




## Establishment and validation of an immunodiagnostic model for prediction of breast cancer

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### ABSTRACT

Serum autoantibodies that react with tumor-associated antigens (TAAs) can be used as potential biomarkers for diagnosis of cancer. This study aims to evaluate the immunodiagnostic value of 11 anti-TAAs autoantibodies for detection of breast cancer (BC) and establish a diagnostic model for distinguishing BC from normal human controls (NHC) and benign breast diseases (BBD). Sera from 10 BC patients and 10 NHC were used to detect 11 anti-TAAs autoantibodies by western blotting. The 11 anti-TAAs autoantibodies were further assessed in 983 sera by relative quantitative enzyme-linked immunosorbent assay (ELISA). Binary logistic regression and Fisher linear discriminant analysis were conducted to establish a prediction model by using 184 BC and 184 NHC (training cohort,  $n = 568$ ) and validated by leave-one-out cross-validation. Logistic regression model was selected to establish the prediction model. Results were validated using an independent validation cohort ( $n = 415$ ). The five anti-TAAs (p53, cyclinB1, p16, p62, 14-3-3 $\xi$ ) autoantibodies were selected to construct the model with the area under the curve (AUC) of 0.943 (95% CI, 0.919–0.967) in training cohort and 0.916 (95% CI, 0.886–0.947) in the validation cohort. In the identification of BC and BBD, AUCs were 0.881 (95% CI, 0.848–0.914) and 0.849 (95% CI, 0.803–0.894) in training and validation cohort, respectively. In summary, our study indicates that the immunodiagnostic model can distinguish BC from NHC and BC from BBD and this model may have a potential application in immunodiagnosis of breast cancer.

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Autoantibody; breast cancer; tumor-associated antigen; benign breast disease; immunodiagnostic model


## 1. Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer-related death among women worldwide<sup>1</sup> According to the data from GLOBOCAN 2018, BC alone accounts for 24.2% of all cancer cases and 15.0% of all cancer deaths among females<sup>1</sup> So far, the etiology of BC has not been completely revealed. As a result, it is imperative to develop strategies for screening, early diagnosis, and monitoring prognosis as well as risk stratification to decrease mortality and increase the possibility of curing the disease. At present, mammography screening for BC is widely available in many countries. However, not all breast cancers can be visualized on screening mammograms<sup>2</sup> Both sensitivity and specificity of mammography are lower, especially in breast tissues that are extremely dense and also in younger women.<sup>3,4</sup> Therefore, it is not recommended for women with average risk and under the age of 50 years<sup>5</sup> In addition, mammography can not only lead to overdiagnosis, but also makes women experience the harms and side effects of treatment.<sup>6,7</sup> In comparison, serological tests based on the tumor-associated biomarkers could be a good way to remedy some defects of mammography.<sup>8</sup>

Recently, with the development of proteomic technologies, a variety of tumor-associated antigens (TAAs) have been identified and employed in immunoassay.<sup>9–11</sup> Meanwhile, many researchers have demonstrated that serum anti-TAAs autoantibodies may be useful as biomarkers for cancer immunodiagnosis.<sup>12–17</sup> In 1982, the anti-p53 autoantibody, for the first time, was reported to be existing in sera from patients with BC<sup>18</sup> Since then, many other anti-TAAs autoantibodies were detected and evaluated in BC diagnosis. Autoantibodies against p53, MUC1, and HER2 were the ones proposed mostly among those autoantibodies. It is universally acknowledged that anti-p53 antibody exists in the sera of various cancer patients and has certain value in the diagnosis of cancer.<sup>19</sup> MUC1 was the most commonly used antigen in BC detection.<sup>20,21</sup> However, Burford et al. investigated a large number of sera which were collected prospectively, and they demonstrated that autoantibodies to MUC1 are unlikely to be useful as a screening test for BC<sup>22</sup> Besides, the diagnostic value of anti-HER2 antibody seems not superior to other reported autoantibodies to the corresponding antigens involved in cell cycle regulation (cyclinB1, cyclinD1, and p16), apoptosis regulation (survivin), cell proliferation (c-myc) and shield mRNAs from natural physiological degradation

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 Supplemental data for this article can be accessed on the [publisher's website](#).

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(IMP1, p62, koc).<sup>23–28</sup> Moreover, previous studies confirmed that the detection of single autoantibody usually offers insufficient diagnostic power, but the detection of multiple autoantibodies to TAAs was shown to be more sensitive.<sup>27,29,30</sup> However, those studies have their own disadvantages, such as less sample size, lack of benign tumor control group, no prediction model or failure to set external validation group.

In this study, the immunodiagnostic values of 11 anti-TAAs (p53, cyclinB1, cyclin D1, p16, p62, IMP1, koc, c-myc, RalA, survivin, 14-3-3 $\xi$ ) autoantibodies were evaluated. The existence of the 11 autoantibodies had been demonstrated in the serum samples from patients with hepatocellular carcinoma, ovarian and gastric cancer, which were collected in the previous study.<sup>29,31–34</sup> Then, an optimal prediction model of multiple autoantibodies was constructed to detect BC with a higher diagnostic value. Both of the Binary logistic regression and Fisher linear discriminant analysis models were established to predict BC based on the relative concentrations of serum autoantibodies. An independent validation cohort was incorporated to verify the prediction model.

## 2. Materials and methods

### 2.1 Serum samples

The case–control study included two cohorts of cases from two different hospitals. The training cohort sera from 184 BC patients were obtained from a hospital in Henan Province, China, from March 2018 to May 2018. The validation cohort consisting of 197 BC patients were recruited from another hospital of Henan Province, China, between February 2011 and June 2014. All cancer sera were collected once patients were diagnosed and before receiving any treatment. Two normal control groups (training cohort 184, validation cohort 109) and patients (training cohort 200, validation cohort 109) with benign breast disease (BBD) were enrolled in this study from December 2017 to March 2018 at a hospital in Henan

province, China. All female cases were age-matched with participants in benign breast diseases and normal control groups. The Institutional Review Board of Zhengzhou University approved the study. All participants enrolled in the study signed an informed consent form. The characteristics of study populations are shown in Table 1.

