



ORIGINAL ARTICLE

Multipanel assay of 17 tumor-associated antibodies for serological detection of stage 0/I breast cancer

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Abstract

Because the production of tumor-associated antibodies (TAA) is a humoral immune response in cancer patients, serum autoantibodies may be detected even in patients with early-stage tumors. Seventeen recombinant proteins with tags in *Escherichia coli* (p53, RalA, p90, NY-ESO-1, HSP70, c-myc, galectin-1, Sui1, KN-HN-1, HSP40, PrxVI, p62, cyclin B1, HCC-22-5, annexin II, HCA25a, and HER2) were applied as capturing antigens in sandwich ELISA to measure serum IgG levels. Sera from 73 healthy donors and 386 patients with breast cancer, including 182 stage 0/I patients, were evaluated using cutoff values for each TAA equal to the mean +3 SD of the serum levels of healthy controls. The positive TAA rates were relatively high for p53 (10%) and RalA (10%). The positive rates of all TAA of stage 0/I were similar to those of all patients. Even in the stage 0/I patients, 24% showed that two or more TAA were positive, and the positive rate of a five-TAA combination assay was 37%. The positivity rate was significantly higher for the non-luminal type than for the luminal type ($P = .003$). Logistic analysis showed that seropositivity (positive for one or more TAA) in breast cancer patients was independent from any TNM factor or disease stage and was significantly associated with histological grade in the multivariate analysis ($P = .007$). TAA in breast cancer patients may be useful for early detection. However, seropositivity of breast cancer reflects the tumor characteristics but not the disease stage.

KEYWORDS

breast cancer, cancer early diagnosis, serological tests, serology, tumor-associated antibody

1 | INTRODUCTION

Breast cancer is the most common cancer affecting Japanese women, with 10.6% (1 in 9) contracting breast cancer in their lifetime.¹ Early diagnosis of the disease is one of the most effective approaches to management of breast cancer. Although a blood test is convenient for screening for breast cancer in an asymptomatic population, there

are only a few efficient biomarkers for detecting early-stage disease because current biomarkers, such as cancer antigen 15-3 (CA15-3), are cancer-derived factors, and their positive rates fundamentally depend on the tumor burden. In contrast, tumor-associated antibodies (TAA) are immunoglobulins specific for tumor antigens and are a humoral response against cancer. The cancer immunosurveillance theory describes how the immune system monitors cancer from its

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genesis. Tumors are recognized and eliminated by the host immune system in the early stages of development. Then, an equilibrium is reached between tumors and the immune response.²

Serological testing measures target tumor antigen-specific immunoglobulins, which are derived from humoral immunity. Because immunoglobulins are stable and abundant in serum, serological positivity is highly reproducible, even in freeze-thaw cycles,³ long-term storage,⁴ and poorly managed storage conditions;⁵ therefore, serological testing is an essential diagnostic tool in clinical settings. Seropositivity for infectious agents indicates the potential presence of a disease, and the same approach could potentially be used for cancer diagnosis. Shimada et al previously established serum p53 antibodies as cancer diagnostic biomarkers,⁶ which achieved a sensitivity of 40% in superficial esophageal cancer patients. In that study, a strong correlation was observed between p53 seropositivity and p53 immunostaining of the tumor, which suggested that accumulated p53 protein in tumor cells induced a serological response against p53. Furthermore, the present study found that 86% of p53-seropositive patients showed negative seroconversion after resections, with the others experiencing recurrence.⁷

We hypothesized that a serological approach would enable early detection of breast cancer. We previously developed an assay using a combination of 17 TAA; namely, p53, RalA, galectin-1, HSP70, HSP40, KM-HN-1, p90, HCC-22-5, NY-ESO-1, Sui1, PrxVI, cyclin B1, c-myc, annexin II, HCA25a, p62, and human epidermal growth factor receptor type 2 (HER2), to assess gastric cancer,⁸ hepatocellular carcinoma,⁹ and colorectal cancer.¹⁰ The findings of those studies showed that TAA biomarkers tended to be positive even in stage 0/I cancer and appeared to be independent of the disease stage. The aim of the present study was to use our previously developed multipanel assay to analyze the sera of 386 patients with breast cancer and to evaluate the clinicopathological and prognostic significance of TAA.

