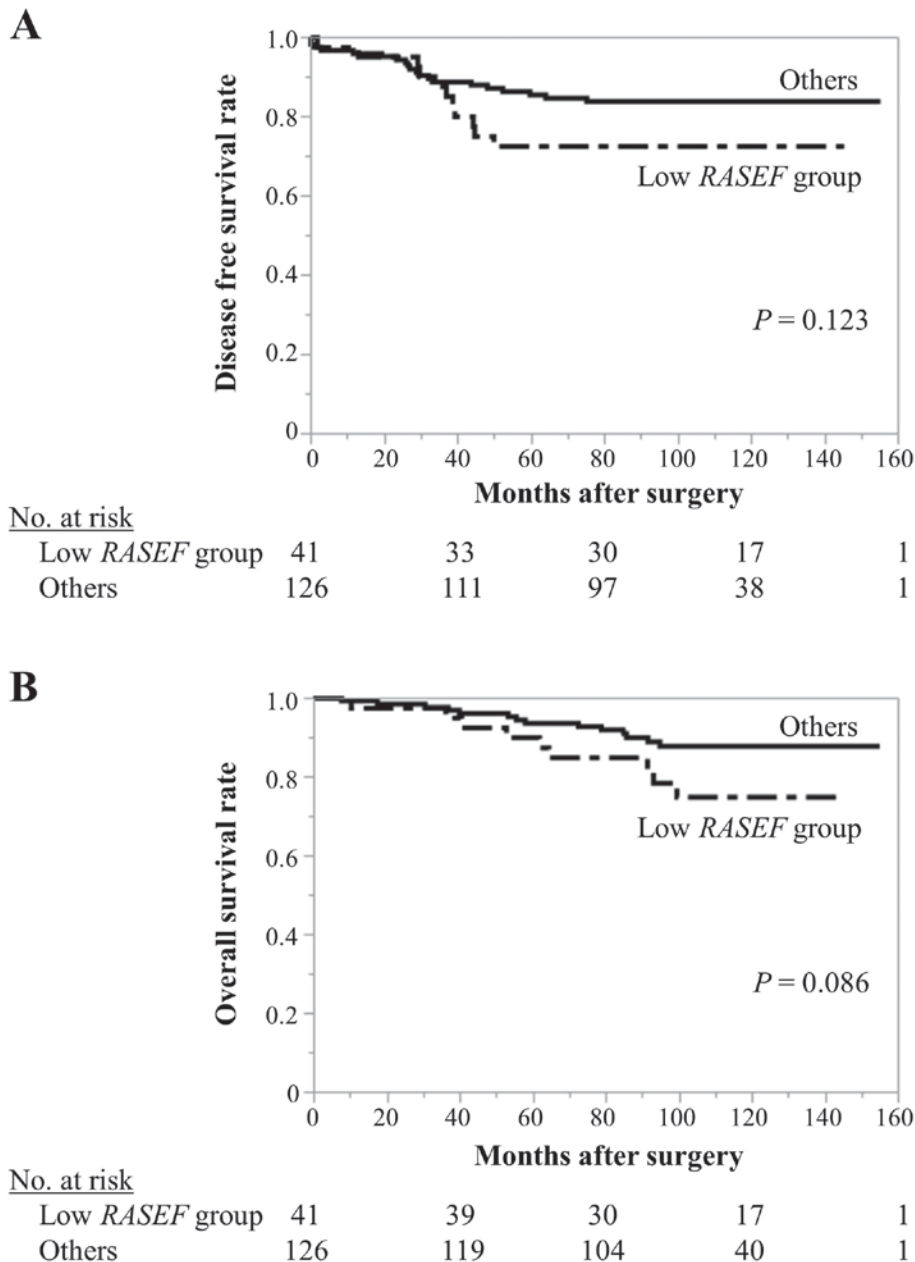| No. at risk					
Low *RASEF* group	41	39	30	17	1
Others	126	119	104	40	1

Figure 3. Prognosis between low *RASEF* group and other patients. Although there were no statistically significant differences, patients with *RASEF* levels in the lowest quartile (low *RASEF* group) tended to experience (A) poorer disease-free survival and (B) overall survival. *RASEF*, RAS and EF-hand domain-containing.

p38, a mitogen-activated protein kinase, is an important mediator of signal transduction for cell survival and apoptosis (28). PgR-positive status in BC has been reported to correlate with high phosphorylated p38 expression (29). In our results, low *RASEF* expression was associated with advanced T-stage. *RASEF* may play tumor suppressive roles by suppressing the proliferation and promoting the apoptosis of BC cells.

ER-positive/PgR-negative specimens tended to exhibit lower *RASEF* mRNA levels than ER-positive/PgR-positive specimens, although this difference was not significant. ER-positive/PgR-positive BC is likely to belong to the 'luminal A-like' subtype, and ER-positive/PgR-negative BC tends to be belong to the 'luminal B-like' subtype (2). Recently, Ki-67 has been widely used to distinguish these two subtypes. However, the threshold for Ki-67 scoring remains controversial. *RASEF*

expression might help to discriminate the 'luminal A-like' and 'luminal B-like' subtypes.

This is the first study to demonstrate an association between *RASEF* mRNA expression and clinicopathological characteristics in BC patients. These findings may potentially be applied to clinical use in the future. For example, *RASEF* levels in surgically resected samples might aid evaluation of BC subtypes and facilitate selection of adjuvant medication. However, this study has some limitations. Because the functional role of *RASEF* in BC cells has not been elucidated, further *in vitro* experiments are needed to determine how *RASEF* interacts with hormone receptor status. Additionally, this is a retrospective study. Evaluation of a large number of patients or a prospective study is warranted to investigate the potential clinical applications of our findings.

To conclude, *RASEF* mRNA expression levels of cell lines and the association between *RASEF* and BC patient specimens were evaluated in this study. We demonstrated an association between *RASEF* mRNA expression levels and hormone receptor status in BC specimens. Low *RASEF* mRNA expression is likely to reflect ER-negative and PgR-negative status.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MK conceived and designed this study. MS conducted the experiments, analyzed the data and wrote the manuscript. MH provided cell lines. TI, NM, YA, YT, KN, DT, SN and TK collected the patients' samples and acquired clinical data. DS, HT, SU, TM, MH, YK and TK interpreted the experimental data and revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the institutional review board of Nagoya University Graduate School of Medicine (reference number: 2016-0224). Written informed consent was obtained from participants for the use of samples and data.

Patient consent for publication

Participants in this study granted written informed consent for publication required by the institutional review board.

Competing interests

The authors declare that they have no competing interests.

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