

A panel containing PD-1, IL-2R α , IL-10, and CA15-3 as a biomarker to discriminate breast cancer from benign breast disease

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Introduction: Programmed cell death protein 1 (PD-1), an immune checkpoint molecule, has recently been recognized as a predictive and prognostic biomarker in several malignant tumors, but its diagnostic value remains largely unknown. We aimed to investigate the differential diagnostic efficiency of PD-1 and other immune molecules and propose a panel of immune molecules combined with cancer antigen 15-3 (CA15-3) to distinguish breast cancer (BC) from benign breast disease (BBD).

Patients and methods: Ninety-one eligible BC patients and 31 BBD patients were enrolled. Pretreatment peripheral blood was collected and tested for mRNA expression of PD-1, cytotoxic T lymphocyte antigen 4, forkhead box P3, transforming growth factor beta, interleukin-10 (IL-10), IL-2 receptor alpha (IL-2R α), and cluster of differentiation 28 by quantitative reverse transcription PCR.

Results: The diagnostic areas under curve (AUCs) of PD-1, IL-2R α , and IL-10 for BC–BBD discrimination were 0.764, 0.758, and 0.743, respectively. The diagnostic efficiencies of these three parameters in distinguishing early-stage or advanced BC from BBD were consistent with a role in BC–BBD discrimination. A panel of PD-1 + IL-10 + IL-2R α + CA15-3 showed the highest AUC (0.862), with a sensitivity of 0.933 and a specificity of 0.724, for BC–BBD discrimination. In addition, for early-stage BC discrimination, this panel also had the highest AUC (0.811), with a sensitivity of 0.933 and a specificity of 0.614, while for advanced BC discrimination, a panel of PD-1 + IL-10 + CA15-3 exhibited the highest AUC (0.896), with a sensitivity of 0.933 and a specificity of 0.783.

Conclusion: These data indicate that the panel containing PD-1, IL-2R α , IL-10, and CA15-3 can effectively discriminate BC from BBD with a high efficiency. After further confirmation, it could be used to complement conventional imaging modalities, especially in discriminating early-stage BC from BBD.

Keywords: breast cancer, immune checkpoint, PD-1, CTLA-4, IL-2R α , diagnosis, biomarker

Introduction

Breast cancer (BC) is the second leading cause of death worldwide and is the most frequently diagnosed cancer in females.¹ Early detection is critically important to optimize treatments and reduce mortality in BC patients. To date, mammography, ultrasonography, and magnetic resonance imaging are the most commonly used methods to detect BC. However, its diagnostic value is relatively limited when patients have early-stage BC and higher breast tissue density.^{2–4} Cancer antigen 15-3 (CA15-3) and carcinoembryonic antigen are common tumor markers for the detection for BC, but their usefulness is limited in advanced BC, and they have low diagnostic sensitivity

and specificity in early-stage cases.⁵ Therefore, there is a great need to develop other methods to complement the abovementioned methods for BC detection, especially in early-stage cases.

Recently, researchers have been trying to establish predictive models to discriminate BC from benign breast disease (BBD) and healthy people in terms of long non-coding RNAs, circulating cell-free DNA (cfDNA), circulating tumor cells, and various metabolites.^{6–9} MicroRNAs, including miR-199a, miR-29c, miR-424, miR-195-5p, miR-495, miR-92a, miR-133a, miR-133b, miR-29b-2, miR-155, miR-197, and miR-205, have shown potential diagnostic efficiency for BC, especially as combinations.^{10–15} In addition, the diagnostic capacity of circulating cfDNA has been demonstrated for the detection of BC.^{16–20} Lipid profiling has been investigated and its differential diagnostic value has been confirmed in distinguishing BC from BBD.^{21,22} In addition, various proteins, including developmental endothelial locus 1 protein, vascular endothelial growth factor, and matrix metalloproteinase 9, have shown diagnostic efficiency in BC.^{23,24} However, the abovementioned existing methods of detection either need high technical requirements and costs or have low sensitivity and specificity.

Immune checkpoint molecules, including programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), have important roles in the anti-tumor activities of immune cells.²⁵ Immune checkpoint blockers have been widely employed to treat various malignant tumors and have created a paradigm shift for cancer treatment.^{26,27} Meanwhile, increasing numbers of studies have shown that PD-1 and CTLA-4 are correlated with tumor response and prognosis in BC, non-small-cell lung cancer, and colorectal cancer.^{28–33} Interleukin-2 receptor alpha (IL-2R α) is one of the IL-2 receptors and contributes to forkhead box P3 (FOXP3) expression on CD4⁺ T cells when combined with IL-2.^{34,35} Previous studies have suggested that levels of soluble IL-2R α were negatively correlated with prognosis in patients with T-cell lymphoma, diffuse large B-cell lymphoma, acute lymphoblastic leukemia, and follicular B-cell non-Hodgkin lymphoma.^{35–38} IL-10, FOXP3, and transforming growth factor beta (TGF- β) were shown to be highly expressed in the peripheral blood and tissues of BC patients and negatively correlated with poor survival.^{39–48} However, the differential diagnostic efficiency of these immune molecules in BC is largely unknown. Here, we investigated the differential diagnostic value of expression of immune checkpoint molecules (PD-1 and CTLA-4), cancer-related immune molecules (FOXP3, TGF- β , IL-10, IL-2R α , and cluster

of differentiation 28 [CD28]), and CA15-3 in the blood specimens, a substantially less invasive approach compared to surgical biopsy. We also proposed a panel of immune molecules combined with CA15-3 to improve diagnostic efficiency by more effectively discriminating BC from BBD.

Patients and methods

Patient selection and sample collection

This study was approved by the ethical committee of Renmin Hospital of Wuhan University. Patients provided their written informed consent. We enrolled BC patients and BBD patients who were admitted to our hospital from November 2015 to March 2017. The inclusion criteria for BC patients were as follows: 1) pathologically confirmed early-stage BC (stage I or II) and ductal carcinoma in situ; 2) pathologically confirmed advanced BC (stage III or IV) before treatment; 3) blood collection before anti-tumor treatment, including surgery, neoadjuvant therapy, and salvage treatment; and 4) age >18 years. The exclusion criteria were as follows: 1) infection or metabolic disease; 2) hematological disease or transplant history; 3) inflammatory disease; and 4) renal or liver disease. We included patients with pathologically confirmed BBD. The exclusion criteria for BBD were the same as those for BC. Among 105 BC patients who enrolled according to the inclusion criteria, 7, 5, 1, and 1 patients were excluded according to exclusion criterion 1)–4), respectively. Among 40 patients with BBD whom we included, 5, 3, 0, and 1 patients were excluded according to exclusion criterion 1)–4), respectively. Finally, 122 blood samples were collected from 91 patients with BC and 31 patients with BBD before anti-tumor treatment and stored in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes.

