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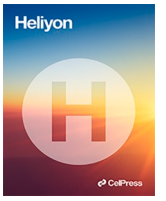

Research article

A novel biomarker GATM suppresses proliferation and malignancy of cholangiocarcinoma cells by modulating the JNK/c-Jun signalling pathways

Yi Yu [a,1], Wei Gan [b,1], Jianping Xiong [a,**], Junhe Li [a,*]

[a] Department of Oncology, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, Jiangxi, China
[b] Department of Thoracic Surgery, Sun Yat-sen University Cancer Centre, Guangzhou City, Guangdong Province, 510060, China

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ABSTRACT

Background: Cholangiocarcinoma (CCA) is the second most common primary malignancy of the liver and is associated with poor prognosis. Despite the emerging role of glycine amidinotransferase (GATM) in cancer development, its function in CCA remains elusive. This study investigated the biological significance and molecular mechanisms of GATM in CCA.

Method: GATM expression was measured using immunohistochemistry and western blotting. Cell proliferation, migration, and invasion were assessed through CCK-8, EdU, clone formation, wound healing, and Transwell assays. Rescue experiments were performed to determine whether the JNK/c-Jun pathway is involved in GATM-mediated CCA development. Immunoprecipitation and mass spectrometry were performed to screen for proteins that interact with GATM. The role of GATM *in vivo* was investigated according to the xenograft experiment.

Result: GATM expression was downregulated in CCA tissues and cells ($p < 0.05$) and had a significant suppressive effect on CCA cell proliferation, migration, and invasion *in vitro* as well as on tumour growth *in vivo* ($p < 0.05$); conversely, GATM knockdown promoted these phenotypes ($p < 0.05$). Notably, GATM inhibited the JNK/c-Jun pathway, and JNK activation abrogated GATM's antitumor effects ($p < 0.05$). Isocitrate dehydrogenase 1 (IDH1) interacts with GATM, and IDH1 knockdown significantly attenuated GATM protein degradation. Overexpression of IDH1 restored the biological function of CCA by reversing the inhibition of JNK/c-Jun pathway phosphorylation by GATM ($p < 0.05$).

Conclusion: GATM acts as a tumour suppressor in CCA by regulating the phosphorylation of the JNK/c-Jun pathway. IDH1 interacted with GATM to regulate CCA progression.

1. Introduction

Cholangiocarcinoma (CCA), the second most common primary hepatic malignancy, accounts for approximately 15 % of all primary liver cancers and 3 % of all gastrointestinal tumours [1]. Predominantly originating from biliary epithelial cells, CCA is categorized as intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA), and distant cholangiocarcinoma (dCCA) [2].

* Corresponding author.
** Corresponding author.
E-mail addresses: ndyfy01874@ncu.edu.cn (J. Xiong), ndyfy02160@ncu.edu.cn (J. Li).
[1] These authors contributed equally to this work and share first authorship.

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Globally, the incidence of CCA is increasing, with an average annual growth of 4 %. Morbidity has increased rapidly by 100 % over the past 10 years, making it one of the most aggressive tumours [3,4]. Surgical resection remains the primary curative treatment for patients with early-stage disease. However, despite complete resection, >50 % of patients experience relapse, leading to a low long-term survival rate. Moreover, the disease is often asymptomatic in its early stages, resulting in more than half of patients being diagnosed with advanced disease and missing opportunities for surgery [5–7]. Delayed clinical diagnosis of iCCA results in a poor prognosis, with a 5-year overall survival rate (OS) of 3 % and a median survival time of patients less than 12 months [8]. Owing to the scarcity of effective biomarkers and molecular-targeted therapies, the clinical outcomes of CCA are generally poor. Therefore, it is imperative to gain a detailed understanding of the molecular mechanisms of CCA to develop effective clinical strategies.