### 2.2 Recombinant TAAs

Two tumor-associated antigens, 14-3-3 $\xi$  and RalA were constructed and purified using the same approach as used in our previous study.<sup>27,31</sup> The rest of TAAs, including p53, cyclin B1, cyclin D1, p16, IMP1, p62, koc, c-myc, survivin, were derived from our previous studies.<sup>31,34</sup>

### 2.3 Western blotting

Ten patients with BC and 10 normal individuals were randomly selected to examine the reactivity of sera autoantibodies to TAAs by western blotting analysis. The protocol for western blotting analysis was performed as described in more detail in our previous study.<sup>14,31</sup>

### 2.4 Relative quantitative enzyme-linked immunosorbent assay (ELISA)

A relative quantitative ELISA, which was previously used by Zhang et al.<sup>35</sup> was adopted to detect serum autoantibodies in patients with BC. In brief, a group of IgG gradients, coupled with one blank control, were arranged on the last two columns of wells in duplicate to provide the standard curve. Next, purified recombinant TAAs were tested by ELISA that described in our previous study.<sup>29</sup> A solution of 3, 3', 5, 5'-tetramethyl benzidine (TMB)-H<sub>2</sub>O<sub>2</sub>-urea was used as the detecting agent and 50  $\mu$ l 2M sulfuric acid was added into each well as the stopping solution. The optical density (OD)

**Table 1.** Characteristics of study participants.

Variable	Training cohort			Validation cohort		
	BC	BBD	NHC	BC	BBD	NHC
Number	184	200	184	197	109	109
Female,n(%)	184(100)	200(100)	184(100)	197(100)	109(100)	109(100)
Age, years						
Mean $\pm$ SD	50 $\pm$ 10	48 $\pm$ 11	50 $\pm$ 11	48 $\pm$ 10	45 $\pm$ 8	46 $\pm$ 8
Range	29-81	20-70	29-81	24-78	24-59	24-62
ER+,n(%)	63(34.2)			119(60.4)		
PR+,n(%)	56(30.4)			107(54.3)		
HER-2+,n(%)	13(7.1)			35(17.8)		
TNM stage,n(%)						
I	68(37.0)			42(21.3)		
II	43(23.4)			50(25.4)		
III	39(21.2)			19(9.6)		
IV	7(3.8)			24(12.2)		
Unknow	27(14.7)			62(31.5)		
Lymph node metastasis,n(%)						
Positive	22(12.0)			24(12.8)		
Negative	157(85.3)			171(86.8)		
Unknow	5(2.7)			2(1.1)		
Histological type,n(%)						
Invasive cancer	159(86.4)			185(93.9)		
noninvasive cancer	9(5.0)			10(5.1)		
Unknow	16(8.7)			2(1.0)		

BC, breast cancer; BBD, benign breast disease; NHC, normal human control

of each well was read at 450 and 620nm by a Microplate reader (Thermo Fisher Scientific).

## 2.5 Statistical analysis

The optical density (OD) of each well in each sample was converted into relative concentrations of autoantibodies using the standard curve. Since the concentration of autoantibodies existing in sera was not distributed normally (Kolmogorov–Smirnov), Kruskal–Wallis H test and Mann–Whitney U test were used for comparison of the three groups ( $p$ -value was obtained by exact test, the  $\alpha$  value was adjusted by Bonferroni correction). The Chi-square test and Fisher exact test were performed to determine the positive rate of individual autoantibody or a panel of autoantibodies among BC, BBD, and NHC. A  $p$ -value  $<0.0167$  (Bonferroni correction, two-tails) was considered statistically significant. A backward stepwise logistic regression (LR) model was conducted to select a panel of autoantibodies based on the training cohort. The two-class Fisher linear discriminant analysis model was also established (stepwise method). The performance of the two models was compared using leave-one-out cross-validation (LOOCV). The area under the receiver operating characteristics (ROC) was constructed to examine the diagnostic value of single autoantibody and the diagnostic performance of the prediction model. Meanwhile, the Youden's index, positive predictive value (PPV), negative predict value (NPV), and  $kappa$  value were calculated as an attempt to evaluate the validity and reliability of the diagnostic test based on sera autoantibodies as biomarkers.

The cutoff value was set at the maximum Yoden index when the specificity was greater than 95%. All statistical

analyses were performed by IBM SPSS Statistics 21.0 and GraphPad Prism 5.

## 3. Results

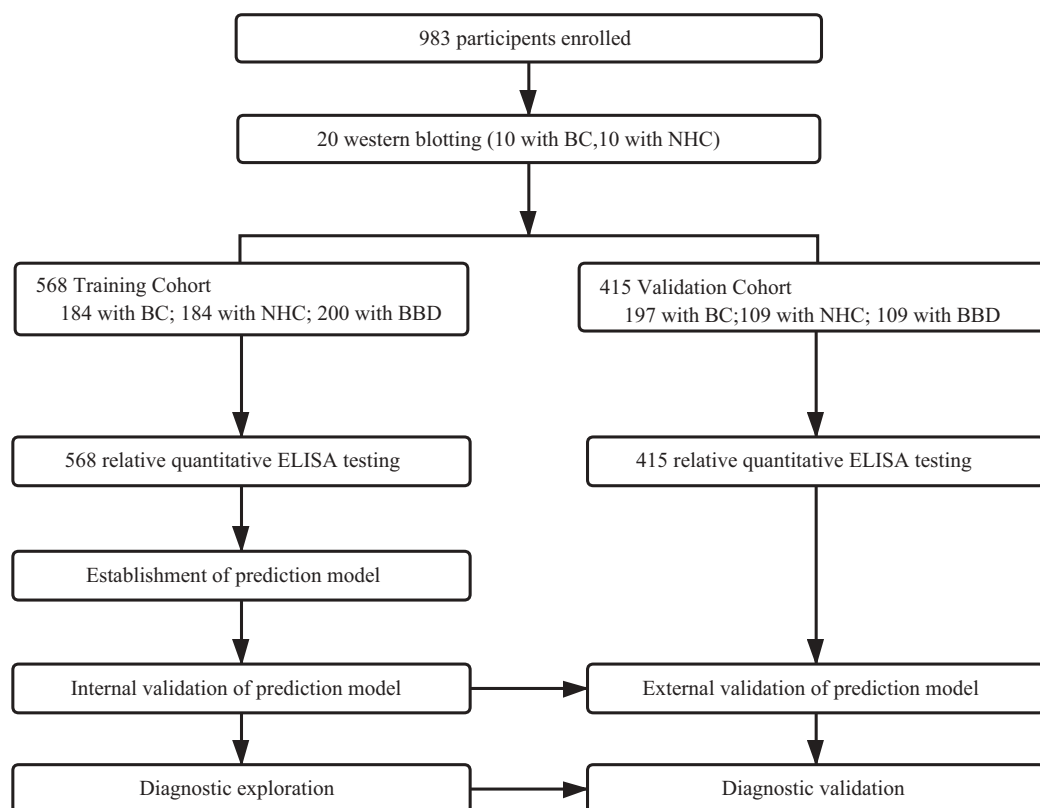
### 3.1 Reactivity of sera autoantibodies among 10 BC and 10 NHC by western blotting

Ten patients with BC and correspondingly age-matched 10 normal controls were randomly selected to explore the reactivity of autoantibodies to 11 TAAs (Figure 1). For the 10 BC patients, the number of positive reactivity of serum autoantibodies ranged from 3 to 6 autoantibodies, more than that of 10 controls showing 1 or 2 autoantibody-positive responses (Figure 2).

