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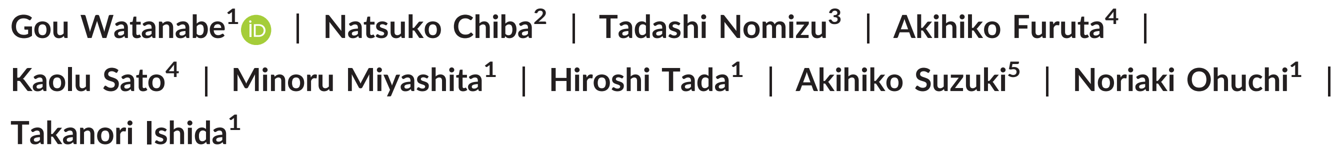
Increased centrosome number in *BRCA*-related breast cancer specimens determined by immunofluorescence analysis



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BRCA-related breast carcinoma can be prevented through prophylactic surgery and an intensive follow-up regimen. However, *BRCA* genetic tests cannot be routinely performed, and some *BRCA* mutations could not be defined as deleterious mutations or normal variants. Therefore, an easy functional assay of BRCA will be useful to evaluate *BRCA* status. As it has been reported that BRCA functions in the regulation of centrosome number, we focused on centrosome number in cancer tissues. Here, 70 breast cancer specimens with known *BRCA* status were analyzed using immunofluorescence of γ-tubulin (a marker of centrosome) foci. The number of foci per cell was higher in cases with *BRCA* mutation compared to wild-type cases, that is, 1.9 (95% confidence interval [CI], 1.5-2.3) vs 0.5 (95% CI, 0.2-0.8) ($P < .001$). Specifically, foci numbers per cell in *BRCA1* and *BRCA2* mutation cases were 1.2 (95% CI, 0.6-1.8) and 2.2 (95% CI, 1.7-2.6), respectively, both higher than those in wild-type cases ($P = .042$ and $P < .0001$, respectively). The predictive value of γ-tubulin foci as determined by area under the curve (AUC = 0.86) for *BRCA* status was superior to BRCAPRO (AUC = 0.69), Myriad Table (AUC = 0.61), and KOHBRA BRCA risk calculator (AUC = 0.65) pretest values. The use of γ-tubulin foci to predict *BRCA* status had sensitivity = 83% (19/23), specificity = 89% (42/47), and positive predictive value = 77% (20/26). Thus, γ-tubulin immunofluorescence, a functional assessment of BRCA, can be used as a new prospective test of *BRCA* status.

KEYWORDS
BRCA1, BRCA2, BRCA-related breast cancer, centrosome, immunofluorescence

1 | INTRODUCTION

Breast cancer is the most common type of cancer and the second leading cause of cancer-related death in women.[1] The Cancer Genome Atlas study revealed that approximately 10% of breast cancer cases had deleterious germline mutations, of which over half were in the *BRCA1* and/or *BRCA2* genes.[2]

Genetic tests for *BRCA* mutation are important for identifying suspected cases of *BRCA*-related breast and ovarian cancer syndrome so that prophylactic surgery and intensive follow-up programs can be recommended to the patient. Furthermore, *BRCA* status predicts patient chemosensitivity to platinum or poly (ADP-ribose) polymerase (PARP) inhibitor.[3,4] Widespread use of PARP inhibitors, thus, necessitates *BRCA* genetic tests. However, genetic tests are costly

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and have problems associated with privacy and genetic counseling. Furthermore, some *BRCA* mutations could not be defined as deleterious mutations or normal variants, and thus, are treated as a variant of unknown significance (VUS). It is therefore important to devise an inexpensive and simple test for predicting *BRCA* mutation status; functional evaluation of the BRCA protein is particularly desired.

Many risk estimation tools for detecting deleterious *BRCA* mutations based on clinicopathological information have been reported, such as BRCAPRO,[5,6] Myriad Table,[7] and the Korean Hereditary Breast Cancer BRCA risk calculator (KOHCal).[8] However, factors such as small family size or a small number of female relatives can prevent accurate assessment of risk.[9]

In this study, we focused on another BRCA function, controlling centrosome duplication;[10-12] it is known that BRCA plays important roles in the DNA repair pathway. Cells normally have either one or two centrosomes. Suppression of BRCA1 or BRCA2 causes centrosome amplification.[13,14] We therefore speculated that an increase in the number of centrosomes may indicate *BRCA* mutations in breast cancer specimens. γ-Tubulin, a centrosome component, could not be detected as foci by immunohistochemistry using 3,3-diaminobenzidine (DAB),[15] whereas immunofluorescence of γ-tubulin was detected as foci in mammalian cells[12] and human breast tissue.[16,17] We used this latter approach in the present study to determine whether the number of γ-tubulin foci could predict *BRCA* status in clinical samples.

