Identification of TMEM178 as a Potential Prognostic Biomarker and Therapeutic Target for Breast Cancer

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Abstract
Background: The transmembrane protein (TMEM) family plays important roles in cancer. However, the expression pattern and biological roles of TMEM178, a member of TMEM family, remains unclear in breast cancer (BRCA).

Methods: Methylation and RNA-seq data were obtained to explore methylation level. Expression of TMEM178, methylation inhibitor 5-Aza-CdR was used to verify the effect of methylation status on the expression of TMEM178. We comprehensively investigated the prognostic outcomes, biological functions and effects on immune cell infiltration of the TMEM178 in BRCA using multiple bioinformatics methods.

Results: The expression of TMEM178 was downregulated and negatively correlated with the level of DNA methylation and DNA methyltransferase (DNMT1, DNMT3A, and DNMT3B) in BRCA. Consistently, TMEM178 mRNA were confirmed to be downregulated, while upregulated in response to treatment with methylation inhibitor 5-Aza-CdR by RT-qPCR. Patients with high expression of TMEM178 have better prognosis and are more sensitive to targeted drug Pazopanib. Immune infiltration analysis showed that the infiltration levels of $CD4^+$ T cell subsets were reduced in BRAC tissues with high TMEM178 expression, and immunosuppressive molecules of T-cell exhaustion were lower expression level.

Conclusion: Hypermethylation of the TMEM178 promoter region was a contributing factor to the downregulation of its expression, and TMEM178 may reflect a prognostic and immunosuppressive situation in BRCA.

Keywords: Methylation modification; $CD4^+$ T subsets; Immunosuppressive molecules

Introduction

The incidence of breast cancer (BRCA) ranks first and the mortality rate ranks fifth, seriously endangering human health (1). However, the exact pathogenesis of BRCA remains unclear. Aberrant methylation of CpG islands in the promoter regions of specific genes have been shown to be closely related to the occurrence and development of BCRA (2). BCRA often display aber-

rant DNA methylation patterns during malignant transformation, hypermethylation of promoter regions of suppressor genes can lead to silencing, whereas hypomethylation of oncogenes can lead to activation (3). Hypermethylation of CpG islands within the promoters of tumor suppressor genes such as PTEN, SFRP2, and BRCA1 represses the expression of these genes, leading to insufficient transcription of target genes involved in cell growth control and apoptosis, which results in uncontrolled proliferation, invasion, and metastasis of BRCA. Therefore, it will provide potential therapeutic targets for BRCA by identifying the key genes involved in the occurrence and development of BRCA and exploring the regulation of methylation modification on its expression.

Transmembrane protein (TMEM) is a type of protein that spans the membrane of a cell or organelle. TMEM family members are aberrantly expressed in tumor tissues or tumor cell lines, such as TMEM45a, TMEM116 and TMEM 7, and they regulate a variety of biological processes, including migration, invasion and apoptosis, thereby affecting the malignant progression of cancer (4). Transmembrane protein 178 (TMEM178, also known as TMEM178A), a member of the TMEM family, is located on chromosome 2p22.1. Previous studies showed the production and accumulation of pro-inflammatory cytokines, and the imbalance of calcium level are important factors to stimulate the progress of cancer (5, 6). TMEM178 reduced the STIM1 puncta formation and the production of inflammatory cytokines IL-6, IL-1 β and TNF α, resulting in impairment of inflammatory response in cancer (7, 8). Concurrently, TMEM178 can also act as a downstream effector of Plcγ2, negatively regulating Ca^{2+} flux and affecting intracellular calcium concentration thereby promoting immune response and inhibiting cell proliferation (5, 9). As shown in the study by Carvalho et al (10), TMEM178 formed fusion genes with DHX57 and MAP4K3 in paediatric high-grade glioma (pHGG) and regulated the proliferation of cancer cells. However, the expression of TMEM178 in BRCA and whether it is regulated

by the level of DNA methylation at promoter regions are unclear.

We aimed to explore the expression of TMEM178 in cancers, and the effects of DNA methylation, methyltransferase and m6A modification on the expression of TMEM178. The role of TMEM178 in tumor immune infiltration, prognosis and drug sensitivity were studied by using multiple bioinformatics tools. Our study facilitated an understanding of the roles of TMEM178 in cancers.

Materials and Methods

Identification of expression levels and functional studies on TMEM178

The expression of TMEM178 in cancers were detected using Oncomine and TIMER based on TCGA cohorts (11, 12). TMEM178 expression in these cancers with few normal samples in the TCGA database was further analysed used the GEPIA2 (13).

To further evaluate the potential functional mechanism of TMEM178, we obtained the top 100 related genes with TMEM178 based on Pearson's correlation coefficients using LinkedOmics database (14). We also performed functional enrichment analysis of the above related genes used GSEA.

Explored the epigenetic factors affect TMEM178 expression

We first used UALCAN (15) to explore the methylation levels of TMEM178 promoter region in different tumors and normal tissues. Subsequently, analyzed the correlation between the expression and methylation levels for TMEM178 in DNMIVD (16). MEXPRESS (17) further analyzed the relationship between the expression of TMEM178 and the methylation status of cpG sites in the promoter region.

DNA methylation is typically mediated by DNA methyltransferases. The expression levels of three typical DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) were obtained from the UALCAN database. The correlation between

these three methyltransferases and TMEM178 expression was evaluated by GEPIA2.

