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ORIGINAL RESEARCH

Comprehensive Insights Into the Role of TRPM4 in Pan-Cancer Progression and Immune Regulation

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Background: Transient receptor potential channel subfamily M member 4 (TRPM4) is a non-selective Na^+ permeable ion channel that regulates disease processes by enhancing sodium entry and membrane depolarization, but its role in tumors remains under-explored. The purpose of this study is to investigate the role of TRPM4 in pan-cancer progression and immune regulation.

Methods: The pan-cancer mRNA expression information of TRPM4 was obtained from TCGA and GTEx, and the protein expression information of TRPM4 was obtained from HPA database. STRING database was utilized to construct the protein-protein interaction network of TRPM4. Gene characterization of TRMP4 was analyzed by GSCA database. The relationship between TRPM4 and immune infiltration characteristics in pan-cancer was analyzed using TCGAplot. Multiple bulk RNA-seq and scRNA-seq datasets treated with PD-(L)1 were used to analyze the relationship between TRPM4 and immunotherapy response. Immunohistochemistry (IHC) and multiplex immunofluorescence (mIHC) were used to validate the expression of TRPM4 in tumor tissue from 19 lung adenocarcinoma patients in relation to the characteristics of immune cell infiltration. In vitro experiments were performed to validate the role of TRPM4 in human breast, lung adenocarcinoma, and esophageal cancer.

Results: TRMP4 expression is higher in most tumors than in normal tissues, and the association with prognosis varies with cancer type. TRPM4 correlates with multiple immune checkpoints as well as the degree of immune cell infiltration. Multiple datasets of anti-PD-(L)1 treatment suggested that high expression of TRPM4 was associated with worse treatment prognosis. The IHC and mIHC found that TRPM4 expression was negatively correlated with the level of M1 macrophage and T cell infiltration. *In vitro* experiments confirmed that knockdown of TRPM4 inhibited proliferation, invasion and migration of human breast, lung and esophageal cancer cells.

Conclusion: TRPM4 plays a complex role in tumor progression and immunotherapeutic response, and targeting TRPM4 may offer promising strategies for inhibiting tumor progression and improving immunotherapy resistance.

Keywords: TRPM4, pan-cancer, biomarker, immunotherapy

Introduction

Malignant tumors, as a serious challenge in global public health, show a continuous increase in their disease burden. According to the global cancer statistics for 2022 released by the International Agency for Research on Cancer of the World Health Organization, there will be 20 million new cases of malignant tumors and nearly 10 million deaths throughout the year.[1] There are significant differences in the cancer spectrum among countries with different levels of geographic and economic development, which are significantly correlated with factors such as accelerated population aging, lifestyle changes, and exposure to environmental carcinogens.[2] Targeted therapies and immunotherapy have profoundly changed clinical practice, and these advances have significantly improved the prognosis of some tumor

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patients, but their efficacy is limited by specific molecular biological features.[3] Therefore, the development of pan-tumor targets can help overcome the limitations of current therapeutic strategies.

Transient receptor potential cation channel subfamily M member 4 (TRPM4) belongs to the Melastatin subfamily members of the TRP channel superfamily and belongs to a calcium-activated, nonselective ion channel, but it does not allow the passage of calcium ions, but rather, when activation, it allows the influx of Na ions thereby altering the membrane potential and lowering the resistance to calcium flow to regulate the calcium levels in its cells.[4] On this basis, TRPM4 can regulate the progression of a variety of diseases, such as cranial brain injury, multiple sclerosis, diabetes, and tumors.[5] Recently Wan et al found that utilizing the compound NC1, which targets TRPM4, can sustainably activate Na ion influx, which in turn induces cellular necrosis characterized by Na overload, also known as NECSO.[6] In addition, several studies have found that NaCl in tumor tissues improves metabolic adaptation and cytotoxicity of CD8T cells and promotes tumor regression.[7,8] A high salt diet (HSD) has been reported to alter $CD4^+$ T cell differentiation, tending to give rise to helper T cell 17 (TH17 cells).HSD reduces the immunosuppressive capacity of myeloid suppressor cells by affecting the differentiation process, thereby enhancing the anti-tumor immune response and leading to slower tumor growth.[9] NaCl modulates the immune in a variety of contexts responses, highlighting its multifaceted immunomodulatory role in cell activation, differentiation and effector functions. And TRPM4, as a key carrier of cellular Na^+ transport, may play an important role in this process.

Past studies have preliminarily revealed the role of TRPM4 in a part of tumors, such as increased TRPM4 expression is closely associated with malignant progression of prostate cancer,[10] and higher levels of TRPM4 are associated with a greater likelihood of recurrence after prostate cancer surgery, suggesting its importance.[11] However, TRPM4 has not been well studied in tumors, and there is a need to fully parse it, especially its role in cancer immunity. Here, we elucidate the significance of TRPM4 in tumors from several aspects based on a series of bioinformatics tools. In vitro experiments confirmed that TRPM4 knockdown was associated with the progression of breast, lung adenocarcinoma, and esophageal squamous carcinoma. Multiplex immunofluorescence confirmed that high expression of TRPM4 was closely associated with lower levels of T cells and M1 macrophages. In conclusion, our results suggest that TRPM4 is an important pro-carcinogenic factor and plays a crucial role in the immune response. It may become a key molecule in assessing the response to immunotherapy.

Materials and Methods

Expression Analysis

Information on TRPM4 expression in normal tissues was obtained from GTEx (https://gtexportal.org/home/). Differential expression of TRPM4 between tumor and normal tissues was obtained by the TCGAplot package.[12] Immunohistochemical information of TRPM4 was obtained from HPA (http://www.proteinatlas.org/).

Protein-Protein Network Construction and Functional Analysis

TRPM4-related protein-protein network co-expression network constructed in STRING database (https://string-db.org/), and the 20 genes with the strongest correlation with TRPM4 were enriched and analyzed. Correlation analysis of TRPM4 expression with pathways involved in tumor progression-related to GSCA[13] (https://guolab.wchscu.cn/GSCA/#/).

Genome Characterization Analysis

Information on copy number variation (CNV), single nucleotide variation (SNV), and methylation levels of TRPM4 in different tumors was obtained from GSCA. TRPM4 gene mutation data were obtained from cbioportal[14] (https://www.cbioportal.org/).

Chemotherapy Drug Sensitivity Analysis

Correlation of TRPM4 expression with different agents Data from GSCA. The drug information in this database was obtained from Genomics of Drug Sensitivity in Cancer (GDSC) and The Cancer Therapeutics Response Portal (CTRP), and the correlation between the expression of TRPM4 and the IC50 of different agents was calculated.

Analysis of Immune Characteristics

Correlation analysis between TRPM4 and immune cell infiltration /immune-related molecules in different tumors performed by TCGAplot package.[12]

Immunotherapy Dataset Validation

Bulk RNA-seq from patients with tumors treated with ICIs was collected from published articles, including ccRCC (n=181),[15] melanoma (n=51, n=153, n=91).[16–18] For animal models treated with ICIs data information from TISMO[19] (http://tismo.cistrome.org).

Single-Cell Sequencing Data Analysis

The scRNA-seq of LUAD (n=6),[20] LUSC (n=6),[20] and CRC (n=39)[21] patients previously treated with ICIs was routinely analyzed using Seurat,[22] including combining samples, removing batch effects, PCA dimensionality reduction, and UMAP dimensionality reduction. T cells, NK cells, B/plasma cells, neutrophils, myeloid cells, fibroblasts, mast cells, endothelial cells, epithelial cells were identified according to known markers. The relationship between TRPM4 expression and pathological responses to ICIs in different datasets was analyzed.

Patient Tumor Specimen Collection

We collected tumor tissue from 19 patients with LUAD who had no distant metastases detected by routine imaging and underwent surgery. All patients signed a paper-based informed consent form. This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (B2024-064-01).

Immunohistochemistry (IHC)

LUAD tissue specimens were collected through surgical resection, promptly fixed in 4% paraformaldehyde, and sectioned to a thickness of 4 μm. IHC staining was performed as follows: the tissue sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide. Antigen retrieval was conducted by heating the sections in Tris-EDTA buffer (pH 9.0). To block nonspecific binding, the slides were incubated with 5% bovine serum albumin (BSA). Subsequently, the sections were incubated with primary antibodies against TRPM4 (Proteintech, Cat#21985-1-AP), followed by an HRP-conjugated secondary antibody (ZSGB-BIO) and visualization with diaminobenzidine (DAB, ZSGB-BIO). Finally, hematoxylin (ZSGB-BIO) was used for counterstaining, after which the slides were dehydrated, mounted, and examined under a microscope. The images were acquired using the Digital Pathology Slide Scanner (KF-PRO-020). Immunohistochemical scoring was independently evaluated by two trained pathologists. Staining intensity was scored as follows: negative (0 points), weakly positive (1 point), positive (2 points), and strongly positive (3 points). The percentage of positive cells was assessed across in the entire visual field (0–100%). The final immunoreactivity score was calculated as the product of staining intensity (0–3) and the percentage of positive cells (0–100%).

