Journal of Translational Medicine

RESEARCH

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MCT4: a key player influencing gastric cancer metastasis and participating in the regulation of the metastatic immune microenvironment

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Abstract

Background MCT4 is a lactate transporter associated with glycolysis, which has been found to be associated with various tumorigenesis and development processes. Gastric cancer is a malignant disease with high incidence and mortality. The role of MCT4 in the occurrence and development of gastric cancer has not been clarified.

Methods In this study, we comprehensively utilized single-cell sequencing and external transcriptome sequencing databases to deeply analyze the mechanism of the impact of MCT4 on gastric cancer and its microenvironment. We verified the function of MCT4 in gastric cancer through in vitro cell line experiments and in vivo experiments using gastric cancer liver metastasis and subcutaneous tumor models. Meanwhile, we collected tumor and normal tissue samples from clinical gastric cancer patients and employed immunohistochemistry and multiplex immunofluorescence techniques to detect the expression and localization of relevant indicators, thereby validating the results of computer simulation analysis and providing a basis for revealing the internal relationship between MCT4 and gastric cancer.

Results The expression of MCT4 is upregulated in gastric cancer patients, and the upregulation is more significant than that in patients with gastric cancer metastasis. MCT4 can mediate the proliferation and migration of gastric cancer cells in vitro. MCT4 can mediate the metastasis of gastric cancer cells in vivo. Multi-omics analysis showed that the expression of MCT4 was related to the composition of the immune microenvironment, and it could mediate the emergence of the inhibitory immune microenvironment. The results of immunofluorescence and immunohistochemistry proved the robustness of the multi-omics analysis.

Conclusion Our study found that MCT4 plays an important role in the occurrence and development of gastric cancer, which may mediate the occurrence of gastric cancer metastasis and shape the immunosuppressive tumor microenvironment.

Keywords SLCA16A3, MCT4, Gastric cancer metastasis, Immune microenvironment, EMT

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Introduction

Gastric cancer remains a major health issue, ranking as the fourth leading cause of cancer-related deaths worldwide [1]. Patients with gastric cancer typically have a poor prognosis, with a high risk of local recurrence and distant metastasis [2]. Currently, research on gastric cancer metastasis has encompassed multiple aspects, including key signaling pathways, metabolic regulation, and the modulation of the immune microenvironment [3]. However, there are currently no effective intervention strategies for metastasis, and research on key targets influencing metastasis remains limited.

The metastasis of gastric cancer can be likened to seeds being sown in soil; thus, the changes in the metastatic ability of the cancer cells (the seeds) and the influence of the microenvironment (the soil) are two critical factors leading to tumor metastasis. The metastatic activity of gastric cancer is regulated by various complex mechanisms, with prominent factors including epithelial-mesenchymal transition (EMT), vascularization, and proliferative capacity all playing significant roles. EMT provides adaptive changes that facilitate the conversion of normal tissue to cancerous tissue. Vascularization supplies rich blood flow and nutrients for metastasis, while proliferative capacity offers strong momentum for the development of tumors following metastasis [4]. Among these factors, various signaling molecules and energy metabolism are regulated, with TGF-β serving as a typical promoting molecule that influences the aforementioned processes [5]. However, the mechanisms and clues regarding its regulation are still lacking. Furthermore, the shaping of the immune microenvironment is another key factor that allows tumors to colonize and survive. The tumor microenvironment comprises a large number of infiltrating immune and non-immune cells, which create a unique microenvironment that may provide a fertile ground for tumor metastasis [6]. Therefore, uncovering these immune influencers and proposing intervention strategies is an important approach to altering the challenges associated with gastric cancer metastasis.

Monocarboxylate transporters (MCTs), also known as monocarboxylate transport proteins, are important members of the solute carrier protein family. Among the 14 identified MCTs, MCT1-MCT4 are expressed in various tissues and catalyze the proton-coupled bidirectional transport of monocarboxylic acids. Among them, MCT1 and MCT4 are the primary members, with MCT1 having been extensively studied [7]. MCT1 and MCT4 primarily function as key proteins regulating lactate, participating in the balance of lactate inside and outside tumor cells [8], with MCT4's role in lactate regulation mainly manifesting in transport and metabolism [9]. Lactate can act as a crucial participant in energy metabolism, regulating energy dynamics in tumors and surrounding cells, thereby influencing cell fate. Additionally, it serves as a signaling molecule involved in gene regulation and transcription. Recent studies have also highlighted that lactate can undergo lactylation modifications on various key genes, impacting their functional expression [10]. MCT4 has been studied in other cancer types, with evidence showing that it can mediate metastasis in liver cancer [11]. However, there are no reported studies on MCT4 in gastric cancer, this study analyzed the role of MCT4 expression in gastric cancer by integrating multiple high-throughput sequencing databases, and further explored the key ways of its role by using single-cell transcriptome sequencing, in vivo and in vitro experiments.

Methods

Acquisition and preprocessing of transcriptome sequencing data

This study obtained multiple RNA-seq expression datasets, including GSE14210, GSE15459, GSE22377, GSE29272, GSE51105, GSE62254, GSE26253, and gastric cancer samples along with their normal tissue counterparts from TCGA (https://portal.gdc.cancer.gov/). Apart from the data from the TCGA database, the remaining tumor expression datasets and corresponding clinical information were downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/).

The expression data were subjected to log2 transformation to stabilize variance, with an addition of 1 to avoid zero values during the log2 conversion. The raw matrix and sequence matrix files for GSE14210, GSE15459, GSE22377, GSE26253, GSE29272, GSE51105, and GSE62254 were downloaded. For the raw data, the R package "affy" (version 4.2.1) was used to extract and normalize the probe expression matrix. Subsequently, the probe expression matrix was converted to a gene expression matrix using the platform annotation file for further analysis.

Immune infiltration analysis

CIBERSORTx, an algorithm designed to estimate the relative abundance of various cell types within heterogeneous cell populations based on gene expression data, was used to assess immune responses in gastric cancer. The abundance of tumor-infiltrating immune cells in groups stratified by MCT4 expression levels was analyzed using 1,140 transcriptome sequencing samples from datasets including GSE26253, GSE62254, and TCGA.

Multivariate cox regression analysis and survival analysis

Based on the coefficients of ligand-receptor pairs and gene expression from the multivariate Cox regression analysis, samples were grouped accordingly. The

"surv_cutpoint" function was applied to evaluate the threshold, categorizing patients into "high-risk" and "low-risk" groups, and Kaplan–Meier survival curves were plotted for prognostic analysis. The log-rank test was performed to determine the significance of differences. Patient survival curves and risk plots were visualized using the R packages "survminer" and "ggrisk." ROC curves were generated using the "survROC" R package. To further assess the robustness of the risk prediction model, patient information was combined with detailed clinical and pathological outcomes from the TCGA dataset to construct a nomogram for model evaluation.

Acquisition and processing of single-cell data

This study utilized single-cell sequencing data from GSE163558, downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). Additionally, single-cell sequencing was performed on liver tissues from HGC-27 cell splenic transplantation tumor models, comparing MCT4 knockout and normal MCT4-expressing cells. The sequencing-related files have been uploaded to the GEO database.

Liver cells were harvested and prepared into a single—cell suspension following published protocols, and libraries were constructed for sequencing. To ensure the quality and reliability of the sequencing data, multiple quality control measures were implemented.

First, CellRanger version 6.0.1 was used to perform bcl2fastq and generate count matrices. The CellRanger mkfastq command was run with default parameters, and the CellRanger count was executed using the GRCh38—3.0.0 reference transcriptome from 10×Genomics and default parameters.

For cell filtering, in addition to removing cells with more than 10% mitochondrial reads and fewer than 4,000 unique molecular identifiers (UMIs), we also checked for cells with an abnormal distribution of gene expression levels. Cells that deviated significantly from the median gene expression levels across all samples were flagged and further examined. If the deviation was due to technical artifacts such as poor cell lysis or excessive background noise, these cells were removed.

Moreover, we evaluated the quality of the library construction by assessing the insert size distribution of the sequencing reads. Libraries with an abnormal insert size distribution, indicating potential problems in the fragmentation or amplification steps, were excluded from further analysis.