2 | MATERIALS AND METHODS

2.1 | Patients

From 2001 to 2014, 68 female Japanese breast cancer patients (including two patients with bilateral breast cancer) underwent breast cancer surgery and genetic testing for *BRCA* mutations at Hoshi General Hospital (Fukushima, Japan) and Ishinomaki Red Cross Hospital (Ishinomaki, Japan). In both hospitals, patients who met the criteria for *BRCA1/2* testing according to the National Comprehensive Cancer Network (NCCN) guidelines were recommended to undergo genetic testing; however, the testing was not covered by Japanese national health insurance. One patient who did not meet the NCCN guidelines underwent genetic testing because of her desire to be tested. All participants were interviewed by experienced genetic counselors to determine the personal and family history of cancer (at least first- and second-degree relatives). The study protocol was approved by the institutional review board at each institution and at Tohoku University Graduate School of Medicine (Sendai, Japan).

2.2 | Mutation detection

Genomic DNA samples from study subjects at Hoshi General Hospital and Ishinomaki Red Cross Hospital were analyzed at Myriad Genetic Laboratories (Salt Lake City, UT, USA). Full sequencing analysis was carried out for probands, and single-site testing for the family-specific mutations (seven patients) was undertaken for relatives of mutation-positive probands.

2.3 | Estimation of *BRCA1/2* mutation probability using available prediction models, BRCAPRO, Myriad Tables, and KOHCal

Our analysis of the predictive value of *BRCA* status compared to pretests was restricted to patients who did not have a family history of deleterious *BRCA* mutations, as seven patients who had such a history were likely to have undergone *BRCA* genetic testing. One patient was excluded from the analysis because of sufficient familial history.

The BRCAPRO model calculated the probability of a *BRCA1* and/or *BRCA2* mutation from patients' personal and first- and second-degree relatives' history of breast and ovarian cancers.[5,6] We used the version implemented in the BayesMendel 2.1-2 package of R statistical software (R Foundation, Vienna, Austria). The Myriad prevalence tables provided a probability of detecting *BRCA* mutations and are based on observations of deleterious mutations in Myriad Genetics Laboratories databases of clinical testing services (http://d1izdzz43r5o67.cloudfront.net/brac/brca-prevalence-tables.pdf).[7] KOHCal was constructed using a logistic regression model based on the Korean Hereditary Breast Cancer study.[8]

2.4 | Immunodetection of γ-tubulin

Experiments were carried out at the Pathology Department of Tohoku University Hospital on unstained tissue specimens (4-μm thickness) mounted on slides, which were provided by Hoshi General Hospital and Ishinomaki Red Cross Hospital. Antigen retrieval was carried out by heating the slides in an autoclave at 120°C for 5 minutes in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate, pH 6.0). After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 minutes at room temperature, samples were incubated with an antibody against γ-tubulin clone GTU-88 (1:600; Sigma, St Louis, MO, USA) followed by Cy-3-conjugated Affinipure F(ab')2 fragment donkey anti-mouse IgG (H + L) (1:100; Jackson Immunoresearch, West Grove, PA, USA) for immunofluorescence or biotinylated rabbit anti-mouse antibody (1:100; Nichirei Bioscience, Tokyo, Japan) for DAB staining for 1 hour at room temperature. Nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA).

2.5 | Microscopy

The number of γ-tubulin foci per cell was counted using a BZ9000 all-in-one fluorescence microscope (Keyence, Osaka, Japan) with an integrated camera and BZ-Analyzer software (Keyence). Foci were counted with Hybrid Cell Counter software in nine to 12 visual fields at 100× magnification. γ-Tubulin foci were captured from 10 lower

to higher depth fields with a 0.4-μm pitch for *Z*-axis scanning. Composite images were generated with the image-joining function of the software and *Z*-axis data were extracted at a single focal point from multiplane images to obtain a fully focused image using the quick full-focusing function of the software.