Multiplex Immunohistochemistry (mIHC)

Multiplex immunohistochemistry staining was performed on paraffin-embedded LUAD tissue sections using the mIHC Kit (PANOVUE, Cat# 10144100050). Tissue samples were sliced into 4-μm-thick sections and mounted on glass slides. Following deparaffinization with xylene and rehydration through a graded ethanol series into distilled water, antigen retrieval was achieved by heating the slides in Tris-EDTA buffer (pH 9.0). To reduce nonspecific antibody binding, sections were incubated with 5% BSA. Sequential staining was conducted in three cycles using primary antibodies against CD80 (1:1,000, HUABIO, M1007-10), CD163 (1:2,000, HUABIO, ET1704-43), and CD3 (1:500, Abcam, ab16669), with each antibody applied for 1 hour at room temperature. Each staining cycle was followed by fluorescent tyramide signal amplification, and nuclear counterstaining was performed with DAPI. The images were captured using the Vectra Polaris™ imaging system.

Cell Culture and Transfection

Human breast cancer (MDA-MB-231), lung cancer (A549), and esophageal cancer (KYSE30) cell lines (Xinyuan Biotech, China) were STR-authenticated and routinely screened for contamination. A549 and KYSE30 were maintained in RPMI-1640 (Gibco), while MDA-MB-231 was cultured in DMEM (Gibco), both supplemented with 10% FBS (Sinsage) at 37°C, 5% CO_2. Cells were transfected with TRPM4-targeting siRNA (si-TRPM4: 5′-GACUGGAAUUGACAUCCCUTT-3′) or negative control siRNA (si-NC: 5′-UUCUCCGAACGUGUCACGUTT-3′) using GP-transfect-Mate (Suzhou Gene Pharma).

Western Blotting

Following 24 h of siRNA transfection, cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease/phosphatase inhibitors and 1 mM PMSF. Protein concentrations were determined by Bradford assay, and equal amounts were separated on 10% Bis-Tris gels (Epizyme) before transfer to PVDF membranes (Invitrogen). After blocking with 5% non-fat milk/PBST for 60 min at room temperature, membranes were incubated overnight at 4°C with primary antibodies: anti-Tubulin (1:5000, Proteintech) and anti-TRPM4 (1:1000, Abcam). HRP-conjugated secondary antibodies were then applied for 1h at room temperature, and signals were detected using an ECL system (Tianneng).

Viability and Proliferation Testing

Proliferation and viability assays were performed at 48h post-siRNA transfection. CCK-8 Assay: Cells (1000/well) were seeded in 96-well plates and cultured for 0–3 days. Viability was measured by adding CCK-8 reagent (DOJINDO) and incubating for 2 h at 37°C, followed by absorbance measurement at 450 nm (Infinite F50, Tecan). For drug NBA (MedChemExpress, HY-128172) treatment, seed cells in 96-well plates at a density of 5×10^3 cells/well and incubate until adherence. After cell attachment, treat with varying drug concentrations. Perform cell viability assays using Beyotime's CellTiter-Lumi™ Luminescent Cell Viability Assay Kit according to the manufacturer's instructions. Measure luminescence intensity every 24 hours using a Promega multimode microplate reader. EdU Assay: Cell proliferation was evaluated using the EdU assay kit (Epizyme) according to manufacturer's instructions after siRNA transfection or TRPM4 inhibitor NBA treatment. Proliferation rates were calculated as the percentage of EdU-positive cells relative to Hoechst-stained nuclei.

Transwell Migration/Invasion Assay

Following 48h siRNA transfection, cells (2×10^5/mL in serum-free medium) were seeded into Matrigel-coated (invasion) or uncoated (migration) trans-well inserts (Corning, 8-μm pores). The lower chamber contained 700μL complete medium as chemoattractant. After 24 h incubation (37°C, 5% CO_2), membranes were fixed (4% PFA), stained (0.1% crystal violet), and imaged (Nikon, 10X). Migrated/invaded cells were quantified from three random fields/well (Image J).

Colony Formation Assay

After treatment (48h of siRNA transfection), MDA-MB-231/A549/KYSE30 cells were trypsinized, counted, and seeded in 6-well plates at densities of 1000 cells/well in complete medium. Cells were maintained at 37°C in a humidified 5% CO2 incubator for 10–14 days, with medium replacement every 3 days to ensure optimal growth conditions. Following the incubation period, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then stained with 0.1% crystal violet for 20 min. After washing and drying, the colonies were photographed and quantified via Image J software.

Cell Apoptosis Analysis

The cells were inoculated into 6-well plates and after adhering, different concentrations of the TRPM4 inhibitor NBA were added for treatment for 48 hours. The degree of apoptosis was detected by flow cytometry according to the instructions of the Annexin V-FITC/PI Apoptosis Kit (Elabscience).

Statistical Analysis

The data were analyzed using GraphPad Prism 9, and the results are expressed as the means ± SD. Statistical differences between two groups were analyzed by unpaired t-tests, while two-way ANOVA followed by Tukey's post hoc test was used for comparisons involving two variables. At least three replications were conducted for each experiment. An analysis of significance requires a p-value of 0.05 or less.

Results

Expression Characterization of TRPM4

We first analyzed the expression of TRPM4 in normal organs and tissues in the GTEx database, and found that TRPM4 was mainly expressed in colon and prostate, while very little was found in the brain as well as blood (Figure 1A). We then compared the mRNA expression of TRPM4 in tumors and normal tissues and found that TRPM4 was significantly elevated in most of the tumors except COAD and READ (Figure 1B). We then queried the protein expression level of TRPM4 in the HPA database and found that TRPM4 was significantly upregulated in a variety of tumors compared to normal tissues, such as LUAD, BRCA, CESC, THCA, OV, PAAD and SKCM (Figure 1C). Subsequently, we also analyzed the prognostic relationship of TRPM4 in different tumors using univariate cox regression, and the forest plot showed that elevated TRPM4 was closely associated with poor prognosis in PAAD, LGG, and UVM, while in BLCA and UCEC, TRPM4 was a significant protective factor (Figure 1D). The above results suggest that TRPM4 is overexpressed in a wide range of tumors and is strongly associated with the prognosis of tumor patients, but the impact varies by tumor type, highlighting its complex role.

Functional Characteristics of TRPM4

We first constructed a TRPM4-related PPI network using the STRING database (Figure 2A), followed by enrichment analysis of key genes in the PPI network, which showed that the main function of TRPM4 was significantly associated with potential changes of Na ion channels (Figure 2B). KEGG results indicated that TRPM4 and inflammation-mediated TRP is closely related to inflammation-mediated TRP (Figure 2C). TRP channels are a family of widely expressed cation channels involved in the regulation of intracellular calcium homeostasis, cell proliferation, apoptosis, migration, and other physiological and pathological processes. TRP channels are involved in the regulation of intracellular calcium homeostasis, cell proliferation, apoptosis, migration, and other physiological and pathological processes by regulating Ca^{2+} signaling and integrating microenvironmental signals, play a complex role in tumor progression in which TRPM4 may play an important role.[23] On Subcellular Localization, TRPM4 is predominantly distributed in the sodium channel complex, nerve fibers, axons, and cell-cell junctions on the cell membrane (Figure 2D). Analysis of key pathways affecting cancer progression showed that Cell cycle, DNA damage, EMT and other pathways were closely associated with TRPM4 expression levels in a variety of tumors, but varied from tumor to tumor, eg, cell cycle was strongly associated with TRPM4 expression levels in a variety of tumors (UVM, UCEC, THYM, STAD, SARC, OV, LUSC, LUAD, COAD, BRCA, BLCA) all showed a trend of negative correlation with TRPM4, while in the EMT pathway, except for LGG and GBM, which are two kinds of brain tumors, the expression of TRPM4 mostly showed a trend of negative correlation with EMT (Figure 2E).

