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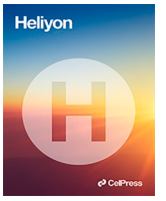

Research article

ncRNAs-mediated overexpression of *TET3* predicts unfavorable prognosis and correlates with immunotherapy efficacy in breast cancer

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ABSTRACT

Breast cancer is the most frequent form of cancer in women and the primary cause of cancer-related deaths globally. DNA methylation and demethylation are important processes in human tumorigenesis. Ten-eleven translocation 3 (*TET3*) is a DNA demethylase. Prior research has demonstrated that *TET3* is highly expressed in various human malignant tumors. However, the exact function and mechanism of *TET3* in breast cancer remain unclear. In this study, we investigated *TET3* expression in breast cancer and its correlation with clinicopathological characteristics of breast cancer patients. The results presented that *TET3* expression was significantly increased in breast cancer and associated with the PAM50 subtype. Subsequently, we performed receiver operating characteristic, survival, and Cox hazard regression analyses. These results suggest that *TET3* expression is associated with a poor prognosis and may be an indirect independent prognostic indicator in breast cancer. We also established a protein-protein interaction (PPI) network of *TET3* and executed enrichment analyses of *TET3* co-expressed genes, revealing their primary association with the cell cycle. Moreover, we identified noncoding RNAs (ncRNAs) contributing to *TET3* overexpression using expression, correlation, and survival analyses. We identified the LINC01521/hsa-miR-29a-3p axis as the primary *TET3* upstream ncRNA-related pathway in breast cancer. Furthermore, *TET3* expression was positively associated with immune cell infiltration, immune cell biomarkers, and eight immune checkpoint gene expressions in breast cancer. *TET3* expression also correlated with patient responses to immunotherapy. Finally, we conducted subcellular localization and immunohistochemical staining analysis of *TET3* in breast cancer. We found that *TET3* localized to the nucleoplasm, vesicles, and cytosol in the MCF-7 cell line, and *TET3* expression was significantly upregulated in breast cancer tissues compared to para-tumor tissues. Our findings indicate that ncRNA-mediated overexpression of *TET3* predicts an unfavorable prognosis and correlates with immunotherapy efficacy in breast cancer.

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1. Introduction

According to Global cancer statistics for 2020, breast cancer is the most prevalent malignant tumor among women worldwide, leading to morbidity and cancer-related deaths [1–3]. Triple-negative breast cancer, a special molecular subtype lacking hormone receptor (HR) and human epidermal growth factor 2-neu (HER2) expression [4], has a poor prognosis due to its aggressive characteristics and limited effective therapeutic targets [5]. It is essential to understand the molecular processes behind breast cancer pathogenesis and find novel prognostic biomarkers to develop targeted treatments and raise the survival rate.

DNA methylation and demethylation are the most common epigenetic processes, allowing the inheritance of phenotypic changes without changing the DNA sequence [6]. Irregular alterations in DNA methylation have been linked to tumorigenesis, primarily through hypomethylation of typically inactive regions and hypermethylation of promoters of tumor suppressor genes, resulting in tumor progression and chemotherapy resistance [7]. Hypomethylation of usually inactive areas and hypermethylation of tumor suppressor gene promoters are abnormal alterations in DNA methylation linked to carcinogenesis, promoting tumor growth and chemotherapy resistance [8].

Since the early 2000s, the ten-eleven translocation (TET) family of DNA demethylase enzymes, including *TET1*, *TET2*, and *TET3*, has played a significant role in oxidizing 5-methylcytosine (5-mC) to form 5-hydroxymethylcytosine (5-hmC), 5-formyl cytosine (5-fC), and 5-carboxy cytosine (5-caC) in DNA using Fe^{2+} and 2-oxoglutarate [9,10]. *TET3*-mediated DNA demethylation significantly influences fertilized egg and embryo development, neurocyte differentiation, embryo survival, growth, and intellectual development. *TET3* is also strongly expressed in various cancerous tumors, including glioma, ovarian cancer, osteosarcoma, and acute myeloid leukemia (AML) [11–15]. However, the *TET3* function in human cancers is still poorly understood compared to *TET1* and *TET2*. *TET3* has varying effects on different cancer types. It benefits esophageal squamous cell cancer but harms glioblastoma [16].

TET gene expression and 5-hmC/5-fC/5-caC levels are important in breast cancer diagnosis and prognosis, indicating the dual-edged nature of the TET/5-mC oxidation pathway [17]. According to Tsai et al., estrogen receptor (ER)-negative breast cancer patients with low 5-hmC levels have a worse prognosis [18], while Wu et al. observed a high 5-hmC level in breast tumors [19]. According to Wu et al., *TET1* and *TET3* collaborate to activate stem-like breast cancer cells via the TNF-α-p38-MAPK signaling axis caused by hypoxia [19]. Duforestel et al. demonstrated that *TET3* is the underlying factor behind glyphosate-induced breast cell tumorigenesis through epigenome reprogramming [20]. After receiving anthracycline therapy, Yang et al. identified high *TET3* and thymyine DNA glycosylase TDG mRNA levels as advantageous independent prognostic factors in breast cancer patients [21]. In summary, the limited research on *TET3* in breast cancer makes its carcinogenic mechanisms unclear and controversial, requiring further investigation.

MicroRNAs (miRNAs) are intrinsic non-coding RNAs that critically influence gene expression regulation in eukaryotic organisms [22]. They negatively downregulate their target gene expressions [23]. Additionally, long non-coding RNA (lncRNA) is essential for carcinogenesis, invasion, metastasis, and treatment resistance. It functions as a competitive endogenous RNA (ceRNA) of miRNA, attaching to it and serving as a miRNA sponge, blocking the miRNA's impact on its target gene and increasing its expression level [24]. In 2013, Song et al. identified that miR-22 targets TET genes in breast tissue by binding to the 3′UTR of TET mRNA, thereby decreasing stability and expression [25]. Other miRNAs, including miR-19a-5p, miR-125a-5p, miR-29b, miR-101, and miR-125b regulate *TET2* mRNA levels [8]. However, there has been insufficient research on the upstream regulation of miRNAs by *TET3*. Furthermore, an association between *TET3* and tumor immune invasion in breast cancer has not been established.

This study aimed to investigate *TET3* expression in multiple human cancer types, focusing on its expression and survival analysis in breast cancer. Additionally, we created a protein-protein interaction (PPI) network for *TET3* and performed enrichment studies on the co-expressed genes in breast cancer. Furthermore, we predicted upstream signaling pathways and *TET3* regulation by non-coding RNA (ncRNAs), including miRNAs and lncRNAs. We also investigated the relationship between *TET3* and immune cell biomarkers, immune cell infiltration, immune checkpoints, and immunotherapy efficacy in breast cancer. Finally, we analyzed the subcellular location of *TET3* in breast cancer cell lines and validated its expression using immunohistochemical staining of a human breast cancer tissue microarray. Our results demonstrate that increased *TET3* expression induced by noncoding RNAs is related to poorer prognosis and reduced immunotherapy efficacy in breast cancer patients.

2. Materials and methods

2.1. Data acquisition and preprocessing

In this study, RNA-seq data from 33 human cancers were downloaded in fragments per kilobase million (FPKM) format. miRNA-seq data of breast cancer were assessed in per million (RPM) format, and clinicopathological data of breast cancer were evaluated from The Cancer Genome Atlas (TCGA) [26]. Two breast cancer datasets, GSE29431 (containing 12 normal tissues and 54 breast cancer tissues) and GSE42568 (containing 17 normal tissues and 104 breast cancer tissues), were obtained from the Gene Expression Omnibus (GEO) database [27]. RNA-seq data were converted from the FPKM format to TPM values to ensure consistency between datasets. The log2 transformation [log2 (TPM+1) or log2 (RPM+1)] and normalization were performed for RNA-seq and miRNA-seq data [28–30].

2.2. TET3 expression analysis in breast cancer

TET3 expression levels were examined in various normal and malignant tissues using the TCGA database. Additionally, the expression profiles from TCGA-BRCA, GSE29431, and GSE42568 datasets were used to confirm *TET3* expression levels in breast cancer. The clinicopathological data of the TCGA-BRCA cohort were analyzed to examine the association between *TET3* expression and

clinicopathological features of breast cancer patients.

2.3. Diagnostic and prognostic values of TET3 in breast cancer

The diagnostic utility of *TET3* was assessed in the overall survival (OS) of breast cancer patients using receiver operating characteristic (ROC) analysis in TCGA-BRCA with the R package pROC [31]. R packages Survminer and survival [32] based on TCGA-BRCA were employed to analyze the relationship between *TET3* expression and three types of survival outcomes (OS, disease-specific survival (DSS), and progression-free interval (PFI)) in breast cancer patients. Breast cancer patients were categorized into high- and low-expression groups according to the median value of *TET3*. Kaplan-Meier survival estimators were used to evaluate the prognoses of the two groups, and log-rank tests were performed to compare the survival outcomes [33]. Additionally, univariate and multivariate Cox hazard regression analyses were performed. Hazard ratios and 95 % confidence intervals (CI) were calculated to determine independent prognostic variables related to OS.