Clinical data collection

The following clinical data were collected from electronic medical records in our hospital: age; pathological type; tumor grade, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 status; and American Joint Committee on Cancer (AJCC) stage according to the 7th edition of the AJCC cancer staging manual.⁴⁹ Therapies included surgery (modified radical mastectomy or breast-conserving surgery), chemotherapy (anthracyclines, paclitaxel, docetaxel, cyclophosphamide, capecitabine, and gemcitabine), targeted therapy (trastuzumab), adjuvant radiotherapy, and endocrine therapy (tamoxifen, letrozole, anastrozole, and exemestane). The non-metastatic patients did not receive the abovementioned therapies before enrollment.

Among 41 metastatic patients, 22 patients received adjuvant endocrine therapy before metastasis; 19 patients were under follow-up before metastasis.

Preparation of peripheral cells

Four milliliters of fresh whole blood stored in EDTA anti-coagulant tubes was mixed with 8 mL red blood cell lysis solution and inverted at room temperature for 10 minutes. Then, we centrifuged the mixture at 1,000 $\times$ g for 5 minutes, removed the supernatant, and kept the cell pellet at the bottom of tube. Next, we used 1 mL red blood cell lysis solution to resuspend the cell pellet and repeated the abovementioned steps until red blood cells were fully lysed. Then, 1 mL phosphate-buffered saline was used to wash left cells in the tube. Finally, we mixed cells with 1 mL TRIzol and resuspended them and stored them at -80°C until RNA isolation.

RNA isolation

Two hundred microliters of chloroform was added to the abovementioned mixture, which was then homogenized by shaking the tube vigorously. After incubation for 3 minutes at room temperature, we centrifuged the mixture at 12,000 $\times$ g for 5 minutes at 4°C . Then, we carefully removed the upper aqueous phase into a new tube and added 500 μL isopropanol for homogenization. The mixture was incubated at room temperature for 10 minutes. After that, we centrifuged the mixture at 12,000 $\times$ g for 10 minutes at 4°C and removed the supernatant, leaving only the RNA pellet. The RNA was washed with 1 mL 75% ethanol and resuspended with RNase-free water.

cDNA synthesis and quantitative reverse transcription PCR

One microliter of oligo(dT)15 was added to 1 μg RNA in a microcentrifuge tube and incubated at 70°C for 10 minutes. After that, the microcentrifuge tube was placed on ice in preparation for reverse transcription. A 20 μL reaction mixture containing 4 μL MgCl_2 , 2 μL reverse transcription 10 $\times$ buffer, 2 μL dNTP mixture, 0.5 μL recombinant RNasin ribonuclease inhibitor, and 0.6 μL avian myeloblastosis virus reverse transcriptase was mixed with RNA, incubated at 42°C for 60 minutes, heated to 95°C for 5 minutes, and subsequently incubated on ice for 5 minutes.

An ABI Prism 7,900-HT Sequence Detection System (96-well, Thermo Fisher Scientific, Waltham, MA, USA) was used to perform quantitative reverse transcription PCR. β -Actin was used as the internal reference. Primers for immune molecules (PD-1, CTLA-4, FOXP3, TGF- β , IL-10,

IL-2R α , and CD28) and β -actin are listed in Table S1. A 20 μL reaction mixture containing 10 μL PCR mixture, 8 μL ddH $_2\text{O}$, and 2 μL cDNA for each immune molecule was prepared for quantitative PCR, which was conducted with the following cycling conditions: 45 cycles of real-time inactivation at 95°C for 2 minutes, denaturation at 95°C for 10 seconds, and annealing at 60°C for 1 minute. The relative mRNA expression of immune molecules was normalized to β -actin expression.

Statistical analysis

Receiver operating characteristic curves were used to evaluate the differential diagnostic value of immune molecules and CA15-3 in terms of area under the curve (AUC), sensitivity (true-positive/(true-positive + false-negative)), and specificity (true-negative/(true-negative + false-positive)). We chose three immune molecules with the largest AUCs, combined with CA15-3, to improve the differential diagnostic efficiency. Binary logistic regression analysis was used to calculate the combined predictors of immune molecules and CA15-3, using Youden index value (sensitivity + specificity – 1). Mann–Whitney test was used to compare the mRNA expression of immune molecules in BC patients with that in BBD patients. All analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered significant.

Results

Baseline characteristics

Ninety-one BC patients and 31 BBD patients were enrolled in this study. Table 1 lists the baseline characteristics of the 91 BC patients. The mean age for BC patients was 51.2 ± 10.7 years. In addition to invasive tumors, there were three ductal carcinomas in situ. There were 44 early-stage BC patients (stages 0–II) and 47 advanced BC patients (stages III–IV). In the BBD group, the mean age was 41.0 ± 9.2 years. We identified four intraductal papillomas, five cases of fibroadenosis, 21 fibroadenomas, and one spindle cell tumor.

Immune molecules in BC and BBD

Levels of seven immune molecules, including immune checkpoint molecules (PD-1 and CTLA-4) and cancer-related immune molecules (FOXP3, TGF- β , IL-10, IL-2R α , and CD28), were upregulated in the peripheral blood of BC patients compared with BBD patients. The relative expression of PD-1 was significantly upregulated in BC patients with a fold change of 16.2 ($P < 0.001$, Figure 1A) versus BBD patients. In addition, relative levels of CTLA-4,

Table 1 Clinicopathological parameters of 91 breast cancer patients

Parameters	Number (%)
Age (years)	
≤50	43 (47.3)
>50	48 (52.7)
Pathological type	
DCIS	3 (3.3)
IDC	61 (67.0)
ILC	7 (7.7)
Invasive duct-lobular cancer	2 (2.2)
Invasive cancer	17 (18.7)
Others	1 (1.1)
Tumor grade	
Grade 1	1 (1.1)
Grade 2	49 (53.8)
Grade 3	17 (18.7)
Unknown	24 (26.4)
ER status	
Positive	65 (71.4)
Negative	26 (28.6)
PR status	
Positive	59 (64.8)
Negative	32 (35.2)
HER-2	
Positive	25 (27.5)
Negative	66 (72.5)
AJCC stage	
0	3 (3.3)
I	10 (11.0)
II	31 (34.1)
III	6 (6.6)
IV	41 (45.1)

