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CLCA1 is Poorly Expressed in Stomach Adenocarcinoma and Correlates with Immune Infiltration

CLCA1 se Expresa Deficientemente en el Adenocarcinoma de Estómago y se Correlaciona con la Infiltración Inmune

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SUMMARY: Calcium-activated chloride channel regulator 1 (CLCA1) is associated with cancer progression. The expression and immunologic function of CLCA1 in stomach adenocarcinoma (STAD) remain unclear. In this investigation, the expression of CLCA1 in STAD tissues and its involvement in the progression and immune response of STAD were examined using databases such as cBioPortal, TISIDB, and UALCAN. In order to validate the expression level of CLCA1 protein in gastric adenocarcinoma, thirty clinical tissue specimens were gathered for immunohistochemical staining. The findings indicated a downregulation of CLCA1 in STAD patients, which was correlated with race, age, cancer grade, Helicobacter pylori infection, and molecular subtype. Through the examination of survival analysis, it was identified that diminished levels of CLCA1 within gastric cancer cases were linked to decreased periods of post-progression survival (PPS), overall survival (OS), and first progression (FP) ($P<0.05$). The CLCA1 mutation rate was lower in STAD, but the survival rate was higher in the variant group. The correlation between the expression level of CLCA1 and the levels of immune infiltrating cells in STAD, as well as the immune activating molecules, immunosuppressive molecules, MHC molecules, chemokines, and their receptor molecules, was observed. Gene enrichment analysis revealed that CLCA1 may be involved in STAD progression through systemic lupus erythematosus (SLE), proteasome, cell cycle, pancreatic secretion, and PPAR signaling pathways. In summary, CLCA1 is anticipated to function as a prognostic marker for patients with STAD and is linked to the immunization of STAD.

KEY WORDS: Stomach adenocarcinoma; Clinical stages; Immunohistochemistry; Bioinformatics; Prognostic factor.

INTRODUCTION

In 2020, the global diagnosis of cancer reached a staggering 19.3 million cases, resulting in nearly 10 million fatalities, as reported by Globoan. Among the multitude of cancers, gastric cancer stands at the fifth position in terms of prevalence and fourth in terms of causing cancer-related deaths on a global scale (Sung *et al*., 2020). In 2022, the U.S. reported 27,294 cases of gastric cancer with 11,898 deaths. China experienced 509,421 new cases of gastric cancer with 400,415 related deaths during the same year (Xia *et al*., 2022). While the occurrence of gastric cancer has exhibited a decline within the confines of the United States, the fatality rate pertaining to metastatic gastric cancer persists at an elevated level, with a median survival span of under a year (Patel & Cecchini, 2020; Xia *et al*., 2022). There is still much need for improvement in the current therapeutic approaches for gastric cancer, including surgery, neoadjuvant radiotherapy, molecular targeted therapy, and immunotherapy. Despite these treatment options, the outcomes for advanced forms of the disease are not satisfactory (Yang *et al*., 2022). With this in mind, it becomes crucial to unearth groundbreaking biomarkers that can aid in the early detection of the disease and ultimately enhance the clinical outlook for patients diagnosed with STAD.

CLCA1 is an autocleavage protein that is part of the human chloride channel auxiliary (CLCA) family. It functions by activating calcium-dependent chloride currents (Yurtsever *et al*., 2012). The primary locations where CLCA1 is expressed are the small and large intestines. It can also be secreted into the bloodstream (Gruber *et al*., 1998). Many

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human cancer types have been extensively studied in relation to CLCA1. According to previous investigations, numerous types of cancer, including hepatocellular carcinoma (He *et al*., 2024), colorectal carcinoma (Li *et al*., 2017), pancreatic carcinoma (Hu *et al*., 2018), and ovarian carcinoma (Musrap *et al*., 2015), exhibit a down-regulation of CLCA1 expression. Underexpression of this particular protein is associated with poor prognosis in these cancers and plays a critical role in controlling tumor development or chemoresistance (Musrap *et al*., 2015; Li *et al*., 2017; Hu *et al*., 2018). Nevertheless, there have been no investigations involving immunohistochemical analyses to evaluate the presence of CLCA1 protein in gastric cancer, and the precise role of CLCA1 in STAD remains uncertain.

To gain a deeper understanding of the role of CLCA1 in gastric cancer, we performed a comprehensive analysis using data from various databases and conducted immunohistochemistry experiments. Our study had several objectives: to investigate the prognostic value of CLCA1 in STAD, to examine its impact on immune cell infiltration, and to explore its correlation with immune checkpoint protein expression. Additionally, we sought to unravel the molecular mechanisms and signaling pathways through which CLCA1 is involved in STAD.

MATERIAL AND METHOD

Data sources. The TCGA database portal, cBioPortal (http://www.cbioportal.org), was utilized for investigating the expression of the CLCA1 in STAD. To further analyze CLCA1 protein expression, the HPA website (https://www.proteinatlas.org) was employed. To investigate a correlation between CLCA1 expression and clinicopathological parameters in the TCGA database, we accessed the UALCAN website (http://ualcan.path.uab.edu) and the TISIDB website (http://cis.hku.hk/TISIDB/). To examine the immune attributes and expression of CLCA1 in STAD, one can utilize the Sangerbox Tool website, accessible at http://www.sangerbox.com/tool. Additionally, for an analysis of the connection between CLCA1 expression and survival in gastric adenocarcinoma, the Kaplan-Meier Plotter Tool website (https://kmplot.com/analysis) can be visited. Moreover, the LinkedOmics website (http://www.linkedomics.org/admin.php) provides the means to investigate co-expressed genes and their functional enrichment in relation to CLCA1.

Sangerbox tools platform. Sangerbox Tools serves as a comprehensive platform to systematically investigate immune infiltration in different forms of cancer (Liu *et al*., 2022). In this investigation, Sangerbox was utilized to examine the expression of the CLCA1 gene in diverse tumor tissues. To gauge the extent of immune cell infiltration in gastric cancer, the CIBERSOR algorithm was employed. It estimated the levels of 22 immune cell types within each sample by considering the ratings of CLCA1 gene expression. After conducting an analysis, we investigated the associations between the CLCA1 gene and 60 marker genes related to two separate categories of immune checkpoint pathways. Furthermore, we examined the Pearson correlations between the CLCA1 gene and 150 marker genes across five sets of immune pathways, including chemokine, receptor, MHC, immunoinhibitor, and immunostimulator.

HPA and UALCAN database analysis. The HPA database utilizes transcriptomics and proteomics techniques to investigate the expression of proteins in different human tissues and organs. To achieve a thorough understanding of protein expression, a combination of RNA and protein immunoassay techniques is used to examine protein expression in 48 normal human tissues, 20 tumor tissues, and 64 cell lines (Xu *et al*., 2021). In this study, particular attention was given to the CLCA1 gene, and its expression was evaluated in both normal and gastric cancer tissues.