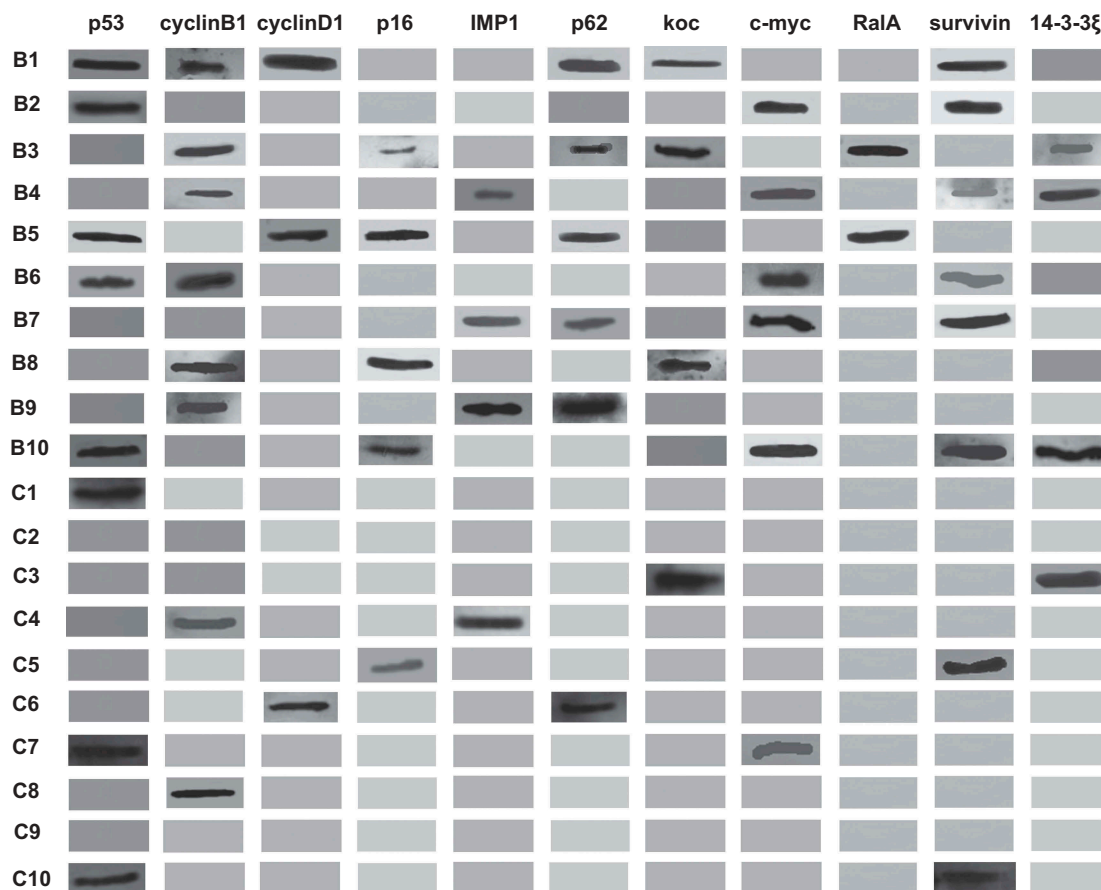
### 3.2 Autoantibodies in BC

A total of 983 serum samples were collected from three groups of participants (Table 1). Eleven purified recombinant proteins were used as coating antigens to detect anti-TAAs autoantibodies in the sera from BC, BBD and NHC groups. The dilution gradients of IgG were explored and demonstrated to have a good fit of a linear relationship between the amounts of coating IgG and OD values. This relationship could be used to convert the original OD values into relative concentrations of autoantibodies (Supplemental Figure 1). The relative concentrations of 11

Anti-TAAs autoantibodies are shown in Figure 3. Eight anti-TAAs (p53, cyclinB1, p16, p62, c-myc, RalA, survivin,



**Figure 1.** Study design. BC, breast cancer; NHC, normal human controls; BBD, benign breast disease; ELISA, enzyme-linked immunosorbent assay.



**Figure 2.** Profiles of autoantibodies against 11 tumor-associated antigens tested by western blotting in 10 patients with breast cancer (B1-B10) and 10 normal controls (C1-C10).

14-3-3ξ) autoantibodies in BC were significantly increased in the two cohorts compared to NHC (Table 2). The five anti-TAAs (p53, cyclinB1, p16, p62, 14-3-3ξ) autoantibodies had significantly different levels between BC and BBD (Table 2). The area under the curve (AUC) of the individual autoantibody ranged from 0.527 to 0.779, and the sensitivity ranged between 2.2% and 41.8% in two cohorts when the specificity was greater than 95% (Supplemental Figure 2 and Supplemental Figure 3).