2.4. PPI network construction and TET3 Co-expressed genes identification

The STRING database is a freely available tool that compiles all PPI [34]. In this investigation, a PPI network was built using the STRING database to investigate possible relationships between *TET3* and other protein-coding genes. The minimum interaction score was set to $\geq$0.4. *TET3* co-expressed genes were obtained using the LinkedOmics database, a web-based platform that analyzes multi-omics data based on TCGA database [35].

2.5. Enrichment analysis

Strict criteria were applied to select co-expressed genes: an absolute value of the correlation coefficient $>$0.5 with an adjusted p-value $<$0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment studies were conducted on the selected genes using the R package clusterProfiler [36]. The results were deemed statistically significant if the adjusted *p*-value was $<$0.05 and the q-value was $<$0.2.

2.6. Prediction and construction of TET3 upstream ncRNAs regulatory network

Seven renowned target prediction databases, including PITA, RNA22, miRmap, Diana-microT, miRanda, PicTar, and TargetScan, were used to predict candidate miRNAs upstream of *TET3* [37]. The possible miRNAs predicted by the four databases were chosen simultaneously. Associations between *TET3* expression and these potential miRNAs were examined using TCGA-BRCA. The miRNA that most likely controls *TET3* expression in breast cancer was discovered and verified using expression and survival data. Additionally, candidate lncRNAs upstream of the most potential miRNAs were predicted using the publicly available starBase database, predicting mRNA-miRNA, miRNA-lncRNA, and mRNA-lncRNA interactions [38]. Similarly, the most potential lncRNA was identified after expression correlation analyses and validated for expression in TCGA-BRCA.

2.7. Associations between TET3 and immune cells infiltration

Different bioinformatics tools and databases were used to evaluate potential relationships between *TET3* and immune cell infiltration levels in breast cancer. The TIMER database, a reliable and comprehensive platform, was employed to evaluate infiltration levels of immune cells and explore their clinical implications [39]. Correlations between genetic copy number variations (CNV) of *TET3* and immune cell infiltration levels were also examined using the "SCNA" module in the TIMER database. The R program GSVA on TCGA-BRCA data was used to analyze the link between *TET3* expression and immune cell infiltration [40]. The GEPIA2 database, a web-based application, was used to analyze expression correlations with TCGA data. The connections between *TET3* expression and immune cell markers were also examined [41].

2.8. Associations between TET3 and immune checkpoint genes

The expression correlations in TCGA-BRCA were analyzed. The R package ggplot2 was used to visualize and evaluate the association between *TET3* and the eight immune checkpoint genes in breast cancer. Results with a *p*-value of 0.05 or below were deemed statistically significant.

2.9. Associations between TET3 and response to immunotherapy

Associations between *TET3* and response to immunotherapy were analyzed using the “immunotherapy” module in the Kaplan-Meier (K-M) Plotter database, a comprehensive bioinformatics tool to evaluate expression and survival parameters of genes, miRNAs, and proteins in tumors [42,43].

2.10. Subcellular location and immunohistochemical staining analysis of TET3 in breast cancer

In this study, the subcellular localization of *TET3* was searched using the Human Protein Atlas (HPA) database. The HPA proteome and transcriptome database encompasses the protein expression patterns in healthy and cancerous tissues [44]. Immunohistochemical staining of *TET3* was performed using a human breast cancer tissue microarray (HBreD050Bc01, Shanghai Outdo Biotech Co. Ltd. Shanghai, China). The images were captured using a light microscope at 10 × and 20 × magnification, and *TET3* expression was scored based on staining positive rate and intensity score [45]. The staining intensity scores ranged from 0 (none) to 3 (strong), while the staining positive rate ranged from 0 % to 100 %. The *TET3* total staining score was determined by multiplying the intensity score by the staining positive rate, ranging from 0 to 300. Furthermore, *TET3* total staining scores were compared between the para-tumor and tumor groups.

2.11. Statistical analysis

Statistical analyses were performed using the previously mentioned online databases. The Xiantao platform (www.xiantao.love), offering a complete set of tools for expression analysis, clustering analysis, interactive network analysis, enrichment analysis, clinical significance analysis, and R-based charting, was used to conduct further studies. A statistically significant threshold was established at a log-rank p-value <0.05 or a p-value <0.05.

3. Results

3.1. Assessment of TET3 expression in breast cancer

We analyzed *TET3* expression in tumors and adjacent normal tissues from 33 types of human cancers using TCGA database. The results explained that *TET3* expression was significantly upregulated in 16 cancer categories, including BRCA, BLCA, CHOL, CESC, ESCA, KIRC, HNSC, KIRP, LUAD, LUSC, LIHC, PCPG, PRAD, THCA, STAD, and UCEC, while it was downregulated in KICH (Fig. 1A). *TET3* expression was validated in breast cancer and normal tissues by analyzing expression profiles from TCGA and GEO databases. *TET3* expression was significantly higher in the tumor tissues than in the normal tissues (Fig. 1B–D). We also analyzed the correlation between *TET3* expression and prediction analysis of microarray 50 (PAM50) subtypes using TCGA-BRCA data, revealing a significant relationship between *TET3* expression and PAM50 subtypes (Fig. 1E). In summary, the PAM50 subtypes and *TET3* expression levels are associated with breast cancer.

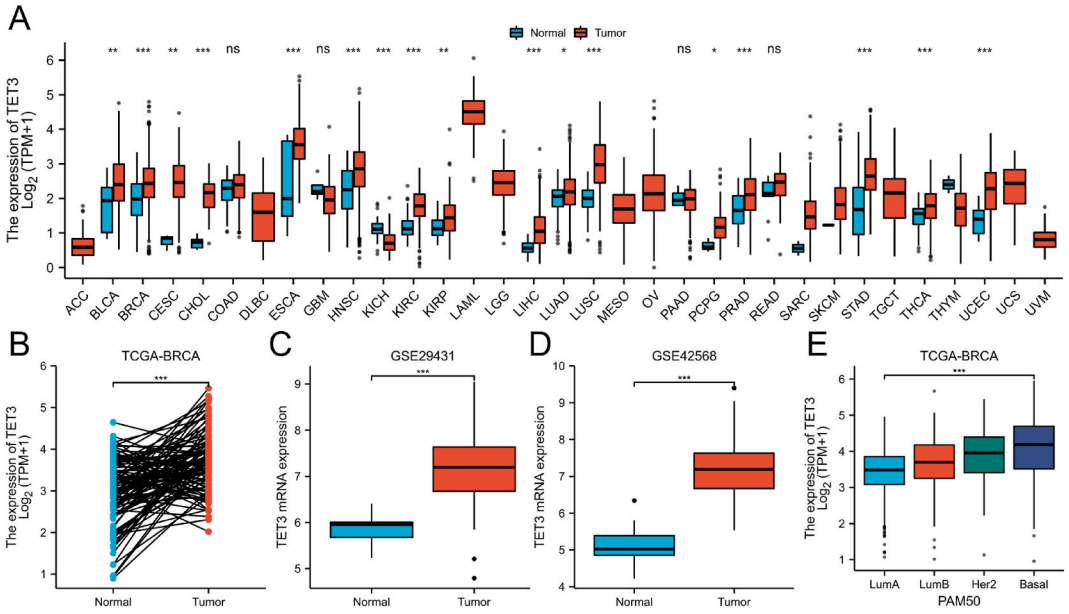

Fig. 1. *TET3* expression in breast cancer. **(A)** *TET3* expression in 33 types of tumor and normal tissues based on TCGA database. **(B)** *TET3* expression in tumor and paired adjacent normal tissues in TCGA-BRCA. **(C)** *TET3* expression in tumor and normal tissues in GSE29431. **(D)** *TET3* expression in tumor and normal tissues in GSE42568. **(E)** Correlation between *TET3* expression and PAM50 subtypes in TCGA-BRCA. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.2. Diagnostic and prognostic values of TET3 in breast cancer

We performed ROC curve analysis to assess *TET3*'s potential as a breast cancer diagnostic biomarker. *TET3* had an AUC of 0.765 (95 % CI = 0.736–0.794, Fig. 2A), indicating high diagnostic accuracy. We examined its connections with patients' clinical outcomes (OS, DSS, and PFI) using TCGA-BRCA data to further research *TET3*'s clinical importance in breast cancer. The results revealed that individuals with higher *TET3* expression had significantly worse clinical outcomes than those with lower *TET3* expression (Fig. 2B–D). We performed univariate and multivariate Cox hazard regression analyses to determine independent prognostic variables related to OS using TCGA database. Univariate analysis revealed that *TET3* expression, T stage, N stage, M stage, pathological stage, and age were significantly associated with OS. *TET3* expression (HR = 1.359, $p = 0.010$), M stage (HR = 2.380, $p = 0.016$), pathologic stage (HR = 2.259, $p = 0.016$), and age (HR = 2.146, $p < 0.001$) were independent prognostic indicators for breast cancer patients in the multivariate analysis (Table 1).