The m6A modification sites of TMEM178 were predicted via SRAMP (18). The correlation between the expression of TMEM178 and the expression of 24 m6A-related genes was analyzed by LinkedOmics. Further divide TCGA-BRCA samples into high and low expression group according to the "Median". The expression of 24 m6A-related genes were analyzed in high and low expression group.

Cell lines and cell culture

BRCA cell lines (MCF-7, MDA-MB-231 and HCC-1937) and normal breast epithelial cell (MCF10A) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). MDA-MB-231 and MCF7 were cultured in DMEM whereas HCC-1937 were grown in RPMI-1640 in 5% CO_2 at 37 °C. Both DMEM and RPMI-1640 medium were supplemented with FBS, penicillin and streptomycin (Biological Industries, Israel). Mammary Epithelial Cell Growth Medium (MEGM; Gibco, USA) was adopted for cultivating MCF-10A.

RT-qPCR

Total RNA was extracted by TRIzol (Invitgen, USA) and reverse transcribed into cDNA by the PrimeScript RT reagent kit (Takara, Japan). RT-qPCR was performed in triplicate in the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using TB Green Premix Ex Taq (Takara, Japan). The expression of target genes was normalized to that of GAPTH. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level. PCR primer pairs were synthesized by Sangon Biotech (Shanghai, China), and the additional File 1 lists the primer sequences.

5-aza-2'-deoxycytidine treatment

MDA-MB-231 were plated (1×10^6 cells/mL) and treated for 3 d with 5-aza-2'-deoxycytidine (5-aza-CdR ,1 μM, 5 μM, and 10 μM; MedChemExpress, USA) or left untreated for an equivalent time. After treatment for 3 days, total RNA was isolated.

Evaluated the clinical value of TMEM178 prognostic risk model in predicting the prognosis

Relationship between TMEM178 expression and overall survival (OS) in patients were analyzed using the HPA (19). The expression level of TMEM178 was searched in GSE1456 to determine its relationship with disease-free survival (DFS). In addition, the prognostic values of TMEM178 expression were further verified by displaying the OS and DFS using the Kaplan-Meier plotter (20).

SurvivalMeth (21) was used to analyze the effect of DNA methylation of TMEM178 on prognosis. The prognosis value of CpG sites on TMEM178 was analyzed by MethSurv (22).

Relationship between TMEM178 and immune cell infiltration in the tumor microenvironment

ssGSEA (23) was performed to assess the level of tumor-infiltrating immune cells in BRCA. Relationship of TMEM178 expression with immunosuppressive molecules was assessed using TISIDB.

Drug sensitivity analysis

Downloading the drug data of NCI-60 cell lines from the CellMiner database (24). Calculating the correlation coefficient between TMEM178 expression and drug sensitivity. In addition, based on the GDSC (25), the drug response of TCGA-BRCA samples was predicted by using R package "pRRophic".

Statistical Analysis

Continuous variables that conformed to the normal distribution were compared with the use of an independent t-test for comparison between groups, while continuous variables with skewed distribution were compared with the Mann-Whitney U test. All statistical data analyses were performed using Graphpad prism software (ver.8.0) except for the online analysis tool.

Results

Identification the expression of TMEM178 in pan-cancer

To determine the differences of TMEM178 expression in tumor and normal tissues, we first performed a pan-cancer analysis using the Oncomine and TIMER database. We observed lower expression of TMEM178 in tissues from 8 tumor types, including BRCA, GBM, KICH, KIRC, KIRP, LUAD, LUSC, and THCA. In contrast, TMEM178 was present at significantly higher levels in CHOL and PRAD (Fig.1A-B). Next, we performed a similar analysis using the GEPIA2. The expression of TMEM178 was down-regulated in 8 tumor types including BRCA, which was consistent with the results of TIMER, but the expression of TMEM178 was not up-regulated in CHOL and PRAD (Fig.1C).
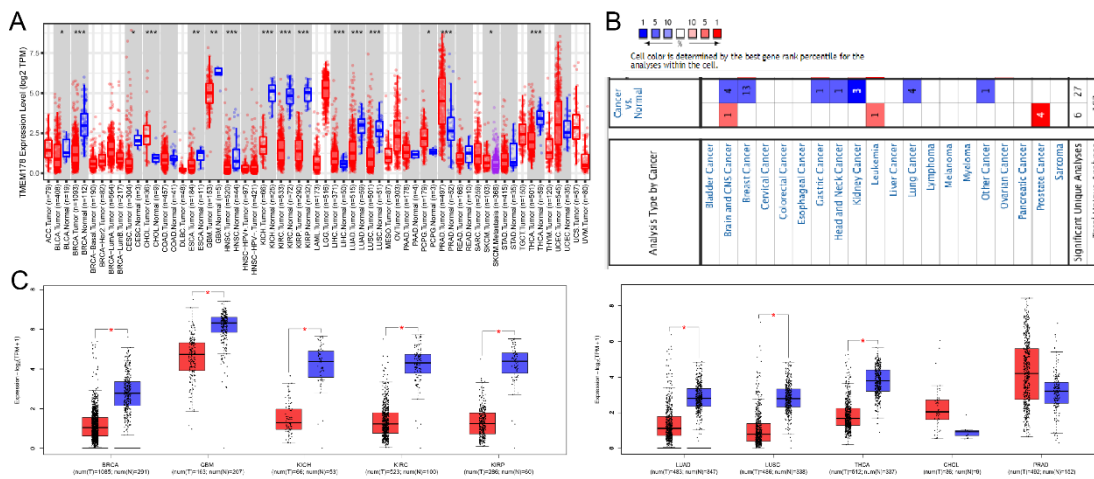
Fig. 1: **The expression levels of TMEM178 in different types of cancers compared to normal tissues.** A: The expression levels of TMEM178 in different type of tumors and normal tissues in TIMER. B: Oncomine showed the expression of TMEM178 in different tumor tissues. C: The TMEM178 expression levels in multiple tumor tissues and normal tissues in the GEPIA2. *$P<0.05$