After a comprehensive round of quality control filtering, a total of 13,195 cells were retained, and the resulting count matrix output was used for all downstream analyses. This rigorous quality control process helped to ensure that the data used for downstream analysis was of high quality and suitable for accurate biological interpretation.

We used the "Seurat" R package to examine the scRNA-seq data to ensure data quality. Subsequently, we performed log normalization on the merged data and used the FindVariableFeatures function to identify the top 2,000 highly variable genes (using variance-stabilizing transformation, "vst"). All genes were scaled using the ScaleData function, and PCA dimensions were reduced for the selected top 2,000 highly variable genes using the RunPCA function. Cells were clustered using the FindNeighbors and FindClusters functions (resolution = 0.2) to identify cell clusters. Visualization was performed using the UMAP method, further reducing dimensions by selecting the top 20 principal components. UMAP is a dimensionality reduction technique that assumes available data samples are uniformly distributed in a topological space (manifold) and that these finite data samples can be approximated and projected into a lower-dimensional space. Gene set scoring was computed using the AddModuleScore function. Differentially expressed genes were calculated using the FindAllMarkers function, followed by GO, KEGG, and GSEA analyses to identify enriched pathways.

Cell culture

The GES-1, MKN-74, AGS, NUGC-4, and HGC-27 cell lines were purchased from MeisenCTCC (catalog numbers: CTCC-002–0010, TCC-ZHYC-0502, CTCC-001–0038, CTCC-007–039, CTCC-002–0006). The GES-1 cell line was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), while the MKN-74, AGS, NUGC-4, and HGC-27 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. All cell lines were maintained at 37 °C in a 5% CO2 atmosphere.

Western blotting

We collected logarithmically growing cells, discarded the cell lysis buffer, and placed the samples on ice for 30 min, shaking every 10 min. The samples were centrifuged (13,300 rpm, for 15 min), and the supernatant was collected. Total protein was quantified using the BCA method, and the remaining samples were stored at −30 °C until further use. Equal amounts of protein were loaded and run on a 10% SDS-PAGE gel, followed by transfer to a membrane to block nonspecific antibodies. After blocking, the membrane was incubated overnight

at 4 °C with a primary antibody (1:1000) in blocking solution. The next day, the primary antibody was removed, and the membrane was washed with PBS before incubating with the appropriate secondary antibody (1:100) at room temperature for 2 h, followed by enhanced chemiluminescence (ECL). β-actin was chosen as an internal control to compare MCT4 protein expression.

Plasmid transfection for MCT knockout or inhibition and construction and evaluation of lentiviruses

MCT4 knockout lentivirus was used to infect HGC27 cells according to the manufacturer's protocol, resulting in target gene knockout.

siRNA plasmids were constructed by Shanghai Jiman Biotechnology Co., Ltd. Transfection was performed following the manufacturer's instructions for 24 h. After 24 h of infection, the cells were maintained in medium containing puromycin (2 μg/ml) for 15 days to generate stable cell lines. Subsequently, qRT-PCR was used to verify the stable MCT4 knockout HGC27 cell lines.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (RC112, Vazyme, Nanjing, China). RNA was reverse transcribed into complementary DNA (cDNA) using the HiScript III RT SuperMix for qPCR (+gDNA Eraser) kit (R323, Vazyme, Nanjing, China). Quantitative real-time PCR was performed using the AceQ® Universal SYBR qPCR Premix Kit (Q511, Vazyme, Nanjing, China). Each experiment was conducted in triplicate for each sample.

CCK-8 assay

When the cells reached 80–90% confluence, they were washed twice with 2 mL of PBS (C0221A, Beyotime). After discarding the PBS, 2 mL of digestion solution containing 0.25% trypsin and 0.02% EDTA (H0518, Bodo) was added for digestion. The remaining digestion solution was incubated for an additional 1–2 min. Cell detachment was confirmed under a microscope to ensure complete separation from the cell wall and to obtain single cells. Cells were counted using a hemocytometer. After counting, the cells were diluted with complete RPMI 1640 medium (G4531-500ML, Servicebio). After 24 h of culture, the original complete medium was discarded. The optical density (OD) at 450 nm was measured using the enzyme-linked immunosorbent assay (ELISA).

Wound healing assay

Ninety percent confluent cells were seeded into a 6-well plate overnight. Two wounds were created per well using a pipette along the adhered cell layer. Cells within the wounds were removed using PBS, and fresh medium without fetal bovine serum (FBS) was added for further culture. At different time points, cell migration was photographed using an inverted microscope, and the area of the wound was measured.

Gastric cancer animal experiments

Gastric Cancer Liver Metastasis Model: Ten male BALB/c nude mice, 5 weeks old, were obtained from Shanghai Super B&K Experimental Animal Co., Ltd. and housed at the Animal Experiment Research Center of Zhejiang Chinese Medical University. MCT4 knockout HGC27 cell lines and control mock HGC27 cells were resuspended in phosphate-buffered saline. After anesthetizing the nude mice, a laparotomy was performed to expose the spleen, where the cell lines were inoculated. Referring to the methods of previous studies [12], 3 weeks later, the mice were euthanized, and the spleen and liver were harvested to calculate the weights of the primary tumors and liver metastatic tumors.

Gastric Cancer Subcutaneous Tumor Model: Ten male BALB/c nude mice, 5 weeks old, were obtained from Shanghai Super B&K Experimental Animal Co., Ltd. and housed at the Animal Experiment Research Center of Zhejiang Chinese Medical University. HGC27 cells were resuspended in phosphate-buffered saline and inoculated subcutaneously. The mice were divided into a normal observation group or VB124 group and a control group. VB124, an MCT4 inhibitor, was administered via intraperitoneal injection at a concentration of 30 mg/kg once daily for 7 days. Refer to the method of the previous study [13], 18 days post-tumor inoculation, the mice were euthanized, and samples were collected.

In terms of evaluating tumor growth, in this study, we utilized a specific formula to precisely assess tumor growth. At each measurement time point, a vernier caliper with a precision of 0.1 mm was employed to measure the length and width of the tumors. The tumor size was calculated according to the formula: $V = 0.5 \times Length \times Width^2$, where "Length" denotes the length of the longest diameter of the tumor, and "Width" represents the maximum diameter length in the direction perpendicular to the longest diameter.

In the above gastric cancer models, the health and behavior of the mice were monitored every 3 days. Five weeks after cell implantation, all mice were anesthetized using 5% isoflurane for 5 min, followed by euthanasia via cervical dislocation. All mice were confirmed dead after euthanasia. The animal experiments were approved by

the Animal Care and Use Committee of Zhejiang Chinese Medical University, and all procedures were conducted in accordance with international guidelines.

Immunohistochemistry staining

Histological analysis was performed using tissue microarrays of gastric cancer tissues and adjacent normal tissues. Immunohistochemistry (IHC) staining was conducted by incubating the samples with antibodies against MCT4 (147765, Aibixin®, Shanghai, China), the M1 macrophage marker CD86 (ab220188, Abcam®, Cambridge, UK) [14], and the M2 macrophage marker CD206 (EPR19518, Abcam®, Cambridge, UK) [15].

Multiplex immunofluorescence staining

According to the manufacturer's instructions, the Opal multiplex staining kit (Akoya Biosciences) was used for multiplex immunofluorescence staining (m-IHC). First, formalin-fixed, paraffin-embedded (FFPE) sections were dewaxed and rehydrated, and fixed with 10% paraformaldehyde (PFA) for 20 min. Then, the slides were washed with TBST and AR6 buffer, and microwaved in AR6 buffer (Akoya Biosciences). The slides were cooled to room temperature (RT), washed with TBST, and blocked with Opal antibody diluent for 10 min. Next, the primary antibody was added and incubated overnight at 4 °C. After washing, polymer HRP (Ms + Rb) secondary antibody was added and incubated at RT for 10 min. After washing again, Opal dye (1:100) was applied for 10 min. The slides were then washed and microwaved. This process was repeated five times to apply different antibodies. Nuclei were stained with DAPI (Akoya Biosciences), and slides were mounted with medium (Abcam). The antibodies used included CD4 + (EPR6855, Abcam®, Cambridge, UK), CD8 + (OX-8, Abcam®, Cambridge, UK), and Ly6G (ab25377, Abcam®, USA), MCT4 (147765, Aibixin®, Shanghai, China), CD68 (ab125212, Abcam®, Cambridge, UK).