The UALCAN database is a web-based tool for analyzing and extracting cancer-related data. It facilitates the analysis of protein expression in CPTAC records (Chandrashekar *et al*., 2022). In order to evaluate the correlation between CLCA1 expression levels and various clinicopathological characteristics in patients with STAD, we utilized the UALCAN database.

cBioPortal database analysis. The cBioPortal is an accessible online source for exploring, examining, and displaying multi-dimensional genomic data of cancer from various databases (Unberath *et al*., 2019). Based on the CLCA1 gene query, datasets related to gastric adenocarcinoma were selected and analyzed using the Oncoprint module to study the CLCA1 gene variants. The expression of the CLCA1 gene in gastric adenocarcinoma (with or without increased expression, mutation, etc.) and its association with survival prognosis were examined by utilizing the CBioPortal database.

TISIDB database analysis. The TISIDB database is a valuable tool for conducting gene expression correlation analysis in relation to tumor-infiltrating lymphocyte abundance and tumor-immune system interactions (Huang *et al*., 2020). In this study, the researchers used the TISIDB database to explore the expression of CLCA1 in various immune and molecular subtypes of human cancers.

The Kaplan-Meier Plotter database. The Kaplan-Meier Plotter, a large and openly accessible database, examines

the link between survival outcomes and gene expression in a wide range of cancer types. This database gathers RNA data from different sources, including the TCGA and GEO databases. To validate the association between CLCA1 mRNA expression and the prognosis of gastric adenocarcinoma, including PPS, OS, and FP, the researchers utilized the Kaplan-Meier Plotter database. The grouping criterion used in this analysis was the best cutoff value.

LinkedOmics database analysis. LinkedOmics is a publicly accessible database that stores multidimensional data on all types of cancer from 32 TCGAs, making it convenient for analyzing clinical data (Vasaikar *et al*., 2018). To assess the co-expression of CLCA1 genes and their functional enrichment, we utilized the LinkedOmics database. Within the "LinkInterpreter" module of LinkedOmics, we performed gene set enrichment analysis (GSEA) for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG). We applied a screening threshold of FDR<0.05 to identify significant enrichments.

Immunohistochemical staining. Thirty tissue specimens of STAD were collected from the Department of Pathology at the First Affiliated Hospital in 2022. Routine pathological tissue examination confirmed all specimens to be gastric adenocarcinoma after surgery. Controls were also collected as paracancerous tissues (>5 cm from the tumor edge). All specimens were confirmed by the Department of Pathology. This study received ethical approval from the Bengbu Medical College Ethics Committee (no.2023127).

The specimens were fixed in 10 % neutral formalin, embedded in paraffin, and then serially sectioned at a thickness of 4 mm. Following this, they were deparaffinized and stained through the use of MaxVisionTM kit immunohistochemistry. After baking, de-waxing with xylene, and dehydrating with a concentration gradient of alcohol, antigen repair was conducted by exposing the slices to citric acid solution at high temperature and pressure for 3 minutes. After natural cooling, the slices were placed in a 3 % H_2O_2 solution for 10 min, and a primary rabbit monoclonal antibody against CLCA1 (ab174319, Abcam, diluted to 1:100) was added at 37 °C for 1 h. To ensure optimal cleanliness, the slices underwent three rounds of washing with phosphate buffered saline (PBS), with each wash lasting for a duration of 3 min. Add the MaxVisionTM HRP reagent (Maixin Biotechnology, Fuzhou, China) and incubate at room temperature for fifteen minutes. Rinse with PBS, and then develop color with diaminobenzidine (DAB). Stain with hematoxylin using gradient ethanol dehydration, followed by Xylene treatment. Seal with neutral gum and observe microscopically.

The results were positively signaled by the presence of yellow granules in the cytoplasm or nucleus. The evaluation involved scoring the combined intensity of cellular staining and the proportion of cells demonstrating positivity. Criteria for determining immunohistochemical results: the reading was blinded and determined by 2 pathologists. Scoring based on the intensity of staining and the rate of positive cells was carried out in this study. The intensity of staining was categorized into four levels: no staining (0 points), light yellow (1 point), brown (2 points), and tan (3 points). Similarly, the rate of positive cells was categorized into five levels: ≤4 % (0 points), 5 % to 24 % (1 point), 25 % to 49 % (2 points), 50 % to 74 % (3 points), and ≥75 % (4 points). The participants were then grouped based on the product of these two scores. Those with a value greater than 4 were classified as having high expression, while those with a value of 4 or less were classified as having low expression.

Statistical methods. Data analysis was conducted using SPSS 27.0 software for statistical purposes. To analyze the disparities in CLCA1 protein expression between gastric adenocarcinoma tissues and their corresponding paracarcinoma tissues, a χ^2 test was employed. For the LinkedOmics database, Spearson's statistical approach was utilized. In order to examine the association between CLCA1 expression and the prognosis of patients with STAD, Kaplan-Meier survival analysis was conducted. Statistical significance was determined at a threshold of P<0.05.

RESULTS

Expression of CLCA1 in STAD and its Correlation with Survival Outcome. Pan-cancer analysis conducted using the online tool Sangerbox Tools indicated differential expression of the CLCA1 gene in seven tumor tissues, including STAD, COAD (Colon adenocarcinoma), COADREAD (Colorectal adenocarcinoma), BRCA (Breast invasive carcinoma), STES (Stomach and Esophageal carcinoma), UCEC (Uterine Corpus Endometrial Carcinoma), READ (Rectum adenocarcinoma), and normal tissues. The tool also disclosed that the expression of the CLCA1 gene in gastric adenocarcinoma is considerably lower compared to that in normal gastric tissues (P<0.05) (Fig. 1). However, a thorough exploration of the HPA revealed that CLCA1 protein expression was absent in both STAD tumor tissues and healthy tissues (Fig. 2A-B).

In order to examine the disparities in survival related to the CLCA1 gene in STAD, the researchers utilized the Kaplan-Meier Plotter database. The findings imply that within STAD, the CLCA1 high expression group displayed longer PPS, OS, and FP compared to the CLCA1 low

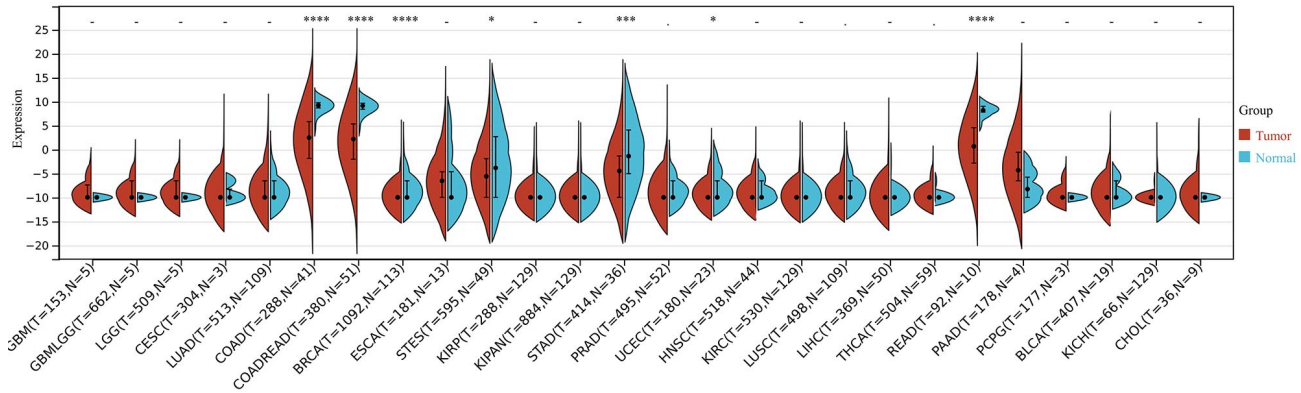

Fig. 1. presents the CLCA1 gene expression found in distinct categories of tumor tissues. * P<0.05, ** P<0.01, *** P<0.001