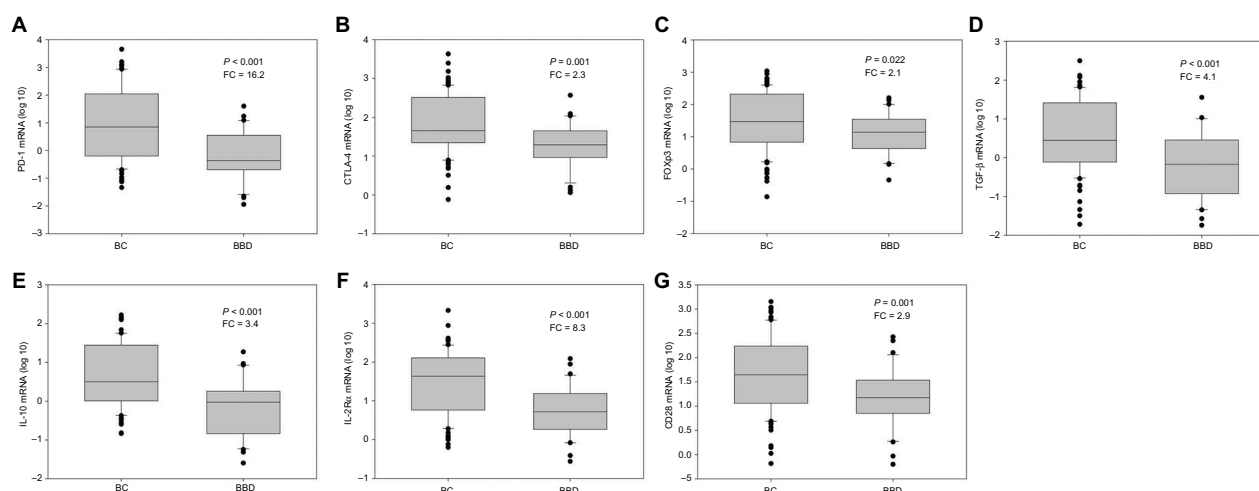
Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; AJCC, American Joint Committee on Cancer.

FOXP3, TGF- β , IL-10, IL-2R α , and CD28 were higher in BC patients than in BBD patients, with fold changes of 2.3 ($P=0.001$, Figure 1B), 2.1 ($P=0.022$, Figure 1C), 4.1 ($P<0.001$, Figure 1D), 3.4 ($P<0.001$, Figure 1E), 8.3 ($P<0.001$, Figure 1F), and 2.9 ($P=0.001$, Figure 1G), respectively. Compared with early-stage BC patients, patients with advanced BC showed no significant differences in the relative expression levels of these immune molecules ($P>0.05$, Figure S1).

Differential diagnostic value of immune molecules in BC

We evaluated the differential diagnostic value of each immune molecule, CA15-3, the combination of the three immune molecules with the largest AUCs, and CA15-3. In 91 BC patients, the AUCs for PD-1, IL-10, and IL-2R α were 0.764 (95% CI: 0.674–0.855), 0.743 (95% CI: 0.646–0.840), and 0.758 (95% CI: 0.668–0.849), respectively (Table 2, Figure 2); the diagnostic sensitivity and specificity for these three immune molecules were 0.448 and 0.933, 0.644 and 0.767, and 0.552 and 0.900, respectively (Table 3). The AUC, sensitivity, and specificity for CA15-3 were 0.707 (95% CI: 0.613–0.800), 0.644, and 0.733, respectively (Tables 2 and 3, Figure 2). The diagnostic efficiencies of other immune molecules are shown in Tables 2 and 3.

A three-parameter combination (PD-1 + IL-10 + CA15-3) showed a diagnostic AUC of 0.859 (95% CI: 0.791–0.926), sensitivity of 0.933, and specificity of 0.713 (Tables 2 and 3, Figure 3). Another three-parameter combination (IL-10 + IL-2R α

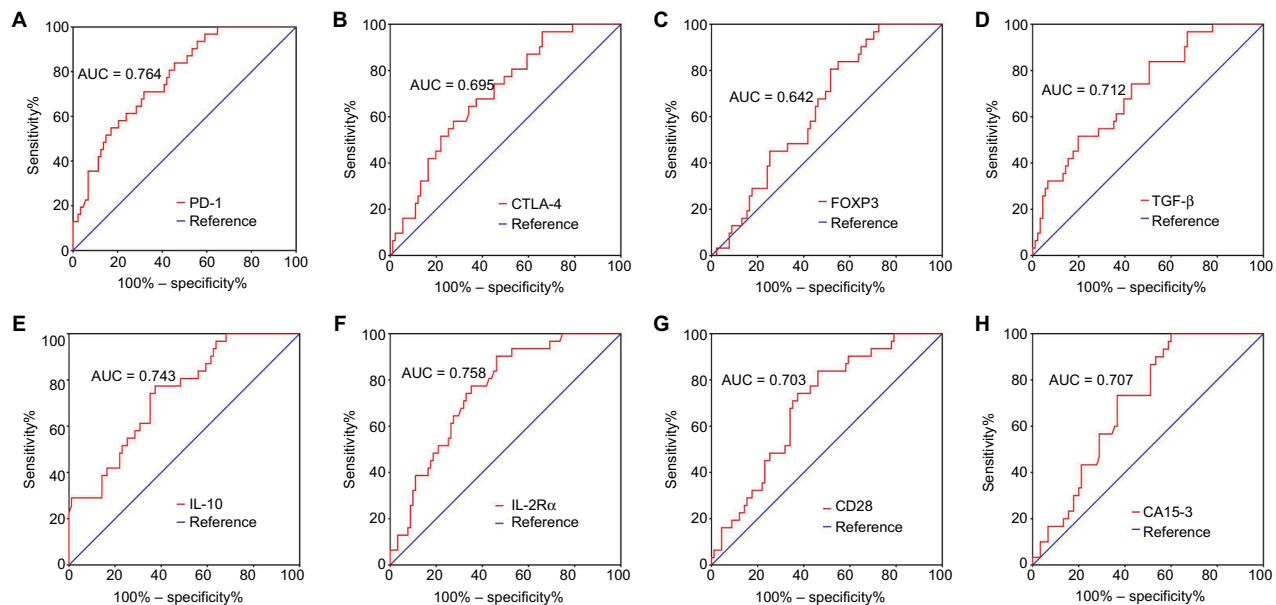
**Figure 1** Relative expression levels of immune molecules in the peripheral blood of patients with BC and BBD.

Note: Levels of (A) PD-1, (B) CTLA-4, (C) FOXP3, (D) TGF- β , (E) IL-10, (F) IL-2R α , and (G) CD28.

Abbreviations: BC, breast cancer; BBD, breast benign disease; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28; FC, fold change.

Table 2 AUCs for immune molecules and in combination with CA15-3 in breast cancer