2 | MATERIALS AND METHODS

2.1 | Recombination of antigens and ELISA

The construction for antigen recombination was based on the full-length cDNA sequences obtained from GenBank. The GenBank accession numbers of 17 antigens (p53, RalA, p90, NY-ESO-1, HSP70, c-myc, galectin-1, Sui1, KN-HN-1, HSP40, PrxVI, p62, cyclin B1, HCC-22-5, annexin II, HCA25a, and HER2) are listed in Table S1. Detailed methods of antigen construction, recombination, and purification were described previously.⁹ Purified antigens were immobilized on 96-well microvalue plates, and 100-fold diluted sera were applied in each well. Captured IgG in sera were detected using HRP-conjugated anti-human IgG antibody. After washing, signals were obtained using substrate solution quenching with HCL, and absorption was measured at 450 nm using a microplate reader. Details of the assay were also described previously.⁸

TABLE 1 Characteristics of the patients in the biobank and from Chiba Cancer Center

	Biobank N = 100	Chiba Cancer Center N = 286	Total N = 386
Stage 0		31	31
Stage I	50	101	151
Stage II	50	97	147
Stage III		34	34
Stage IV		23	23

Note: The Eighth Edition of the AJCC Cancer Staging was used to identify the TNM stage.

TABLE 2 Clinical profiles of Chiba Cancer Center patients

Number of patients in Chiba Cancer Center, N = 286 (number of patients with non-luminal breast cancer, N = 62)	
Stage 0	31
Stage I	101 (18)
Stage II	97 (25)
Stage III	34 (12)
Stage IV	23 (7)
T0	31
T1	116 (27)
T2	97 (27)
T3	34 (5)
T4	8 (3)
N0	176 (33)
N1	60 (16)
N2	17 (6)
N3	12 (7)
M0	262 (55)
M1	24 (7)
ER status	
Positive	224 (18)
Negative	31 (44)
PgR status	
Positive	173 (12)
Negative	82 (50)
HER2 status	
Positive	40 (40)
Negative	197 (22)
Histological grade	
1	73 (6)
2	111 (24)
3	39 (24)

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor. Non-luminal breast cancer is HER2-positive breast cancer or breast cancer negative for ER, PgR, and HER2.

2.2 | Patients and serum samples

Serum samples of 73 healthy controls with no history of cancer were obtained from the residual samples of employee yearly health checkups in 2008 at Medical & Biological Laboratories, Nagoya Japan (MBL). Serum samples of 386 patients with breast cancer (100 samples were obtained from BioBank Japan¹¹ and 286 samples were obtained from Chiba Cancer Center) were also included in our analysis (Tables 1 and 2). Healthy control samples and Chiba Cancer Center samples were collected into serum separator tubes and centrifuged at 3000 g for 5 minutes. All accumulated serum samples were stored at -80°C until the experiment. Preanalytical freeze thaw cycles were performed fewer than two times. The clinical information obtained for the biobank samples included only sex and stage. All 286 patients treated at Chiba Cancer Center were women with a median age of 56 years (range, 29–89). They were treated between July 2008 and August 2009, and serum samples were obtained preoperatively or to assess the indication for neoadjuvant chemotherapy. Tumor characteristics were referenced from the patients' medical records, including: the results of immunohistochemistry (IHC) for estrogen receptor (ER), progesterone receptor (PgR), and HER2; FISH for HER2; and histological grade. IHC and the interpretation of clinical subtype were based on American Society of Clinical Oncology/College of American Pathologist guideline recommendations and the statement of the St. Gallen International Expert Consensus Conference.¹² Histological grade was analyzed on the basis of the Nottingham histological score.¹³ The Eighth Edition of the AJCC Cancer Staging for breast cancer was applied to identify the TNM stage.¹⁴ Informed consent for this study, which was approved by the Ethics Committee of Toho University School of Medicine (A18103_A17052_A16035_A1

6001_26095_25024_24038_22047), Toho University Omori Hospital (M19213), and Chiba Cancer Center (H30-220), was obtained from all patients. This study has been registered in the UMIN Clinical Trials Registry (UMIN000014530). The procedure for taking samples from the healthy controls was approved by the ethics committee of MBL.

2.3 | Statistical analysis and cutoff value optimization

Comparisons of continuous variable, such as the value of each TAA or number of positive TAA in the two groups, were performed using the Mann-Whitney *U* test. Survival analysis according to the TAA status was performed using the log-rank test. To identify the clinical factors that influence recurrence and seroactivity in Chiba Cancer Center patients, logistic analysis was performed. All statistical procedures were performed using EZR statistical software, and two-tailed *P* values lower than .05 indicated statistical significance.¹⁵ The cutoff value of each TAA assay was set at the mean +3 SD of the health controls.¹⁰