## Explored the epigenetic DNA methylation on the regulation of TMEM178 expression

A significant increase in the methylation level within promoter regions of TMEM178 in BRCA, KIRC, KIRP, LUAD, LUSC and THCA (Fig. 2A). Additionally, there were negative correlations between TMEM178 expression and promoter methylation status in BRCA, KIRC, KIRP, LUAD and THCA (Fig. 2B). Methylation status of multiple CpG sites in the promoter region were identified to be negatively correlated with the expression of TMEM178, including 3 CpG sites for BRCA and KIRC, 4 for LUAD and THCA and 5 for KIRP, as shown in Supplementary Table 1. The methylation of the TMEM 178 may influence its expression in these several cancer types.

DNA methylation is generally catalyzed by typical DNA methyltransferase (DNMT1, DNMT3A, and DNMT3B). Therefore, assessed the relationship between TMEM178 expression and three DNA methyltransferases. Only in BRCA, the expression of TMEM178 was negatively correlated with all three DNA methyltransferases (Table 1). Consistently, these DNA methyltransferases were highly expressed in BRCA (Fig. 2C). The expression of TMEM178 in BRCA is negatively correlated with the level of DNA methylation in the promoter region and the expression of DNA methyltransferase, we further studied the relation-

ship between the expression of TMEM178 and DNA methylation in BRCA.
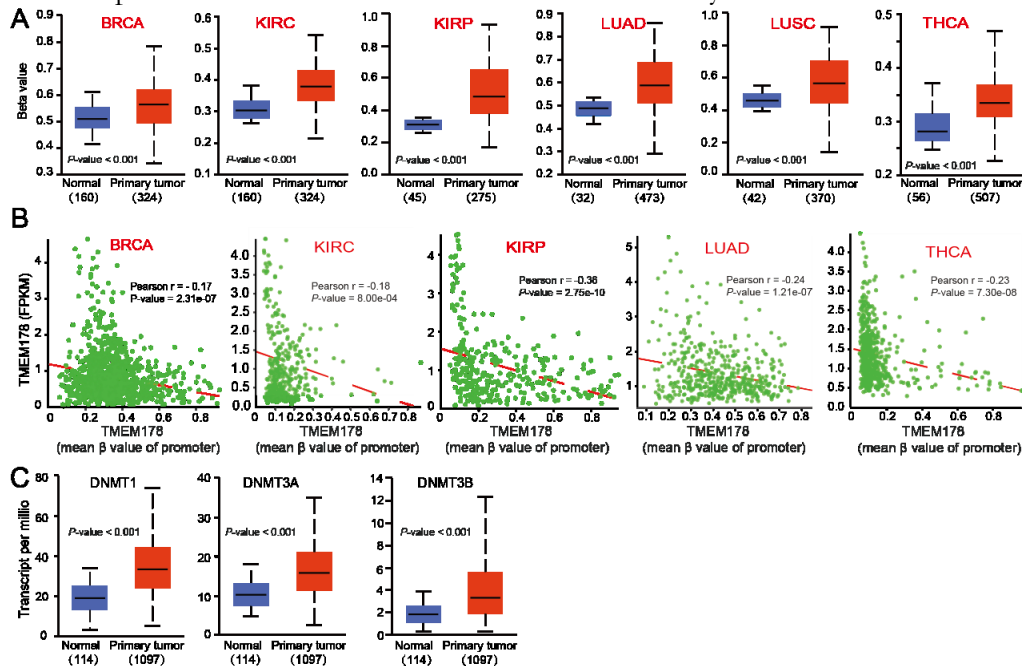
Fig. 2: The relationship between the expression of TMEM178 and its methylation and DNA methyltransferase. A: Promoter methylation of TMEM178 in cancers and normal tissues. B: The correlation between methylation and expression of TMEM178. C: The expression of DNMT1, DNMT3A, and DNMT3B in BRCA

Table 1: Correlation between TMEM178 and DNA Methyltransferase

DNMTs	BRCA		KIRP		KICH		LUAD		THCA	
	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*
DNMT1	-0.14	2.2E-06	-0.17	0.0028	0.059	0.58	0.15	0.00031	-0.044	0.3
DNMT3a	-0.12	1.7E-05	0.032	0.57	0.61	1.5E-10	0.28	3.6E-11	0.24	3.8E-09
DNMT3b	-0.15	3.1E-07	-0.17	0.0025	0.19	0.075	0.064	0.14	0.52	0

Experimental verification of the effect of DNA methylation on TMEM178 expression

The expression of TMEM178 in BRCA cells (MCF-7, MDA-MB-231 and HCC1937) was dramatically down-regulated than in normal breast epithelial cell (MCF-10A) (Fig.3A). To determine whether hypermethylation of the TMEM178 promoter inhibited transcription, the expression was compared before and after treating cells with the methylation inhibitor 5-Aza-CdR. After treatment with 5-Aza-CdR, expression of TMEM178 was increased obviously (Fig. 3B).

