Original paper

Prognosis poor, immune infiltration of colon adenocarcinoma associated with low expression levels of calcium-activated chloride channel

Xueying Zhao[1,2], Yunfan Chen[1], Liyuan Wang[1], Dongli Sui[1], Jin Lu[1]

[1]Basic Medicine College, Bengbu Medical University, Bengbu, Anhui, China
[2]Anhui Key Laboratory of Computational Medicine and Intelligent Health, Bengbu Medical University, Donghai Avenue, Bengbu, Anhui, China

The calcium-activated chloride channel (*CLCA4*) in colon adenocarcinoma (COAD) and immunological infiltration have not been extensively studied.
This work thoroughly employed several datasets to assess the expression, prognosis, and association between immune infiltration and clinicopathological characteristics of *CLCA4* in cancer, as well as look into potential signalling pathways. The human protein atlas (HPA), TIMER, UALCAN, TISIDB, GSCA, SangerBox, GeneMANIA, and LinkedOmics were among the datasets that were used.
The findings demonstrated that, in comparison to normal tissues, COAD tissues had lower levels of *CLCA4* expression. The prognosis was worse for those whose levels of *CLCA4* expression were lower. For validation, immunohistochemistry (HPA) was used. Positive correlations between *CLCA4* mRNA expression and its copy number variation (CNV) were observed, and *CLCA4* CNV was linked to immunological infiltration. Subsequent investigation demonstrated the association between immune cell markers, immune checkpoint genes, and immunological infiltration with *CLCA4*. The overall survival and disease-free survival of M0 patients were considerably better than those of M1 patients, and the groups with tumour stages M0 and M1 had notably different levels of *CLCA4* expression. Its substantial enrichment in ion channel activity, transmembrane transporter activity, digestion, and other biological processes was revealed by gene ontology analysis. Oxidative phosphorylation, pancreatic secretion, Parkinson's and Alzheimer's diseases, renin secretion, and other signalling pathways were the primary associations found for *CLCA4*.
It is evident that the immunological microenvironment and functions like ion transport, metabolism, and intestinal digestion are all impacted by *CLCA4* expression.

Key words: colon adenocarcinoma, *CLCA4*, immune infiltration, metastasis, prognosis biomarker.

Introduction

The third most common cause of mortality from cancer worldwide is colorectal malignancies. It is startling to learn that 20% of colorectal cancer (CRC) patients who receive a new diagnosis have already experienced metastatic disease [1]. According to Fabregas *et al*. [2], there has been a rise in the occurrence of CRC among young adults. This highlights the need for improved screening and treatment measures. Even though immunotherapy has shown clinical success in treating CRC, there are still potential immune-related adverse events that need to be considered [3]. The effectiveness of immune checkpoint inhibitors (ICIs) in treating cancer, especially anti-PD-1 therapies, has demonstrated encouraging

results. Nevertheless, the efficacy of these treatments is restricted in individuals diagnosed with CRC and having microsatellite stable CRC, as highlighted in a study conducted by Liu *et al.* in 2021 [4]. This indicates that there is still much to be learned about the immune microenvironment of CRC.

Calcium-activated chloride channel (*CLCA4*) has been shown to play a significant role in both carcinogenesis and development through a growing body of research. Wei *et al*. established a correlation between the down-regulation of *CLCA4* and the initiation and progression of CRC [5]. This suggests that *CLCA4* may be a useful molecular biomarker for the identification of CRC in its basic stages [6]. As a tumour suppressor, *CLCA4* plays a role in the growth of a variety of cancerous tumour types. Through PI3K/AKT signalling, it prevents the epithelial-mesenchymal transition and restrains the invasive and migratory abilities of bladder, hepatocellular, and colorectal cancer [7–9]. Meanwhile, a worse prognosis for HNSCC patients has been linked to *CLCA4*, a crucial regulator influencing the biological behaviour of HNSCC cells [10]. According to recent research [11], miR-19a targets *CLCA4*, and miR-19a overexpression raises the risk of CRC by lowering *CLCA4* levels. Nevertheless, a clear understanding of the molecular mechanism underlying the connection between tumour immune infiltration and *CLCA4* remains elusive.

Using many databases, we investigated *CLCA4* levels in colon adenocarcinoma (COAD) in this work, as well as *CLCA4*'s potential involvement in colon adenocarcinogenesis, clinicopathological features, and the relationship between *CLCA4* and tumour immune infiltration. The purpose of this study is to gather more data regarding *CLCA4* as a possible biomarker, which is particularly relevant for COAD patients to enable prompt and informed diagnosis and treatment decisions.

Material and methods

Collection of data sets

Following a search across multiple publicly accessible databases, information regarding COAD from The Cancer Genome Atlas (TCGA) database was located, and the *CLCA4* gene was obtained and examined.

Calcium-activated chloride channel expression analysis

We looked at the levels of *CLCA4* expression in both normal human tissues and various cancer types using 2 independent datasets: the human protein atlas (HPA) dataset (https://www.proteinatlas.org) and TIMER https://cistrome.shinyapps.io/timer/). We performed differential analysis of *CLCA4* mRNA and protein expression as well as patient survival prognosis analysis in COAD using several online tools, including the Assistant for Clinical Bioinformatics (https://www.aclb.com/), UALCAN (http://ualcan.path.uab.edu), TISIDB (http://cis.hku.hk/TISIDB), and GEPIA (http://gepia.cancer-pku.cn/). Validation by immunohistochemistry: the information came from the HPA database, and the antibody utilised was HPA 017045. Furthermore, the location of *CLCA4* in the cell lines HeLa, RT-4, and U2OS was evaluated using the antibody HPA064770.

Relationship between calcium-activated chloride channel and copy number variation

Using the GSCA dataset (http://bioinfo.life.hust.edu.cn/GSCA/#/), we looked at the relationship between *CLCA4* and copy number variation (CNV). Next, the TIMER dataset was utilised to investigate the connection between its CNV and immune infiltration to examine the effect of different copy states of *CLCA4*, including arm-level deletion (–1), diploid/normal (0), and arm-level gain (1), on the immune infiltration as compared to normal tissues. Additionally, we looked at the connection between COAD patients' ESTIMATE scores and *CLCA4* expression using the SangerBox database (http://www.sangerbox.com).

An in-depth examination of tumour-infiltrating immune cells

Utilising the gene module provided by TIMER, our initial focus was on exploring the correlation between *CLCA4* and the abundance of immune infiltration as well as immunological markers. Afterwards, we examined the link between *CLCA4* and tumour-infiltrating lymphocytes (TILs) using the TISIDE dataset as a way to understand the types of TILs that may be regulated by *CLCA4* in COAD.

Immune checkpoint analysis

The study assessed immune checkpoint gene expression in COAD tissues and normal tissues using the Assistant for Clinical Bioinformatics dataset. These genes included *SIGLEC15*, *CTLA4*, *LAG3*, *PDCD1LG2*, *CD274*, *HAVCR2*, *TIGIT*, and *PDCD1*. The association between *CLCA4* and these genes was also examined, and the TIDE algorithm was used to forecast how ICIs will affect *CLCA4* high and low expression samples.

Association between calcium-activated chloride channel and clinical features

The connection between *CLCA4* expression and various clinicopathological factors, including gender, age, race, and tumour-node-metastasis stage, was examined using the TCGA data from the Assistant for Clinical Bioinformatics.