3 | RESULTS

A total of 386 patients with breast cancer, 100 from biobank and 286 from Chiba Cancer Center, were enrolled in this study (Table 1). A total of 182 patients had stage 0/I tumors, and 204 patients had stage II/III/IV tumors. The detailed clinicopathological findings of the patients with breast cancer at Chiba Cancer Center are summarized in Table 2. Patients with invasive breast cancer were categorized as having luminal breast cancer or non-luminal breast cancer

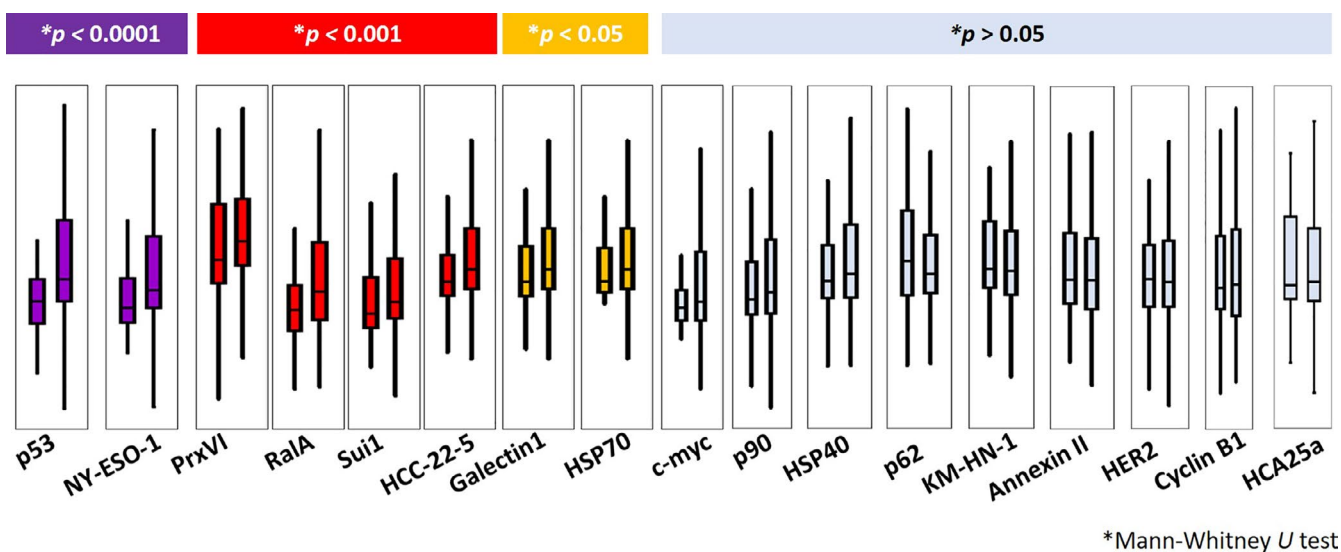


FIGURE 1 Comparison of each tumor-associated antibody (TAA) value between healthy controls and breast cancer patients. Box-whisker plots of each TAA value showing healthy controls ($N = 73$, left) and breast cancer patients ($n = 386$, right). Statistical analysis for comparison of the values between healthy controls and breast cancer patients was performed using the Mann-Whitney *U* test

depending on the IHC data for ER, PgR, and HER2 (also FISH). Luminal breast cancer was positive for ER or PgR and negative for HER2. Non-luminal breast cancer includes HER2 positive tumors or tumors with negative for ER, PgR, and HER2. The percentages of patients with luminal breast cancer and non-luminal breast cancer were 73% ($n = 174$) and 27% ($n = 62$), respectively. Although the

TMN staging did not significantly differ across these three clinical subtypes, non-luminal breast cancer tended to have a higher histological grade. In total, 48 of 286 patients experienced recurrence, and 39 deaths were recorded. Thus, the median progression-free survival was 85 months, and the median overall survival was 87 months.