Parameters	Breast cancer (total)			Early-stage breast cancer (N = 44)			Advanced breast cancer (N = 47)		
	AUC	95% CI	P	AUC	95% CI	P	AUC	95% CI	P
PD-1	0.764	0.674–0.855	<0.001*	0.761	0.654–0.869	<0.001*	0.725	0.614–0.837	0.001*
CTLA-4	0.695	0.594–0.796	0.001*	0.698	0.580–0.817	0.004*	0.687	0.568–0.807	0.006*
FOXP3	0.642	0.540–0.744	0.021*	0.652	0.528–0.775	0.028*	0.611	0.485–0.736	0.104
TGF- β	0.712	0.610–0.815	0.001*	0.706	0.587–0.825	0.003*	0.703	0.585–0.820	0.003*
IL-10	0.743	0.646–0.840	<0.001*	0.730	0.616–0.844	0.001*	0.738	0.628–0.849	<0.001*
IL-2R α	0.758	0.668–0.849	<0.001*	0.756	0.647–0.866	<0.001*	0.740	0.629–0.851	<0.001*
CD28	0.703	0.603–0.803	0.001*	0.711	0.595–0.828	0.002*	0.672	0.551–0.793	0.012*
CA15-3	0.707	0.613–0.800	0.001*	0.607	0.477–0.737	0.121	0.791	0.688–0.893	<0.001*
PD-1 + CA15-3	0.847	0.778–0.916	<0.001*	0.780	0.677–0.883	<0.001*	0.880	0.802–0.959	<0.001*
IL-10 + CA15-3	0.835	0.76–0.91	<0.001*	0.764	0.656–0.873	<0.001*	0.891	0.821–0.960	<0.001*
IL-2R α + CA15-3	0.841	0.769–0.912	<0.001*	0.783	0.680–0.886	<0.001*	0.875	0.797–0.954	<0.001*
PD-1 + IL-10 + CA15-3	0.859	0.791–0.926	<0.001*	0.799	0.700–0.898	<0.001*	0.896	0.826–0.965	<0.001*
PD-1 + IL-2R α + CA15-3	0.854	0.786–0.921	<0.001*	0.792	0.692–0.891	<0.001*	0.883	0.806–0.960	<0.001*
IL-10 + IL-2R α + CA15-3	0.860	0.791–0.928	<0.001*	0.805	0.706–0.903	<0.001*	0.893	0.824–0.962	<0.001*
PD-1 + IL-10 + IL-2R α + CA15-3	0.862	0.795–0.929	<0.001*	0.811	0.713–0.910	<0.001*	0.896	0.827–0.964	<0.001*

Note: *P<0.05.**Abbreviations:** AUC, area under curve; CA15-3, cancer antigen 15-3; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28.**Figure 2** ROC analyses of immune molecules and CA15-3 to distinguish BC (N = 91) from BBD (N = 31).**Notes:** Differential diagnostic value of (A) PD-1, (B) CTLA-4, (C) FOXP3, (D) TGF- β , (E) IL-10, (F) IL-2R α , (G) CD28, and (H) CA15-3.**Abbreviations:** ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28; AUC, area under curve.

+ CA15-3) exhibited a diagnostic AUC of 0.860 (95% CI: 0.791–0.928), sensitivity of 0.900, and specificity of 0.736 (Tables 2 and 3; Figure 3). Finally, the AUC, sensitivity, and specificity for a four-parameter combination (PD-1 + IL-10 + IL-2R α + CA15-3) were 0.862 (95% CI: 0.795–0.929), 0.933, and 0.724, respectively (Tables 2 and 3; Figure 3). Diagnostic values for other combined models are shown in Tables 2 and 3.

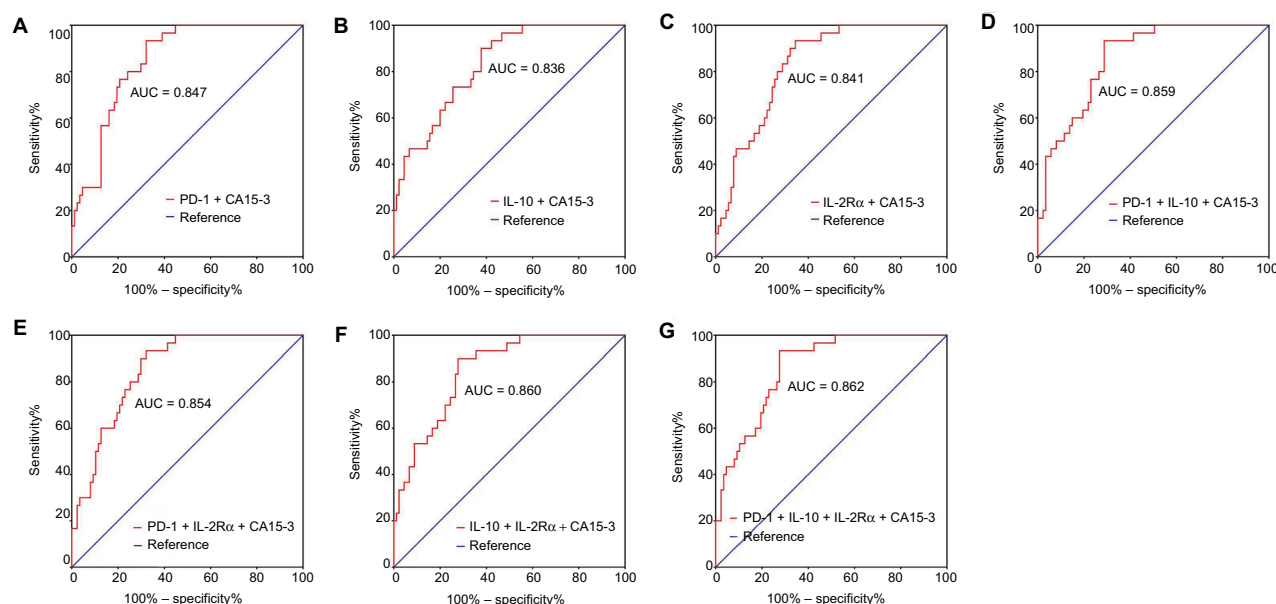
Differential diagnostic value of immune molecules in early-stage BC

In early-stage BC, the AUC, sensitivity, and specificity for CA15-3 were 0.607 (95% CI: 0.477–0.737), 0.523, and 0.733, respectively (Tables 2 and 3; Figure 4). The AUCs for PD-1, IL-10, and IL-2R α were 0.761 (95% CI:

Table 3 Diagnostic sensitivity and specificity for immune molecules and in combination with CA15-3 in breast cancer

Parameters	Breast cancer (total)			Early-stage breast cancer (N = 44)			Advanced breast cancer (N = 47)		
	Sensitivity	Specificity	Youden index	Sensitivity	Specificity	Youden index	Sensitivity	Specificity	Youden index
PD-1	0.448	0.933	0.381	0.591	0.800	0.391	0.478	0.933	0.411
CTLA-4	0.563	0.733	0.296	0.682	0.667	0.349	0.826	0.467	0.293
FOXP3	0.494	0.800	0.294	0.477	0.800	0.277	0.478	0.833	0.311
TGF- β	0.506	0.833	0.339	0.545	0.833	0.378	0.348	0.967	0.315
IL-10	0.644	0.767	0.411	0.659	0.733	0.392	0.630	0.767	0.397
IL-2R α	0.552	0.900	0.452	0.568	0.900	0.468	0.500	0.900	0.400
CD28	0.552	0.833	0.385	0.568	0.833	0.401	0.652	0.700	0.352
CA15-3	0.644	0.733	0.377	0.523	0.733	0.256	0.652	1.000	0.652
PD-1 + CA15-3	0.933	0.678	0.611	0.767	0.705	0.472	0.933	0.783	0.716
IL-10 + CA15-3	0.900	0.632	0.532	0.433	0.955	0.388	0.967	0.717	0.684
IL-2R α + CA15-3	1.000	0.368	0.368	0.933	0.545	0.478	0.933	0.783	0.716
PD-1 + IL-10 + CA15-3	0.933	0.713	0.646	0.933	0.591	0.524	0.933	0.783	0.716
PD-1 + IL-2R α + CA15-3	0.933	0.678	0.611	0.833	0.636	0.469	0.933	0.783	0.716
IL-10 + IL-2R α + CA15-3	0.900	0.736	0.636	0.933	0.614	0.547	1.000	0.696	0.696
PD-1 + IL-10 + IL-2R α + CA15-3	0.933	0.724	0.657	0.933	0.614	0.547	0.933	0.783	0.716

Abbreviations: CA15-3, cancer antigen 15-3; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28.