Glycine amidinotransferase (GATM), a mitochondrial enzyme, catalyses the transfer of a guanidino group from l-arginine to glycine, producing an intermediate precursor of creatine—guanidinoacetic acid [9–11]. GATM expression is most pronounced in the kidneys, liver, pancreas, and brain [12]. In these organs, GATM is essential for the synthesis of creatine, which is important for cellular energy production and metabolic processes. In the kidneys and liver, GATM supports creatine metabolic functions, while in the pancreas, it aids in metabolic regulation. In the brain, GATM is crucial for maintaining normal neurological function [13,14]. Recessive loss-of-function mutations in GATM can lead to a rare inborn disorder of creatine synthesis—cerebral creatine deficiency syndrome—characterised by severe neurological impairment developmental delays, intellectual disability, and motor dysfunction, including impaired coordination and muscle weakness [15,16]. Patients often exhibit early onset developmental delay, mild-to-moderate intellectual disability, and myopathy [17]. However, the relationship between GATM expression and specific human malignancies remains unclear. In colorectal cancer, high level of GATM promotes liver metastasis through creatine-mediated epithelial-mesenchymal transition (EMT) [18]. Moreover, GATM acts as a candidate tumour suppressor and has been implicated in the therapeutic efficacy of immune checkpoint inhibitors in metastatic renal cell carcinoma [19]. Nevertheless, no study has assessed the correlation between GATM and CCA, rendering the exact role and underlying mechanism of GATM in CCA progression unclear.

Isocitrate dehydrogenase 1 (IDH1) catalyses the production of α-ketoglutarate and NAPDH from isocitrate, which are involved in cellular energy metabolism and biosynthesis [20]. Mutated IDH1 produces 2-hydroxyglutaric acid which leads to epigenetic changes and promotes tumorigenesis [21,22]. IDH1 mutations are found in many tumours such as glioma, CCA, and acute myeloid leukaemia [23–25]. The wild-type IDH1 also plays an important role in tumour development, such as in lung and pancreatic cancers. It is involved in metabolic processes that support tumor growth and progression. Specifically, wild-type IDH1 contributes to the regulation of cellular metabolism and energy production, which are crucial for the sustenance and proliferation of tumor cells [26,27]. Overexpression of the wild-type IDH1 promotes non-small cell lung cancer (NSCLC) progression and gemcitabine resistance by activating serine metabolism in tumour stem cells. In pancreatic cancer, the upregulation of the wild-type IDH1 enhances energy metabolism and oxidative stress in cancer cells and promotes tumour growth. The wild-type IDH1 may promote the proliferation, migration, and invasion of iCCA cells by increasing the expression of acetaldehyde dehydrogenase 1 [28]. However, research on the internal mechanisms of action of IDH1 in cancer remains limited.

The c-Jun N-terminal kinase (JNK) is a mitogen-activated protein kinase (MAPK) that can promote inflammation, proliferation, invasion, and angiogenesis through the JNK/c-Jun signalling pathway. The extent of these effects depends on the specific circumstances and the duration of stimulation [29]. Recent studies have highlighted the critical role of the JNK signalling pathway of proliferation, differentiation, and carcinogenesis in the development of CCA [30,31]. Considering the potential significance of the JNK/MAPK signalling pathway in CCA treatment, further studies are warranted to explore this possibility.

This study aimed to clarify the roles and mechanisms of GATM in CCA and to assess its prognostic predictive value. Initial findings revealed that GATM was downregulated in CCA and is a potential prognostic factor. Subsequent investigations into the functional role of GATM in cell proliferation and invasiveness of CCA, both *in vitro* and *in vivo*, indicated that GATM could inhibit CCA progression. Furthermore, exploration of the underlying mechanisms suggested that GATM modulates the JNK/c-Jun pathway in CCA. IDH1 interacts with GATM protein and negatively regulates GATM protein levels. IDH1 restores the biological function of CCA by partially counteracting the inhibition of JNK/c-Jun pathway phosphorylation by GATM.

2. Materials and methods

2.1. Dataset analyses

Transcriptome profiling and corresponding clinicopathologic information of 36 CCA cases and 9 non-cancerous samples were retrieved from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/). Tumor Immune Estimation Resource (TIMER2.0) (http://timer.cistrome.org/) [32] was used to analyze the expression of GATM across cancers. GEPIA (http://gepia.cancer-pku.cn/) was performed to determine and evaluate the expression of GATM in CCA cancer. The criteria for significance were defined as follows: an adjusted $P < 0.05$ and $|\log2\ FC| > 1$. The Kaplan-Meier analysis was conducted with the "survival" R package to assess the prognostic role of GATM.

