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Ergotamine modulates CTSK-associated glutamine metabolism and M2 macrophage polarization in hepatocellular carcinoma

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Abstract

Introduction: *Tumor-associated macrophages (TAMs) are a key component of the tumor microenvironment (TME) in hepatocellular carcinoma (HCC). Cathepsin K (CTSK) has been implicated in promoting TAM polarization toward an anti-inflammatory phenotype and tumor progression. However, its role and mechanism in HCC-associated TAMs remain unclear.*

Material and methods: *The expression of CTSK in HCC was assessed using bioinformatics, western blot, and qRT-PCR. HCC cells with CTSK knockdown were generated to investigate the effects of CTSK on the M2 polarization of TAMs, using assays including CCK-8, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA). A glutamine metabolism assay kit was used to investigate the effect of CTSK on glutamine metabolism. Rescue experiments were conducted to elucidate the role of glutamine metabolism in macrophage M2 polarization. The interaction between the small molecule drug ergotamine and CTSK was evaluated using molecular docking and Cellular Thermal Shift Assay (CETSA). The influence of ergotamine on macrophage M2 polarization was further clarified through gene overexpression and pharmacological studies.*

Results: *CTSK expression was substantially upregulated in HCC tissues and cells and was associated with increased M2 macrophage polarization. CTSK overexpression enhanced glutamine metabolism-related parameters, while inhibition of glutamine metabolism attenuated CTSK-mediated effects on macrophage polarization. Ergotamine interacted with CTSK and reduced M2 macrophage polarization in HCC.*

Conclusions: *This study suggests that CTSK contributes to M2 macrophage polarization in HCC, potentially via modulation of glutamine metabolism. Ergotamine, a small-molecule compound, may modulate CTSK-associated effects and warrants further investigation as a potential regulator of the HCC tumor microenvironment. These findings provide insights into the role of CTSK in HCC-associated macrophage polarization.*

Key words: *CTSK, ergotamine, glutamine metabolism, macrophage M2 polarization.*

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Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in men globally [1, 2]. Immunotherapy has greatly changed the clinical management of HCC, especially with the introduction of immune checkpoint inhibitors. These therapies have shown remarkable potential in enhancing patient survival and quality of life. However, a significant portion of patients do not benefit from these treatments due to drug resistance and high recurrence rates [3-5], which highlights the need for research into the pathological mechanisms of HCC and the development of new therapeutic strategies to improve patient outcomes.

The tumor microenvironment (TME) has been increasingly recognized as a key cause of the loss of immune function in HCC [6]. The TME is a complex ecosystem that includes endothelial cells, stromal cells, immune cells,

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and a range of cytokines; the interactions among these components can alter the microenvironment of HCC cells, promoting cancer cell proliferation and curbing the effectiveness of anti-tumor therapies [7, 8]. Tumor-associated macrophages (TAMs) are a dominant immune cell type within the TME that display great functional plasticity, as they polarize into two phenotypes that shape the course of HCC [9]. M1 macrophages typically unleash a cascade of pro-inflammatory cytokines, including interleukin (IL)-12, that amplify T cell responses and suppress tumor growth; on the other hand, M2 macrophages secrete cytokines such as IL-10, which dampen immune reactions and foster tumor progression through enhanced migration and invasion [10]. The plasticity of macrophages has been a subject of in-depth research. In the advanced stages of cancer, there is often a phenotypic switch from M1 to M2 macrophages, which is clinically relevant because a high M2/M1 ratio or M2 polarization is associated with poor cancer prognosis [11]. Therefore, uncovering the mechanisms of TAM polarization may offer new avenues for HCC treatment.

In recent years, a large body of work has established that glutamine metabolism plays a pivotal role in cancer cells. Tumor cells import glutamine via dedicated transporters; once inside the cell, it is deaminated by glutaminase to glutamate, which is then converted to α-ketoglutarate (α-KG) and fed into the tricarboxylic acid (TCA) cycle, thereby promoting initiation, progression, and dissemination of malignancies [12-14]. On the basis of these findings, targeting glutamine metabolism appears to be a promising therapeutic strategy. Moreover, with the widespread use of immune-checkpoint inhibitors, the impact of glutamine metabolism within the TME has attracted intense interest. For example, in ovarian and liver cancers, pharmacologic blockade of glutamine metabolism prevents TAMs from polarizing toward the immunosuppressive M2 phenotype [15, 16]. However, in HCC, the influence of glutamine metabolism on TAM polarization, along with the availability of effective intervention targets, remains unclear.

Cathepsin K (CTSK) is a highly efficient lysosomal cysteine protease from the papain superfamily. It is the most active mammalian collagenase in its class. CTSK is a key regulator of bone resorption and tissue remodeling. It modulates the turnover of the extracellular matrix (ECM), facilitating collagen degradation and ECM reorganization, functions that are essential for maintaining skeletal health and tissue homeostasis [17]. Beyond its activity in bone biology, CTSK is a contributing factor in cancer progression. Its overexpression can cause the ubiquitination and degradation of SIAH1, ultimately driving the proliferation of HCC cells [18]. Furthermore, the rise of CTSK levels has been shown to boost epithelial-mesenchymal transition, remarkably propelling the growth and metastasis of castration-resistant prostate cancer cells [19]. CTSK can also modulate the function of TAMs, particularly the M2 variety. For instance, CTSK can drive M2 polarization of TAMs *via* an mTOR-dependent pathway, prompting these cells to secrete IL-10 and IL-17. These cytokines, in turn, activate the NF-κB signaling axis and thereby accelerate invasion and metastasis of colorectal cancer cells [20]. However, whether CTSK is involved in regulating M2 polarization of HCC macrophages and whether its regulatory effect is related to glutamine metabolism remain to be elucidated.

