Original Article

Presence of Autoantibodies against Ras-like GTPases in Serum in Stage I/II Breast Cancer

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ABSTRACT

Background: Ras-like GTPases, RalA, and RalB, are members of the Ras superfamily of small GTPases. Their aberrant activation is a major cause of human tumorigenesis induced by oncogenic Ras. Serum anti-RalA antibodies (s-RalA-Abs) are induced in patients with esophageal carcinoma, but there have been no reports regarding their presence in patients with breast cancer.

Methods: Serum samples from 100 patients with breast cancer and 73 healthy individuals were analyzed using an enzyme-linked immunosorbent assay system specifically developed for s-RalA-Abs. Positive rates of s-RalA-Abs and serum p53 antibodies (s-p53-Abs) were evaluated for two cut-off optical density values of 0.26 and 0.33 (i.e., the control mean + 2 and 3 standard deviations), respectively.

Results: Overall positive rates for s-RalA-Abs at the two cut-offs were 24% and 11%, respectively, with no statistically significant differences between stage I and stage II cancers. The positive rate increased significantly in a combination assay of s-p53-Abs with s-RalA-Abs, and s-RalA-Abs was shown to be inversely associated with s-p53-Abs.

Conclusions: s-RalA-Abs in combination with s-p53-Abs may be a candidate serum antibody marker for breast cancer.

Toho J Med 3 (4): 125–130, 2017

KEYWORDS: RalA, serum autoantibody, tumor marker, breast cancer, ELISA

Introduction

Breast cancer remains the leading cause of cancer death among women in less developed countries, but a substantial number of these deaths could be prevented by broadly applying effective early detection tests.[1)] Although carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) are standard serum markers for detecting tumor antigens, they have been shown not to be positive in patients with early stage breast cancers.[2)] Various alternative autoantibody detection kits have been developed to detect early stage of cancers.[3)] Because of its high sensitivity of serum p53 antibody (s-p53-Abs), testing for s-p53-Abs has been approved by the Japanese Ministry of Health and Welfare as a tumor marker for breast cancer.[4, 5)]

Recently, various autoantibodies against tumor antigens

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DOI: 10.14994/tohojmed.2017-008
Received Sept. 4, 2017: Accepted Oct. 23, 2017
Toho Journal of Medicine 3 (4), Dec. 1, 2017.
ISSN 2189–1990, CODEN: TJMOA2

have been reported as potential biomarkers for breast cancer.[6, 7] Among these are the Ras-like GTPases, RalA, and RalB, which are members of the Ras superfamily of small GTPases known to be aberrantly induced during tumorigenesis by oncogenic Ras.[8] RalA and RalB have been implicated in diverse cellular functions in cancers.[9] RalA has been reported to induce serum anti-RalA antibodies (s-RalA-Abs) in patients with hepatocellular carcinoma,[10] prostate carcinoma,[11] and esophageal carcinoma.[12]

To date, there have been no reports that have analyzed s-RalA-Abs in patients with breast cancer, and neither have there been any reports evaluating a combination assay for s-RalA-Abs and s-p53-Abs. In this study, we tested serum samples from patients with stage I and II breast cancer and healthy individuals for the presence of s-RalA-Abs and s-p53-Abs, and evaluated the clinical significance of a combination assay for s-RalA-Abs and s-p53-Abs.

Materials and Methods

Collection of sera

Serum samples of 100 patients with stage I or II breast cancer were obtained from BioBank Japan. Serum samples were also obtained from 73 healthy donors. Patients with other malignancies synchronous or metachronous (within five years) to the carcinoma *in situ* were excluded. Each serum sample was centrifuged at 3,000 × g for 5 min and the resulting supernatant was stored at −80℃ until further use, avoiding repeated thawing and freezing of the samples. This study was approved by the Institutional Review Board of the Toho University School of Medicine (# 22-112, #22-047) and written informed consent was obtained from all subjects. Unlinkable anonymization was applied to the samples.