**Figure 3** ROC analyses of panels of combining immune molecules with CA15-3 to distinguish BC (N = 91) from BBD (N = 31).

Note: Differential diagnostic value of panels of (A) PD-1 + CA15-3, (B) IL-10 + CA15-3, (C) IL-2R α + CA15-3, (D) PD-1 + IL-10 + CA15-3, (E) PD-1 + IL-2R α + CA15-3, (F) IL-10 + IL-2R α + CA15-3, and (G) PD-1 + IL-10 + IL-2R α + CA15-3.

Abbreviations: ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-1, programmed cell death 1; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; AUC, area under curve.

0.654–0.869), 0.730 (95% CI: 0.616–0.844), and 0.756 (95% CI: 0.647–0.866), respectively (Table 2; Figure 4); the diagnostic sensitivity and specificity for these three immune molecules in early-stage BC were 0.591 and 0.800, 0.659 and 0.733, and 0.568 and 0.900, respectively (Table 3).

A three-parameter combination (IL-10 + IL-2R α + CA15-3) exhibited a diagnostic AUC of 0.805 (95% CI: 0.706–0.903), sensitivity of 0.933, and specificity of 0.614 (Tables 2 and 3; Figure 5). Another three-parameter combination (PD-1 + IL-10 + CA15-3) showed a diagnostic AUC of 0.799 (95% CI: 0.700–0.898), sensitivity of 0.933, and specificity

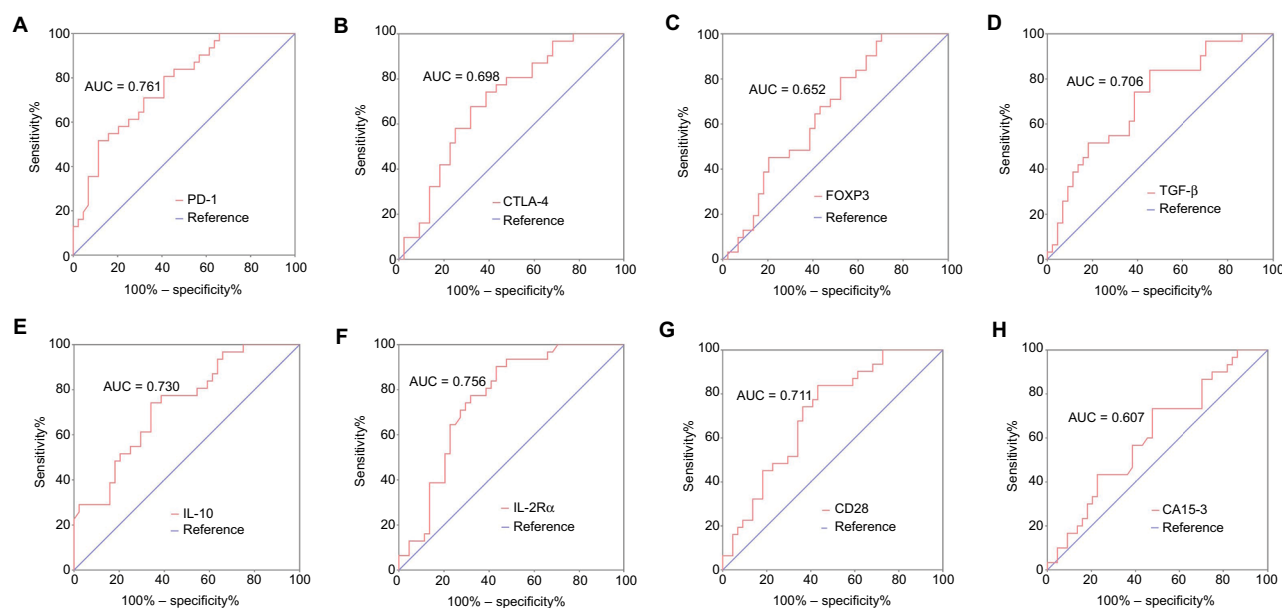


Figure 4 ROC analyses of immune molecules and CA15-3 to distinguish early-stage BC (N = 44) from BBD (N = 31).

Note: Differential diagnostic value of (A) PD-I, (B) CTLA-4, (C) FOXP3, (D) TGF- β , (E) IL-10, (F) IL-2R α , (G) CD28, and (H) CA15-3.

Abbreviations: ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-I, programmed cell death I; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28; AUC, area under curve.

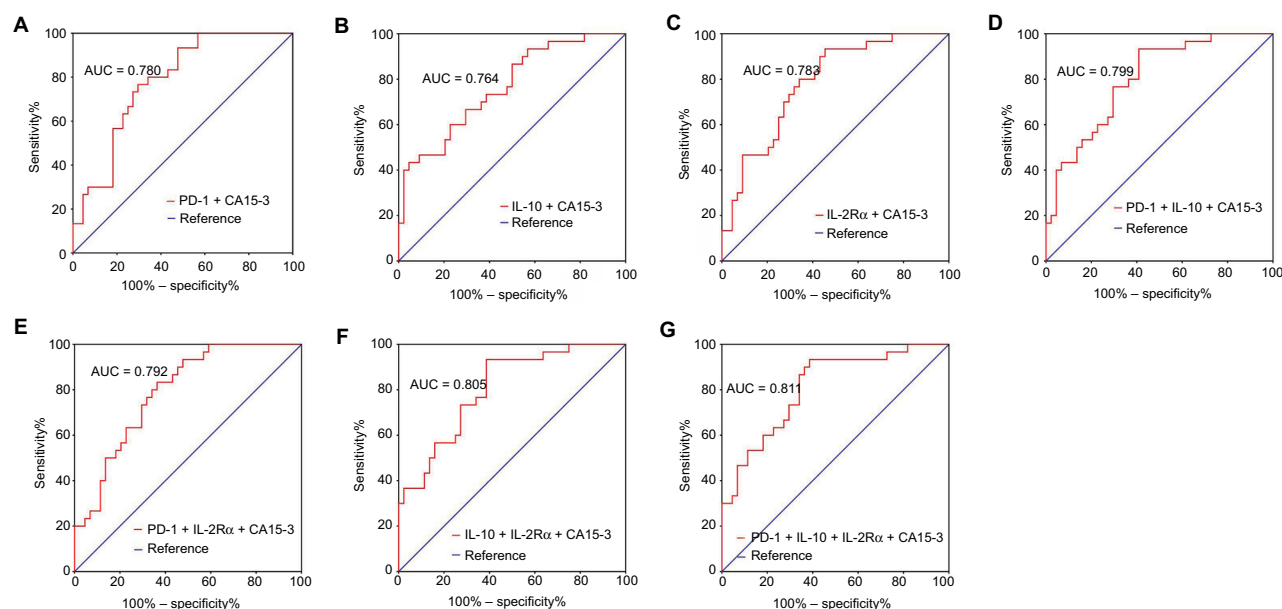


Figure 5 ROC analyses of panels of combining immune molecules with CA15-3 to distinguish early-stage BC (N = 44) from BBD (N = 31).