In this study, focusing on HCC, we employed bioinformatics tools and *in vitro* experiments to show that CTSK is upregulated in HCC. Further *in vitro* experiments revealed that CTSK promotes the M2 polarization of macrophages in HCC. More importantly, whereas previous studies have centered on the effects of traditional factors such as cytokines and signaling pathways on macrophage polarization, our work uncovered a distinctive mechanism whereby CTSK drives the transition of M0 macrophages into the M2 phenotype by regulating glutamine metabolism. These findings underscore the important role of metabolic pathways in the polarization of immune cells. Additionally, molecular docking and CETSA experiments indicated that the small molecule drug ergotamine interacts with CTSK. Treatment with ergotamine inhibits glutamine metabolism and attenuates the M2 polarization of HCC macrophages. This research not only clarifies how CTSK regulates M2 macrophage polarization *via* glutamine metabolism but also provides a basis for future research on the potential therapeutic application of ergotamine in HCC.

Material and methods

Bioinformatics

We analyzed mRNA expression data from the TCGA database for HCC, covering 50 non-tumor adjacent tissues and 374 tumor tissues. Based on previous literature, we identified CTSK as a candidate gene of interest. We then used TIMER2.0 to analyze the correlation between CTSK expression and M2 macrophage infiltration, finding a positive correlation. Next, we performed gene set enrichment analysis (GSEA) to identify enriched pathways associated with CTSK. Using an R package, we further explored the correlation between CTSK and key genes in these pathways. Finally, we conducted batch molecular docking for the CTSK protein and identified ergotamine as a potential drug based on its binding affinity.

Cell culture

Human normal liver epithelial cells (THLE-2), human HCC cells (Hep-3B and HuH-7), and human monocytic leukemia cells (THP-1) were sourced from ATCC (USA). The HuH-7 HCC cell line was obtained from the Chinese Academy of Sciences Cell Bank, and the HCCLM3 HCC cell line was purchased from Shanghai Yaji Biotechnology Co., Ltd. (China). For cell culture, THLE-2 cells were

maintained in their specific medium (Pricella, CM-0833, China) supplemented with 10% fetal bovine serum (FBS, Bovogen, SFBS, USA). THP-1 cells were cultured in RPMI-1640 medium (Oumarsi, OMD81717, China) with 10% FBS. The remaining cells were cultured in DMEM medium (Oumarsi, OMDCM-014, China) containing 10% FBS. All cells were incubated at 37°C with 5% CO_2 and passaged every 2 days to maintain healthy growth.

Transfection with siRNA and plasmid vectors

The si-NC, si-CTSK, pcDNA3.1 empty vector (oe-NC), and pcDNA3.1-CTSK expression plasmid (oe-CTSK) were all sourced from HANBIO (China). Lipofectamine 3000 reagent (Invitrogen, L3000015, USA) was diluted in Opti-MEM medium (Gibco, 31985062, USA) and mixed thoroughly. The siRNA or overexpression plasmid was then diluted in Opti-MEM medium to create a pre-mix. After combining the two solutions and incubating at room temperature for 15 minutes, the mixture was added to the cells and incubated at 37°C for 24 hours. Cells were subsequently treated with either 1 mM GPNA hydrochloride (abbreviation: GPNA, Selleck, S6670, USA) or 100 μM ergotamine tartrate (abbreviation: ErgT; USP, 1241506, USA) for 24 hours, as required by the experimental design [21, 22].

qRT-PCR

We extracted total RNA from cells using TRIzol Reagent (Invitrogen, 15596018CN, USA). RNA concentration was measured using a microvolume spectrophotometer (Hengmei, China). The RNA was reverse-transcribed into cDNA using the SynScript III RT SuperMix for qPCR kit (Tsingke, TSK314S, China). qRT-PCR was performed using the ArtiCanATM SYBR qPCR Mix (Tsingke, SQ0101, China). Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference. Primer sequences used were (5′-3′): CTSK-F: ACACCCACTGGGAGCTATG, CTSK-R: GACAGGGGTACTTTGAGTCCA, GAPDH-F: GGAGCGAGATCCCTCCAAAAT, and GAPDH-R: GGCTGTTGTCATACTTCTCATGG.