2.6 | Quantification of γ-tubulin foci per cell

Non-cancer cells were excluded from the analysis by serial H&E staining, and cancer cells were counted based on DAPI staining by the cell (hybrid cell count) separation method (Keyence) with manual correction. γ-Tubulin foci were counted using color extraction and size selection (0.2-20.0 μm^2). The total number of γ-tubulin foci divided by DAPI-positive cells in fully focused composite images is expressed as foci/cell.

2.7 | Statistical analysis

The relationship between clinicopathological parameters and *BRCA* status was analyzed with Fisher's exact test. The Kruskal–Wallis test was used to evaluate the relationship between family history of breast or ovarian cancer and *BRCA* status. The predictive value of foci per cell and pretests for *BRCA* status was analyzed with the Wilcoxon test. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were estimated. To determine optimal thresholds, the point on the ROC curve with a maximum Youden index (sensitivity−[1−specificity]) and the point with the shortest distance value from the 0.1 point of the ROC curve were calculated. Statistical analyses and AUC estimates were carried out using JMP pro11 software (SAS Institute, Cary, NC, USA). Statistical significance was defined as $P < .05$.

3 | RESULTS

3.1 | Patient characteristics and *BRCA* mutation status

Patient characteristics by *BRCA* status are summarized in Table 1. Of 68 patients, including two with bilateral breast cancer, seven (10.3%) had *BRCA1* mutations, 17 (25.0%) had *BRCA2* mutations, and one (1.5%) had mutations in both genes (Table S1). Only one patient had a normal variant, *BRCA1* G275D, which was classified as wild-type *BRCA* as in previous reports,[18,19] and two *BRCA1* intronic mutations (one patient with bilateral breast cancer: exon 9-62, 1 bp deletion). Variant of unknown significance was not observed. All of the patients with suspected *BRCA*-related cancer underwent *BRCA* genetic testing without coverage by national health insurance as a matter of clinical practice; therefore, nearly all patients (96.6%) met *BRCA1/2* testing criteria of NCCN guidelines (https://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf). Among these patients, familial history of breast or ovarian cancer was the only statistically significant factor related to *BRCA* mutation status ($P < .001$).

3.2 | Increased number of γ-tubulin foci with deleterious mutation in *BRCA*

Representative images of γ-tubulin foci in wild-type and mutant *BRCA1/2* cases are shown in Figure 1. γ-Tubulin foci could not be detected by DAB immunohistochemistry (Figure 1B,G,L), but were observed by immunofluorescence (Figure 1C,H,M). The magnified views of Figure 1(C,H,M) are shown in Figure 1(D,I,N) and the views for counting using a hybrid cell counter are shown in Figure 1(E,J,O), respectively. All of the areas of the foci/cell number were higher for *BRCA1* L63X (foci/cell = 1.10, Figure 1H) and *BRCA2* 2423del4 (foci/cell = 6.52; Figure 1M) compared to wild-type *BRCA* (foci/cell = 0.53; Figure 1C).

As shown in Figure 2, the number of γ-tubulin foci/cell was higher in cases with *BRCA1* and/or *BRCA2* mutation than in wild-type *BRCA1* and *BRCA2* (1.9 [95% confidence interval (CI), 1.5-2.3] vs 0.5 [95% CI, 0.2-0.8]) ($P < .001$). The number of foci/cell in cases of *BRCA1* and *BRCA2* mutations was 1.2 (95% CI, 0.6-1.8) and 2.2 (95% CI, 1.7-2.6), respectively, which was higher than that for wild-type *BRCA1* and *BRCA2* ($P = .042$ and $P < .0001$, respectively).

As shown in Figure 3, the number of γ-tubulin foci/cell was higher in cases with *BRCA1* and/or *BRCA2* deleterious mutations than in cases with *BRCA1* and *BRCA2* normal variant mutations (2.0 [95% CI, 1.4-2.7] vs 0.3 [95% CI, 0.02-0.55]) ($P = .049$).

3.3 | Comparison of pretests for BRCA status: BRCAPRO, Myriad Tables, KOHCal, and γ-tubulin immunofluorescence

γ-Tubulin immunofluorescence and existing pretests were compared in terms of their predictive value for *BRCA* mutation status. We analyzed 60 patients who did not have a family history of deleterious *BRCA* mutations. γ-Tubulin immunofluorescence had superior predictive value ($P < .0001$), followed by BRCAPRO ($P = .0073$) (Table 2).