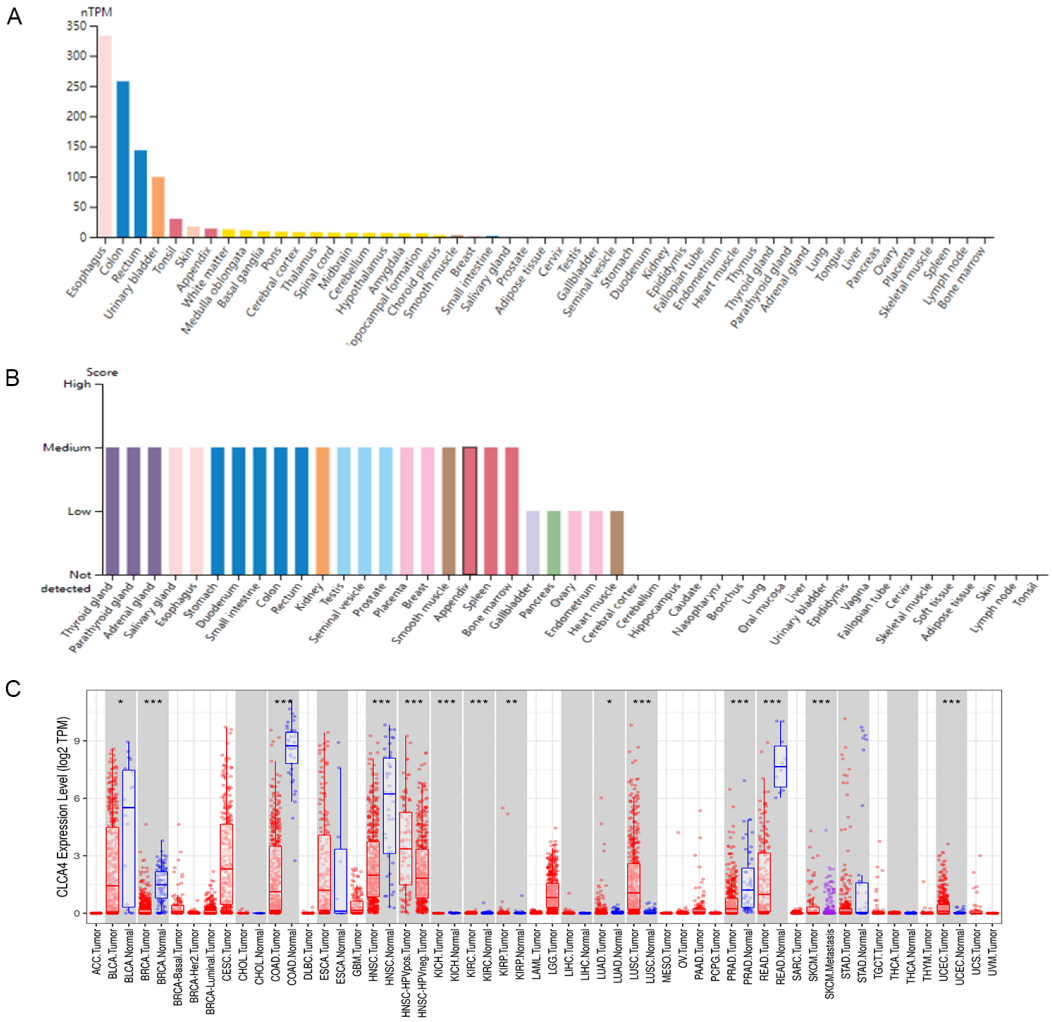

Fig. 1. Calcium-activated chloride channel (*CLCA4*) expression in a variety of human tissues. A) *CLCA4* mRNA expression levels in several human body tissues that are typical. B) Protein expression levels in several human body tissues that are typical. C) *CLCA4* mRNA expression in pan-cancer

Analysis of calcium-activated chloride channel co-expressed proteins and their functional enrichment

CLCA4-related genes were identified by GeneMANIA (http://genemania.org/) and subsequently subjected to functional enrichment analysis with the SangerBox dataset. Furthermore, we used the gene set enrichment analysis (GSEA) from the LinkedOmics (https://www.linkedomics.org/) dataset to confirm the possible function of *CLCA4*.

Statistical methods

Significance was tested by the Wilcox test for 2 samples and the Kruskal-Wallis test for 3 samples (*$p < 0.05$,**$p < 0.01$,***$p < 0.001$).

Results

Calcium-activated chloride channel levels in normal tissues and pan-cancer tissues

Using the HPA dataset, the amounts of *CLCA4* mRNA and protein in healthy human tissues were examined. The findings are presented in Figure 1A, indicating that *CLCA4* mRNA expression is tissue-specific, with particularly strong expression observed in the oesophagus, colon, and rectum. However, the concordance between *CLCA4* protein level expression (Fig. 1B) and its mRNA expression data was low, which may also be related to *CLCA4* antibody specificity and needs further confirmation. In the following study, we conducted an analysis of *CLCA4* expression across various cancer types using TIEMR. *CLCA4* was expressed at low levels in BRCA, COAD, BLCA, HNSC, KICH, KIRC, KIRP, LUAD, LUSC, READ, PRAD, SKCM, and UCEC (Fig. 1C).

Expression and prognostic analysis of calcium-activated chloride channel in colon adenocarcinoma

We further validated the low expression of *CLCA4* mRNA in COAD by utilising the GEPIA, UALCAN, and Assistant for Clinical Bioinformatics ($p < 0.05$) datasets (Figs. 2A–C). The study of CPTAC data in UALCAN revealed that COAD had lower *CLCA4* protein expression than normal tissues ($p < 0.05$)

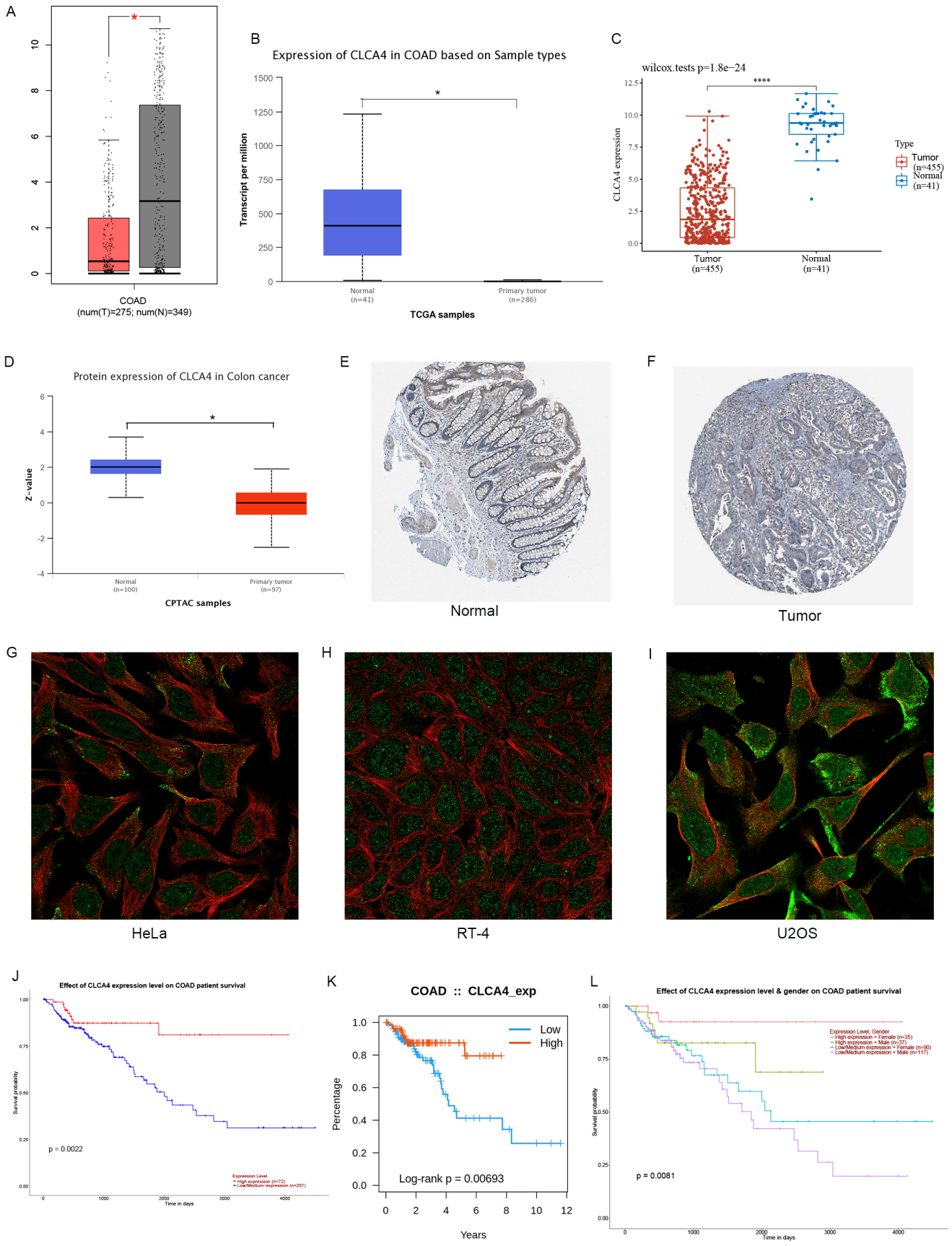

Fig. 2. Calcium-activated chloride channel (*CLCA4*) expression and prognostic importance in colon cancer. (A–C) Variations in *CLCA4* mRNA expression in colon adenocarcinoma (COAD) and normal tissues across several datasets. A) The UALCAN dataset. B) The GEPIA dataset. C) The Assistant for Clinical Bioinformatics. D) The profile of *CLCA4* protein expression (UALCAN). E–F) Representative human protein atlas-based immunohistochemistry pictures. G–I) Using the HeLa, RT-4, and U2OS cell lines, the localisation of *CLCA4* protein expression was observed. J) UALCAN was utilised to examine the correlation between *CLCA4* and the overall survival of COAD patients. K) TISIDB was utilised to examine the correlation between *CLCA4* and the overall survival of COAD patients. L) Variations by gender in the prognosis of COAD patients