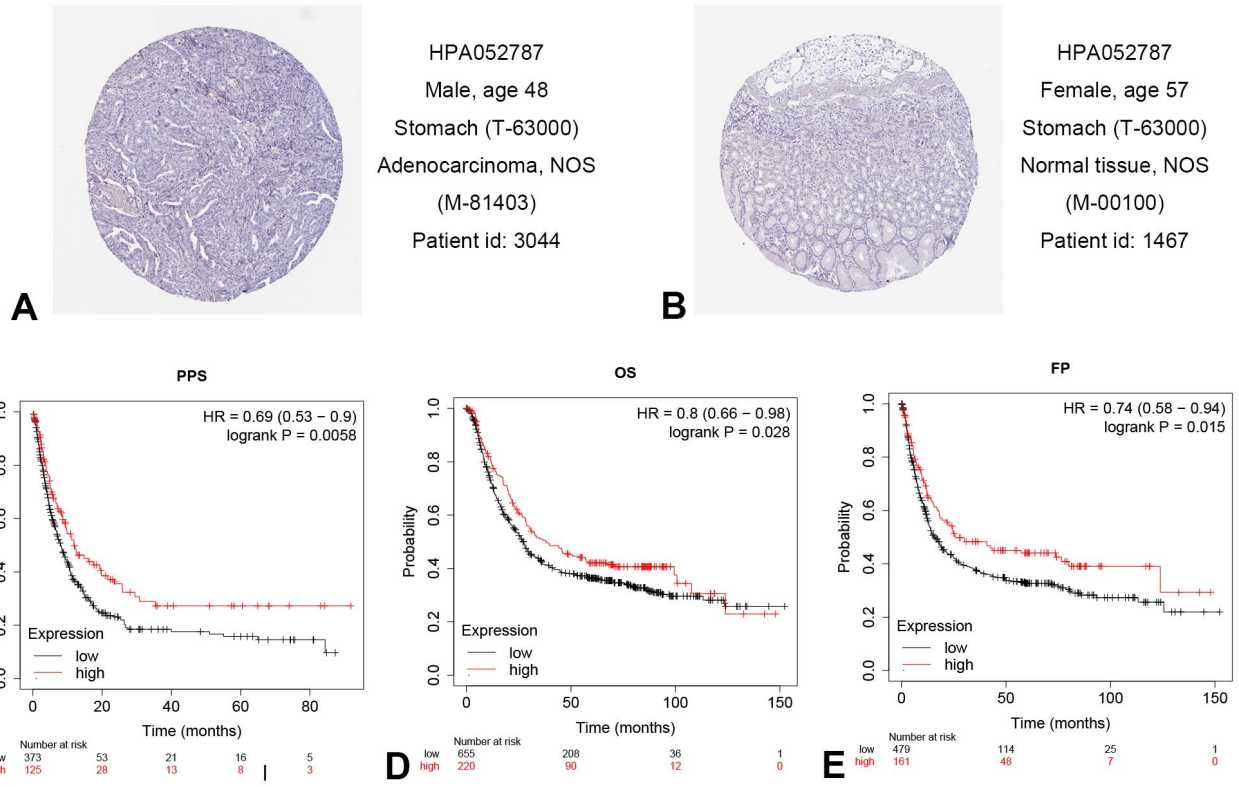

Fig. 2. The expression pattern of the CLCA1 gene in STAD and its association with clinical features. (A-B) Assessment of CLCA1 protein expression through immunohistochemistry. (D-F) Relationships between CLCA1 expression levels and OS, PPS, and FP in STAD.

expression group (P<0.05) (Fig. 2C-E). These outcomes indicate that individuals with diminished CLCA1 expression experienced a more unfavorable prognosis.

CLCA1 Protein Expression in Clinical Samples. The protein level of CLCA1 in STAD tumors was verified by immunohistochemistry. Immunohistochemical staining results showed (Fig. 3) that CLCA1 protein was weakly expressed in STAD tissues, almost all of which were nuclear-positive, whereas in paracarcinoma tissues the gastric epithelium was almost negative, some of the secretory glands were strongly positive, mainly located in the cytoplasm, and the intestinal chemotaxis epithelial cells were strongly positive and brownish-yellow granular-like. The positive rate of CLCA1 protein expression in STAD tissues was significantly lower than that in paracarcinoma group, and the difference was statistically significant (P< 0.05), as shown in Table I.

Table I. Expression of CLCA1 protein in STAD and paracancerous tissues [n (%)].

Sample	n	CLCA1 expression		χ^2	P value
		High expression	Low expression		
Tumor tissue	30	3 (10.0)	27 (90.0)	16.484	0.000
Paracancerous tissue	30	18 (60.0)	12 (40.0)		

Differential expression of CLCA1 gene in various clinical subgroups of STAD. Subsequently, we investigated the connection between the expression levels of CLCA1 mRNA and various clinical factors in STAD with the assistance of the UALCAN database. The findings demonstrated variations in CLCA1 expression amongst different clinical subgroups of STAD, which exhibited a correlation with cancer grade (grade 1 versus grade 3), patient ethnicity (Caucasian versus African American), age (21-40 versus 41-60 years; 21-40 years versus 61-80 years), and *H. pylori* infection status (with *H. pylori* infection versus without *H. pylori* infection; with *H. pylori* infection versus not available). No significant correlations were found between tumor stage, patient sex, histological subtype, lymph node status, and TP53 mutation (Fig. 4A-I).

The expression levels of CLCA1 in the TISIDB database showed a correlation with cancer grade, as illustrated in Figure 5A. However, there was no correlation observed with tumor stage, as indicated in **Figure 5B.** These findings were consistent with the analysis conducted using the UALCAN database. Furthermore, our study examined the correlation between CLCA1 expression and both immunological subtypes and molecular subtypes in patients with STAD. The results of our study demonstrate that there is no notable link between low levels of CLCA1

