Solute carrier family 4 member 4 (SLC4A4) is associated with cell proliferation, migration and immune cell infiltration in colon cancer

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Abstract

Background

Solute Carrier Family 4 Member 4 (SLC4A4) is a membrane protein-coding gene for a Na^+/HCO_3^- cotransporter and plays a crucial role in regulating pH, bicarbonate secretion and homeostasis. However, the prognostic and immunological role of SLC4A4 in colon cancer remains unknown.

Method

In this study, expression profiles of SLC4A4 were retrieved from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, to which a variety of bioinformatic analyses were performed. Sangerbox, Xiantao, ESTIMATE and TIMER online tools were used to delve into the relationship between SLC4A4 expression and immune cell infiltration. The role of SLC4A4 in the proliferation and migration of colon cancer cells was verified by CCK8, EdU and wound healing assays. The related molecules and pathways that SLC4A4 may affect were validated by bioinformatic prediction and western blotting analysis.

Results

The expression levels of SLC4A4 were significantly lower in colon cancer tissues than in normal tissues and its low expression was positively correlated with poor prognosis. TIMER and ESTIMATE showed that SLC4A4 broadly influenced immune cell infiltration. Experiments in vitro demonstrated that SLC4A4 inhibited partial epithelial-mesenchymal transition (EMT) phenotypes.

Conclusions

To conclude, our study revealed that SLC4A4 is lowly expressed in colon cancer tissues, and SLC4A4 may inhibit the progression of colon cancer via regulating partial EMT phenotypes and immune cell infiltration, which may provide new perspectives for the development of more precise and personalized immune anti-tumor therapies.

Introduction

Colon cancer is one of the most common malignancies of the gastrointestinal tract and the third leading cause of cancer-related deaths worldwide[1]. According to the World Health Organization (WHO), 940,000 colon cancer cases occur annually in the world, and nearly 500,000 people die from it each year. Furthermore, unfortunately, due to the lack of obvious clinical symptoms of colon cancer, most patients are already at a locally advanced stage when diagnosed[2]. Despite continuous efforts have been put on

the diagnosis and treatment of colon cancer, the 5-year rate of advanced colon cancer remains less than 30% due to post-operation recurrence and metastasis. Therefore, it is of great necessity to investigate in depth the mechanisms of colon cancer progression and identify new potential biomarkers for the individualized treatment decisions and prognostic assessment of patients with colon cancer.

Increasing evidence suggests that abnormal metabolism occurs throughout tumor development and influences tumor progression and therapeutic resistance[3–5]. Pyruvate, the end product of glycolysis, is a key molecule in both cell anabolism and catabolism. Previous studies have shown that abnormal metabolism in tumor cells is not only associated with the silencing or activation of signaling pathways[6–8], but can also regulate the expression of driver genes, such as partial epithelial-mesenchymal transition (p-EMT)[9]. Furthermore, researchers have affirmed that tumor metabolites are not only involved in the vital activities including lipid uptake, synthesis and hydrolysis, but also directly involved in the biological behavior of malignant tumors[10, 11]. Therefore, the investigation of genes related to pyruvate metabolism might be beneficial in guiding tumor therapy.

SLC4A4 (Solute Carrier Family 4 Member 4) is a membrane protein-coding gene for a $Na^+/HCO3^-$ cotransporter and is reported to be responsible for regulating pH regulation, bicarbonate secretion and homeostasis[12–14]. Contrasting evidence showed that the role of SLC4A4 in regulating cancer biology is tumor cell type specific and SLC4A4 can both promote and inhibit cancer cell proliferation, invasion and migration traits. Liu et al. found that SLC4A4 is up-regulated in prostate cancer and its overexpression was closely associated with the cancer development and progression [15]. While Huang et al. declared that down-regulated SLC4A4 might contribute to extra-thyroid metastasis in papillary thyroid carcinoma through JNK/P38 MAPK signaling pathway [16]. Few studies reported that SLC4A4 might function as a gene signature in guiding colorectal cancer prognosis. SLC4A4 can also participate in and influence biological processes such as mismatch repair, base excision repair and DNA replication, which are involved in colon tumorigenesis [12]. However, the mechanism of SLC4A4 in regulating colon cancer progression and the association between SLC4A4 expression and tumor immune microenvironment have not been well studied.

In this study, we performed comprehensive bioinformatics on datasets collected from public databases including The Cancer Genome Atlas (TCGA) database (http://portal.gdc.cancer.gov/) and cBioPortal database (https://www.cbioportal.org/) to characterize the genetic variants and transcriptome of SLC4A4 alterations and found that SLC4A4 was lowly expressed in colon cancer tissues and its low expression was positively correlated with poor prognosis of patients with colon cancer. Then, we preliminarily validated our findings in clinical specimens and explored the function of SLC4A4 in colon cancer-derived cell lines. CCK8 and EdU assays were performed to investigate the effect of SLC4A4 on colon cancer cell proliferation. Wounding healing assay was used to detect the effect of SLC4A4 on colon cancer cell migration. Moreover, mechanistic studies shed light on the critical role of SLC4A4 in suppressing partial EMT and its correlation with immune infiltration phenotypes. Therefore, SLC4A4 might serve as a new promising immune therapeutic and prognostic biomarker in guiding colon cancer treatment.

Materials and methods

Retrieval of data from public databases

Gene expression data based on RNA sequencing and clinical data of patients with colon cancer were retrieval from the COAD (colon adenocarcinoma) project of TCGA (https://portal.gdc.cancer.gov/), which containing 478 cases of primary colon cancer. The baseline profile of these 478 cases was shown in Table 1. In addition, RNA sequencing and clinical information file GSE17536 were downloaded from the Gene Expression Omnibus database (GEO) (https://www.ncbi.nlm.nih.gov/geo/).

Table 1 Baseline profile of patients with colon cancer in the TCGA database (n=478)

Characteristics		Total (%)
Age, n (%)	<= 65	194 (40.6%)
	> 65	284 (59.4%)
Gender, n (%)	Female	226 (47.3%)
	Male	252 (52.7%)
Weight, n (%)	<= 90kg	189 (69.2%)
	> 90kg	84 (30.8%)
Height, n (%)	< 170cm	127 (49.6%)
	>= 170cm	129 (50.4%)
BMI, n (%)	<= 25	87 (34%)
	> 25	169 (66%)
Pathologic T stage, n (%)	T1	11 (2.3%)
	T2	83 (17.4%)
	T3	323 (67.7%)
	T4	60 (12.6%)
Pathologic N stage, n (%)	N0	284 (59.4%)
	N1	108 (22.6%)
	N2	86 (18%)
Pathologic M stage, n (%)	M0	349 (84.1%)
	M1	66 (15.9%)
Pathologic stage, n (%)	Stage I	81 (17.3%)
	Stage II	187 (40%)
	Stage III	133 (28.5%)
	Stage IV	66 (14.1%)
CEA level, n (%)	<= 5	196 (64.7%)
	> 5	107 (35.3%)

Abbreviations: TCGA, The Cancer Genome Atlas Program; kg, kilogram; cm, centimeter; BMI, Body Mass Index; T, tumor; N, node M, metastasis; CEA, carcinoembryonic antigen

Identification of differentially expressed genes (DEGs), and co-expressed genes

Pyruvate metabolism related genes were obtained from the MSigDB database, and 116 DEGs were selected using R software. 199 SLC4A4 co-expressed genes were retrieved from Metascape (https://metascape.org/).

SLC4A4 gene expression analysis

SLC4A4 gene expression levels in various tumors were analyzed using “limma” R package in R software, Sangerbox online platform (https://vip.sangerbox.com/) and Xiantao online platform (https://www.xiantaozi.com/). P-value ≤0.05 was selected as the threshold value.

Survival analysis

SLC4A4 gene expression and overall survival (OS), disease specific survival, progression free survival and disease-free survival were analyzed using the SURVIVAL package in R software. The Kaplan Meier (http://kmplot.com/analysis) and the GEPIA online database were used to validate the survival curve analysis of the SLC4A4 gene.

Construction and validation of 5-gene signature risk model in colon cancer

Univariate Cox regression analysis was used to screen for pyruvate metabolism related genes in colon cancer that were significantly associated with prognosis by “survival” R package. Moreover, Multivariate factors Cox regression analysis identified the key pyruvate metabolism related genes in colon cancer. Based on the analysis of 5-gene signature, we divided patients into high and low-risk by the median value. Then the 5-gene signature model was validated in TCGA cohort and GEO cohort. Kaplan-Meier (KM) survival curves and risk score distribution model was plotted respectively by “survival” R package. R packages “pheatmap” and “glmnet” were used show the relationship between expression of pyruvate metabolism related genes and 5-gene signature.

Enrichment analysis

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and GSEA enrichment analysis were performed by the “clusterProfiler” in R software. GO contains three branches: molecular function (MF), biological process (BP), and cellular component (CC). The KEGG database was used to systematically analyze the intracellular metabolic pathways and functions of SLC4A4. GSEA analysis was performed based on nominal values and normalized enrichment scores (NES) using a cluster analysis R package. Gene Set Cancer Analysis (GSCA), an integrated platform for genomic, pharmacogenomic, and immunogenomic gene set cancer analysis, was used for pathways analysis of SLC4A4 hub interactive genes.

Immune cell infiltration analysis

To evaluate the microenvironment of colon cancer, “ESTIMATE” R package was used to acquire the tumor mutation burden (TMB), microsatellite instability (MSI), immune score, and estimate score. The Tumor

Immunity Evaluation website (TIMER, http://timer.cistrome.org/) is a publicly available website that covers a total of 10897 samples from 32 cancer types in the TCGA database and aims to analyze the infiltration of immune cells in tumor tissue. Correlation between SLC4A4 expression and immune cell abundance in colon cancer was assessed by the TIMER database.

We divided samples into high- and low- SCL4A4 expression groups based on median values. Immune infiltration algorithms were CIBERSORT, CIBERSORT abs, EPIC, MCP-counter, Quantiseq, TIMER, and xcell, and heat maps of relevant immune cells were drawn by R language software.

TISIDB (http://cis.hku.hk/TISIDB/in-dex.php) is an integrated database used for the interaction of tumor-immunity and genes. TISIDB was employed for the SLC4A4 gene expression analysis of various molecular subtypes of tumor samples obtained from TCGA. The HPA (https://proteinatlas.org/) contains information on human gene expression profiles at the level of normal and tumor tissue proteins and also relates to information on immune cells. In this study, we explored the immune cell expression of SLC4A4 in colon cancer.

