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ORIGINAL ARTICLE

T-helper Transcription Factor Profiling in Peripheral Blood Mononuclear Cells: A Non-invasive Approach to Predicting Disease Stage in Breast Cancer

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Abstract

Breast cancer remains a leading cause of mortality among women, highlighting the need for improved diagnostic and treatment approaches. This study aims to analyze the expression levels of key immunologic factors in the peripheral blood mononuclear cell (PBMC) population of breast cancer patients and assess their relationship with various disease characteristics. A total of 48 treatment-naive breast cancer patients were enrolled, with blood samples collected prior to surgery for PBMC isolation. Gene expression of Foxp3, RORγt, GATA3, and T-bet was measured using quantitative real-time PCR. Gene expressions of Foxp3, RORγt, and GATA3 were significantly elevated in breast cancer patients compared to controls. Logistic regression revealed a strong association between elevated RORγt levels and larger tumor sizes. Subgroup analysis indicated that Foxp3 related to lymphovascular invasion (LVI), RORγt correlated with lymph node involvement and tumor size, GATA3 was associated with tumor size alone, and T-bet was linked to disease stage. ROC analysis demonstrated T-bet and Foxp3 as sensitive indicators for disease stage, while RORγt was notable for lymph node involvement. The study indicates that T-helper cell-related transcription factors in PBMCs reflect important clinical characteristics of breast cancer, supporting the role of T cell immune responses in disease progression. PBMCs emerge as a promising and accessible resource for diagnostic information in breast cancer.

Extended author information available on the last page of the article

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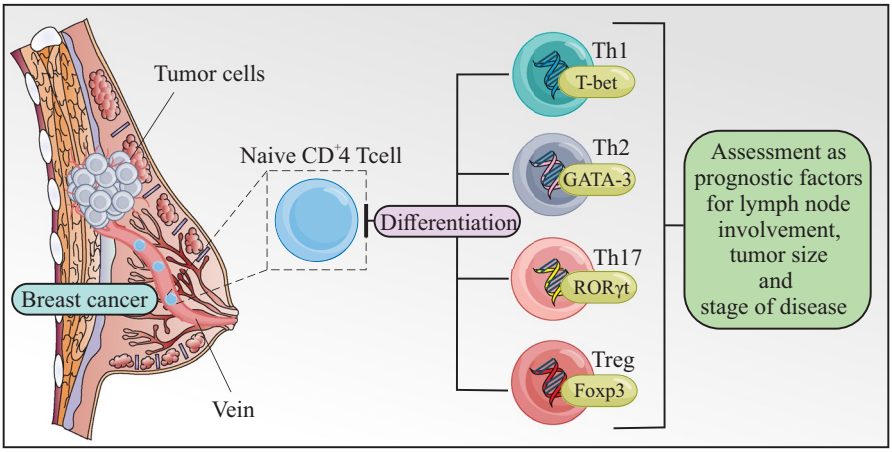
Graphical Abstract



Keywords Breast cancer · PBMC · Foxp3 · RORγt · GATA3 · T-bet

Introduction

Breast cancer is the most prevalent malignancy among women worldwide, representing a significant public health challenge due to its complex biology and varied clinical outcomes.[1] Immunological factors play a crucial role in tumor progression, particularly through the mechanisms of cell-mediated immunity. Recent research has highlighted the importance of T helper cell (Th) differentiation and its impact on the tumor microenvironment (Cardoso et al. 2019; Abbasi-Dokht et al. 2024; Nafissi et al. 2022).

CD4+T cells are critical components of the immune response, differentiating into several subtypes, notably Th1, Th2, Th17, and regulatory T cells (Tregs). Each of these subsets exerts distinct effects on tumor behavior (Gil Del Alcazar et al. 2017; Baharlou et al. 2016). Th1 cells are known for their anti-tumoral properties, enhancing the activity of cytotoxic T cells and promoting strong immune responses against tumors. They express key cytokines, including interferon-gamma (IFN-γ), and their development is regulated by the transcription factor T-box transcription factor TBX21 (T-bet) (Mullen et al. 2001). Conversely, Th2 cells, induced by GATA-binding protein 3 (GATA-3), tend to promote immune suppression and may facilitate tumor growth (Yagi et al. 2011; Takaku et al. 2015). The balance between Th1 and Th2 cells is often disrupted in cancer, leading to an immunosuppressive

[1] Global Cancer Observatory. International Agency for Research on Cancer, Lyon, France. https://gco.iarc.fr/ (accessed on 1 August 2024).

environment that favors tumor progression (de Visser and Joyce 2023; Xiao et al. 2023).

Th17 cells, regulated by retinoid orphan nuclear receptor gamma t (RORγt), have also garnered attention in cancer research due to their pro-inflammatory effects (Chen et al. 2007). Their exact role in breast cancer remains complex, as they can exhibit both tumor-promoting and anti-tumor properties depending on the tumor context (Marques et al. 2021). Meanwhile, Tregs, which develop through the activation of the Forkhead Box Protein P3 (Foxp3) transcription factor, are critical for maintaining immune tolerance but can hinder anti-tumor immunity and contribute to tumor progression (Hashemi et al. 2020).

The expression levels of these key transcription factors—Foxp3, RORγt, GATA3, and T-bet—in peripheral blood mononuclear cells (PBMCs) may provide insights into the immune response in breast cancer. Previous studies have primarily focused on tumor tissue analyses, but PBMCs offer a less invasive and potentially informative alternative for assessing immune status and tumor dynamics (Goto et al. 2018).

This study aims to investigate the effects of breast carcinoma on the differentiation of CD4+ T cells into the aforementioned subtypes. By analyzing the expression profiles of Foxp3, RORγt, GATA3, and T-bet in PBMCs from breast cancer patients, we seek to understand how these transcription factors correlate with important clinical parameters such as lymph node involvement, tumor size, and disease stage. The findings from this study could provide valuable insights into the role of immune cell differentiation in breast cancer and highlight potential biomarkers for predicting disease outcomes.