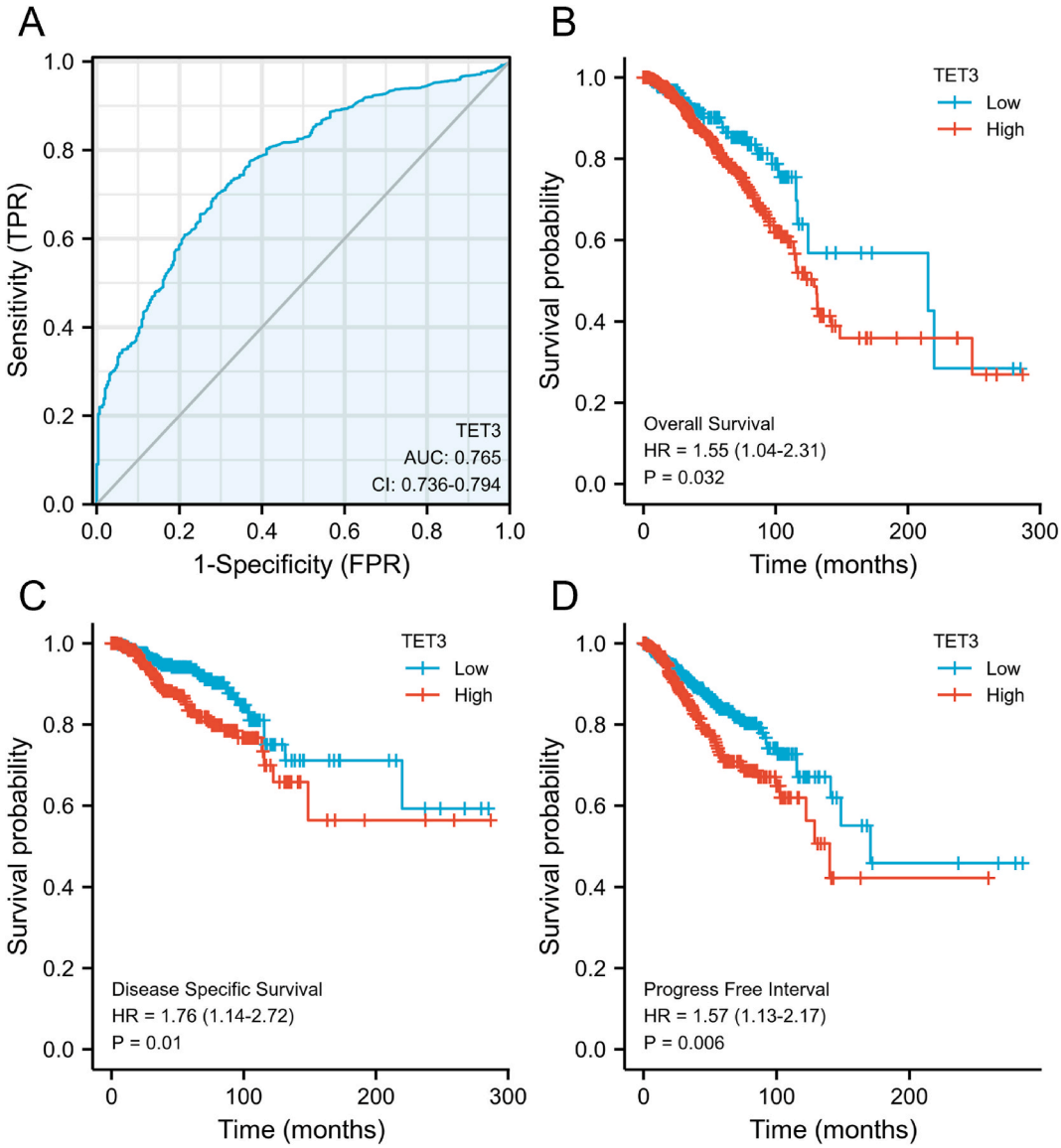

Fig. 2. Diagnostic and prognostic values of *TET3* in breast cancer. **(A)** Receiver operating characteristic analysis (ROC) curve of *TET3* in TCGA-BRCA. **(B–D)** Kaplan–Meier curves for breast cancer patients with high *TET3* expression suggested poor overall survival (OS), disease specific survival (DSS), and progress free survival (PFI), respectively.

Table 1
Univariate and multivariate Cox hazard regression analyses of *TET3* expression and clinicopathologic characteristics associated with OS in breast cancer patients based on TCGA database.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*p*-value	HR (95 % CI)	*p*-value
TET3 expression (Low vs. High)	1.264 (1.028–1.554)	**0.026**	1.359 (1.075–1.718)	**0.010**
T stage (T1-2 vs. T3-4)	1.608 (1.110–2.329)	**0.012**	0.976 (0.579–1.647)	0.928
N stage (N0-1 vs. N2-3)	2.163 (1.472–3.180)	**<0.001**	1.140 (0.619–2.099)	0.674
M stage (M0 vs. M1)	4.254 (2.468–7.334)	**<0.001**	2.380 (1.174–4.825)	**0.016**
Pathologic stage (I-II vs. III-IV)	2.391 (1.703–3.355)	**<0.001**	2.259 (1.161–4.396)	**0.016**
Age (≤60 vs. >60)	2.020 (1.465–2.784)	**<0.001**	2.146 (1.483–3.105)	**<0.001**
ER status (Negative vs. Positive)	0.712 (0.495–1.023)	0.066		
PR status (Negative vs. Positive)	0.732 (0.523–1.024)	0.068		
HER2 status (Negative vs. Positive)	1.593 (0.973–2.609)	0.064		

Bold font: $P < 0.05$.

3.3. PPI network and Co-expressed genes of TET3 in breast cancer

Next, we constructed a PPI network of *TET3* in breast cancer using the STRING database to explore potential correlations between *TET3* and other protein-coding genes. Network analysis revealed that *TET3* was associated with OGT, SIN3A, DNMT1, TDG, *TET2*, PRMT5, DNMT3A, DNMT3B, WHSC1L1, and NANOG, with correlation scores ranging from 0.720 to 0.950 (Fig. 3A). We also used the LinkedOmics database to obtain co-expressed genes of *TET3* in breast cancer. *TET3* expression was positively associated with 8166 genes and negatively associated with 6888 genes (FDR <0.05; Fig. 3B). The top 50 genes positively and negatively correlated with *TET3* expression in breast cancer are presented as heat maps (Fig. 3C and D). Additionally, we selected 496 genes for enrichment analysis with |cor| > 0.5 and FDR <0.05 (Supplementary Table 1).