Genomic Characterization of TRPM4

Genomic variation is one of the critical factors affecting tumor progression.[24] We first analyzed the CNV variants of TRPM4, including Heterozygous Amplification, Heterozygous Deletion, Homozygous Amplification, Homozygous Deletion. ACC, KICH, BLCA, UCS, CESC, GBM, LUSC, and ESCA had more than 25% Heterozygous Amplification of the TRPM4 gene, and for Heterozygous Deletion, TRPM4 variants in UCS, LUSC, ESCA, SARC, TGCT, LUAD, OV, and LGG had more than 25%, while the other two variants, Homozygous Amplification, and Homozygous Deletion, occurred at a very low percentage (Figure 3A). For SNV, only UCEC, SKCM, STAD, COAD, and LUAD had relatively high mutation frequencies of TRPM4 (Figure 3B). Methylation level is also a key factor affecting gene expression,[25] and only 10 tumors showed significant changes in methylation level (Figure 3C), but all had

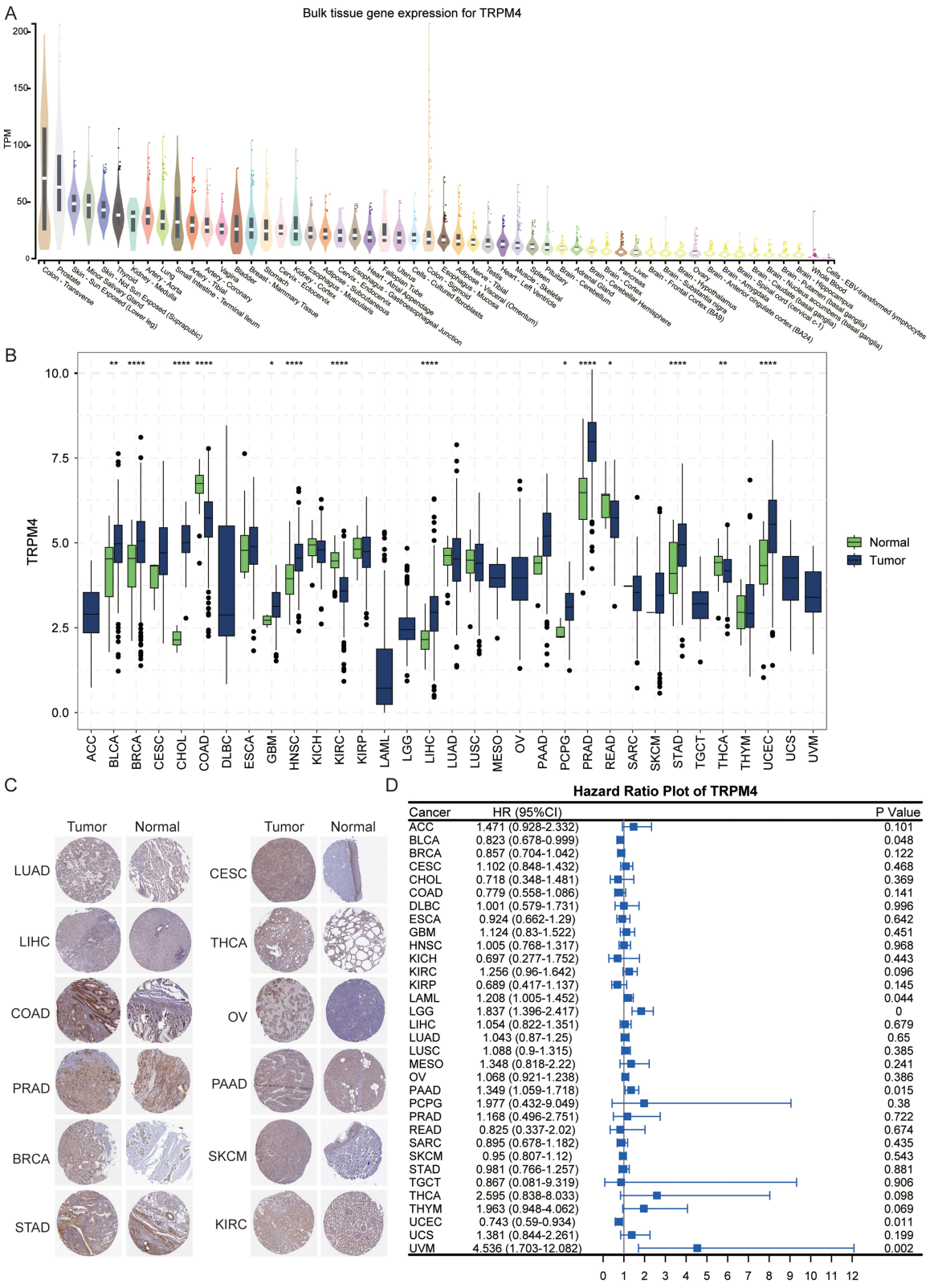

Figure 1 Expression characteristics of TRPM4. (**A**) The expression level of TRPM4 in normal tissues. (**B**) Differential expression of TRPM4 in tumors and normal tissues. (**C**) Differences in protein levels of TRPM4 between tumor and normal tissues. (**D**) Univariate cox regression analysis of TRPM4 in different tumors. *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$.

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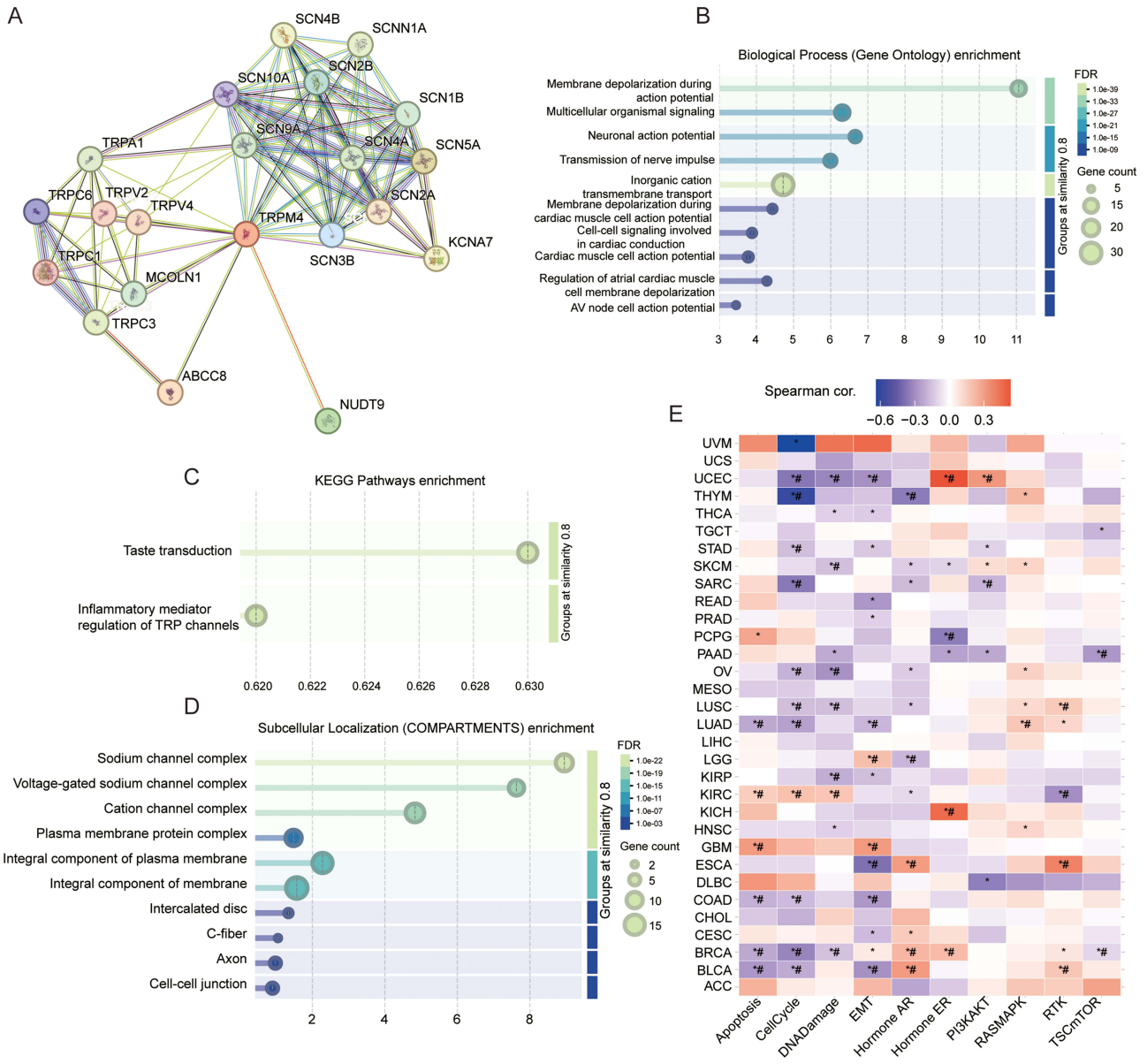

Figure 2 Functional analysis of TRPM4. (**A**) Construction of PPI network related to TRPM4. (**B**) GO enrichment analysis. (**C**) KEGG enrichment analysis. (**D**) Subcellular Localization enrichment analysis. (**E**) Correlation analysis between tumor promoting pathways and TRPM4 expression. *: *P* value < 0.05; #: FDR < 0.05.

relatively low methylation. We also analyzed the overall genetic mutations of TRPM4 (Figure 3D), on the whole, the mutation frequency in different tumors did not exceed 10%, and the alteration frequency of TRPM4 that occurred more frequently were mutations, such as SKCM, STAD and UCEC, while the rest of the mutation types were less frequent.