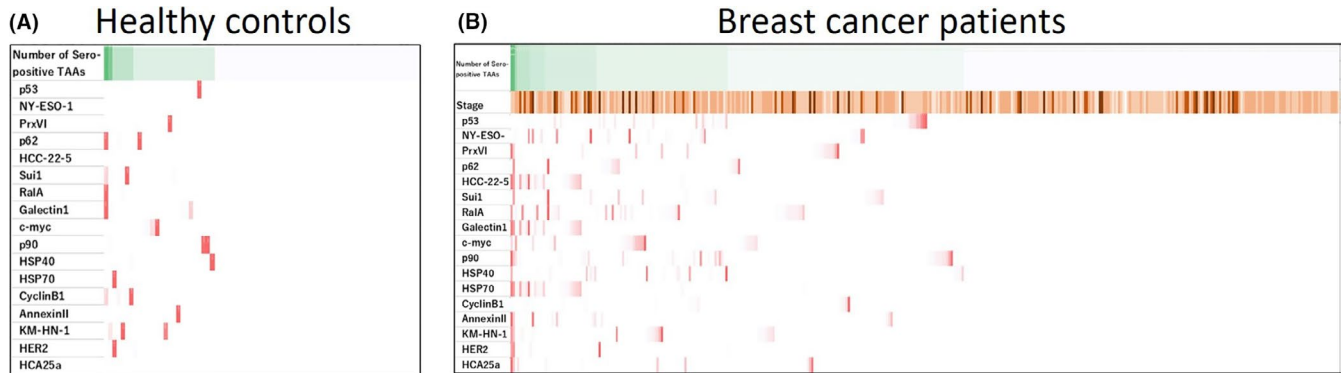


FIGURE 2 Heatmap showing each tumor-associated antibody (TAA) value in every individual. Every individual was sorted according to the positive number of TAA. Increasing color depth in the heatmap represents increasing value, such as the positive number of TAA (green), a TAA value greater than the cutoff value (red), or tumor stage (brown). The deep green or white bar above the heatmap represents the number of positive TAA. The heatmap with red bars shows the positivity for each TAA, and the increasing depth of red color indicates increasing value (lower than the cutoff value is shown in white). (A) Heatmap representing healthy controls ($N = 73$). (B) Heatmap representing breast cancer patients ($N = 386$). Brown color represents the stage of the disease (darkest brown is stage IV)