Purification of the recombinant RalA

A full-length RalA cDNA, cloning in pET28 plasmids (Novagen Inc., Madison, WI), was provided by Dr. Jian-Ying Zhang (The University of Texas, El Paso, TX). The details of this procedure have been described previously.[12] The recombinant protein was expressed in *Escherichia coli* BL21-CodonPlu (DE3)-RIL (Stratagene, La Jolla, CA) and dissolved in 8 M urea-phosphate buffered saline (PBS). The RalA extract was applied to Ni Sepharose 6 Fast Flow medium (GE Healthcare UK Ltd., Amersham, UK). The column was washed with 50 mM imidazole in 8 M urea-PBS, and the purified RalA recombinant protein was then eluted with 500 mM imidazole in 8 M urea-PBS. The expression and purity of the recombinant protein were examined using 12.5% SDS-PAGE. We confirmed that the constructed plasmid contained the inserted gene by using DNA-sequencing analysis.

Enzyme-linked immunosorbent assay to detect s-RalA-Abs

Serum samples from the patients and healthy controls were analyzed by enzyme-linked immunosorbent assay (ELISA) using RalA, as described previously.[12] Briefly, the purified recombinant proteins were placed in 96-well microtiter plates (Nunc MaxiSorp; Nunc Nalgene International Corp., Rochester, NY). RalA was diluted in PBS to a final concentration of 1.0 μg/ml and added to the plates (100 μl/well) ; these were then incubated overnight at 4℃. PBS was used as control. After two washes with PBS, the proteins were blocked with 200 μl PBS containing 1% bovine serum albumin (BSA) and 5% sucrose at room temperature for 3 h. All sera were diluted (1:100) in PBS containing 0.15% Tween-20, 1% casein, and 0.2 mg/ml *E. coli* extract; 100 μl of the diluted serum was then added to each RalA- or PBS-coated well and incubated at room temperature for 60 min while agitating at 250 rpm. After washing four times with PBS containing 0.05% Tween-20, 100 μl of HRP-conjugated antihuman IgG (1:5000; MBL, Nagoya, Japan) diluted in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxiphenylacetic acid was added to each well as a secondary antibody. The plates were incubated at room temperature for 60 min while agitating at 250 rpm. The wells were washed four times with PBS containing 0.05% Tween-20, and autoantibodies were detected by adding 100 μl of 3,3',5,5'-tetramethylbenzidine substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding 0.25 N H_2SO_4 (100 μl/well). Absorbance was measured at 450 nm using a SUNRISE Microplate Reader (Tecan Japan Co., Ltd., Kawasaki, Japan). The RalA signals were evaluated by calculating the difference between the absorbance values for the wells containing RalA and PBS.

The cut-off values of optical density were fixed according to the mean + 2 standard deviations (SDs) of the values in healthy donors. For combination assay with s-RalA-Abs and s-p53-Abs, we evaluated the significance of the other cut-off value of optical densitiy according to the mean + 3 SDs of the values in healthy donors.

Purification of recombinant p53 protein

For the expression and purification of recombinant protein, full-length cDNA of p53 (GenBank accession Number: AB082923) was amplified by polymerase chain reaction.