Note: Differential diagnostic value of panels of (A) PD-I + CA15-3, (B) IL-10 + CA15-3, (C) IL-2R α + CA15-3, (D) PD-I + IL-10 + CA15-3, (E) PD-I + IL-2R α + CA15-3, (F) IL-10 + IL-2R α + CA15-3, and (G) PD-I + IL-10 + IL-2R α + CA15-3.

Abbreviations: ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-I, programmed cell death I; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; AUC, area under curve.

of 0.591 (Tables 2 and 3; Figure 5). The AUC, sensitivity, and specificity for a four-parameter combination (PD-1 + IL-10 + IL-2R α + CA15-3) were 0.811 (95% CI: 0.713–0.910), 0.933, and 0.614, respectively (Tables 2 and 3; Figure 5).

Differential diagnostic value of immune molecules in advanced BC

In advanced BC, CA15-3 exhibited a differential diagnostic AUC of 0.791 (95% CI: 0.688–0.893), sensitivity of 0.652,

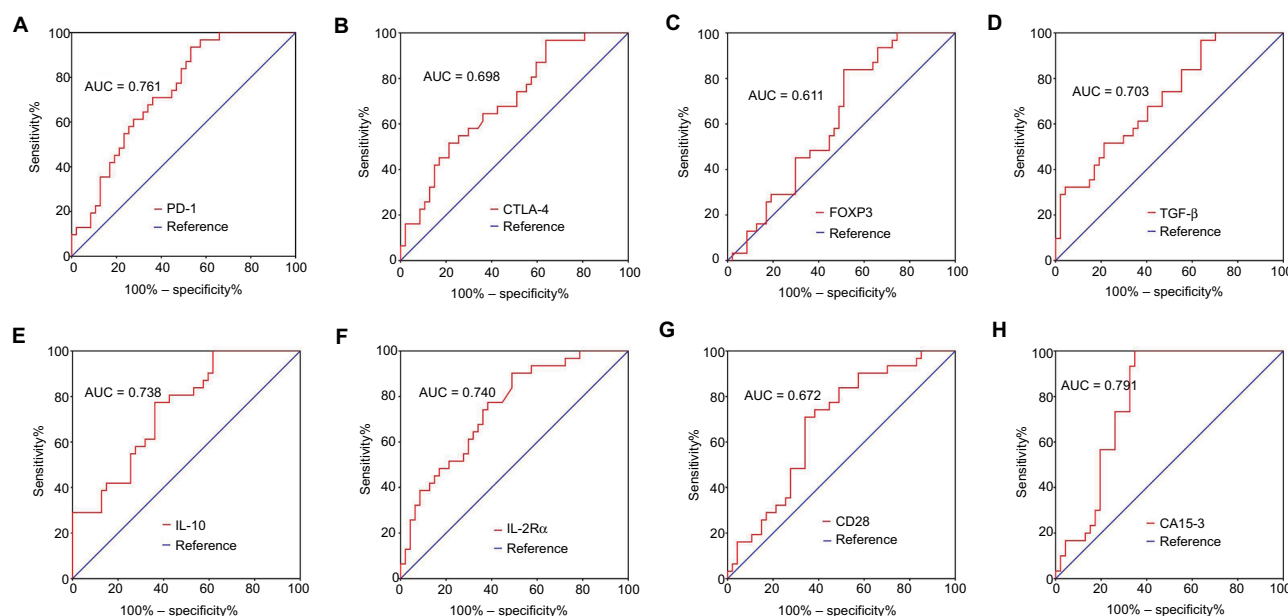


Figure 6 ROC analyses of immune molecules and CA15-3 to distinguish advanced BC (N = 47) from BBD (N = 31).

Note: Differential diagnostic value of (A) PD-1, (B) CTLA-4, (C) FOXP3, (D) TGF- β , (E) IL-10, (F) IL-2R α , (G) CD28, and (H) CA15-3.

Abbreviations: ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28; AUC, area under curve.

and specificity of 1.000 (Tables 2 and 3; Figure 6). The AUCs for PD-1, IL-10, and IL-2R α were 0.725 (95% CI: 0.614–0.837), 0.738 (95% CI: 0.628–0.849), and 0.740 (95% CI: 0.629–0.851), respectively (Table 2; Figure 6); the diagnostic sensitivity and specificity for these three immune molecules were 0.478 and 0.933, 0.630 and 0.767, and 0.500 and 0.900, respectively (Table 3).

The AUC, sensitivity, and specificity for a three-parameter combination (PD-1 + IL-10 + CA15-3) were 0.896 (95% CI: 0.826–0.965), 0.933, and 0.783, respectively (Tables 2 and 3; Figure 7). The AUC, sensitivity, and specificity for another three-parameter combination (IL-10 + IL-2R α + CA15-3) were 0.893 (95% CI: 0.824–0.962), 1.000, and 0.696, respectively (Tables 2 and 3; Figure 7). The four-parameter combination (PD-1 + IL-10 + IL-2R α + CA15-3) showed a diagnostic AUC of 0.896 (95% CI: 0.827–0.964), sensitivity of 0.933, and specificity of 0.783 (Tables 2 and 3; Figure 7).

Discussion

To the best of our knowledge, this study is the first to investigate the differential diagnostic efficiencies of immune checkpoint molecules in discriminating BC from BBD. Specifically, we combined immune molecules with CA15-3 to form a panel that could be used as a substantially less invasive approach compared to surgical biopsy to discriminate early-stage and advanced BC from BBD. We found that 1) relative mRNA levels of PD-1, CTLA-4, FOXP3, TGF- β , IL-10, IL-2R α , and

CD28 were significantly upregulated in the peripheral blood of BC patients versus BBD patients; 2) the diagnostic AUCs for PD-1 in BC (AUC = 0.764), early-stage BC (AUC = 0.761), and advanced BC (AUC = 0.725) were similar; the diagnostic AUCs for IL-2R α in BC, early-stage BC, and advanced BC were 0.758, 0.756, and 0.740, respectively; and the diagnostic AUCs for IL-10 in BC, early-stage BC, and advanced BC were 0.743, 0.730, and 0.738, respectively; 3) for BC at all stages, a panel of PD-1 + IL-10 + IL-2R α + CA15-3 showed the highest AUC of 0.862 with a sensitivity of 0.933 and a specificity of 0.724; for early-stage BC, this panel also showed the highest AUC of 0.811 with a sensitivity of 0.933 and a specificity of 0.614, while for advanced BC, a panel of PD-1 + IL-10 + CA15-3 showed the highest AUC of 0.896 with a sensitivity of 0.933 and a specificity of 0.783.