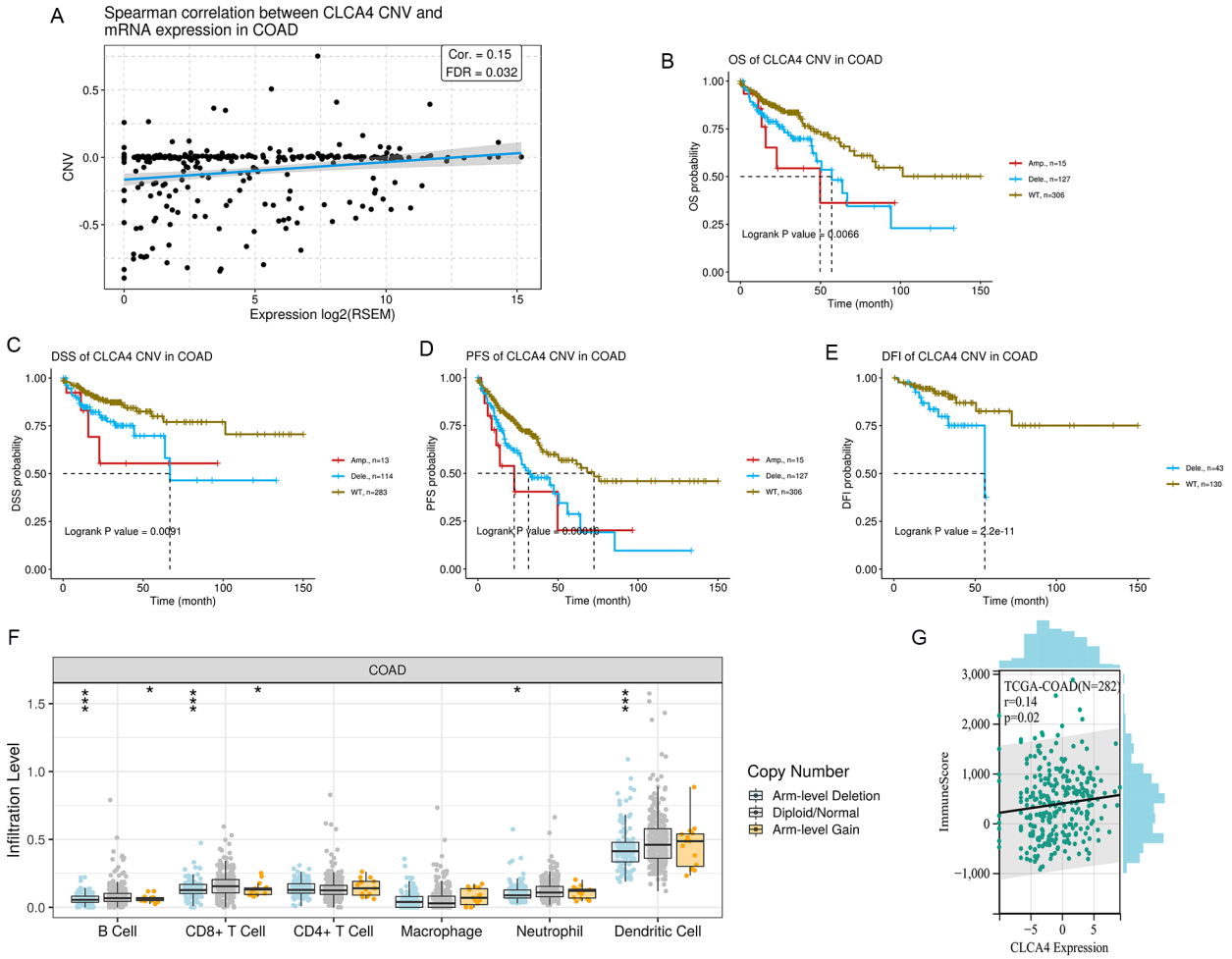

Fig. 3. Correlation between calcium-activated chloride channel (*CLCA4*) copy number variation (CNV) and *CLCA4* mRNA expression and its prognostic analysis. A) Correlation analysis of *CLCA4* CNV and *CLCA4* mRNA expression. B–E) OS, DSS, PFS, DFI of *CLCA4* CNV in colon adenocarcinoma (COAD). F) Relationship between *CLCA4* copy numbers in COAD and immune infiltration. G) Spearman's correlation coefficient of *CLCA4* and immune infiltration score in COAD

(Fig. 2D). Poorer overall survival (OS) in COAD patients was found to be associated with decreased *CLCA4* expression, according to prognostic analysis using UALCAN (Fig. 2J) and TISIDB (Fig. 2K). Additionally, there were differences in the prognosis survival rates of men and women in the groups with low and high *CLCA4* expression ($p < 0.05$) (Fig. 2L). Immunohistochemistry results showed moderate or weak *CLCA4* staining in normal colon tissues (Fig. 2E), whereas no *CLCA4* staining was detected in COAD tissues (Fig. 2F). Furthermore, *CLCA4* expression was mostly seen in the plasma membrane, as well as in the nucleoplasm and aggregates, in HeLa, RT-4, and U2OS cell lines (Figs. 2G–I). These results further demonstrate that *CLCA4* mRNA expression shows low concordance with protein expression, in approximate agreement with the above results.

Calcium-activated chloride channel mRNA expression and prognosis are correlated with copy number variation expression

Using the GSCA database, we closely examined the relationship between *CLCA4* CNV and *CLCA4* to comprehend the mechanism of *CLCA4* aberrant expression. The study's findings indicate a positive correlation between *CLCA4* and CNV in COAD patients (Cor = 0.15, FDR = 0.032) (Fig. 3A). Additionally, the study found that patients in different *CLCA4* CNV groups had varying survival prognoses (OS, DSS, PFS, and DFI) (Figs. 3B–E).

We examined *CLCA4* somatic-based CNV to better comprehend the possible function of the *CLCA4* gene and its effect on immune cell infiltration. Our results showed that the CNV of *CLCA4* signatures, such as arm-level deletion, diploid/normal, and arm-level gain, had a significant impact on the quantities of B-cells, CD8+ T-cells, neutrophils, and dendritic cell infiltration in COAD (Fig. 3F). SangerBox evaluation in COAD patients showed a statistically significant positive correlation ($r = 0.14$, $p = 0.02$) between immune infiltration and *CLCA4* (Fig. 3G). This study reveals that the tumour immune microenvironment (TIME) of COAD patients may be significantly influenced by *CLCA4*.

Immune infiltration analysis

To evaluate the impact of *CLCA4* on the immune environment of COAD, we utilised the TIMER data-