2.2. Cell culture and transfection

The human biliary epithelial cells (HIBEpiC) and human cholangiocarcinoma cell lines (HCCC 9810, RBE) were purchased from Procell Life Science and Technology (Wuhan, China). HIBEpiC cells were cultured with a complete culture medium (Procell Life Science and Technology). CCA cell lines (HCCC 9810, RBE) were in RPMI-1640 (Solarbio, China) supplemented with 10 % fetal bovine serum (FBS, Procell Life Science&Technology), and cultured in a humidified incubator maintained at 37 °C and 5 % CO2.

The GATM, IDH1 overexpression plasmid, and GATM, IDH1 siRNA were synthesized and purchased from GenePharma (Shanghai, China). The cell transfection was conducted based on the previous report [33]. Cells were seeded in advance and transfected with TurboFect transfection reagent (R0532; Thermo Scientific Scientific, United States). The sequences of the siRNAs were shown at Table S1. GATM overexpression lentivirus and corresponding control lentivirus were synthesized by Gene Chemical Technology Company (Shanghai, China). We used lentivirus production to infect RBE cells based on manufacturer's instructions and establish stable cell lines such as LV-GATM and LV-NC with puromycin (2 μg/mL).

2.3. Western blotting analysis

Western blotting was conducted based on previous study [34]. The protein was extracted from cholangiocarcinoma cells with 4 °C precooled RIPA buffer including the 10 % cocktail of protease inhibitor as well as 10 % phosphatase inhibitor (CWBIO, Jiangsu, China). Then the protein was separated by 10 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The protein marker applied in the procedure of protein separated was purchased from ThermoFisher Scientific (26616; ThermoFisher Scientific, United States). Membranes were blocked at room temperature with 5 % skim milk, followed by incubating with primary antibodies against GATM (1:1000; 12801-1-AP; Proteintech), IDH1 (1:1000; 12332-1-AP; Proteintech), JNK (1:1000; T40073; Abmart), phosphorylated-JNK (1:1000; #4668; Cell Signaling Technology), c-Jun (1:1000; A02038-2; Boster), phosphorylated-c-Jun (1:1000; BM4845; Boster), GAPDH (1:5000; 60004-1-Ig; Proteintech) at 4 °C overnight. Then membranes were washed using Tris-buffered saline Tween buffer (TBST, T1082, Solarbio, China) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (ZSGB, China) at room temperature for 1 h. After the final washes with TBST, we detected the resulting signals using chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Immunohistochemistry

The specimen section of human cholangiocarcinoma tissues and corresponding normal tissues were obtained from the First Affiliated Hospital of Nanchang University. This study gained approval by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. (2023)CDYFYYLK(02-010)). The specimen suffered from deparaffinized, rehydrated, and then processed for antigen retrieval in citrate buffer. The activity of endogenous peroxidase was blocked by 3 % H_2O_2. Tissue sections were incubated with primary antibody overnight, followed by Horseradish peroxidase (HRP)-conjugated secondary antibody (PV-6000) incubation for 15 min and Diaminobenzidine (DAB; ZLI-9018) staining. The primary antibody included GATM (1:200; 12801-1-AP; Proteintech), E-cadherin (1:2500; 20874-1-AP; Proteintech), N-cadherin (1:100; 22018-1-AP; Proteintech), Vimentin (1:2500; 10366-1-AP; Proteintech). Two pathologists graded staining results in a blinded manner. The scores are based on the ratio of staining intensity and positive staining according to previous standards [35].

2.5. Quantitative real-time PCR

Total RNA was extracted from CCA cells with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocol as previous described [36]. cDNA was reverse transcribed by the PrimeScriptTM RT reagent Kit with gDNA Erase (RR047A, Takara, Dalian, China). Real-time quantitative PCR (RT-PCR) was conducted on an ABI Step One Plus system (Applied Biosystems, Foster City, CA, USA) with TB GreenTM Premix Ex Taq II (RR820A, Takara, Dalian, China). The comparative expression of genes was normalized to GAPDH, which was calculated using the $2-\Delta\Delta CT$ method. The primer sequences were presented in Table S2.