Cell culture and transfection

LOVO and RKO cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biosharp) supplemented with 10% fetal bovine serum (FBS; VivaCell) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. SLC4A4 was overexpressed by pcDNA3.1(+) integrated with SLC4A4 full sequences. The plasmids were purchased from GenePharma and were transfected as demanded into RKO or LOVO cells with the application of Lipofectamine 2000 (Invitrogen).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The cells were washed with PBS and all RNA was extracted according to the instructions in the RNA extraction kit. total RNA extracted was dissolved in diethyl pyro carbonate (DEPC) H_2O at a concentration adjustable to 0.5ug/ul. first, total RNA was mixed with oligosaccharide extracts of 18 deoxyribonucleic acids (OLIGO (DT)) and degraded at 65°C for 5 min, then immediately annealed for 2 minutes. Ice bath. Deoxyribonucleic acid (DNTPS), M-MLV and nuclease inhibitors were then added to the reaction system and placed into PCR at 42°C for 30 minutes in the form of cDNA template. This was followed by real-time detection of the extent of target gene mRNA expression using a quantitative real-time PCR mixture. The reaction system was first pre-denatured at 95°C for 30S, then denatured using 95°C for 5S, annealed at 60°C for 30S to extend the amplification for 40 cycles and finally a dissociation procedure was added to detect the solubility profile. The relative expression levels of the target gene and the internal reference gene GAPDH were obtained by $2^{-\Delta\Delta CT}$ calculation method. The primer sequences were listed in Table S1.

Western blotting

Colon cancer cells from the empty cell control, transfected Vector and OE-SLC4A4 groups were digested, centrifuged and collected, then the cells were resuspended in PBS and lysed with BeyoClick Cell Lysis

Solution. Cell debris was removed by centrifugation of the cell lysate at 4°C and 12,000 r/min. The supernatant was collected, added to 5-SDS buffer and heated to 99°C for 10 minutes to obtain denatured protein samples. Protein samples were separated by SDS polyacrylamide gel electrophoresis and then transferred to polyvinyl fluoride (PVDF) membranes. PVDF membranes were sealed with 5% skimmed milk for 1 hour at room temperature, then primary antibody dilutions were added and incubated overnight at 4°C, followed by secondary antibody dilutions for 1 hour at room temperature. Finally, after washing the PVDF membranes, the proteins on the membranes were developed by adding chemical reflective substrates and the protein bands were obtained using a gel imager. Tubulin was selected as an internal loading control. All primary and secondary antibodies are presented in Table S2. All western blot assays were repeated at least three times.

Cell counting kit-8 (CCK-8) assay

Colon cancer LOVO and RKO cell lines, Vector group and OE-SLC4A4 group were evenly plated on 96 cell plates with a cell density of 3000 per well. at 0h, 24h , 48h , 72h and 96h after cells adhered to the wall, appropriate reagent concentrations were added to detect CCK8 reagent and incubated in the incubator for two hours, followed by enzyme labeling using Detection. OD values of colon cancer cells in the experimental and control groups were measured at 450 nm and the data analyzed. The experiment was repeated three times.

EdU assay

The EdU assay was performed to assess DNA synthesis in colon cancer cells. Colon cancer cell lines LOVO and RKO, Vector group and OE-SLC4A4 group colon cancer cells were incubated with 25uM of EdU for 2 hours and then stained using the BeyoClick™ EdU Cell Proliferation Assay Kit according to the manufacturer's protocol. Images were obtained by using a fluorescent microscope and analyzed by Image J software.

Wound healing assay

LOVO and RKO cells were inoculated in 6-well culture plates and transfected with Vector group and OE-SLC4A4 group colon cancer cells respectively after the cells were plastered. Cells continued to be plastered until they aggregated to 80-90%. A straight line was drawn at the bottom of the well with a 10uL pipette tip, followed by a horizontal line in the vertical direction of the first line. After labelling, wash three times with PBS to remove the exfoliated cells and then add fresh medium. At 0 and 24 hours of labelling, photographs were taken of the same scratch location using an inverted phase contrast microscope and the scratch area was measured using ImageJ software.

Statistical analysis

All statistical analysis was performed by R software (version 4.0.5; http://www.Rproject.org) and Graphpad Prism 9. Data were expressed as mean ± standard deviation, and t-test was used for

comparison between two groups and one-way ANOVA for comparison between multiple groups. Differences were considered significant at values of $P<0.05$.

Results

Screening and identification of SLC4A4 as an independent protective factor of prognosis prediction in colon cancer.

First, we retrieved the raw data of 150 pyruvate metabolism related genes from the MSigDB database. In order to explore the comprehend functions of pyruvate metabolism related genes, GO analysis was performed and the results showed that the top five enriched BP terms were “pyruvate metabolic process”, “glycolytic process”, “ATP generation from ADP”, “ADP metabolic process” and “nucleoside diphosphate phosphorylation”. The top five enriched CC terms were “nuclear envelope”, “host cellular component”, “host cell”, “nuclear pore” and “mitochondrial matrix”. The top five enriched MF terms were “structural constituent of nuclear pore”, “lyase activity”, “monosaccharide binding”, “carbohydrate kinase activity” and “oxidoreductase activity, acting on the aldehyde or oxo group of donors” (Figure 1A). KEGG pathway analysis revealed that pyruvate metabolism related genes were involved in glycolysis/gluconeogenesis, carbon metabolism, citrate cycle (TCA cycle), RNA degradation, and HIF-1 signaling pathway (Figure 1B). Then, expression analysis of 150 pyruvate metabolism related genes was performed and 116 differentially expressed genes were selected, which were subsequently subjected to univariate and multivariate Cox regression analysis and the results indicated that the pyruvate metabolism related genes SLC4A4 and PPARCG1A were independent protective factors, and INSR and SLC16A8 were independent risk factors in colon cancer (Figure 1C, 1D). Therefore, ENO3, INSR, PPARGCA1, SLC16A8 and SLC4A4 were selected as pyruvate metabolism hub genes for subsequent analysis. With the expression of ENO3, INSR, PPARGCA1, SLC16A8 and SLC4A4, we generated a 5-gene signature risk model and found out that higher 5-gene signature represented poor prognosis. To verify the validity of 5-gene signature, Kaplan-Meier curves for the training cohorts in TCGA and validation cohorts in GEO database were shown in Figure S1A&S1D. The 5-gene signature was positively correlated with poor prognosis. Then, we divided patients into high and low risk groups according to the best cut-off value of 5-gene signature. The distribution of the survival data and 5-gene signature for each patient, as well as the heatmaps of ENO3, INSR, PPARGCA1, SLC16A8 and SLC4A4 were shown in Figure S1B S1C S1E S1F, in which patients with higher 5-gene signature usually had shorter survival time. Moreover, we detected the relationship between expression levels of ENO3, INSR, PPARGCA1, SLC16A8 and SLC4A4 with prognosis of colon cancer with Kaplan Meier analysis, and the results showed that the differences between the expression of INSR and PPARGC1A and OS were not significant, high expressions of ENO3 and SLC16A8 were associated with worse OS, whereas high expression of SLC4A4 was correlated to better OS, disease specific survival and progression free survival status (Figure 1E-L). On the basis of above results of Cox regression analysis and Kaplan Meier analysis, SLC4A4 was an independent protective factor of prognosis prediction and was selected for in-depth study to investigate its expression and function in colon cancer.

The correlation of SLC4A4 with clinicopathological factors and validation of its expression in clinical specimens of colon cancer.

We obtained raw data of RNA-seq profile and clinical information from the Genomic Data Commons (GDC) of the TCGA database, including 478 primary colon cancer samples, and the baseline data of training set are shown in Table 1. Using Xiantao online tools, the expression levels of SLC4A4 with clinicopathological factors were analyzed, and the results showed that the expression of SLC4A4 was significantly lower in colon cancer samples than in normal colon tissues (p<0.001, Figure 2A). The expression of SLC4A4 in colon cancer was also lower than in paired normal tissues (p<0.001, Figure 2B). The expression of SLC4A4 was lower in T4 than in normal tissues (p<0.001) while there was no difference between stage T4 and T1&T2&T3 (p=0.34, Figure 2C). And there were significant differences between stage N1&N2 and N0 (p<0.05, Figure 2D), stage M1 and M0 (p<0.05, Figure 2E), pathological stage and & & (p<0.05, Figure 2F). Further analysis of relationship between expression levels of SLC4A4 and clinicopathological factors in TCGA cohort revealed that the expression levels of SLC4A4 was significantly correlated to N stage (p=0.018), M stage (p=0.042) and pathological stage (p=0.016) (Table S3). Therefore, SLC4A4 correlates with a better prognosis and clinicopathological factors in colon cancer.

Analysis of potential upstream factors of SLC4A4.

Genetic and epigenetic variants significantly contribute to the regulation of carcinogenesis and immune tolerance. We examined the frequency of SLC4A4 mutations in the TCGA database (10,967 samples/10,953 patients in 32 studies) using cBioPortal and the results showed no significant differences in mutation rates in COAD (Figure S2A), with SLC4A4 mutations accounting for 3.2% of all mutations. Then we subsequently analyzed the DNMIVD database to test whether methylation factors influence the expression of SLC4A4 in colon cancer, and the results showed no significant effect of methylation on the difference in SLC4A4 expression in colon cancer (Figure S2B), no significant difference in methylation between colon tumors and normal tissue (Figure S2C), and no significant correlation between SLC4A4 and methylation (Figure S2D). The prognostic differences between the two groups were investigated by grouping patients according to whether SLC4A4 were methylated, and the results showed that the prognosis of different human cancer cases with SLC4A4 methylation was similar to human cancer cases without SLC4A4 methylation, including overall survival (p=0.429), disease-free survival (p=0.509) and progression-free survival (p=0.281) (Figure S2E-G). Then we compared SLC4A4 expression levels across cell lines for pre- and post-cytokine treated samples via the TISMO online database. The cytokine treatments included in this module included four methods, IFNγ, IFNβ, TNFα and TGFb1. The results showed that there were significant differences in SLC4A4 expression levels after the three cytokine treatments, IFNγ, IFNβ and TNFα (Figure S3A). We predicted the TFs and miRNAs of SLC4A4 and TFs of miRNAs predicted through TransmiR and PROMO databases, and then intersected TFs and miRNAs and finally obtained 12 miRNAs and 28 TFs (Figure S3B). The results indicated that 28 TFs such as FOXP3, IRF1 and HNF1B acted on 12 miRNAs such as has-miR-92b-3p and has-miR-211-5p

to regulate the expression of SLC4A4 through the feed-forward loops, which provide transcriptional–post transcriptional regulatory network.