Western blot (WB)

Proteins were extracted from cells using RIPA lysis buffer (Solarbio, R0010, China) and quantified to 2 μg/μl with the BCA protein assay kit (Beyotime, P0009, China). The samples were denatured at 98°C for 10 minutes. Proteins were separated by 8-10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). After blocking with 5% skim milk for 1 hour at room temperature, the membranes were incubated with primary antibodies against CTSK (Abcam, ab187647, UK) or GAPDH (Abcam, ab181602, UK) overnight at 4°C. Following incubation with HRP-conjugated secondary antibody (Abcam, ab6721, UK) for 1 hour at room temperature, the membranes were washed three times with TBST (Solarbio, T1082, China) and protein signals were detected using a highly sensitive ECL chemiluminescent kit (Beyotime, P0018S, China).

Cell Counting Kit-8 (CCK-8)

We evaluated cell viability using the CCK-8 assay kit (Elabscience, E-CK-A362, China). Cells in the logarithmic growth phase were seeded into 96-well plates at a density of 2000 cells per well. After a 24-hour incubation period, 10 μl of CCK-8 reagent was added to each well and the plates were incubated for 2 hours. Absorbance at 450 nm was measured using a microplate reader (Scientific, USA) to determine cell viability.

Cellular thermal shift assay (CETSA)

To treat the cells, ErgT was applied for 24 hours. The cells were then washed three times with PBS and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in PBS to form a cell suspension. This suspension was divided into eight EP tubes and heated in a metal bath at temperatures ranging from 45°C to 80°C for 3 minutes. After rapid freezing in liquid nitrogen twice, the lysates were centrifuged at 12,000 rpm for 15 minutes to separate the soluble fraction from the precipitate. The supernatant was collected for WB analysis.

Measurement of glutamine metabolic activity

Transfected cells were seeded into 6-well plates at 1×10^6 cells per well and incubated for 24 hours. The cells were subsequently treated with GPNA or ErgT for 24 hours. After treatment, they were washed three times with PBS and collected into EP tubes. Magnetic beads were added to the cells, which were then lysed using a grinder. The lysates were centrifuged at 12,000 rpm for 15 minutes, and the supernatant was collected for further analysis. We assessed the levels of glutamine uptake, glutamate content, and α-KG in the cells using the Glutamine Content Assay Kit (Elabscience, E-BC-K853-M, China), Glutamate Content Assay Kit (Elabscience, E-BC-K903-M, China), and α-KG Content Assay Kit (Abcam, ab83431, UK).

Determination of the NADP/NADPH ratio

After treating the cells as described above, we collected the cell supernatant by centrifugation and transferred it to a new EP tube. Using the NADP+/NADPH Assay Kit (Elabscience, E-BC-K803-M, China), we measured the levels of NADP+ and NADPH.

Co-culture of HCC cells with M0 macrophages

After THP-1 cells were resuspended, the density was adjusted to 1×10^6 cells/ml. PMA (MCE, HY-18739, USA)

was then added to a final concentration of 100 ng/ml. The treated cells were placed in the incubator and monitored for morphological changes. Differentiation into M0 macrophages was typically achieved within approximately 24 h. The M0 macrophages were then cultured in RPMI-1640 medium for 24 hours to stabilize. For the co-culture, HCC cells were treated with GPNA or ErgT for 24 hours post-transfection. 1×10^6 HCC cells were placed in the upper chamber of a Transwell, and 1×10^6 M0 macrophages were placed in the lower chamber. The co-culture was maintained for 24 hours, and the cells were collected for analysis.

Immunofluorescence

After the co-culture, the cells were washed three times with PBS. M0 macrophages were fixed with 4% paraformaldehyde (Servicebio, G1101, China) to preserve their structure. The cells were permeabilized with 0.2% Triton X-100 (Beyotime, P0096, China) for 10 minutes at room temperature. The cells were blocked with 5% BSA (Elabscience, E-IR-R107, China) for 1 hour. The coverslips were incubated with anti-CD206 (Abcam, ab64693, UK), anti-CD68 (sc-20060, Santa Cruz Biotechnology, USA), and anti-CD80 (Abcam, ab225674, UK) overnight at 4°C. After washing with PBS, the coverslips were incubated with goat anti-rabbit IgG-Alexa Fluor 488 (Abcam, ab150077, UK) or Goat Anti-Mouse IgG H&L (Alexa Fluor 647) (Abcam, ab150115, UK) for 1 hour at room temperature in the dark. The coverslips were washed with PBS, stained with DAPI (Biosharp, BL2345A, China) to label nuclei, and imaged using the EVOS M5000 imaging system (Invitrogen, USA).

Enzyme-linked immunosorbent assay (ELISA)

Co-cultured cells were washed with PBS three times. The M0 macrophages were collected, lysed with RIPA buffer, and centrifuged to collect the supernatant. The supernatant was then analyzed for IL-10 (E-EL-H6154), TGF-β1 (E-EL-0162), and IFN-γ (E-EL-H0108) levels using ELISA kits from Elabscience, China.

Data interpretation and analysis

We used GraphPad Prism 8 for all statistical analyses. Data are presented as mean ±SEM. Student's *t*-test was used to determine significance, with $p < 0.05$ as the cutoff, unless otherwise stated.