A previous study found that increased PD-1(+) cells in tumor tissue were associated with poor survival in BC patients.³³ In addition, increased PD-1 expression in peripheral blood mononuclear cells was correlated with high tumor stage in renal cell carcinoma.⁵⁰ We found increased PD-1 mRNA expression in the peripheral blood of BC patients compared with BBD patients with a fold change of 16.2, which was consistent with previous studies. In addition, we found that the differential diagnostic efficiency of PD-1 in total and early-stage BC was better than that of CA15-3. As a co-inhibitory molecule, PD-1 is highly expressed in immune cells and inhibits the anti-tumor response after

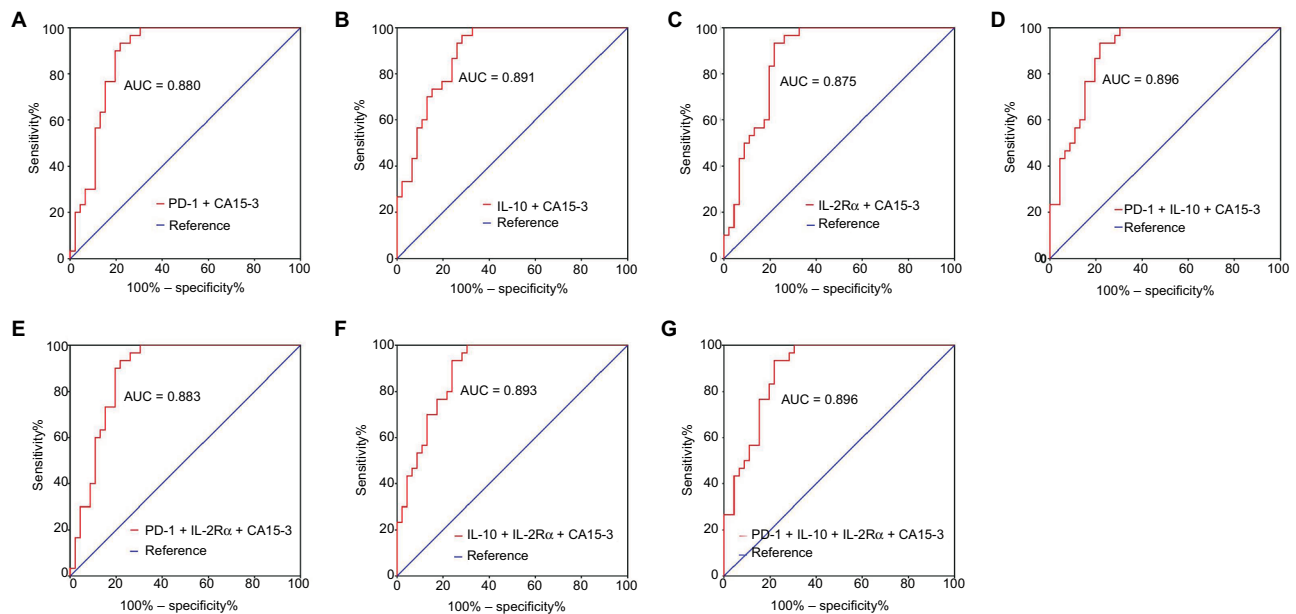


Figure 7 ROC analyses of panels of combining immune molecules with CA15-3 to distinguish advanced BC (N = 47) from BBD (N = 31).

Note: Differential diagnostic value of panels of (A) PD-1 + CA15-3, (B) IL-10 + CA15-3, (C) IL-2R α + CA15-3, (D) PD-1 + IL-10 + CA15-3, (E) PD-1 + IL-2R α + CA15-3, and (F) IL-10 + IL-2R α + CA15-3, and (G) PD-1 + IL-10 + IL-2R α + CA15-3.

Abbreviations: ROC, receiver-operating characteristic; CA15-3, cancer antigen 15-3; BC, breast cancer; BBD, breast benign disease; PD-1, programmed cell death 1; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; AUC, area under curve.

tumor occurrence and development, which may explain our findings.⁵¹

IL-2R α has been extensively investigated in lymphoma owing to its negative correlation with patients' survival.^{35–38} In this study, we found elevated relative expression of IL-2R α with a fold change of 8.3 in BC versus BBD. More importantly, we found a better diagnostic AUC for IL-2R α than for CA15-3 in BC patients. In addition, IL-10 showed similar results to those of IL-2R α , consistent with previous findings that IL-10 is highly expressed in the peripheral blood of malignant breast tumors.^{52,53} The underlying mechanism is that IL-10, as an anti-inflammatory cytokine, promotes tumor progression and induces immunosuppression.⁵²

Due to the fact that single- or two-molecule combinations did not work well, we chose three molecules with the largest AUCs and CA15-3 to establish a panel to improve diagnostic efficiency. In the total BC group, a panel containing four parameters (PD-1 + IL-10 + IL-2R α + CA15-3) had the highest diagnostic AUC of 0.862, sensitivity of 0.933, and specificity of 0.724. Shan et al¹⁷ established a panel of six immune molecules to discriminate BC and BBD, which showed an AUC of 0.789, sensitivity of 0.824, and specificity of 0.781. In addition, another study revealed that plasma cfDNA integrity could provide an AUC of 0.80, sensitivity of 0.910, and specificity of 0.682 in discriminating non-small-cell lung cancer from benign lung nodules.¹⁹ Our

panel exhibited a better differential diagnostic value, which may be accounted for by the significance of checkpoint molecules and other immune molecules in tumor occurrence and invasion. In advanced BC patients, a panel containing three parameters (PD-1 + IL-10 + CA15-3) showed the same diagnostic power with the panel containing four parameters (PD-1 + IL-10 + IL-2R α + CA15-3). Considering the cost, we suggest that the three-parameter panel may be a complement for conventional imaging modalities to detect advanced BC.

For early-stage BC detection, a recent study revealed that a model containing 15 lipid species had great differential diagnostic power, with an AUC of 0.926, sensitivity of 0.833, and specificity of 0.927.²¹ Another previous study showed that the diagnostic value of developmental endothelial locus-1 protein was high, with an AUC of 0.924, sensitivity of 0.849, and specificity of 0.758 in distinguishing BC from BBD and non-cancer patients;²³ although they did not split BBD and other non-cancer patients, the diagnostic efficiency was still promising. In the current study, we found that our panel of PD-1 + IL-10 + IL-2R α + CA15-3 showed an inferior AUC of 0.811, sensitivity of 0.933, and specificity of 0.614 when compared with the two abovementioned studies. However, considering technical issues and costs, our panel is easy to use and feasible.