Functional enrichment analysis of SLC4A4

To gain insight into the potential functions of SLC4A4 in colon cancer and the regulatory networks involved, a series of functional enrichment analysis was performed. The volcano plot showed 1121 genes associated with SLC4A4, including 896 up-regulated associated genes such as FAM11B, CA2, CDKN2BAS et al. and 225 down-regulated associated genes such as BYSL, DDAH2, WDR46 (Figure 3A). Heat map showing the top 50 up-regulated and 50 down-regulated SLC4A4-related genes (Figure 3B). Then we analyzed SLC4A4 co-expressed genes using Metascape, and the results indicated that SLC4A4 co-expressed genes were enriched in cargo concentration in the ER, ribosome biogenesis, regulation of viral-induced cytoplasmic pattern recognition and metabolism of RNA (Figure 3C, 3E). The PPI network identified densely connected regions between proteins and three molecular complex detection (MCODE) were extracted, including MCODE1, MCODE2, and MCODE3. MCODE1 is consisted of RPL37A, RRS1, WDR74, RPL37, RPL31, RPL30, and EIF6. MCODE2 is consisted of CTNNBL1, PRPF6, POLR2J, GTF2F2, and PQBP1. MCODE3 is consisted of SEC24D, TMED10, LMAN1, and SERPINA1 (Figure 3D). Then the top 100 DEGs of SLC4A4 were identified, and a heatmap indicated the correlation between them (Figure 4A). Gene set enrichment analysis (GSEA) revealed that DEGs of SLC4A4 were mainly enriched in cell cycle regulatory processes, including mitotic G1 phase and G1/S transition, DNA replication, cell cycle mitotic G2/M checkpoint, and cell cycle checkpoint, indicating that SLC4A4 may play an important role in proliferation of colon cancer (Figure 4B). GO analysis were subsequently performed on 300 SLC4A4 DEGs, which suggesting that the top five molecular function (MF) terms were hormone activity, UDP-glycosyltransferase activity, peptidoglycan binding, beta-1,3-galactosyltransferase activity and galactosyltransferase activity. The top five biological process (BP) terms were isoprenoid metabolic process, terpenoid metabolic process, diterpenoid metabolic process, retinoid metabolic process and retinol metabolic process. The top five cellular component (CC) were cluster of actin-based cell projections, brush border, brush border membrane, zymogen granule and zymogen granule membrane (Figure 4C). Moreover, KEGG pathways analysis was carried out to investigate comprehend functions of SLC4A4 DEGs and the results showed that SLC4A4 DEGs were involved in neuroactive ligand-receptor interaction, pancreatic secretion, retinol metabolism and nitrogen metabolism (Figure 4D).

SLC4A4 is correlated with immune cell infiltration and tumor microenvironment

Immune cell infiltration plays a crucial role in tumor progression. To investigate the relationship between SLC4A4 with immune cell infiltration, CIBERSORT, CIBERSORT-ABS, EPIC, MCPCOUNTER, QUANTISEQ, TIMER and XCELL algorithms were performed on SLC4A4 high expressed group and low expressed group. The heatmap indicates the correlation of SLC4A4 with immune infiltration phenotypes, including T cells, B cells, $CD8^+$ T cells, macrophages, mast cells, neutrophils and NK cells (Figure 5A). The subsequent analysis of relationship between expression levels of SLC4A4 with immune cell infiltration showed that high expression levels of SLC4A4 are associated with higher expression of $CD4^+$ T cell

(p=0.019, TIMER, Figure 5B; p=2.5e^{-09}, EPIC, Figure 5C), CD8^{+} T cell (p=3.4e^{-05}, TIMER, Figure 5D; p=0.00047, EPIC, Figure 5E) and NK cell (p<2.22e^{-16}, MCPCOUNTER, Figure 5F; p=0.031, QUANTISEQ, Figure 5G). Besides, high expression of SLC4A4 are associated with higher ESTIMATE score (p=0.0015), immune score (p=1.3e^{-06}) and TMB (p=0.022) (Figure 5H-5J). Moreover, the correlation network indicates that SCL4A4 was positively correlated with FAS, CD274 and CTLA4 (Figure 5K).

SLC4A4 inhibits colon cancer cell proliferation and migration

Next, to explore the role of SLC4A4 in regulating colon cancer cell proliferation and migration, a series of experiments were performed in vitro. We firstly upregulated the expression of SLC4A4 in LOVO and RKO cells and the efficiency of overexpression was verified by qRT-PCR and western blotting (p=0.0275, Figure 6B&6C; p=0.0209, Figure 6E&6F) and qRT-PCR (p<0.0001, Figure 6A; p<0.0001, Figure 6D) analysis. The wound healing assay revealed that upregulation of SLC4A4 inhibits the migrative ability of LOVO (p<0.0001, Figure 6I&6J) and RKO (p<0.001, Figure 6K&6L) cells. Next, CCK8 assay was performed that the results indicated that SLC4A4 promotes the cell viability of colon cancer cells. The subsequent EdU assay also revealed that upregulation of SLC4A4 promotes the cell viability in LOVO (p<0.01, Figure 6M&6N) and RKO (p<0.001, Figure 6O&6P) cells.

SLC4A4 suppresses partial EMT signaling in vitro.

To further investigate the mechanisms of SLC4A4 in suppressing colon cancer progression, we firstly screened genes interacting with SLC4A4 using GeneMANIA online tool, and the results showed that there were 20 genes correlating with SLC4A4, including CA4, SLC4A7, CA2, MAPK7, et al. (Figure 7A). Then we analyzed the top 5 genes with the strongest correlations in cancer-related pathways by GSCALite , which revealed that SLC4A4 had strong inhibitory effects on EMT, apoptosis, cell cycle, DNA damage response, while had the strongest activate effects on RKT, suggesting that SLC4A4 affects oncogenesis through multiple pathways (Figure 7B). Since above bioinformatic analysis indicated the potentially inhibitory effect of SLC4A4 on EMT, we detected the protein levels of EMT signaling pathway related proteins through western blotting assays. And the results showed that the upregulated of SLC4A4 significantly increased the protein levels of E-cadherin (p=0.0017, Figure 7C&7D; p=0.0030, Figure 7E&7F) and decreased the protein levels of Twist1 (p=0.0015, Figure 7C&7D; p=0.0009, Figure 7E&7F), while there was no obvious change in N-cadherin in both LOVO and RKO cells. These findings suggest that SLC4A4 inhibits colon cancer proliferation and migration through suppressing partial EMT signaling pathway.

Expression and prognostic analysis of SLC4A4 and its correlation with immune cell infiltration in pan-cancer

Next, to investigate the role of SLC4A4 in pan-cancer, a series of bioinformatic analysis were performed. Figure 8A showed that the expression levels of SLC4A4 were significantly upregulated in tumors including GBM, GBMLGG, LGG, STES, PRAD, STAD, SKCM, LAML, PCPG, and CHOL, while downregulated in tumors including BRCA, KIRP, COAD, COADREAD, HNSC, LUSC, LIHC, WT, BLCA, THCA, READ, PAAD, TGCT, ALL, ACC, KICH, CESC, ESCA, KIPAN, and OV. Survival analysis revealed that SLC4A4 comes with better

prognosis in tumors including GBMLGG, KIRC, MESO, COAD, COADREAD, NB, and SKCM (Figure 8B). Then we explored the correlation of SLC4A4 with immune cell infiltration in pan-cancer using Sangerbox online tool, and the heatmap showed the correlation of expression level of SLC4A4 with immune regulators including chemokines, receptors, MHC, immune inhibitors and immune stimulators in pan-cancer (Figure 8C). Besides, Figure 8D suggested the SLC4A4 was positively associated with immune checkpoints including CD276, IL4, IL13, CTLA4 et al. and negatively correlated to immune checkpoints including CD70, IL1A, CX3CL1 et al. in pan-cancer.

To investigate the immune cell phenotypic heterogeneity of SLC4A4, we performed single cell RNA-seq analysis of immune cells from colon cancer patients through THE HUMAN PROTEIN ATLAS (https://www.proteinatlas.org/) online website. After data pre-processing and quality control, we analyzed in the Monaco database to obtain immune cell type expression maps, which showed that the expression of SLC4A4 in the bar graph (Figure 9A) was closely related to Gama Delta (γδ) T cells, T cells, and NK cells, and the heat map (Figure 9B) further showed that the expression of SLC4A4 was mostly associated with γδ T cells. To identify cell subpopulations in colon cancer patients, we performed SC Transform standardization to regress UMI counts, gene counts, percentage of mitochondrial reads and cell cycle phases in immune cells, followed by integrated normalization of the dataset using Seurat. Following SingleR annotation, 52 immune cell expression clusters were predicted from the samples (Figure 9C) and correlated Gotreemap was done for γδ T cells (Figure 9D), showing that SLC4A4 expression was closely associated with cell membrane, plasma membrane and T cell receptor complexes in cellular components, and in biological processes, with negative regulation of translocation, positive regulation of calcium ion transport, and positive regulation of natural killer cell-mediated cytotoxicity, cytolysis, and adaptive immune responses.

Exploring the correlation of SLC4A4 with tumor mutation load (TMB) and microsatellite instability (MSI) and its role in pan-cancer immunotherapy.

To understand the effectiveness of SLC4A4 in predicting immune checkpoint inhibitor (ICI) therapy, we assessed the correlation of expression levels of SLC4A4 and the two biomarkers commonly used clinically for immunotherapy prediction: tumor mutational load (TMB) and microsatellite instability (MSI). The results showed that SLC4A4 expression was positively correlated with TMB values in COAD, COADREAD, USCN, OV, MESO and KIVH, and negatively correlated with TMB in CHOL, LUAD, THYM, LUSC and GBMLGG (Figure 10A). In addition, a positive correlation between SLC4A4 and MSI expression was found in TGCT, COAD, COADREAD, CHOL, UVM and READ; and a negative correlation was found in KICH, KIPAN, PAAD, UCS and LUSC (Figure 10B). The results suggest that SLC4A4 may have the ability to predict the efficacy of ICIs in the corresponding cancers. To this end, we further investigated the role of SLC4A4 expression levels in pan-cancer immunotherapy. In anti-PD-1 treatment group, the difference between the correlation of SLC4A4 and prognosis was not significant. Higher expression of SLC4A4 was negatively correlated with prognosis of patients in Anti-PD-1 treatment Nivolumab only group, whereas positively associated with better prognosis of patients in Anti-PD-1 treatment Pembrolizumab only group. In Anti-PD-L1 treatment, Anti-PD-L1 treatment Atezolizumab only, and Anti-CTLA4 treatment groups,

SLC4A4 expression was positively correlated with prognosis (Figure 10C-H). Then we used the TISMO database(http://tismo.cistrome.org/) to verify the correlation between SLC4A4 with immunotherapy, through comparing SLC4A4 expression levels in different tumor models and ICB treatments and comparing SLC4A4 expression before and after ICB treatment and in responders and non-responders. Four ICB treatments were included in the module, including anti-PD1, anti-PDL1, anti-PDL2 and anti-CTLA4. The results suggested that the expression levels of SLC4A4 were significantly correlated to immunotherapies including anti-PD1, anti-PDL1 and anti-CTLA4 (Figure S4). To conclude, SLC4A4 is significantly associated with TMB and MSI and plays a crucial role in pan-cancer immunotherapy.