Statistical analysis

All data analyses were performed on the R platform (version 4.4.1). Student's t-test or Wilcoxon rank-sum test was used to compare continuous variables between two subgroups. One-way ANOVA or Kruskal–Wallis test was employed to compare differences among three groups. Pearson correlation was used to assess the correlation between normally distributed variables, while Spearman correlation was applied for non-normally distributed variables. The Benjamini and Hochberg (BH) method was used to estimate the false discovery rate for multiple testing. The "survminer" R package was utilized for Kaplan–Meier analysis and log-rank tests to evaluate survival differences between groups. GO, KEGG, and GSEA analyses were conducted using the clusterProfiler R package (org package in R). Spearman correlation was calculated using the cor.test function in the R stats package. All analyses were performed using default parameters.

Result

MCT4 is highly expressed in gastric cancer and is associated with malignant behaviors of the disease

To assess the impact of the MCT family on gastric cancer, we first evaluated the transcriptomics of the MCT family in cancer and adjacent normal tissues using the TCGA database. The results, shown in Fig. 1A, indicate a trend of high expression for several transporters, including SLC16A1, SLC16A3, SLC16A5, and SLC16A13, in gastric cancer. Furthermore, we conducted an expression analysis of multiple transcripts of SLC16A3 in normal and cancerous tissues using paired samples from GSE29272 and GSE14210 (Fig. 1B, D) as well as unpaired samples from GSE15459, GSE22377, GSE51105, and GSE62254 (Fig. 1C, E). The results consistently indicated that MCT4 is elevated in gastric cancer tumor tissues. We validated this finding using the HPA database (Fig. 1F). To further clarify whether MCT4 is associated with tumor progression, we analyzed single-cell datasets (GSE163558) for gastric cancer and adjacent tissues. We found distinct epithelial cell subpopulations exhibiting different characteristics between cancer and adjacent tissues (Fig. 1G). Further analysis revealed a unique epithelial subpopulation (labeled as cell b) that emerged in gastric cancer (Fig. 1H). Functional analysis showed that the uniquely expressed epithelial subpopulation in tumors highly responds to TGF- β signaling, dominating responses related to hypoxia, tumor proliferation, EMT, and DNA repair, among other tumor-specific biological effects (Fig. 1I). Moreover, this subpopulation also highly expressed MCT4 and other tumor progression markers, particularly CDH1 and Ki67 (Fig. 1J). Next, we assessed the expression of MCT4 in the TCGA dataset in relation to tumor proliferation, EMT, and angiogenesis. We found significant correlations, particularly with EMT and angiogenesis (Fig. 1K). These results suggest that MCT4 is involved in the progression of gastric cancer and is associated with several key biological effects.

The expression of MCT4 is more closely related to the recurrence and metastasis of gastric cancer

To validate the data analysis results, we conducted protein detection of MCT4 using 46 paired gastric cancer tissue paraffin chips. The results, shown in Fig. 2A,

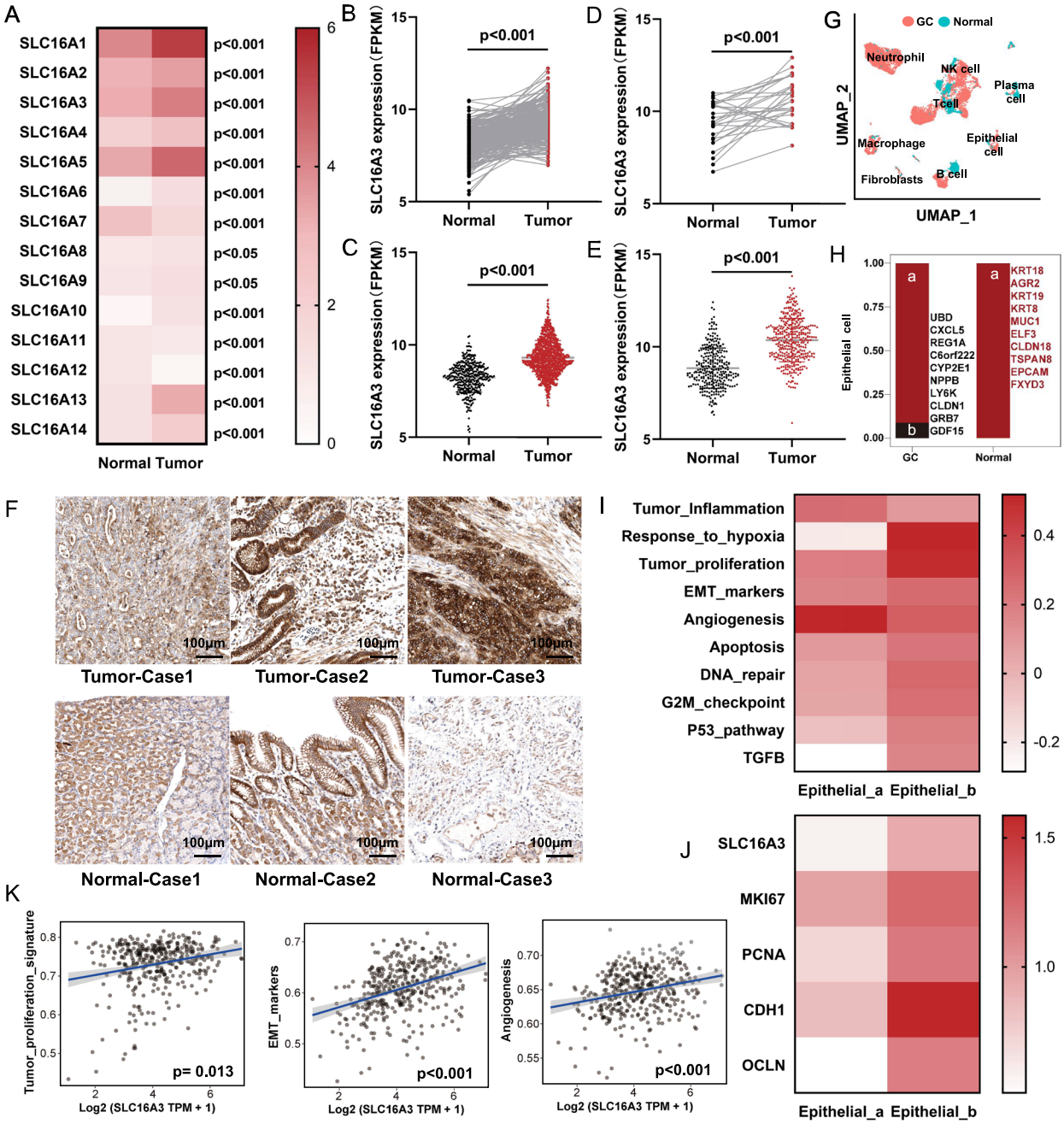

Fig. 1 MCT4 is highly expressed in gastric cancer and is associated with malignant behavior of gastric cancer. **A** Expression of SLC family genes in multiple transcriptome sequencing datasets. **B** MCT4 expression in paired samples of GSE29272. **C**, **D** Expression of MCT4 in GSE15459, GSE22377, GSE51105 and GSE62254. **E** Expression of MCT4 in paired samples of GSE14210. **F** Immunohistochemical results of MCT4 expression in normal and gastric cancer tissues in HPA database. **G** Grouping of normal and gastric cancer samples in GSE163558 gastric cancer single cell data. **H** Gene expression and composition of different epithelial cells in GC tissues and normal tissues. **I** Characteristic gene enrichment analysis of different epithelial cells in GC tissues and normal tissues. **J** Evaluation of typical tumor-related features in different epithelial cells in GC tissues compared to normal tissues. **K** Evaluation of the correlation between MCT4 expression and tumor proliferation, EMT, and vascular growth in TCGA data

confirm that MCT4 is expressed in gastric cancer, consistent with the previous findings. We further evaluated MCT4 expression in cancerous versus adjacent tissues and in metastatic versus non-metastatic cases. The results indicate that MCT4 expression increases from T1 to T4 tumor stages, although no significant differences were observed between T3 and T4 stages. At the same time, there was no significant association with the tumor's site of occurrence (Fig. 2B–F). However, we found that MCT4 was expressed at higher levels in metastatic patients compared to non-metastatic patients (Fig. 2D). Furthermore, we evaluated MCT4 expression in various gastric cancer pathological tissues, confirming that MCT4 exhibited a high expression pattern in metastatic gastric cancer patients (Fig. 2G–H).