### 3.3 Establishment of immunodiagnostic model to distinguish BC from BBD or NHC

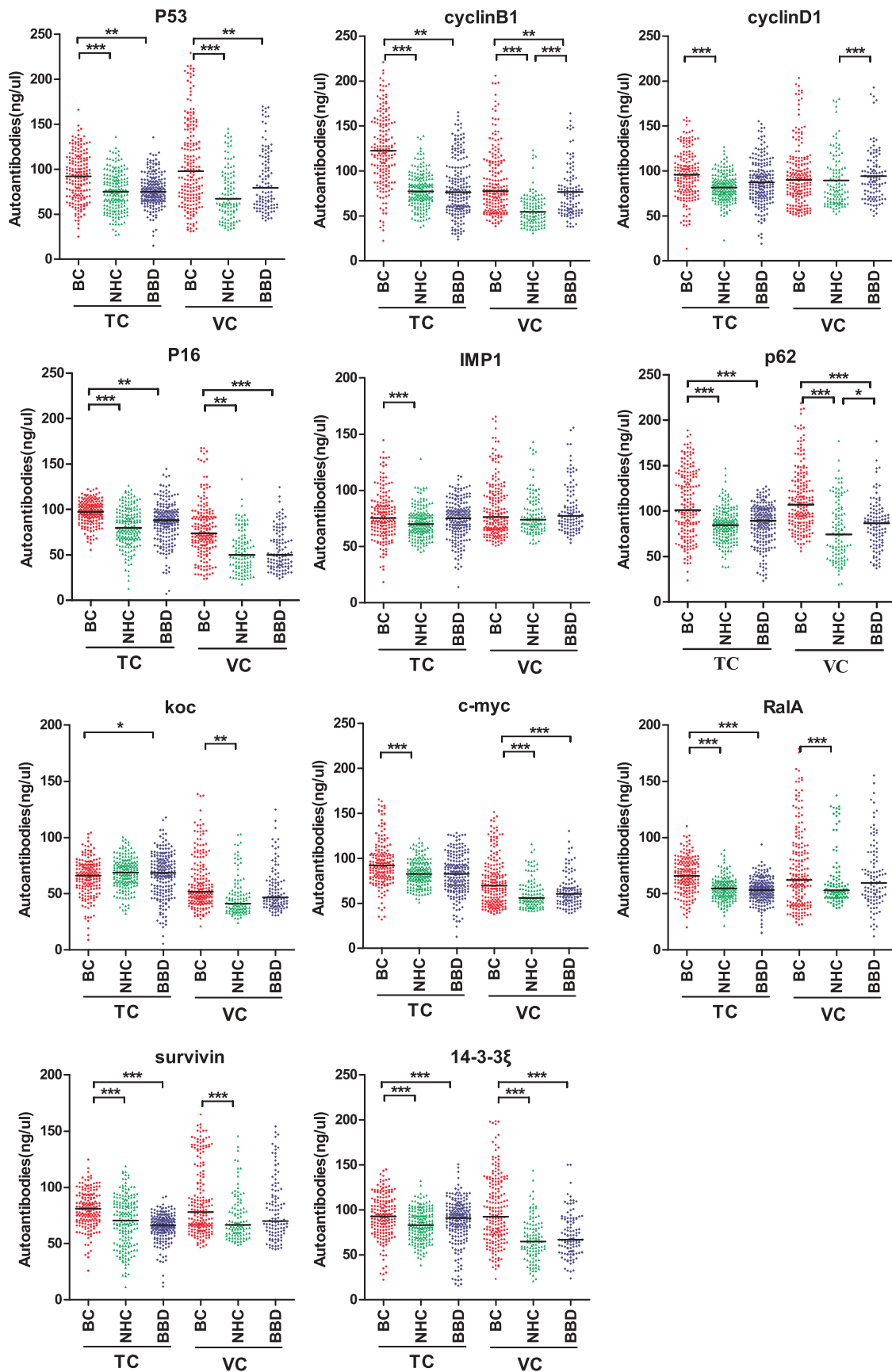
The serum samples of 184 BC and 184 NHC in the training cohort were selected to establish LR model and Fisher linear discriminant analysis model (Figure 1). The dependent variable was whether a participant was considered as BC or not. Eight anti-TAAs autoantibodies with different expression levels between BC and NHC were used as independent variables. The logistic regression model with five anti-TAAs autoantibodies was produced as follows:  $\text{Logit}(p = \text{BC}) = -9.833 + 0.024 \times p53 + 0.040 \times \text{CyclinB1} + 0.019 \times p16 + 0.028 \times p62 + 0.022 \times 14-3-3\xi$ . Furthermore, based on the eight autoantibodies, a Fisher linear discriminant analysis model was also constructed to separate participants into the BC group and a NHC group. The six anti-TAAs (p53, CyclinB1, p16, p62, 14-3-3ξ, survivin) autoantibodies were identified to be

potential biomarkers in BC. The Classification function (Cf) for distinguishing BC from healthy people was obtained as:  $\text{Cf } 1 = 0.035 \times p53 + 0.041 \times \text{CyclinB1} + 0.045 \times p16 + 0.065 \times p62 + 0.028 \times 14-3-3\xi + 0.075 \times \text{survivin} - 10.631$  and  $\text{Cf } 2 = 0.052 \times p53 + 0.066 \times \text{CyclinB1} + 0.066 \times p16 + 0.092 \times p62 + 0.043 \times 14-3-3\xi + 0.075 \times \text{survivin} - 19.947$ .

LOOCV was employed in both models, and results are shown in Table 3. The LR model was chosen for subsequent analysis due to its higher separation ability between BC and NHC with a PPV of 90.0% compared with Fisher linear discriminant analysis model with a PPV of 88.0%. Finally, predicted probabilities of the 184 BC and 184 NHC were computed and used for ROC analysis in order to find the optimal threshold ( $p = .692$ ). The model had an AUC of 0.943 to discriminate individuals with BC from NHC with a sensitivity of 80.4%, specificity of 95.1% and the accuracy of 87.8% (Figure 4(a)). In the differentiation of BC and BBD in the training cohort, the LR model showed the AUC of 0.881, a sensitivity of 59.5%, specificity of 88.5%, and a 74.5% accuracy, at  $p = .692$  cutoff value (Figure 4(b)).

### 3.4 The external validation of the immunodiagnostic model

Classification performance of the LR model for BC was further assessed in the validation cohort, which is an external validation group. Discrimination between BC and NHC gave



**Figure 3.** Scatter plots of sera relative concentration levels of individual autoantibody among breast cancer patients ( $N_{TC} = 184$ ,  $N_{VC} = 197$ ), benign breast disease ( $N_{TC} = 200$ ,  $N_{VC} = 109$ ) and normal individual ( $N_{TC} = 184$ ,  $N_{VC} = 109$ ) in two cohorts (TC, training cohort; VC, validation cohort). BC, breast cancer; NHC, normal human controls; BBD, benign breast disease.

\*:  $P < .05$ , \*\*:  $P < .01$ , \*\*\*:  $P < .001$  (Kruskal–Wallis H test, Mann–Whitney U test).



**Table 2.** Serum relative concentration levels of individual autoantibody among breast cancer patients, breast benign disease and normal individual.