RT-qPCR was performed to quantify the mRNA expression levels of DNMT1, DNMT3a, and DNMT3b. An increase in mRNA of DNMT1,

DNMT3a and DNMT3b was observed in BRCA cells (Fig. 3C).

### Explored the epigenetic m6A modification on the regulation of TMEM178 expression

SRAMP predicted three high confidence m6A sites in TMEM178 mRNA sequences (Fig. 4A). We further explored the relationships between TMEM178 expression and the expression of 24 m6A-related genes. The correlation analysis revealed that TMEM178 was significantly negatively correlated with the level of m6A writer gene (RBM15) and 8 m6A Reader genes (YTHDF1, YTHDF3, IGF2BP1, IGF2BP2, IGF2BP3, HNRNPA2B1, FMR1 and LRPPRC) (Fig. 4B). LinkedOmics database was utilized to further

verify the relationship between TMEM178 expression and the above m6A-related genes (Supplementary Fig.1). The above m6A-related genes may affect the m6A modification site of TMEM178 and regulate its expression.
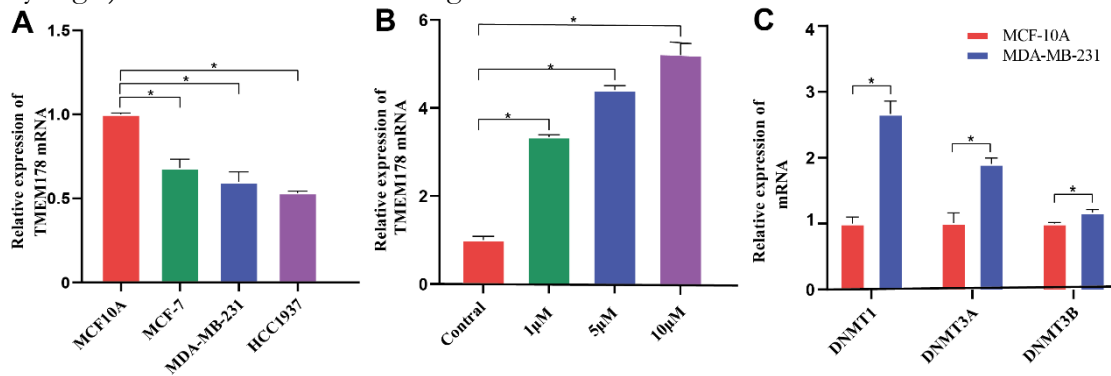
Fig. 3: The expression of TMEM178, DNMT1, DNMT3A, and DNMT3B. A: The expression of TMEM178 in MCF-7, MDA-MB-231, HCC1937 and MCF-10A. B: The expression of TMEM178 in MDA-MB-231 with and without 5-Aza-CdR treatment. C: The expression of DNA methyltransferase DNMT1, DNMT3A and DNMT3B.*$P < 0.05$
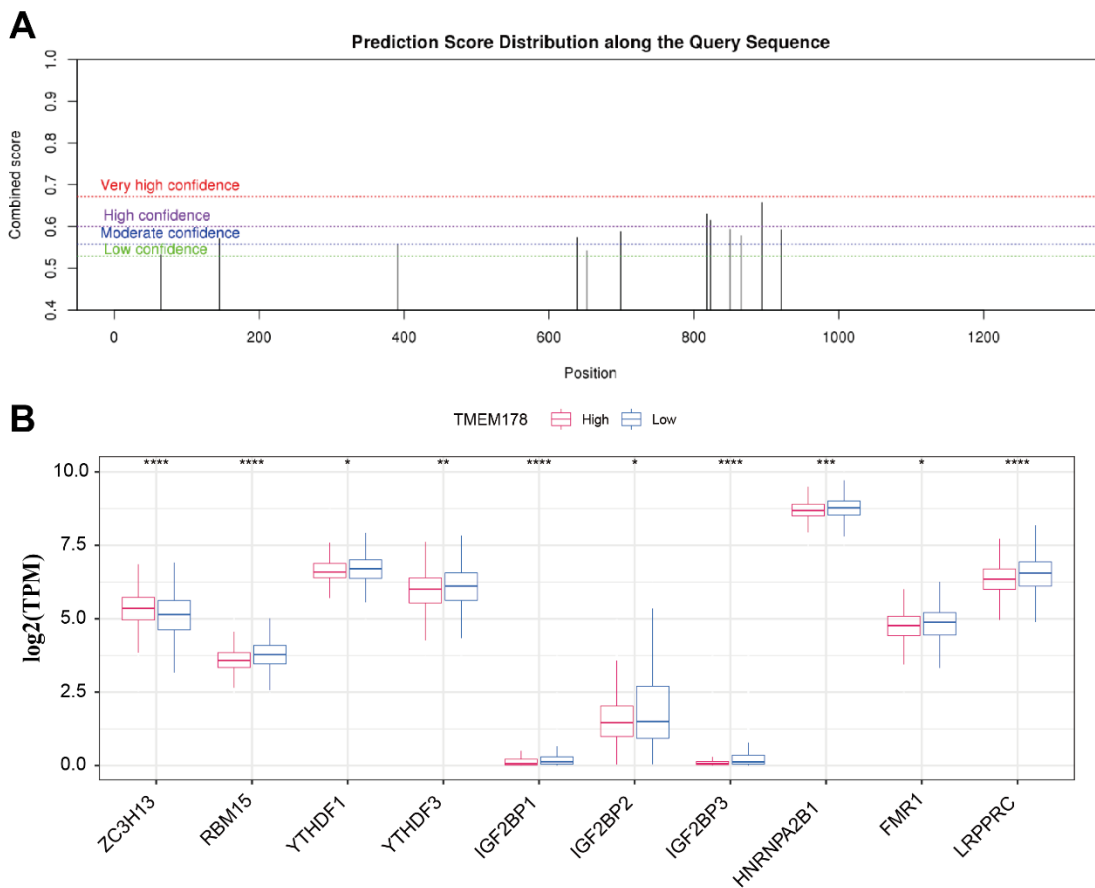