Materials and Methods

Patient Selection

In the present study, a cohort was selected from 115 breast cancer patients admitted to Khatam Al-Anbiya Hospital in Tehran, Iran, between April 2023 and March 2024. All patients underwent standard imaging techniques, including mammography and ultrasound. To confirm and classify their cancer, core needle biopsies were performed, evaluating key markers such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Ki-67 indices.

The criteria for exclusion included: (1) previous receipt of neoadjuvant therapy, (2) history of other malignancies, (3) presence of rheumatologic diseases, (4) use of immunosuppressive medications, and (5) any history of infection within the past two months. During the recruitment process, 67 patients were excluded due to one or more of these criteria, or due to inconclusive or negative biopsy findings. Consequently, 48 patients were selected to participate in the study. Additionally, a control group consisting of 20 age-matched healthy women was also recruited (Fig. 1). Comprehensive data regarding the study population is presented in Table 1.

The study protocol, including all associated procedures, was reviewed and approved by the Ethics Committee of Iran University of Medical Sciences. All

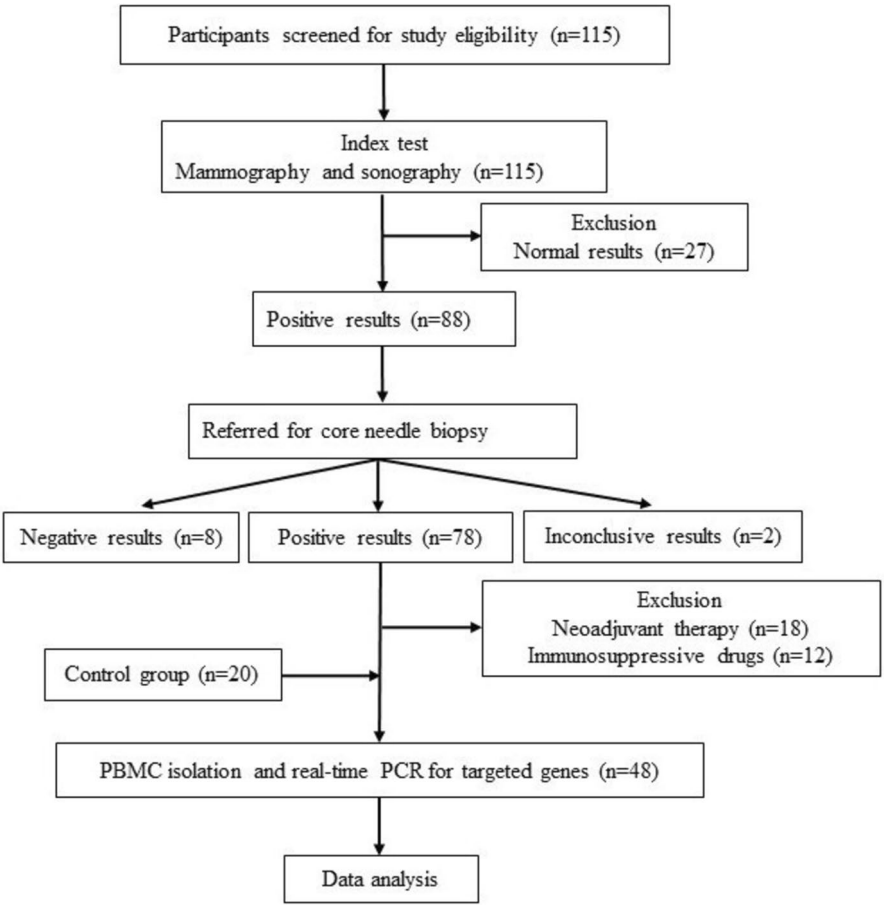


Fig. 1 The patient flowchart outlines the identification and enrollment process for eligible patients with newly diagnosed breast cancer. Patients were enrolled based on index test results and treatment history. Those with negative or inconclusive index test results were excluded, while patients with positive results underwent further investigation via realtime PCR

participants were fully informed about the study's procedures and provided written consent in accordance with ethical guidelines (IR.IUMS.REC.1402.540).

PBMC Isolation

To achieve this, a 10 mL venous blood sample was collected from patients (post-diagnosis and prior to their scheduled surgery) as well as from the control group, using heparinized tubes. PBMCs were isolated using Ficoll Isopaque (Pars Azmaye Teb, Iran). Specifically, the collected blood sample was diluted with an equal volume (1:1 ratio) of phosphate-buffered saline (PBS, pH 7.4), which was

Table 1 Demographic, clinical, and laboratory characteristics of breast cancer patients

Variables	Patients ($n=48$)	Variables	Patients ($n=48$)
Age	54.4 ± 11	Multicenteric tumor (%)	
Tumor side (%)		Negative	45 (93.8)
Right	22 (45.8)	Positive	3 (6.3)
Left	23 (47.9)	Vascular invasion (%)	
Both	3 (6.3)	Negative	24 (50.0)
Family history (%)		Positive	24 (50.0)
Negative	38 (79.2)	Perineural invasion (%)	
Positive	10 (20.8)	Negative	29 (60.4)
Histology (%)		Positive	19 (39.6)
IDC	42 (87.5)	Calcification (%)	
ILC	5 (10.4)	Negative	34 (70.8)
Other types	1 (2.1)	Positive	14 (29.2)
ER (%)		Necrosis (%)	
Negative	4 (8.3)	Negative	30 (62.5)
Positive	44 (91.7)	Positive	18 (37.5)
PR (%)		Sentinel (%)	
Negative	8 (16.7)	Free	19 (39.6)
Positive	40 (83.3)	Positive	7 (14.6)
HER-2 (%)		Not sentinel	22 (45.8)
Negative	43 (89.6)	Pathological T stage (%)	
Positive	5 (10.4)	T1 (≤ 2 cm)	29 (60.4)
Ki67% (%)		T2 (> 2 cm, ≤ 5 cm)	19 (39.6)
Low (≤ 15%)	31 (64.6)	Pathological N stage (%)	
Moderate (15–30%)	8 (16.7)	N0	25 (52.1)
High (> 30%)	4 (8.3)	N1 (1–3)	18 (37.5)
%DCIS (%)		N2 (4–9)	3 (6.3)
Negative	20 (41.7)	N3 ≥ 10	2 (4.2)
Positive	28 (58.3)	TNM stage (%)	
Tumor grade (%)		I	21 (43.8)
I (well differentiation)	5 (10.4)	II	21 (43.8)
II (mod differentiation)	38 (79.2)	III	6 (12.5)
III (poor differentiation)	5 (10.4)		

Values are presented as mean ± standard deviation or number (%)

DCIS ductal carcinoma in situ; *IDC* Invasive ductal carcinoma; *ILC* Invasive lobular carcinoma; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2; *TNM* tumor, node and metastasis

then gently added to the separation media. The samples underwent centrifugation at 600×*g* for 20 min, after which the PBMC layer that formed was carefully harvested and washed with PBS. TRIzol (Sinaclon, Iran) was added to the isolated PBMCs, which were then stored at – 80 °C for future experiments.

RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA extraction was conducted using the phenol–chloroform method. In brief, 200 µL of chloroform was added to the isolated PBMCs. After briefly vortexing the mixture, the tubes were placed on ice for 5 min to facilitate phase separation. The supernatant was carefully transferred to a new tube, and cold isopropanol was added to precipitate the RNA. Following a 15-min incubation on ice, the samples were centrifuged, and the supernatant was discarded.

Subsequently, 1 mL of cold 75% ethanol was added to the RNA pellet, which was then centrifuged for 8 min at a speed of 12,000×*g*. The RNA pellet was left to air dry at room temperature before being resuspended in 40 µL of diethylpyrocarbonate (DEPC) treated water.

The quantity and quality of the extracted RNA were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). The isolated RNA was then utilized for cDNA synthesis, employing a cDNA synthesis kit (Parstous, Iran). Gene expression was quantified with a Rotor-Gene Q thermal cycler (Qiagen, Germany) using SYBR Green PCR Master Mix (Ampliqon, Denmark). A complete list of the primers used for this experiment—including those for Foxp3, GATA3, T-bet, RORγt, and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—is provided in Table 2. Finally, the relative expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method (Abbasi-Dokht et al. 2023). Further details are provided in supplementary data.

Statistical Analysis

The data in the current study are presented as frequency (percentage) or mean ± SEM. To determine differences in gene expression between the patient and control groups, the distributions of data were initially assessed using the Kolmogorov–Smirnov test. Based on the results, statistical analysis was conducted using the two-tailed Mann–Whitney *U* test. To explore the relationship between gene expression and various clinical manifestations in the patient group, both univariate and

Table 2 Forward and reverse primers of GAPDH, Foxp3, RORγt, GATA3, and T-bet genes for real-time PCR amplification

Gene	Sequence (5′ → 3′)
GAPDH Forward	CAAATTCCATGGCACCGTCA
GAPDH Reverse	GACTCCACGACGTACTCAGC
Foxp3 Forward	AAGTTCCACAACATGCGACC
Foxp3 Reverse	AAGGCAAACATGCGTGTGAA
RORγt Forward	GTGCCTTGCACTTTTCCGAG
RORγt Reverse	GCTTTGGCGATGAGTCTTGC
GATA3 Forward	CAGCATGAAGCTGGAGTCGT
GATA3 Reverse	GTAGTGTCCCGTGCCATCTC
T-bet Forward	CAAGGGGGCGTCCAACAAT
T-bet Reverse	TCTGGCTCTCCGTCGTTCA

multivariate binary logistic regression analyses were performed. Additionally, receiver operating characteristic (ROC) curve analyses were employed to evaluate the predictive potential of the studied transcription factors for different clinical indices of breast cancer. All statistical tests were carried out using IBM SPSS Statistics (Version 27) and GraphPad Prism (Version 9), with a p-value of < 0.05 considered statistically significant.

Results

Clinical and Pathological Characteristics Modulate the Gene Expression Patterns Associated with T Cell Differentiation

The findings indicate that PBMCs from breast cancer patients exhibited significantly elevated expression levels of the Foxp3 (Fig. 2A), RORγt (Fig. 2B), and GATA3 (Fig. 2C) genes in comparison to PBMCs obtained from healthy individuals ($p < 0.05$ for all). Notably, the expression of the T-bet gene did not demonstrate any significant differences between the two groups (Fig. 2D).

Further investigation into gene expression across different patient subgroups revealed that Foxp3 levels were substantially higher in patients exhibiting lymphovascular invasion (LVI) as opposed to those without LVI ($p < 0.05$). A comparable trend was observed when comparing patients with lymph node involvement to those without ($p < 0.05$) (Fig. 3A). Conversely, the expression of RORγt was significantly reduced in patients with lymph node involvement relative to their counterparts without such involvement ($p < 0.05$). Furthermore, patients presenting with larger tumor sizes exhibited lower RORγt expression when contrasted with those having smaller tumors ($p < 0.05$) (Fig. 3B). Similarly, GATA3 expression followed a parallel trend, with significantly reduced levels in patients with larger tumors ($p < 0.05$) (Fig. 3C). Notably, variations in T-bet expression were primarily linked to the disease stage, as patients with advanced-stage tumors displayed markedly decreased levels ($p < 0.05$) (Fig. 3D).

CD4+ T Cell-Specific Transcriptional Signatures have the Potential to Predict Tumor Size and Stage of Disease in Breast Cancer Patients

Logistic regression analysis was used to investigate the association between gene expression variables and pathological N stage, T stage, and TNM stage in breast cancer patients. Results showed that while in univariate analysis only GATA3 was associated with tumor size, in multivariate analysis, after taking into consideration the gene expression of all factors together, Foxp3, RORγt and GATA3 all showed a significant association (OR 0.91, $p = 0.01$; OR 5.75, $p = 0.03$; OR 0.96, $p = 0.02$; for Foxp3, RORγt and GATA3, respectively). Moreover, T-bet gene expression showed a negative relationship with tumor stage (OR 0.84, $p = 0.04$). None of the factors showed any significant association with lymph node involvement (Table 3).