TAA	Training cohort [Median (P25 ~ P75)]			cutoff	P	Validation cohort [Median (P25 ~ P75)]			cutoff	P
	BC	BBD	NHC			BC	BBD	NHC		
p53 <sup>a,b</sup>	93.5(70.2 ~ 112.2)	75(65.1 ~ 87.1)	74.8(58.8 ~ 90.3)	133.5	0.000	98.0(68.2 ~ 139.2)	79.4(60.1 ~ 113.6)	67.1(52.8 ~ 93.2)	132.0	0.000
p62 <sup>a,b</sup>	101.0(77.9 ~ 138.7)	89.1(71.9 ~ 101.7)	84.6(71.9 ~ 96.2)	136.9	0.000	107.0(87.4 ~ 132.6)	86.5(66.6 ~ 98.1)	74.1(50.5 ~ 102.9)	127.2	0.000
p16 <sup>a,b</sup>	97.3(89.2 ~ 105.4)	87.8(74.2 ~ 98.3)	79.7(67.3 ~ 96.0)	94.9	0.000	73.7(59.6 ~ 92.0)	50.0(37.6 ~ 71.4)	50.0(35.7 ~ 68.2)	103.4	0.000
cyclinB1 <sup>a,b</sup>	119.9(95.2 ~ 142.2)	76.5(58.4 ~ 103.9)	77.4(67.5 ~ 91.4)	88.4	0.000	77.6(59.8 ~ 108.0)	72.0(55.8 ~ 89.3)	54.4(44.1 ~ 68.0)	94.6	0.000
cyclinD1	95.8(77.5 ~ 113.2)	87.2(72.4 ~ 100.8)	81.5(72.1 ~ 90.7)	145.6	0.000	90.2(72.6 ~ 108.9)	89.7(71.3 ~ 108.0)	79.7(66.1 ~ 103.4)	137.1	0.176
koc	99.1(82.8 ~ 113.2)	103.0(79.8 ~ 121.0)	103.2(89.5 ~ 116.2)	87.7	0.106	51.7(43.3 ~ 71.7)	46.7(40.3 ~ 61.5)	41.2(35.0 ~ 51.7)	85.8	0.035
c-myc <sup>a</sup>	119.8(102.6 ~ 136.9)	107.6(87.4 ~ 126.5)	107.5(96.6 ~ 122.6)	97.7	0.000	69.5(52.6 ~ 89.0)	60.6(51.9 ~ 70.5)	56.0(49.3 ~ 69.8)	112.0	0.000
RalA <sup>a</sup>	98.8(83.1 ~ 116.0)	79.9(70.0 ~ 90.0)	82.2(72.0 ~ 90.1)	126.6	0.000	62.3(42.4 ~ 88.1)	56.5(46.7 ~ 81.4)	50.0(43.5 ~ 63.4)	123.4	0.000
IMP1	75.3(63.8 ~ 88.3)	75.0(64.9 ~ 84.0)	72.1(62.5 ~ 78.3)	118.9	0.003	76.3(65.1 ~ 97.1)	77.4(69.0 ~ 95.1)	74.1(50.5 ~ 102.9)	99.8	0.358
survivin <sup>a</sup>	121.4(105.6 ~ 138.8)	99.6(89.6 ~ 108.6)	106.0(75.6 ~ 130)	118.0	0.000	78.1(65.1 ~ 110.7)	70.1(59.4 ~ 95.0)	66.5(59.0 ~ 81.6)	105.4	0.000
14-3-3 $\xi$ <sup>a,b</sup>	92.7(78.0 ~ 109.4)	91.0(76.6 ~ 102.7)	83.0(69.9 ~ 96.7)	107.0	0.000	92.4(71.7 ~ 128.4)	66.8(53.6 ~ 89.8)	64.7(51.1 ~ 82.5)	109.7	0.000

TAA, tumor-associated antigen; BC, breast cancer; BBD, benign breast disease; NHC: normal human control; P25, 25th percentile; P75, 75th percentile.

cutoff value was set at the maximum Yoden index when the specificity was greater than 95%.

<sup>a</sup>Comparison of autoantibody level of BC group with that of NHC group or BBD group was significantly different.

<sup>b</sup>Comparison of autoantibody level of BC group with that of BBD group was significantly different.

**Table 3.** LOOCV results predicted by the two models of BC.

	LR model		LDA	
	Predicted BC	Predicted NHC	Predicted BC	Predicted NHC
BC	161	20	161	22
NHC	23	164	23	162
Se (%)	87.5		87.5	
Sp (%)	89.1		88.0	
PPV (%)	90.0		88.0	
NPV (%)	87.7		87.6	
Accuracy (%)	88.3		87.8	

LR, logistic regression; LDA, Fisher linear discriminant analysis; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

an AUC of 0.916 with a sensitivity of 78.2%, a specificity of 89.0%, and a diagnostic accuracy of 82.4% at a cut-off value of  $p = .692$  (Figure 4(c)). In addition, the model was able to distinguish BC from BBD with the AUC of 0.849 yielding a sensitivity of 69.5%, a specificity of 84.4%, and a diagnostic accuracy of 74.8% (Figure 4(d)).

### 3.5 The immunodiagnostic model for early stage and under the age of 50 years detection

The patients from both training and validation cohorts were divided into early-stage (stage I, II) and late-stage (stage III, IV) of BC. For the early-stage BC detection in the training cohort, the model showed a high AUC value (0.949) for the comparison between BC and NHC with the sensitivity of 72.1% and specificity of 96.7%. The AUC for discriminating BC from BBD was 0.890 with the sensitivity of 53.2% and specificity of 94.0%. Similar results were observed in the validation cohort. The AUC was 0.912 with the sensitivity of 69.5% and specificity of 89.9% when comparing BC to NHC. The AUC was 0.851 with the sensitivity of 54.4% and specificity of 93.6% when the control group was BBD.

Additionally, the diagnostic value of the model was determined between the patients whose ages were under 50 years and above 50 years. For patients under the age of 50 years, the AUC remained high within the training cohort with a value of 0.963 when comparing BC to NHC. The autoantibodies panel exhibited a sensitivity of 71.3%, a specificity of 96.7%. The AUC was 0.898 with the sensitivity of 51.5% and specificity of 95.0% when BC was compared to BBD. In the validation

cohort, the model showed an AUC of 0.927 in BC versus NHC with the sensitivity of 73.8% and specificity of 89.9%. The AUC was 0.865 in BC versus BBD with the sensitivity of 59.8% and specificity of 90.8%. All the results above are shown in Table 4, as well as the diagnostic value of the model in late-stage and that in the patients older than 50 years.