Fig. 4: m6A modification regulates the expression of TMEM178. A: Prediction score of m6A distribution in TMEM178 mRNA sequence. B: The differential expression of m6A associated genes in the high and low TMEM178 expression groups

Evaluated the clinical value of TMEM178 expression/DNA methylation in predicting the prognosis

To determine the prognostic value of TMEM178 expression and methylation status in BRCA, we performed Kaplan-Meier analyses. Both the OS (Fig. 5A) and RFS (Fig. 5B) were markedly lower in low TMEM178 expression group. Similarly, Kaplan-Meier Plotter results also illustrated that low TMEM178 expression was correlated with a poor OS and RFS (Fig. 5C, D). Next, we investi-gated the correlation between the prognostic values and DNA methylation of TMEM178. The statistical analysis revealed that patients in hypo-methylation group had higher OS rate (Fig. 5E), the hypermethylation of CpG cg04104114 was significantly associated with poor prognosis of patients (Fig. 5F).

Taken together, TMEM178 was closely related to the prognosis of patients, which can potentially be used as a biomarker for the BRCA.
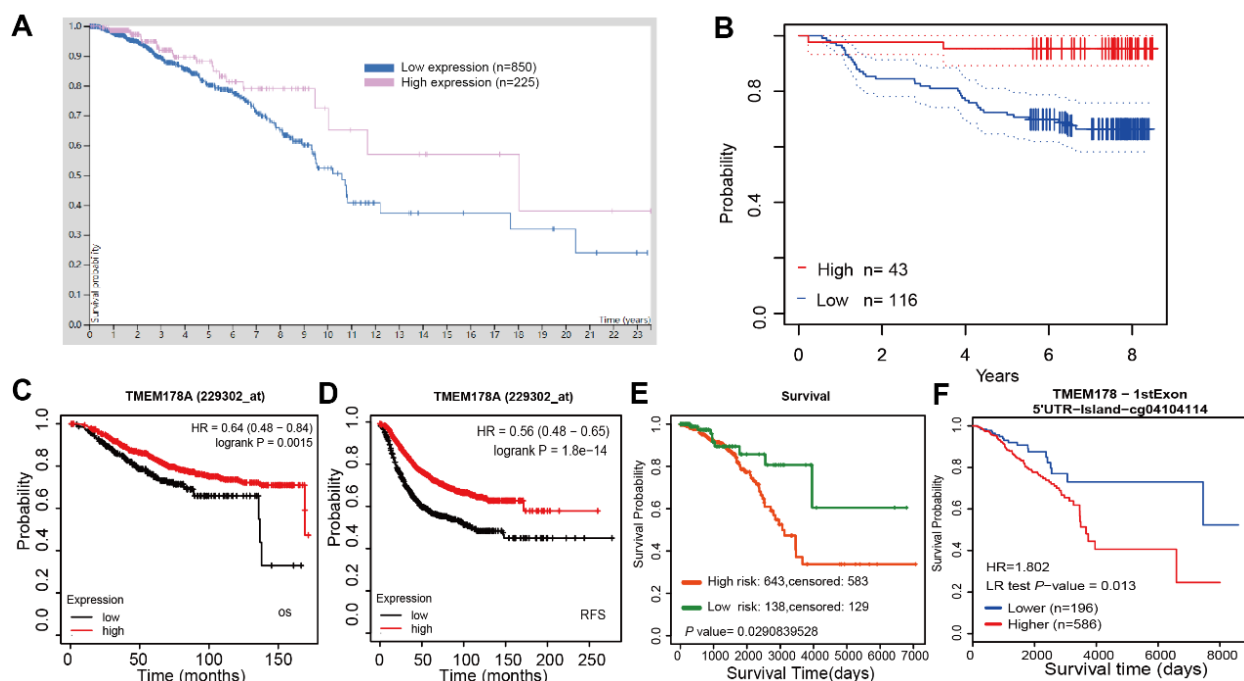

Fig. 5: Prognostic value of the TMEM178 expression/DNA methylation in BRCA. A: Survival curves of OS based on HPA. B: RFS survival curves in GSE1456. C, D: OS and RFS using Kaplan-Meier Plotter. E: OS survival curves of hypomethylation and hypermethylation of TMEM178. F: The OS of cg04104114 of TMEM178 in BRCA

