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ORIGINAL RESEARCH

Diagnostic Efficacy and Clinical Significance of Lymphocyte Subsets, Granzyme B and Perforin in the Peripheral Blood of Patients with Invasive Breast Cancer Following Neoadjuvant Chemotherapy

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Purpose: Breast cancer, a predominant contributor to cancer-related mortality worldwide, is increasingly managed through the application of neoadjuvant chemotherapy (NAC). Analyzing the dynamic changes in peripheral blood lymphocyte subsets, granzyme B and perforin are crucial for investigating their roles in tumorigenesis, development and treatment; this study aimed to use these analyses to diagnose malignant breast tumor, assess the anti-tumor immunity and predict chemotherapy efficacy in breast cancer patients.

Patients and Methods: To address this objective, a total of 582 peripheral blood samples were collected from healthy controls (n=47), benign breast disease patients (n=401) and breast cancer patients (n=134). Lymphocyte subsets, along with granzyme B and perforin expression, were assessed using flow cytometry. Changes before and after NAC were also monitored.

Results: Breast cancer patients exhibited reduced proportions and absolute counts of $CD3^+$ and $CD8^+$ T cells, increased NK cell percentage and $CD4^+/CD8^+$ ratio, and higher levels of granzyme B and perforin in $CD3^+$, $CD8^+$ T cells and NK cells. Post-NAC, the percentages of $CD3^+$, $CD4^+$, $CD8^+$ T cells and NK cells increased, along with a higher $CD4^+/CD8^+$ ratio, while B cell percentages decreased compared to pre-NAC. Furthermore, the effective group showed higher percentages of $CD3^+$, $CD8^+$ T cells and lower percentages of B cells than the ineffective group post-NAC. Incidentally, Granzyme B and perforin expression in $CD3^+$ and $CD8^+$ T cells was elevated following postoperative chemotherapy.

Conclusion: These findings indicated that peripheral blood lymphocyte subsets, along with granzyme B and perforin levels, could serve as potential biomarkers for differentiating benign from malignant breast tumors, assessing anti-tumor immunity and predicting chemotherapy efficacy.

Keywords: breast cancer, lymphocyte subsets, granzyme B, perforin, diagnostic biomarker, neoadjuvant

Introduction

Breast cancer is one of the most prevalent malignancies worldwide and a leading cause of cancer-related mortality.[1–3] Despite advancements in survival through various clinical treatments, including surgical resection, chemotherapy, radiotherapy, immunotherapy, endocrine therapy and targeted therapy,[4,5] the five-year survival rates for breast cancer patients remain suboptimal.[6] Neoadjuvant chemotherapy (NAC) has increasingly become a pivotal approach in the management of breast cancer, extending its application to early-stage operable cases.[7,8] Notably, the response to NAC varies across different breast cancer subtypes, such as HER2-positive and triple-negative breast cancer (TNBC), with

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distinct mechanisms of resistance and therapeutic response emerging. These include alterations in hypoxia-inducible factor-1α (HIF-1α) signaling within the tumor microenvironment, the induction of cancer stem cells (CSCs) and the activation of epidermal growth factor receptor (EGFR), all of which play pivotal roles in modulating treatment outcomes.[9–11] Nonetheless, tumor progression during NAC is often linked to unfavorable prognostic outcomes,[12–14] underscoring the critical need to identify underlying biomarkers that can predict treatment efficacy and to identify patients who are most likely to derive benefit from NAC.

Advances in tumor immunity have underscored the crucial role of the immune system in breast cancer biology.[15–17] In the intricate tumor microenvironment (TME), variations in lymphocyte counts, proportions, effector molecule expression such as granzyme B and perforin, and immune cell interactions critically affect development, progression, therapeutic efficacy and prognosis of tumors. In terms of diagnostic value, studies have found that $CD3^+$ T cells, $CD4^+$ T cells and natural killer (NK) cells, as well as the $CD4^+/CD8^+$ T cell ratio, were significantly reduced in patients with non-small cell lung cancer (NSCLC) compared to the control group.[18] Regarding predictive efficacy, research has confirmed that ratios of lymphocyte subsets, $CD4^+$ T cells, $CD8^+$ T cells and B cells are significantly higher in NSCLC responders compared to non-responders receiving chemotherapy or combination immunotherapy.[19] For prognostic value, early decreases in peripheral blood $CD4^+$ T cell counts may serve as a biomarker for poor prognosis in gastrointestinal cancer patients undergoing immune checkpoint inhibitor (ICI) therapy.[20] Additionally, perforin and granzyme B, key components of cytotoxic T cell and NK cell granules, were found to be elevated in lip squamous cell carcinoma.[21] Similarly, these markers were increased in NSCLC patients following treatment with ICI, potentially enhancing anti-tumor immunity through directly killing tumor cells.[22] In summary, emerging evidences suggested a potential relationship between circulating lymphocytes and diagnostic as well as therapeutic efficacy in tumor. However, the roles and clinical impacts of changes in lymphocyte subsets and the expression of granzyme B and perforin in breast cancer are not yet fully understood.

Here, we designed clinical cohorts to investigate the comparison of peripheral blood lymphocyte subsets, the expression levels of granzyme B, and perforin in patients with benign breast diseases and breast cancer, monitoring these changes before and after chemotherapy to identify potential peripheral blood biomarkers for diagnosing malignant breast tumor, predicting chemotherapy efficacy and reflecting anti-tumor immunity.

Patients and Methods

Patients Cohort and Study Design

In this study, a total of 582 peripheral blood samples were collected from participants at Zhanjiang Central Hospital, Guangdong Medical University, between August 2022 and December 2023. As shown in Figure 1, participants were divided into four cohorts based on different objectives. Peripheral blood samples were collected from 47 healthy controls (46.70 ± 14.91 years), 401 patients with benign breast diseases (38.55 ± 11.10 years) and 134 invasive breast cancer patients (54.73 ± 11.29 years). Among the breast cancer patients, 30 received neoadjuvant chemotherapy, 57 underwent surgery alone and 47 received adjuvant chemotherapy after surgery. Detailed information on all breast cancer patients, including those who received postoperative chemotherapy, is provided in Supplementary Information Tables S1 and S2. The study adhered closely to the ethical guidelines of the Helsinki Declaration (2013 revision) and received approval from the Ethics Review Committee of Zhanjiang Central Hospital, Guangdong Medical University (approval No. PJ[IIT-2021-007-02]). The patients' data were thoroughly anonymized to ensure privacy and confidentiality.

Inclusion Criteria

Breast Cancer Group

(1) Both imaging and pathological examination fulfilled the diagnostic criteria for invasive breast cancer; (2) No surgery, chemotherapy, radiotherapy, or other anti-cancer treatments were administered before the initial peripheral blood sample collection; (3) Availability of complete clinical, imaging and pathological medical records.