Discussion

Pyruvate is a crucial substrate in the process of organic anabolism and catabolism and participates in the tricarboxylic acid (TCA) cycle. Tumor cells still could produce pyruvate and convert it into lactic acid even under aerobic conditions. Considerable studies have identified that abnormal mitochondrial pyruvate metabolism is closely correlated to the occurrence, development and metastasis of tumors [17-19].

SLC4A4, a sodium-dependent HCO_3^- transporter, is a class of membrane integrins that mediate transmembrane transport of specific substrates [13], which has been discovered to be aberrantly expressed in a variety of malignant tumors including breast cancer, prostate cancer, pancreatic cancer, renal cell cancer and colorectal cancer [12, 15, 16, 20-24]. Previous studies revealed that SLC4A4 inhibited colon cancer cell proliferation and metastasis, and low expression levels of SLC4A4 were significantly associated with worse OS of patients with colon cancer, suggesting that SLC4A4 may play an important role in the process and prognosis of colon cancer [25, 26]. However, to the best of our knowledge, the specific mechanisms of SLC4A4 in regulating colon cancer progression and prognosis has not been well studied. In this study, SLC4A4 was found lowly expressed in colon cancer tissues compared with normal tissues, and its low expression was positively correlated with worse prognosis of patients with colon cancer. Moreover, clinicopathologic factors analysis revealed that patients with low expression level of SLC4A4 were accompanied with higher risk of lymph node metastasis and distant metastasis, suggesting that SLC4A4 may be involved in the development and metastasis of colon cancer. CCK8, EdU and wounding healing assays in vitro further verified that SLC4A4 could inhibit colon cancer cell proliferation and migration.

Tumor proliferation and metastasis are key factors affecting patient prognosis. However, due to the complexity of tumor microenvironment, no significant breakthrough has been made in the study of the mechanisms of tumor invasion and metastasis. EMT is the transformation of tumor cells from epithelial morphology to mesenchymal cell type, which usually leads to invasion and metastasis of tumor cells [27, 28]. Recent studies have shown that EMT plays a crucial role in regulating tumor cell invasion and metastasis [29-34]. One of the main features of EMT is a reduce in intercellular junctions, a decrease in the expression levels of the cell membrane protein E-cadherin and an increase in the expression levels of N-

cadherin. At the meantime, the expression levels of Twist-binding transcription factors in EMT cells were also increased. Partial EMT is a common feature of cancer and have been found to be widely expressed in pancreatic cancer cells, breast cancer cells and colon cancer cell lines [32, 35-39]. Partial EMT may be transitional or special types of tumor cells, and these epithelial cells rarely fully transformed into mesenchymal cells, and they are characterized by the widespread expression of epithelial and mesenchymal markers or the absence of epithelial markers. Partial EMT make tumor cells more plastic to epithelial filling, which is critical for tumor metastasis, recurrence, and therapeutic resistance. In this study, it was discovered that SLC4A4 upregulated the expression level of E-cadherin protein and downregulated Twist1 protein in colon cancer cells but did not significantly regulate the expression level of N-cadherin protein. Therefore, the inhibitory effect of SLC4A4 on the proliferative and migrative ability of colon cancer cells may be realized through regulating partial EMT pathways.

Immunosuppressive mechanisms in the tumor microenvironment play a protective barrier role of cancer cells [40-42]. Activating the dormant immune system by making the tumor itself an antigen through various immunomodulators may be a promising approach for tumor therapy. In this study, we explored the immune cell infiltration of SLC4A4 in colon cancer and found that SLC4A4 extensively affected immune cell infiltration, and further analysis revealed that the number of B cells, dendritic cells and $CD8^+$ T cells were positively correlated with SCL4A4 expression. B cells in the tumor microenvironment not only trigger humoral immunity and secrete inflammatory factors, but also recognize, process and present antigens. In addition, B cells maintain active interactions with other immune cells, including T cells and all immune cells that express Fc receptors. When T cell clusters such as $CD4^+$ T cells and $CD8^+$ T cells coexist with tumor-infiltrating B cells, patients with tumors could achieve better clinical outcomes. Dendritic cells activate $CD8^+$ T cells through cross-presentation of exogenous antigens, a step that is thought to be crucial for activating $CD8^+$ T cells against tumors. Previous studies have found that a reduction in the number of dendritic cells and $CD8^+$ T cells leads to a poor prognosis in patients with colon adenocarcinoma [43-48]. The γδ T cells, a kind of innate immune T cells, has attracted increasing attention in recent years because of their cytotoxic effect on most tumor cells without major histocompatibility complex (MHC) restriction [49]. An increasing number of basic studies have focused on the development, antigen recognition, activation, and antitumor immune response of γδ T cells. In the tumor microenvironment (TME), these lymphocytes express a variety of recognition receptors that have good prognostic value in a variety of malignancies, including leukemia [50]. According to several reports, γδ T cells have been shown to trigger immune responses through direct and indirect mechanisms, for example, through cytolytic synapses with target cells, as well as recruitment and stimulation of other immune cells required for the immune response. Establishing an anti-tumor response [51, 52]. These lymphocytes can induce tumor regression through cell-to-cell interactions or by secreting several soluble molecules that inhibit tumor expansion (e.g., IFN-γ and TNF). These effector molecules induce increased antitumor activity in other cytotoxic cells or positively regulate MHC-I expression in cancer cells [53]. In addition, γδ T cells stimulate somatic hypermutation and isotype switching in B cells [54-56] and may induce antibody-mediated immunity. Their effector functions also include macrophage activation and the recruitment and

activation of $CD8^+$ cytotoxic T cells and NK cells [57-60]. The immune effects of these lymphocytes also include stimulation of dendritic cell (DC) maturation, which in turn enhances their cytotoxic activity [61, 62]. Notably, cancer cells tend to express a variety of stress-inducing molecules or metabolic antigens that are recognized by the γδ TCR and helper receptors to mediate an effective response against the tumor [63-65]. γδ T cells have great potential to modulate local immunity and reshape the tumor ecological niche. However, there are few studies reporting the correlation between SLC family and γδ T cells. Our study firstly revealed that the expression levels of SLC4A4 in colon cancer was positively associated with γδ T cells. This study analyzed the expression and clinical significance of SLC4A4 in colon cancer through the TCGA database and found that SLC4A4 was closely associated with multiple signaling pathways in malignancy as well as immune cell infiltration.

These findings illustrate that SLC4A4 inhibits the proliferation and migration of colon cancer via regulating partial EMT phenotypes and positively correlates with immune cell infiltration. Meanwhile, there are still some limitations in the study. More clinical data and experiments in vitro and vivo after knockdown of SLC4A4 are needed to further validate our results.

Conclusion

To conclude, our study provides valuable insights into the prognosis of colon cancer through bioinformatic analysis and experiments verification in vitro that SLC4A4 expression is downregulated in colon cancer. SLC4A4 inhibits the proliferation and migration of colon cancer via regulating partial EMT phenotypes. Furthermore, SLC4A4 correlates with immune cell infiltration and γδ T cells. These findings may help to elucidate the role of SLC4A4 in tumorigenesis and progression and provide new perspectives for the development of more precise and personalized immune anti-tumor therapies in the future.

Declarations

Acknowledgments

Not applicable.

Patient consent for publication

Not applicable.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

The original manuscript was both prepared and written by Chengqing Yu and Haoran Li. Chen Zhang, Yuchen Tang, Yujie Huang, Haodong Lu and Kanghui Jin were responsible for the laboratory experiments and data compilation. Jian Zhou and Jian Yang monitored the project. All authors made contributions to the article and approved the submitted version.

Data Availability Statement

These data are as presented within the paper. Raw data supporting the conclusions of this paper will be made available to the corresponding authors on request, but without unnecessary reservations.