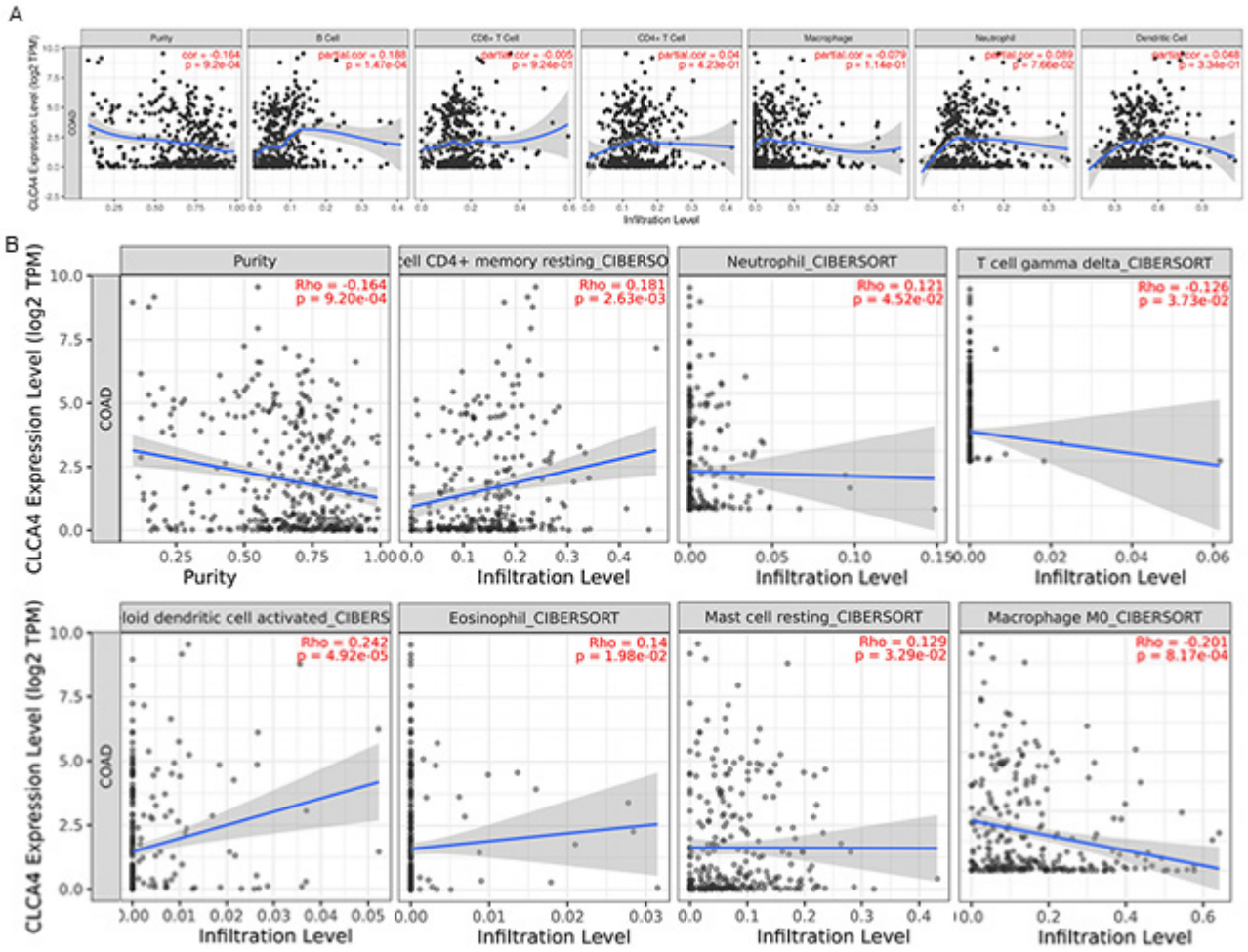

Fig. 4. Immune infiltration levels in colon adenocarcinoma (COAD) and their relationship with calcium-activated chloride channel (*CLCA4*). A) *CLCA4* and COAD immune cell infiltration levels in relationship (TIMER). B) Results of CIBERSOR analysis of *CLCA4* correlation with immune cell subtypes

set to examine the association between *CLCA4* and 6 different levels of immune cell infiltration.

As shown in Figure 4A, our results show a significant positive connection between *CLCA4* and the degree of B- cell infiltration (p = 1.47e–04). Further evaluating the correlation between *CLCA4* and immune cell subtypes based on CIBERSOR algorithm, the results showed that *CLCA4* was positively correlated with neutrophil (rho = 0.121, p = 4.52e–02), memory resting CD4+ T-cell (rho = 0.181, p = 2.63e–03), eosinophil (rho = 0.14, p = 1.98e–02), and resting mast cell (rho = 0.129, p = 3.29e–02), γ δ T-cell (rho = –0.126, p = 3.73e–02) and negatively correlated with macrophage M0 (rho = –0.201, p = 8.17e–04) and activated myeloid dendritic cell (rho = 0.242, p = 4.92e–05) (Fig. 4B).

TISIDB analysis showed a substantial connection between *CLCA4* and 28 tumour-infiltrating cells in pan-cancer, with the exception of Act CD8, Tcm CD8, Tcm CD4, Tem CD4, Tgd, Treg, Mem B, NK, CD56bright, CD56dim, and NKT (Fig. 5A). Specifically, Act B (p = 1.91e–07), Act CD4 (p = 0.00788), Act DC (p = 1.05e–06), Eosinophil (p = 9.09e–05), iDC (p = 1.27e–05), Imm B (p = 4.42e–06), Macrophage (p = 0.0433), Mast (p = 1.16e–05), MDSC (p = 0.0414), Monocyte (p = 2.54e–05), Neutrophil (p = 3.25e–18), pDC (p = 0.0188), Tem CD8 (p = 0.00493), Tfh (p = 0.0182), Th1 (p = 0.000453), Th2 (p = 0.0367), and Th17 (p < 2.2e–16) were positively correlated with *CLCA4* (Figs. 5B–R). This shows that the modulation of various TIL types in COAD tissues may be influenced by *CLCA4* gene regulation.

Calcium-activated chloride channel and immune cell marker expression levels are related

The research discovered a positive correlation between *CLCA4* and several immune cell markers in COAD, as Table I illustrates. These markers included neutrophils (CCR7, CEACAM8), B-cells (CD19, CD79A), CD8+ T-cells (CD8A), CD4+ T-cells (CD4), dendritic cells (HLA-DPB1, HLA-DQB1, HLA-DPA1, CD1C), monocytes (CD86), T-cell exhaustion (*CTLA4*, *LAG3*), M1 macrophages (NOS2, PTGS2), and M2 macrophages (MS4A4A).

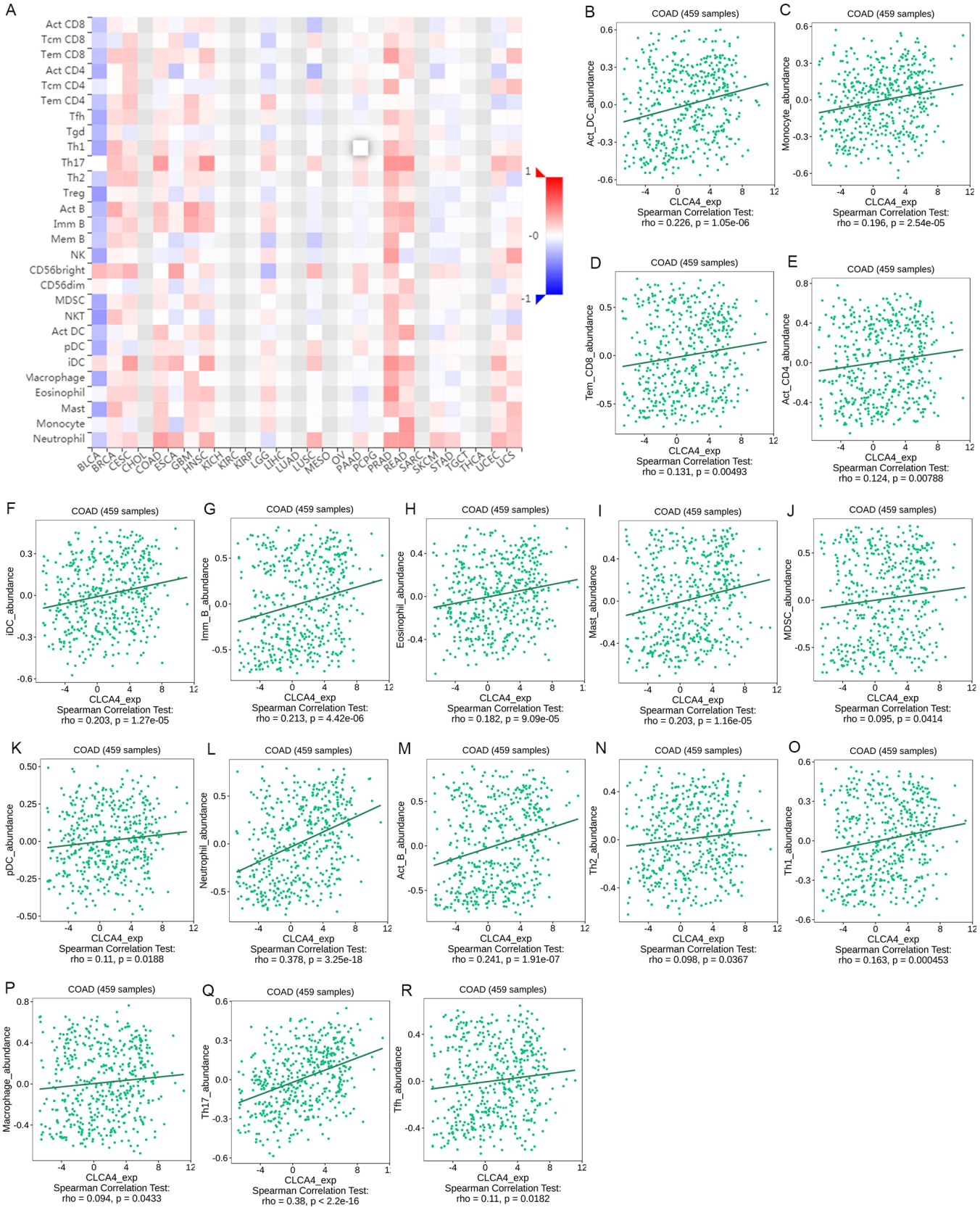

Fig. 5. A link between calcium-activated chloride channel (*CLCA4*) expression and lymphocytes invading tumours. A) *CLCA4* expression in pan-cancer was linked to 28 different kinds of tumour-infiltrating lymphocytes. (B–R) Tumour-infiltrating lymphocytes controlled by *CLCA4* in colon adenocarcinoma tissues

Table I. Immune cell markers in colon adenocarcinoma and calcium-activated chloride channel – correlation