To further reveal the clinical associations of MCT4 with gastric cancer, we integrated seven datasets comprising 1,231 gastric cancer cases to comprehensively assess its correlation with clinical factors. The results, as shown in Fig. 3A, indicated that MCT4 had no significant association with tumor TNM staging, stage, or gender. However, it was significantly correlated with gastric cancer recurrence and various tumor subtype classifications, including EMT, MSI, and TP53+. Further analysis of MCT4 on patient prognosis revealed that patients with high MCT4 expression had poorer overall survival (Fig. 3B). In assessments of progression-free survival and tumor progression, MCT4 demonstrated enhanced prognostic potential (Fig. 3C, D), consistent with previous results. We used TCGA data to stratify patients based on MCT4 expression into different levels, and survival analysis showed that patients with low expression (levels 1–3) had significantly improved survival compared to those with high expression (levels 4–5) (Fig. 3E). The results from the nomogram also supported this finding (Fig. 3F).

In vivo and in vitro experiments demonstrated that MCT4 can influence the metastasis of gastric cancer

We examined the expression of MCT4 in four gastric cancer cell lines and found that MCT4 was expressed in all cell lines. To verify the function of MCT4, we silenced MCT4 in AGS gastric cancer cells (supplementary Fig. 1A-C). The results showed that silencing MCT4 significantly affected their migration ability (Fig. 4A–C). Repeated CCK8 assay found that knocking out MCT4 significantly inhibited the proliferation of HGC-27 (Fig. 4D–F). Cell migration assay found that knocking out MCT4 significantly inhibited migration ability of HGC-27 cell (Fig. 4G). Further, we also confirmed that knocking out MCT4 significantly inhibited the migration ability of HGC-27 cell line through cell scratch experiments. Further, we verified our results through cell experiments, which showed that the modeling of AGS cell lines with MCT4 silenced and HGC-27 cell lines with MCT4 knocked out did not significantly change the size of transplanted tumors compared with the control group (Fig. 4I, J). However, we found that MCT4 knockout did not affect the number of splenic implant tumors, but significantly reduced the number and size of liver metastases, which is consistent with our previous in vitro experiments (Fig. 4K, supplementary Fig. 1D). This suggests that, similar to previous analyses, the effect of MCT4 on gastric cancer metastasis is greater than the effect on the primary tumor itself.

MCT4 influences tumor metastasis by affecting the immune microenvironment

The microenvironment is a key factor influencing gastric cancer metastasis, a notion supported by researchers. To investigate the mechanism by which MCT4 promotes gastric cancer metastasis, we performed single-cell sequencing on liver tissues from spleen liver metastasis models of HGC-27 cells with MCT4 knockout and normal MCT4 expression. The results are shown in Fig. 5, where we used unsupervised clustering analysis to categorize all cells into seven major types (Fig. 5A), with characteristic genes illustrated in Fig. 5B. Further analysis of subgroups revealed that MCT4 knockout led to significant changes in the immune microenvironment, notably an increase in hepatic macrophage content, as well as a marked upregulation of B cells and neutrophils. This indicates that the knockout of MCT4 has a profound impact on the formation of the metastatic microenvironment. We further assessed the tumor immune-related functions of each cell subgroup. It was found that the knockout of MCT4 significantly enhanced the immune activation capacity within the microenvironment (Fig. 5E), and there was a marked upregulation in tumor growth inhibition capacity and chemokine-related abilities (Fig. 5F, G). Furthermore, we conducted enrichment analysis based on the differentially expressed genes between the two groups of cells before and after knockout. The results showed that the KO group exhibited a significant upregulation in activities related to natural killer cell-mediated cytotoxicity, immune receptor activity, and other immune-related processes (Fig. 5H, I). The above results collectively indicate that MCT4 is involved in the regulation of the immune microenvironment and may influence tumor recurrence and metastasis through neutrophils, T cells, macrophages, and other immune cells.

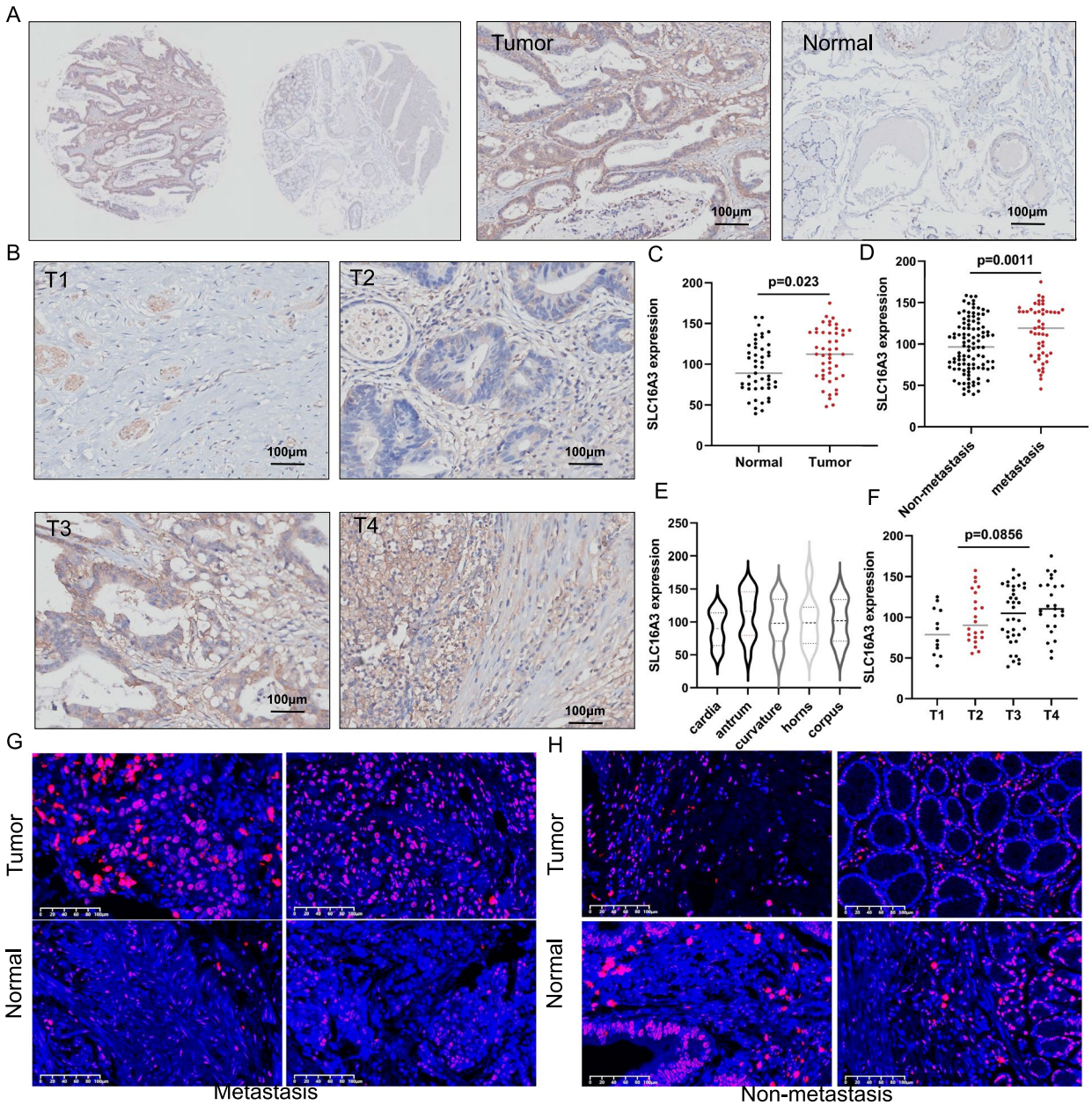

Fig. 2 Clinical validation of MCT4 expression and recurrence and metastasis of gastric cancer. **A** Immunohistochemical staining results of MCT4 in normal and gastric cancer tissues. **B**–**F** MCT4 expression in different pathological stages. The expression of MCT4 was significantly increased in patients with metastatic gastric cancer. The expression of MCT4 increased gradually with the deepening of pathological stage, but the difference was not significant. **G** Immunofluorescence staining results of MCT4 in normal and gastric cancer tissues of patients with metastasis. **H** Immunofluorescence staining results of MCT4 in normal and gastric cancer tissues of non-metastatic patients

MCT4 affects tumor metastasis in relation to T cell and immune cell infiltration

Next, we grouped 1170 samples according to the expression of MCT4 in the TCGA, GSE62254, and GSE26253 datasets, and performed immunoinfiltration correlation analysis (Fig. 6A). After counting the cumulative cell proportion, we confirmed that MCT4 expression is associated with multiple immune cells. Further, we compared the proportions of immune cells between the two groups and found that the proportions of putative cells of B cells, T cells, NK cells, M0 macrophages, M2 macrophages, dendritic cells, and mast cells in patients with high MCT4 expression were significantly different from those in patients with low MCT4 expression (Fig. 6C).