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Figures

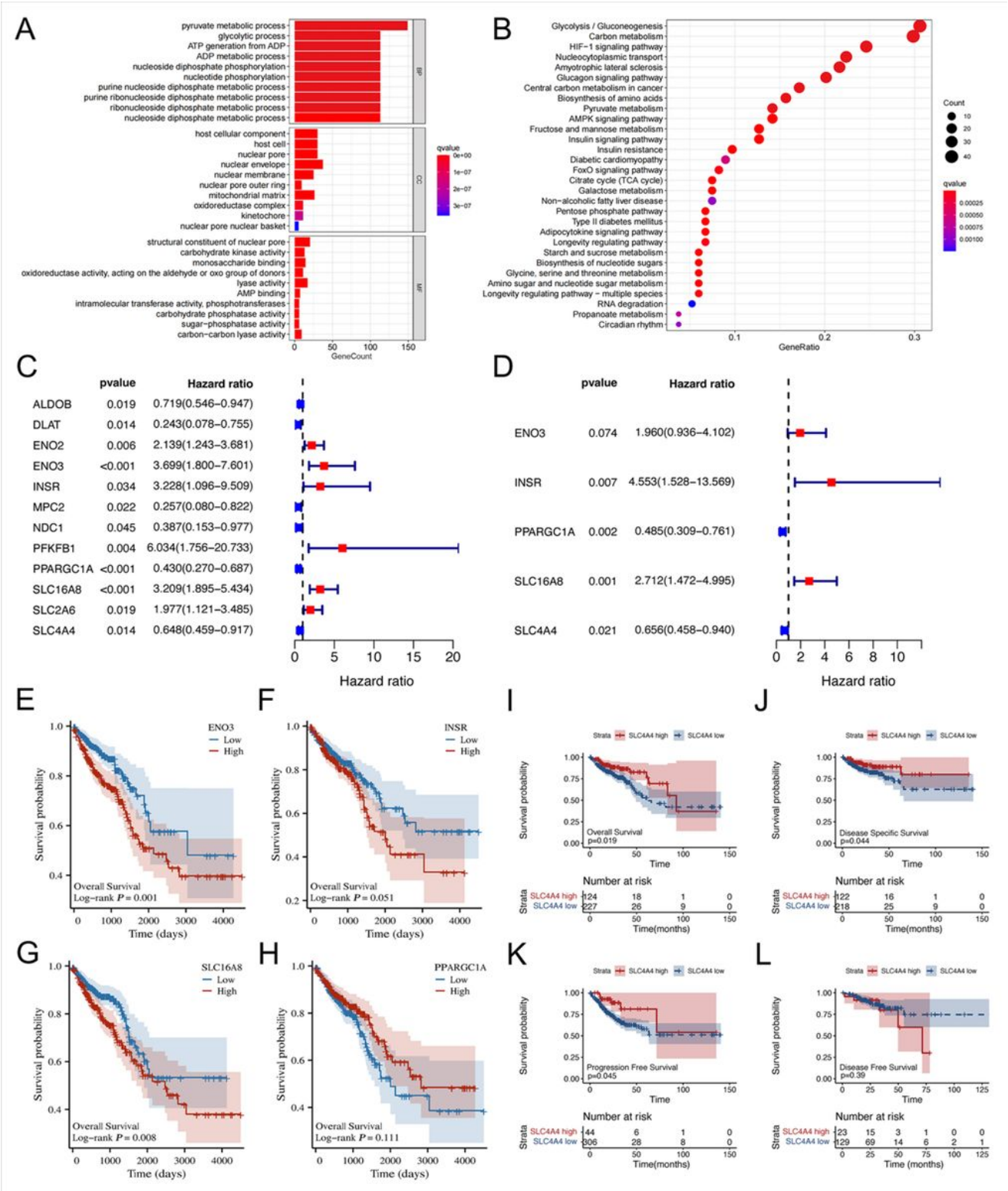

Figure 1

Screening and identification of SLC4A4 as an independent protective factor of prognosis prediction in colon cancer (A) GO analysis of 150 pyruvate metabolism-related genes. (B) KEGG analysis of 150 differentially expressed pyruvate metabolism-related genes. (C, D) Univariate and multivariate cox regression analysis of 116 differentially expressed pyruvate metabolism-related genes. (E-H) Kaplan-Meier analysis showed the prediction probability of overall survival of patients with colon cancer according to differential expression of ENO3, INSR, SLC16A8 and PPARGC1A in the TCGA cohort. (I-L) All cases from TCGA cohort were stratified into high- and low- SLC4A4 groups based on the best cut off value. Kaplan-Meier analysis suggested that patients with upregulated SLC4A4 experienced a longer overall survival, disease specific survival and progression free survival time and there was no difference in disease free survival time between high- and low- SLC4A4 groups.

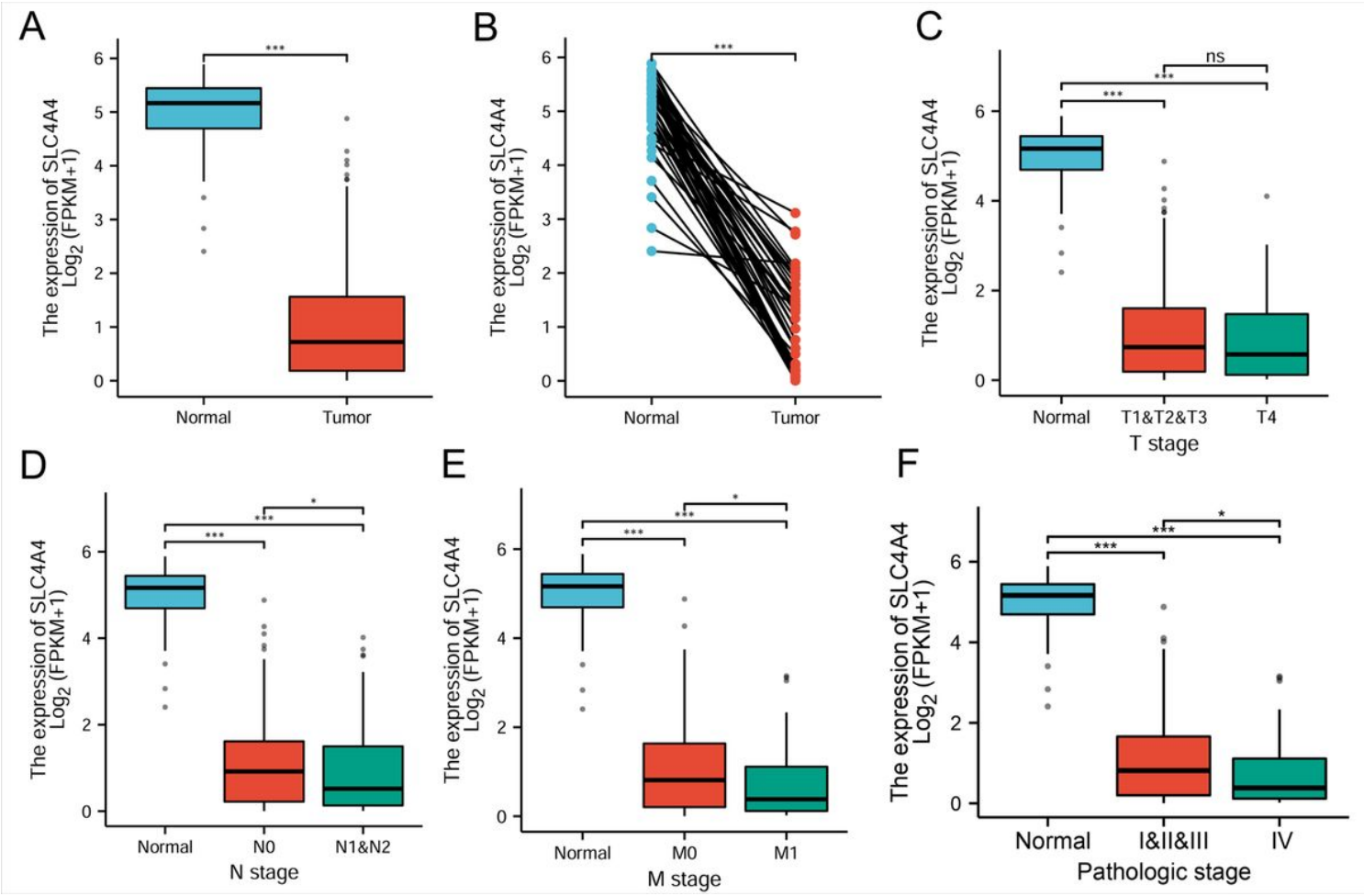

Figure 2

Investigating the correlation of SLC4A4 with clinicopathological factors in TCGA cohort and validation of its expression in clinical specimens of colon cancer (A) The analysis of SLC4A4 expression in colon cancer compared with unpaired normal tissues in RNA-seq in TCGA-COAD cohort. (B) The analysis of SLC4A4 expression in colon cancer compared with paired normal tissues in RNA-seq in TCGA-COAD cohort. (C) The expression of SLC4A4 between normal, T1&T2&T3 and T4 in TCGA-COAD cohort. (D) The expression of SLC4A4 between normal, N0 and N1&N2 in TCGA-COAD cohort. (E) The expression of

SLC4A4 between normal, M0 and M1 in TCGA-COAD cohort. (F) The expression of SLC4A4 between normal, & & and in TCGA-COAD cohort.

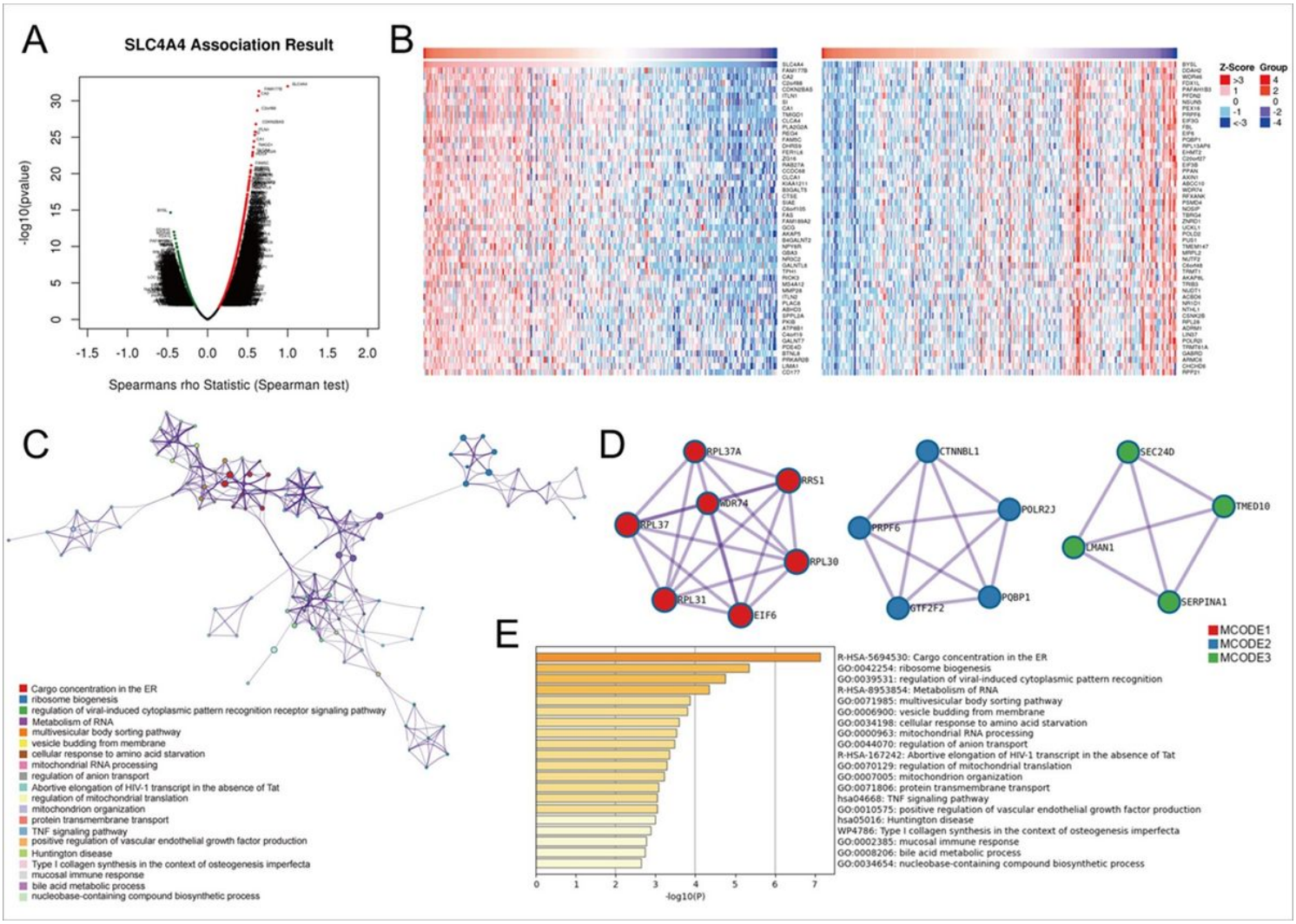

Figure 3

Functional enrichment analysis of SLC4A4 related genes (A) A volcano plot of SLC4A4 related genes in colon cancer. (B) Heatmap showing the top 50 SLC4A4 related genes in TCGA-COAD cohort. (C) A network diagram showing the related pathways of 199 co-expressed genes through Metascape. (D) Three significant MCODES showing the densely connected regions between proteins according to the relationship of co-expressed genes. (E) The GO enrichment of co-expressed genes.