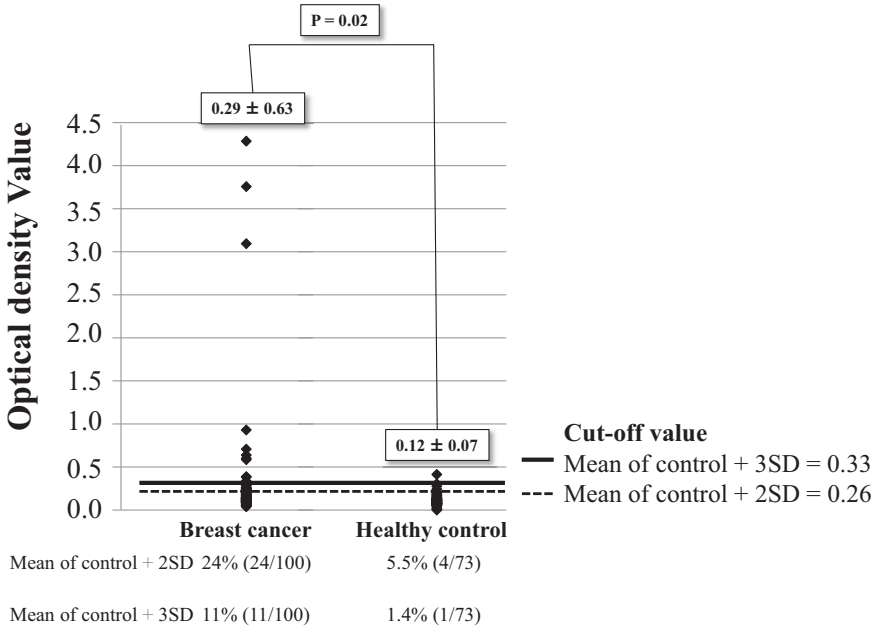

Fig. 1 The distribution of anti-RalA antibody titers in 100 patients with breast cancer and 73 healthy controls. Two optical density cut-off values were applied: the mean value for the healthy controls + 2 standard deviations (SDs) (= 0.26) and + 3 SDs (= 0.33).

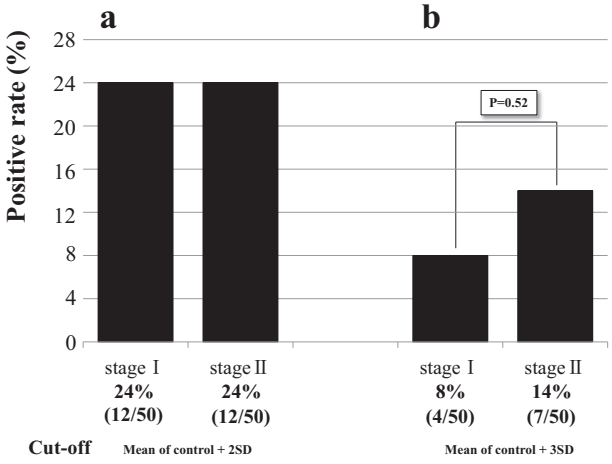

Fig. 2 Comparison of positive rates of serum anti-RalA antibodies in patients with breast cancer by tumor stage. a. Applying the cut-off value of healthy control mean + 2 SDs. b. Applying the cut-off value of healthy control mean + 3 SDs.

Amplified gene was inserted between BamHI and HindIII site in pFastBac Dual vector (Invitrogen, Carlsbad, CA). The inserted gene was confirmed by DNA sequencing analysis. The recombinant p53 protein was expressed in Sf 21 cells, using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, CA). The cells were dissolved in RIPA buffer and the target protein was fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). p53 antigen was purified by elution from the gels. The expression and purity of recombinant protein was examined in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA to detect serum p53 antibodies

Sera from a total of 100 breast cancer patients and 73 healthy controls were analyzed by ELISA, as described previously.[4] Briefly, purified recombinant proteins were coated on to 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY). p53 was diluted in PBS to a final concentration of 0.15 μg/ml and added to the plates (100 μl/well), which were then incubated overnight at 4℃. PBS was used as control. After two washes with PBS, the proteins were blocked with 200 μl of PBS containing 1% BSA and 5% sucrose at room temperature for 3 h. All human sera were diluted (1:100) in PBS containing 0.15% Tween 20, 1% casein, and 0.2 mg/ml *E. coli* extract. Then, 100 μl of the diluted sera was added to each p53 or PBS-coated well and incubated at room temperature, 250 rpm for 60 min. After washing with PBS containing 0.05% Tween-20 (PBST) 4 times, 100 μl of horseradish peroxidase-conjugated antihuman IgG (1:5000; MBL, Nagoya, Japan) diluted in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxiphenylacetic acid was added to each well as a secondary anti-