2.6. Transcriptomic RNA-sequencing (RNA-seq)

The RNA of RBE cells in the control (OE-NC) group and GATM overexpressed (GATM) group was extracted using TRIzol reagent. Prepared RNA samples were performed transcriptome sequencing at Beijing Genomics Institute and analyzed on BGISEQ-500 [37]. Further bioinformatics analysis was carried out by the Beijing Genomics Institute. The significance of the differentially expressed genes (DEGs) was determined as the combination of $|\log2(FC)| > 0.5$ and adjusted p-value <0.05.

2.7. Cell count kit-8 assay

Cells were cultivated in the 96-well plates using 1640 medium with 10 % FBS at 37 °C and 5 % CO_2. Approximately 3×10^3 cells per 100 μL were added to each well, with five replicate wells set for each group. After cells were fully adhered, then processed with 10 μL Cell Count Kit-8 (CCK-8) reagent for 2 h at 37 °C. Finally, cells were assayed for absorbance at 450 nm for 5 days using a Varioskan LUXTM multimode microplate reader (Thermo Scientific). This experiment was conducted in triplicate independently.

2.8. EdU pulse-chase incorporation

The EdU (5-ethynyl-2′-deoxyuridine) assay was performed to measure the proliferation of cells by the manufacturer's protocol of the Clicl-iT EdU DNA Cell Proliferation Kit (UElandy, Suzhou, China). Each set of cells was seeded into 96-well plates with a density of 8×10^3 cells/well followed by culturing in 1640 containing 10 % FBS for 12 h. Then the cells were treated with EdU (20 μM) for 2 h at 37 °C, in which the nucleic acids were dyed with DAPI (4′,6-diamidino-2-phenylindole). Cell images were captured by an inverted

fluorescence microscope (Nikon) and counted by Image J software. Cell proliferation was evaluated by the average of EdU-positive cells in three fields within each sample.

2.9. Colony formation assay

Cells were cultured at 2×10^3 cells/well in 6-well plates. After 2 weeks, colonies were fixed, stained and scored using ImageJ software.

2.10. Wound-healing assay

Cells were seeded with 5×10^5 cells/well in 6-well plates until fully confluent. Each well of cells was scratched with a 200 μl pipette tip and washed three times with phosphate-buffered saline. After incubation in serum-free medium for 48 h, the wound was imaged at 0 and 48 h to measure the percent wound closure. Three microscopic fields were selected randomly for counting wound closure ranges according to previous study [34].

2.11. Transwell assay

The transwell assay was conducted to detect the effect of GATM on migration and invasion of CCA cell. Before the invasiveness assay, the liquefied Matrigel gel was diluted as a ratio of 8:1 by bovine serum albumin (BSA) solution (10 g/L), with 50 μL diluted reagent added to the Transwell chamber for 30 min. Then 50 μL BSA solution was applied to hydrate the Matrigel gel. The upper chamber was transferred with 2×10^5 CCA cells contained in 200 μl serum-free medium. The lower chamber was added with 20 % serum-positive medium as the chemoattractant. After 48 h, the migrated and invaded cells were carefully fixed and stained. Five random views were selected for imaging using microscopic, then cell counting by Image J software. The experiments were carried out in triplicate manner.

2.12. JNK/c-Jun signaling activation

To validate whether GATM regulated the biological behavior of CCA cells in a JNK/c-Jun dependent manner, the JNK activator anisomycin (HY-18982, MCE) was used to stimulate JNK/c-Jun pathway in GATM upregulated CCA cells [38,39]. RBE cells that transfected with GATM plasmid treated with anisomycin (20 μM/mL) incubated at 37 °C for 30 min.