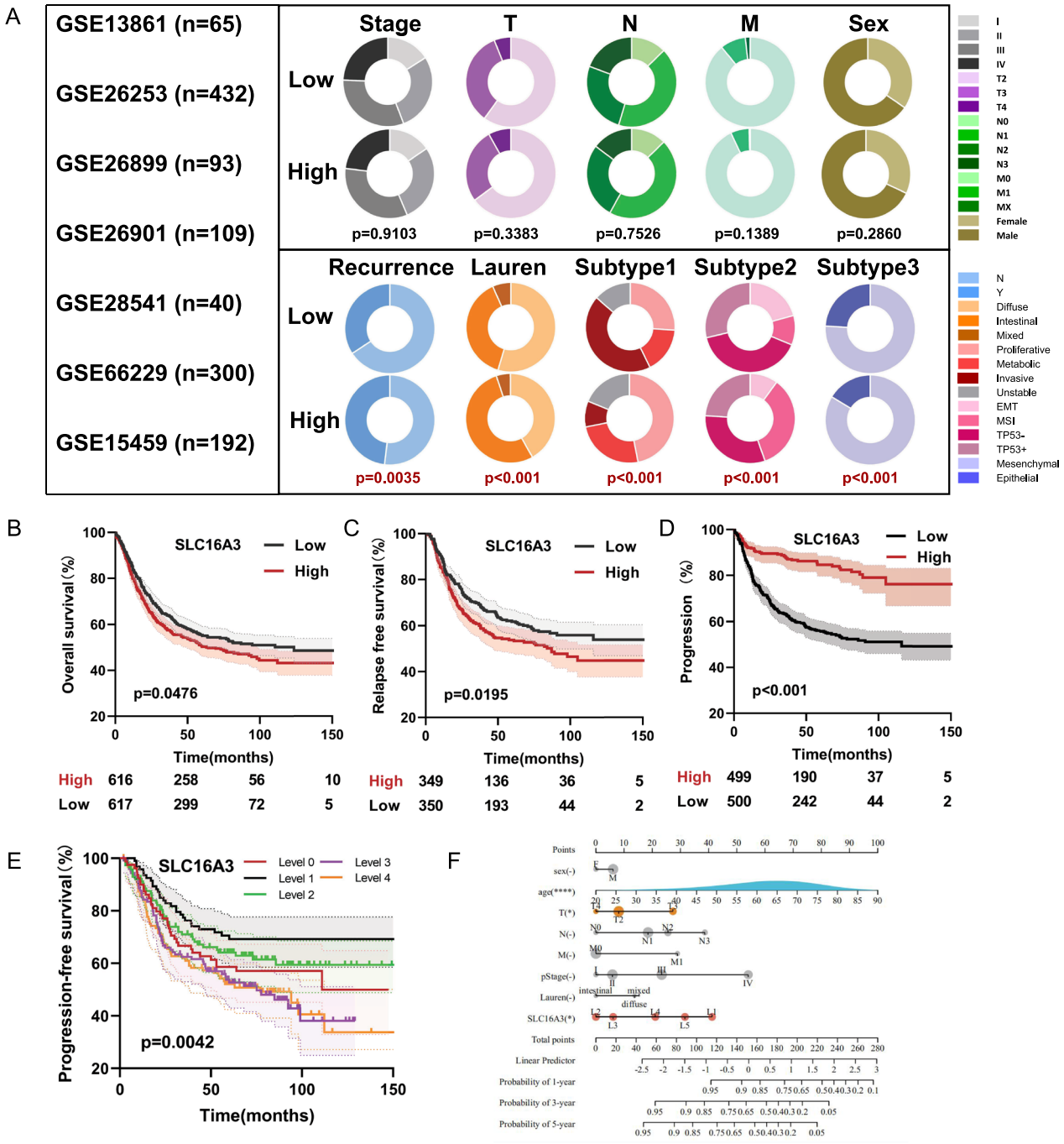

Fig. 3 MCT4 expression and clinical validation of recurrence and metastasis in gastric cancer. **A** Correlation analysis between MCT4 expression and clinical features in patients with gastric cancer in seven datasets. **B** Survival analysis with MCT4 expression level as grouping method and survival period as outcome. **C** Survival analysis based on MCT4 expression level and disease-free survival as outcome. **D** Survival analysis based on MCT4 expression level and progression-free outcome. **E** The patients with gastric cancer were divided into five groups according to the expression level of MCT4, with progression-free stage as the outcome for survival analysis. **F** The gastric cancer transcriptome sequencing data in the TCGA database was used to make a nomogram