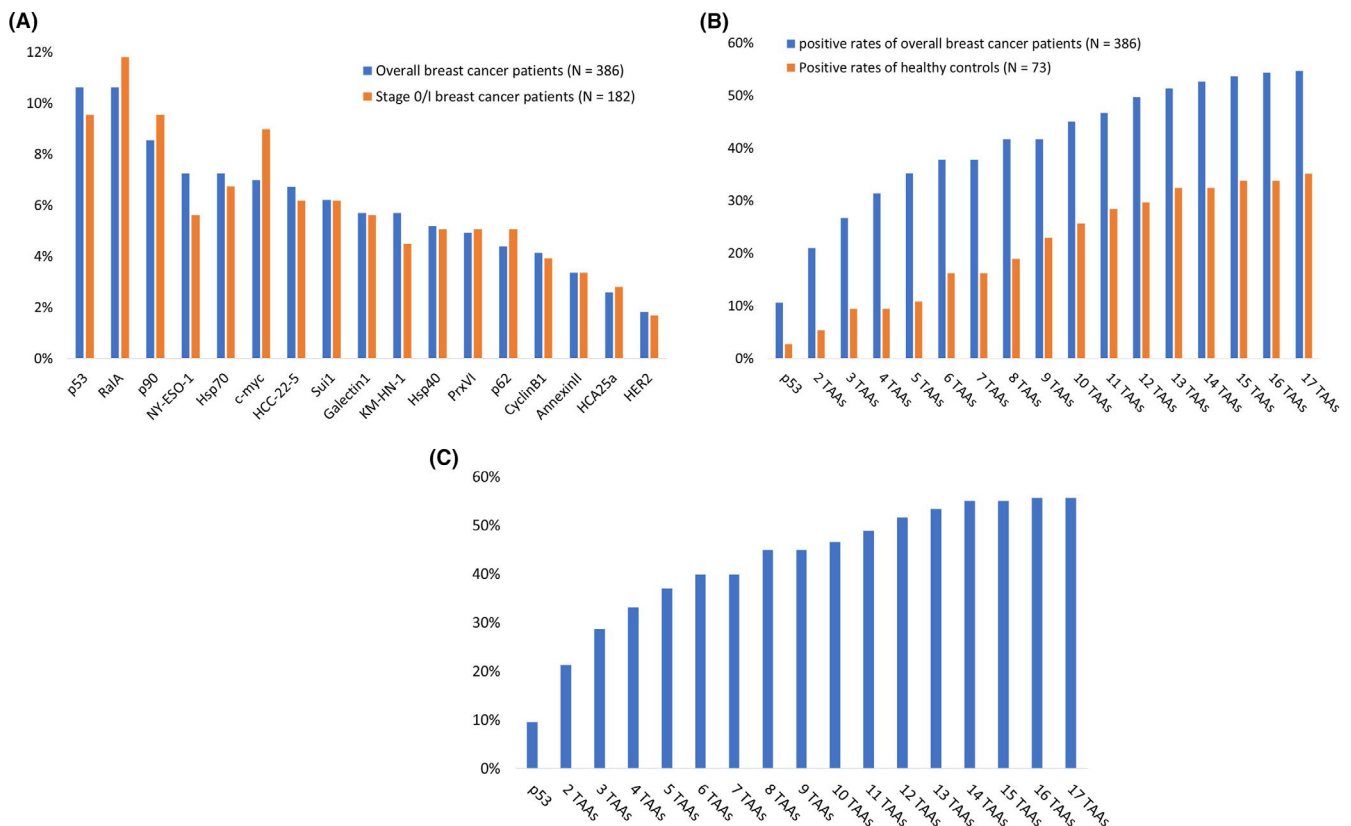
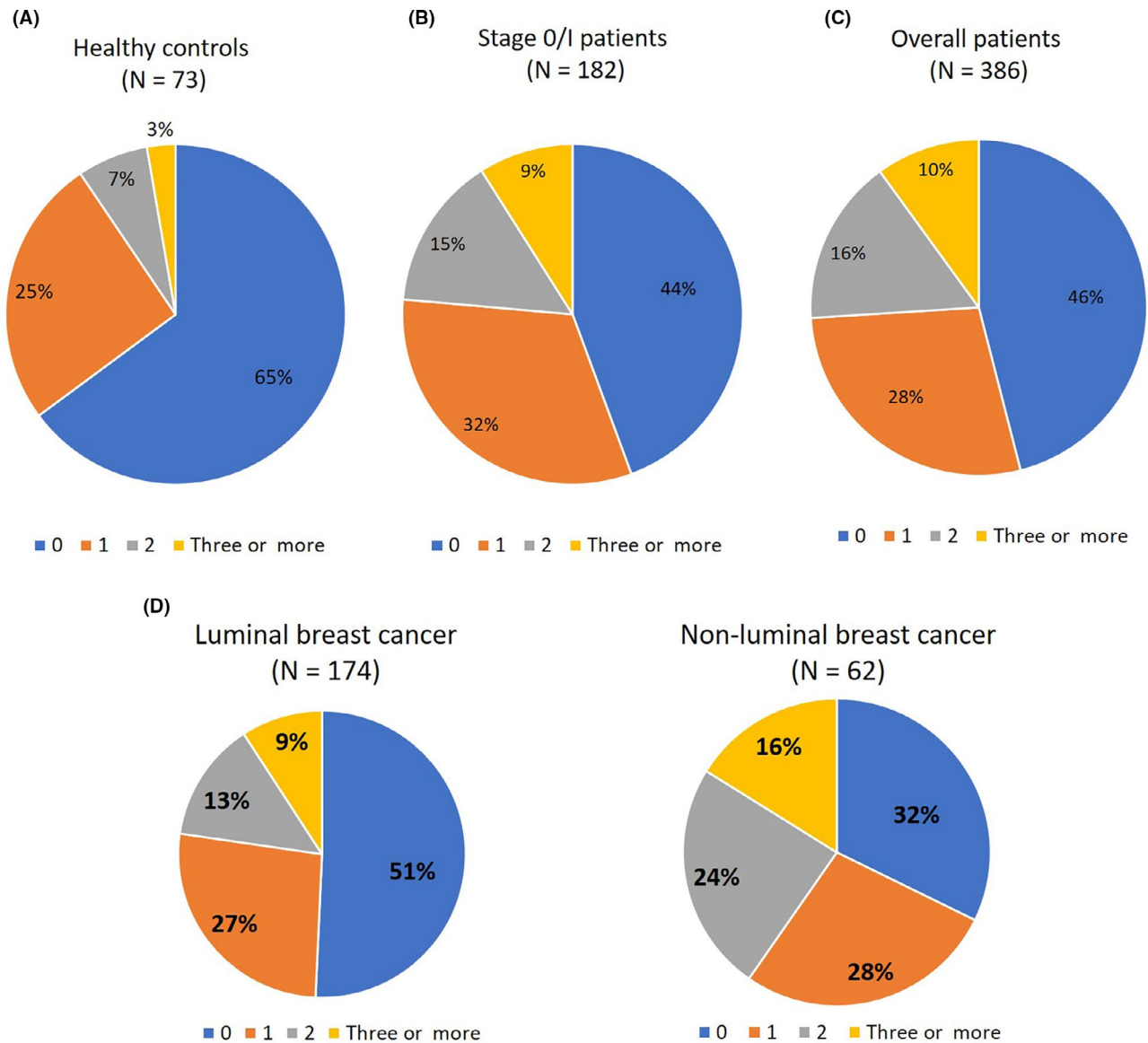


FIGURE 3 Positive rates of tumor-associated antibodies (TAA) above the cutoff value greater than the mean $+3$ SD. (A) Positive rate of each TAA in overall breast cancer patients ($N = 386$, blue bar) and stage 0/I breast cancer patients ($N = 182$, orange bar). (B) Positive rates of multipanel TAA assays in healthy controls ($n = 73$, orange bar) and overall breast cancer patients ($N = 386$, blue bar). (C) Positive rates of multipanel TAA assays in stage 0/I breast cancer patients ($N = 182$)



* $p = 0.003$

* The numbers of positive TAAs in the two subtypes were compared by performing the Mann-Whitney U test.