We next analyzed ROC curves to determine the appropriate cut-off point for predicting *BRCA* mutation status (Figure 4). γ-Tubulin immunofluorescence had the highest AUC (0.86; Figure 4D). The AUC (0.97; Figure 4F) of the *BRCA2* mutation was higher than that of the *BRCA1* mutation (0.79; Figure 4E). The cut-off value for γ-tubulin immunofluorescence in *BRCA1/2* mutations was 0.95 according to the maximum Youden index, with a sensitivity of 83% (19/23), specificity of 89% (42/47), and positive predictive value of 77% (20/26).

4 | DISCUSSION

The ability to identify *BRCA*-related cancer patients among many breast cancer patients is important, because these patients are suitable for targeted therapy and prophylactic surgery as well as intensive follow-up to prevent breast cancer-related death.[4,20-23] γ-Tubulin immunodetection could be a useful pretest for patients who are considering *BRCA* genetic testing, as it is inexpensive and

TABLE 1 Patient characteristics by *BRCA* status

	BRCA status						
	Non-carriers[a] (n = 45)		*BRCA1* mutation carriers[b] (n = 7)		*BRCA2* mutation carriers[b] (n = 17)		
	No. of patients	%[c]	No. of patients	%[c]	No. of patients	%[c]	*P*-value
Age at diagnosis, years	48.7		42.8		52.3		.4200
NCCN HBOC testing criteria							
Met	42	93.3	7	100.0	17	100.0	
Unmet	3	6.7	0	0.0	0	0.0	
BRCA sequencing							
Full sequencing	44	97.8	6	85.7	12	70.6	
Single-site	1	2.2	1	14.3	5	29.4	
Pathological type							
Invasive ductal carcinoma	38	84.5	6	87.5	13	76.5	
Invasive lobular carcinoma	1	2.2	0	0	0	0	
Ductal carcinoma in situ	6	13.3	1	14.3	4	23.5	
Invasive size, mm	22.8		38.3		22.3		.0600
LN status							
Negative	27	61.4	4	57.1	8	47.1	.4400
Positive	17	38.6	3	42.9	9	52.9	
Stage							
0	6	13.3	1	14.3	4	23.5	.6600
1	15	33.3	1	14.3	1	5.9	
2	15	33.3	4	57.1	8	47.1	
3	8	17.8	0	0.0	3	17.6	
4	1	2.0	1	14.3	1	5.9	
ER							
Negative	17	40.5	5	71.4	3	17.6	.1900
Positive	25	59.5	2	28.6	14	82.4	
Unknown	3		0		0		
PR							
Negative	20	47.6	5	71.4	10	58.8	.5100
Positive	22	52.4	2	28.6	7	41.2	
Unknown	3		0		0		
HER2/neu							
Negative	22	81.5	5	71.4	14	87.5	.9800
Positive	5	18.5	2	28.6	2	12.5	
Unknown	18		0		1		
ER and HER2 status							
ER+, HER2−	14	56.0	3	42.9	11	68.8	
ER+, HER2+	0	0.0	0	0.0	2	12.5	
ER−, HER2+	2	8.0	2	28.6	0	0.0	
ER−, HER2−	9	36.0	2	28.6	3	18.7	
Unknown	20		0		1		
Familial history of breast or ovarian cancer							
None	4	8.9	2	28.6	0	0.0	<.0001
1	17	37.8	0	0.0	0	0.0	
2	17	37.8	1	14.3	1	5.9	
≥3	7	15.5	4	57.1	16	94.1	

ER, estrogen receptor; HBOC, hereditary breast and ovarian cancer; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; LN, lymph node; NCCN, National Comprehensive Cancer Network.
[a]Including one normal variant (BRCA1 G275D) and two intronic mutation patients.
[b]Numbers of *BRCA1* and *BRCA2* mutation carriers included one patient with both mutations.
[c]Calculated by excluding the number of unknown cases.