Immune cell	Biomarker	*P*-value	*P*-star
Neutrophil	CCR7	7.23e–08	***
	ITGAM	8.33e–01	
	CEACAM8	2.2e–15	***
B-cell	CD19	2.01e–10	***
	CD79A	7.18e–11	***
$CD4^+$ T-cell	CD4	3.66e–03	**
$CD8^+$ T-cell	CD8A	1.69e–02	*
	CD8B	3.61e–01	
Dendritic cell	CD1C	3.49e–08	***
	HLA-DPA1	5.9e–03	**
	HLA-DPB1	3.2e–02	*
	HLA-DQB1	4.36e–02	*
	ITGAX	2.4e–01	
	NRP1	4.63e–01	
	ITGAX	2.4e–01	
Monocyte	CD86	4.07e–02	*
TAM	CCL2	2.28e–01	
	CD68	8.27e–02	
	CSF1R	9.62e–02	
M1 macrophage	NOS2	1.07e–10	***
	IRF5	5.96e–01	
	PTGS2	9.4e–05	***
M2 macrophage	CD163	2e–01	
	MS4A4A	2.82e–02	*
	VSIG4	2.2e–01	
Treg	FOXP3	1.54e–02	*
	CCR8	1.04e–01	
	STAT5B	4.76e–01	
	TGFB1	3.56e–01	
Th1	TBX21	2.38e–01	
	STAT4	1.79e–04	***
	STAT1	6.56e–01	
	IFNG	1.38e–01	
	TNF	2.6e–04	***
Th2	GATA3	2.92e–01	
	STAT6	2.64e–01	
	STAT5A	6.79e–01	
	IL13	3.57e–03	**
Th17	GATA3	8.55e–04	***
	STAT6	1.51e–06	***

Table I. Cont.

IMMUNE CELL	BIOMARKER	*P*-VALUE	*P*-STAR
T-cell exhaustion	*PDCD1*	7.2e–02	
	CTLA4	4.37e–03	**
	LAG3	2.39e–02	*
	HAVCR2	1.24e–01	
	GZMB	7.28e–01	
Tfh	BCL6	3.14e–01	
	IL21	3.46e–01	

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Analysis of immune checkpoints and the connection with calcium-activated chloride channel

The expression of immunological checkpoints, such as *CD274*, *HAVCR2*, *TIGIT*, *SIGLEC15*, *CTLA4*, *LAG3*, and *PDCD1LG2*, in COAD tissues and healthy tissues was also assessed using the Assistant for Clinical Bioinformatics. The results showed that only *SIGLEC15* ($p = 5.04e{-}12$), *CTLA4* ($p = 5.64e{-}09$), *LAG3* ($p = 1.34e{-}08$), and *PDCD1LG2* ($p = 5.13e{-}03$) (Figs. 6A, B). A favourable correlation between *CLCA4* and *CTLA4*, *CD274*, *LAG3*, and *PDCD1LG2* was found ($p = 0.024$, 0.011, 0.029, and 0.033, respectively) when we additionally examined the association between *CLCA4* and immunological checkpoints (Figs. 6C–J). Additionally, TIDE scores were higher in COAD patients who had low *CLCA4* expression (Fig. 6K), which suggests that immune checkpoint blockade therapy (ICB) was ineffective and that these patients had short survival times after starting ICB therapy.

Calcium-activated chloride channel levels and clinicopathological traits in colon adenocarcinoma patients

Using the Assistant for Clinical Bioinformatics, we investigated the relationship between clinicopathological features and *CLCA4*. The findings demonstrated that *CLCA4* expression substantially predicted the M stage ($p < 0.05$). Other pathological variables, such as gender, age, race, T and N stages, and clinical stage, showed no statistically significant connection. The difference between T3 and T4 was, nevertheless, significant ($p < 0.05$) (Figs. 7A–G). The overall survival and disease-free survival of M0 patients were considerably better than those of M1 patients, according to further study of the prognosis between M0 and M1 groups (Figs. 7H–I).

Functional enrichment analysis

Twenty *CLCA4*-related genes are shown in the GeneMANIA network, which has 21 genes overall (plus *CLCA4*) and 179 total links (Fig. 8A). The biological functions of these genes were explored by enriching them for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. According to what we discovered, the GO functional enrichment analysis showed enrichment mostly in the following areas: plasma membrane part, ion transmembrane transporter activity, transmembrane transporter activity, and ion channel activity. There are other biological processes, such as passive transmembrane transporter activity and substrate-specific channel activity (Fig. 8B). These genes are primarily abundant in the metabolism-related pathways: renin secretion, pancreatic secretion, cGMP-PKG, NOD-like receptor, cAMP, and other signalling pathways (Fig. 8C).

We utilised the LinkedOmics dataset to validate *CLCA4*'s potential role. A volcano map (Fig. 9A) illustrates the co-expression of *CLCA4* genes in COAD. We also created a heat map to visualise the top 50 genes associated with *CLCA4* (Figs. 9B, C). According to the GSEA analysis, the biological processes that are enriched are related to mitochondrial gene expression, mitochondrial respiratory chain complex assembly, translational elongation, nicotinamide adenine dinucleotide (NADH) dehydrogenase complex assembly, digestion, and coronary vasculature development. As demonstrated in Figure 9D, the KEGG pathway enrichment analysis identified enrichment in multiple pathways, including oxidative phosphorylation, Parkinson's disease, Alzheimer's disease, ribosome, and non-alcoholic fatty liver disease, as well as alcoholic fatty liver disease (NAFLD), drug metabolism, retinol metabolism, chemical carcinogenesis, fatty acid degradation, pancreatic secretion, extracellular matrix (ECM)-receptor interac-

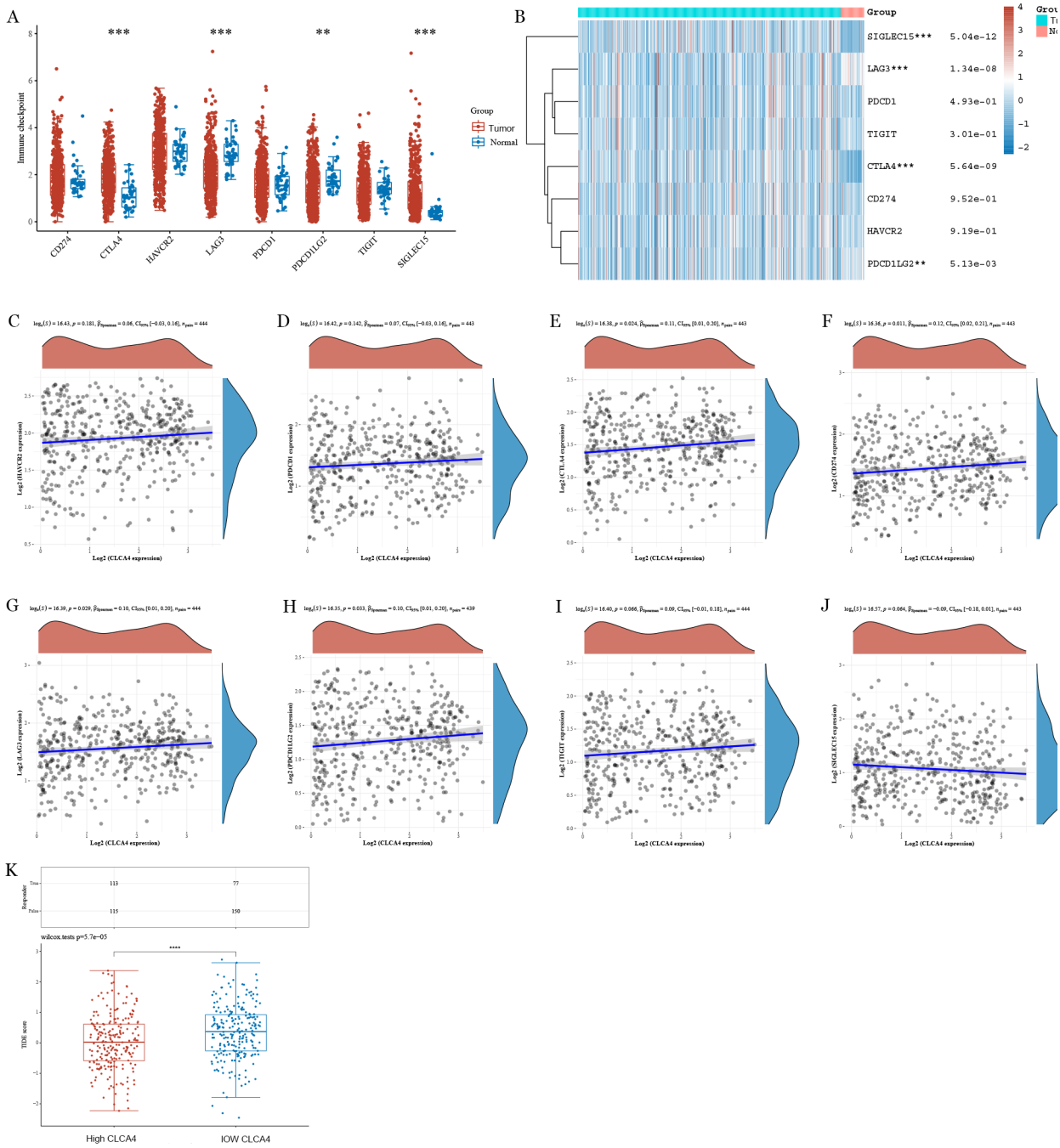

Fig. 6. Immune checkpoint gene expression. A) Immune checkpoint gene expression distribution in normal and colon adenocarcinoma (COAD) tissues. B) Heatmap of immune checkpoint-related gene expression. C–J) Correlation between *CLCA4* and immune checkpoint-related genes in COAD. K) Different reactions to ICB therapy amongst individuals with high and low *CLCA4* expression

tion, and other types of O-glycan biosynthesis (Figs. 9E–I). These discoveries illuminate the possible importance of *CLCA4* in COAD and give information on the disease's underlying molecular pathways.