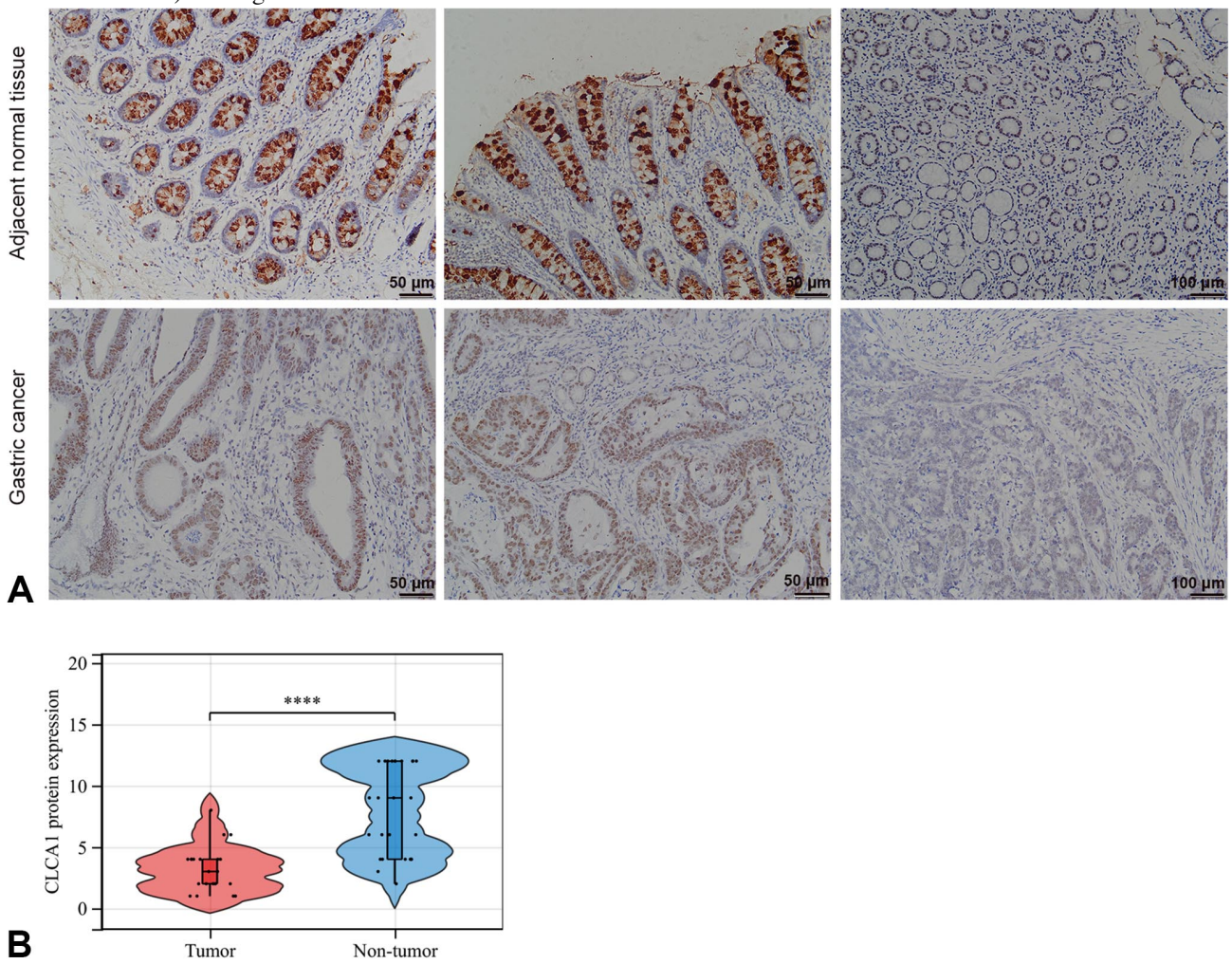

Fig. 3. Expression of CLCA1 protein in gastric adenocarcinoma and paracancerous tissues (immunohistochemistry left 2 columns 200 ×, right 1 column 100 ×). (A) Results of CLCA1 immunohistochemical staining. (B) Analysis of CLCA1 immunohistochemical results.

expression and immunological subtypes in patients with STAD (Fig. 5C). Nonetheless, we did find a significant association between low CLCA1 expression levels and the molecular subtypes of STAD patients (Fig. 5D). These findings indicate that the expression levels of CLCA1 may play a role in the molecular characteristics of STAD, but do not appear to have a direct impact on immunological subtypes.

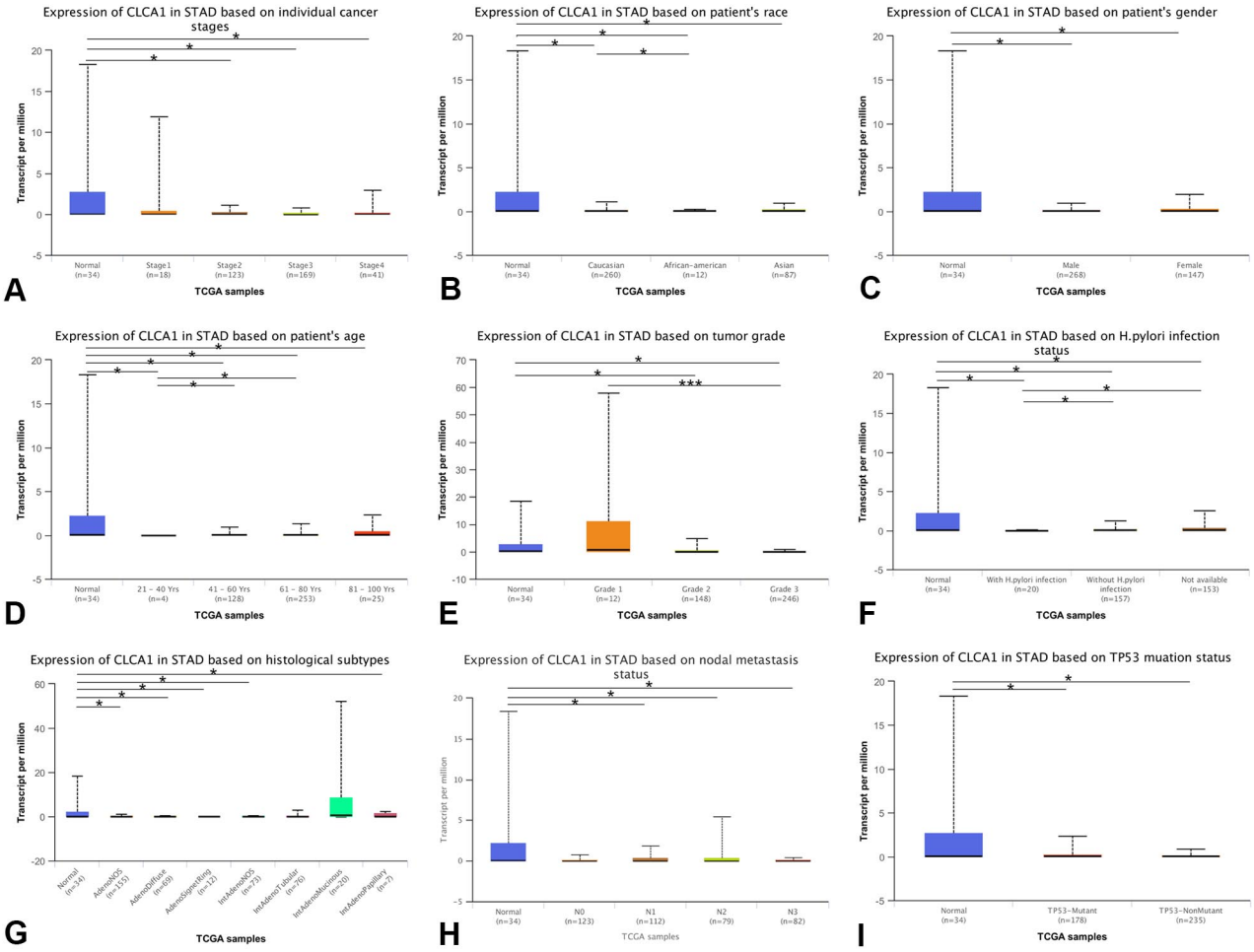

Fig. 4. The UALCAN analysis examined the varying expression of CLCA1 across different clinical subgroups in patients with STAD. The factors considered in the analysis included: (A) the stage of cancer, (B) the ethnic background of patients, (C) the sex of patients, (D) the age of patients, (E) the grade of tumors, (F) the presence or absence of Helicobacter pylori infection, (G) the histological subtype of tumors, (H) the status of lymph node metastasis, and (I) the presence or absence of TP53 mutation.