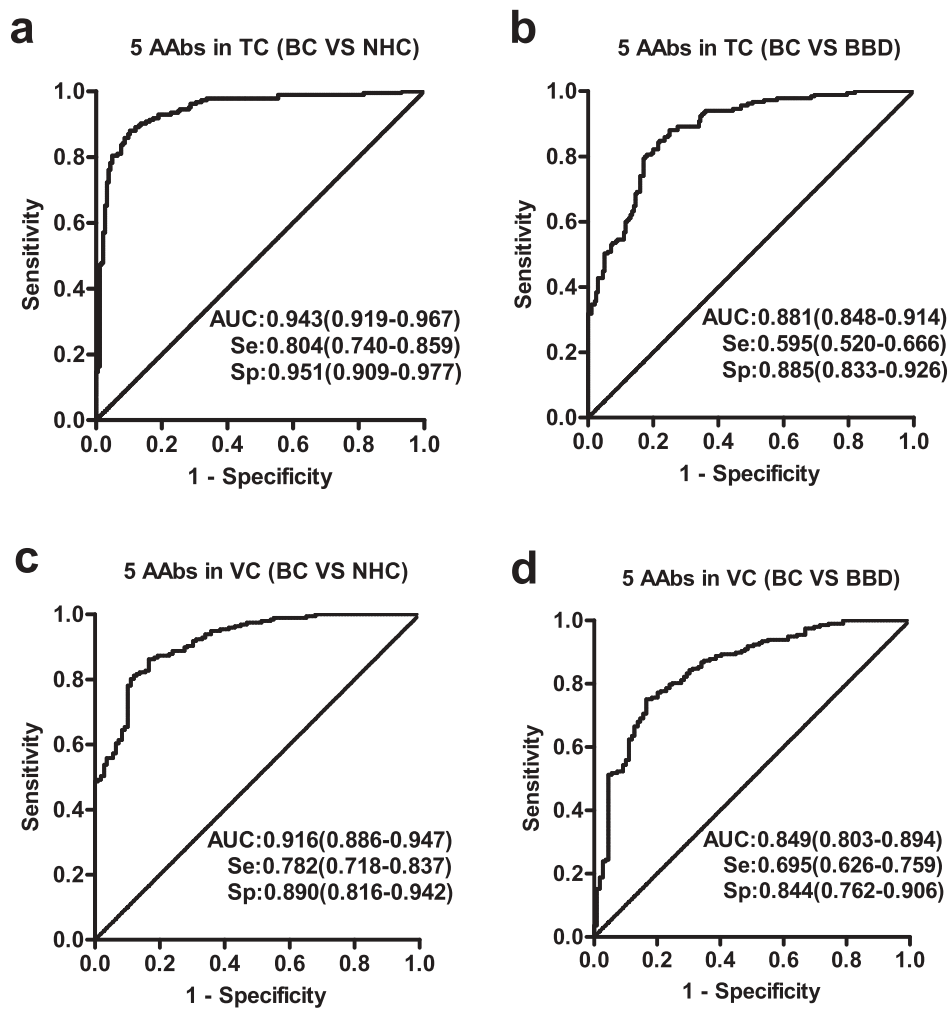
### 3.6 Positive reactivity of the immunodiagnostic model among subgroups

For the immunodiagnostic model, if the predicted probability was greater than 0.692, a sample could be classified as BC. As shown in Figure 5(a,b), in both training and validation cohorts, the positive response of every single autoantibody in BC group, ranging from 10.2% to 57.6%, was significantly different from that in NHC (ranged from 1.0% to 4.9%) and that in BBD (ranged between 2.0% and 25.7%). Additionally, among the training cohort, 80.4% of 184 BC patients were identified to have positive reactivity to five TAAs of the model compared to 11.5% in BBD and 4.9% in NHC. In the other cohort, 78.2% of 197 BC patients were identified as having positive reactivity to the five TAAs compared to 15.6% in BBD and 11.0% in NHC.

The model showed no significant difference when data were subdivided by clinical stage, prognostic index, lymph node status, histological type or family history of tumor (Figure 5(c-j)). Interestingly, the autoantibodies to p16 were significantly different between stage I and other stages (II, III and IV) in validation cohort (Supplemental Figure 4). In addition, the model also showed no differences between patients of early-stage and late-stage. Nevertheless, the difference of positive reactivity between patients at the ages of under and above 50 years was statistically significant in the training cohort (Table 5).

## 4. Discussion

In this study, Western Blotting was first performed to confirm the ELISA results which were previously obtained. The results from western blotting analysis visually revealed the differences of frequency of autoantibodies between cancer patients and normal controls<sup>14</sup> For all 10 BC patients, there were 3–6 anti-TAAs



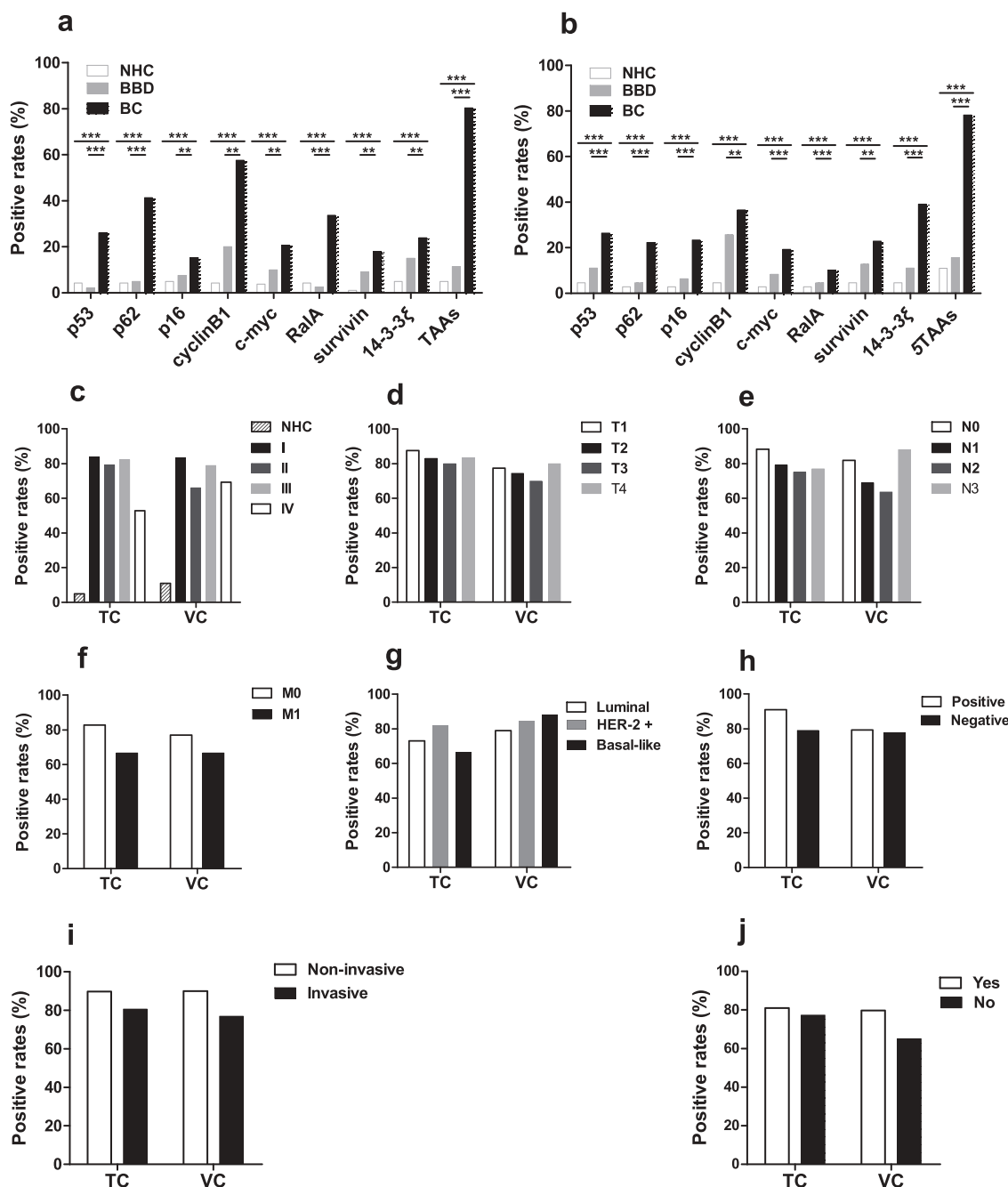
**Figure 4.** Performance of the prediction model with five anti-TAAs autoantibodies to detect breast cancer. Receiver operating characteristic (ROC) curves from training cohort (a, b) and validation cohort (c, d). Performance shown for distinguishing individuals with BC from NHC (a, c) or BBD controls (b, d). TC, training cohort; VC, validation cohort; BC, breast cancer; NHC, normal human controls; BBD, benign breast disease; Se, sensitivity; Sp, specificity.