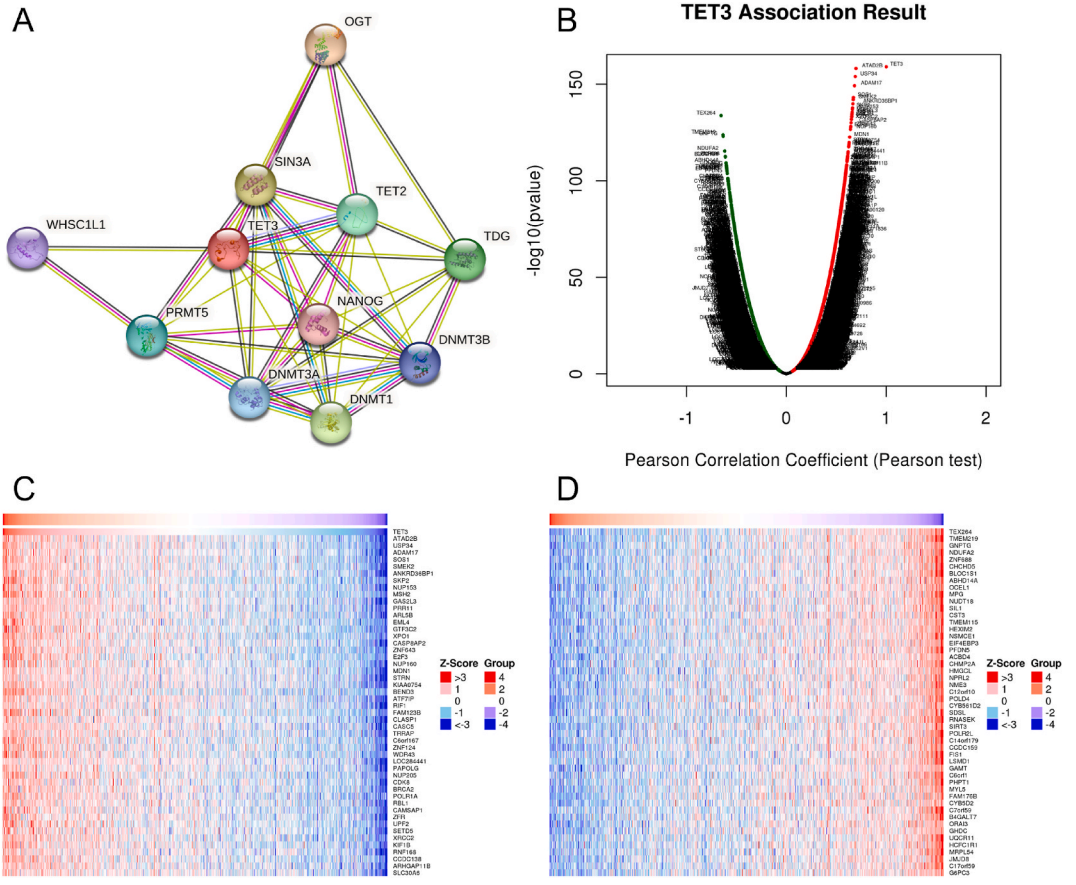

Fig. 3. Construction of *TET3* Protein-Protein Interaction (PPI) network and identification of *TET3* co-expressed genes. **(A)** PPI network of *TET3*. **(B)** Volcano plot of genes highly relevant to *TET3* detected in breast cancer by Pearson test. **(C)** Top 50 positively co-expressed genes of *TET3* in breast cancer. **(D)** Top 50 negatively co-expressed genes of *TET3* in breast cancer.

3.4. Enrichment analysis of TET3 Co-expressed genes in breast cancer

We used the R package clusterProfiler to perform GO and KEGG pathway enrichment analyses on the chosen *TET3* co-expressed genes to better understand the biological importance of *TET3* in breast cancer. We filtered the genes under $|cor| > 0.5$ and FDR <0.05. The *TET3* co-expressed genes were involved in 321 GO biological processes (BP), 55 cellular components (CC), 41 molecular functions (MF), and 12 KEGG pathways. The bubble (Fig. 4A) and bar (Fig. 4B) charts display the top five GO BP, CC, and MF ranked by gene ratio and adjusted p-value. Similarly, the bubble (Fig. 4C) and bar (Fig. 4D) charts present the top five KEGG pathways ranked by gene ratio and adjusted p-values. The GO enrichment analysis results revealed that the primary biological processes involving *TET3* co-expressed genes include ATPase activity, helicase activity, chromosomal region, spindle, organelle fission, and DNA replication. The KEGG pathway enrichment analysis indicated that most co-expressed *TET3* genes were linked to the cell cycle.

3.5. Prediction and analysis of TET3 upstream ncRNAs regulatory network in breast cancer

According to recent evidence, ncRNAs are crucial for controlling gene expression [46]. We anticipated eight miRNAs that might bind to *TET3* in the upstream region to examine the possible regulation of *TET3* by ncRNAs. We created a miRNA-*TET3* regulation network using Cytoscape to enhance the data's visual appeal (Fig. 5A). *TET3* and related upstream miRNAs should have a negative association because upstream miRNAs negatively regulate target gene expression at the post-transcriptional stage. Therefore, we conducted expression correlation analyses between *TET3* and predicted upstream miRNAs using TCGA-BRCA data. Fig. 5B illustrates the substantial inverse relationship between hsa-miR-29a-3p and *TET3* expression levels. Afterward, we investigated the hsa-miR-29a-3p expression and prognostic significance in breast cancer. We detected marked downregulation of hsa-miR-29a-3p expression in breast cancer (Fig. 5C and D), which was negatively correlated with patient prognosis (Fig. 5E). The starBase database was used to predict upstream lncRNAs of hsa-miR-29a-3p and obtained 45 candidate lncRNAs. This allowed us to investigate the upstream control of *TET3* expression (Supplementary Table 2). According to the ceRNA hypothesis, lncRNA-target miRNA or lncRNA-target mRNA correlations in the ceRNA network should be negative or positive. Consequently, we examined the 45 lncRNA

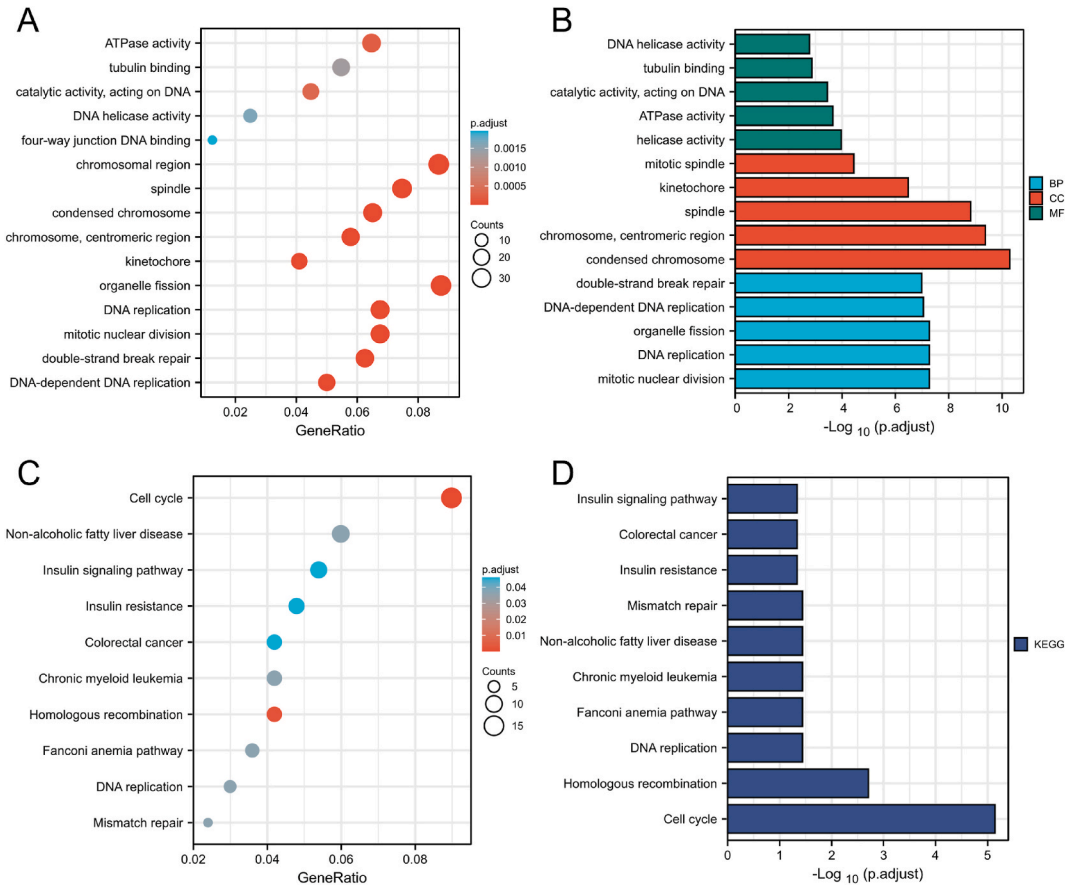

Fig. 4. Enrichment analysis of *TET3* co-expressed genes. **(A–B)** Enrichment analysis of gene ontology (GO) terms for *TET3* co-expressed genes, displayed in bubble chart and bar chart, respectively. **(C–D)** Enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for *TET3* co-expressed genes, displayed in bubble chart and bar chart, respectively.