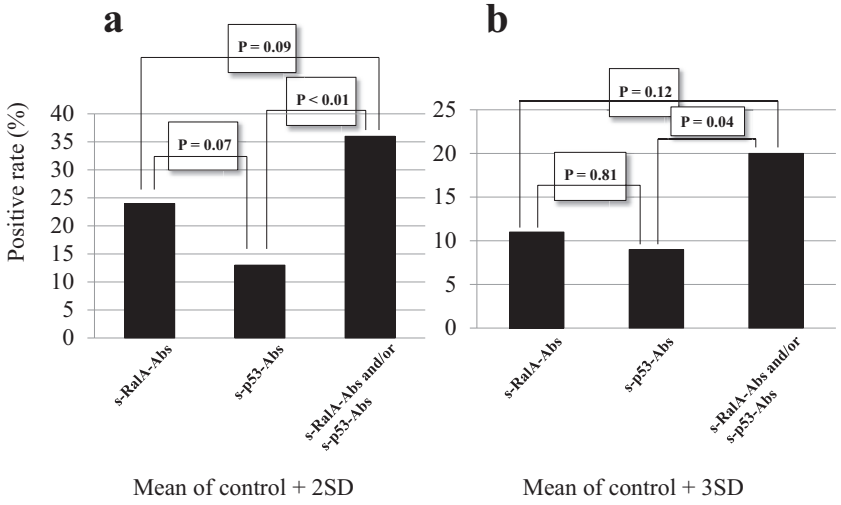

Fig. 3 Comparison of positive rates of the two serum tumor markers and the combination of these in patients with breast cancer. a. Applying the cut-off value of healthy control mean + 2 SDs. b. Applying the cut-off value of healthy control mean + 3 SDs.

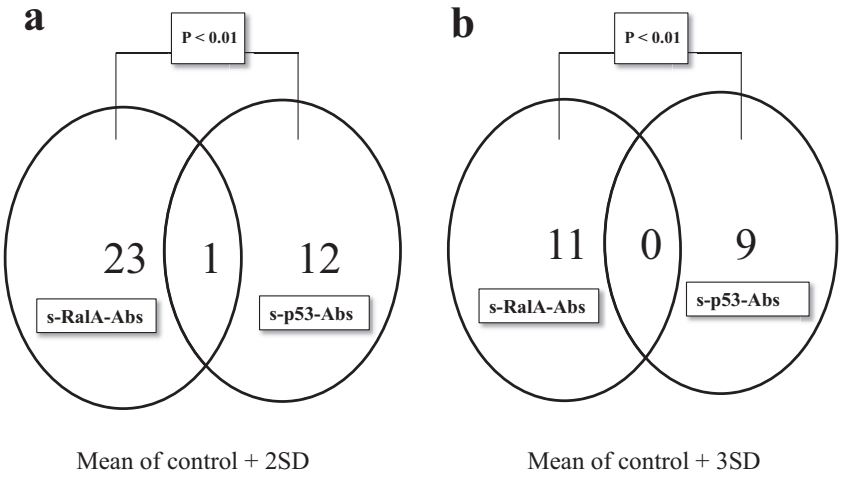

Fig. 4 Relationship between positive findings of serum autoantibodies in patients with breast cancer. a. Applying the cut-off value of healthy control mean + 2 SDs. b. Applying the cut-off value of healthy control mean + 3 SDs.

body and incubated at room temperature, 250 rpm for 60 min. The wells were washed four times with PBST buffer, and autoantibodies were detected by addition of 100 µl of 3, 3', 5, 5'-tetramethylbenzidine substrate. After incubation at room temperature for 30 min, the reaction was stopped by the addition of 0.25 N sulfuric acid (100 µl/well). Absorbance value was measured at 450 nm using a SUNRISE Microplate Reader (Tecan Japan Co., Ltd., Kawasaki, Japan). p53 signals were evaluated by calculating the difference in absorbance values between the wells containing p53 and PBS.