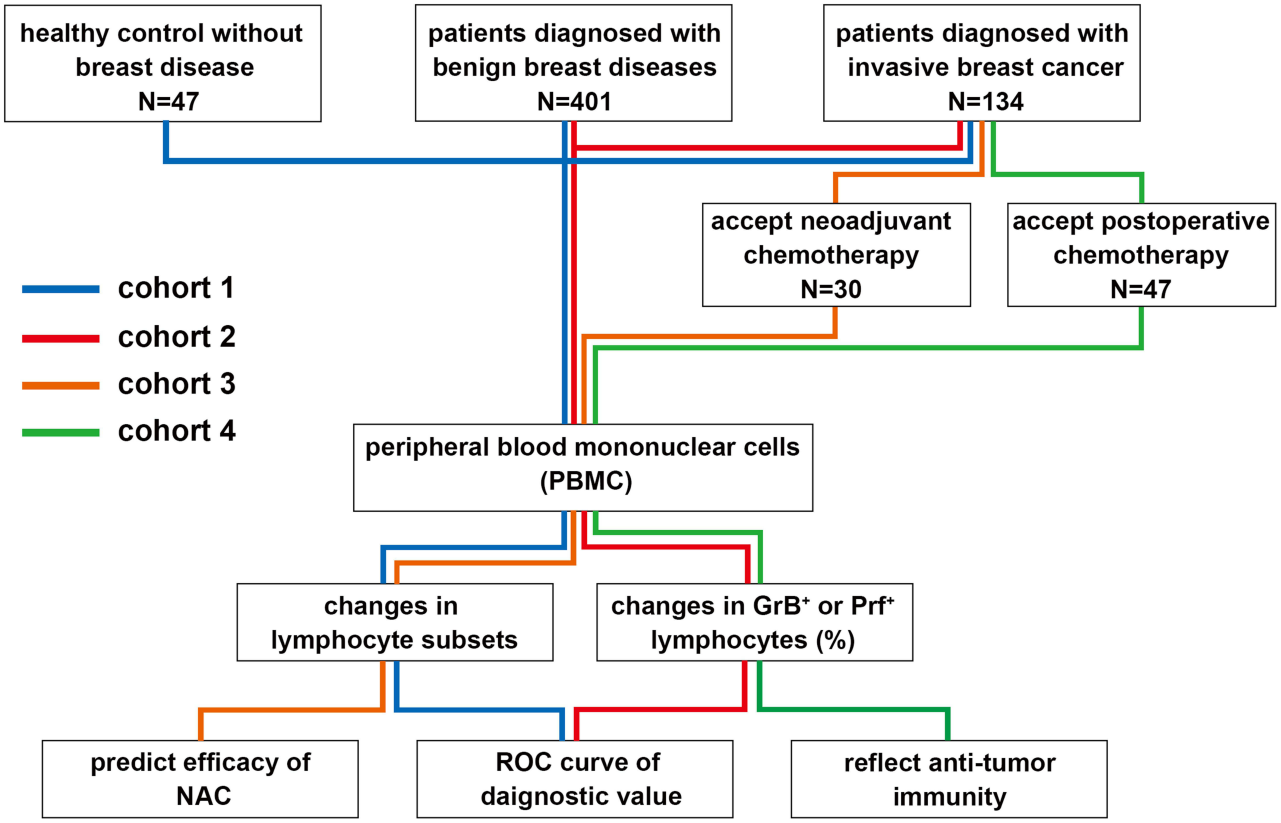

Figure 1 The flow chart of this study. Blue line: cohort 1: Compare the levels of lymphocyte subsets among healthy controls, benign breast disease patients, and breast cancer patients to evaluate their efficacy in distinguishing between benign and malignant breast tumors. Red line: cohort 2: Compare the expression levels of granzyme B and perforin between benign breast diseases and breast cancer patients, and use ROC curves to distinguish between tumor malignancy. Orange line: cohort 3: Investigate changes in lymphocyte subsets before and after NAC in breast cancer patients and identify biomarkers to predict efficacy of chemotherapy. Green line: cohort 4: Evaluate changes in the positive rates of granzyme B and perforin in breast cancer patients before and after adjuvant chemotherapy to assess their reflection of anti-tumor immunity. **Abbreviations**: NAC, neoadjuvant chemotherapy; GrB, granzyme B, Prf, perforin.

Benign Breast Disease Group

Imaging scans and pathological evaluation confirmed the diagnosis of benign breast disease (including Breast fibroadenoma, Breast adenosis, Intraductal papilloma, etc.) and ruled out the possibility of malignant breast tumor.

Healthy Control Group

Healthy female candidates with no breast-related conditions as confirmed by imaging scans (MRI, ultrasound and X-ray) as well as visual and palpation examinations.

Exclusion Criteria

(1) Patients with significant dysfunction of the heart, lungs or other major organs; (2) Patients with infectious, autoimmune diseases or immune-compromising conditions; (3) Patients with no comprehensive clinical, imaging or pathological data.

Peripheral Blood Samples and Flow Cytometry Analysis

In brief, whole blood samples were obtained from candidates prior to therapy using ethylenediaminetetraacetic acid (EDTA) tubes (Kindly, Shanghai) through venipuncture, and then analyzed for lymphocyte subsets using flow cytometry. According to the manufacturer's protocol, blood cells were incubated with monoclonal antibodies for 15 min in conditions void of light at room temperature. Lysis solution (RaiseCare) was used for dissolving red blood cells. Subsequently, 50 μL of absolute counting fluorescent microbeads (Beckman Coulter) were added to the test samples and mixed thoroughly for the absolute counting of lymphocyte subsets. The samples were analyzed using a flow

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cytometer (Navios EX System, Beckman Coulter) and the lymphocyte subset percentages, as well as absolute counts, were calculated using Kaluza software. The formula for absolute cell count (cells/μL) was: (Total number of counted cells / Total number of counted fluorescent beads) × Concentration of fluorescent beads. Five lymphocyte subsets, total T cells ($CD3^+$ $CD45^+$), T helper cells ($CD3^+$ $CD4^+$), T cytotoxic cells ($CD3^+$ $CD8^+$), NK cells ($CD3^-$ $CD16^+$ $CD56^+$), B cells ($CD3^-$ $CD19^+$), were assessed for both percentages and absolute counts using specific antibodies. These included tetraCHROME antibody cocktails: CD45-FITC/CD56-PE/CD19-ECD/CD3-PC5 (6607073, Beckman Coulter) and CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 (6607013, Beckman Coulter), along with anti-CD16-APC (RaiseCare), anti-granzyme B (RaiseCare) and anti-perforin (RaiseCare). For intracellular staining of Granzyme B and perforin, cells were first fixed using fixation buffer at 4°C for 20 minutes, followed by permeabilization with 1× Permeabilization Wash Buffer (RaiseCare) at 4°C for another 20 minutes. Subsequently, the samples were incubated with anti-Granzyme B and anti-Perforin antibodies at room temperature in the dark for 20 minutes. Finally, cells were resuspended in 300 μL 1×PBS and analyzed using flow cytometry. To ensure specificity and eliminate non-specific fluorescence, appropriate fluorescence minus one (FMO) controls and isotype controls were included in all experiments. Gating strategy of flow cytometry analysis were described in the Supplementary Material Figure S1 and S2.