2.13. Co-immunoprecipitation (CO-IP) and protein stability assay

The protein was lysed from cells by NP-40 buffer (KGB5206-50). Then the protein was mixed with primary antibody (GATM, A6598, ABclonal; IDH1, 12332-1-AP, Proteintech; IgG, 30000-0-AP, Proteintech) and incubated overnight at 4 °C. Protein A/G magnetic beads (HY-K0202, MCE) were added to the complexes for 2 h at 4 °C. The magnetic beads were eluted with 1 × SDS loading buffer for further experiments. To investigate the protein stability, cells were treated with 20 μM MG132 (HY-13259, MCE) and 100 μg/mL Cycloheximide (CHX, HY-12320, MCE) followed by western blotting.

2.14. Immunofluorescence assay

Cells seeded well were fixed in 4 % paraformaldehyde, permeabilized with Triton X-100, and blocked with BSA. Next, the cells were incubated with primary antibodies (GATM, A6598, ABclonal; IDH1, 12332-1-AP, Proteintech) overnight at 4 °C. Then cells were added with fluorescent secondary antibody (Anti-Rabbit IgG (Elab Fluor®488), E-AB-1055, Elabscience; Anti-Rabbit IgG (Elab Fluor®594), E-AB-1060, Elabscience) for 1 h and stained with DAPI for 40 min. Images were captured by confocal microscopy.

2.15. Animal experiments

All animal experiments were conducted with protocols approved by the Animal Center of The First Affiliated Hospital of Nanchang University (approval no. CDYFY-IACUC-202303QR007). Four-week-old female nude mice were purchased from Charles River Laboratories (Zhejiang, China) in the study. Xenograft tumour experiments were conducted referred to previous study [40]. Nude mice were injected subcutaneously with 1×10^7 LV-NC or LV-GATM RBE cells suspended in 100 μL PBS into the left axilla ($n = 6$/group). Tumour growth was recorded at 5-day intervals. The formula of tumour volume was as follow: tumour volume (mm^3) = [length × $(width)^2$]/2. Xenograft tumours were excised, measured and weighed at 30 days.

2.16. Statistical analysis

The statistical analysis was developed using R software (version 4.2.1) and GraphPad Prism 10. Values were shown as the mean ± standard deviation. Mean differences among groups were determined using student's two-tailed *t*-test, one-way ANOVA, and two-way ANOVA. Survival outcomes were assessed by Kaplan-Meier analysis and log-rank tests. Statistical significance was indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All experiments were performed at least three times independently.

3. Results

3.1. GATM is downregulated in human CCA and correlates with prognosis

TIMER2.0 was used to evaluate the variation in GATM expression between normal and tumourigenic tissues across multiple tumour types. (Fig. S1). Pan-cancer analysis showed that GATM was differentially expressed in multiple tumours, including CCA. According to the GEPIA database analysis, GATM was significantly downregulated in cholangiocarcinoma (CHOL) tissues compared to normal tissues (Fig. 1A). To explore the correlation between GATM and CCA prognosis, we used the R package 'survival' to analyze CCA datasets from TCGA. Low expression of GATM correlated with Stage IV, T3, and lymphatic metastasis (Fig. 1B–D), which are important predictive factors of CCA prognosis. We found that patients with low GATM expression exhibited worse overall survival (Fig. 1E). We detected GATM expression in CCA cells and tissues and found that the mRNA and protein expression of GATM was downregulated in CCA cells compared to that in normal cells (Fig. 1F–G). Additionally, immunohistochemistry analysis showed a reduction in GATM expression in tumour tissues compared to that in normal tissues for two CCA patients. (Fig. 1H). Overall, our results suggested that GATM was downregulated in CCA tissues and cells and correlated with poor prognosis in this patient population.