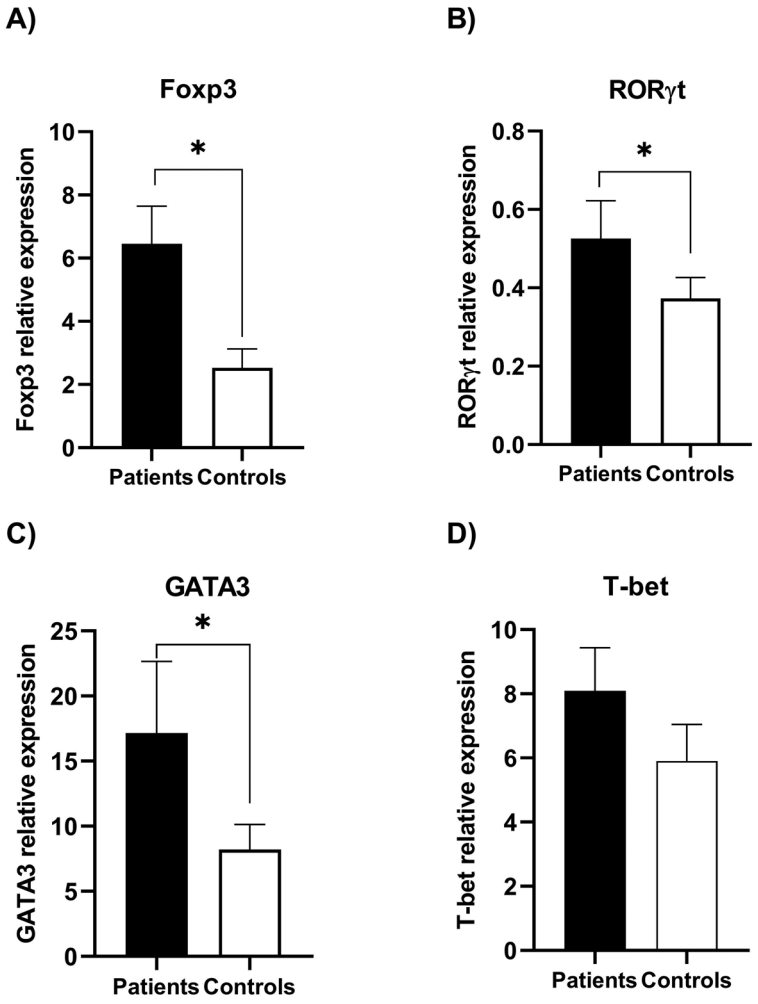


Fig. 2 The gene expression of Foxp3 (**A**), RORγt (**B**), GATA3 (**C**) and Tbet (**D**) in PBMCs of patients with breast cancer in compared to heathy controls. Results were analyzed with nonparametric two-tailed Mann–Whitney *U* test. Values are the mean ± SEM; $*p < 0.05$

ROC curve analysis was conducted to assess the diagnostic performance of Foxp3, RORγt, GATA3, and T-bet in predicting lymph node involvement, tumor size, and stage in breast cancer patients. Analyzing the gene expression data revealed that RORγt has potential diagnostic value to differentiate of lymph node involvement (AUC = 0.62, $p = 0.04$) (Fig. 4A). Additionally, Foxp3 gene expression levels showed potential in predicting the size of the tumor (AUC = 0.62, $p < 0.05$) (Fig. 4B). Furthermore, both Foxp3 and T-bet demonstrated sensitivity in predicting the stage of the cancer (AUC = 0.76, $p = 0.002$ and AUC = 0.68, $p = 0.04$ for Foxp3 and T-bet, respectively) (Fig. 4C).

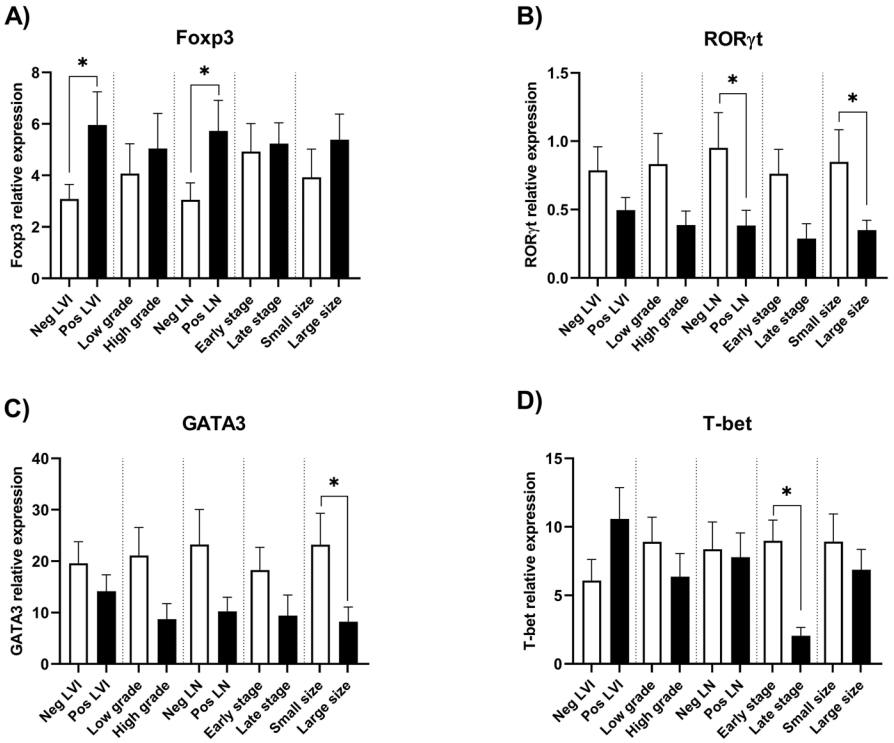


Fig. 3 The gene expression of Foxp3 (**A**), RORγt (**B**), GATA3 (**C**), and Tbet (**D**) in PBMCs of patients with breast cancer was analyzed across different clinicopathological parameters. Results were analyzed with nonparametric two-tailed Mann–Whitney *U* test. Values are the mean ± SEM; $*p < 0.05$

Discussion

Recent progress in cancer therapies largely stems from extensive research into the structure and interactions within the tumor microenvironment (TME). While direct analysis of tumor tissue yields valuable insights, it often overlooks crucial systemic and immune responses that play a significant role in cancer progression. Studies show that the TME can influence distant tissues and organs, as well as circulating immune cells, setting up a supportive system for tumor development (González-Silva et al. 2020; Klemm et al. 2020). In this context, PBMCs emerge as a promising and relatively underexplored alternative for studying these systemic alterations. Investigating PBMCs can provide a broader understanding of the pathological mechanisms across various cancer types. PBMCs house most immune cells responsible for the body's response to cancer, with T helper cells being particularly crucial as they regulate adaptive immune responses.