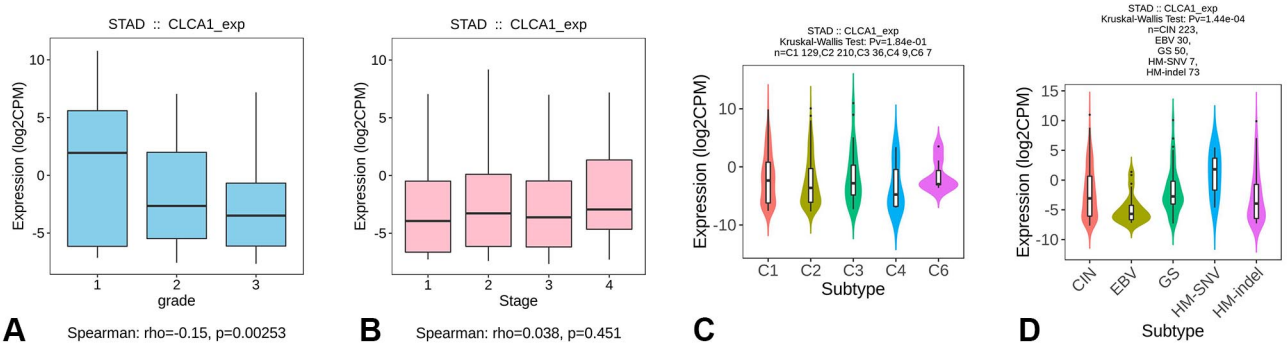

Fig. 5. TISIDB analysis of CLCA1 gene expression in STAD with respect to clinical characteristics. (A-B) Associations between CLCA1 expression and cancer grade and stage in STAD patients. (C-D) Distribution of CLCA1 expression among immune and molecular subtypes. Note: C1. Wound healing; C2. IFN-gdominant; C3. Inflammatory; C4. Lymphocyte depleted; C6. TGF-bdominant.

CLCA1 gene variants in STAD. Using the cBioPortal platform, we searched for cases of gastric adenocarcinoma and collected RNA sequencing data from 1365 samples. We found that 23 cases exhibited CLCA1 gene mutations, resulting in a total mutation rate of 1.7 %. Among the observed cases, there were 2 instances of amplification, 17 instances of missense mutation, and 4 instances of truncating mutation **(Fig. 6A).** These findings suggest that missense mutation is the most prevalent type of CLCA1 gene variation in STAD, although its mutation rate is not particularly high. Utilizing the Cancer Types Summary module, an examination was conducted on the Alteration Frequency of CLCA1 across six datasets. In TCGA Nature 2014, gastric adenocarcinoma was genetically altered in 2.37 % of 295 cases. In TCGA PanCancer Atlas, the alteration frequency was 1.82 % of 440 cases. In TCGA Firehose Legacy, it was 1.46 % of 478 cases. Furthermore, in Pfizer and UHK Nat Genet 2014, it was 1 % of 100 cases (Fig. 6B). Among the incident cases, Mucinous Stomach Adenocarcinoma was found in 4.55 % of 44 cases; Tubular Stomach Adenocarcinoma in 2.53 % of 158 cases; Intestinal Type Stomach Adenocarcinoma in 2.47 % of 81 cases; and Stomach Adenocarcinoma in 1.68 % of 895 cases (Fig. 6C).

In this study, we analyzed the high-frequency mutations in CLCA1 genes and their associations with mutated and unmutated groups. The mutation frequencies of H2BW2*, ARL6*, EPS15*, ENTREP2*, C14ORF180*, IQCB1*, RNF222*, TST*, MS4A5*, and GAS2L1* genes were found to be notably higher in the group with mutations when compared to the group without mutations (Fig. 6D). According to the data presented in Figure 6E, there was a statistically significant difference in median months of survival observed between patients with gene mutations and those without when analyzing survival curves. The observed higher survival and larger population of patients with prolonged survival in the mutation group may be attributed to the identification of appropriate targeted drug therapy enabled by the gene mutation, which significantly prolongs patient survival.

The results indicate that modifications and variations in the CLCA1 gene do occur in tumor tissues and may have a crucial impact on tumor formation and development.

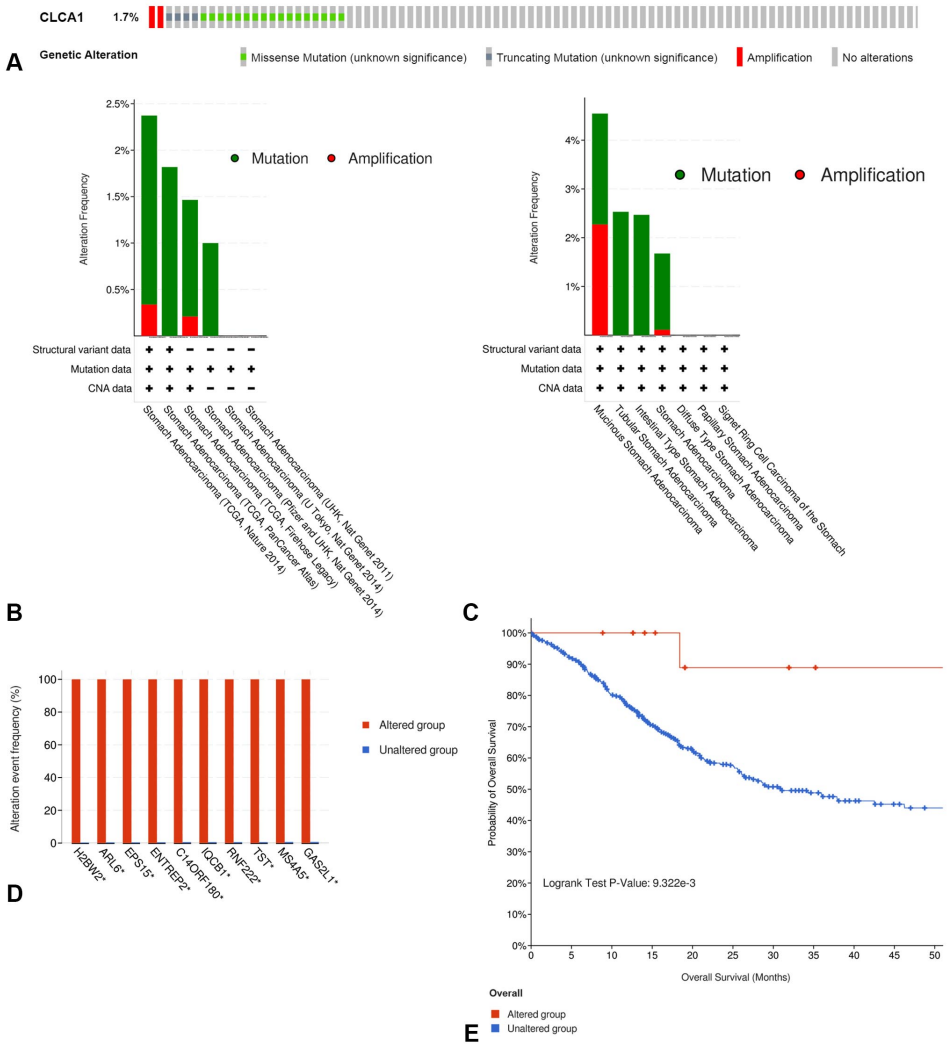

Fig. 6. CLCA1 gene mutation analysis in a sample set of 1365 gastric adenocarcinoma cases. (A) Waterfall map illustrating mutations in the CLCA1 gene in gastric adenocarcinoma. (B-C) Comparison of mutation types in gastric adenocarcinoma cases in the cBiPortal database. (D) Comparison of mutation frequencies in other highly mutated genes. (E) Comparison of differences in median survival months between patients with and without mutations in the gene (OS).