Immunological Characterization of TRPM4

Tumor mutational load (TMB) and microsatellite instability (MSI) are markers for ICIs treatment,[26] and we first assessed the association between TRPM4 expression and TMB/MSI. For TMB, TRPM4 expression was significantly associated with ACC, DLBC, ESCA, OV, and THYM (Figure 4A), whereas for MSI, TRPM4 was significantly associated with MSI scores of COAD, LUAD, STAD, and UCEC (Figure 4B). We then further analyzed the relationship between TRPM4 and immune scores, and TRPM4 was significantly negatively correlated with tumor Stromal Score, Immune Score, and ESTIMATE Score in most tumors, and positively correlated in LGG, LIHC, OV, and UVM (Figure 4C). The level of immune cell infiltration significantly affected the prognosis of patients, and we analyzed the correlation between TRPM4

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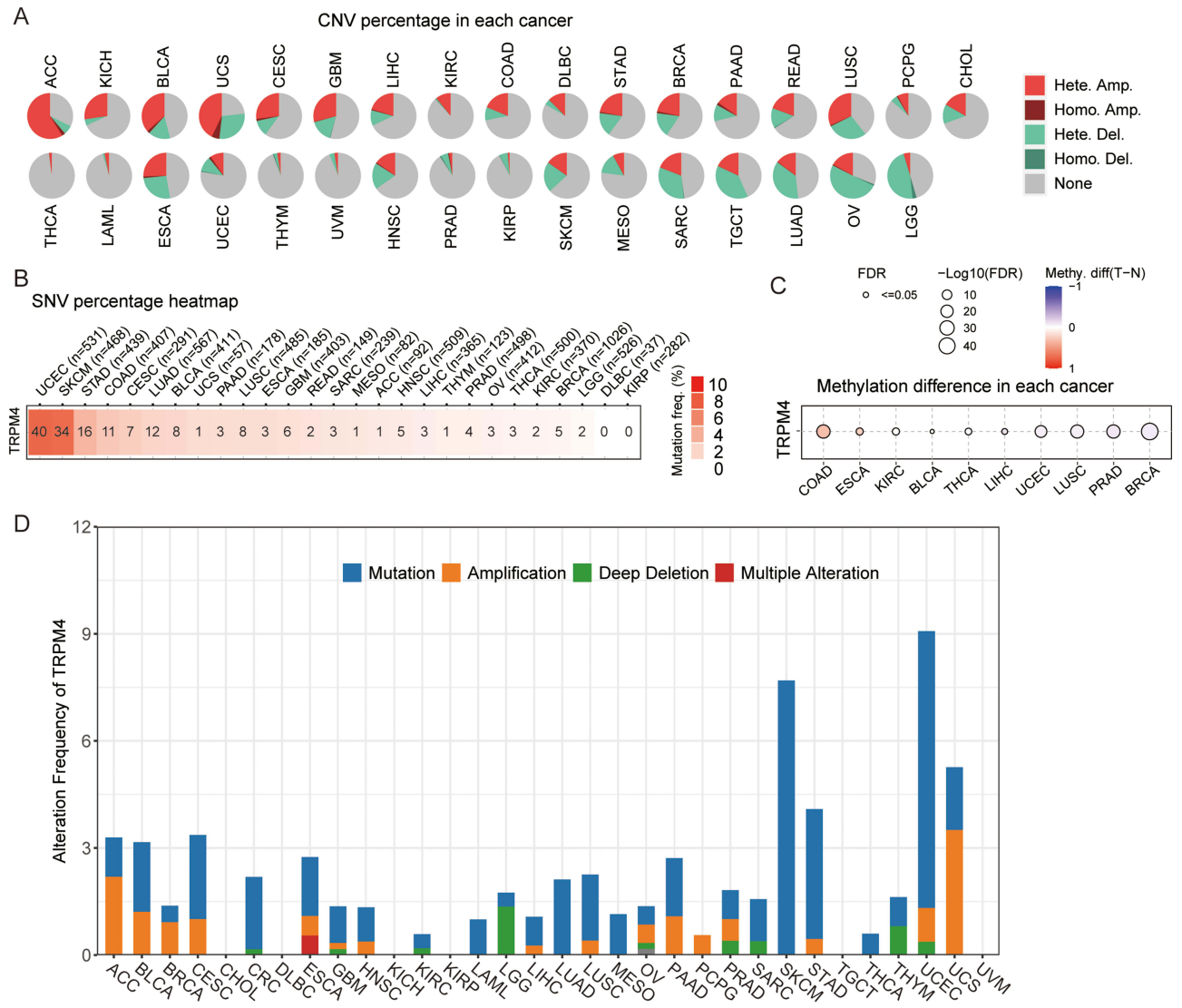

Figure 3 Genomic characteristics of TRPM4. Univariate (**A**) CNV characteristics of TRPM4. (**B**) SNV characteristics of TRPM4. (**C**) Methylation level of TRPM4. (**D**) The genetic mutation status of TRPM4.

and 22 types of immune cell infiltration, and for M1 macrophages, CD4T cells, and CD8T cells, TRPM4 was mostly positively correlated (Figure 4D). We further explored the relationship between TRPM4 and chemokine ligands/receptors, key immune checkpoints, and the TNF family that affect immune cell infiltration, and found that the correlation between these immune molecules and TRPM4 varied significantly among different tumors, but in the same tumors, there was a high degree of concordance in their relationships. For example, CCL family and CXCL family showed significant positive correlation with TRPM4 in BLCA, BRCA, COAD, KIRP, PRAD and READ, but significant negative correlation with TRPM4 in LAML, LIHC, PAAD, UCS and UVM (Figure 5A). And the ligand CCR of CCL, the ligand CXCR of CXCL, immune checkpoints and TNF family showed similar properties (Figure 5B–D). This suggests that TRPM4 may be closely associated with tumor hot and cold, and thus have a significant impact on patient prognosis and treatment response.

Prediction of Response to Drug Therapy

Given that the effect of TRPM4 on tumors may have an impact on their therapeutic response, we analyzed the relationship between TRPM4 and multiple bulk RNA-seq datasets treated with ICIs, and the results showed that

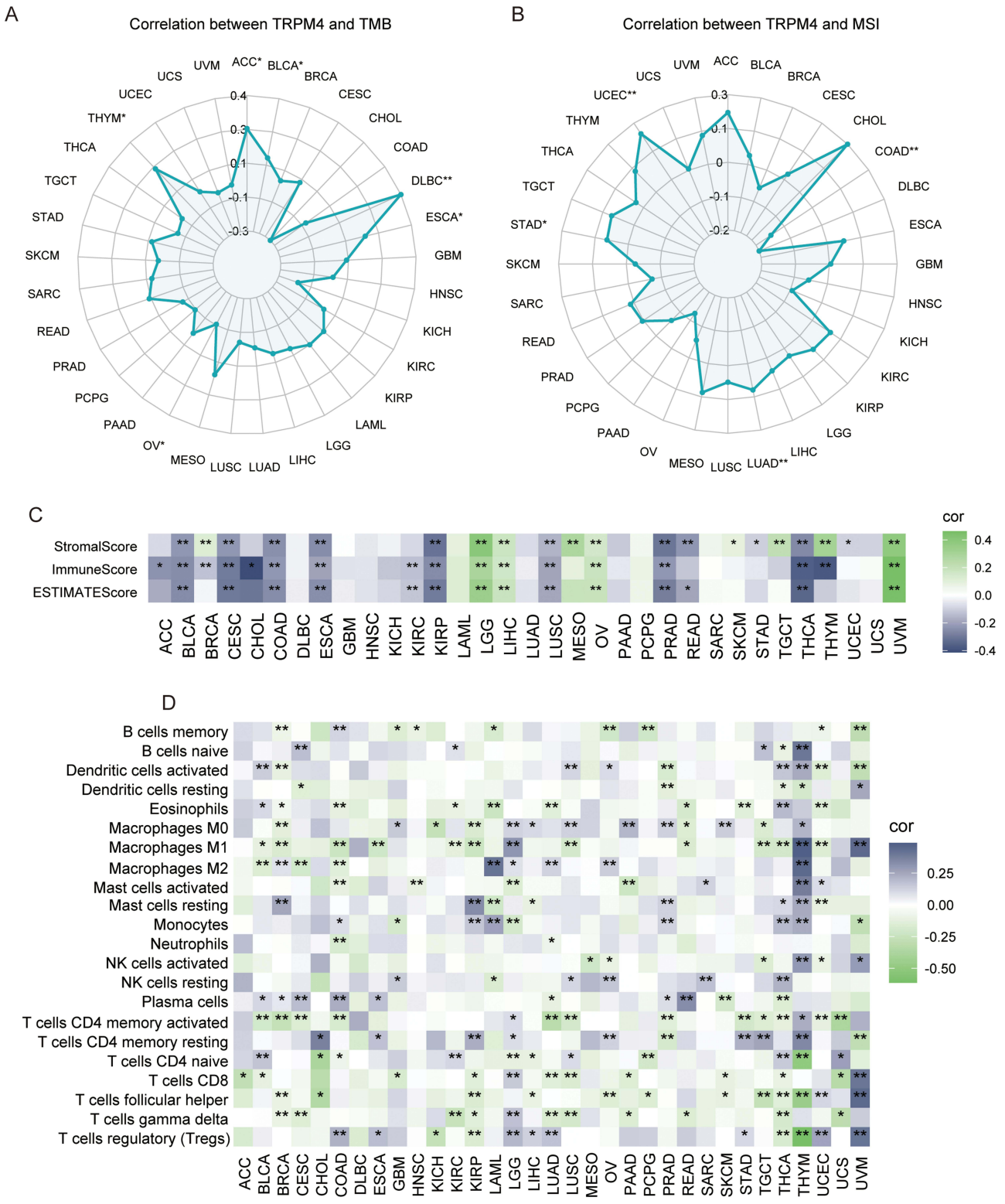

Figure 4 Immune characteristics of TRPM4. (**A**) The correlation between TRPM4 and TMB. (**B**) The correlation between TRPM4 and MSI. (**C**) The correlation between TRPM4 and immune score (**D**). The correlation between TRPM4 and immune cell infiltration levels. *:$P < 0.05$, **:$P < 0.01$.