In our current study, we examined the gene expression levels of four pivotal transcription factors—T-bet, GATA3, RORγt, and Foxp3—within the PBMCs of breast cancer patients. Our findings revealed a significant upregulation of GATA3, RORγt,

Table 3 Logistic regression analysis of PBMCs-associate variables to predict pathological N stage, T stage and TNM stage

	Univariate analysis			Multivariate analysis		
	B	*p*	OR (95% CI)	B	*p*	OR (95% CI)
LN involvement (neg vs. pos)						
Foxp3	−0.01	0.09	0.98 (0.97–1.01)	−0.01	0.09	0.98 (0.97–1.01)
RORγt	−0.28	0.10	0.75 (0.53–1.06)	–	–	
GATA3	−0.01	0.06	0.98 (0.97–1.01)	–	–	–
Tbet	−0.01	0.79	0.99 (0.97–1.02)	–	–	–
Tumor size (T1 vs. T2)						
Foxp3	−0.03	0.10	0.96 (0.91–1.01)	**−0.09**	**0.01**	**0.91 (0.84–0.98)**
RORγt	−0.33	0.11	0.71 (0.48–1.10)	**1.75**	**0.03**	**5.75 (1.18–27.85)**
GATA3	**−0.01**	**0.03**	**0.98 (0.96–0.99**	**−0.35**	**0.02**	**0.96 (0.93–0.99)**
Tbet	−0.01	0.36	0.98 (0.96–1.01)	–	–	–
Stage (early vs. late)						
Foxp3	−0.20	0.15	0.81 (0.61–1.1)	–	–	–
RORγt	−0.39	0.31	0.67 (0.30–1.46)	–	–	–
GATA3	−0.01	0.36	0.99 (0.97–1.01)	–	–	–
Tbet	**−0.16**	**0.04**	**0.84 (0.71–0.99)**	**−0.16**	**0.04**	**0.84 (0.71–0.99)**

Bold significant p-values (typically <0.05) in the logistic regression analysis

OR odds ratio; *LN* lymph node; *Neg* negative; *Pos* positive

and Foxp3 in the breast cancer cohort. Notably, these three transcription factors correlated with tumor size, while T-bet was linked to tumor staging. Additionally, some of the transcription factors exhibited potential diagnostic utility regarding lymph node involvement and tumor size and stage.

In immune cells, T-bet directly promotes the transcription of key genes such as IFN-γ and CXC-chemokine receptor (CXCR) 3, which are integral to the Th1 immune response—this pathway is vital for activating macrophages and CD8+T cells (Miller and Weinmann 2010). Study involving patients with triple-negative breast cancer (TNBC) found T-bet expressed in tumor-infiltrating lymphocytes in approximately 25% of cases, with a positive correlation to CD8 expression. Increased T-bet levels also correlated with improved patient survival, suggesting its potential as a prognostic factor (Mori et al. 2019). Similarly, another study of breast cancer patients indicated that T-bet overexpression in tumor tissue was associated with a favourable prognosis despite indicators of more adverse clinical characteristics like higher histological grade and larger tumor size (Mulligan et al. 2016). T-bet's role has also been explored in the context of response to therapies such as trastuzumab and taxanes, which have been shown to promote a T-bet-driven Th1 immune response and enhance patient survival rates (Ladoire et al. 2011). While research generally emphasizes T-bet's activity in immune cells, evidence suggests that T-bet may also be expressed in breast cancer tissue, potentially influencing tumor growth and correlating with poorer prognosis, while expression in immune infiltrates may signal a better outlook (Yu et al. 2014).

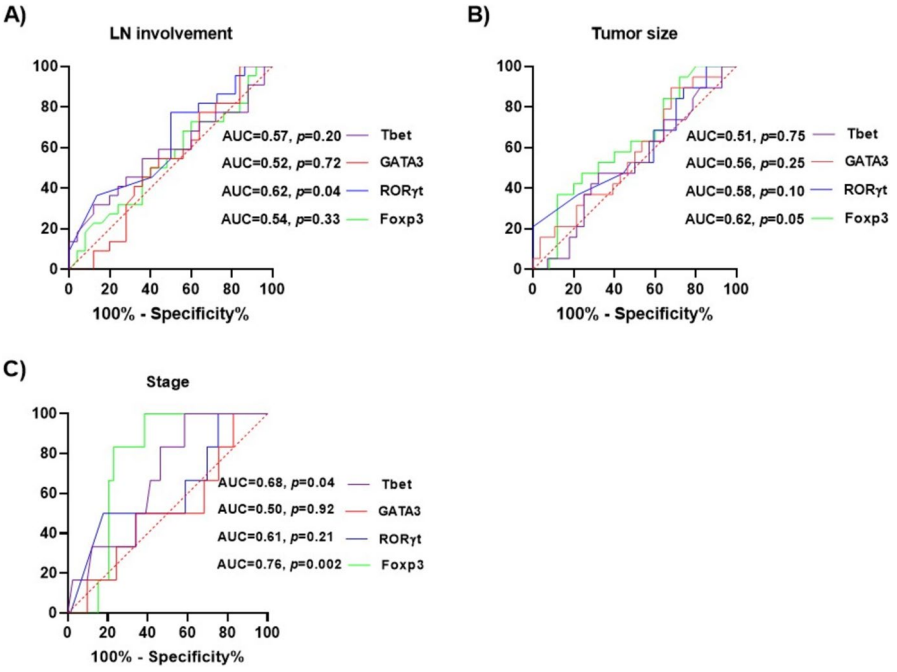


Fig. 4 Receiver operating characteristics (ROC) curve analyses of PBMCs- associated variables. (**A**) As diagnostic biomarkers differentiating LN-negative (0 involved nodes) vs. LN-positive (>1 involved node) cases; (**B**) diagnostic biomarkers differentiating small tumors (≤2 cm) vs. larger tumors (>2 cm); and (**C**) diagnostic biomarkers differentiating early-stage vs. late-stage disease. *AUC* area under the curve; *LN* lymph node

In our study, we found that T-bet expression in PBMCs negatively correlated with tumor histological grade and was notably diminished in patients with advanced-stage tumors, indicating a reduction in Th1 immune response associated with tumor progression.