Neoadjuvant Chemotherapy and Efficacy Evaluation

The details of the chemotherapy drugs, including specific agents and their administration frequencies, for the 30 breast cancer patients who received NAC, can be found in Supplementary Information Table S3. The efficacy of NAC in breast cancer patients was evaluated based on changes in the primary tumor size pre- and post-NAC, as assessed by imaging in accordance with the RECIST 1.1 criteria. Clinical responses were categorized as Complete Response (CR), Partial Response (PR), Stable Disease (SD) and Progressive Disease (PD). CR: complete disappearance of all target lesions with no new lesions. PR: a ≥30% reduction in the longest diameter of the tumor compared to baseline. SD: a reduction of <30% or an increase of <20% in the longest diameter compared to baseline. PD: a ≥20% increase in the longest diameter of the tumor or the appearance of new lesions. Notably, CR and PR were classified as effective, while SD and PD were deemed ineffective.

Statistical Analysis

A *t*-test was employed for comparisons between two groups. For analyses involving three groups, a one-way analysis of variance (ANOVA) was conducted, with subsequent post-hoc evaluations performed using Tukey's multiple comparisons test. A receiver operating characteristic (ROC) curve was constructed to evaluate the prognostic accuracy of the biomarker, with the area under the ROC curve (AUC) computed to quantify the biomarker's discriminatory performance. The optimal cutoff value for the biomarker was determined using the Youden index, which provides a criterion-independent measure for maximizing the diagnostic efficiency. Statistical analyses were performed with SPSS Statistics 26.0 and GraphPad Prism 8.0.2 software. For variables that were normally distributed, quantitative data were reported as the mean±standard deviation (SD). Conversely, for variables that were non-normally distributed, the data were presented as the median with the interquartile range (IQR). A value of $P < 0.05$ was deemed statistically significant throughout the analyses.

Results

Comparison of Lymphocyte Subsets in the Peripheral Blood Among Healthy Controls, Benign Breast Disease Patients, and Breast Cancer Patients

We employed flow cytometry to assess the percentages and absolute counts of peripheral blood lymphocyte subsets in healthy controls, patients with benign breast disease and patients with breast cancer; detailed statistical data were presented in Table 1. When compared to benign breast diseases, patients with breast cancer exhibited lower proportions of $CD3^+$ and $CD8^+$ T cells (70.22(9.90) versus 72.31(9.07) %, P = 0.008; 21.33(8.74) versus 24.49(8.52) %, P < 0.001, respectively). Similarly, the absolute counts of $CD3^+$ and $CD8^+$ T cells were significantly lower, with $CD3^+$ T cells at 1210(575) versus 1300(488) cells/μL (P = 0.041) and $CD8^+$ T cells at 354(204) versus 422(212) cells/μL (P < 0.001).

Table 1 Comparison of Lymphocyte Subsets in Healthy Controls, Benign Breast Disease and Breast Cancer Patients

Lymphocyte subsets	Breast cancer N=134	BBD N=401	Healthy control N=47	P^a value	P^b value
$CD3^+$ T cell (%)	70.22 (9.90)	72.31 (9.07)	73.28 (8.35)	0.008	0.025
$CD3^+$ T cell absolute count (cells/μL)	1210 (575)	1300 (488)	1384 (549)	0.041	0.013
$CD4^+$ T cell (%)	41.93 (9.22)	41.21 (9.02)	41.55 (5.74)	0.341	0.680
$CD4^+$ T cell absolute count (cells/μL)	703 (371)	718 (300)	766 (303)	0.999	0.788
$CD8^+$ T cell (%)	21.33 (8.74)	24.49 (8.52)	24.99 (2.95)	<0.001	<0.001
$CD8^+$ T cell absolute count (cells/μL)	354 (204)	422 (212)	473 (196)	<0.001	<0.001
$CD4^+/CD8^+$ T cell ratio	2.06 (1.07)	1.65 (0.91)	1.61 (0.32)	<0.001	<0.001
NK cell (%)	13.48 (9.66)	11.68 (7.67)	11.86 (6.10)	0.014	0.070
NK cell absolute count (cells/μL)	225 (203)	204 (168)	205 (124)	0.345	0.441
B cell (%)	13.84 (6.73)	13.71 (5.91)	13.51 (6.05)	0.943	0.993
B cell absolute count (cells/μL)	238 (164)	243 (144)	263 (112)	0.799	0.870

Notes: a: comparison between the breast cancer group and the BBD group; b: comparison between the breast cancer group and the healthy control group.
Abbreviation: BBD, benign breast disease.

However, the proportion of NK cells was higher (13.48(9.66) versus 11.68(7.67) %, P = 0.014). Additionally, an increased $CD4^+/CD8^+$ ratio was observed (2.06(1.07) versus 1.65(0.91), P < 0.001). In a comparable manner, when compared to healthy controls, breast cancer patients also demonstrated the same trends in peripheral blood $CD3^+$ and $CD8^+$ T lymphocyte percentages and absolute counts, as well as the $CD4^+/CD8^+$ ratio (Figure 2A, C,E,F). On the other hand, no significant differences were observed in the proportion and absolute count of B cells and $CD4^+$ T cells when comparing breast cancer patients to either those with benign breast disease or healthy individuals (P > 0.05) (Figure 2B