TRPM4 was an effective predictor of the prognosis of patients treated with ICIs in multiple datasets (Figure 6A), and the higher the TRPM4, the The higher the TRPM4, the worse the prognosis of patients treated with ICIs. For chemotherapy and targeted therapies, we analyzed the correlation between TRPM4 and a variety of drugs, and after analyzing the drug

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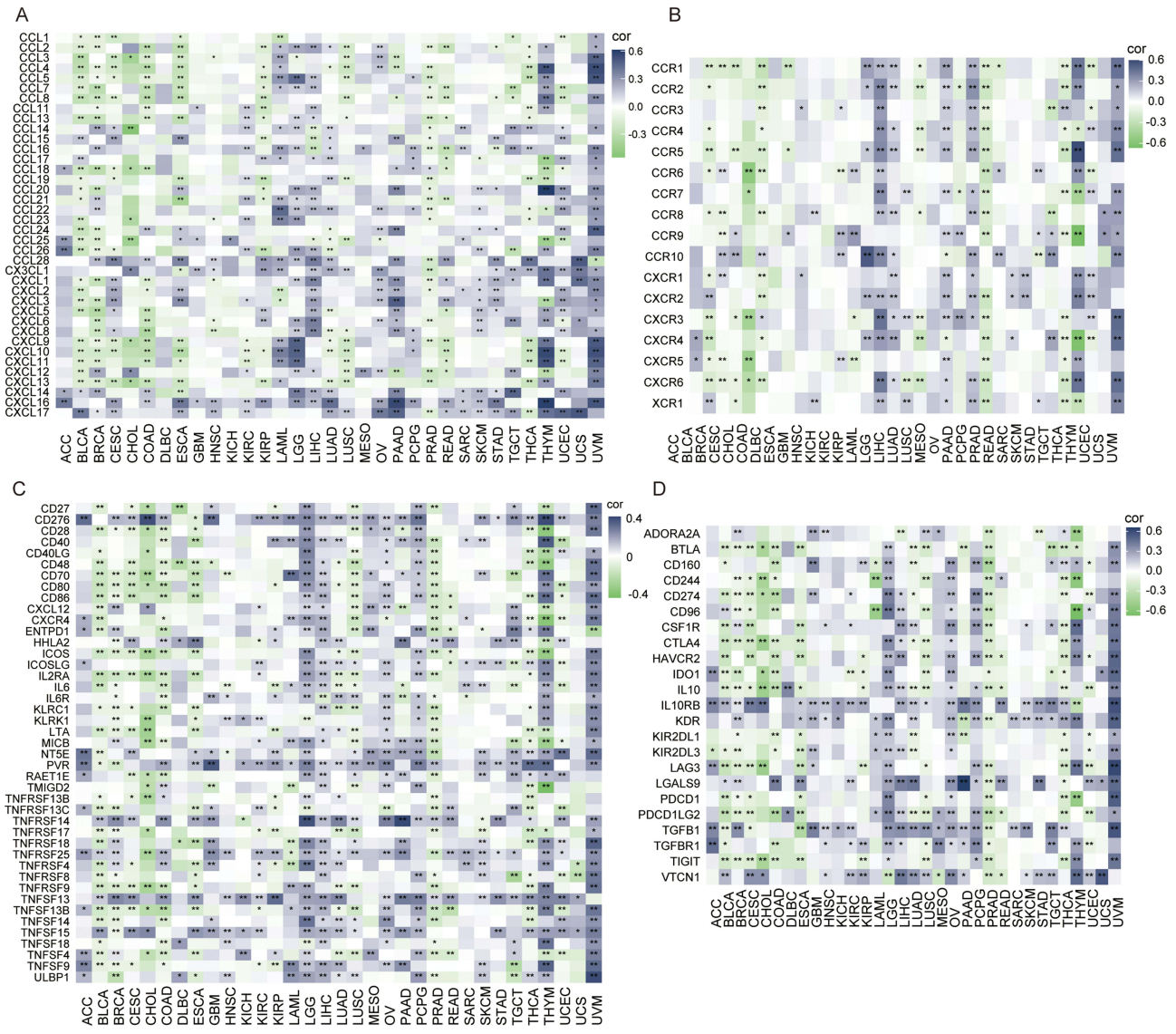

Figure 5 The correlation between TRPM4 and immune related molecules. (**A**) The correlation between TRPM4 and chemokines. (**B**). The correlation between TRPM4 and chemokine receptors. (**C**) The correlation between TRPM4 and immunostimulators. (**D**). The correlation between TRPM4 and immunoinhibitors. *:$P < 0.05$, **:$P < 0.01$.

information in the CTRP database, we found that, except for erlotinib, the therapeutic response of most drugs had a significant positive correlation with the expression of TRPM4 (Figure 6B). In the GDSC database, response prediction was positively correlated with TRPM4 expression only for Bortezomib, DMOG, and MS-275, and significantly negatively correlated for the remaining drugs (Figure 6C). In addition, we further analyzed the animal models treated with ICIs and found that there was a trend of decreasing TRPM4 in response compared to baseline levels (Figure 6D), suggesting that TRPM4 may be an important marker for predicting response to immunotherapy.

TRPM4 Predicts Response to Immunotherapy by scRNA-Seq

We collected 3 scRNA-seq datasets treated with ICIs, including LUAD, LUSC, and CRC, based on classical marker we identified immune cells, mesenchymal cells, and epithelial cells. In LUAD, TRPM4 was predominantly expressed in epithelial cells and macrophages (Figure 7A and B), and TRPM4 expression was significantly higher in the SD group than in the PR group (Figure 7C). Similar results were validated in LUSC and CRC (Figure 7D–I), and these results further suggested that TRPM4 could be used as a marker for immunotherapy response.