Discussion

Clinical trials have shown that the molecular and pathological characteristics of the tumour determine the response to treatment and can improve OS [1]. Further investigation into the molecular mechanisms of colon carcinogenesis could offer novel avenues for the treatment of colon cancer. In COAD tissues, we observed low expression of *CLCA4* in this investigation. This finding was based on information obtained from various datasets, including the Assistant for Clinical Bioinformatics, TIMER, TISIDE, and HPA. Furthermore, we found that the downregulation of *CLCA4* was linked to a poorer prognosis in COAD patients. Consequently, *CLCA4* mRNA and protein expression were found to vary between normal human tissues, with oesophageal and colonic tissues expressing *CLCA4*

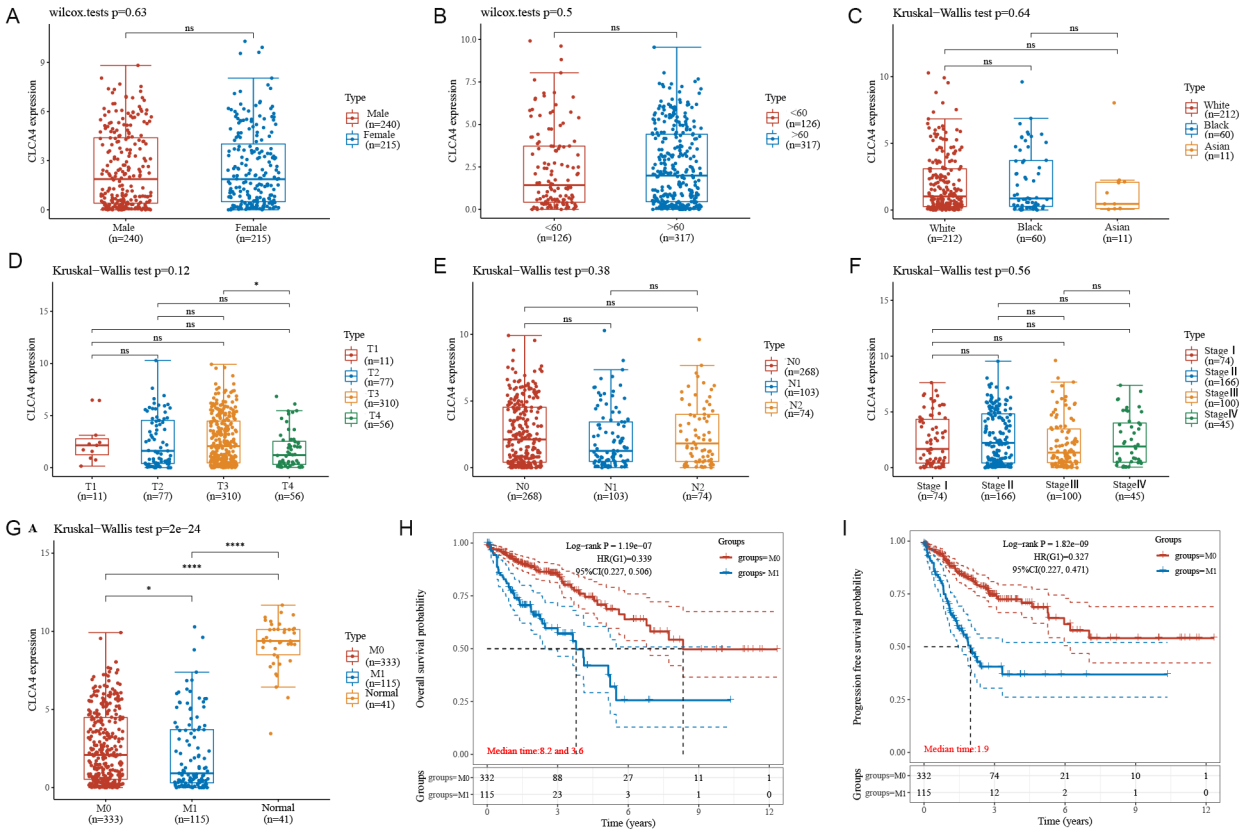

Fig. 7. The relationship between calcium-activated chloride channel (*CLCA4*) and clinicopathologic characteristics. Correlation between *CLCA4* and sex (A), age (B), race (C), T-stage (D), N-stage (E), TNM-stage (F), and M-stage (G). Prognostic analysis of M0 vs. M1 groups: 0S (H) and disease-free survival (I)

mRNA most highly, followed by the rectum and bladder, with the nervous system expressing *CLCA4* mRNA weakly, which is in disagreement with what Agnel *et al.* previously reported [12]. They concluded that *CLCA4* is widely expressed in the human nervous system, but the strongest signal is from the colon, which is twice as strong as the neural signal. Furthermore, our findings indicate that the *CLCA4* protein is abundantly expressed in various parts of the body, including the gastrointestinal tract and several glands, such as the thyroid, parathyroid, adrenal, testis, prostate, seminal vesicles, and breast. However, we observed a disparity between *CLCA4* RNA and protein expression in some of these regions, which we speculate may be brought on by variations in antibody specificity.

Genomic analysis plays a crucial role in detecting somatic variants because it can help identify potentially effective treatments [1]. In particular, analysis revealed that for COAD, CNV is positively correlated with *CLCA4* and is associated with patient survival. Subsequently, we found that these variations significantly affected the counts of B-cells, CD8+ T-cells, neutrophils, and dendritic cells infiltrating COAD. More investigation was done on the possible connection between immune cell infiltration and *CLCA4* in COAD.

There is emerging evidence that immune cells that penetrate tumours influence tumour growth as well as patient prognosis [13]. In this work, we discovered that *CLCA4* is linked to B-cell infiltration and affects several other immune cell types, such as M0 macrophages, activated myeloid dendritic cells, neutrophils, resting memory CD4+ T-cells, eosinophils, resting mast cells, and γ δ T-cells. B-cells are known to express many MHC II molecules and play a substantial role in humoral immunity. These cells are also recognised as key antigen-presenting cells that contribute to tumour immunity. Surprisingly, research has revealed that a high amount of B-cell infiltration in tumours might forecast a good prognosis for cancer patients [13]. Not surprisingly, *CLCA4* has been shown to positively correlate with several other subsets of lymphocytes, including Act B, Act CD4, Act DC, Eosinophil, iDC, Imm B, Macrophage, Mast, MDSC, Monocyte, Neutrophil, pDC, Th1, Th2, and Th17. As part of our adaptive immune response, $CD4^+$ T-cells play a significant role. As soon as they are activated, they divide into a variety of subpopulations, such as Th1 and Th2 cells, Tfh cells, Th17 cells, and Treg [14] – Th17 in particular, the presence of which in the mouse gut has been linked in the literature to the development of intestinal adeno-