Immune characterization of CLCA1 in the tumor microenvironment. To investigate the immune properties of CLCA1 in STAD TME, we evaluated the potential correlation between CLCA1 gene and 22 classes of immune cell infiltration scores in STAD tumors. After analyzing the data, we have determined that there is a negative correlation between the expression of the CLCA1 gene and the presence of CD8+ T cells, T follicular helper (Tfh) cells, Macrophages M0, and Macrophages M1 in STAD. On the other hand, we have observed a positive correlation between the CLCA1 and the presence of B cells naive, Plasma cells, CD4+ memory resting T cells, Monocytes, and Eosinophils in STAD. This correlation is evident in the analysis of 388 samples (Fig. 7A).

Moreover, the expression levels of CLCA1 showed close correlation with the expression levels of various immune checkpoint (ICP) genes in STAD. It was negatively correlated with immunosuppressant CTLA4 and immunostimulants CD40LG, GZMA, and CCL while positively correlated with immunostimulant SELP (Fig. 7B).

In this study, we examined the correlation between CLCA1 and immunomodulatory factors in STAD. Our findings revealed an inverse association between CLCA1 and the chemokines, such as CCL27, CCL5, XCL2, CXCL11, and CCL25. Additionally, we observed a direct

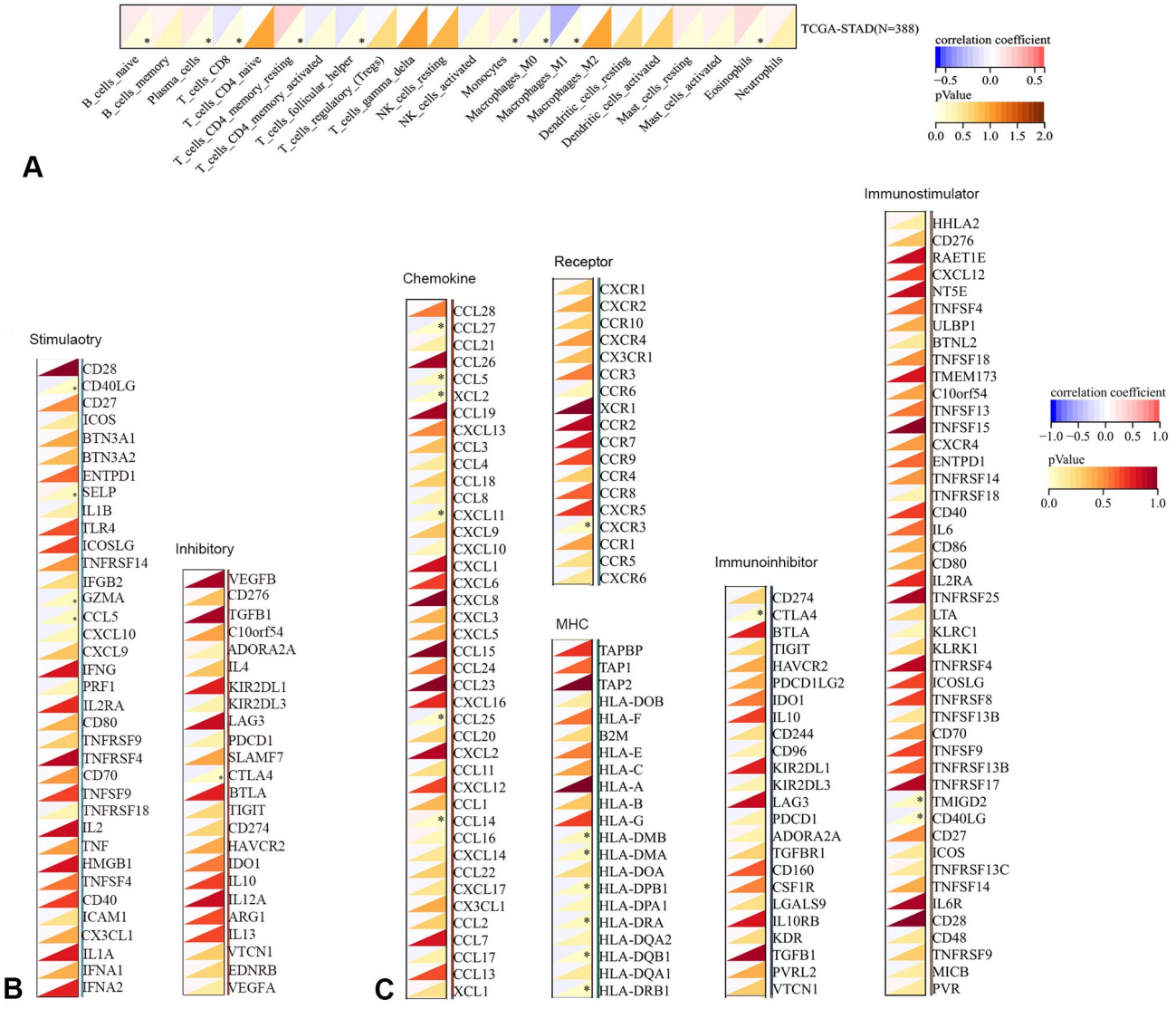

Fig. 7. The correlation between the levels of CLCA1 expression and the infiltration of immune cells, as well as the immune checkpoint genes and immunomodulatory genes, was investigated in STAD. (A) The extent of infiltration by immune cells was assessed. (B) Two categories of immune checkpoint genes were analyzed, including those with inhibitory and stimulatory functions. (C) Immunomodulatory genes (Chemokines, Receptors, MHC, Immunosuppressants, and Immunostimulants).

correlation between CLCA1 expression and the level of CCL14. Additionally, we observed a negative correlation with the receptor molecule CXCR3. Negative correlation with HLA-DMB, HLA-DMA, HLA-DPB1, HLA-DRA, HLA-DQB1, HLA-DRB1 in MHC, and negative correlation with the immunostimulators TMIGD2 and CD40LG (Fig. 7C).

This indicates that CLCA1 might control the function of these immunomodulatory or immune checkpoint genes across various signaling pathways, presenting a possibility for immunotherapy.

Enrichment Analysis. The obtained results suggested a significant association between CLCA1 and cancer prognosis and immunity. To further validate the potential function of CLCA1 in STAD tumor tissues, we obtained the co-expressed gene network of CLCA1 via the LinkedOmics database (Fig. 8A).