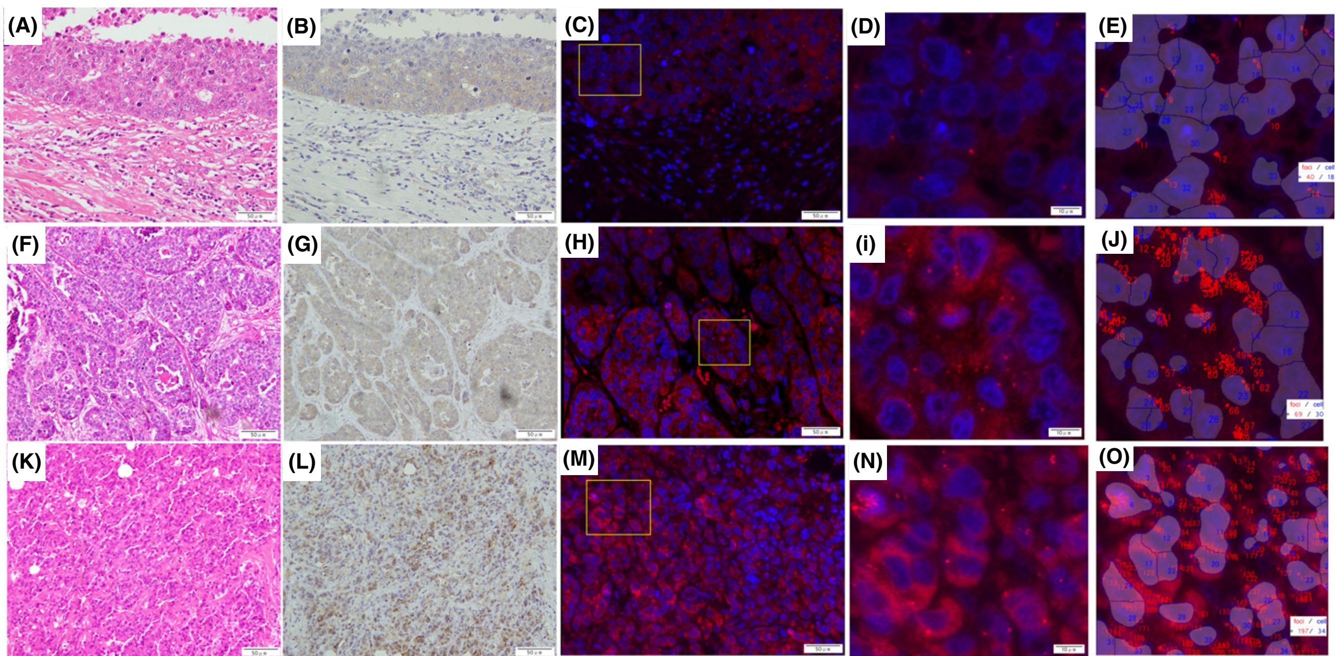

FIGURE 1 Representative cases for detection of breast cancer susceptibility gene *BRCA* by immunofluorescence analysis. (A-C) Wild-type *BRCA* breast cancer tissue sample with H&E staining (A), 3,3-diaminobenzidine immunostaining of γ-tubulin (B), and immunofluorescence (C). (D) Enlarged view of area in the yellow square in (C). (E) Numbers of γ-tubulin foci (red) and DAPI-stained cells (blue) were counted using a hybrid cell counter. (F-J) *BRCA1* L63X and (K-O) *BRCA2* 2423del4 breast cancer specimens with the same staining and views as those shown in (A-E)