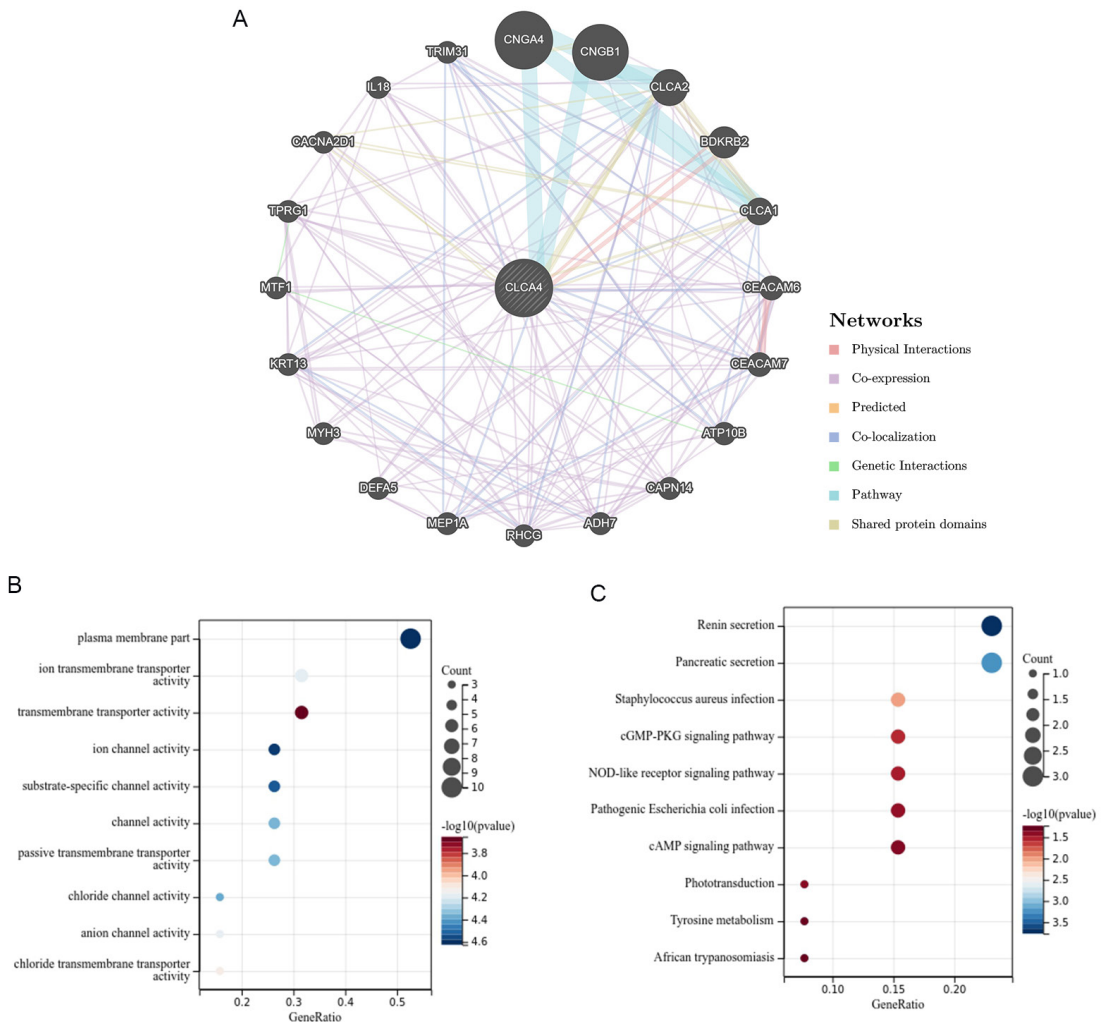

Fig. 8. Calcium-activated chloride channel Kyoto Encyclopedia of Genes and Genomesco-expressed gene enrichment study. A) Building the *CLCA4* co-expressed gene network with GeneMANIA. Gene ontology analysis (B) and Kyoto Encyclopedia of Genes and Genomes analysis (C) of the co-expressed gene *CLCA4*

mas in mice [15]. There is mounting evidence indicating that IL-17 and its primary source, Th17, are highly concentrated in CRC and have a negative correlation with patient prognosis. Furthermore, IL-17A was previously demonstrated to enhance the levels of PD-L1, reducing the efficacy of immunotherapy. IL-17 may be a potential target for sensitizing tumour cells to ICI [16]. Furthermore, we found several immune cell markers, including *STAT3* and STAT4, to have a favourable correlation with *CLCA4* expression. *STAT3* affects the clinical outcome of CRC patients and is implicated in the invasion of many immune cell types, such as B- cells, CD8+ T-cells, CD4+ T-cells, and macrophages [17]. Based on this, we propose that *CLCA4* may be involved in CD4+ T-cell-mediated anti-tumour immunity within the COAD tumour microenvironment, but further study is needed to fully grasp the underlying process.

Immune checkpoint molecules that are produced on immune cells may impede their ability to perform their functions, which could lead to an insufficient anti-tumour immunological response and let the tumour evade the immune system. Our research indicates a positive correlation between *CLCA4* and immune checkpoints *CTLA4*, *CD274*, *LAG3*, and *PDCD1LG2*. Furthermore, COAD patients with reduced *CLCA4* expression had higher TIDE scores, indicating that immune checkpoint blockade treatment was possibly ineffective for them. Metastasis, which frequently affects the liver, is the primary factor in deaths due to cancer in persons with CRC [18]. Subsequent analysis of the relationship between *CLCA4* and clinicopathological features showed that it was significantly correlated with the M stage. Conversely, no statistically significant link was discovered between *CLCA4* and age, gender, race, or clinical stage.

GeneMANIA produced a network of 21 genes that interact with each other. The biological functions associated with the *CLCA4* genes were analysed through GO functional enrichment. According

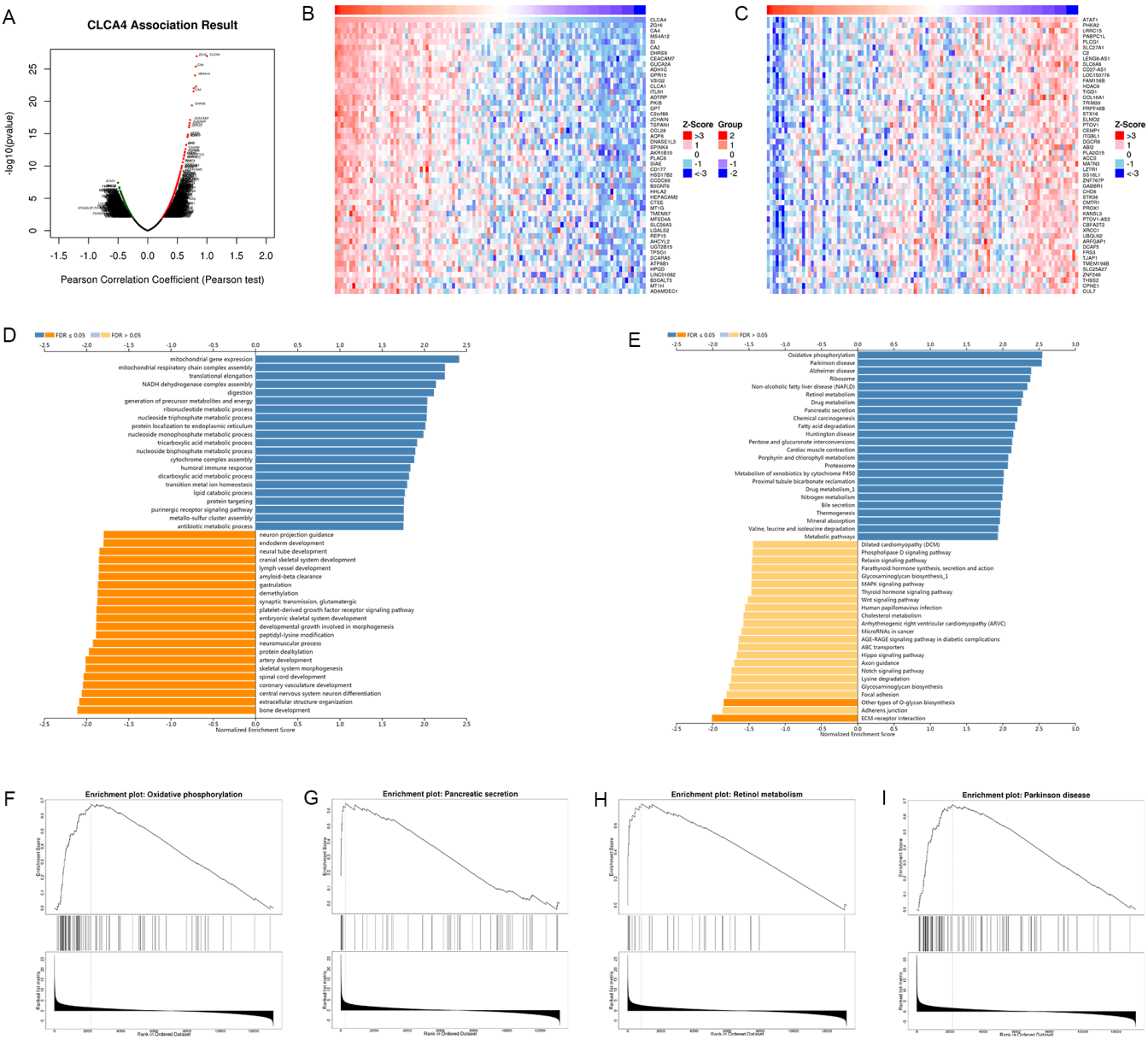

Fig. 9. The potential role of calcium-activated chloride channel (*CLCA4*) in colon adenocarcinoma (COAD). A) The correlation of *CLCA4* with differentially expressed genes in COAD is illustrated in the volcano plot. The top 50 genes linked with *CLCA4* both negatively (C) and positively (B). D) Gene ontology study of *CLCA4*'s biological functions in COAD. E–I) Analysis of pathway enrichment: pancreatic secretion, retinol metabolism, oxidative phosphorylation, and Parkinson's disease