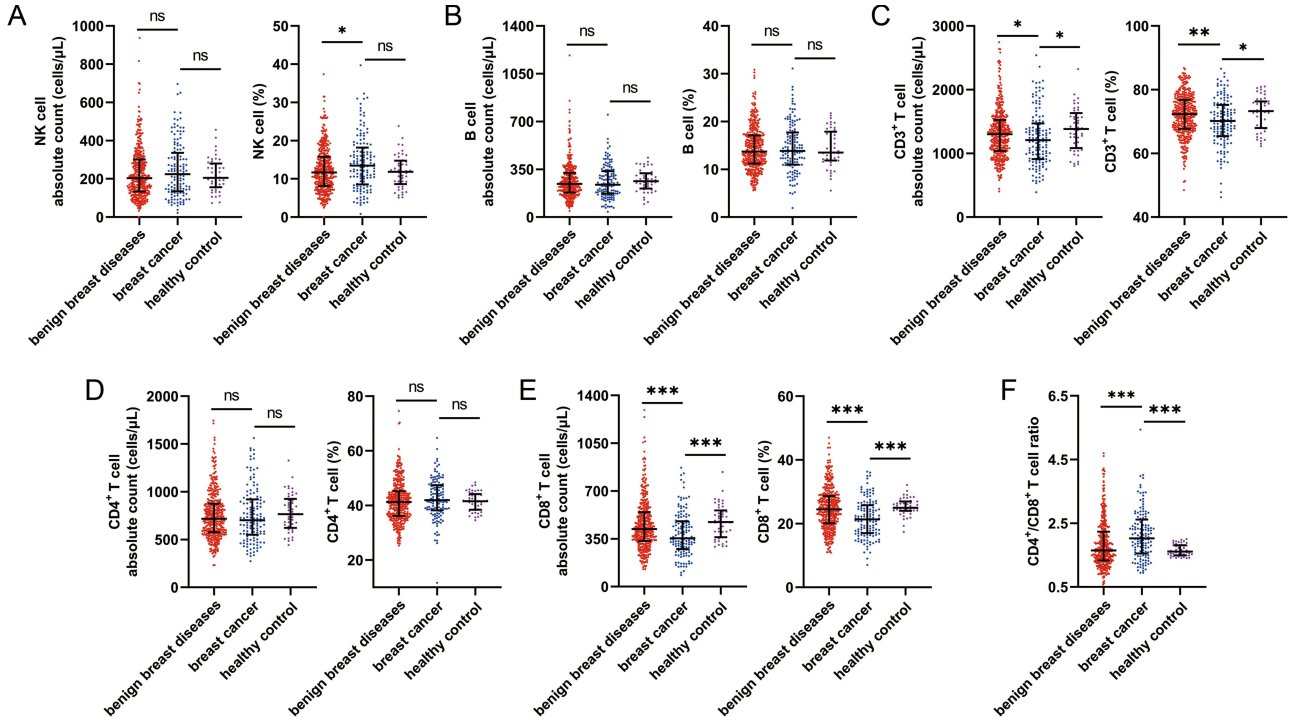

Figure 2 Comparison of peripheral blood lymphocyte subsets among healthy controls, BBD and breast cancer groups: (**A**) NK cells absolute count and percentage; (**B**) B cells absolute count and percentage; (**C**) $CD3^+$ T cells absolute count and percentage; (**D**) $CD4^+$ T cells absolute count and percentage; (**E**) $CD8^+$ T cells absolute count and percentage; (**F**) $CD4^+/CD8^+$ T cell ratio. (*P < 0.05, **P < 0.01, ***P < 0.001, ns: P > 0.05).

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and D). By the way, there were no differences in percentages or absolute counts of lymphocyte subsets between patients with benign breast diseases and healthy controls ($P > 0.05$).

Diagnostic Value of the Lymphocyte Subsets in the Peripheral Blood in Breast Cancer

Above results suggested that the ratio of $CD4^+/CD8^+$ T cells, the proportion and absolute count of $CD3^+$ and $CD8^+$ T cells, and the NK cell percentage were potential diagnostic tools for breast cancer. Therefore, we generated ROC curves to estimate the diagnostic performance of different lymphocyte subsets for breast cancer, as shown in Figure 3. The areas under the curve (AUC) for ratio of $CD4^+/CD8^+$ T cells, the proportion and absolute count of $CD3^+$ and $CD8^+$ T cells, and NK cell percentage were 0.629 (95% CI 0.575–0.682), 0.576 (95% CI 0.519–0.634), 0.559 (95% CI 0.499–0.618), 0.635 (95% CI 0.581–0.689), 0.608 (95% CI 0.551–0.664), 0.571 (95% CI 0.513–0.629), respectively. Sensitivity, specificity, cut-off value and Youden index were calculated to assess the diagnostic performance of different peripheral blood lymphocyte subsets for malignant breast tumors, with detailed statistical data presented in Table 2. Among the six biomarkers, the $CD3^+$ T cell absolute count showed the highest sensitivity at 76.6% but the lowest specificity at 35.8%, resulting in a Youden index of 0.124. In contrast, NK cell percentage had the lowest sensitivity at 51.5% but the highest specificity at 63.1%, with a Youden index of 0.146. Compared to other indicators, the $CD8^+$ T cell percentage demonstrated relatively high sensitivity (63.1%) and specificity (60.4%), along with the highest Youden index of 0.235.

Dynamic Changes of Lymphocyte Subsets in Breast Cancer Patients Before and After Neoadjuvant Chemotherapy

Studies have shown that neoadjuvant chemotherapy affects the percentages and absolute counts of peripheral blood lymphocyte subsets in cancer patients. In our study, this was evident as shown in Table 3, after neoadjuvant chemotherapy, breast cancer patients showed a significant increase in the percentage of $CD3^+$ T cells from 69.01±8.08 to 82.94 ±7.90% ($P < 0.001$), $CD4^+$ T cells from 41.64±6.79 to 50.23±7.11% ($P < 0.001$), $CD8^+$ T cells from 21.52±5.74 to 29.68 ±7.30% ($P < 0.001$) and NK cells from 12.64(13.23) to 18.41(13.31)% ($P = 0.047$). Additionally, the $CD4^+/CD8^+$ ratio increased from 2.11±0.67 to 2.32±0.62 ($P = 0.008$), while the percentage of B cells decreased from 13.56(8.41) to 2.86 (6.21)% ($P < 0.001$) (Figure 4A-F). Conversely, after chemotherapy, absolute counts of lymphocyte subsets decreased as follows: total lymphocytes from 1672(495) to 881(363) cells/μL, $CD3^+$ T cells from 1057(447) to 650(349) cells/μL,

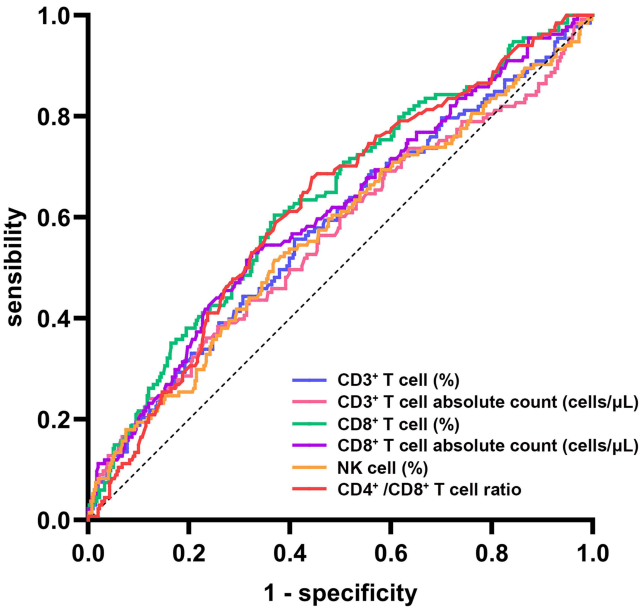

Figure 3 ROC curve of absolute counts and percentages of peripheral blood lymphocyte subsets in distinguishing breast tumor malignancy.

Table 2 Diagnostic Value of Absolute Counts and Percentages of Peripheral Blood Lymphocyte Subsets in Distinguishing Benign and Malignant Breast Tumor

Lymphocyte subsets	Sensibility (%)	Specificity (%)	*P* value	AUC	95% CI	Youden index	Cut-off value
$CD3^+$ T cells (%)	59.1	55.2	0.008	0.576	0.519–0.634	0.143	70.64
$CD3^+$ T cell absolute count (cells/μL)	76.6	35.8	0.041	0.559	0.499–0.618	0.124	1032
$CD8^+$ T cells (%)	63.1	60.4	<0.001	0.635	0.581–0.689	0.235	22.54
$CD8^+$ T cell absolute count (cells/μL)	67.6	53.0	<0.001	0.608	0.551–0.664	0.206	360
NK cells (%)	51.5	63.1	0.014	0.571	0.513–0.629	0.146	13.46
$CD4^+/CD8^+$ T cell ratio	68.7	55.6	<0.001	0.629	0.575–0.682	0.243	1.74

Table 3 Changes in Peripheral Blood Lymphocyte Subsets Among Breast Cancer Patients Before and After Neoadjuvant Chemotherapy (NAC)