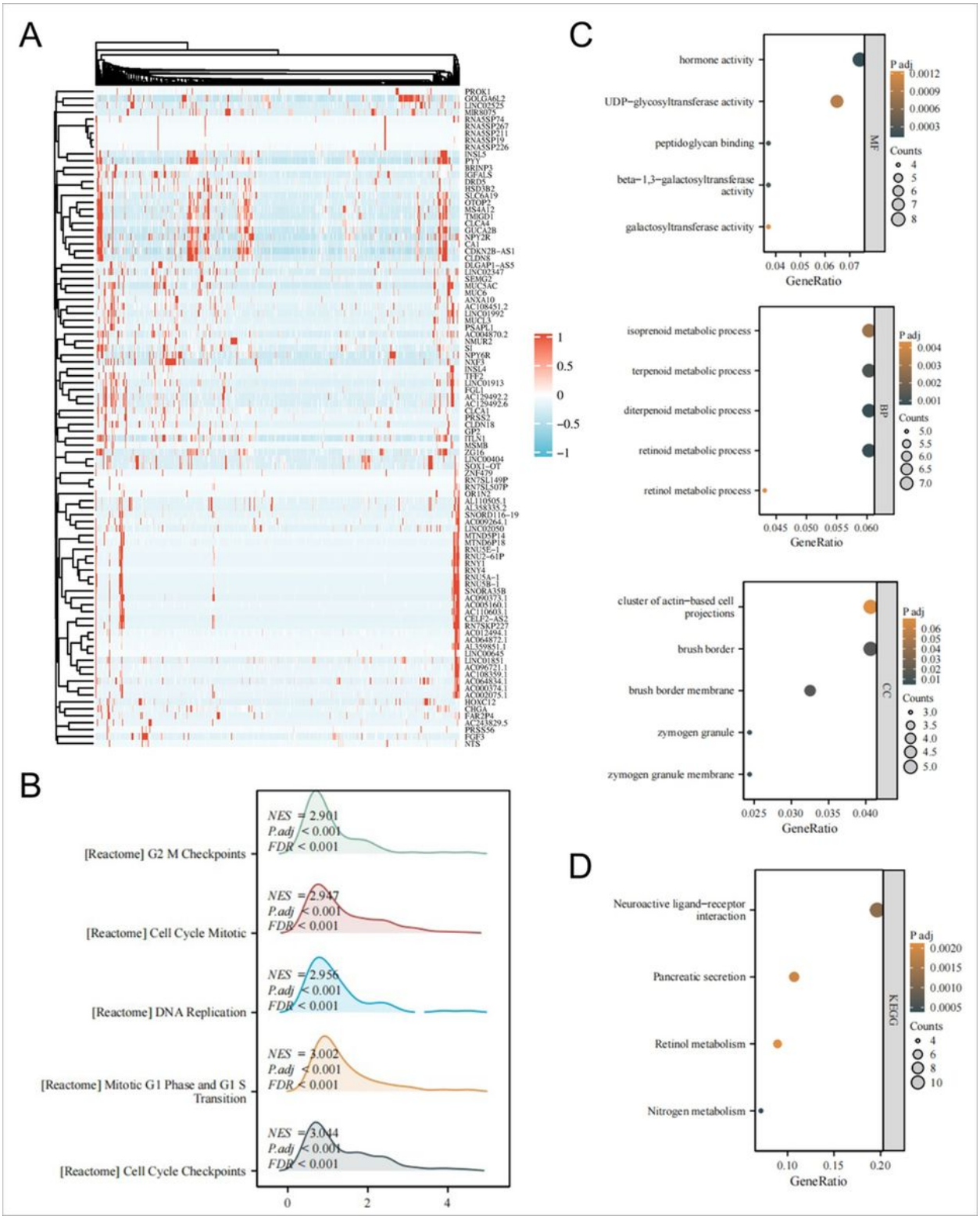

Figure 4

Functional annotation of SLC4A4 differentially expressed genes (A) Heatmap showing the expression of top 100 SLC4A4 differentially expressed genes between colon cancer and adjacent normal tissues in TCGA-COAD cohort. (B) GSEA analysis on functional cluster of differentially expressed genes in colon cancer. (C) The GO enrichment of biological process (BP), cellular component (CC), and molecular

function (MF) terms of 300 differentially-expressed genes. (D) The KEGG enrichment of 300 differentially-expressed genes.

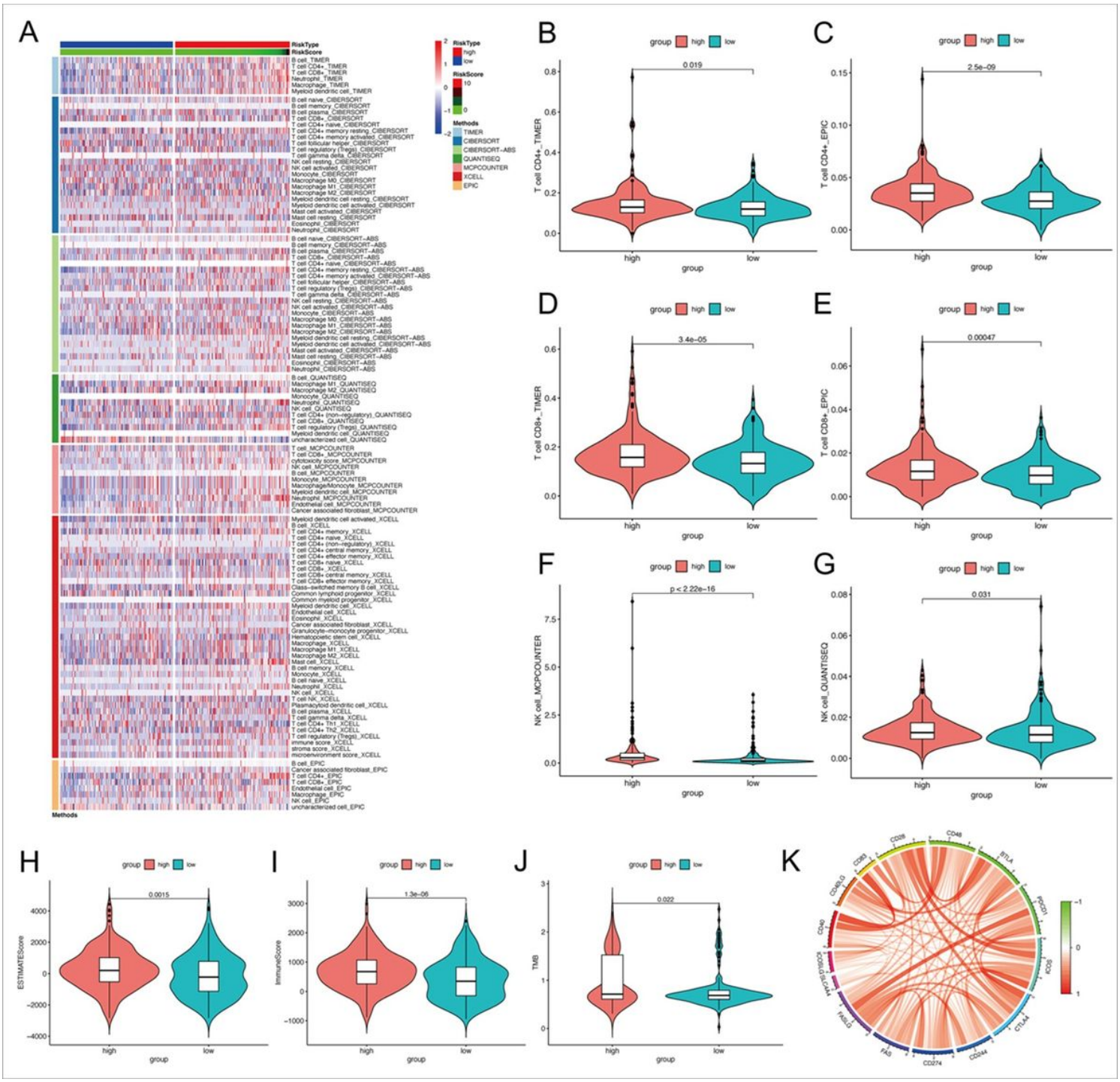

Figure 5

Correlation of SLC4A4 expression levels with immune cell infiltration, tumor microenvironment and immune checkpoint gene expression levels (A) Heatmap showing the correlation of SLC4A4 expression levels with immune cell infiltration using the Sangerbox online platform. (B-G) Box plots showing the correlation of SLC4A4 expression levels with immune cell infiltration in colon cancer. (H-J) Box plots

showing the correlation of SLC4A4 expression levels with tumor microenvironment. (K) Correlation of SLC4A4 expression levels with immune checkpoint genes expression levels.