**Table 4.** Diagnostic value of the prediction model with five anti-TAAs autoantibodies for different stages and ages of BC patients.

Group	n	AUC	95%CI	P-value	Se(%)	Sp(%)	YI	+ LR	-LR	Kappa	PPV(%)	NPV(%)	Accuracy (%)
<b>Training cohort</b>													
Early-stage(I+ II)	111	0.949 <sup>a</sup>	0.924–0.974	0.000	72.1	96.7	0.7	21.8	0.3	0.7	93.0	85.2	87.4
		0.890 <sup>b</sup>	0.850–0.923	0.000	53.2	94.0	0.5	8.9	0.5	0.5	83.1	78.1	79.4
Late-stage(III+IV)	46	0.919 <sup>a</sup>	0.862–0.975	0.000	47.8	97.8	0.5	21.7	0.5	0.5	84.6	88.2	87.8
		0.839 <sup>b</sup>	0.770–0.908	0.000	50.0	97.0	0.5	16.7	0.5	0.5	90.0	87.6	88.2
age≤50	101	0.963 <sup>a</sup>	0.938–0.988	0.000	71.3	96.7	0.7	21.6	0.3	0.7	92.3	86.0	87.7
		0.898 <sup>b</sup>	0.863–0.934	0.000	51.5	95.0	0.5	10.3	0.5	0.5	83.9	79.5	80.4
age>50	83	0.924 <sup>a</sup>	0.889–0.958	0.000	61.4	96.7	0.6	18.6	0.4	0.6	89.5	84.8	85.7
		0.860 <sup>b</sup>	0.812–0.908	0.000	41.0	98.5	0.4	27.3	0.6	0.5	91.9	80.1	81.6
BBD	200												
NHC	184												
<b>Validation cohort</b>													
Early-stage(I+ II)	92	0.912 <sup>a</sup>	0.875–0.949	0.000	69.5	89.9	0.6	6.9	0.3	0.6	84.3	74.8	80.6
		0.851 <sup>b</sup>	0.798–0.904	0.000	54.4	93.6	0.5	8.5	0.5	0.5	87.7	70.8	75.7
Late-stage(III+IV)	43	0.893 <sup>a</sup>	0.835–0.951	0.000	44.2	97.2	0.4	15.8	0.6	0.4	86.4	81.5	82.2
		0.811 <sup>b</sup>	0.734–0.887	0.000	27.9	96.3	0.2	7.5	0.7	0.3	75.0	77.2	77.0
age≤50	122	0.927 <sup>a</sup>	0.896–0.958	0.000	73.8	89.9	0.6	7.3	0.3	0.6	89.1	75.4	81.4
		0.865 <sup>b</sup>	0.817–0.912	0.000	59.8	90.8	0.5	6.5	0.4	0.5	87.8	66.4	74.0
age>50	75	0.898 <sup>a</sup>	0.854–0.942	0.000	57.3	91.7	0.5	6.9	0.5	0.5	82.7	75.8	77.1
		0.827 <sup>b</sup>	0.766–0.887	0.000	36.0	95.4	0.3	7.8	0.7	0.3	84.4	68.4	71.2
NHC	109												
BBD	109												

<sup>a</sup>BC VS NHC

<sup>b</sup>BC VS BBD; YI, Youden's index; Se, sensitivity; Sp, specificity; +LR, positive likelihood ratio; -LR, negative likelihood ratio; PPV, positive predictive value; NPV negative predictive value.



**Figure 5.** Positive rates of autoantibodies against eight TAA or combined five TAA in individual sera of BC patients, BBD patients and NHC (a, b). The cutoff value was set at the maximum Yoden index when the specificity was greater than 95%. Positive rates of the prediction model with five anti-TAA autoantibodies among subgroups of BC patients based on tumor stage (c-f), molecular subtypes (g), lymph node metastasis (h), histological type (i) and family history of tumor (j). TC, training cohort; VC, validation cohort; BC, breast cancer; NHC, normal human controls; BBD, benign breast disease.

\*:  $P < .05$ , \*\*:  $P < .01$ , \*\*\*:  $P < .001$  (Chi-square tests).

**Table 5.** Positive reactivity of the prediction model with five anti-TAA autoantibodies among subgroups.

Group	Training Cohort			Validation Cohort		
	Positive (%)	$\chi^2$	$P$	Positive (%)	$\chi^2$	$P$
Stage						
Early-stage(I+ II)	91(82.0)	0.749	0.691	68(73.9)	0.422	0.516
Late-stage(III+IV)	35(76.1)			34(79.1)		
Age						
Age $\leq$ 50	87(86.1)	4.629	0.031*	99(81.1)	1.622	0.197
Age $>$ 50	61(73.5)			55(73.3)		

\* $P < 0.05$ .

autoantibodies showing positive reactivity in each patient, and for 10 normal controls, only 1 or 2 sera showed positive reactivity for anti-TAA autoantibodies. This observation has provided us a clue that multiple autoantibodies detection could achieve higher sensitivity in the diagnosis of BC than a single autoantibody. These findings were further supported by published data that the application of a panel of TAA could enhance the detection of autoantibodies.<sup>29,31,36,37</sup> Moreover, the ELISA results of the 10 BC patients and 10 controls showed similar results to those of Western Blot (Supplemental Table 1–2).