Lymphocyte subsets	NAC (before) N=30	NAC (after) N=30	*P* value
$CD3^+$ T cell (%)	69.01±8.08	82.94±7.91	<0.001
$CD4^+$ T cell (%)	41.64±6.79	50.23±7.11	<0.001
$CD8^+$ T cell (%)	21.52±5.74	29.68±7.30	<0.001
NK cell (%)	12.64 (13.23)	18.41 (13.31)	0.047
B cell (%)	13.56 (8.41)	2.86 (6.21)	<0.001
$CD4^+/CD8^+$ T cell ratio	2.11±0.67	2.32±0.62	0.008
Lymphocyte absolute count (cells/μL)	1672 (495)	881 (363)	<0.001
$CD3^+$ T cell absolute count (cells/μL)	1057 (447)	650 (349)	<0.001
$CD4^+$ T cell absolute count (cells/μL)	673±205	408±158	<0.001
$CD8^+$ T cell absolute count (cells/μL)	312 (146)	189 (145)	<0.001
NK cell absolute count (cells/μL)	198 (242)	95 (113)	<0.001
B cell absolute count (cells/μL)	217 (180)	27 (71)	<0.001

$CD4^+$ T cells from 673±205 to 407±157 cells/μL, $CD8^+$ T cells from 312(146) to 189(145) cells/μL, NK cells from 198(242) to 95(113) cells/μL and B cells from 217(180) to 27(71) cells/μL, with all P value < 0.001 (Figure 4G-L). Subsequently, to screen for potential biomarkers predicting the efficacy of neoadjuvant chemotherapy, we divided patients who underwent neoadjuvant chemotherapy into effective and ineffective groups and compared lymphocyte subsets before and after treatment. As illustrated in Figure 4M-P, there were no significant differences in lymphocyte subsets between the effective and ineffective groups before neoadjuvant chemotherapy. Nevertheless, after chemotherapy, the effective group had higher percentages and absolute counts of $CD8^+$ T cells ($P < 0.001$; $P = 0.015$, respectively), a higher percentage of $CD3^+$ T cells ($P = 0.003$) and a lower percentage of B cells ($P = 0.034$) compared to the ineffective group, suggesting that patients with these peripheral blood lymphocyte subset characteristics may benefit from neoadjuvant chemotherapy.

Comparison of Granzyme B and Perforin Expression in Lymphocyte Subsets of Patients with Breast Cancer and Benign Breast Disease

Cytotoxic T cells and NK cells, characterized by effector molecules such as granzyme B and perforin, have been found to effectively kill cancer cells and serve as important biomarkers for the diagnosis and prognosis of cancer. Therefore, after identifying differences in lymphocyte subsets between patients with breast cancer and those with benign breast conditions, we further investigated variations in effector lymphocytes expressing granzyme B and perforin using flow cytometry. Table 4 and Figure 5A-F presented data showing that the percentages of granzyme B and perforin expression were higher in patients with breast cancer compared to those with benign breast diseases, specifically, the expression of granzyme B was 37.88(16.31) versus 22.91(9.41)% in $CD3^+$ T cells ($P < 0.001$), 56.45(17.27) versus 37.55(16.31)% in $CD8^+$ T cells ($P < 0.001$), 90.37(7.24) versus 86.64(12.28)% in NK cells ($P = 0.020$), respectively; the expression of

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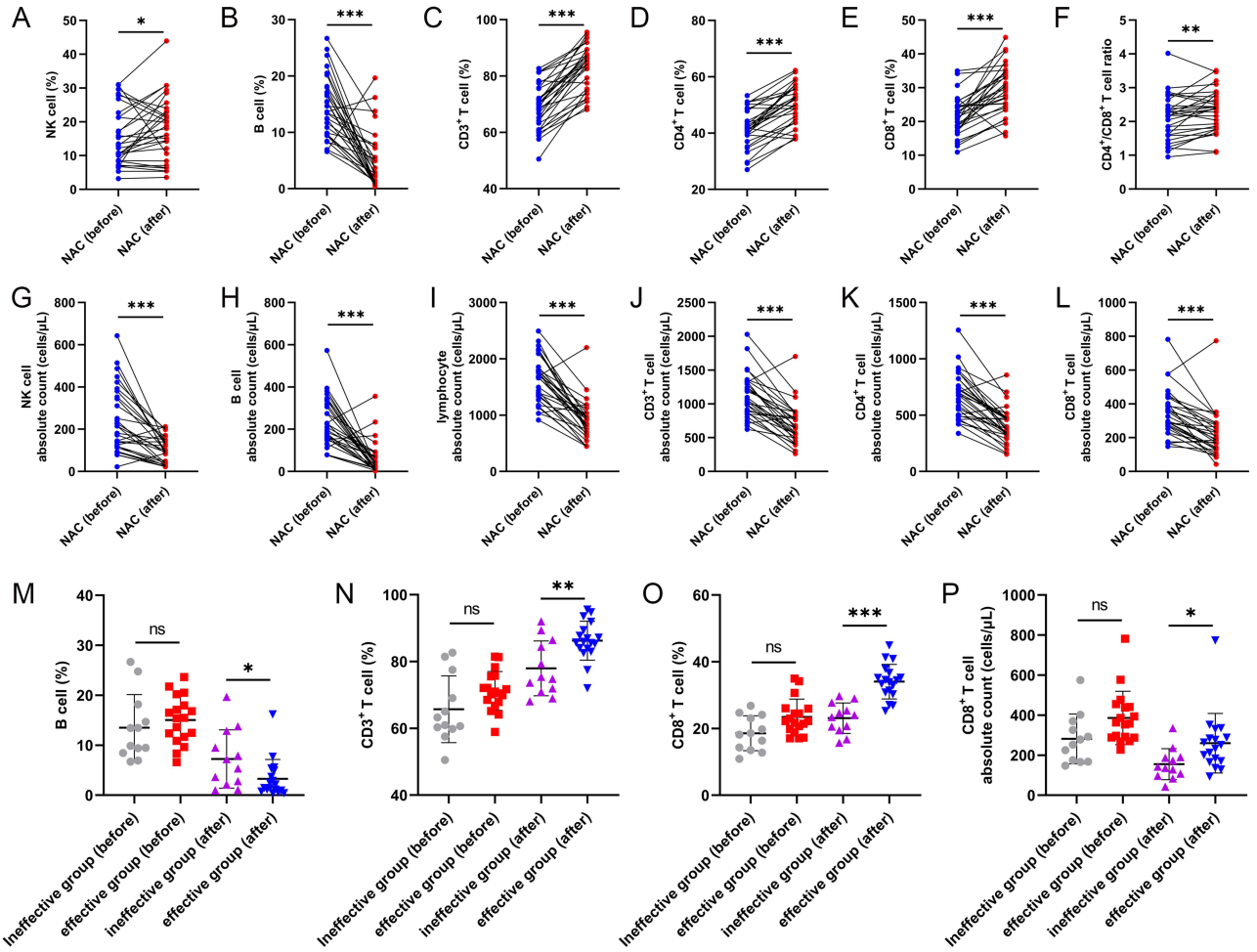

Figure 4 Change of percentage and absolute count of peripheral blood lymphocyte subsets in breast cancer patients before and after neoadjuvant chemotherapy (NAC). (**A**) NK cells percentage; (**B**) B cells percentage; (**C**) $CD3^+$ T cells percentage; (**D**) $CD4^+$ T cells percentage; (**E**) $CD8^+$ T cells percentage; (**F**) $CD4^+/CD8^+$ T cell ratio; (**G**) NK cells absolute count; (**H**) B cells absolute count; (**I**) lymphocyte absolute count; (**J**) CD3+ T cells absolute count; (**K**) CD4+ T cells absolute count; (**L**) CD8+ T cells absolute count; (**M**), (**N**), (**O**), (**P**) Statistically significant differences in lymphocyte subset levels are observed between ineffective (N=12) and effective group (N=18) of breast cancer patients after undergoing NAC, while no differences were evident before NAC. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: $P > 0.05$).
Abbreviation: NAC, neoadjuvant chemotherapy.

perforin was 39.59(15.56) versus 26.32(10.32)% in $CD3^+$ T cells ($P < 0.001$), 57.93(17.05) versus 39.99(15.46)% in $CD8^+$ T cells ($P < 0.001$), 92.54(6.23) versus 90.53(7.16)% in NK cells ($P = 0.039$), respectively. The results indicated that aberrant expression of granzyme B and perforin in lymphocytes may be linked to malignant behavior in breast disease, potentially identifying novel therapeutic targets and diagnostic biomarkers.