Overall, we proved the diagnostic value of PD-1 and established a panel containing PD-1, IL-2R α , IL-10, and

CA15-3 to distinguish BC from BBD. However, there were some limitations. First, a validation group for above-mentioned results is absent in this study. Second, we only enrolled 91 BC patients and 31 BBD patients in the present study. The sample size was small, especially in terms of the BBD patient numbers, which was usually larger than 50 in similar studies. The limited sample size may affect the results. Third, some unknown factors for patients, such as eating habits, amount of exercise every day, sleep, and emotion, may influence the expression of immune molecules. Further studies need to take them into consideration and conduct subgroup analyses to address this limitation. Finally, 22 metastatic BC patients received adjuvant endocrine therapy before enrollment; endocrine therapy and its associated comorbidities, such as osteoporosis, hypercholesterolemia, cardiovascular events, and depression, may affect patients' immune status. In addition, therapies for above-mentioned comorbidities may affect expression of immune markers.

Conclusion

We established a panel containing PD-1, IL-2R α , IL-10, and CA15-3 to discriminate BC from BBD, which could serve as a complement for conventional imaging modalities, especially in detecting early-stage BC. Further large-scale investigations are needed to confirm its diagnostic value and improve the identification of BC by combining the panel with other parameters.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

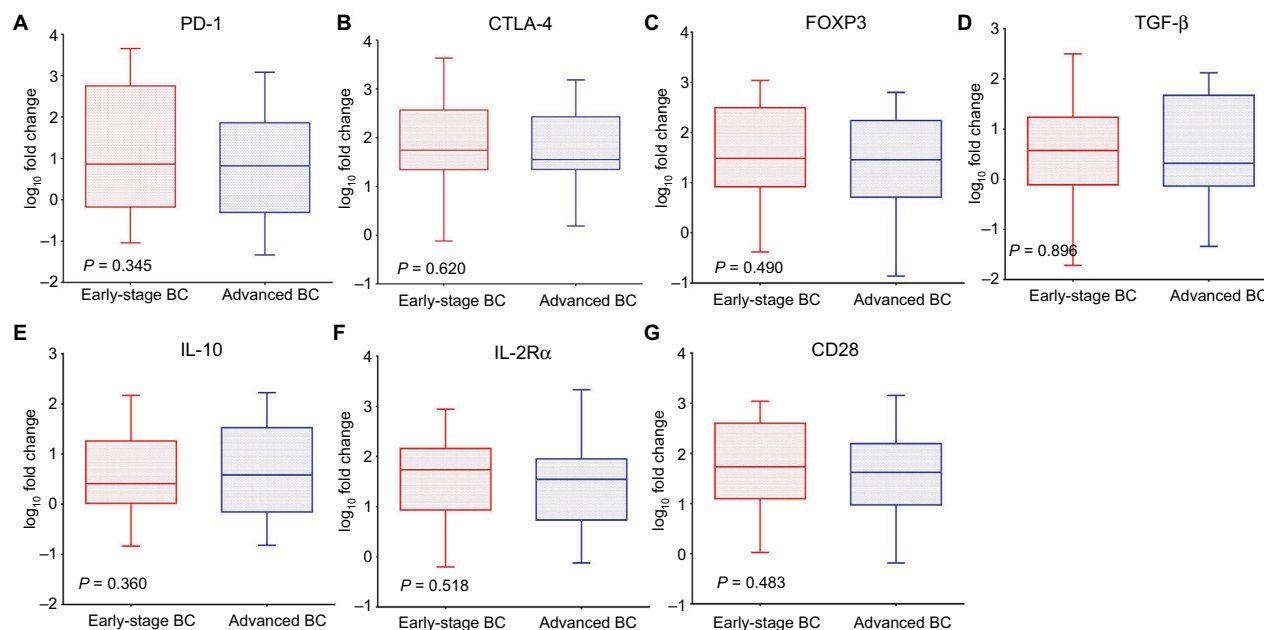


Figure S1 Relative expression levels of immune molecules in the peripheral blood of patients with early-stage BC and advanced BC.

Note: Levels of (A) PD-1, (B) CTLA-4, (C) FOXP3, (D) TGF-β, (E) IL-10, (F) IL-2Rα, and (G) CD28.

Abbreviations: BC, breast cancer; PD-1, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF-β, transforming growth factor-beta; IL-10, interleukin-10; IL-2Rα, interleukin-2 receptor alpha; CD28, cluster of differentiation 28.

Table S1 Primers for PD-I, CTLA-4, FOXP3, TGF- β , IL-10, IL-2R α , CD28, and β -actin

Molecules	Primers
PD-I-F	5'-GGTGTGAGGCCATCCACAA-3'
PD-I-R	5'-CCATTCTGTCTGGAGCCTCTG-3'
PD-I-probe	5'-AGCCGATTAGCCATGGACAGTTG-3'
CTLA4-F	5'-GCTGACAGCCAGGTGACTGAA-3'
CTLA4-R	5'-TGAGCTCCACCTTGACAGATG-3'
CTLA4-probe	5'-CTGTGCGGCAACCTACATGATGG-3'
FOXP3-F	5'-AAGAGCCAGAGGACTTCCTCAA-3'
FOXP3-R	5'-CATGGCACTCAGCTTCTCCTT-3'
FOXP3-probe	5'-CAGGCGGACCATCTTCTGGATGA-3'
TGF- β -F	5'-CAACACATCAGAGCTCCGAGAA-3'
TGF- β -R	5'-GCTGAGGTATCGCCAGGAAT-3'
TGF- β -probe	5'-CTGCGTCTGCTGAGGCTCAAGTTA-3'
IL-10-F	5'-TGCCAAGCCTTGTCTGAGATG-3'
IL-10-R	5'-CCACGGCCTTGCTCTTGTT-3'
IL-10-probe	5'-CAGACATCAAGGCGCATGTGAAC-3'
IL-2R α -F	5'-ATGGCTGCAACCATGGAGAC-3'
IL-2R α -R	5'-TCTGTTCCCGGCTTCTTACC-3'
IL-2R α -probe	5'-TGATCAGCGTCCTCCTCCTGAGTG-3'
CD28-F	5'-CGCAGTGGCTCATGCTTGTA-3'
CD28-R	5'-TCTCATGCCTCAGCCTCTTG-3'
CD28-probe	5'-AGATCAGGACCAGCCTGGTCAAGA-3'
β -Actin-F	5'-ACGTTGCTATCCAGGCTGTG-3'
β -Actin-R	5'-CGCTCGGTGAGGATCTTCAT-3'
β -Actin-probe	5'-CGTACCACTGGCATCGTGATGGA-3'

Abbreviations: PD-I, programmed cell death 1; CTLA-4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; TGF- β , transforming growth factor-beta; IL-10, interleukin-10; IL-2R α , interleukin-2 receptor alpha; CD28, cluster of differentiation 28.

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