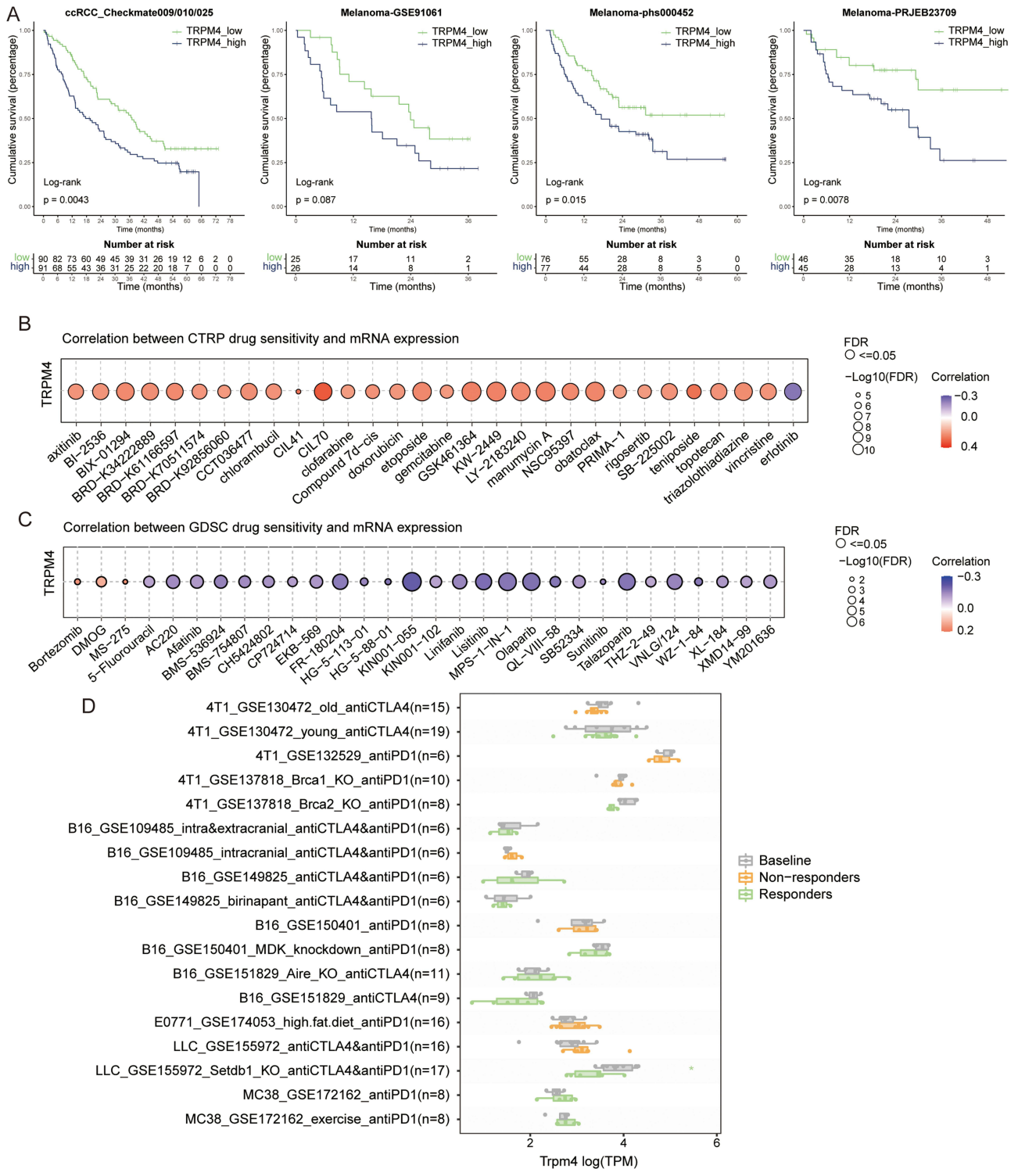

Figure 6 The relationship between TRPM4 expression and drug sensitivity. (**A**) Predictive prognosis of TRPM4 in different immunotherapy cohorts. (**B**) Correlation between CTRP drug sensitivity and mRNA expression. (**C**) Correlation between GDSC drug sensitivity and mRNA expression. (**D**) Expression of TRPM4 in different treatment response groups in mouse immunotherapy models. 4T1, E0771: breast cancer; B16: melanoma; LLC, lung cancer; MC38: colon cancer. *: Responders vs Baseline, $P < 0.05$.

Validation of TRPM4 in LUAD Patients

We performed IHC on the tumors of these 19 patients (Figure 8A) and scored them for IHC. To further elucidate the effect of TRPM4 on immune cell infiltration, we further did mIHC on these specimens and examined the levels of immune infiltration of T cells (CD3), M1 macrophages (CD80), and M2 macrophages (CD163) (Figure 8B), and found that the expression score of

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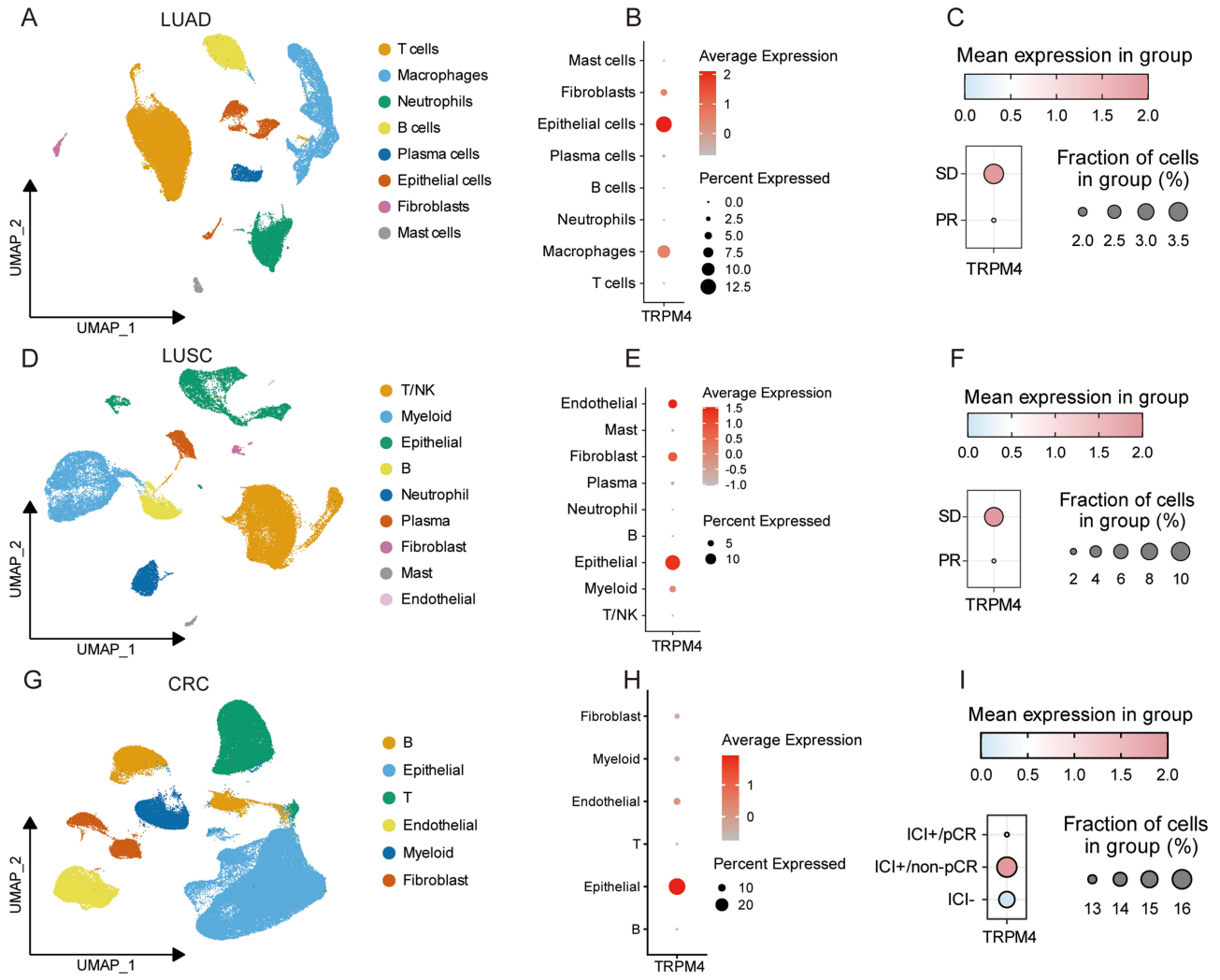

Figure 7 Expression of TRPM4 in scRNA datasets. (**A**) UMAP dimensionality reduction of LUAD. (**B**) Bubble plot of TRPM4 expression in different cells from LUAD. (**C**) The expression of TRPM4 in different treatment response groups from LUAD. (**D**) UMAP dimensionality reduction of LUSC. (**E**) Bubble plot of TRPM4 expression in different cells from LUSC. (**F**) The expression of TRPM4 in different treatment response groups from LUSC. (**G**) UMAP dimensionality reduction of CRC. (**H**) Bubble plot of TRPM4 expression in different cells from CRC. (**I**) The expression of TRPM4 in different treatment response groups from CRC.

TRPM4 and the levels of CD3 and CD80 showed a significant negative correlation with the levels of CD3 and CD80 and a significant positive correlation with the level of CD163 (Figure 8C). These results suggest that the reason why TRPM4 affects the therapeutic response of ICIs is closely related to the regulation of different immune cell infiltration.

Functional Validation of TRPM4 in Tumors

We further analyzed the role of TRPM4 in MDA-MB-231 (breast cancer), A549 (lung adenocarcinoma) and KYSE30 cells (esophageal cancer). TRPM4 was knocked down using siRNA and validated by WB (Figure 9A and B). The low expression of TRPM4 significantly inhibited the proliferation level as well as clone formation of the three tumor cells (Figure 9C–G), and TRPM4 knockdown reduced the invasive and migratory ability of tumor cells (Figure 9H–K). These findings emphasized the critical role of TRPM4 in tumor progression. Then, we further utilized the TRPM4 inhibitor NBA[5] to treat MDA-MB-231, A549, and KYSE30. The results show that NBA has a time- and dose-dependent effect on cell viability (Figure 10A). Among them, 100μM of NBA can significantly inhibit the growth of cells. The results of EDU proliferation assay showed that the NBA dose-dependently inhibited cell proliferation (Figure 10B). Finally, we selected a concentration of 50μM for NBA to investigate its effect on cell apoptosis. The results showed that NBA can inhibit cell apoptosis (Figure 10C).