Correlation Between TMEM178 and immune characteristics of TME

We explored whether TMEM178 can affect im-mune infiltration levels in BRCA. The results showed TMEM178 reflected various CD4$^+$T cell subsets infiltration status. TMEM178 expression was significantly negative associated with the in-filtration abundances of Act CD4$^+$T, Treg, Tfh and Th2. Next, we found that the expression of TMEM178 was significantly higher in NK cells and significantly lower in MDSCs with immuno-suppression (Fig 6A). In addition, we explored the relationships between TMEM178 and immu-nosuppressive molecules. TMEM178 was nega-tively correlated with CD274, CTLA4, HAVCR2, IDO1, LAG3 and TIGIT (Fig 6B). TMEM178 may be related to the immunosuppressive micro-environment.
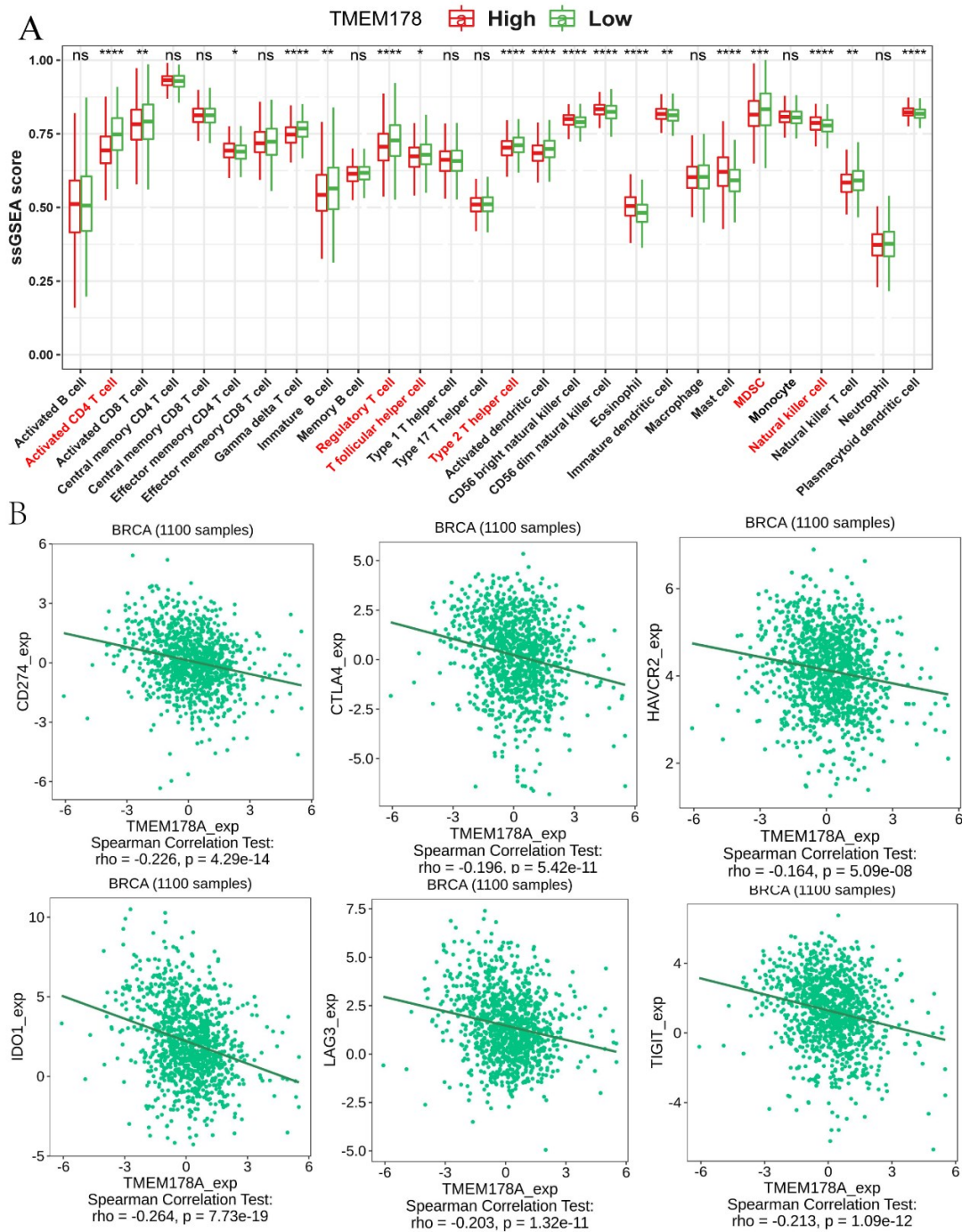
Fig. 6: Relationship of TMEM178 with immune infiltration in BRCA A: The correlation between TMEM178 and tumor-infiltrating lymphocytes. B: The correlation between TMEM178 and immunosuppressive molecules

Identification the biological function of TMEM178 transcription/DNA methylation in BRCA

We collected the first 100 genes associated with TMEM178 expression and methylation levels