Results

High expression of CTSK in HCC tissues and cells

We identified CTSK as a candidate gene of interest in HCC by analyzing mRNA expression data from the TCGA database and reviewing relevant literature. A *t*-test revealed that CTSK was markedly upregulated in HCC tissues (Fig. 1A). WB and qRT-PCR showed that CTSK protein and mRNA levels were elevated in three HCC cell lines (HCCLM3, Hep-3B, and HuH-7) compared to normal liver epithelial cells (THLE-2) (Fig. 1B, C). Given the high expression of CTSK in HuH-7 cells and its lower expression in HCCLM3 cells, we decided to use HuH-7 cells to knock down CTSK and HCCLM3 cells to overexpress CTSK in our later experiments.

Knocking down CTSK inhibits M2 macrophage polarization in HCC

As shown by previous studies, CTSK promotes M2 macrophage polarization and tumor progression [23]. We used TIMER2.0 to evaluate the correlation between CTSK and M2 macrophages in HCC and found a positive correlation (Fig. 2A). This finding suggests that CTSK may be involved in regulating M2 polarization in HCC. Then we constructed a CTSK knockdown model in HuH-7 cells and verified it using qRT-PCR and WB (Fig. 2B, C). The CCK-8 assay showed that CTSK knockdown reduced cell viability (Fig. 2D). To investigate the effect of CTSK on macrophage polarization, we co-cultured the cells with M0 macrophages. Immunofluorescence showed that CTSK knockdown significantly reduced CD206 expression compared to the control group and increased the expression of the M1 macrophage marker CD80 (Fig. 2E, Supplementary Fig. 1). We then used ELISA to quantify the anti-inflammatory cytokines IL-10 and TGF-β1, as well as the pro-inflammatory cytokine IFN-γ, in the co-culture supernatants. Knockdown of CTSK markedly reduced IL-10 and TGF-β1 levels, whereas IFN-γ was significantly upregulated (Fig. 2F-H). Collectively, these findings indicate that CTSK knockdown inhibits M2 macrophage polarization in the HCC co-culture model.

CTSK promotes M2 macrophage polarization *via* modulation of glutamine metabolism

To uncover the pathways through which CTSK mediates M2 macrophage polarization in HCC, we performed bioinformatics analysis. The results indicated that CTSK was associated with the glutamine metabolism pathway (Fig. 3A). Additionally, CTSK was found to be positively correlated with several key genes in glutamine metabolism, including GFPT2, SLC38A1, GLS, and ASNS (Fig. 3B). To further investigate the role of CTSK in glutamine metabolism, we constructed a CTSK overexpression model and applied the glutamine metabolism inhibitor GPNA. qRT-PCR and WB analysis revealed that CTSK overexpression led to an increase in CTSK levels, whereas GPNA treatment did not affect CTSK expression (Fig. 3C, D). Next, we quantified intracellular glutamine metabolism and observed that CTSK overexpression increased

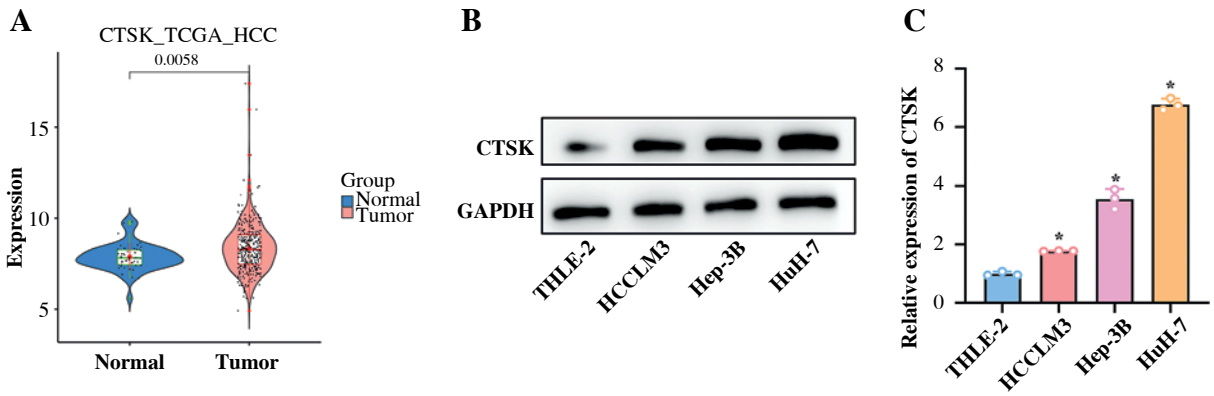
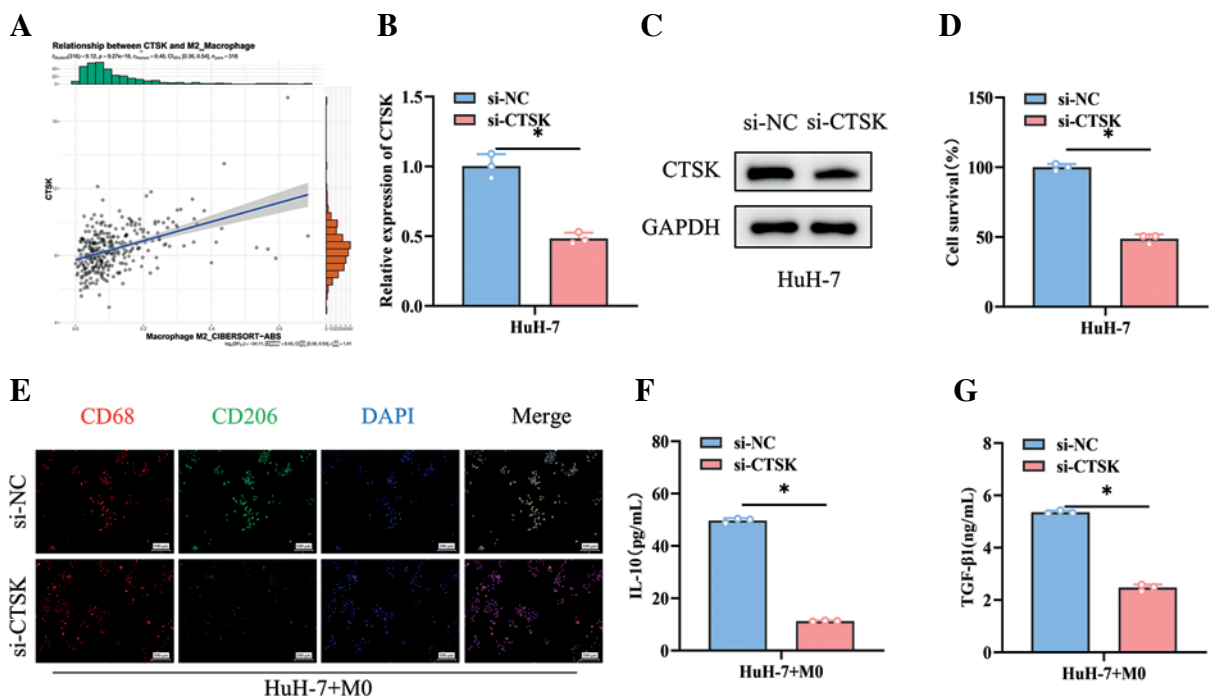