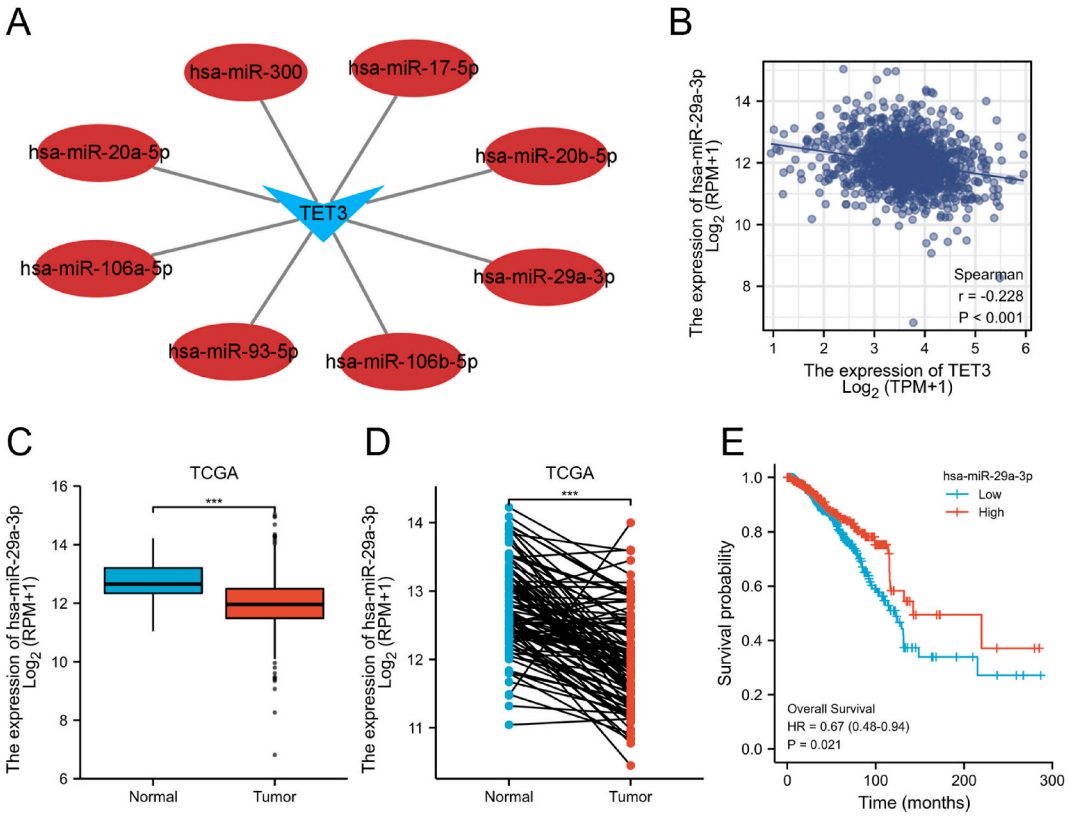

Fig. 5. Identification of hsa-miR-29a-3p as a potential upstream miRNA in regulation of *TET3* expression in breast cancer. **(A)** The miRNA-*TET3* regulatory network visualized by Cytoscape software. **(B)** Correlation between *TET3* expression and hsa-miR-29a-3p expression in breast cancer. **(C)** The expression of hsa-miR-29a-3p in breast cancer analyzed based on tumor and normal tissues in TCGA-BRCA. **(D)** The expression of hsa-miR-29a-3p in breast cancer analyzed based on paired tumor and adjacent normal tissues in TCGA-BRCA. **(E)** The prognostic value of hsa-miR-29a-3p in breast cancer based on TCGA database. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

expression levels using TCGA-BRCA data and identified expression correlations between the hsa-miR-29a-3p/*TET3* and 45 lncRNAs. According to the findings, only LINC01521, significantly more expressed in tumor tissue than in normal tissue, should be used (Fig. 6A and B), was positively related to *TET3* and negatively related to hsa-miR-29c-3p (Table 2, Fig. 6C and D). The LINC01521/hsa--miR-29a-3p/*TET3* axis may be the upstream axis regulating *TET3* expression in breast cancer.

3.6. Associations between TET3 and immune cells infiltration in breast cancer

We used the TIMER database to explore associations between the *TET3* expression level and the invasion of six immune cells in breast cancer. Fig. 7A presents a significant positive correlation between the *TET3* expression level and the infiltrating level of B cells ($p = 8.26 \times 10^{-8}$), $CD8^+$ T cells ($p = 4.16 \times 10^{-20}$), $CD4^+$ T cells ($p = 6.22 \times 10^{-12}$), macrophages ($p = 2.36 \times 10^{-5}$), neutrophils ($p = 1.23 \times 10^{-15}$), and dendritic cells ($p = 6.82 \times 10^{-12}$) in breast cancer. The numbers of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, dendritic cells, and neutrophils infiltrating breast cancer strongly correlated with *TET3* copy number variation (CNV) (Fig. 7B). We also utilized the R package GSVA to examine the connection between *TET3* expression and immune cell infiltration using TCGA-BRCA. The infiltrating level of 15 immune cells, including B cells, $CD8^+$ T cells, cytotoxic cells, eosinophils, iDC, mast cells, NK CD56bright cells, NK cells, pDC, T helper cells, Tcm, Tem, Tgd, Th17 cells, and Th2 cells, differ statistically between the high- and low-*TET3* expression groups. Fig. 7C depicts this difference. Additionally, we conducted an expression correlation analysis of *TET3* and immune cell biomarkers in breast cancer using the GEPIA2 database. Table 3 presents the significant association between *TET3* and various biomarkers, including $CD4^+$ T cell biomarker (CD4), $CD8^+$ T cell biomarker (CD8A), M1 macrophage biomarkers (NOS2, PTGS2, and IRF5), M2 macrophage biomarkers (CD163, CD206, and MS4A4A), neutrophil biomarkers (CCR7, CEACAM8, and ITGAM), dendritic cell biomarkers (HLA-DQB1, HLA-DRA, ITGAX, and NRP1), mast cell biomarkers (TPSB2 and TPSAB1), NK cell biomarkers (CD57, KIR2DL1, KIR2DL3, KIR2DL4, and KIR3DL2), Th1 cell biomarkers (T-bet, STAT1, STAT4, IFN-γ, and TNF-α), Th2 cell biomarkers (GATA3, STAT6, STAT5A, and IL13), Th17 cell biomarkers (STAT3 and IL17A), Tfh cell biomarkers (BCL6 and IL21), and Treg cell biomarkers (FOXP3, TGFβ, CCR8, and STAT5B).

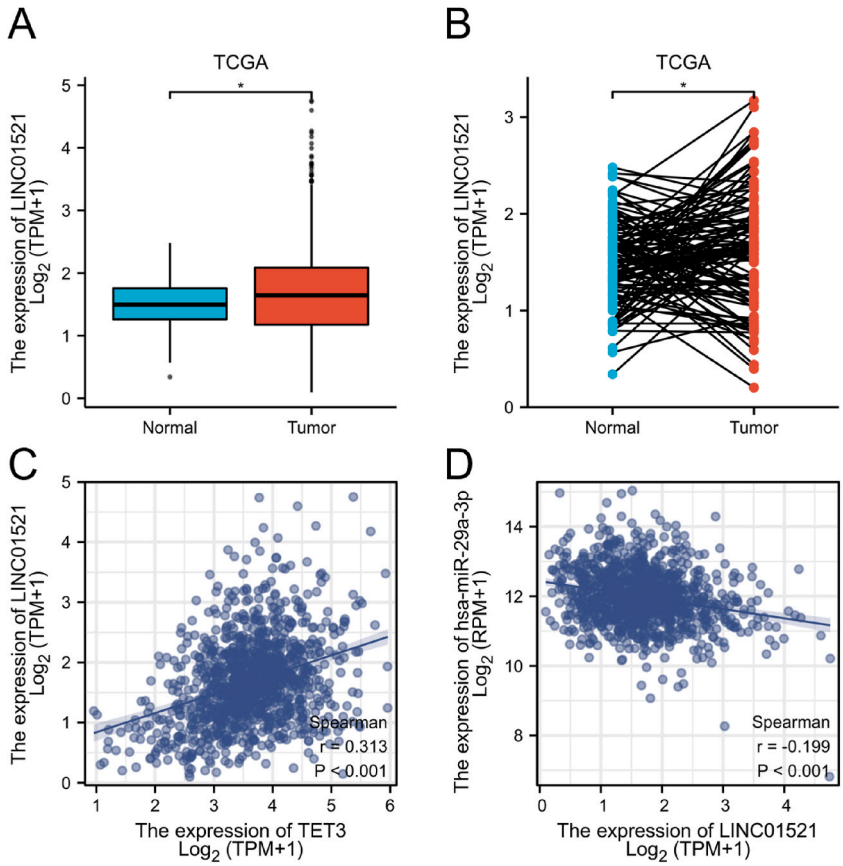

Fig. 6. Identification of LINC01521 as a potential upstream lncRNA of hsa-miR-29a-3p in breast cancer. **(A)** The expression of LINC01521 in breast cancer analyzed based on tumor and normal tissues in TCGA-BRCA. **(B)** The expression of LINC01521 in breast cancer analyzed based on paired tumor and adjacent normal tissues in TCGA-BRCA. **(C)** Correlation between LINC01521 expression and *TET3* expression in breast cancer. **(D)** Correlation between LINC01521 expression and hsa-miR-29a-3p in breast cancer. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
Correlation analysis between lncRNA and hsa-miR-29a-3p or lncRNA and *TET3* in breast cancer performed by starBase database.

lncRNA	mRNA/miRNA	R value	*p* value
LINC01521	*TET3*	0.313	3.51E-31***
LINC01521	hsa-miR-29a-3p	−0.199	4.93E-11***

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.7. Associations between TET3 and immune checkpoints and immunotherapeutic response

We examined the relationship between *TET3* and immunological checkpoints, such as HAVCR2, TIGIT, CTLA4, LAG3, PDCD1, CD274, PDCD1LG2, and SIGLEC15, to investigate the potential function of *TET3* in tumor immunity.