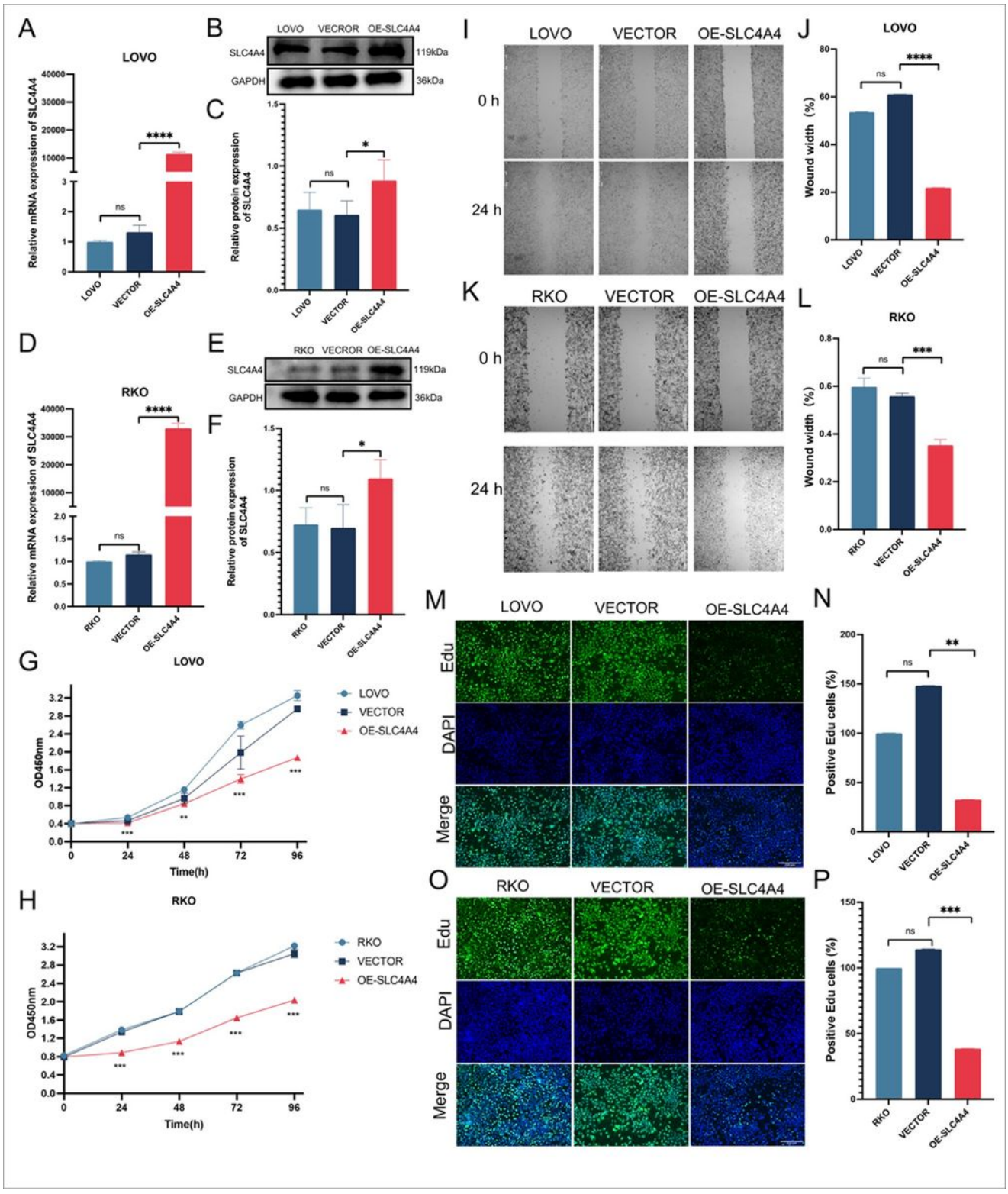

Figure 6

SLC4A4 promote colon cancer cells viability and migration in vitro (A-F) SLC4A4 was overexpressed by transient transfection in LOVO and RKO cells. Their expression levels were re-examined by qRT-PCR and

western blotting. (I-L) Images and quantitative analysis of wound healing assay in normal control, VECTOR and SLC4A4 overexpression group, suggesting that upregulation of SLC4A4 suppresses LOVO and RKO cells migration. (G, H) CCK-8 assay showing that the upregulation of SLC4A4 suppresses LOVO and RKO cells viability. (M-P) Images and quantitative analysis of EdU showing that the upregulation of SLC4A4 suppresses LOVO and RKO cells viability.

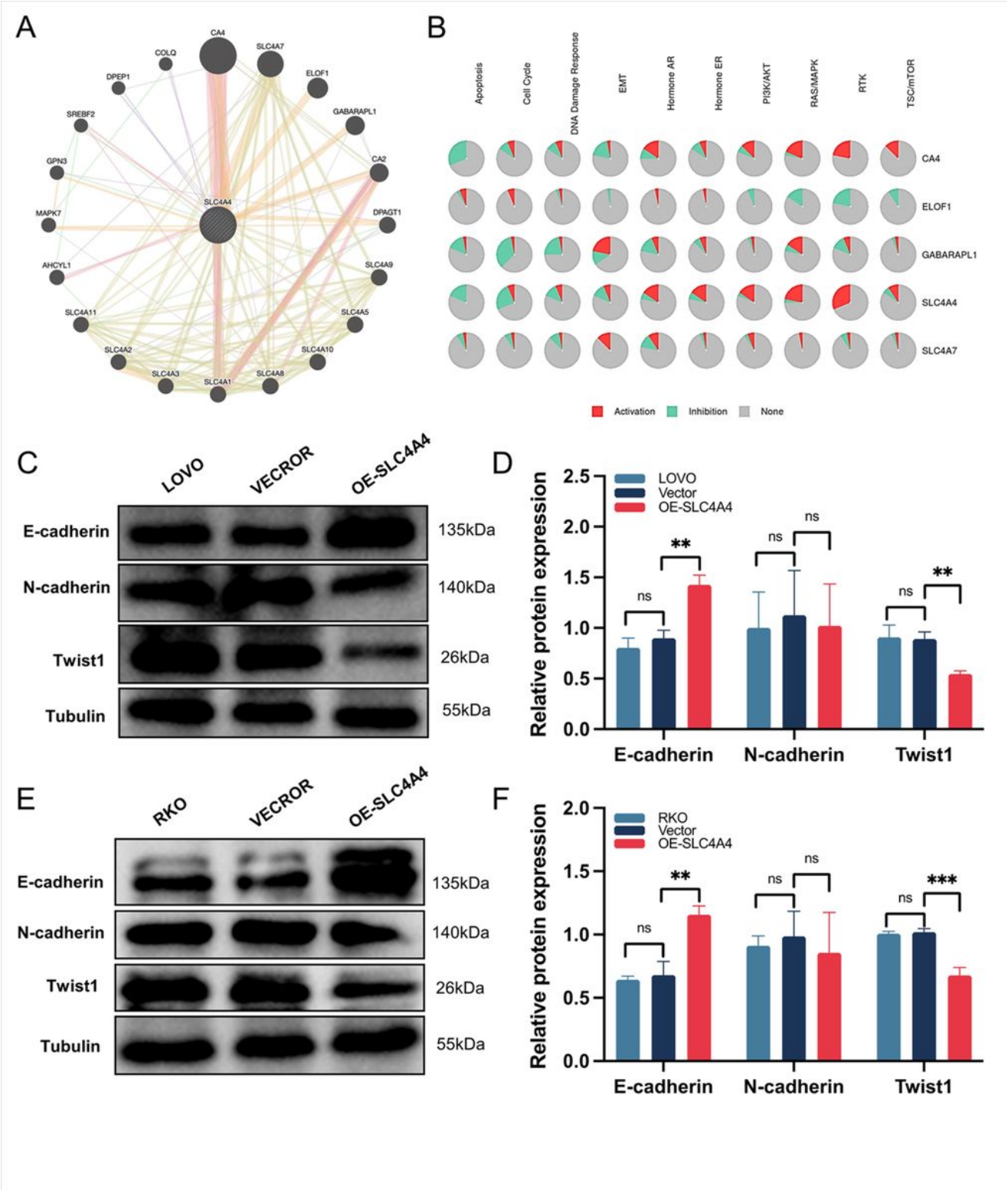

Figure 7

SLC4A4 suppresses partial EMT signaling in vitro (A) A network diagram showing the correlation of SLC4A4 with related genes. (B) GSCA pathway analysis suggested that SLC4A4 was correlated with EMT signaling. (C-F) Western blotting analysis indicates that upregulation of SLC4A4 increases the protein levels of E-cadherin and decreases the protein levels of Twist1 in LOVO and RKO cells. Tubulin protein was used as the internal control. n=3. **p < 0.01. ***p < 0.001.