Fig. 1. High expression of cathepsin K (CTSK) in hepatocellular carcinoma (HCC) tissues and cells. **A**) CTSK expression in HCC tumor tissues and adjacent non-tumor tissues was analyzed using the TCGA database; black dots represent the CTSK gene expression values in individual samples; red dots represent the median of each group; green dots represent statistical outliers. **B, C**) Western blot and qRT-PCR were used to measure CTSK protein and mRNA levels in human normal liver epithelial cells (THLE-2) and three HCC cell lines (HCCLM3, Hep-3B, HuH-7). *$p < 0.05$, $n = 3$



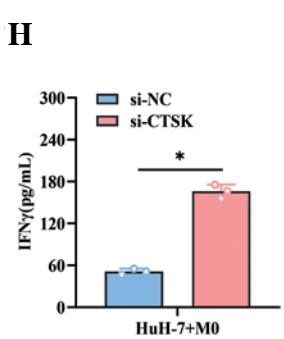


Fig. 2. Knocking down cathepsin K (CTSK) inhibits M2 macrophage polarization in hepatocellular carcinoma (HCC). **A**) TIMER2.0 was used to analyze the correlation between CTSK and M2 macrophage immune infiltration. **B, C**) CTSK mRNA expression and protein levels were detected in si-NC and si-CTSK groups *via* qRT-PCR and Western blot. **D**) The cell viability of HuH-7 cells in si-NC and si-CTSK groups was assessed using CCK-8. **E**) The expression of CD68 and CD206 in the co-culture system was detected by immunofluorescence. **F-H**) The levels of IL-10, TGF-β1, and IFN-γ in the co-culture system were measured by ELISA. *$p < 0.05$, $n = 3$