Table 4 Comparison of Granzyme B and Perforin Expression in Peripheral Blood Lymphocytes Between Benign Breast Disease and Breast Cancer Patients

Positive rate	Breast cancer N=70	BBD N=70	*P* value
$CD3^+$ GrB^+ T cell (%)	37.88 (16.31)	22.91 (9.41)	<0.001
$CD8^+$ GrB^+ T cell (%)	56.45 (17.27)	37.55 (16.31)	<0.001
GrB^+ NK cell (%)	90.37 (7.24)	86.64 (12.28)	0.020
$CD3^+$ Prf^+ T cell (%)	39.59 (15.56)	26.32 (10.32)	<0.001
$CD8^+$ Prf^+ T cell (%)	57.93 (17.05)	39.99 (15.46)	<0.001
Prf^+ NK cell (%)	92.54 (6.23)	90.53 (7.16)	0.039

Abbreviations: GrB, granzyme B; Prf, perforin; BBD, benign breast disease.

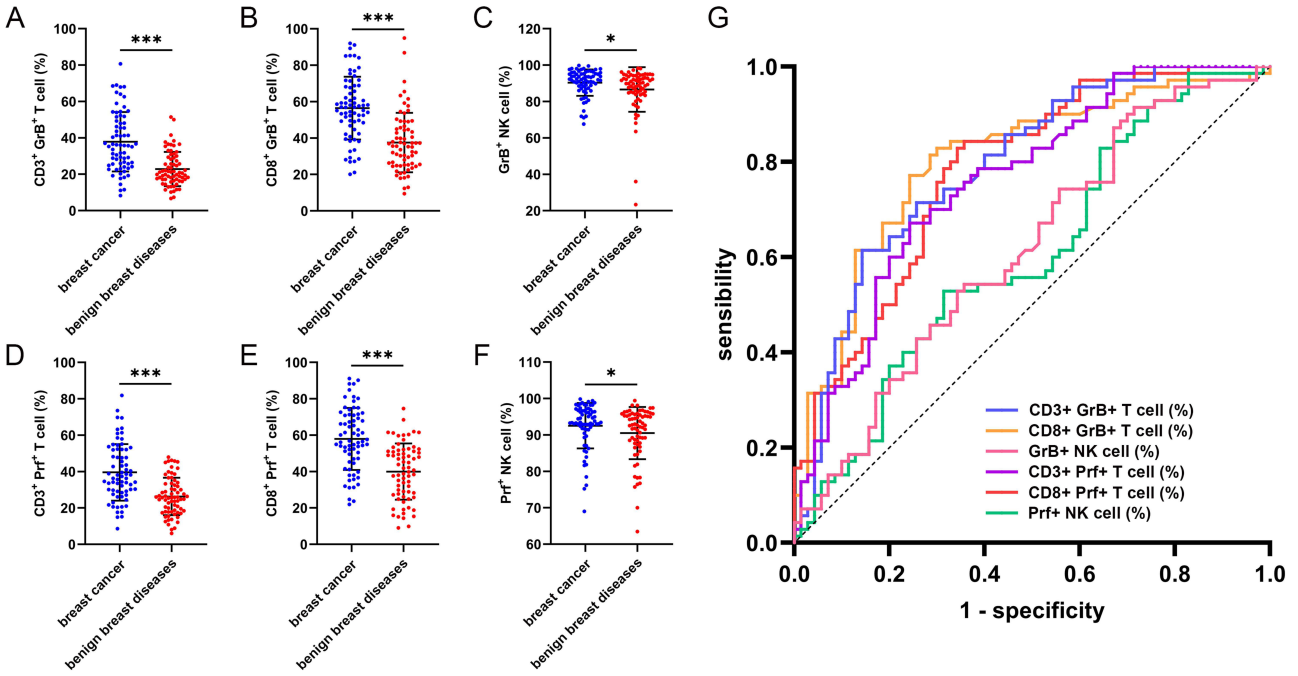

Figure 5 Compare granzyme B and perforin expression in peripheral blood lymphocytes between patients with benign breast disease and breast cancer, and construct ROC curves to evaluate their positive rates in distinguishing malignant breast tumor. (**A**) $CD3^+$ GrB^+ T cell (%); (**B**) $CD8^+$ GrB^+ T cell (%); (**C**) GrB^+ NK cell (%); (**D**) CD3+ Prf^+ T cell (%); (**E**) $CD8^+$ Prf^+ T cell; (%); (**F**) Prf^+ NK cell (%); (**G**) ROC curve of positive rate of granzyme B and perforin of peripheral blood lymphocyte subsets. (*P < 0.05, ***P < 0.001).

Abbreviations: GrB, granzyme B; Prf, perforin.

Diagnostic Significance of Positive Rates of Granzyme B and Perforin in Peripheral Blood Lymphocyte Subsets in Breast Cancer

Currently, the contribution of granzyme B and perforin expression levels in lymphocyte subsets to the diagnosis of malignant breast tumor remains undefined. Based on this, ROC curve analysis was used to assess the diagnostic efficiency of granzyme B and perforin expression in lymphocyte subsets, with results presented in Table 5 and Figure 5G. The area under the curve (AUC) values for positive rates of granzyme B in different lymphocytes were 0.786 in $CD3^+$ T cells (95% CI 0.715–0.864), 0.800 in $CD8^+$ T cells (95% CI 0.725–0.875) and 0.614 in NK cells (95% CI 0.521–0.707), with the ideal cut-off values of 22.38, 49.50 and 95.13%, respectively. In the same way, the AUC values for perforin were quantified as 0.758 in $CD3^+$ T cells (95% CI 0.680–0.837), 0.780 in $CD8^+$ T cells (95% CI 0.704–0.855) and 0.601 in NK cells (95% CI 0.507–0.695), with optimal cut-off values established at 29.05, 53.67 and 92.80%, respectively. Notably, the AUC value for granzyme B in $CD8^+$ T cells was the highest, with a Youden index of 0.529, indicating that it may exhibit the best performance for diagnosing malignancy.