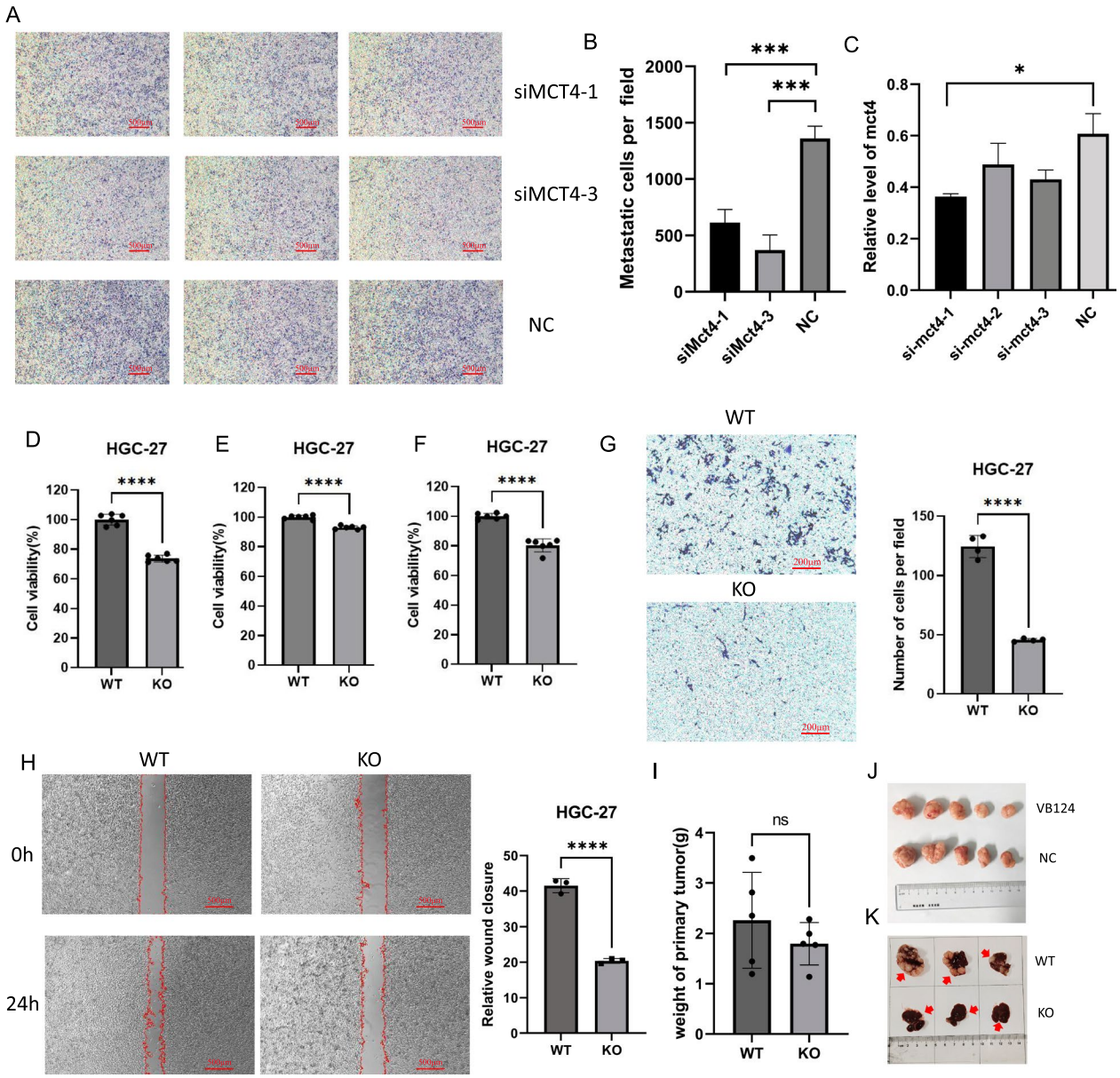

Fig. 4 In vitro and in vivo experiments demonstrated the effect of MCT4 expression on gastric cancer cells. **A** The cell migration ability of AGS gastric cancer cell lines with low MCT4 and normal AGS gastric cancer cell lines was measured using small interfering RNA. **B**, **C** Cell migration ability of AGS gastric cancer cell lines with low MCT4 and normal AGS gastric cancer cell lines knocked with small interfering RNA. **D**–**F** CCK8 proliferation capacity of HGC-27 cells after MCT4 knockdown. **G** Cell migration of HGC-27 cells after MCT4 knockout. **H** Cell scratch assay of HGC-27 cells after MCT4 knockout. **I** Statistical map of tumor weight of HGC-27 cells after MCT4 knockout. There was no significant difference in tumor weight in situ between the two groups after MCT4 knockout. **J** VB124 interfered with the expression of MCT4 in HGC-27 cells for experimental tumor transplantation in vivo. There was no significant difference in tumor size in situ after VB124 interfered with MCT4 expression. **K** HGC-27 cells after MCT4 knockout were used to model the liver of mice implanted with splenic tumor liver metastasis. After MCT4 knockout, the number of liver metastases in KO group decreased significantly

The proportion of CD4 T cell activation, NK cell activation and mast cell activation were significantly decreased in patients with high expression of MCT4. Through correlation analysis, we also found that MCT4 expression was associated with the activation of multiple immune cells (Fig. 6D). To support this hypothesis, we performed multiple fluorescent staining of gastric cancer tissue samples and found that MCT4-positive areas were inversely associated with infiltration of T cell markers such as CD4 and CD8 (Fig. 7A). Similarly, using LY6G and CD68

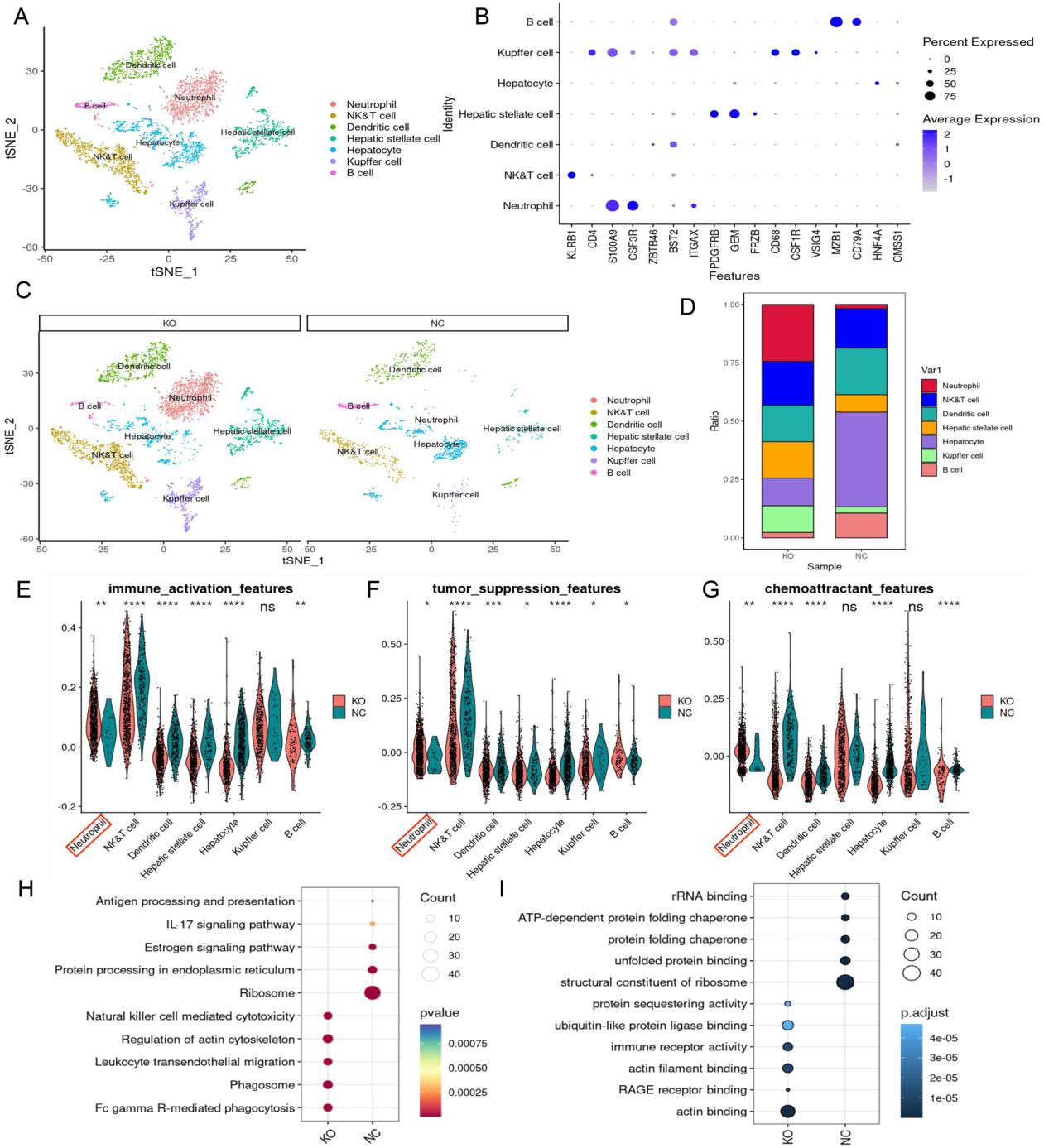

Fig. 5 Single cell sequencing analysis revealed the effect of MCT4 knockout on tumor microenvironment. **A** Unsupervised cluster TSNE of single cell sequencing results of liver and tumor in mice with HGC-27 cells modeled after MCT4 knockout. **B** Bubble map of marker genes for identification of cell types. **C** Unsupervised clustering TSNE plots of KO group and NC group. **D** Histogram of cell composition in KO group and NC group. **E**–**G** Statistical violin diagram of differences in immune activation, tumor inhibition and chemokine-related levels of different cell subsets. **H**, **I** Differential function of neutrophil subsets in different subgroups KEGG and GO enrichment analysis Bubble map

to label neutrophils and macrophages, we found that high expression of MCT4 significantly inhibited LY6G expression, while having no effect on the expression and enrichment of the macrophage marker CD68. In addition, we used CD86 and CD206 to label typical M1 and M2 macrophage markers, respectively, and associated

them with MCT4 expression. We found that the expression of MCT4 was significantly negatively correlated with the expression of CD206 in gastric cancer tissues, but not with the expression of CD86. This effect was not evident in adjacent tissues (Fig. 7C). Taken together, these results suggest that MCT4 expression affects the immunoinvasive state of tumors, especially the infiltration of T cells and neutrophils, but also the polarization of macrophages.