The findings indicate a significant positive correlation between the expression level of *TET3* and CD274 ($r = 0.244$, $p = 2.23 \times 10^{-16}$), CTLA4 ($r = 0.170$, $p = 1.31 \times 10^{-8}$), HAVCR2 ($r = 0.083$, $p = 0.006$), PDCD1LG2 ($r = 0.282$, $p = 1.28 \times 10^{-21}$), and TIGIT ($r = 0.168$, $p = 1.91 \times 10^{-8}$), whereas a significant negative correlation between *TET3* and SIGLEC15 ($r = -0.117$, $p = 9.51 \times 10^{-5}$) in breast cancer (Fig. 8A–H). Additionally, we evaluated the associations between *TET3* expression and response to immunotherapy using the K-M Plotter database. The high-*TET3* expression group had a higher survival probability than the low-*TET3* expression group in cohorts treated with anti-PD-L1 ($P = 0.0071$) or anti-CTLA-4 ($P = 0.048$), but no significant difference was observed in cohorts treated with anti-PD-1 (Fig. 8I–K).

3.8. Subcellular location and immunohistochemical staining analysis of TET3 in breast cancer

Subcellular location analysis revealed that *TET3* is primarily localized in the nucleoplasm, vesicles, and the MCF-7 cell line cytosol (Fig. 9A and B). We conducted immunohistochemical labeling of *TET3* in a human breast cancer tissue microarray to examine its *TET3*

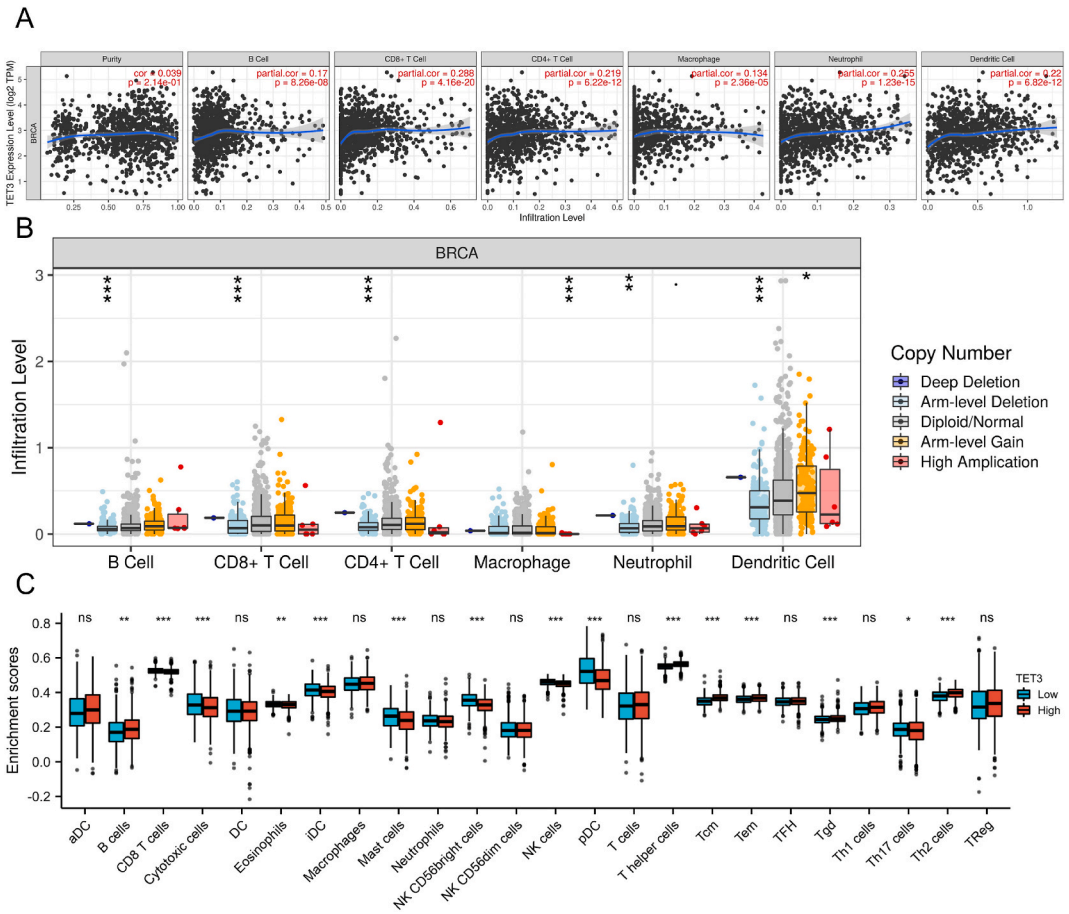

Fig. 7. Correlation between *TET3* expression and tumor immune cell infiltration in breast cancer. **(A)** Correlation between *TET3* expression and infiltration levels of six different immune cells in breast cancer based on TIMER database. **(B)** Infiltration levels of six different immune cells in different *TET3* genetic copy number variation (CNV) groups based on TIMER database. **(C)** Enrichment scores of twenty-four different immune cells in the high and low *TET3* expression groups in breast cancer based on TCGA database. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

function in breast cancer. Fig. 9C–E reveal that tumor tissues had considerably higher *TET3* expression levels than para-tumor tissues based on the staining positive rate and intensity score ($p = 0.025$).

4. Discussion

Global cancer statistics for 2020 have highlighted that breast cancer is the primary malignant tumor affecting women worldwide, with the highest incidence rate and cancer-related mortality [1,2]. Triple-negative breast cancer, a unique molecular subtype that lacks HR and HER2 expression [4], is known for its aggressive characteristics and limited therapeutic options, contributing to poor prognosis [5].

TCGA (cancergenome.nih.gov) data indicate that loss-of-function mutations in all three TET genes were found across many cancer types, demonstrating that TETs serve as tumor suppressor genes [47].

TET2 is mostly expressed in hematopoietic organs, whereas *TET1* is primarily found in the thymus, lungs, and ovary [48,49]. In contrast, *TET3* is abundant in oocytes and fertilized eggs [50] and is highly expressed in several malignant tumors, including AML [13], ovarian cancer [12], osteosarcoma [15], and glioma [14].

Our investigation found that breast cancer tissues expressed *TET3* at greater levels than normal tissues. Moreover, our findings proved that the *TET3* expression was significantly elevated in AML and could function as an independent prognostic factor [51]. Our ROC curve analysis also explained that *TET3* had a high predictive power, with an AUC value of 0.765. The TET protein family catalyzes the oxidation of 5 mC to 5hmC, 5-formylcytosine (5 fC), and 5caC [52–54]. Tumor formation, progression, and invasion can occur due to abnormal methylation of tumor suppressor genes resulting from low levels of 5hmC. Low 5hmC levels are also an important diagnostic indicator for early diagnosis and suggest a poor prognosis in some cancers [55]. Furthermore, some studies have reported increased TET protein and 5hmC levels in breast cancer [56,57]. Tsai et al. discovered that ER-negative breast cancer patients had a worse prognosis when their 5hmC levels were low [18]. Yang et al. reported high l*TET3* and TDG mRNA levels as positive

Table 3
Correlation analysis between *TET3* and biomarkers of immune cells in breast cancer performed by GEPIA2 database.