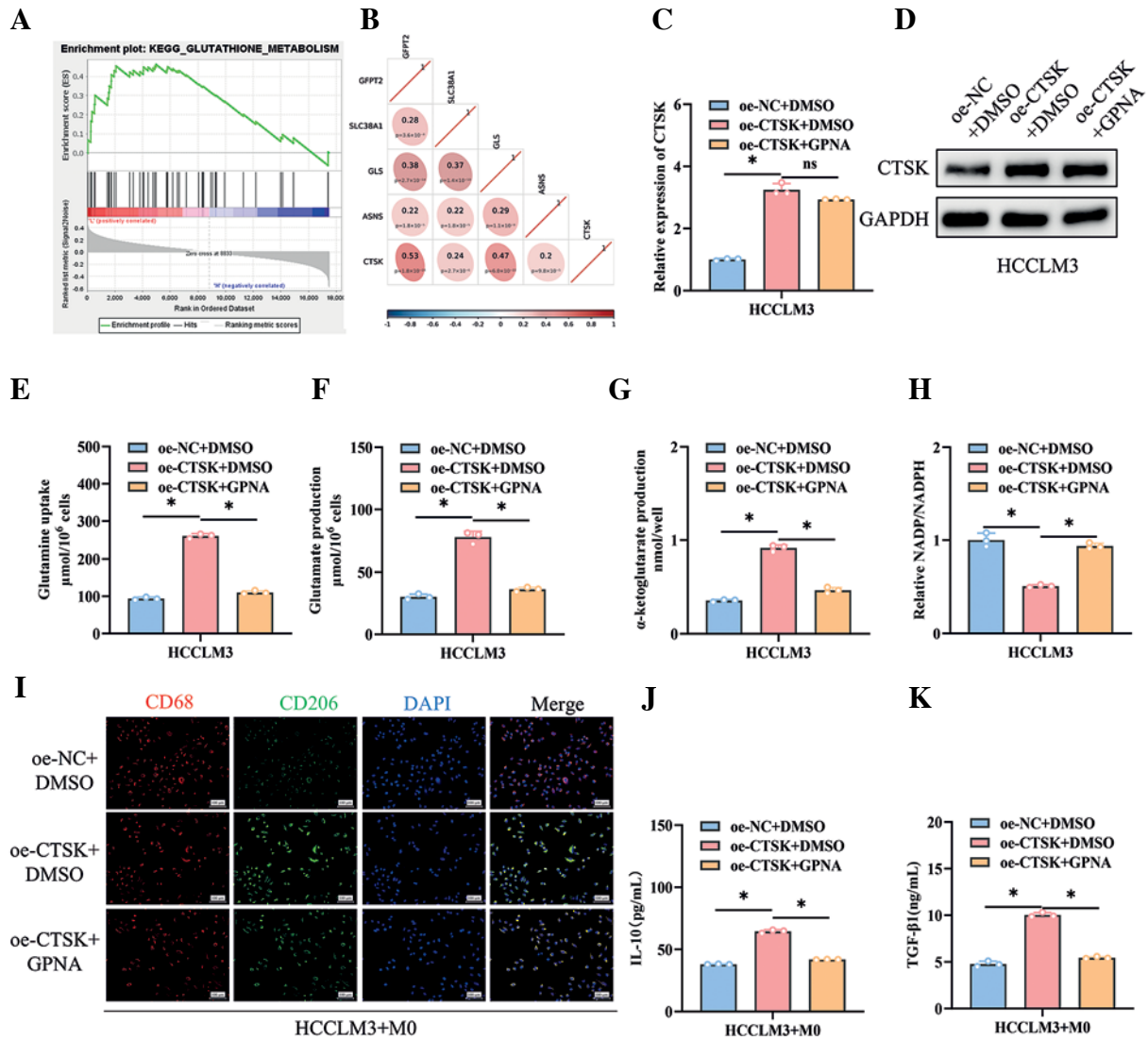


Fig. 3. Cathepsin K (CTSK) promotes M2 macrophage polarization *via* modulation of glutamine metabolism. **A**) GSEA plot showing enrichment of glutamine metabolism-related pathways associated with CTSK expression. **B**) Spearman correlation analysis between CTSK and key genes involved in glutamine metabolism. **C, D**) Detection of CTSK mRNA expression and protein levels by qRT-PCR and Western blot. **E-H**) Detection of glutamine uptake, glutamate content, α-KG content, and NADP+/NADPH ratio using corresponding kits. **I**) Detection of CD68 and CD206 expression in the co-culture system by immunofluorescence. **J, K**) Detection of IL-10 and TGF-β1 levels in the co-culture system by ELISA. $*p < 0.05$, $n = 3$

glutamine uptake, as well as glutamate and α-KG levels. Conversely, treatment with the glutamine metabolism inhibitor GPNA reversed these changes (Fig. 3E-G). During glutamine metabolism, glutamine is sequentially converted to α-KG, which then enters the TCA cycle; the generation and utilization of NADPH constitute important steps in this process [24]. We measured the NADP+/NADPH ratio. As depicted in Figure 3H, overexpression of CTSK reduced the NADP+/NADPH ratio, and treatment with the glutaminase inhibitor GPNA reversed this effect, bringing the NADP+/NADPH ratio back to levels close to the control group. These findings suggest that CTSK upregulation enhances glutamine metabolism. Further analysis revealed that CTSK overexpression promoted M2 macrophage polarization and upregulated IL-10 and TGF-β1 levels in the co-culture system, whereas inhibition of glutamine metabolism had the opposite effect (Fig. 3I-K). Overall, the data demonstrate that CTSK contributes to M2 macrophage polarization in HCC, at least partly through its effects on glutamine metabolism.

Ergotamine interacts with CTSK to regulate glutamine metabolism and inhibit M2 macrophage polarization in HCC

We conducted molecular docking on the CTSK protein to identify potential interacting compounds and found that ergotamine had the lowest predicted binding affinity score. We generated 2D (Fig. 4A) and 3D (Fig. 4B) diagrams to visualize the interactions, revealing that ergotamine formed a hydrogen bond with Asn47 and engaged in hydrophobic interactions with Ser83, Asp85, and Glu84. A CETSA experiment showed that ErgT treatment enhanced the thermal stability of CTSK (Fig. 4C), supporting an interaction between ergotamine and CTSK. It was further found that ErgT treatment attenuated the effects of CTSK overexpression on glutamine uptake, glutamate, and α-KG levels, and it also raised the NADP+/NADPH ratio (Fig. 4D-G). Moreover, ErgT treatment reduced CTSK-mediated effects on M2 macrophage polarization and IL-10 and TGF-β1 levels in the co-culture system (Fig. 4G-J). These findings indicate that ergotamine interacts with CTSK, modulates glutamine metabolism, and attenuates M2 macrophage polarization.