To control systematic errors and selection bias, 11 autoantibodies were tested in two BC groups from different hospitals, and were also tested in two different control groups. Among the 11 anti-TAAs autoantibodies, significantly increased levels of eight anti-TAAs (p53, cyclin B1, p16, p62, c-myc, RalA, survivin, and 14-3-3 $\xi$ ) autoantibodies were testified between cancer group and NHC group both in training cohort and validation cohort. Many reported data presented the evidence for the existence in breast cancer sera of autoantibodies that used in the present study.<sup>19,27,38</sup> Ye et al. examined 42 BC cases and 81 normal controls, and they showed the sensitivities of autoantibodies to cyclin B1 (17.1%), survivin (22.0%), p53 (12.2%)<sup>30</sup> Additionally, this study also showed that the serum levels of five autoantibodies to p53, cyclin B1, p16, p62, and 14-3-3 $\xi$  were significantly different between cancer group and BBD group. This finding provided the foundation for the following study of a panel of autoantibodies in the identification of BC and BBD.

Even though the significant difference of certain autoantibodies existed between two BC groups and two control groups, the result showed that the sensitivity of single autoantibody in BC was low, which will limit its clinical application.<sup>30,31</sup> Therefore, based on the results of western blotting, multiple autoantibodies were examined as a panel to reach the higher sensitivity. In addition, to establish a diagnostic model with classification, multivariate statistical analysis was used, which can improve the diagnostic accuracy and predict the probability of developing BC. It can also increase the specificity of BC detection. This study chose the Fisher linear discriminant analysis model and LR model and compared their predictive effects for finding the ideal classifier in BC prediction.<sup>39,40</sup> The LOOCV approach was used to compare the performances of two prediction models and to ensure a statistically independent prediction of the multivariate classification model.<sup>41</sup> Finally, it was noticed that the LR model had higher PPV with fewer amounts of autoantibodies (5 TAAs as a panel). The 5 TAAs (p53, cyclinB1, p16, p62, and 14-3-3 $\xi$ ) panel showed rather high sensitivity and specificity in this study. Liu et al. showed that expression of autoantibodies against the panel (p16, c-myc, p53, and ANXA-1) was significantly higher in the breast cancer group (102 breast cancer patients and 146 age-matched healthy volunteers) with the sensitivity of 33.3% (90% specificity)<sup>38</sup> Ye et al. examined 42 BC cases and 81 controls, and they showed the sensitivity of 61.0% and a specificity of 86.6% in BC by the combination of ten TAAs (Imp1,p62, Koc, p53, c-myc, survivin, p16, cyclinB1, cyclinD1, and p16)<sup>30</sup> This study takes advantage of large sample size and the 2 cohorts (one for training and one for validation).

In order to make a solid conclusion, the case-control study included two cohorts of patients from different hospitals. Both healthy individuals and benign diseases age-matched with BC patients were selected as controls. Hermann1 et al. presented the evidence that CA15-3 showed significant differences in BBD and BC. The AUC for discriminating between BC and BBD achieved 0.71 with a sensitivity of 33.8% at 95% specificity.<sup>42</sup> Liu et al. evaluate the immunodiagnostic values of autoantibodies to a panel of 6 TAAs in 49 sera from breast

cancer patients, 35 sera from patients with benign breast disease, and 38 sera from normal controls. They indicated the positive reactions of autoantibody reaching a sensitivity of 67.3% and a specificity of 92.2% (Imp1, p16, Koc, survivin, cyclinB1, and c-myc)<sup>26</sup> Furthermore, reported data of previous studies set different cutoff values for various markers to distinguish BC from NHC.<sup>14,36</sup> However, it will increase the difficulty of using multiple markers in the detection of cancer. For an optimal combination of various autoantibodies, using only one cutoff value to discriminate BC from normal control or distinguish BC from BBD would facilitate the screening of BC. The advantage of this study is the achievement of the aforementioned problem.

Additionally, the present study had provided some relevant exploration findings of potential biomarkers for detection and screening of breast cancer in early-stage BC.<sup>43</sup> The immunodiagnostic model showed that in early-stage (stage I and II) AUC had more accuracy than that in the late stage (stage III and IV) in the diagnosis of BC no matter what the control group was (NHC or BBD). In addition, this study also revealed that the positive reactivity of the five anti-TAAs autoantibodies was significantly different between patients at the age under and above 50 years in the training cohort. This finding suggests that the immunodiagnostic model is more capable of predicting BC among young women. Peer et al. screened 23000 women and followed up. The data from them showed that for patients under age 50, the sensitivity was 64% for cancers within one year after screening. The sensitivity was lower than those of the 50–69 and >70 age groups, with 85% and 80% sensitivity, respectively.<sup>44</sup> High density of breast tissue impairs the sensitivity of mammography, especially in women between 40 and 49 years of age (about 74% of them with high breast density) and 45% of women in their 60s have high breast density.<sup>6</sup> Therefore, the immunodiagnostic model could make up the low sensitivity of mammography in young women, especially at high breast density.<sup>2,4,6</sup> Furthermore, the results of this study demonstrated that at stage I of BC, the level of anti-p16 autoantibody was significantly higher than that in stage II, stage III and stage IV. The anti-p16 autoantibody was firstly reported by our group in 2006<sup>45</sup> The p16 protein is a cyclin-dependent kinase inhibitor, and also a negative regulator of the mammalian cell cycle.<sup>46</sup> Despite it is not specific for BC, the finding indicates that the anti-p16 antibody could be a biomarker in the early detection of BC.<sup>45</sup> However, this is a retrospective case-control study, the pre-diagnostic sera of BC were unavailable. Therefore, there is a need to confirm autoantibody signatures and its diagnostic values on a large prospective study.<sup>2</sup> In addition, more works are needed to further confirm the clinical utility of the five anti-TAAs autoantibodies.

In summary, this study suggests that the immunodiagnostic prediction model with the combination of five anti-TAAs autoantibodies could identify BC from NHC as well as BBD. It has a potentially important role in future prediction and diagnosis of BC as effective biomarkers for early diagnosis are lacking. The data from this study also reveal that as a supplement approach of mammography, the model could improve the power of immunodiagnosis in breast cancer. Recently, we noticed some other approaches to the detection of autoantibodies to TAAs, such as

whole genome derived peptide arrays and protein microarray technology.<sup>47–50</sup> The future directions would detect autoantibody signatures using those approaches, and we anticipate obtaining a panel of new biomarkers.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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