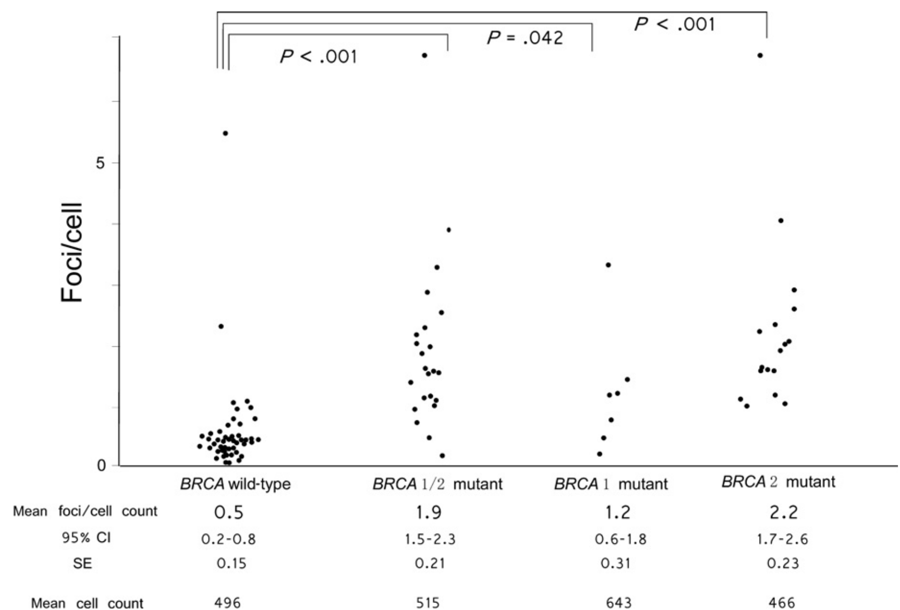

FIGURE 2 Scatter plot of foci/cell by status of *BRCA* breast cancer susceptibility gene in breast cancer tissue samples. The number of γ-tubulin foci stained per cell was counted using Hybrid Cell Counter software. CI, confidence interval SE, standard error

convenient and can be undertaken at any general hospital. The AUC (0.86) of γ-tubulin immunofluorescence for predicting *BRCA* mutation status was superior to that of BRCAPRO (0.69), Myriad Tables (0.61), and KOHCal (0.65); these tests were previously reported to be more accurate with AUCs of 0.76, 0.71-0.72, and 0.76, respectively.[9,24,25] These pretests that use clinicopathological information might be more affected by ethnic differences than our method.[26]

The immunohistochemical analysis of breast cancer specimens, especially of estrogen receptor and human epidermal growth factor receptor 2 expression, is important for determining the appropriate treatment by targeted therapy. These overexpressed proteins are easy to evaluate by immunohistochemistry; on the contrary, evaluation of tumor suppressor proteins such as p53 and BRCA is challenging. We previously focused on protein function to determine gene status by immunohistochemistry, and have reported combined immunohistochemical detection of p53 and its downstream proteins for predicting *TP53* mutation.[27] Shimomura et al reported that loss of BRCA1 expression was associated with an increase in the number

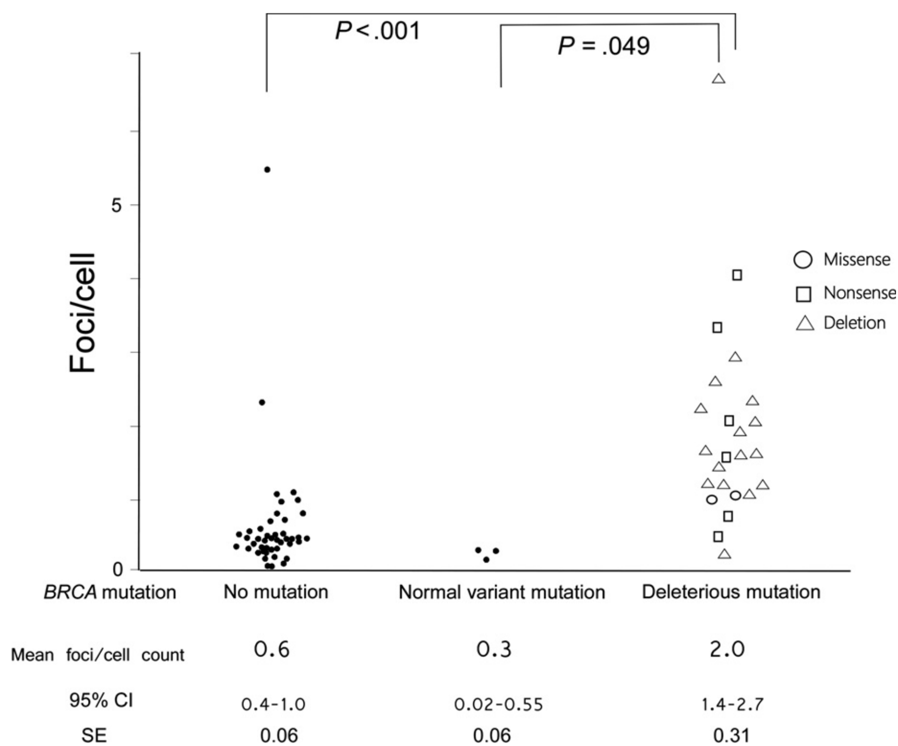

FIGURE 3 Scatter plot of foci/cell by type of mutation of *BRCA* breast cancer susceptibility gene. The number of γ-tubulin foci stained per cell was counted using Hybrid Cell Counter software. CI, confidence interval SE, standard error