(Fig.S2). GO BP analysis identified distinct biological processes were enriched in related genes of TMEM178, with the most prominent being the pathways related to cell cycle, such as negative regulation of cell cycle process, mitotic cell
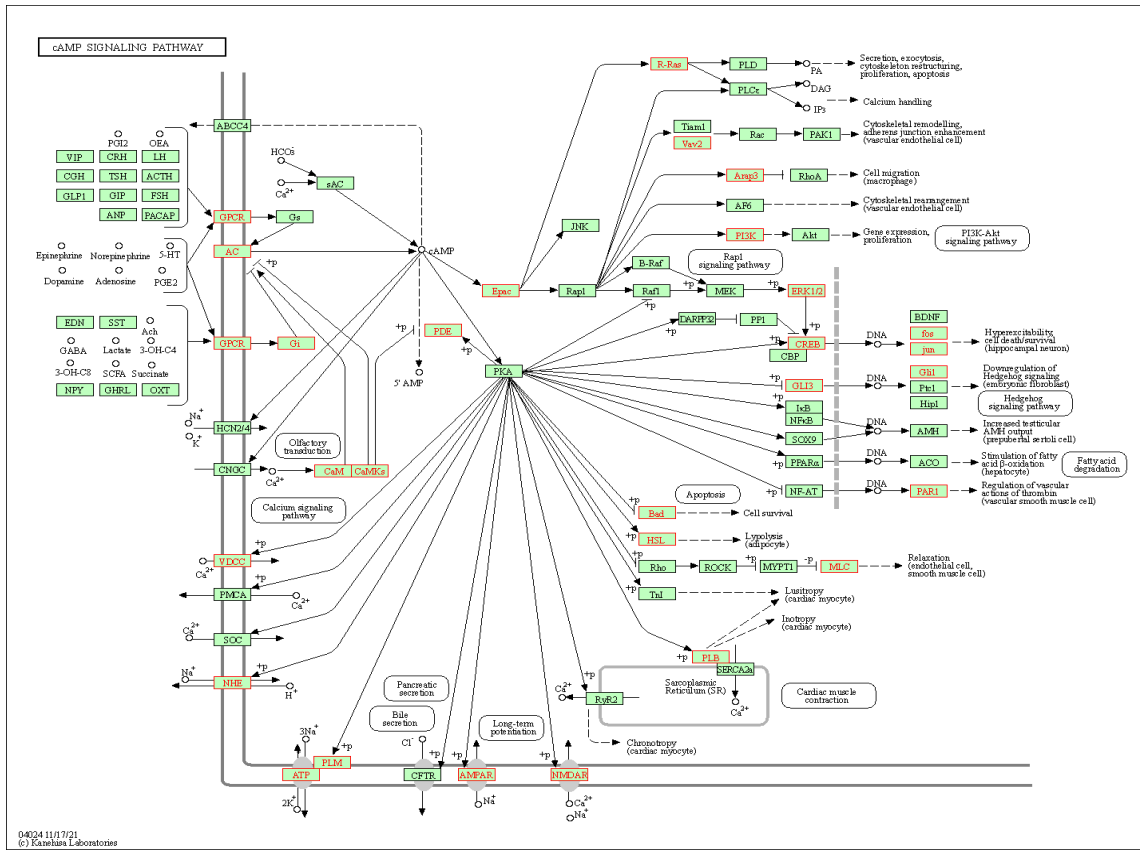
cycle phase transition and cell cycle G2/M phase transition (Fig. 7A-B). These results suggested TMEM178 was a potential cell cycle regulator. GSEA analysis also has consistent results (Fig. 7C).

KEGG analysis revealed that the related genes of TMEM178 were significantly enriched in cAMP signaling pathway (Fig. 8).

Relationship between TMEM178 expression and drug sensitivity

The expression of TMEM178 was negatively correlated with the drug sensitivity of Pazopanib, Clofarabine and Tandutinib (Fig. 9A). In addition, BRCA patients with lower TMEM178 DNA methylation and higher espression had lower Pazopanib IC50 (Fig. 9B, C). TMEM178 was associated with drug sensitivity to Pazopanib in BRCA patients.

Fig. 7: GO Analysis (Biological process, BP) of genes connected with TMEM178 expression/DNA methylation. A: BP of genes that correlated with TMEM178 expression. B: BP of genes that correlated with TMEM178 DNA methylation. C : GSEA analysis of genes that correlated with TMEM178 expression

Fig. 8: The related genes of TMEM178 were enriched in cAMP signaling pathway shown in red
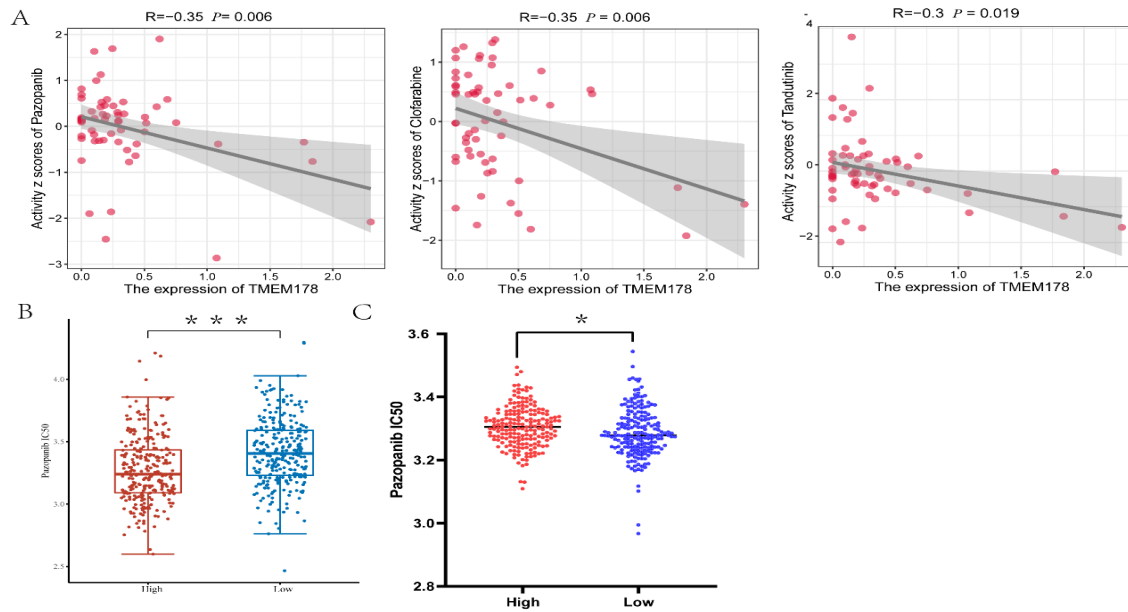