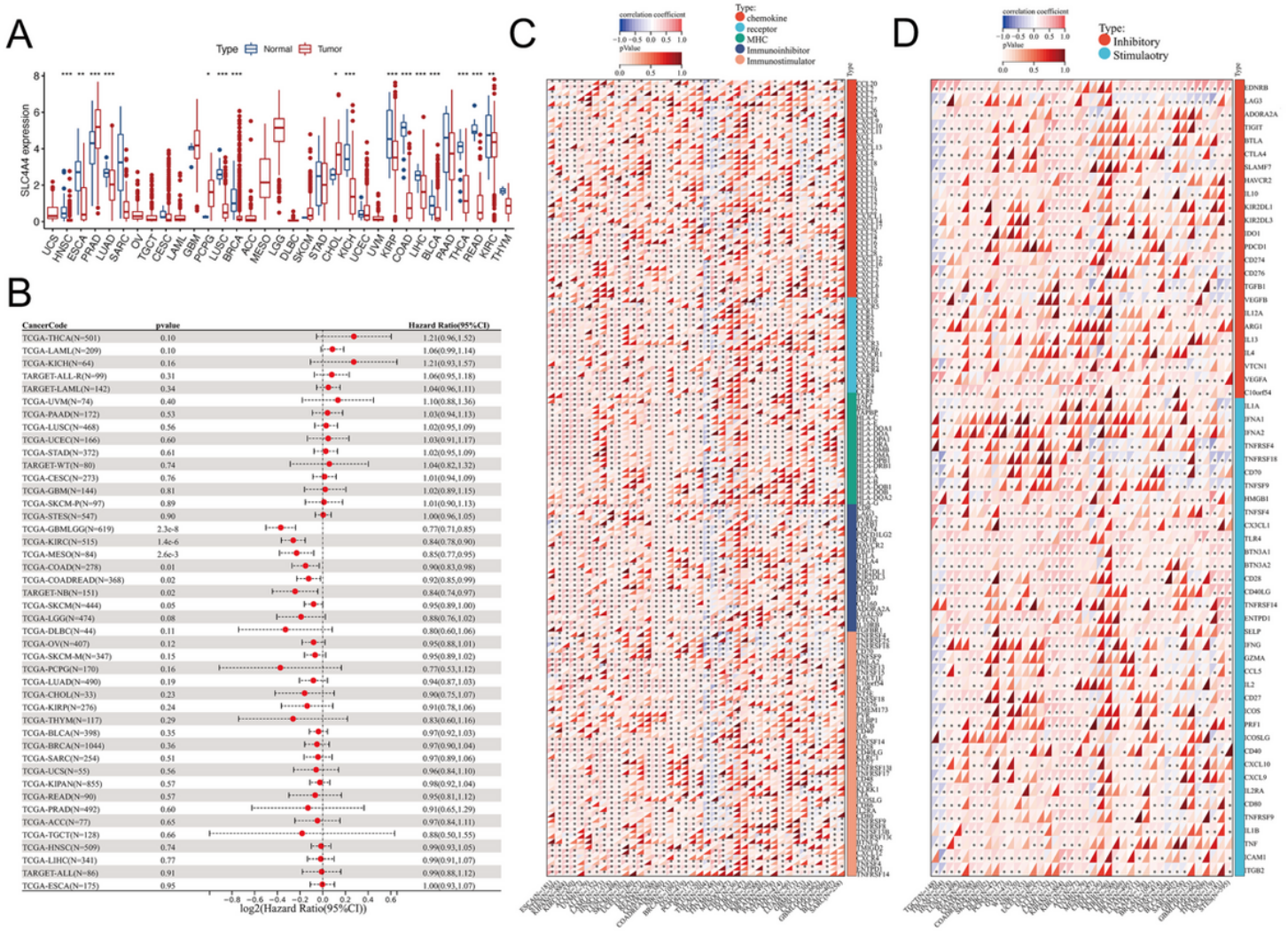

Figure 8

Expression and prognostic analysis of SLC4A4 and its correlation with immune infiltration in pan-cancer (A) Box plot of SLC4A4 expression in 33 kinds of cancers from a TCGA cohort. (B) Prognostic analysis of SLC4A4 in pan-cancer using the Sangerbox online platform. (C) Heatmap showing the correlation of SLC4A4 with immune regulator genes. (D) Heatmap showing the correlation of SLC4A4 with immune checkpoints. *p < 0.05. **p < 0.01. ***p < 0.001.

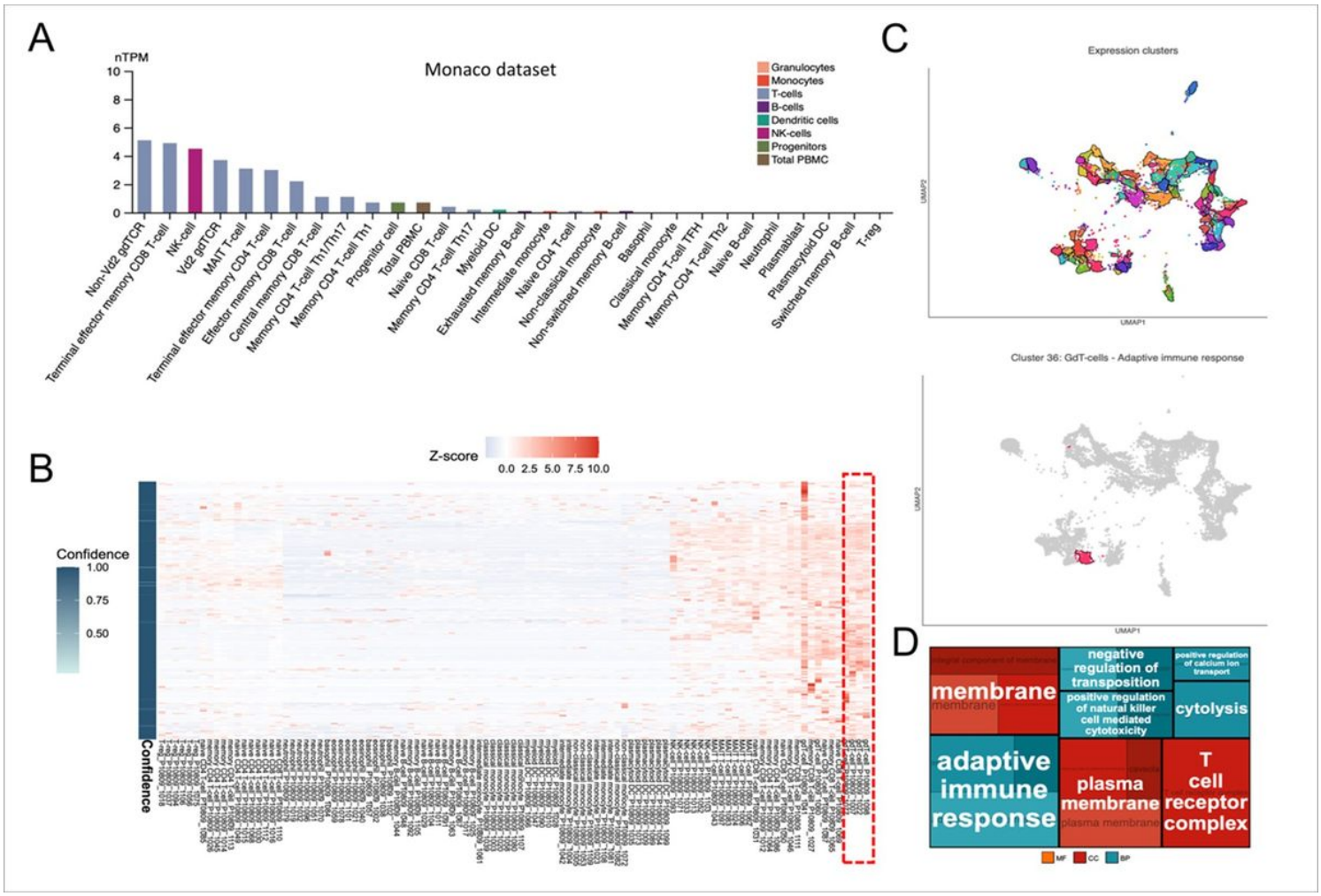

Figure 9

SLC4A4 is closely associated with Gamma Delta (γδ) T cells (A) A bar chart showing that SLC4A4 is closely associated with γδ T cells, terminal effector memory CD8 T-cell and NK cell using proteinatlas online platform. (B) A heatmap suggested that SLC4A4 is mostly associated with γδ T cells using proteinatlas online platform. (C) Single cell analysis indicates the correlation of SLC4A4 with γδ T cells. (D) A tree map showing the GO pathway analysis of γδ T cells.

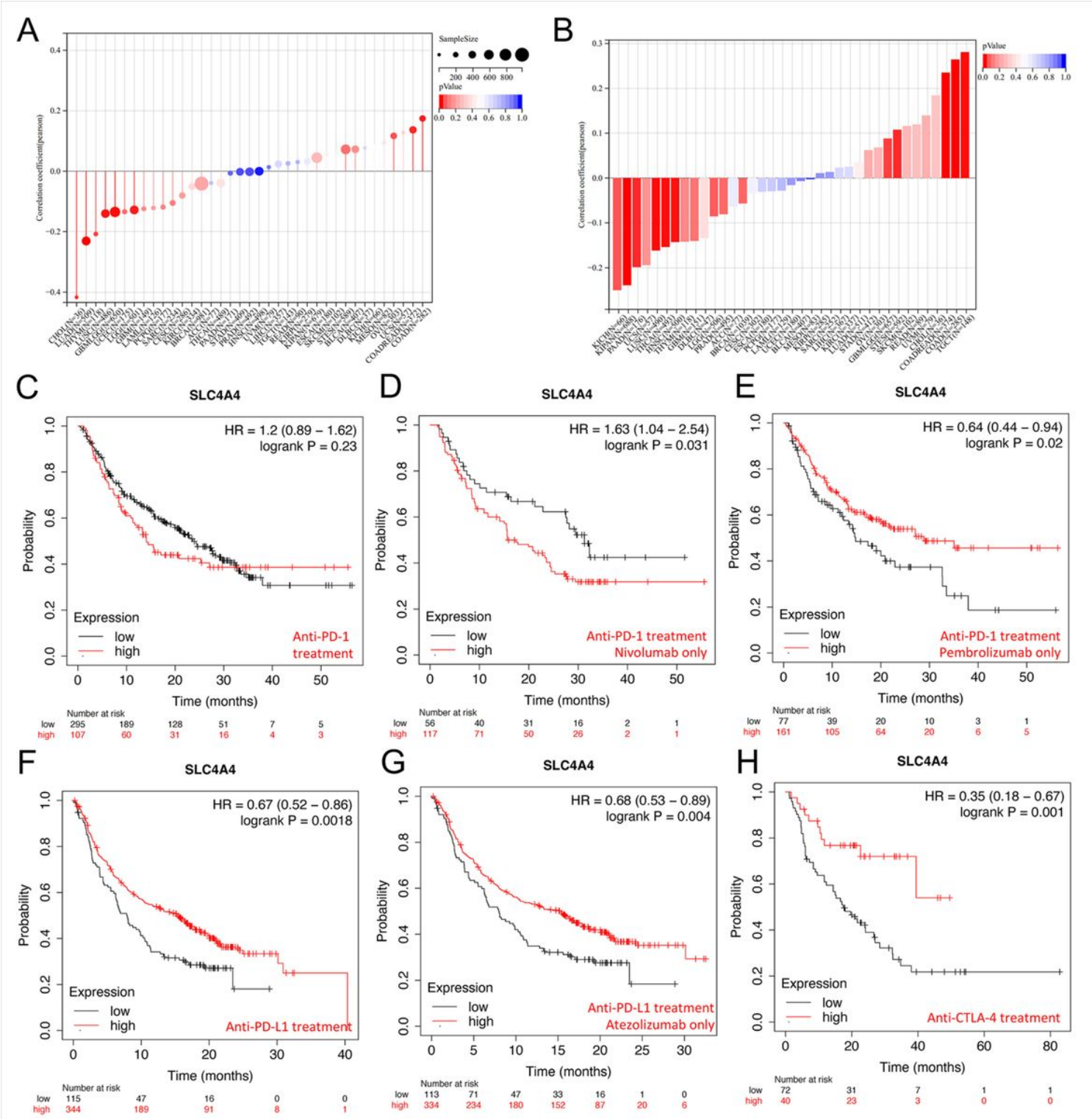

Figure 10

Exploring the correlation of SLC4A4 with tumor mutation load (TMB) and microsatellite instability (MSI) and its role in pan-cancer immunotherapy (A) The correlation of SLC4A4 with TMB in pan-cancer. (B) The correlation of SLC4A4 with MSI in pan-cancer. (C) The expression level of SLC4A4 with or without Anti-PD-1 treatment. (D) The expression level of SLC4A4 with or without Anti-PD-1 (Nivolumab only) treatment. (E) The expression level of SLC4A4 with or without Anti-PD-1 (Pembrolizumab only) treatment.

(F) The expression level of SLC4A4 with or without Anti-PD-L1 treatment. (G) The expression level of SLC4A4 with or without Anti-PD-L1 (Atezolizumab only) treatment. (H) The expression level of SLC4A4 with or without Anti-CTLA-4 treatment.

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