Statistical analyses

Data are expressed as the mean ± SD. Fisher's exact (two-sided) probability test and Mann-Whitney *U*-test were used to determine the significant differences between the two groups. *P*-values <0.05 were considered significant. All statistical analyses were performed using EZR for Windows (32-bit version).

Results and Discussion

Serum titers of RalA antibodies in the breast cancer patients

The optical density values of the s-RalA-Abs titers were significantly higher for the patients with breast cancer than for the healthy donors (0.29 ± 0.63 vs. 0.12 ± 0.07, P = 0.02; Fig. 1). With a cut-off value of optical density for normal s-RalA-Abs titers set at 0.26 (the mean+2 SDs of the values in healthy donors), the positive rate of s-RalA-Abs was 24% for patients with breast cancer (same for both stage I and II; Fig. 2a) and 5.5% for healthy controls. Similarly, with a cut-off optical density value for normal s-RalA-Abs titers of 0.33 (the mean+3 SDs of the values in healthy donors), the positive rate of s-RalA-Abs was 11% in patients with breast cancer and 1.4% in healthy controls. Although the positive rate of the stage II patients (14%) was relatively higher than that of the stage I patients (8%), the difference was not statistically significant (P = 0.52; Fig. 2 b).

Combination assay of s-RalA-Abs and s-p53-Abs in breast cancer

Fig. 3 shows a comparison of the rates of positive findings of serum autoantibodies in patients with breast cancer. Using the cut-off of control mean+2 SDs, the overall positive rate for s-RalA-Abs was higher than that for s-p53-Abs, though without statistical significance (P = 0.07; Fig. 3a). However, the positive rate for s-RalA-Abs and/or s-p53-Abs was significantly higher than that for s-p53-Abs alone (36% vs. 13%, P < 0.01). Using the cut-off of control mean+3 SDs, the positive rate for s-RalA-Abs and/or s-p53-Abs was significantly higher than that for s-p53-Abs alone (20% vs. 9%, P = 0.04). It was also higher than for s-RalA-Abs alone, although without statistical significance (20% vs. 11%, P = 0.12; Fig. 3b).

Fig. 4 shows the relationship between the presence of s-RalA-Abs and s-p53-Abs in patients with breast cancer. Notably, s-RalA-Abs-positive patients are more likely revealed to be negative for s-p53-Abs. Using the cut-off value of control mean+2 SDs, only one patient exhibited both positive s-RalA-Abs and positive s-p53-Abs. Using the cut-off value of control mean+3 SDs, all s-RalA-Abs-positive patients were s-p53-Abs-negative; no patient was positive for both s-RalA-Abs and s-p53-Abs. Nanami et al.[13] reported a similar pattern of results for these two serum autoantibodies, which could result from the interaction between RalA and p53 in esophageal carcinoma patients.[12]

Because all samples were obtained from the BioBank, there was no information about other conventional serum markers. Thus, a limitation of this study was that there were no clinicopathological data, immunohistochemical staining, or comparisons with CEA and/or CA15-3 to evaluate the clinical usefulness of s-RalA-Abs for patients with breast cancer. Based on the results of s-RalA-Abs research in patients with esophageal cancer,[12] conventional serum markers may be independent of s-RalA-Abs. A combination assay with CEA, CA15-3, and s-RalA-Abs may therefore increase positive rates in detecting breast cancers.

In conclusion, s-RalA-Abs in combination with s-p53-Abs may be a useful serum marker for patients with breast cancer. Further prospective studies are needed to evaluate the sensitivity and specificity of s-RalA-Abs combined with other serum tumor markers in patients with breast cancer.

This research was partly supported by a Grant-in-Aid for Scientific Research (nos. 16K10519 and 16K10520) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest: Hideaki Shimada received research grants and technical lecture fees form Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. Akiko Kuwajima is an employee of Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. The other authors have no conflicts of interest.

Ethical Statement: This study was approved by the Institutional Review Board of the Toho University School of Medicine (#22-112, #22-047). Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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