Table 5 Diagnostic Value of Positive Rate of Granzyme B and Perforin of Peripheral Blood Lymphocyte Subsets in Distinguishing Benign and Malignant Breast Tumor

Positive rate	Sensibility (%)	Specificity (%)	*P* value	AUC	95% CI	Youden Index	Cut-off Value
$CD3^+$ GrB^+ T cell (%)	61.4	85.7	<0.001	0.786	0.715–0.864	0.471	22.38
$CD8^+$ GrB^+ T cell (%)	82.9	70.0	<0.001	0.800	0.725–0.875	0.529	49.50
GrB^+ NK cell (%)	91.4	28.6	0.020	0.614	0.521–0.707	0.200	95.13
$CD3^+$ Prf^+ T cell (%)	67.1	75.7	<0.001	0.758	0.680–0.837	0.428	29.05
$CD8^+$ Prf^+ T cell (%)	84.3	64.3	<0.001	0.780	0.704–0.855	0.486	53.67
Prf^+ NK cell (%)	52.9	68.6	0.039	0.601	0.507–0.695	0.215	92.80

Abbreviations: GrB, granzyme B; Prf, perforin.

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Dynamic Changes of Positive Rate of Granzyme B and Perforin in Breast Cancer Patients Before and After Chemotherapy

Granzyme B and perforin served as indicators of anti-tumor immune activity following immune checkpoint inhibitor therapy, with elevated levels frequently correlating with clinical benefit and favorable prognosis. However, the relationship between granzyme B and perforin expression and anti-tumor immunity in breast cancer patients receiving postoperative chemotherapy remained unclear. In this study, we assessed the changes in peripheral blood levels of granzyme B and perforin in 47 breast cancer patients undergoing adjuvant chemotherapy. As shown in Figure 6, Chemotherapy resulted in increased expression of granzyme B and perforin in T lymphocyte subsets, with granzyme B levels in $CD3^+$ T cells rising from 21.51(13.69) to 29.23(15.66)% ($P < 0.001$) and in $CD8^+$ T cells from 40.10(20.81) to 52.87(15.92)% ($P < 0.001$), while perforin levels increased in $CD3^+$ T cells from 23.72(11.48) to 32.06(13.37)% ($P = 0.003$) and in $CD8^+$ T cells from 42.86(25.03) to 53.31(19.29)% ($P = 0.018$), respectively. Additionally, no significant changes of granzyme B and perforin were observed in NK cells ($P > 0.05$). These findings suggested that chemotherapy might promote the activation of anti-tumor immunity in breast cancer patients.

Discussion

The maintenance of a normal immune state relies on the dynamic equilibrium and coordination of various immune cells, particularly the subsets of peripheral blood lymphocytes, which collectively support normal immune function, especially

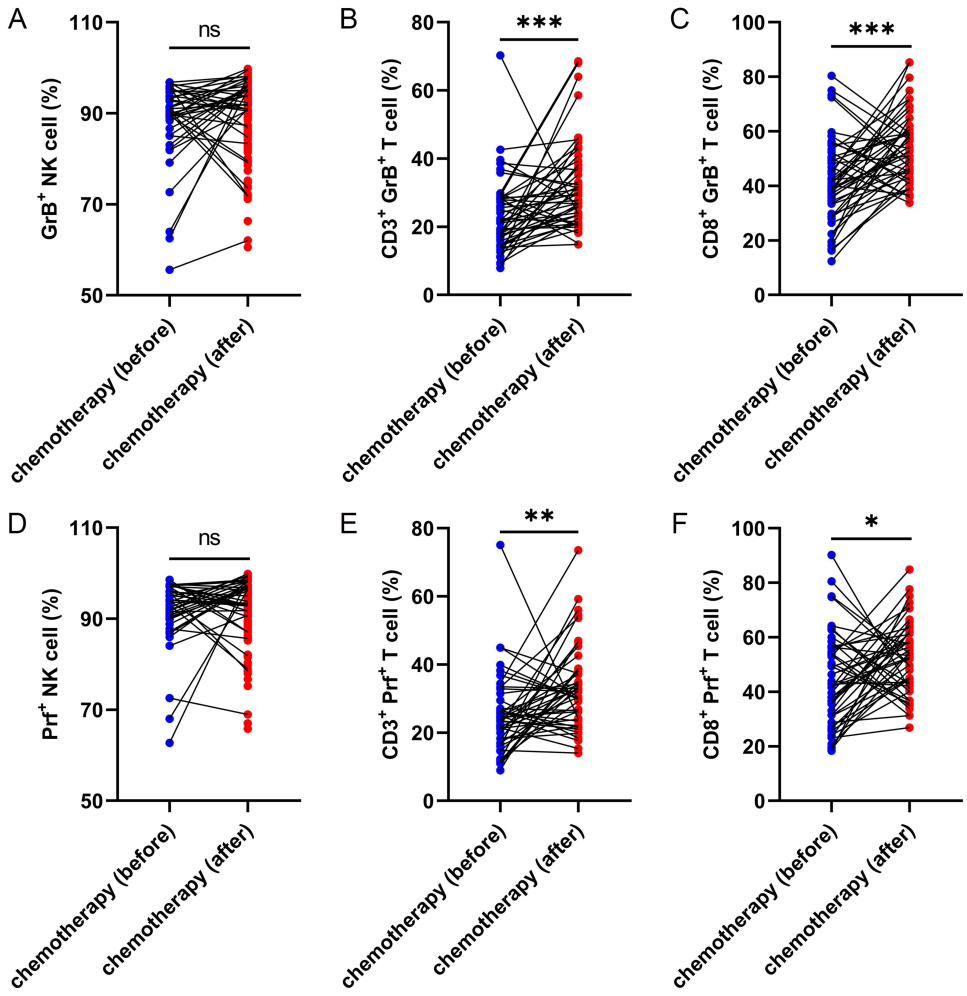

Figure 6 Changes in the positive rates of granzyme B and perforin in peripheral blood lymphocyte subsets among breast cancer patients before and after postoperative (adjuvant) chemotherapy. (**A**) GrB^+ NK cell (%); (**B**) $CD3^+$ GrB^+ T cell (%); (**C**) $CD8^+$ GrB^+ T cell (%); (**D**) Prf^+ NK cell (%); (**E**) $CD3^+$ Prf^+ T cell (%); (**F**) $CD8^+$ Prf^+ T cell (%).(*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: $P > 0.05$).