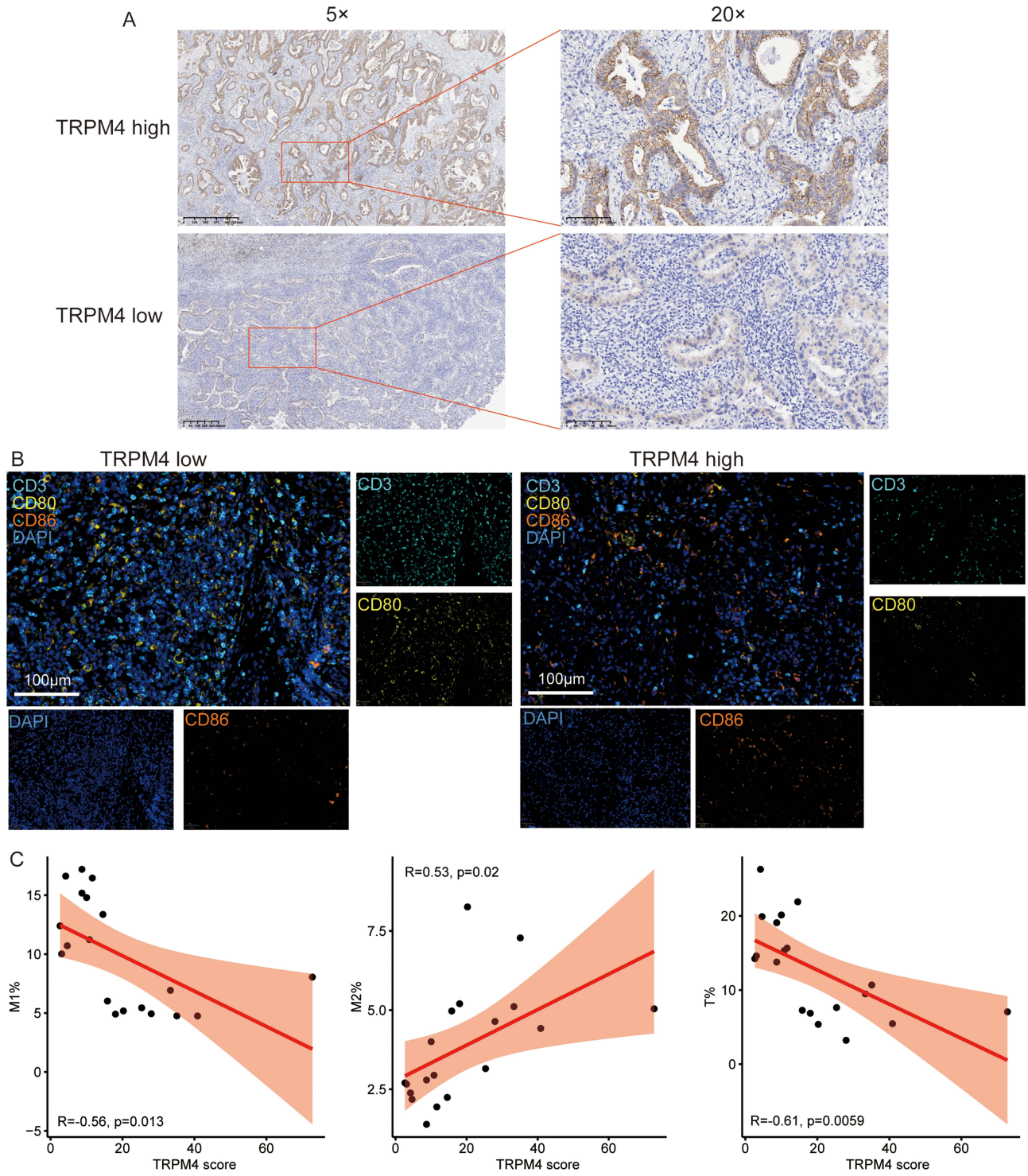

Figure 8 Expression of TRPM4 in tumors of LUAD patients. (**A**) Representative IHC images of high and low expression of TRPM4. (**B**) Representative mIHC images of high and low expression of TRPM4. (**C**) The correlation between the immunohistochemical score of TRPM4 and the corresponding proportion of immune cells.

Discussion

TRPM4 is a calcium-activated nonselective cation channel that plays a multidimensional regulatory role in tumorigenesis and development. Studies have shown that TRPM4 exhibits abnormally high expression in a variety of malignant tumors and is significantly correlated with the poor prognosis of patients.[27–29] The mechanisms by which TRPM4 affects tumors

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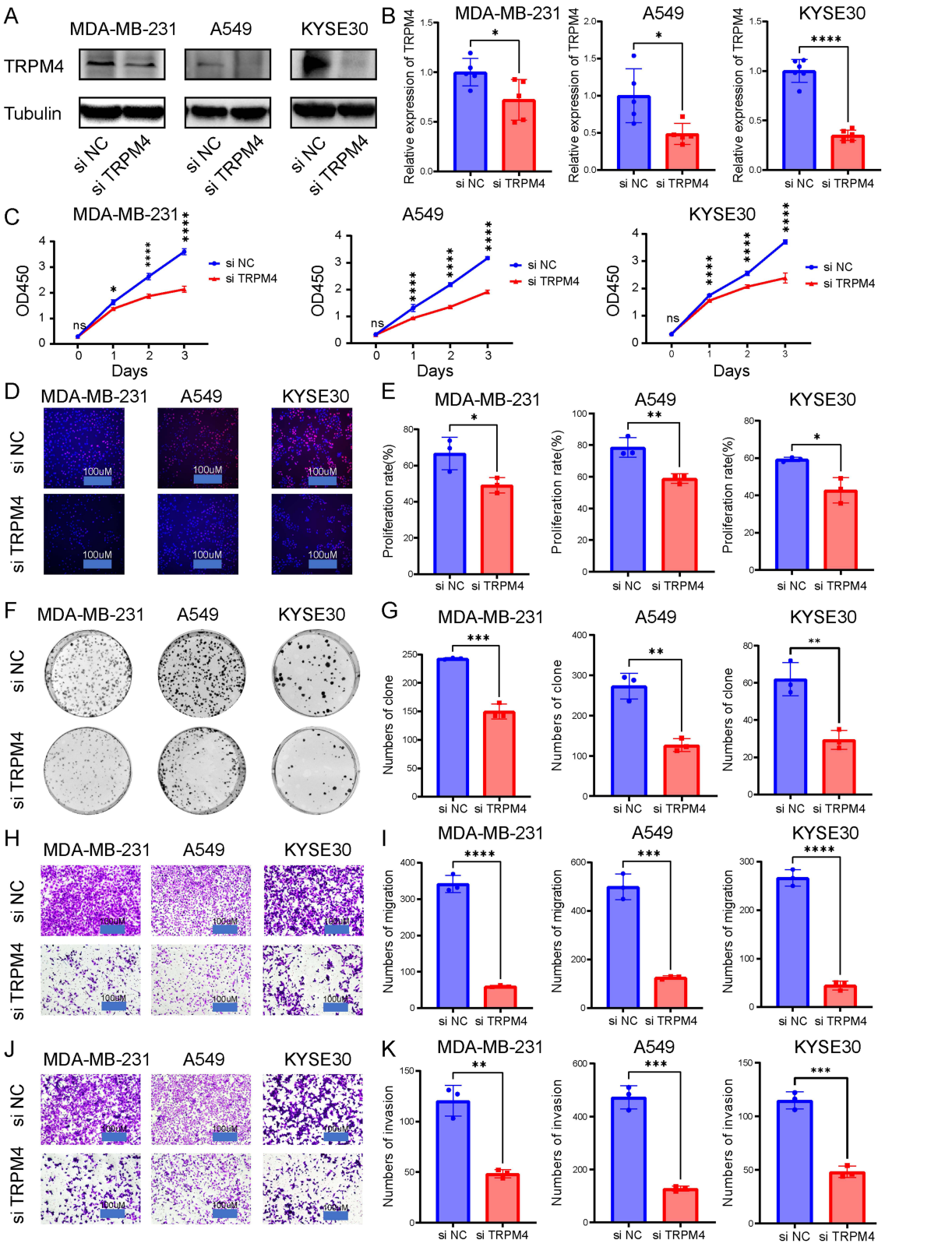

Figure 9 Effect of TRPM4 knockdown on cell function of breast cancer, lung cancer and esophageal cancer. (**A**) Western blot data confirm diminished TRPM4 levels after knockdown. (**B**) Quantitative analysis of TRPM4 knockdown (n = 5). (**C**) CCK-8 data demonstrated that inhibition of TRPM4 expression compromises proliferation capacity of tumor cells. (**D**) TRPM4 knockdown significantly reduced the percentage of tumor cells in a proliferative state according to EDU studies. (**E**) Quantitative analysis of EDU (n = 3). (**F**) Plate cloning showed that TRPM4 knockdown significantly inhibited the ability to produce clones. (**G**) Quantitative analysis of plate cloning (n = 3). (**H**) Tumor migration was reduced by TRPM4 knockdown. (**I**) Quantitative analysis of tumor migration (n = 3). (**J**) Tumor invasion was reduced by TRPM4 knockdown. (**K**) Quantitative analysis of tumor invasion (n = 3). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