Discussion

Hepatocellular carcinoma is a complex cancer with an immunosuppressive microenvironment that limits ef-

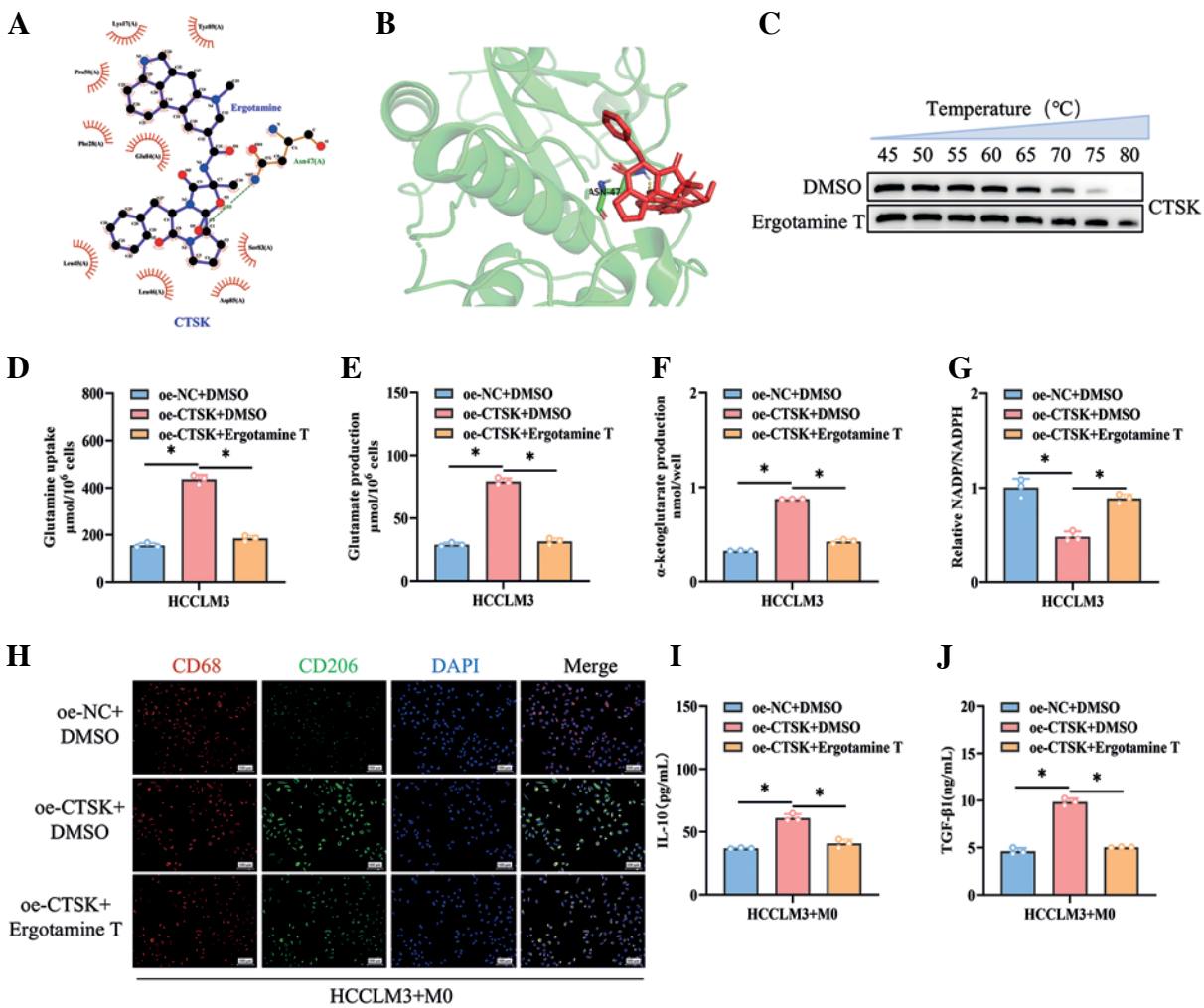


Fig. 4. Ergotamine interacts with cathepsin K (CTSK) to regulate glutamine metabolism and inhibit M2 macrophage polarization in hepatocellular carcinoma (HCC). **A, B**) 2D and 3D diagrams showing the predicted hydrogen bond and hydrophobic interactions between ergotamine and CTSK. **C**) CETSA was used to assess the interaction between ErgT and CTSK. **D-G**) Corresponding kits were used to detect glutamine uptake, glutamate content, α-KG content, and NADP+/NADPH ratio. **H**) CD68 and CD206 expression in the co-culture system was detected by immunofluorescence. **I, J**) IL-10 and TGF-β1 levels in the co-culture system were detected by ELISA. *$p < 0.05$, $n = 3$

fective treatment options and often leads to poor patient outcomes [25, 26]. TAMs are highly abundant in HCC and are central to its immune evasion. As research into the role of TAMs in cancer advances, they are increasingly recognized as a promising target for new therapeutic strategies [11, 26]. However, the mechanisms that regulate TAM polarization and infiltration in HCC remain incompletely understood. In this study, we elucidated the role of CTSK as an important regulator of macrophage M2 polarization. We found that ergotamine, through its interaction with CTSK, inhibits glutamine metabolism and attenuates M2 macrophage polarization in HCC.