FIGURE 4 The circle graph shows the distributions of the number of positive tumor-associated antibodies (TAA). Distribution of positive TAA in (A) healthy controls (N = 73), (B) stage 0/I breast cancer patients and (C) overall breast cancer patients (N = 386). (D) Distribution of positive TAA in patients with luminal breast cancer (N = 174) and non-luminal breast cancer (N = 62) at Chiba Cancer Center. The number of positive TAA in the two subtypes compared by performing the Mann-Whitney U test

3.1 | Tumor-associated antibody value comparisons between patients with healthy controls and breast cancer

The serum values of eight TAA (p53, NY-ESO-1, PrxVI, RalA, Sui1, HCC22-5, galectin-1, and HSP70) were significantly higher in breast cancer patients than in healthy controls (Figure 1).

3.2 | Tumor-associated antibody sensitivity and specificity of breast cancer patients

The TAA positivity in every breast cancer patient and healthy control is described in Figure 2. Among 17 TAA, p53 and RalA were the most frequently detected and were present in >10% of patients (Figure 3A). Sensitivity was greater with the higher number

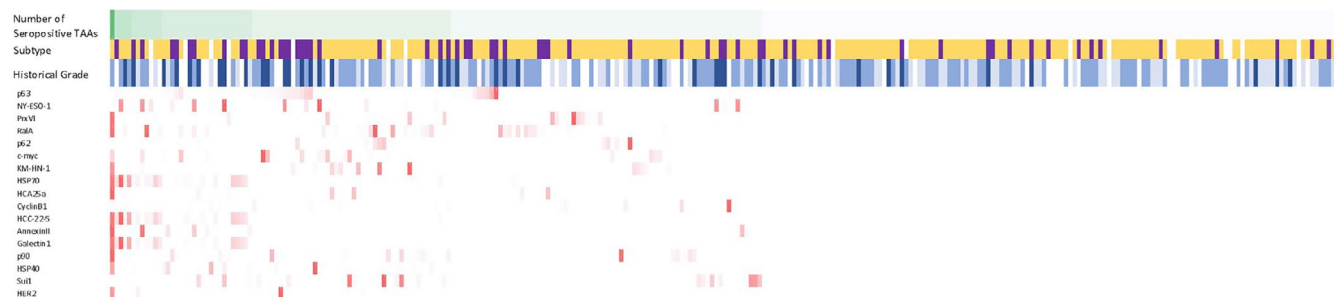


FIGURE 5 Each tumor-associated antibody (TAA) value of every Chiba Cancer Center breast cancer patient and their tumor characteristics (N = 286). All patients were sorted by the positive number of TAA (green bar). Tumor characteristics of each patient are included in the heatmap. The yellow bar represents the luminal type, and the purple bar represents the non-luminal type. Histological grade is reflected by the color depth. Highest histological grade (= 3) is represented by the deepest blue, the lowest histological grade (= 1) by light blue, intermediate histological grade (= 2) by middle blue, and white indicates unknown or non-invasive breast cancer. The red bar in every patient indicates positivity for each TAA and increasing color depth indicates increasing value (lower than the cutoff value is indicated by white)

of contributing TAA, but specificity decreased (Figure 3B). For the five-TAA combination assay (positive for any of p53, RalA, p90, NY-ESO-1, and HSP70), the positive rate was 38% in the cancer patients and 11% in the healthy controls (Figure 3B). The number of positive TAA was significantly larger in the non-luminal breast cancer patients than in the luminal breast patients (Figure 4D).

3.3 | Tumor-associated antibody positivity in patients with stage 0/I breast cancer

Each of the TAA-positive rates in the stage 0/I breast cancer patients and the distribution numbers of the positive TAA were similar to those for all of the breast cancer patients (Figures 3C and 4B). When using the five-TAA combination assay, the positive rate in the cancer patients was 37% (Figure 3C).