It's important to highlight that, while T-bet is recognized as essential for Th1 development, it also plays a significant role in the maturation of various lymphocyte types, including CD8+ T cells. Thus, T-bet levels may not exclusively reflect Th1 activity but could represent a broader type 1 inflammatory response (Kallies and Good-Jacobson 2017).

GATA3 serves as a zinc-finger transcription factor critical for Th2 cell differentiation and mammary gland development, and it is the most predominant transcription factor in the mammary luminal epithelium. Recent studies have highlighted GATA3's potential as a diagnostic marker, particularly in luminal A and B breast cancer subtypes, though its effectiveness is less pronounced in non-luminal or TNBC variants (Stolnicu et al. 2020; Ni et al. 2018). While the precise role of GATA3 in breast cancer remains to be fully elucidated, data suggest it may have a protective role—as patients with GATA3 mutations tend to show improved prognoses and some studies implicate GATA3 in DNA repair and prevention of

cellular (Takaku et al. 2015; Wang et al. 2024). Conversely, research involving triple-positive breast cancer (TPBC) patients identified a negative correlation between GATA3 gene expression and the levels of tumor-infiltrating lymphocytes, coupled with a positive association with disease stage (Chen et al. 2024). Our study is among the first to investigate GATA3 gene expression in PBMCs from breast cancer patients, revealing a significant increase in GATA3 levels among the patient group and a notable decline in cases with larger tumors. This aligns with findings from studies on GATA3 levels in tumor tissues; however, there is a crucial need for more research into GATA3 expression in immune cells to further elucidate its role in breast cancer pathogenesis and progression.

RORγt is recognized as the primary driver of Th17 cell differentiation (Ivanov et al. 2006). A heightened RORγt/CD3 ratio, reflecting increased Th17 cell infiltration into the TME, has been proposed as a prognostic marker for survival and is associated with metastasis in colorectal cancer (Yoshida et al. 2016). Research has also shown elevated RORγt expression in PBMCs of liver cancer patients, marking an aberrant inflammatory profile within circulating immune cells (Lin et al. 2015). While RORγt expression primarily occurs in immune cells, studies examining its impact across various cancers have largely focused on Th17 cells. However, despite extensive research, consensus remains elusive regarding the pro- or anti-tumor roles of Th17 activity. For instance, cytokines produced by Th17 cells, such as IL-17 and IL-21, have been implicated in promoting angiogenesis, tumor progression, and metastasis in breast and colorectal cancers (Du et al. 2012; Stolfi et al. 2011). In contrast, other studies highlight the protective effects of Th17 cells and their secreted cytokines in melanoma and various carcinomas (Tian et al. 2021; Muranski et al. 2008). This inconsistency has prompted exploration of both agonist and antagonist strategies for RORγt in several diseases, including cancer (Pastwińska et al. 2023; Gege 2021). Our data suggest a relationship between RORγt expression in PBMCs and breast cancer progression, as we discovered elevated levels in the patient cohort closely tied to larger tumor sizes.

Foxp3 is another crucial transcription factor that governs Treg cell development. The clinical implications of Foxp3+ Treg presence do not adhere to a universal standard and appear to vary by cancer type. For example, in breast cancer, non-small cell lung cancer, and gastric cancer, tumor-infiltrating Foxp3+ Treg cells have been linked to adverse clinical outcomes, whereas they correlate positively with prognosis in cancers such as head and neck squamous cell carcinoma and colorectal, as well as esophageal malignancies (Meyiah and Elkord 2024). Furthermore, these Treg cells have been implicated in metastatic spread in breast cancer, being present at secondary sites like the liver and brain, where they likely contribute to an immunosuppressive environment that curtails anti-tumor responses (Zou et al. 2023). The microenvironment in which these cells operate can significantly influence their overall effect on cancer progression. Recent findings have revealed that a higher presence of circulating Foxp3+ Treg cells might be detrimental to survival in colorectal cancer, while their localization in the TME could indicate a more favorable prognosis (Al-Mterin et al. 2022).

Current reports on Foxp3 gene expression in the PBMCs of breast cancer patients generally supports a role that favors tumor promotion. One study demonstrated a

linear increase in Foxp3 expression across control, non-metastatic, and metastatic groups, with the highest levels observed in the metastatic cohort (Kawaguchi et al. 2017). Another investigation involved PBMCs from breast cancer patients incubated with the T cell activator phytohemagglutinin for 32 h, revealing markedly elevated Foxp3 expression across both stimulated and non-stimulated conditions compared to healthy controls (Khalife et al. 2018). Our findings mirrored this trend, showing significantly higher Foxp3 levels in the patient population. Furthermore, patients presenting with lymphovascular invasion (LVI) and lymph node involvement also displayed heightened Foxp3 gene expression, suggesting a correlational link between Foxp3 expression and disease advancement.

In our study, ROC curve analysis was employed to evaluate the diagnostic efficacy of Foxp3, RORγt, GATA3, and T-bet in predicting lymph node involvement, tumor size, and stage in breast cancer patients. The results indicate that Foxp3 exhibits the highest predictive power with an AUC of 0.76, suggesting its significant potential as a biomarker for aggressive disease behavior. This finding is consistent with the work of Li et al., who identified Foxp3 as a vital component in the tumor microenvironment's dynamics (Li et al. 2022). Furthermore, T-bet and RORγt also demonstrated meaningful associations with lymph node status and cancer staging, with AUC values of 0.68 and 0.62, respectively. This reinforces the observations from previous studies highlighting T-bet's role in cancer progression (Fang et al. 2017; Li et al. 2014).