Figures 8B-C displays the employment of heat maps to showcase the top 50 genes that display both positive and negative correlation with CLCA1. Following this, gene set enrichment analysis (GSEA) was employed to detect the gene ontology (GO) of the genes that are co-expressed with CLCA1, and by analyzing the categories of biological processes in which the GO functioned, The discovery revealed that the primary functions of CLCA1 and its co-expressed genes centered around controlling digestion and initiating translation. Additionally, they played a significant role in the metabolic pathway of tRNAs, the expression of genes in mitochondria, and the assembly of protein-DNA complexes' subunits (Fig. 8D). Afterwards, we performed an analysis of KEGG pathways, which revealed that genes exhibiting co-expression with CLCA1 were significantly enriched in various biological phenomena including chemical carcinogenesis, systemic lupus erythematosus, proteasome, pyrimidine metabolism, cell cycle, pancreatic secretion, ribosomal, and PPAR signaling pathways (Fig. 8E).

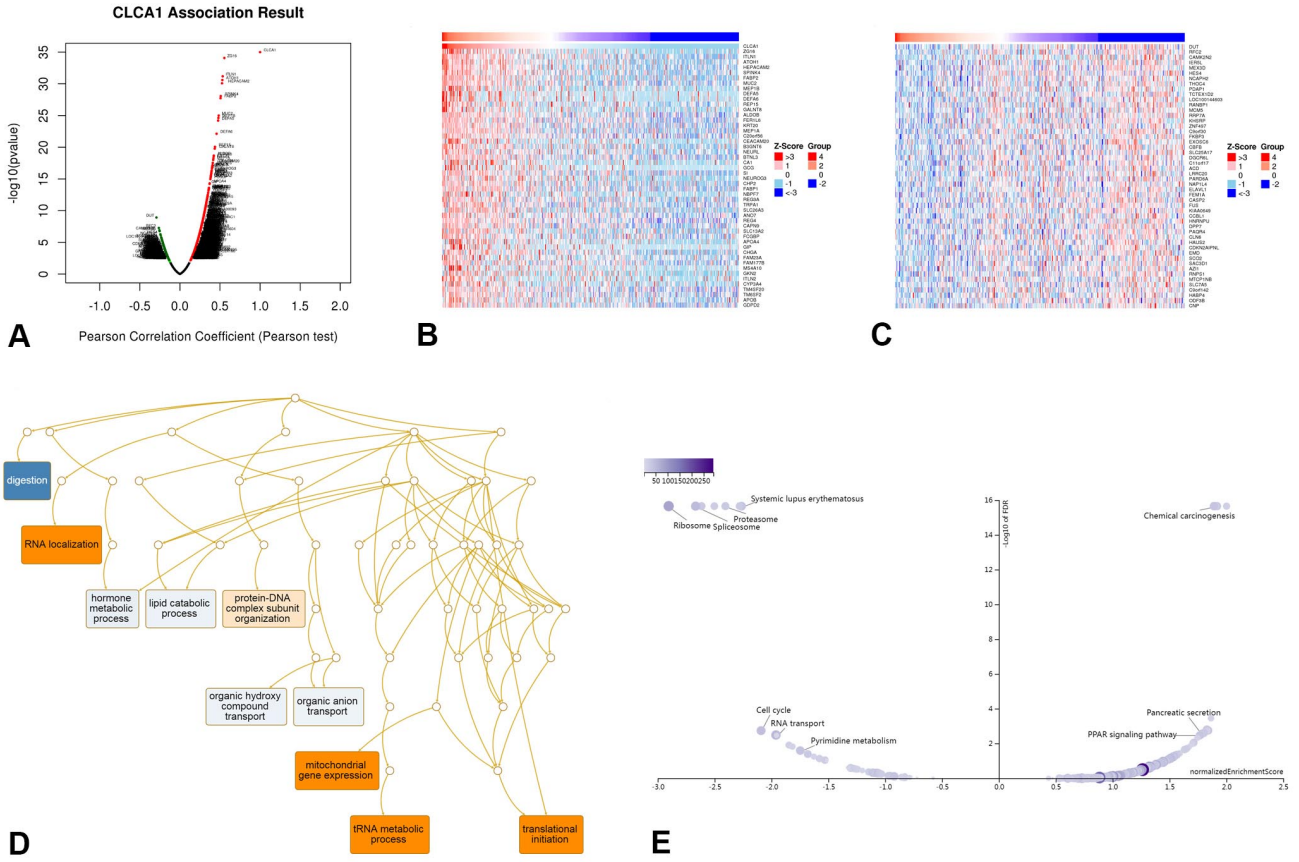

Fig. 8. Analysis of co-expressed genes with CLCA1 in STAD using the LinkedOmics database. (A) Volcano plot showing highly correlated genes for CLCA1 in STAD. (B, C) Heatmaps showing the top 50 positive (B) and negative (C) co-expressed genes for CLCA1 in STAD. (D) Directed acyclic graph (DAG) showing CLCA1 GO analysis (biological process) in STAD. (E) Volcano diagram showing the KEGG pathway of CLCA1 in STAD.

DISCUSSION

The prevalence of gastric cancer is high and it is a malignant tumor that affects the gastrointestinal tract. Diagnosing gastric cancer at an early stage is challenging due to the non-specific initial symptoms. As a consequence, a majority of patients receive their diagnosis when the disease has already advanced to the intermediate or advanced stages (Machlowska *et al*., 2020). There are multiple treatment alternatives, yet the outcomes continue to be dissatisfactory due to a substantial morbidity and mortality rate (Hu *et al*., 2022). In recent years, the application of bioinformatics has laid the foundation for the search of effective molecular markers and promoted the research progress of targeted tumor therapy. Hence, it is imperative to delve deeper into the prospective pathogenesis of STAD from a fresh outlook and discover novel biomarkers for initial focused molecular interventions to diminish fatality. In this investigation, we conducted an extensive analysis of the expression, genetic modifications, and associated clinical importance of CLCA1 in STAD employing public databases and IHC.

At present, the CLCA1 gene's expression remains unreported in gastric cancer. Utilizing bioinformatics technology, our analysis unearthed low CLCA1 expression in various cancers, such as colorectal, breast, endometrial, gastric, and esophageal cancers. Immunohistochemical validation demonstrated the down-regulation of CLCA1 in STAD. The low levels of CLCA1 expression demonstrated correlations with molecular subtypes, race, age, cancer grade, histologic subtype, and unfavorable prognosis among patients with STAD. In addition, the CLCA1 protein exhibited a lower mutation rate in gastric adenocarcinoma and a higher survival rate in the variant group. These results indicate that the importance of CLCA1 expression level is crucial in the advancement of STAD.