3.4 | Logistic analysis of recurrence and seropositivity in the Chiba Cancer Center patients

The TAA-positive values for every breast cancer patient of Chiba Cancer Center (N = 286) are shown in Figure 5. All breast cancer patients were assigned to two groups according to seropositivity (those in the seropositive group were positive for one or more TAA and those in the seronegative group were negative for any TAA). Relapse-free survival was not observed in the Chiba Cancer Center total breast cancer patients (Figure 6A). Although seropositivity appeared to be correlated with favorable relapse-free survival in non-luminal breast cancer patients without distant metastasis (Figure 6B), there was no difference in the overall survival rates between these two groups (Figure 6C). To evaluate the association between seropositivity and recurrence, logistic regression analysis for recurrence in patients without distant metastasis as criterion variables was performed. Univariate analysis with clinical factors and recurrence showed that tumor size, lymph-node metastasis, ER status, and PgR status were significant factors (Table S2a). Multivariate analysis for recurrence was further performed, and advanced breast

cancer (more than stage IIIA) was the only factor associated with recurrence (Table S2b). Then, another logistic analysis for seropositivity as a criterion variable was performed. Univariate analysis showed that histological grade, ER status, and PgR status were significant factors (Table 3a). Multivariate analysis identified histological grade as the only factor associated with seropositivity (Table 3b).

4 | DISCUSSION

A combination assay of 17 TAA was performed in the serum of 386 breast cancer patients and 73 healthy controls, which allowed autoantibody profiling in the patients with breast cancer. The serum levels of 17 TAA were significantly higher in the breast cancer patients, indicating the presence of cancer, than in the healthy controls. Under the cutoff value condition of +3 SD of the mean serum levels in the healthy controls, 54% of the 386 patients were found to be positive for at least one of the autoantibodies. Furthermore, 37% of 182 patients with stage 0/I tumors were positive in the combination assay of five TAA (positive for any of p53, RalA, p90, NY-ESO-1, and HSP70), and the specificity of this combination assay was 11%. Logistic regression analysis showed that seropositivity was not associated with any TNM factors of breast cancer. In contrast, histological grade was the only factor associated with seropositivity.

The most common biomarker in breast cancer diagnosis is CA15-3, a cleaved form of MUC-1 protein. Because the sensitivity of this marker in the early stages of disease is low, the clinical utility of this marker is basically limited to surveillance of recurrence¹⁶ or monitoring during treatment.¹⁷

To validate the premise of our theory that TAA are humoral immune responses against cancer, each TAA value was compared between breast cancer patients and healthy controls (Figure 1). This result indicated that humoral immunity reacted to cancer to a different degree for each case. Furthermore, the TAA-positive rates were not significantly different between the stage 0/I patients and overall breast cancer patients, a finding that was similar to the finding in our previous colorectal cancer study.¹⁰ Our previous reports about serological studies in cancer demonstrated that serum TAA values were