The utilization of PBMCs related to the lymph node involvement enhances the precision of our findings, allowing us to gain deeper insights into the immune landscape shaped by the tumor environment. These results underscore the practical utility of these transcription factors in clinical settings, potentially improving patient stratification and guiding personalized treatment plans.

This study, despite its valuable insights, highlights several areas for future investigation. The lack of comparison between PBMC data and tumor-infiltrating lymphocytes (TILs) limits our understanding of how the tumor microenvironment influences systemic immune responses. Additionally, analyzing PBMCs in bulk, rather than at the single-cell level, may overlook specific behaviors of T cell subpopulations linked to cancer progression. Furthermore, excluding patients who have received treatment prevents us from assessing how chemotherapy affects immune system reorganization. The relatively small sample size of patients also restricts our ability to explore molecular differences between breast cancer subtypes, such as triple-negative breast cancer. Lastly, to validate the predictive models presented in this study, larger studies with more diverse populations are needed. These limitations underscore the importance of continued research to enhance our understanding of the immune landscape in breast cancer and improve patient outcomes.

Conclusion

In conclusion, our study sheds light on the important role of key transcription factors involved in influencing the differentiation of CD4+T cells in breast cancer patients—Foxp3, RORγt, GAT3, and T-bet—in breast cancer progression. Our

findings reveal that changes in the expression of these factors are closely linked to critical clinical parameters, including lymph node involvement, tumor size, and disease stage. This insight highlights their potential as valuable biomarkers, which could pave the way for enhanced diagnostic capabilities and targeted therapeutic strategies in the management of breast cancer. By understanding how these transcription factors contribute to the immune landscape of tumors, we can move toward more personalized and effective treatment approaches for patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10528-025-11133-z.

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Author Contributions Rasoul Baharlou, Nahid Nafissi and Maryam Rezaee conceived and planned the experiments. Fatemeh Kheiri carried out the experiments. Rasoul Baharlou wrote the manuscript. Maryam Rezaee, Fatemeh Kheiri and Saeed Azad Armaki contributed to sample preparation. Rasoul Baharlou, Nahid Nafissi abd Fatemeh Faraji contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

Ethics Approval and Consent to Participate This study was reviewed and approved by scientific advisory and ethical committees of Iran University of medical sciences (IR.IUMS.REC.1402.540) and before signing written informed consent forms, all patients were given complete explanation about the study procedures and protocol.

References

Abbasi-Dokht T, Vafaeinezhad A, Khalesi N, Malek F, Haghmorad D, Baharlou R (2023) T-cell immune responses and immunological factors associated with coronavirus disease 2019 progression as predictors for the severity of the disease in hospitalized patients. Int Arch Allergy Immunol 184(6):557–566

Abbasi-Dokht T, Malek F, Nafissi N, Mohammadlou M, Sheikh M, Akbari S et al (2024) Assessing angiogenesis factors as prognostic biomarkers in breast cancer patients and their association with clinicopathological factors. Biomarkers 29(1):36–43

Al-Mterin MA, Murshed K, Alsalman A, Abu-Dayeh A, Elkord E (2022) Associations of different immune checkpoints-expressing CD4(+) Treg/T cell subsets with disease-free survival in colorectal cancer patients. BMC Cancer 22(1):601

Baharlou R, Atashzar MR, Vasmehjani AA, Rahimi E, Khoshmirsafa M, Seif F et al (2016) Reduced levels of T-helper 17-associated cytokines in the serum of patients with breast cancer: indicators for following the course of disease. Central Eur J Immunol 41(1):78–85

Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT et al (2019) Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Ann Oncol 30(8):1194–1220

Chen Z, Laurence A, O'Shea JJ (2007) Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. Semin Immunol 19(6):400–408

Chen X, Zhao W, Huang Y, Luo S, Tang X, Yi Q (2024) Association of GATA3 expression in triple-positive breast cancer with overall survival and immune cell infiltration. Sci Rep 14(1):17795

de Visser KE, Joyce JA (2023) The evolving tumor microenvironment: from cancer initiation to metastatic outgrowth. Cancer Cell 41(3):374–403

Du JW, Xu KY, Fang LY, Qi XL (2012) Interleukin-17, produced by lymphocytes, promotes tumor growth and angiogenesis in a mouse model of breast cancer. Mol Med Rep 6(5):1099–1102

Fang J, Li X, Ma D, Liu X, Chen Y, Wang Y et al (2017) Prognostic significance of tumor infiltrating immune cells in oral squamous cell carcinoma. BMC Cancer 17:1–9

Gege C (2021) Retinoic acid-related orphan receptor gamma t (RORγt) inverse agonists/antagonists for the treatment of inflammatory diseases-where are we presently? Expert Opin Drug Discov 16(12):1517–1535

Gil Del Alcazar CR, Huh SJ, Ekram MB, Trinh A, Liu LL, Beca F et al (2017) Immune escape in breast cancer during in situ to invasive carcinoma transition. Cancer Discov 7(10):1098–1115

González-Silva L, Quevedo L, Varela I (2020) Tumor functional heterogeneity unraveled by scRNA-seq technologies. Trends Cancer 6(1):13–19

Goto W, Kashiwagi S, Asano Y, Takada K, Takahashi K, Hatano T et al (2018) Predictive value of improvement in the immune tumour microenvironment in patients with breast cancer treated with neoadjuvant chemotherapy. ESMO Open 3(6):e000305

Hashemi V, Maleki LA, Esmaily M, Masjedi A, Ghalamfarsa G, Namdar A et al (2020) Regulatory T cells in breast cancer as a potent anti-cancer therapeutic target. Int Immunopharmacol 78:106087

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ et al (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126(6):1121–1133

Kallies A, Good-Jacobson KL (2017) Transcription factor T-bet orchestrates lineage development and function in the immune system. Trends Immunol 38(4):287–297