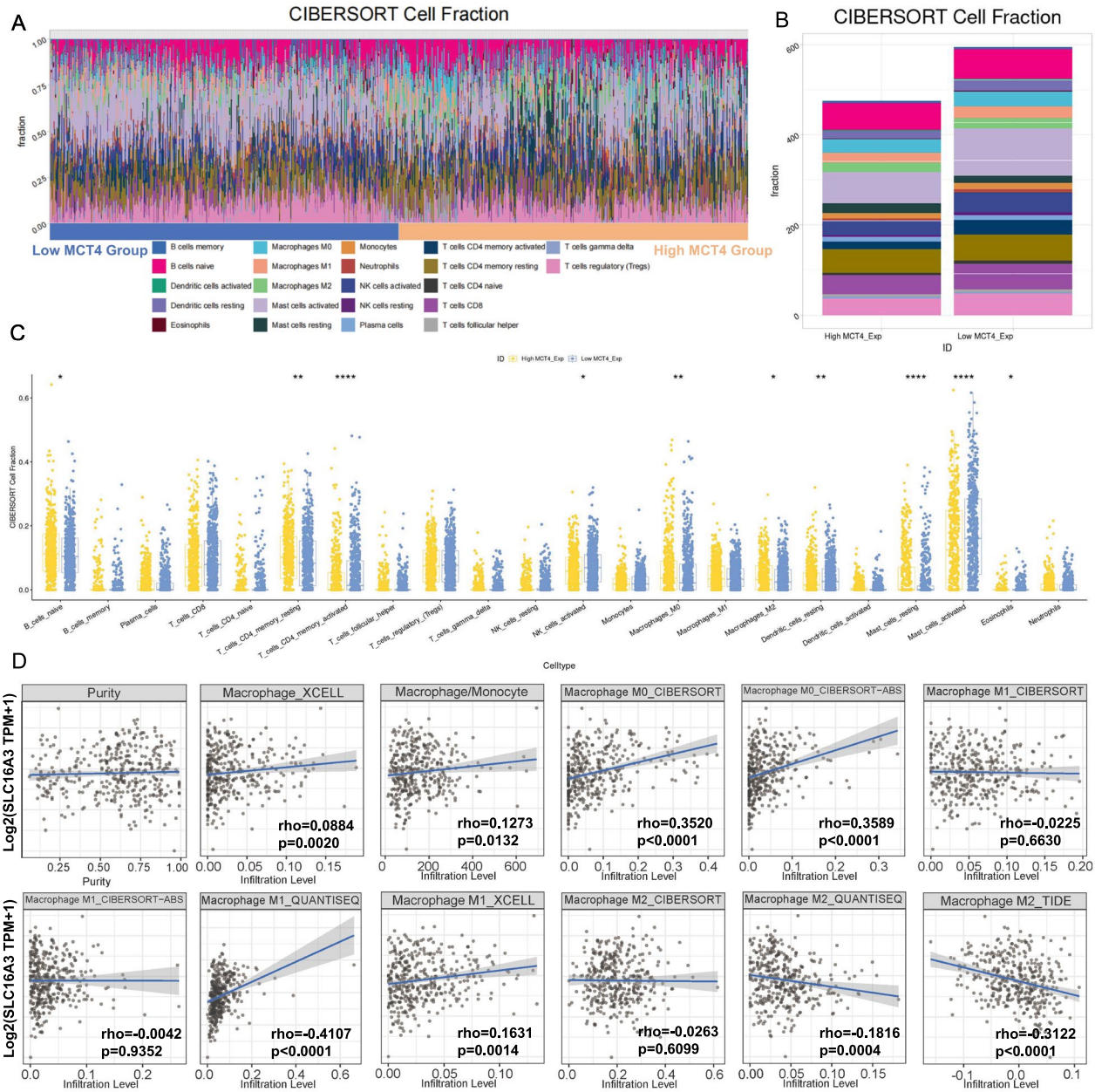

Fig. 6 Gastric cancer transcriptome sequencing data were analyzed for immunoinfiltration of different MCT4 expression states. **A** Accumulation histogram of immune infiltration in gastric cancer patients with different MCT4 expression status. **B** Histogram of the sum of the proportion of putative cells in immunoinfiltration in gastric cancer patients with different MCT4 expression states. **C** Statistical analysis of the proportion of putative cells in immunoinfiltration in gastric cancer patients with different MCT4 expression status. **D** Correlation analysis diagram of MCT4 expression status and the proportion of multiple cells. Cibersort, Xcell and other algorithms were used to evaluate the cell proportion and tumor purity in the transcriptome sequencing results of gastric cancer patients. The results showed that the expression of MCT4 was significantly correlated with macrophage infiltration

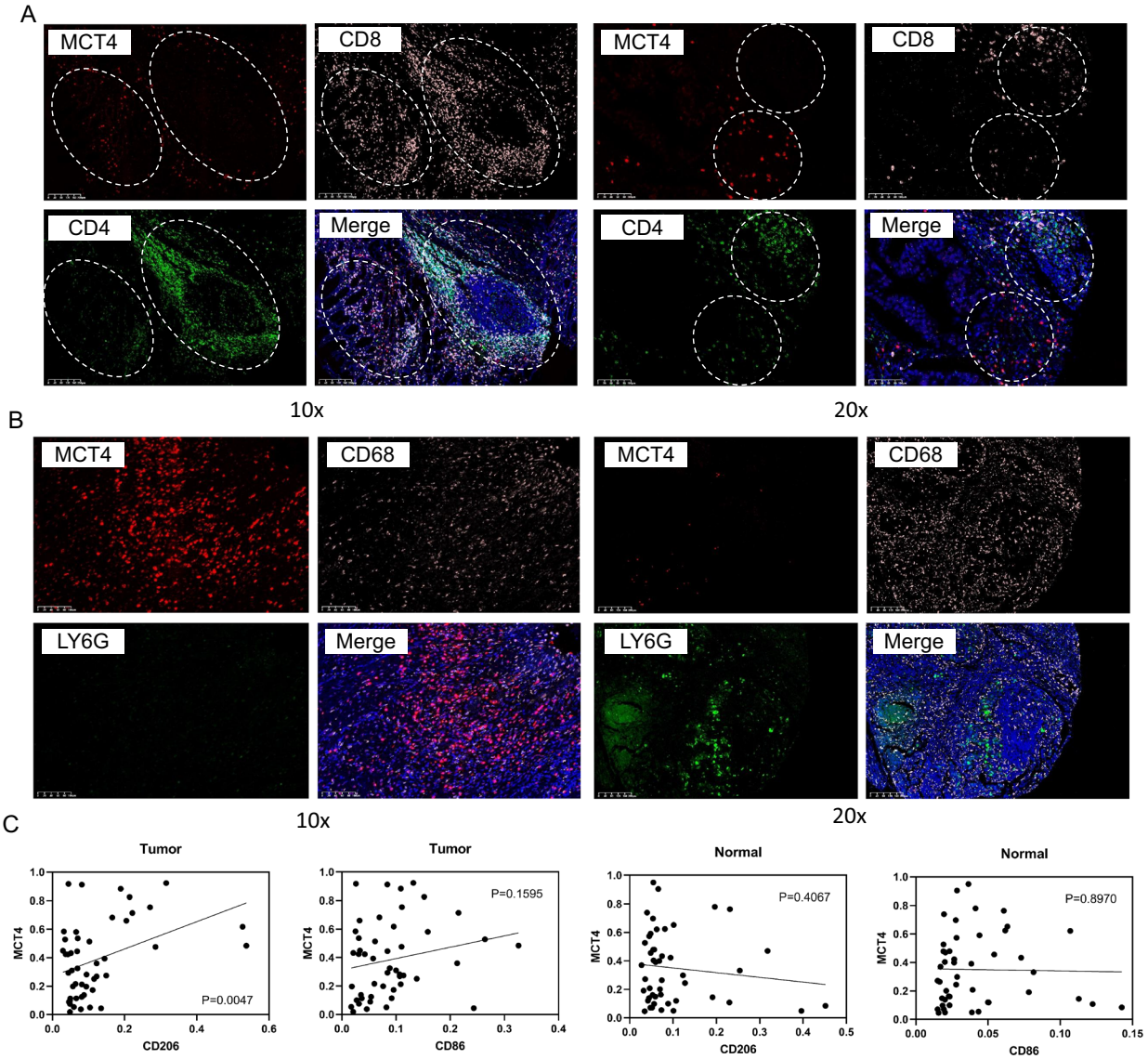

Fig. 7 Immunohistochemistry and multiple immunofluorescence demonstrated that MCT4 regulates the immune microenvironment. **A** Multiple immunofluorescence demonstrated the relationship between MCT4 expression and T cell infiltration. **B** Multiple immunofluorescence demonstrated the relationship between MCT4 expression and neutrophil infiltration. **C** Immunohistochemistry demonstrated the relationship between MCT4 expression and CD206 and CD86 in tumor patients and CD206 and CD86 in normal patients