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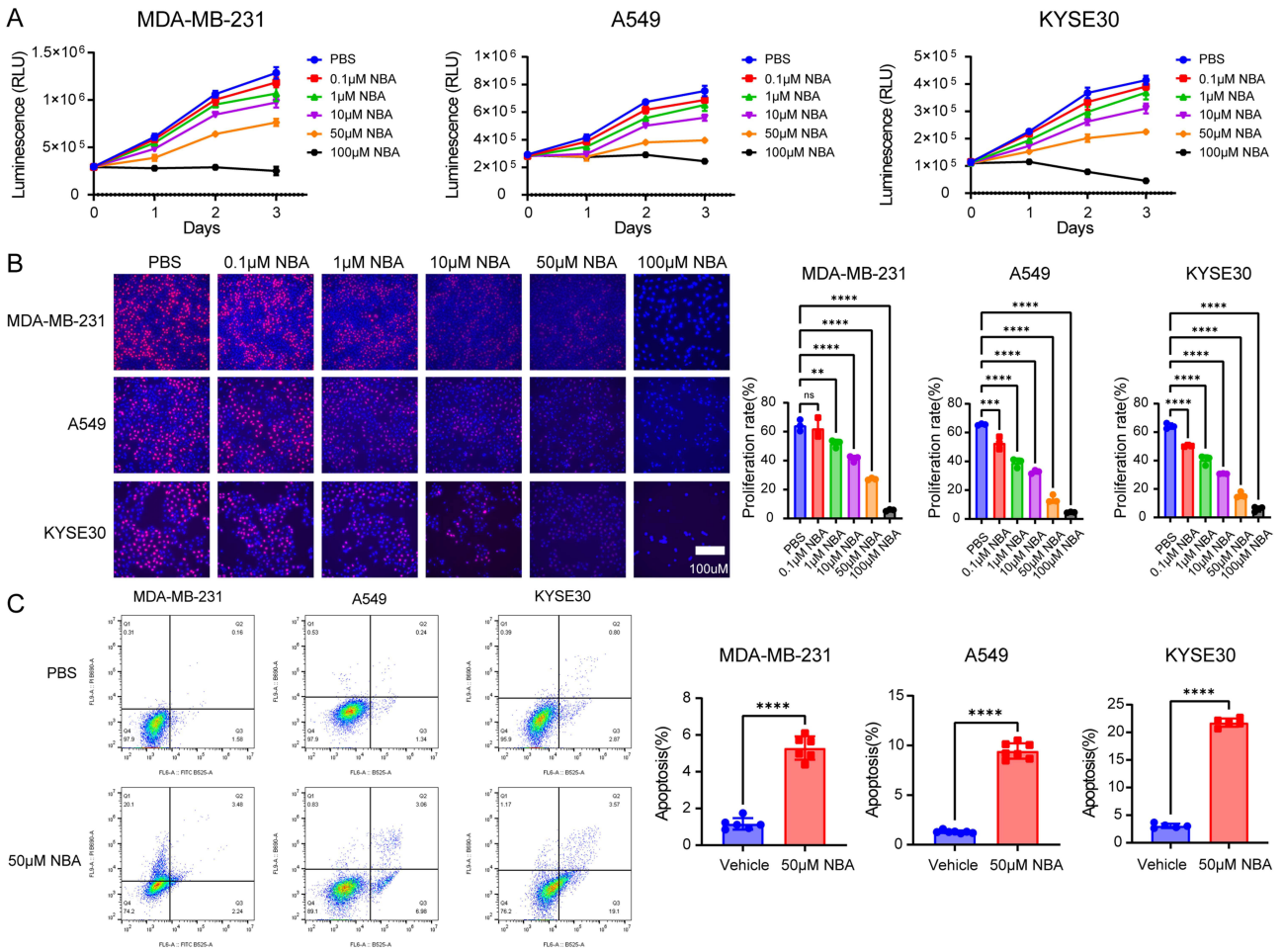

Figure 10 The effects of TRPM4 inhibitor NBA on MDA-MB-231, A549 and KYSE30 cells. (**A**) The curve of the cell's luminescence intensity over time (n=3). (**B**) Representative images of EDU detection of cells after 48 hours of treatment with different concentrations of NBA and statistical analysis of the results of EDU detection (n=3). (**C**)Cell apoptosis and statistical analysis of the proportion of apoptotic cells after 48 hours of treatment with 50 μM NBA (n=5~7). The one-way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis. Data were presented as the mean ± SD, SD: standard deviation. **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

involve cell proliferation regulation,[30] promotion of metastatic invasion[31,32] and immune cell migration and activation.[33–35] A recent study found that sustained activation of the TRPM4 ion channel can lead to sustained sodium inward flow and thus cause cellular energy depletion and cell death, which has been labeled as sodium death, a process that does not depend on other modes of death, and in which TRPM4 is a central molecule. However, this process has not yet revealed the relationship between TRPM4-mediated sodium death and tumors.

In this study, we revealed the multidimensional tumor biology of TRPM4 gene by systematic pan-cancer analysis. At the molecular expression level, TRPM4 showed synchronous abnormal high expression of mRNA and protein products in a variety of malignant tumor tissues. Clinical prognostic analysis revealed that the expression level of TRPM4 correlated with patients' overall survival, but its prognostic predictive efficacy exhibited significant tumor type dependence. Notably, correlation analysis showed that the expression of this gene was significantly correlated with the levels of immune checkpoint molecules, chemokines, and immune cell infiltration, and varied from tumor to tumor, suggesting that it might be involved in shaping the tumor microenvironment (TME), and that TRPM4 effectively distinguishes between responders and non-responders after immunotherapy. Based on scRNA-seq, it was found that the expression profile of this gene was mainly enriched in tumor cells, and furthermore, TRPM4 was highly expressed mainly in non-responding patients, suggesting that it may serve as a promising marker for immunotherapeutic response. We also validated immune cell infiltration in tumor tissues of LUAD patients and found that TRPM4 expression was negatively

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correlated with the degree of T cells and M1 macrophages infiltration, which may be an important reason for the poor therapeutic efficacy of ICIs in patients with high TRPM4 expression. Hence targeting TRPM4 may be a key molecule to improve the treatment of ICIs. Ghosh et al revealed that drug-resistant cancer cells strongly down-regulated TRPM4 under the induction of necrosis-inducing drugs BHPI and ErSO, and that TRPM4 deletion completely limited the tumor-killing effect of necrosis-inducing drugs.[36] This result seems to be in contradiction with our findings, probably due to the complex interactions between immune activation and necrosis induction, and thus further studies are needed to uncover the relevant mechanisms and to improve the therapeutic efficacy of ICIs by using the regulation of TME by TRPM4.

At the level of functional mechanism, similar to previous studies, TRPM4 knockdown significantly inhibited the proliferative ability and reduced the migration and invasion potential of human breast, lung and esophageal cancer cells, further confirming its pro-tumorigenic effects. And the TRPM4 inhibitor NBA can also inhibit the growth of these three tumors and promote their apoptosis. Previous studies have reported that TRPM4 directly binds to a variety of adhesion molecules and its deficiency affects cell migration and damage repair.[37] Although TRPM4 channels do not directly allow Ca^{2+} to pass through, they can indirectly regulate Ca^{2+} flow by affecting membrane potential through influencing Na^{+} inward flow.[38] Sustained Na^{+}/Ca^{2+} inward flow contributes to the development of therapeutic drug resistance,[39] and the use of ion channel inhibitors significantly improves the efficacy of a variety of chemotherapeutic treatments, targeted therapies, and immunotherapies.[40–43] Zhang et al developed a novel ion-mediated immunotherapy agent that precisely controls K^{+}/Ca^{2+} homeostasis at the organelle level. This therapeutic approach reshapes the immunosuppressive tumor microenvironment (TME), awakens systemic immune responses, and induces long-term immune memory effects, effectively inhibiting the growth of primary/distant tumors, in situ tumors, and metastatic tumors in various mouse models.[44] TRPM4, as a key regulator of Na^{+}/Ca^{2+} homeostasis, holds great promise for enhancing the efficacy of other tumor treatment modalities through the use of its inhibitors, such as ICIs, chemotherapy. The previous review summarized the inhibitors targeting TRPM4, but considering the expression characteristics of TRPM4, screening for tumor-specific targeted drugs is a future research direction.

In this study, we systematically analyzed the molecular characteristics of TRPM4 and its clinical significance at the pan-cancer level for the first time, and revealed the key regulatory role of TRPM4 in tumor malignant progression and TME remodeling by integrating multi-omics data. However, it should be noted that the limitations of this study are mainly reflected in the following two aspects: first, the current conclusions are based on the bioinformatics analysis of retrospective cohorts, and the practical value needs to be verified in prospective clinical cohorts; second, the specific molecular mechanisms by which TRPM4 regulates immune cell infiltration are still unclear, especially the regulatory network between calcium signaling and immune checkpoint molecules, which needs to be targeted to specific Further studies on specific tumors are needed to clarify the effect of TRPM4 on immune cells, which will help the development of TRPM4 inhibitors and lay a molecular theoretical foundation for subsequent translational studies of TRPM4-targeted therapy.

Conclusion

These findings systematically elucidate the multiple biological functions of TRPM4 as an oncogene, which promotes tumor progression through the regulation of cell-autonomous proliferative capacity and non-cell-autonomous immune regulatory mechanisms. This study not only provides a new molecular perspective for understanding tumor heterogeneity, but also lays a theoretical foundation for the development of precision therapeutic strategies based on TRPM4 targeting.

Data Sharing Statement

The code and raw data generated in this study are available from the corresponding author (Zhesheng Wen) upon reasonable request.

Ethical Approval

This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (B2024-064-01). All samples were collected with the written consent of the patients and their families and strictly followed the ethical guidelines of the Helsinki Declaration.

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Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; All authors took part in drafting, revising or critically reviewing the article; All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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