Immune Cell	Biomarker	R-value	*p*-value
B cell	CD19	0.036	2.40E-01
	CD79A	0.049	1.10E-01
$CD4^+$ T cell	CD4	0.140	5.20E-06***
$CD8^+$ T cell	CD8A	0.088	3.70E-03**
	CD8B	0.059	5.30E-02
M1 Macrophage	NOS2	0.250	1.80E-16***
	IRF5	0.090	2.90E-03**
	PTGS2	0.190	2.30E-10***
M2 Macrophage	CD163	0.089	3.50E-03**
	CD206	0.250	1.80E-16***
	VSIG4	0.048	1.10E-01
	MS4A4A	0.150	3.30E-07***
Neutrophil	CEACAM8	0.063	3.70E-02*
	ITGAM	0.170	9.40E-09***
	CCR7	0.110	2.30E-04***
Dendritic cell	HLA-DPB1	−0.051	9.40E-02
	HLA-DQB1	−0.069	2.30E-02*
	HLA-DRA	0.087	4.30E-03**
	HLA-DPA1	0.058	5.70E-02
	CD1C	0.0026	9.30E-01
	CD141	−0.013	6.60E-01
	NRP1	0.270	3.90E-20***
	ITGAX	0.160	8.70E-08***
NK cell	CD7	−0.022	4.80E-01
	CD57	0.160	7.70E-08***
	KIR2DL1	0.078	9.90E-03**
	KIR2DL3	0.075	1.30E-02*
	KIR2DL4	0.073	1.60E-02*
	KIR3DL1	0.044	1.40E-02
	KIR3DL2	0.088	3.60E-03**
	KIR3DL3	0.049	1.10E-01
	KIR2DS4	−0.0014	9.60E-01
Mast cell	TPSB2	−0.150	4.80E-07***
	TPSAB1	−0.170	3.30E-08***
	CPA3	−0.018	5.40E-01
	HDC	−0.049	1.10E-01
	MS4A2	0.043	1.60E-01
Th1	T-bet	0.073	1.50E-02*
	STAT1	0.330	2.90E-29***
	STAT4	0.120	4.00E-05***
	IFN-γ	0.140	4.00E-06***
	TNF-α	0.210	9.90E-13***
Th2	GATA3	−0.063	3.90E-02*
	STAT6	0.210	2.80E-12***
	STAT5A	0.091	2.80E-03**
	IL13	0.097	1.40E-03**
Th17	STAT3	0.350	1.10E-31***
	IL17A	0.064	3.60E-02*
Tfh	BCL6	0.110	2.00E-04***
	IL21	0.220	2.30E-13***
Treg	FOXP3	0.200	2.70E-11***
	CCR8	0.360	5.50E-34***
	STAT5B	0.210	3.10E-12***
	TGFβ	−0.110	2.70E-04***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

independent prognostic factors in breast cancer patients receiving anthracycline chemotherapy [21]. Based on TCGA-BRCA data, we also examined *TET3* expression and clinical outcomes in breast cancer patients. According to Kaplan-Meier curves, patients with higher *TET3* expression had worse OS, DSS, and PFI rates than those with lower *TET3* expression. Additionally, Cox regression analysis revealed that M stage, age, and pathological analysis were independent predictors. Another study found that the TNM stage was associated with disease-free survival (DFS) and OS in patients receiving anthracycline chemotherapy, and higher *TET3* levels were associated with good DFS, but the correlation with OS was not statistically significant [21]. Therefore, *TET3* could be a novel tumor marker for predicting the diagnosis and clinical outcome prognosis of breast cancer.

We discovered that *TET3* has distinct expression patterns and a propensity for prediction in breast cancer. Therefore, it is essential to understand how *TET3* affects breast cancer. We discovered that *TET3* is concentrated in the cell cycle using GO and KEGG enrichment analyses. Aberrant changes in DNA methylation promote tumorigenesis and chemotherapy resistance [7]. DNA

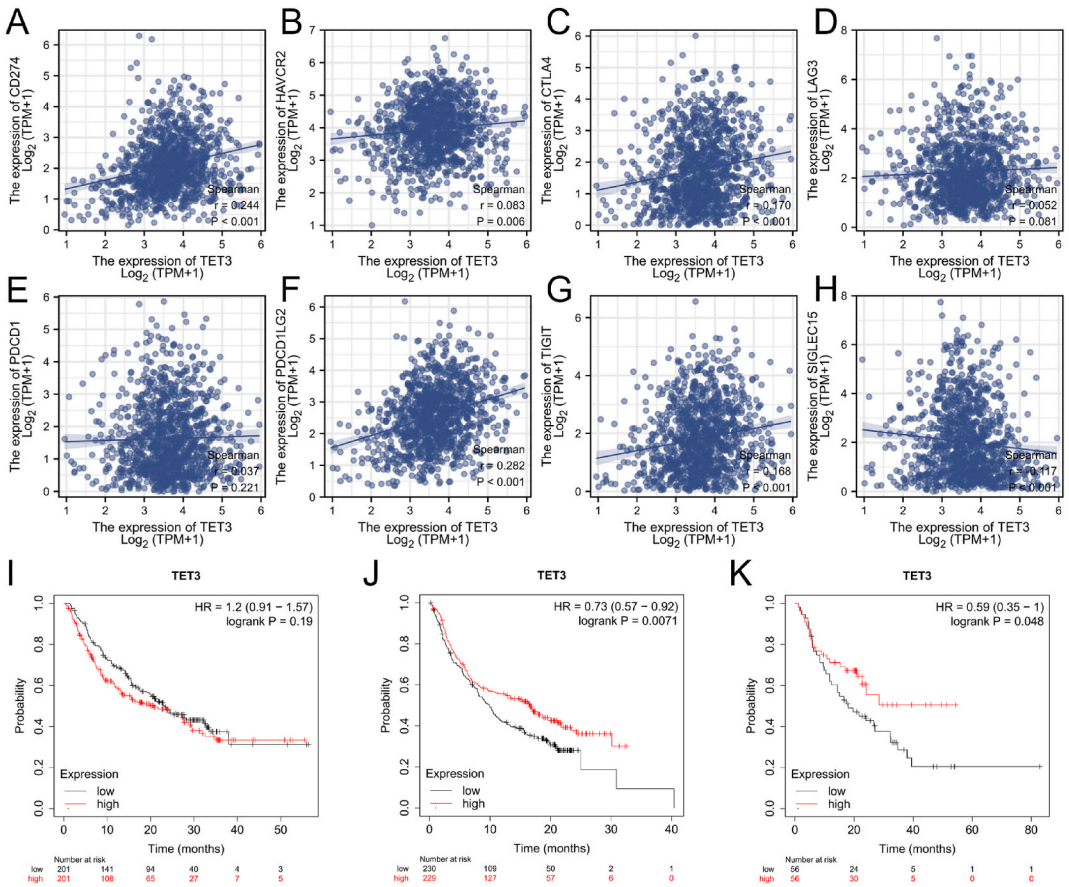

Fig. 8. Relevance to immune checkpoints and immunotherapy efficacy of *TET3* in breast cancer. **(A–H)** Correlation between *TET3* expression and eight immune checkpoint genes based on TCGA-BRCA. **(I–J)** Kaplan–Meier curves for the high and low *TET3* expression groups in cohorts treated with anti-PD-1, anti-PD-L1 and anti-CTLA-4, respectively.

methylation effectively occurs during the S phase of the cell cycle [58,59]. DNA methyltransferase has been approved for treating myelodysplastic syndrome (MDS), chronic monocytic leukemia (CMML), and AML [60,61]. The TET-mediated oxidation of 5 mC to 5hmC, 5 fC, and 5caC activates DNA demethylation in mammals [52–54]. TET catalyzes the oxidative decarboxylation of α-KG using molecular oxygen as a substrate, generating an active high-valent enzyme-bound Fe(IV)-oxo intermediate that converts 5 mC to 5hmC [62].

α-KG activation occurs via isocitrate dehydrogenase 1(IDH1), IDH2, and IDH3, which are activated by isocitrate [63]. Therefore, IDH overexpression can contribute to 5hmc production via TET [64]. Following transcription, microRNAs regulate the mRNA of TET. Currently known microRNAs that regulate TET include the miR-29 family members, regulating *TET1* expression, such as miR-26a, miR-767, miR-494, and miR-520b [65–67]. MiR-26 suppresses TET expression during gastric carcinogenesis [68]. The elimination of the miR-22-induced phenotype by ectopic TET protein expression and the induction of phenotypes similar to miR-22 overexpression in EMT and stemness upon inhibition of TET proteins suggest that the TET family is an important target mediating the role of miR-22 in breast cancer and metastasis [25]. In hepatocytes, miR29a promotes SOCS1-MMP9 signaling axis-mediated tumor metastasis by inhibiting TET proteins [69]. Our analysis revealed that miR-21 directly regulates PTEN and PTENp1 and modulates TET expression, increasing PTENp1 promoter methylation levels [70]. Our findings demonstrate that miR-106, miR-17, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-29a, and miR-300 are associated with the TET expression regulation. In contrast to hsa-miR-29a-3p, we discovered that LINC01521 is significantly expressed in tumor tissues and positively correlates with *TET3*. These results suggest that the LINC01521/hsa-miR-29a-3p/*TET3* axis may be the most promising upstream axis for regulating *TET3* expression in breast cancer.

TET proteins are involved in various developmental processes in immune cells [71]. TET proteins in regulatory T cells (Tregs) can obstruct efficient anti-tumor immunity in cancer patients and anti-cancer immune surveillance in healthy persons, accelerating tumor development [72]. *TET2* and *TET3* in Treg cells are necessary to preserve immunological homeostasis and stability. Mice lacking *TET2* and *TET3* developed inflammatory illness [73]. Yamamoto et al. revealed that TET proteins have more favorable characteristics for immunotherapy in $CD4^+$ T cells and $CD8^+$ T cells than CD4 CD8 double-positive T cells [74].