Cathepsin K, a key member of the peptidase C1 family, is involved in bone resorption and the remodeling of the ECM under normal physiological conditions [27, 28]. Over the past few years, research has unveiled a strong connection between the abnormal expression of CTSK and the progression of various cancers. In breast and lung cancers, CTSK is typically upregulated and has been reported to promote tumor invasion and metastasis through ECM degradation and activation of the mTOR pathway [17]. Conversely, in cervical cancer, CTSK is usually expressed at low levels, but its activation has been reported to induce downstream p65 and NF-κB signaling and promote tumor growth [29]. These findings indicate that although the expression levels of CTSK vary in different cancers, dysregulation of CTSK may influence tumor progression in a context-dependent manner. In HCC, elevated CTSK expression has been associated with worse clinical outcomes [18]. Our study further supports this association, revealing that CTSK is upregulated in HCC tissues and cells, suggesting its potential involvement in HCC progression.

Extensive research has revealed that the involvement of CTSK extends beyond ECM degradation and remodeling to potentially include regulation of immune cell function, especially macrophages [20]. One notable example is a bioinformatics study that identified a positive correlation between CTSK overexpression and macrophage infiltration in pancreatic cancer [30]. Furthermore, cellular experiments have corroborated that CTSK can bolster the migratory and invasive properties of castration-resistant prostate cancer by promoting macrophage polarization towards the M2 phenotype [23]. However, these studies have primarily focused on the association between CTSK, TAM polarization, and tumor progression, while the molecular mechanisms underlying CTSK-mediated M2 polarization remain incompletely understood.

New evidence reveals that CTSK may promote TGF-β1-induced SMAD3 activation *via* Sorting nexin 9 (SNX9), thereby enhancing downstream glutaminase 1 (GLS1) expression and glutamine metabolism [31]. These findings highlight the potential role of CTSK in regulating glutamine metabolism. Similarly, in our study, bioinformatics analysis and *in vitro* experiments indicated an association between CTSK expression and the glutamine metabolism pathway. We also confirmed that CTSK overexpression increased indicators of glutamine metabolism, suggesting that CTSK may contribute to the regulation of glutamine metabolism in HCC. Glutamine metabolism and the polarization of TAMs are closely interconnected [32, 33], especially in liver cancer. Research has shown that modulating glutamine metabolism can promote the M2 polarization of macrophages, which can, in turn, accelerate tumor growth and progression [15]. In the current study, we examined the correlation between CTSK's regulation of M2 macrophage polarization and glutamine metabolism. The findings indicated that CTSK contributes to M2 macrophage polarization in HCC, potentially through modulation of glutamine metabolism. This discovery highlights the role of CTSK in regulating glutamine metabolism in HCC and supports a link between glutamine metabolism and M2 macrophage polarization.

Further to these findings, our research investigated potential small-molecule drugs that could modulate M2 macrophage polarization associated with CTSK in HCC. Through molecular docking techniques, we found that ergotamine showed a predicted interaction with CTSK, suggesting its potential as a candidate therapeutic compound. Ergotamine, typically associated with migraine relief, has a lesser-known but significant impact on cancer [34]. Research from 1980 onwards has demonstrated its ability to inhibit tumor growth [35]. More recently, it has been reported that ergotamine can affect tumor malignancy through mechanisms such as induction of anoikis [36]. Given its anti-tumor properties, we explored the potential of ergotamine in HCC. Our research suggests that ergotamine interacts with CTSK and attenuates CTSK-associated glutamine metabolism and M2 macrophage polarization in HCC. Compared with existing studies, we note that some have already demonstrated the roles of CTSK and its inhibitor balicatib in regulating HCC cell proliferation [18]. Our work extends these findings by not only supporting the involvement of CTSK in HCC but also suggesting a potential mechanism in which ergotamine modulates CTSK-associated glutamine metabolism and macrophage polarization in HCC.

Overall, our investigation suggests that ergotamine interacts with CTSK, reducing glutamine metabolism-related changes in HCC cells and attenuating M2 polarization of M0 macrophages. These results highlight the potential role of CTSK in regulating macrophage polarization in HCC and provide initial evidence supporting further investigation of ergotamine as a potential modulator of the HCC tumor microenvironment. Despite the encouraging results, our study has some limitations. First, we did not fully uncover the biological effects and underlying mechanisms of ergotamine-mediated CTSK regulation in the context of HCC progression. Second, due to the scarcity of clinical specimens and matched primary cells, we were unable

to validate the clinical relevance, therapeutic potential, or safety profile of ergotamine. Future studies should address these limitations to further evaluate the potential application of CTSK-targeted strategies in HCC treatment.

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Disclosures

Ethical approval is not required for this study, in accordance with local or national guidelines. Patient consent was not required, in accordance with local or national guidelines.

The authors declare no conflict of interest.

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