Kawaguchi K, Suzuki E, Yamaguchi A, Yamamoto M, Morita S, Toi M (2017) Altered expression of major immune regulatory molecules in peripheral blood immune cells associated with breast cancer. Breast Cancer 24(1):111–120

Khalife E, Khodadadi A, Talaeizadeh A, Rahimian L, Nemati M, Jafarzadeh A (2018) Overexpression of regulatory T cell-related markers (FOXP3, CTLA-4 and GITR) by peripheral blood mononuclear cells from patients with breast cancer. Asian Pac J Cancer Prev 19(11):3019–3025

Klemm F, Maas RR, Bowman RL, Kornete M, Soukup K, Nassiri S et al (2020) Interrogation of the microenvironmental landscape in brain tumors reveals disease-specific alterations of immune cells. Cell 181(7):1643–60.e17

Ladoire S, Arnould L, Mignot G, Apetoh L, Rébé C, Martin F et al (2011) T-bet expression in intratumoral lymphoid structures after neoadjuvant trastuzumab plus docetaxel for HER2-overexpressing breast carcinoma predicts survival. Br J Cancer 105(3):366–371

Li S, Li Y, Qu X, Liu X, Liang J (2014) Detection and significance of TregFoxP3+ and Th17 cells in peripheral blood of non-small cell lung cancer patients. Arch Med Sci 10(2):232–239

Li J, Zhang X, Liu B, Shi C, Ma X, Ren S et al (2022) The expression landscape of FOXP3 and its prognostic value in breast cancer. Ann Transl Med 10(14):801

Lin ZW, Wu LX, Xie Y, Ou X, Tian PK, Liu XP et al (2015) The expression levels of transcription factors T-bet, GATA-3, RORγt and FOXP3 in peripheral blood lymphocyte (PBL) of patients with liver cancer and their significance. Int J Med Sci 12(1):7–16

Marques HS, de Brito BB, da Silva FAF, Santos MLC, de Souza JCB, Correia TML et al (2021) Relationship between Th17 immune response and cancer. World J Clin Oncol 12(10):845–867

Meyiah A, Elkord E (2024) What is the relevance of FoxP3 in the tumor microenvironment and cancer outcomes? Expert Rev Clin Immunol 20(8):803–809

Miller SA, Weinmann AS (2010) Molecular mechanisms by which T-bet regulates T-helper cell commitment. Immunol Rev 238(1):233–246

Mori H, Kubo M, Kai M, Yamada M, Kurata K, Kawaji H et al (2019) T-bet(+) lymphocytes infiltration as an independent better prognostic indicator for triple-negative breast cancer. Breast Cancer Res Treat 176(3):569–577

Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM et al (2001) Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 292(5523):1907–1910

Mulligan AM, Pinnaduwage D, Tchatchou S, Bull SB, Andrulis IL (2016) Validation of intratumoral T-bet+ lymphoid cells as predictors of disease-free survival in breast cancer. Cancer Immunol Res 4(1):41–48

Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A et al (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood 112(2):362–373

Nafissi N, Mohammadlou M, Akbari ME, Mahdavi SR, Sheikh M, Borji M et al (2022) The impact of intraoperative radiotherapy on breast cancer: focus on the levels of angiogenic factors. World J Surg Oncol 20(1):191

Ni YB, Tsang JYS, Shao MM, Chan SK, Cheung SY, Tong J et al (2018) GATA-3 is superior to GCDFP-15 and mammaglobin to identify primary and metastatic breast cancer. Breast Cancer Res Treat 169(1):25–32

Pastwińska J, Karwaciak I, Karaś K, Bachorz RA, Ratajewski M (2023) RORγT agonists as immune modulators in anticancer therapy. Biochim Biophys Acta Rev Cancer 1878(6):189021

Stolfi C, Rizzo A, Franzè E, Rotondi A, Fantini MC, Sarra M et al (2011) Involvement of interleukin-21 in the regulation of colitis-associated colon cancer. J Exp Med 208(11):2279–2290

Stolnicu S, Tunde C, Cadar A, Boros M (2020) Differences in GATA3 expression among histological/molecular subtypes and grades in infiltrating breast carcinoma (IBC) are important in the diagnosis of metastatic breast carcinoma. Pol J Pathol 71(1):62–65

Takaku M, Grimm SA, Wade PA (2015) GATA3 in breast cancer: tumor suppressor or oncogene? Gene Expr 16(4):163–168

Tian EM, Yu MC, Feng M, Lu LX, Liu CL, Shen LA et al (2021) RORγt agonist synergizes with CTLA-4 antibody to inhibit tumor growth through inhibition of Treg cells via TGF-β signaling in cancer. Pharmacol Res 172:105793

Wang X, Bai F, Liu X, Peng B, Xu X, Zhang H et al (2024) GATA3 functions downstream of BRCA1 to promote DNA damage repair and suppress dedifferentiation in breast cancer. BMC Biol 22(1):85

Xiao Y, Huang Y, Jiang J, Chen Y, Wei C (2023) Identification of the prognostic value of Th1/Th2 ratio and a novel prognostic signature in basal-like breast cancer. Hereditas 160(1):2

Yagi R, Zhu J, Paul WE (2011) An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation. Int Immunol 23(7):415–420

Yoshida N, Kinugasa T, Miyoshi H, Sato K, Yuge K, Ohchi T et al (2016) A High RORγT/CD3 ratio is a strong prognostic factor for postoperative survival in advanced colorectal cancer: analysis of helper T cell lymphocytes (Th1, Th2, Th17 and regulatory T cells). Ann Surg Oncol 23(3):919–927

Yu H, Yang J, Jiao S, Li Y, Zhang W, Wang J (2014) T-box transcription factor 21 expression in breast cancer and its relationship with prognosis. Int J Clin Exp Pathol 7(10):6906–6913

Zou Y, Ye F, Kong Y, Hu X, Deng X, Xie J et al (2023) The single-cell landscape of intratumoral heterogeneity and the immunosuppressive microenvironment in liver and brain metastases of breast cancer. Adv Sci (Weinh) 10(5):e2203699

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