to the GO enrichment study, they were highly enriched for ion channel activity and transmembrane transporter activity. Additionally, the evaluation of KEGG signalling pathways showed that they were enriched in certain signalling pathways, such as renin secretion, pancreatic secretion, and the cAMP signalling pathway. Furthermore, the GSEA's findings demonstrated that the GO's biological activities were mostly related to digestion, coronary vascular development, mitochondrial respiratory chain complex assembly, translation extension, NADH dehydrogenase complex assembly, and structural organisation. During the analysis of signalling pathways, the focus was primarily on various pathways such as oxidative phosphorylation, Parkinson's disease, Alzheimer's disease, NAFLD, drug metabolism, retinol metabolism, chemical carcinogenesis, fatty acid degradation, pancreatic secretion, and other pathways that were deemed relevant. According to Moreno-Sánchez *et al*. [19], most cancer cells do not experience impairment in their mitochondrial function. Cancer cells experience aerobic glycolysis, a metabolic process that converts glucose into carbon dioxide. This occurs through the oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid cycle. As a result of this process, NADH is created, which encourages

oxidative phosphorylation to increase adenosine triphosphate synthesis while reducing lactate formation [20]. Recent research suggests that cancers may have diverse cellular metabolisms. Based on the research conducted by Sonveaux *et al*. [21], particular cells might utilise the additional lactate produced as an energy source for the process of mitochondrial oxidative phosphorylation. Interestingly, a growing body of research indicates that insulin plays a major role in the development of tumours *via* regulating signalling networks [22]. Numerous studies have linked the renin-angiotensin system (RAS) to the development of CRC. In particular, research indicates that dysregulation of the RAS may contribute to the pathophysiology of CRC [23, 24]. A growing number of epidemiological studies indicate a remarkable inverse correlation between Alzheimer's and Parkinson's disease. According to Hang *et al*., this correlation may be influenced by the metabolites of gut bacteria, which accelerate the development of Alzheimer's disease/Parkinson's disease and cancer [25]. On the other hand, when there is gut microbial dysbiosis, it causes excessive activation of CD8+ T-cells and triggers chronic inflammation and premature T-cell failure. This ultimately results in a higher susceptibility to colon tumours and a subsequent decrease in anti-tumour immunity [26]. Thus, it appears that the functional annotation of *CLCA4* co-expressed genes suggests that they are involved in intestinal digestion, ion transport, and metabolism, which in turn affect colorectal carcinogenesis and progression by altering the intestinal microenvironment.

Conclusions

In conclusion, the expression of *CLCA4* may impact the immune microenvironment, ultimately influencing the occurrence and progression of COAD and subsequently affecting the prognosis of COAD patients. As such, *CLCA4* may function as a diagnostic predictor for COAD. A constraint of this research is the requirement for a substantial quantity of clinical specimens to verify the association between immune cell counts and *CLCA4* expression. Additionally, it is critical to do additional research to better comprehend the immunological systems involved in the emergence of COAD and to pinpoint prospective therapeutic targets. This information could aid in defining the factors that influence immune therapy response.

Disclosures

1. The database used in this study can be found on the internet. Our study did not require ethical board approval because it did not contain human or animal trials.
2. We appreciate the platform provided by the Cancer Genome Atlas database, datasets like HPA, and contributors who uploaded valuable datasets.
3. The study was supported by the Anhui Provincial Education Department's Key project (2022AH051530) and the Bengbu Medical University's Natural Science Foundation's Key project (2021byzd031), and Anhui Key Laboratory of Computational Medicine and Intelligent Health (Bengbu Medical University) (AHCM2023Z005).
4. Conflicts of interest: None.

References

1. Biller LH, Schrag D. Diagnosis and treatment of metastatic colorectal cancer: a review. JAMA 2021; 325: 669-685.
2. Fabregas JC, Ramnaraign B, George TJ. Clinical updates for colon cancer care in 2022. Clin Colorect Cancer 2022; 21: 198-203.
3. Lou E. Immunotherapy success for microsatellite stable colorectal cancers-searching for the horizon. JAMA Oncol 2023; 9: 615-617.
4. Liu C, Liu R, Wang B, et al. Blocking IL-17. A enhances tumor response to anti-PD-1 immunotherapy in microsatellite stable colorectal cancer. J Immunother Cancer 2021; 9: e001895. Erratum: J Immunother Cancer 2021; 9.
5. Wei L, Chen W, Zhao J, et al. Downregulation of *CLCA4* expression is associated with the development and progression of colorectal cancer. Oncol Lett 2020; 20: 631-638.
6. Han J, Zhang X, Liu Y, et al. *CLCA4* and MS4A12 as the significant gene biomarkers of primary colorectal cancer. Biosci Rep 2020; 40: BSR20200963.
7. Liu Z, Chen M, Xie LK, et al. *CLCA4* inhibits cell proliferation and invasion of hepatocellular carcinoma by suppressing epithelial-mesenchymal transition via PI3K/AKT signaling. Aging (Albany NY) 2018; 10: 2570-2584.
8. Hou T, Zhou L, Wang L, et al. *CLCA4* inhibits bladder cancer cell proliferation, migration, and invasion by suppressing the PI3K/AKT pathway. Oncotarget 2017; 8: 93001-93013.
9. Chen H, Liu Y, Jiang CJ, et al. Calcium-activated chloride channel A4 (*CLCA4*A4) plays inhibitory roles in invasion and migration through suppressing epithelial-mesenchymal transition via PI3K/AKT signaling in colorectal cancer. Med Sci Monit 2019; 25: 4176-4185.
10. Li B, Jiang YP, Zhu J, et al. MiR-501-5p acts as an energetic regulator in head and neck squamous cell carcinoma cells growth and aggressiveness via reducing *CLCA4*. Mol Biol Rep 2020; 47: 2181-2187.
11. Li H, Huang B. miR-19a targeting *CLCA4* to regulate the proliferation, migration, and invasion of colorectal cancer cells. Eur J Histochem 2022; 66: 3381.
12. Agnel M, Vermat T, Culouscou JM. Identification of three novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea. FEBS Lett 1999; 455: 295-301.
13. Wei Q, Miao T, Zhang P, et al. Comprehensive analysis to identify GNG7 as a prognostic biomarker in lung adenocarcinoma correlating with immune infiltrates. Front Genet 2022; 13: 984575.
14. Zhang Q, Jazwinski SM. A novel strategy to model age-related cancer for elucidation of the role of Th17 inflammaging in cancer progression. Cancers (Basel) 2022; 14: 5185.
15. Wu S, Rhee KJ, Albesiano E, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med 2009; 15: 1016-1022.

16. Li S, Na R, Li X, et al. Targeting interleukin-17 enhances tumor response to immune checkpoint inhibitors in colorectal cancer. Biochim Biophys Acta Rev Cancer 2022; 1877: 188758.
17. Li D, Jiao Y, Gao W, et al. Comprehensive analysis of the prognostic and immunotherapeutic implications of STAT family members in human colorectal cancer. Front Genet 2022; 13: 951252.
18. Zarour LR, Anand S, Billingsley KG, et al. Colorectal cancer liver metastasis: evolving paradigms and future directions. Cell Mol Gastroenterol Hepatol. 2017;3:163-173.
19. Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, et al. Energy metabolism in tumor cells. FEBS J 2007; 274: 1393-1418.
20. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324: 1029-1033.
21. Sonveaux P, Végran F, Schroeder T, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest 2008; 118: 3930-3942.
22. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008; 8: 915-928. Erratum: Nat Rev Cancer 2009; 9: 224.
23. Neo JH, Ager EI, Angus PW, et al. Changes in the renin angiotensin system during the development of colorectal cancer liver metastases. BMC Cancer 2010; 10: 134.
24. Mehranfard D, Perez G, Rodriguez A, et al. Alterations in gene expression of renin-angiotensin system components and related proteins in colorectal cancer. J Renin Angiotensin Aldosterone Syst 2021; 2021: 9987115.
25. Hang Z, Lei T, Zeng Z, et al. Composition of intestinal flora affects the risk relationship between Alzheimer's disease/Parkinson's disease and cancer. Biomed Pharmacother 2022; 145: 112343.
26. Yu AI, Zhao L, Eaton KA, et al. Gut microbiota modulate CD8 T cell responses to influence colitis-associated tumorigenesis. Cell Rep 2020; 31: 107471.
27. Siegel RL, Miller KD, Wagle NS, et al. Cancer statistics, 2023. CA Cancer J Clin 2023; 73: 17-48.

Address for correspondence

Xueying Zhao
Bengbu Medical University
Bengbu, China
e-mail: zhxy2020@163.com