Fig. 9: Analysis of TMEM178 and drug sensitivity. A: The correlation between IC50 of Pazopanib, Clofarabine and Tandutinib and TMEM178. B: The relationship between TMEM178 and drug sensitivity. C: The relationship between methylation level of TMEM178 and drug sensitivity

Discussion

BRCA is the most common malignant tumor, and its morbidity and mortality are increasing gradually in recent years. However, the molecular pathogenesis of BRCA remains poorly defined due to its heterogeneity. Studies have shown that abnormal expression of genes are considered to be a key factor leading to the carcinogenesis of various tumors, including BRCA (26). It was reported to be abnormally expressed of TMEM in tumor tissues and was closely related to tumor progression and prognosis in tumors (27). TMEM178 also belongs to the TMEM family. Carvalho et al (10) reported that it formed fusion genes with DHX57 and MAP4K3 in pHGG and regulated the proliferation of cancer cells. However, TMEM178 has been rarely reported in BRCA. In our study, the expression of TMEM178 was downregulated in BRCA, and patients with high TMEM178 expression had a relatively better prognosis, it's a potential prognostic marker of BRCA.

Hypermethylation of CpG island in the promoter region has been identified as an important cause for downregulation of TMEM expression (4). In our study, the promoter region of TMEM178 was hypermethylation and negatively correlated with expression in breast cancer. Cell treatment with DNA methylation inhibitor 5-aza-CdR up-regulates expression of TMEM178. TMEM178 expression was inactivated by aberrant CpG island DNA hypermethylation in its promoter region. In a variety of tumors overexpression of DNMTs results in hypermethylation and oncogenic activation (28). We demonstrated a high expression of DNMT1, DNMT3A and DNMT3B, and negatively correlated with the expression of TMEM178. These results further reveal overexpressed DNMTs may mediate the downregulation of TMEM178 expression in BRCA.

Gene dysregulation is an important reason for drug resistance of cancer (29). Thus, to identify and intervene the key factors that induce drug resistance is very important to improve drug sen-

sitivity. Pazopanib is an oral small-molecule tyrosine kinase inhibitor, play an anticancer role by primarily inhibits both angiogenic and oncogenic signaling pathways (30). Pazopanib-resistance sarcoma cells have reduced lncRNA HAR1B expression and lncRNA HAR1B over-expression leads to increased sensitivity of pazopanib (31). In this study, BRCA patients with low TMEM178 methylation level or high expression of TMEM178 might be more sensitive to pazopanib agents. We speculated that the up-regulation of methylation in the promoter region of TMEM178 leaded to down-regulation of its expression, thus reducing the sensitivity of BRCA patients to Pazopanib. TMEM178 may be a marker of Pazopanib for individualized medication and efficacy monitoring.

The TME is in an immunosuppressive state associated with a severe dysregulation of the immune response through numerous mechanisms, including accumulation of abundant immunosuppressive cytokines, presence of immunosuppressive cells (32). We studied the relationship between TMEM178 and immunosuppressive cells. The low expression of TMEM178 was tightly associated with the increased infiltration of MDSCs and decreased infiltration of NK cell. Research shows that negative effect on anti-tumor activity of immune system is exerted by MDSCs, while NK cells can effectively enhance anti-tumor immunity (33). It suggested the expression of TMEM178 may regulate anti-tumor immunity by affecting the infiltration level of MDSCs and NK in BRCA. Some CD4$^+$ T cell subsets, particularly Th2 and Tregs, are known to negatively affect the antitumor response by decreasing antigen presentation and dampening T-cell effector functions, respectively (34). Our results then presented that when TMEM178 was highly expressed in BRCA, the infiltration level of Treg, Tfh and Th2 significantly reduced. Besides, we found that the expression of TMEM178 was negatively correlated with the expression of many inhibitory immune checkpoints, including CD274, CTLA4, HAVCR2, IDO1, LAG3, PDCD1LG2 and TIGIT, while studies showed that inhibitory immune checkpoints were related to T-cell exhaustion

(35). Restoration of exhausted CD4^{+} T-cell function by checkpoint blockade may contribute significant clinical benefit in tumors, by improving direct CD4^{+} antitumor activity (36). Successful anti-cancer therapies have been associated with reductions in the level of these markers on the surface of CD4^{+} T-cells (37). These results suggested that TMEM178 could be a promising immune-related therapeutic biomarker in BRCA.

Conclusion

The downregulation of TMEM178 expression in BRCA was associated with hypermethylation of promoter region by bioinformatics analysis and in vitro experiments. In addition, this study also provides evidence that TMEM178 is associated with patient prognosis, cell cycle, drug sensitivity and tumor-immunity in BRCA.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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