TET2 and *TET3* expression progressively increase in B cells during maturation and activation. In contrast to T cells [75], similar to T cells, the lack of *TET2* and *TET3* in B cells disrupts B cell homeostasis and spontaneously develops germinal center-derived B-cell

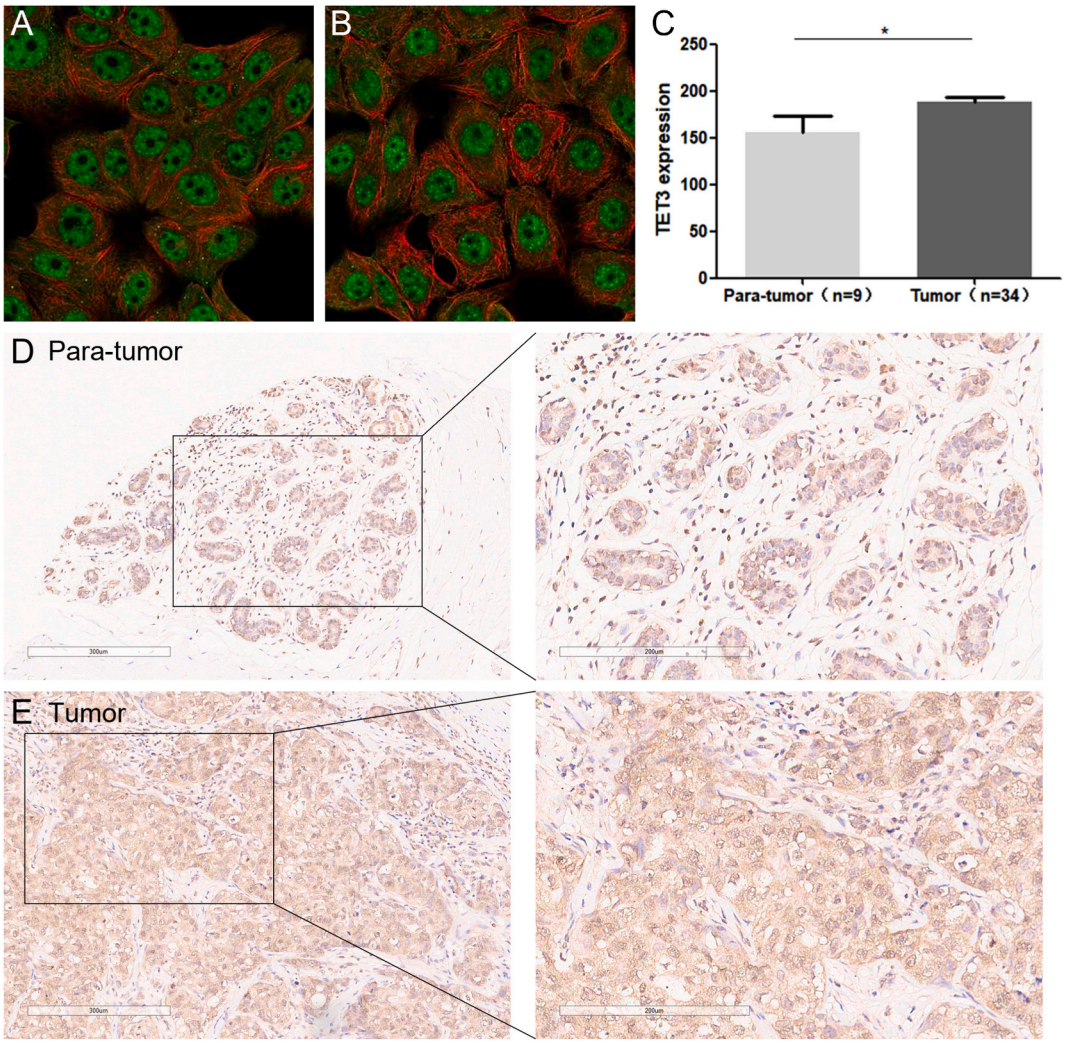

Fig. 9. Subcellular location and immunohistochemical staining analysis of *TET3* in breast cancer. **(A–B)** Subcellular location of *TET3* in MCF-7 cell line based on HPA database. **(C)** Comparison of *TET3* expression in human normal breast tissues and breast cancer tissues. **(D)** Protein expression of *TET3* analyzed by immunohistochemical staining assay in human normal breast tissues. **(E)** Protein expression of *TET3* analyzed by immunohistochemical staining assay in human breast cancer tissues. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

lymphomas [76]. Moreover, the DNA methyltransferase (DNMT1) maintenance is upregulated in TET-deficient B cells, resulting in a minimal but considerable rise in DNA methylation in areas connected to the G-quadruplex and R-loop. Additionally, our analysis of immune infiltration suggested differential *TET3* expression in these cells, demonstrating the critical involvement of TET proteins in B cell growth.

We investigated the relationship between *TET3* expression and immunological checkpoints using the K-M Plotter database to investigate *TET3*'s potential role in tumor immunotherapy. The high-*TET3*-expressing group had a greater likelihood of survival than the low-*TET3*-expressing group in cohorts treated with anti-PD-L1 ($P = 0.0071$) or anti-CTLA-4 ($P = 0.048$), but not in the anti-PD-1 cohort. This observation is consistent with Xu et al. [77]. This study suggests that TET activity might operate as a biomarker to assess the effectiveness of the patient response to anti-PD-L1 treatment. Moreover, our study highlights the possibility of stimulating TET activity as adjuvant immunotherapy for solid tumors. However, our study highlights a potential role for *TET3*, in addition to PD-L1, in anti-CTLA-4 immunotherapy due to a lack of studies on TET in this area.

We investigated *TET3*'s subcellular distribution and discovered it was mostly located in the nucleoplasm, vesicles, and cytosol. Among these, vesicles can transfer their contents between cells [78,79], and exosomes are a particularly active area of research [80]. Exosomes contain various nucleic acids, including mRNA, miRNAs, and other ncRNA [81], which can negatively regulate gene expression. For instance, a recent study found that exosomal miR-231 released from MCF-105A and MDA-MB-10 breast cancer cell lines reduced ZO-1 gene expression in endothelial cells, promoting metastasis in lung cancer and the brain [82]. Moreover, exosomal miR-29a activates immune cells by targeting mRNA and binding to Toll-like receptors (TLRs). Our results support the potential influence of *TET3* on immunotherapy by regulating the miR-29a-3p/*TET3* pathway.

Circulating exosomal miR-21 levels have been studied as biomarkers to estimate the recurrence and mortality of colorectal cancer at the TNM stage [83]. Thus, *TET3* and miR-29a may also function as predictive biomarkers of breast cancer.

This study enhances our comprehension of the association between *TET3* and breast cancer. Nevertheless, some limitations remain. First, most of our results rely on bioinformatic analysis, indicating that few experiments have been conducted to verify our findings. Second, due to technical limitations, we could not build a user-friendly platform for the complex prognostic predictor involving *TET3* and other clinical covariates that can facilitate the wide use of our results for clinicians [84]. In the future, we will focus on constructing such platforms. Third, it is necessary to analyze more immunotherapy-related cohorts or conduct large clinical trials in the future to validate the relationship between *TET3* and response to immunotherapy. Finally, we discovered an upstream regulatory mechanism of *TET3* in breast cancer: the LINC01521/hsa-miR-29a-3p/*TET3* axis. However, the potential regulatory mechanism of *TET3* in carcinogenesis must be validated through in-depth studies.

5. Conclusion

In this study, we investigated the expression, clinical significance, biological function, upstream regulation, and immunotherapeutic features of *TET3* in breast cancer. Our findings revealed that *TET3* was overexpressed in breast cancer and correlated with an unfavorable prognosis. Furthermore, we identified the LINC01521/hsa-miR-29a-3p/*TET3* axis as a potential regulator of *TET3* in breast carcinogenesis. Our findings suggest that *TET3* may play a significant role in regulating the tumor's immune environment, making it a potential predictive biomarker for the effectiveness of immunotherapy.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yiyuan Liu: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jinyao Wu:** Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Lingzhi Chen:** Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Juan Zou:** Software, Formal analysis, Data curation. **Qiuping Yang:** Writing – review & editing, Writing – original draft, Data curation. **Huiting Tian:** Writing – review & editing, Investigation, Formal analysis. **Daitian Zheng:** Writing – review & editing, Writing – original draft, Investigation. **Zeqi Ji:** Writing – original draft, Formal analysis, Data curation. **Jiehui Cai:** Writing – review & editing, Writing – original draft. **Zhiyang Li:** Supervision, Resources, Project administration, Conceptualization. **Yexi Chen:** Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24855.

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