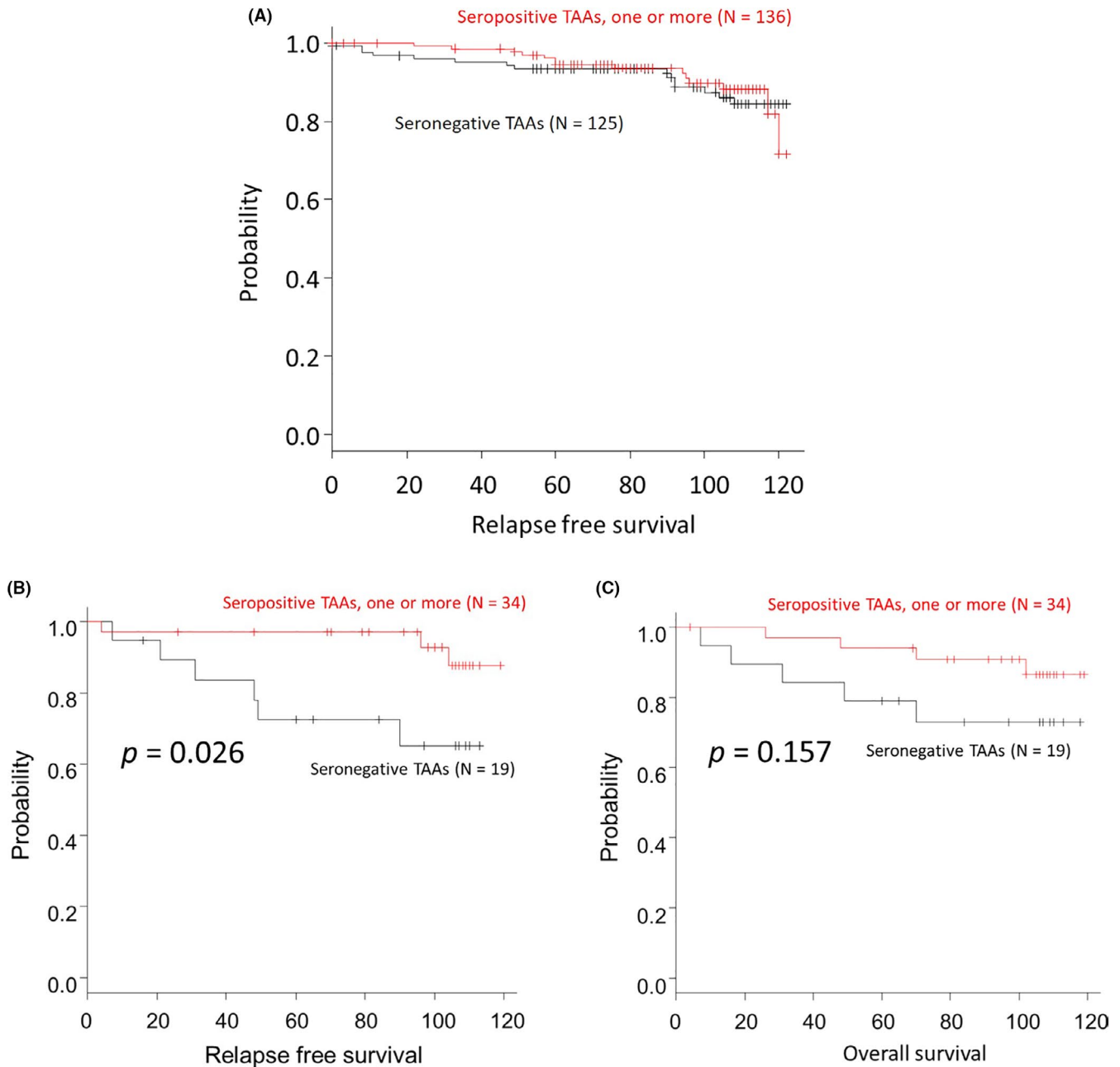


FIGURE 6 Log-rank test for seropositivity (seropositive was positive for one or more tumor-associated antibodies [TAA] and seronegative was negative for all TAA). (A) Relapse-free survival and seropositivity in overall patients at Chiba Cancer Center. (B) Relapse-free survival and seropositivity in patients with non-luminal breast cancer at Chiba Cancer Center (N = 53) (C) Overall survival and seropositivity in patients with non-luminal breast cancer without distant metastasis at Chiba Cancer Center (N = 53)

not associated with disease stage.⁸⁻¹⁰ The present study indicated that seropositivity for cancer mainly depended on the tumor characteristics. Even though seropositivity for the disease was associated with high histological grade, the seropositive group showed favorable relapse-free survival in non-luminal breast-cancer patients. These seemingly inconsistent findings reflected the high sensitivity to chemotherapy in high-grade tumors.¹⁸

A limitation of this study was that the serological diagnostic approach was relatively difficult in breast cancer. The seropositivity of the 17-TAA combination assay was 70% in the colorectal cancer

cohort and 54% in the breast cancer cohort.¹⁰ This contrast reflected that most breast cancers were low-grade tumors, which tended to be seronegative in our analysis. Approximately 80% of breast cancers at Chiba Cancer Center were low-to-middle histological grades in our analysis.

In conclusion, serological testing with 17 TAA to evaluate antibody profiles in patients with breast cancer achieved a positive rate of 38% based on a five-TAA assay even in stage 0/I patients. Our study also demonstrated that the serological approach could reflect the characteristics of a tumor.

TABLE 3 Logistic analysis of the relationship between seropositivity (seropositive for one or more tumor-associated antibodies) and clinical factors in the Chiba Cancer Center patients

Factor	Odds	95% CI	P value
(a) Univariate analysis of seropositivity and clinical factors			
Age	1.27	.799-2.03	.311
Tumor size (≥5 cm)	1.12	.429-2.94	.812
Lymph node metastasis (≥N1)	.761	.457-1.27	.296
Distant metastasis (= M1)	1.28	.548-2.98	.571
Stage (>T3N1)	.744	.221-2.51	.633
ER positive	.433	.224-.84	.0132
HER positive	2.1	1.02-4.30	.0431
PgR positive	.633	.374-1.07	.0891
Histological grade (= 3)	3.33	1.54-7.23	.00231
(b) Multivariate analysis of seropositivity and clinical factors			
Histological grade (= 3)	3.37	1.39-8.15	.00713
ER positive	.67	.225-2.01	.473
PgR positive	.94	.406-2.18	.886
Lymph node metastasis (≥N1)	.703	.393-1.26	.234
Age	1.05	.598-1.85	.864

CI, confidence interval; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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