Discussion

The MCT family is an important transport family responsible for the transport of various substances. Previous studies have focused significantly on MCT1, confirming its complex relationships with multiple tumors [16–18]. As a member of this family, MCT4 has been shown in studies to play a significant role in liver cancer [19–21], particularly in mediating metastasis. However, research on MCT4 in gastric cancer is relatively limited. Some innovative studies have revealed potential links between MCT4 and gastric cancer [22]. However, there has been little exploration of issues related to gastric cancer metastasis. In this study, we assessed MCT4 levels at both gene and protein levels using various databases and detection methods. In addition to discovering its high expression in gastric cancer, we also observed a potential link between MCT4 and gastric cancer metastasis. We validated this result through in vitro and in vivo experiments, demonstrating a strong correlation between MCT4 and gastric cancer metastasis.

It is widely recognized that gastric cancer metastasis is influenced by multiple factors, including various

signaling pathways, biological processes, metabolic changes, and the regulation of signaling molecules. In this study, we observed a correlation between high expression of MCT4 and various biological phenomena, particularly TGF-β, EMT, and angiogenesis. TGF-β, as a classic inducer of the Wnt signaling pathway [23–25], often leads to changes such as EMT and angiogenesis upon activation [26]. Currently, pathways such as the TGF-β/SMAD pathway are particularly focused on due to their significant role in gastric cancer metastasis. Small molecular inhibitors targeting TGF-β have been designed to evaluate clinical efficacy [27]. Our study suggests that there may be a correlation between TGF-β and MCT4 [28]; however, the specific activation mechanisms remain unclear. To date, the number of identified activation pathways for TGF-β is limited, including pH and reactive oxygen species (ROS) [29, 30]. Given that MCT4 is a key member of the lactate transporter family, investigating whether lactate can promote the activation of TGF-β and subsequently mediate its downstream functions has become a significant focus of this study.

In addition to changes within the tumor cells themselves, the immune microenvironment is also a critical factor contributing to gastric cancer metastasis [31, 32]. The malignant tumor cells, when disseminated through the bloodstream or peritoneal cavity, can regrow in suitable "soil" thereby promoting tumor metastasis [33, 34]. In our study, we found that MCT4 not only contributes to a decrease in the migratory capabilities of gastric cancer cells themselves, but also exhibits a significant regulatory effect on the overall microenvironment following its knockout. This includes key immune modulators such as T cells, macrophages, and B cells, which have been the focus of extensive research [19, 35]. These immune cells appear to be influenced by the knockout of MCT4, exhibiting changes in their enrichment and activation. During the enrichment of these immune cells, metabolic byproducts have consistently acted as critical regulators. Numerous studies have demonstrated that lactate plays an important role either as an energy regulator or as a key player in lactylation modifications [10]. Given the significant roles of MCT4 and MCT1 in the dynamic regulation of lactate, we have reason to suspect that changes in MCT4 may lead to alterations in overall lactate or metabolic byproducts, thereby influencing the activation and differentiation of various immune cells.

In summary, we have confirmed the special role of MCT4 in the process of gastric cancer metastasis through approaches such as single—cell transcriptome sequencing, clinical sample staining, and animal experiments. The discovery of the crucial role of MCT4 in gastric cancer metastasis holds great promise for clinical translation. If MCT4 is indeed a key player in promoting gastric cancer metastasis, it can serve as a novel biomarker for predicting the metastatic potential of gastric cancer. Clinicians may measure the expression level of MCT4 in tumor biopsies of newly diagnosed gastric cancer patients. High MCT4 expression may indicate a higher risk of metastasis, thus enabling more aggressive treatment strategies from the outset. For instance, patients with high MCT4 expression could be considered for adjuvant therapies targeting MCT4 or related pathways immediately after surgery, instead of waiting for signs of metastasis to emerge. In terms of therapeutic development, identifying MCT4 as a potential target provides new directions for drug design. Moreover, MCT4 may be a key pathway affected by many natural compounds [36–39]. Therefore, it becomes of great significance to detect the changes in MCT4 and its downstream expression pathways after drug administration. This can help us better understand the therapeutic mechanisms of these compounds and optimize treatment regimens to improve the prognosis of gastric cancer patients.

In summary, our findings indicate that MCT4 plays a significant biological role in gastric cancer, influencing the metastatic potential beyond its impact on primary tumors. Mechanistically, MCT4 affects the migratory capability of tumor cells while also modulating the tumor microenvironment [40]. These results underscore the significant potential of MCT4 as an intervention target in gastric cancer. However, it's important to acknowledge the limitations of this study. Currently, one of the major limitations is the lack of direct evidence regarding the role of MCT4 in the lactate—TGF-β signaling pathway. Although we've observed a correlation between MCT4 expression and various biological phenomena related to TGF-β, such as EMT and angiogenesis, we have yet to directly demonstrate how MCT4—mediated lactate transport impacts TGF-β activation. This is a significant gap in our understanding, as the exact mechanism could provide crucial insights into developing more targeted therapies. Another significant limitation lies in the survival analysis. In our survival analysis, we identified an association between MCT4 expression and patient survival outcomes. However, we recognize that there are likely numerous confounding factors that could have influenced these results. Firstly, patient—specific factors such as age, overall health status, and comorbidities were not fully accounted for in our analysis. Older patients or those with multiple comorbidities may have a poorer prognosis regardless of MCT4 expression levels. For example, patients with pre—existing cardiovascular diseases or diabetes may have a weakened immune system and reduced tolerance to cancer treatments, which could independently affect survival. Secondly, treatment—related factors are potential confounders. The type,

intensity, and duration of treatment received by patients can vary widely. Some patients may have received more aggressive chemotherapy regimens, while others may have only received palliative care. These differences in treatment can have a profound impact on survival, and it is possible that they could have masked or distorted the true relationship between MCT4 expression and patient outcomes. Moreover, how MCT4 affects a variety of immune cells, resulting in changes in their enrichment and activation, is a subject that merits further investigation, and a significant amount of work is needed to clarify the underlying mechanisms. In conclusion, while our study has made significant progress in understanding the role of MCT4 in gastric cancer, addressing these limitations, especially those related to survival analysis, is crucial for a more accurate interpretation of the relationship between MCT4 and patient outcomes and for the development of more effective treatment strategies.

Conclusion

MCT4 is of great significance for gastric cancer, which can mediate the metastasis of gastric cancer, reshape the microenvironment of gastric cancer, and change the composition of the immune microenvironment of gastric cancer.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06279-8.

Supplementary material 1.

Author contributions

Conceptualization: T.J., J.Z.; methodology: S.Z., M.Z., Y.W.; formal analysis: L.X., W.F.; investigation: Y.L., T.J.; data curation: S.Z., Yuanlin.L., Y.W., Shuo.Z.; visualization: J.Z., S.Z., Yuanlin.L., G.Z., T.J.; writing—original draft preparation: T.J., J.Z., Shuo.Z.; writing—review and editing: All authors read and approved the final manuscript. T.J., J.Z., and S.Z.; contributed equally to this work.

Funding

This work was supported by Zhejiang Provincial Natural Science Foundation of China (No. LQ23H270013); Key Support Project of Regional Innovation and Development Joint Fund of National Natural Science Foundation of China (No. U23A20499); National Natural Science Foundation of China (No. 82204950); Key project of the National Nature Foundation of China (No. 82030119) and Zhejiang Chinese Medicine University Chunyan Special Fund for the development of traditional Chinese medicine(No. CY202302).

Availability of data and materials

All data has been recorded in the method of acquisition.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Received: 9 October 2024 Accepted: 20 February 2025
Published online: 05 March 2025

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