of centrosomes in breast cancer specimens, as determined by immunofluorescence analysis;[17] they also found that centrosome numbers were higher in three breast cancer patients with *BRCA1* mutation compared to those with normal BRCA1 expression. Martins et al reported that the centrosome number was higher in normal and cancerous breast tissue from *BRCA1* mutation carriers than in non-carriers.[16] However, there have been no studies evaluating the predictive value of centrosome numbers for *BRCA* mutation status using immunofluorescence analysis.

TABLE 2 Predictive value of available pretests and immunofluorescence of γ-tubulin for *BRCA* status

	BRCA status		
	Non-carriers[a] (n = 44)	*BRCA1/2* mutation carriers (n = 16)	*P*-value
γ-Tubulin foci/cell			
Mean (95% CI)	0.6 (0.4-0.9)	1.7 (1.2-2.2)	<.0001
Standard error	0.13	0.21	
BRACAPRO			
Mean (95% CI)	12.8 (5.6-20.0)	42.5 (30.5-54.5)	.0073
Standard error	3.6	6	
Myriad table			
Mean (95% CI)	10.3 (7.6-13.0)	16 (11.6-20.4)	.1167
Standard error	1.3	2.2	
KOHcal			
Mean (95% CI)	29.9 (3.1-23.7)	42.5 (5.1-32.2)	.0984
SE	3.1	5.2	

CI, confidence interval; KOHcal, KOHBRA (The Korean Hereditary Breast Cancer) BRCA risk calculator.
[a]Including one normal variant (*BRCA1* G275D) and two intronic mutation patients.

BRCA genetic testing is rapidly spreading worldwide because of the low cost of next-generation sequencing, availability of targeted therapy for BRCA-related cancer, and *BRCA* patent invalidity. However, many problems exist with the genetic testing, especially regarding the evaluation of VUS mutations. The Breast Cancer Information Core (https://research.nhgri.nih.gov/bic/), the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and the Leiden Open Variation Database – International Agency for Research on Cancer (http://www.lovd.nl/2.0/index_list.php) are open access public databases for genome mutations. However, the databases still contain many VUS mutations. For this reason, a functional assessment of BRCA protein is needed for assisting the judgment of a VUS as a deleterious or normal variant mutation. In this study, three cases with *BRCA* normal variant mutations did not have increased centrosome numbers, the same as cases without *BRCA* mutations. However, further study is needed for this issue.

Tumors with the molecular features of *BRCA* mutation are known as having "BRCAness".[28] BRCAness is not only the inactivation of a protein related to DNA repair pathways, but also methylation of *BRCA1* or *BRCA2*.[29] The Cancer Genome Atlas study showed 60% of basal-like breast cancers involved BRCA inactivation, including methylation and mutation.[2] BRCAness evaluation is important for determining individualized treatment regimens that can include PARP inhibitors or DNA cross-linking agents. Several studies have attempted to identify BRCAness using transcriptional biomarkers[30,31] or by assaying homologous recombination.[32,33] The homologous recombination deficiency score is highly correlated with defects in BRCA1/2 and is associated with the response to platinum therapy,[33]