The biological functionality of various cancers has been shown to be influenced significantly by infiltrating immune cells in the tumor microenvironment, thus making a valuable contribution to the response observed in immunotherapy (Miyazaki *et al*., 2018). Based on research findings, the level of infiltration by diverse immune cells significantly correlates with CLCA1 gene expression, which exhibits a strong connection to immunotherapy response. For instance, immune cells such as CD8+ T cells, M1-type macrophages, and Tfh play a pivotal role in this association. Numerous clinical investigations have exhibited the pivotal role of Tfh cells in assessing the outlook of individuals diagnosed with cancer. The high level of infiltration of Tfh cells is related to a poorer prognosis (Zhang *et al*., 2022). Moreover, multiple investigations propose that an increased infiltration of CD8+ T cells is linked to an unfavorable prognosis (Thompson *et al*., 2017). The findings of these investigations exhibit consistent outcomes with the present study, suggesting a negative correlation between CLCA1 and Tfh cells as well as CD8+ T cells. In addition, we evaluated CLCA1 expression in relation to MHC molecules, immunosuppressants, immunostimulants, and chemokines, receptors. Remarkably, the expression of CLCA1 was discovered to have associations with various chemokines like CCL27 and CCL5, as well as XCL2, CXCL11, CCL25, and CCL14. Moreover, receptor molecules such as CXCR3, immunosuppressants like CTLA4, immunostimulants such as CD40LG and TMIGD2, along with MHC molecules like HLA-DMB, HLA-DMA, HLA-DPB1, HLA-DRA, HLA-DQB1, and HLA-DRB1 demonstrate close relationships. Previous studies have demonstrated the significant involvement of chemokines in gastric cancer's development and progression. Among the human chemokines, CCL5, CCL25 and CCL27 exhibit a close association with CD8+ T cell infiltration (Li *et al*., 2021). The vital involvement of CCL5 protein in tumor advancement via autocrine or paracrine mechanisms is well-established (Zhang *et al*., 2023). Furthermore, multiple investigations have indicated a direct association betwixt enhanced quantities of CCL5 in neoplastic tissues and the quantity as well as the functionality of CD8+ T lymphocytes with cytotoxic properties (Zumwalt *et al*., 2015).

The observation made in our investigation reveals that the expression of CLCA1 exhibited a connection with the immune checkpoint genes including CTLA4, CD40LG, SELP, GZMA, and CCL5. As a crucial molecular regulator of T-cell activities within tumors, GZMA restrains tumor proliferation, triggers programmed cell death, orchestrates antigen-induced CD8+ T lymphocyte cytotoxicity, and confers safeguard against tumor assault on mice (Shimizu *et al*., 2019). In recent studies, cancer researchers have shown keen interest in GZMA as a possible prognostic biomarker (Huo *et al*., 2023). SELP, an adhesion molecule and selectin protein, has emerged as an expression in various cell types which involves immune cells, endothelial cells, cancer cells, and platelets. Its involvement in orchestrating the immune response against cancer has also been firmly established (Yeini & Satchi-Fainaro, 2022). Thus, the exploration of molecules that may act synergistically or antagonistically with CLCA1 in gastric adenocarcinoma remains a focus of future research.

The biological functions of CLCA1 in STAD have yet to be fully understood. In this study, we carried out an enrichment analysis of CLCA1 and its co-expression in order to anticipate the potential pathways in which CLCA1

may be involved in STAD. Analysis of KEGG enrichment indicated that the reduction in CLCA1 expression in STAD was linked to various signaling pathways such as SLE, the proteasome, pyrimidine metabolism, the cell cycle, secretion of pancreatic enzymes, ribosomes, and peroxisome proliferator-activated receptors (PPAR). The cell cycle is a vital regulatory mechanism for cell growth and proliferation. Deviations in this pathway significantly contribute to tumor invasion and metastasis (Krabbe *et al*., 2016). Furthermore, there is an association between the occurrence of stomach cancer and systemic lupus erythematosus (SLE) (Sun *et al*, 2021). In support of this connection, Clarke *et al*. (2021) conducted a meta-analysis and found that individuals with SLE had a significantly higher likelihood of developing gastric cancer. It was suggested that future clinical practice might significantly benefit from novel therapies targeting pathways common to SLE and malignancy (Capone *et al*., 2015).

This study proposes that gastric cancer may be diagnosed and prognosticated by considering CLCA1 as a tumor suppressor and a potential marker. A substantial correlation has been observed between the expression of CLCA1 and immune checkpoints, along with the extent of infiltration by immune cells. Nevertheless, additional experiments are required to validate these findings. These discoveries have the potential to generate novel concepts for fundamental research on immunotherapy for gastric adenocarcinoma.

Nevertheless, there exist certain constraints within the scope of this investigation. To begin with, the quantity of available clinical tissue samples was limited and their verification was solely based on immunohistochemical staining. Therefore, it is imperative to increase the sample size and combine various experimental methods in the future to further elucidate the expression of CLCA1. Secondly, the database analysis in this study revealed that the mutation rate of CLCA1 protein in gastric adenocarcinoma is exceptionally low and closely related to immune cell infiltration. CLCA1's impact on the biological behavior of gastric adenocarcinoma should be explored with in vitro cellular experiments in the future.

AUTHOR CONTRIBUTIONS

The manuscript was drafted and the data were analyzed by ZXY. LJ conceived the study, designed it, and analyzed the data. CZN conducted the experiments and analyzed the data. CYF and LXH also contributed to data analysis. The manuscript received approval from all authors after reading. ZXY and LJ should be recognized as co-first authors due to their equal contributions to this study.

ZHAO, X.; CHEN, Y.; LU, X.; CHENG, Z. N. & LU, J. CLCA1 se expresa deficientemente en el adenocarcinoma de estómago y se correlaciona con la infiltración inmune. *Int. J. Morphol., 42(1)*:173-184, 2024.

RESUMEN: El regulador 1 del canal de cloruro activado por calcio (CLCA1) está asociado con la progresión del cáncer. La expresión y la función inmunológica de CLCA1 en el adenocarcinoma de estómago (STAD) aún no están claras. En esta investigación, se examinó la expresión de CLCA1 en tejidos STAD y su participación en la progresión y respuesta inmune de STAD utilizando bases de datos como cBioPortal, TISIDB y UALCAN. Para validar el nivel de expresión de la proteína CLCA1 en el adenocarcinoma gástrico, se recolectaron treinta muestras de tejido clínico para tinción inmunohistoquímica. Los hallazgos indicaron una regulación negativa de CLCA1 en pacientes con STAD, que se correlacionó con la raza, la edad, el grado del cáncer, la infección por *Helicobacter pylori* y el subtipo molecular. Mediante el examen del análisis de supervivencia, se identificó que los niveles reducidos de CLCA1 en los casos de cáncer gástrico estaban relacionados con períodos reducidos de supervivencia posterior a la progresión (PPS), supervivencia general (OS) y primera progresión (FP) (P <0,05). La tasa de mutación CLCA1 fue menor en STAD, pero la tasa de supervivencia fue mayor en el grupo variante. Se observó la correlación entre el nivel de expresión de CLCA1 y los niveles de células inmunes infiltrantes en STAD, así como las moléculas activadoras inmunes, moléculas inmunosupresoras, moléculas MHC, quimiocinas y sus moléculas receptoras. El análisis de enriquecimiento genético reveló que CLCA1 puede estar involucrado en la progresión de STAD a través del lupus eritematoso sistémico (LES), el proteasoma, el ciclo celular, la secreción pancreática y las vías de señalización de PPAR. En resumen, se prevé que CLCA1 funcione como un marcador de pronóstico para pacientes con STAD y está vinculado a la inmunización de STAD.

PALABRAS CLAVE: Adenocarcinoma de estómago; Estadios clínicos; Inmunohistoquímica; Bioinformática; Factor pronóstico.

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