Abbreviations: GrB, granzyme B; Prf, perforin.

in the context of anti-tumor immunity.[23–26] In immune suppression states, such as those induced by immunosuppressive agents during the transplantation period or by pathological conditions like primary immunodeficiencies (PIDs), disrupting the immune equilibrium significantly increases cancer risk, underscoring the complex relationship between immune responses and the onset, progression, metastasis and prognosis of malignancies.[27–30] Numerous studies have confirmed that lymphocyte subsets are effective for assessing immune function status and serve as crucial diagnostic, therapeutic and prognostic biomarkers, underscoring their significant impact in clinical practice.[31–35]

Peripheral blood lymphocytes, categorized into distinct subsets based on various cluster differentiation antigens, maintain normal immune functions through dynamic changes within their physiological range and complex interactions between cells.[36–38] CD3 antigen, a surface marker present on mature T lymphocytes, is a universal indicator for assessing the normal range of T lymphocytes and the efficacy of cellular immune responses.[39] Mature T lymphocytes are further distinguished into $CD4^+$ T helper cells and $CD8^+$ T cytotoxic cells according to the differential expression of CD4 or CD8 antigens on their surface.[40] From a functional perspective, $CD4^+$ T cells are capable of directly inducing the death of tumor cells by secreting cytokines such as IFN-γ and TNF-α, or indirectly exerting anti-tumor immunity by activating innate immune cells like NK cells or by reducing tumor angiogenesis.[41–43] $CD8^+$ T cells eliminate tumor cells by secreting granzymes and perforin, and they also possess the capacity to differentiate into T cytotoxic lymphocytes that directly mediate anti-tumor responses.[44] The $CD4^+/CD8^+$ T cells ratio serves as an indicator of the dynamic equilibrium within T cell subsets, where a reduced ratio often indicates immune dysregulation, particularly in anti-tumor immunity, whereas a normal ratio is crucial for sustaining immune homeostasis.[45] Studies have shown that in breast cancer, the percentage of peripheral blood $CD8^+$ T cells is reduced while the percentage of $CD4^+$ T cells is increased, leading to an elevated $CD4^+/CD8^+$ ratio,[46] which is consistent with our results demonstrating that despite unchanged percentages and absolute counts of $CD4^+$ T cells, the significant decrease in $CD8^+$ T cells results in an increased $CD4^+/CD8^+$ ratio. It is important to note that while tumor development is typically linked to immune suppression, evidenced by a reduced $CD4^+/CD8^+$ ratio, an elevated $CD4^+/CD8^+$ ratio may indicate an early clinical stage of the tumor, whereas a rise in $CD8^+$ T cells, coupled with a reduced $CD4^+/CD8^+$ ratio, may signify progression to an advanced stage.[47,48]

Additionally, previous studies have demonstrated that chemotherapy regulates anti-tumor immunity in cancer patients by remodeling the immune microenvironment.[49,50] Since evaluating NAC efficacy mainly relies on post-treatment imaging or pathological assessments, which may delay the identification of resistant patients and worsen outcomes, there is an urgent need for a rapid, simple biomarker to predict chemotherapy efficacy, guide treatment decisions, and extend overall survival.[51] Therefore, we conducted a prospective cohort study on 30 breast cancer patients receiving NAC and found that NAC reduced absolute counts of peripheral blood lymphocyte subsets but increased the percentages of $CD3^+$, $CD4^+$, $CD8^+$T cells, and NK cells, while decreasing the percentage of B cells. This may be due to the NAC agent inducing bone marrow suppression, which decreases absolute lymphocyte counts, while simultaneously reducing tumor burden and tumor cell-mediated immune suppression, thereby potentially activating the immune system and increasing the percentages of $CD3^+$, $CD4^+$, $CD8^+$ T cells, and NK cells, a result that is supported by data from research conducted by Zhang et al.[52] Moreover, despite no differences in baseline lymphocyte subset percentages or absolute counts between the effective and ineffective groups prior to NAC, post-treatment analysis revealed that the effective group had increased percentages of $CD3^+$ and $CD8^+$ T cells, an increased absolute count of $CD8^+$ T cells, and a decreased percentage of B cells compared to the ineffective group, in line with findings from Meng et al,[53] indicating that baseline lymphocyte subset status may not predict NAC efficacy, thereby underscoring the need for dynamic and multiple assessments of lymphocyte subset changes to accurately predict NAC outcomes.

Granzyme B, in conjunction with perforin, is crucial for NK and CTL-mediated tumor cell apoptosis, with co-expression enhancing cytotoxicity.[31,54,55] However, there is a paucity of research on perforin and granzyme B in the diagnostic context of malignant breast tumors. In our study, we found significantly elevated levels of granzyme B in peripheral blood $CD8^+$ T cells and both granzyme B and perforin in NK cells in breast cancer patients compared to those with benign breast conditions, indicating that tumor cells activate the cytotoxic response of T cells and NK cells, leading to increased levels of granzyme B and perforin that induce tumor cell apoptosis. Similarly, consistent with our findings, increased expression of perforin and granzyme B in peripheral blood NK cells has been reported in ovarian cancer patients.[56] Given that the interplay between treatment and the immune microenvironment is reciprocal, current biomarkers for assessing anti-tumor immune function

following treatment remain inadequate. While the expression levels of granzyme B and perforin have been employed to assess anti-tumor immunity following immunotherapy in NSCLC,[22] it remains unclear whether changes in these markers accurately reflect anti-tumor immunity in breast cancer patients after chemotherapy. Our findings, in alignment with data from Mazzaschi et al,[22] demonstrated that chemotherapy in breast cancer patients leads to an increase in the percentage of granzyme B and perforin in $CD3^+$ and $CD8^+$ T cells, suggesting a significant enhancement of cytotoxic T cell anti-tumor immunity and highlighting the potential of granzyme B and perforin as more precise and readily detectable biomarkers in clinical practice. However, the clinical application of granzyme B and perforin as diagnostic biomarkers faces several challenges. Assay standardization is a major issue, as variations in detection methods and protocols can affect reproducibility. Additionally, the cost of these assays may limit their widespread use in routine clinical practice, especially for large-scale screenings. Moreover, individual immune variability and complex cell interactions complicate the interpretation of these biomarkers, necessitating a multifaceted approach to diagnosis and monitoring. These challenges must be addressed for granzyme B and perforin to be reliably integrated into clinical practice for breast cancer diagnosis and treatment assessment.

Nevertheless, this study had several limitations. Firstly, all data were derived from a single-center study that also lacked prognostic data, thus necessitating larger multi-center studies and long-term follow-up to collect survival data to support and validate the impacts of lymphocyte subsets, granzyme B and perforin in diagnosing malignancies, predicting the efficacy of NAC, and assessing prognosis. Secondly, the data from breast cancer patients receiving chemotherapy were obtained from prospective cohorts with insufficient sample sizes for further analysis based on different chemotherapy regimens, tumor stages and comorbidities, requiring larger sample sizes for validation and exploration. Finally, although lymphocyte subsets, granzyme B, and perforin demonstrated non-invasive and reproducible properties in breast cancer diagnosis, their diagnostic efficacy remained inadequate to fully guide clinical decisions, making it essential to incorporate other novel factors, such as radiomics data and additional peripheral blood indicators, into the model to enhance diagnostic performance. Notably, our long-term goal is to develop a comprehensive model that integrates immune characteristics, tumor gene expression profiles, and patient clinicopathological features to more accurately diagnose malignancies and predict treatment efficacy.

Conclusion

In summary, our work analyzed the differences in lymphocyte subsets, granzyme B and perforin expression in the peripheral blood of breast cancer patients versus those with benign breast diseases, and constructed ROC curves to assess the diagnostic efficacy for malignant breast diseases. This provides valuable insights for clinical oncologists to assess the anti-tumor immunity and predict the efficacy of chemotherapy in breast cancer patients.

Data Sharing Statement

Data are available from corresponding author upon reasonable request.

Ethics Approval and Informed Consent

The research, approved by the Central People's Hospital of Zhanjiang's ethics committee (approval No. PJ[IIT-2021-007-02]), adhered to the World Medical Association Declaration of Helsinki. All participants provided informed consent according to the committee's guidelines.

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Disclosure

The authors have declared that no competing interests exist.

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