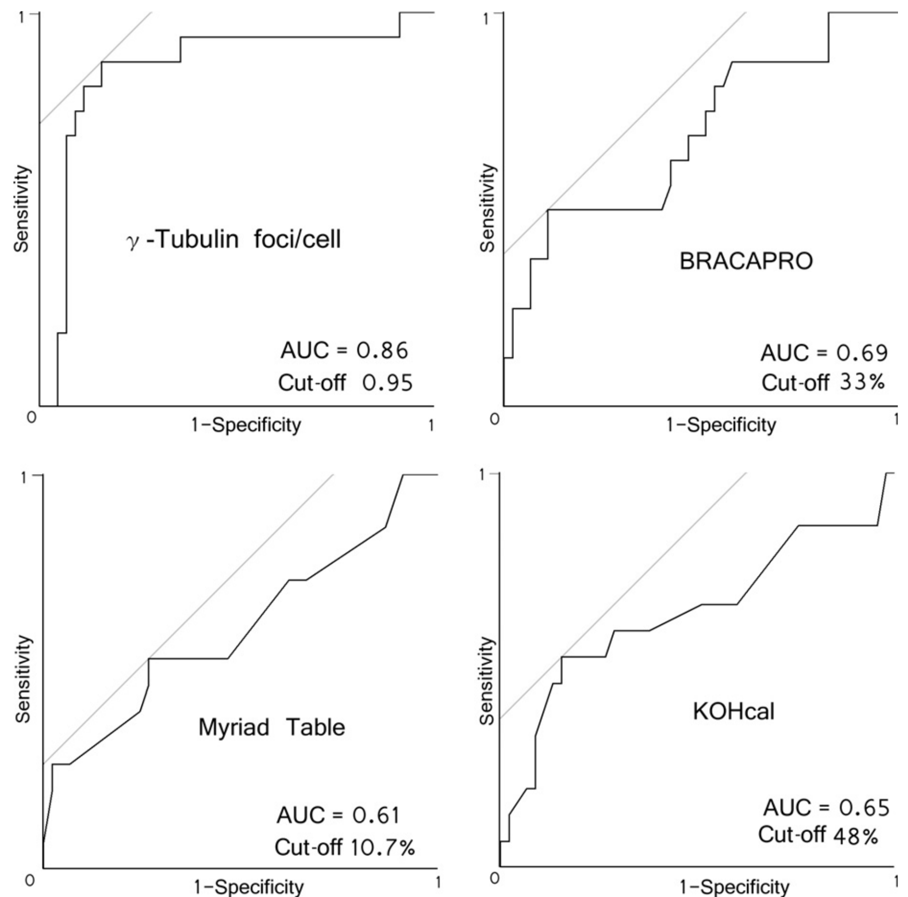

FIGURE 4 Receiver operating characteristic (ROC) curves of γ-tubulin immunodetection and existing pretests for predicting *BRCA* mutation status in breast cancer tissue samples. BRCAPRO (A), Myriad Table (B), The Korean Hereditary Breast Cancer *BRCA* risk calculator (KOHCal) (C), and γ-tubulin foci/cell (E) were evaluated using ROC curves for *BRCA1/2* mutations. γ-Tubulin foci/cell for *BRCA1* (E) and *BRCA2* (F) were evaluated. The cut-off point was determined by the maximum Youden index. AUC, area under the ROC curve

but this scoring system is complex. In this study, some cases of increased centrosome number in *BRCA* wild-type individuals (23% of false-positive results) might be considered to represent BRCAness.

Centrosome amplification often occurs in human cancers, especially in breast cancer.[34] Triple negative breast cancers strongly correlate with centrosomal amplification using immunofluorescence analysis of γ-tubulin, the same as our method.[35,36] Based on their biological functions, both BRCA1 and BRCA2 play important roles in the proper control of centrosome duplication: BRCA1 is a ubiquitin ligase of γ-tubulin that regulates centrosome number;[13] and BRCA2 has a centrosomal localization signal that functions in centrosome positioning, amplification, and cohesion.[37-39] Numerous studies have revealed that inhibition of BRCA function causes centrosome aberrations in human breast cell lines, mice, and human breast tissue.[13,17,40] However, many proteins function to control centrosome duplications.[41] Therefore, the 23% false-positive rate in our study may involve disruption of other centrosomal proteins.

There is a debate as to whether all deleterious mutations of *BRCA1/2* genes equally disrupt both functions: controlling centrosome duplication and homologous recombination.[42] We and Cochran et al have reported that most *BRCA* deleterious mutations abrogate centrosome duplication and are well correlated with DNA repair function (Table S2).[8,10,43] However, the 17% false-negative rate in our study might retain controlling centrosome duplication with *BRCA* mutation.

Our study has two limitations. First, although the foci/cell were counted using an objective evaluation method and two previous reports support an increased number of specimens with *BRCA* mutations among breast cancer specimens,[16,17] our findings need to be validated by additional data. Second, centrosomes are regulated by multiple factors, including BRCA1/2.[11] We need to investigate the other factors that contribute to increased centrosome number with wild-type *BRCA*.

In conclusion, an increased number of centrosomes can be used to predict the presence of *BRCA* mutation, thus potentially assisting patients in deciding whether to pursue genetic testing.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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