3.2. Modulation of GATM expression affected the malignant behaviours of CCA cells in vitro

In this study, we investigated the effect of GATM on the phenotype of CCA cells. The transfection efficiency of both GATM knockdown and overexpression in HCCC 9810 and RBE cells was assessed through western blotting and qRT-PCR (Fig. 2A–B, Fig. 3A–B, and Fig. S2). Subsequently, colony formation (Figs. 2C and 3C, Figs. S3A, S4A), EdU (Figs. 2D and 3D, Figs. S3B, S4B), and CCK-8 assays (Figs. 2E and 3E) were performed to assess the effect of GATM on the proliferation of CCA cells. These findings indicated that downregulation of GATM expression significantly enhanced the proliferation of CCA cells *in vitro*. Conversely, GATM upregulation inhibited the proliferation of CCA cells. Additionally, Transwell assays revealed that silencing GATM led to increased migration and invasion (Fig. 2F, Figure S3C-3D) of CCA cells *in vitro*, whereas upregulation of GATM yielded the opposite effects (Fig. 3F, Fig. S4C). Wound healing assays showed that GATM knockdown promoted migration (Fig. 2G, Fig. S3E), and increased GATM expression inhibited the behaviour of CCA cells (Fig. 3G, Fig. S4D).

3.3. GATM overexpression inhibited the growth of CCA in vivo

The subcutaneous xenograft tumour experiment was conducted to evaluate the effects of GATM on tumour growth. CCA cancer cells infected with either LV-NC or LV-GATM were inoculated into nude mice (n = 6/group), and tumours were monitored for 30 days. The efficiency of GATM upregulation was confirmed through western blotting (Fig. 4A). During the observation period, the LV-GATM group showed a significant reduction in tumour volume compared with that of the LV-NC group. (Fig. 4C). Thirty days after implantation, the tumours were examined or harvested from the nude mice to measure their volume and weight (Fig. 4B). Notably, both the volume and weight of the tumours were significantly reduced in the LV-GATM group compared to those in the LV-NC group (Fig. 4C–D). Immunohistochemistry analysis of subcutaneous tumours showed GATM overexpression in LV-GATM groups. E-cadherin expression was significantly increased in the LV-GATM group, and N-cadherin and vimentin expression were decreased compared to those in the LV-NC group (Fig. 4E). These results revealed that GATM may be involved in inhibiting the EMT transition of CCA *in vivo*. These findings provide evidence that GATM overexpression suppresses tumour growth *in vivo*.

3.4. GATM regulated the phosphorylation of the JNK/c-Jun signalling

To investigate the mechanism by which GATM regulates CCA progression, we conducted RNA-seq analysis to compare the transcriptome profiles of RBE cells overexpressing GATM and control cells. A total of 201 differentially expressed genes (DEGs) were screened with the criteria $|\log2(FC)|>0.5$ and adjusted p-value<0.05, including 139 upregulated and 62 downregulated genes (Fig. 5A). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed the top 20 pathways (Fig. 5B). The MAPK pathway was identified as closely related to cell proliferation and migration, suggesting that this pathway may be vital for GATM-mediated regulation of CCA progression. Western blotting analysis results confirmed that the GATM knockdown increased the expression of phosphorylated JNK and c-Jun (Fig. 5C, Fig. S5A). Additionally, the GATM overexpression group showed significant inhibition of JNK and c-Jun phosphorylation (Fig. 5D, Fig. S5B). However, there were no significant differences in the total levels of JNK and c-Jun in relation to the expression of GATM (Fig. 5C–D).

3.5. GATM regulated the proliferation and invasiveness of CCA cells via inhibition of the JNK/c-Jun signalling pathways

To determine whether GATM regulates the proliferation and aggression of CAA cells in a JNK-c-Jun-dependent manner, we used the JNK activator anisomycin to induce the JNK/c-Jun pathway in GATM-overexpressing cells. JNK-c-Jun signalling was induced by anisomycin (Fig. 6A). Activation of JNK-c-Jun significantly reversed the inhibitory effect of upregulated GATM on the proliferation of RBE cells compared to GATM overexpression alone. The results indicated that the number and size of colonies in the JNK-c-Jun-activated group were significantly higher than those in the GATM overexpressed group (Fig. 6B, Fig. S6A). CCK8 assay and EdU staining results suggested that the induction of JNK-c-Jun promoted CCA cell proliferation (Fig. 6C–D, Fig. S6B). Furthermore, Transwell assays indicated that the migration and invasive ability of CCA cells were enhanced following JNK-c-Jun activation (Fig. 6E,

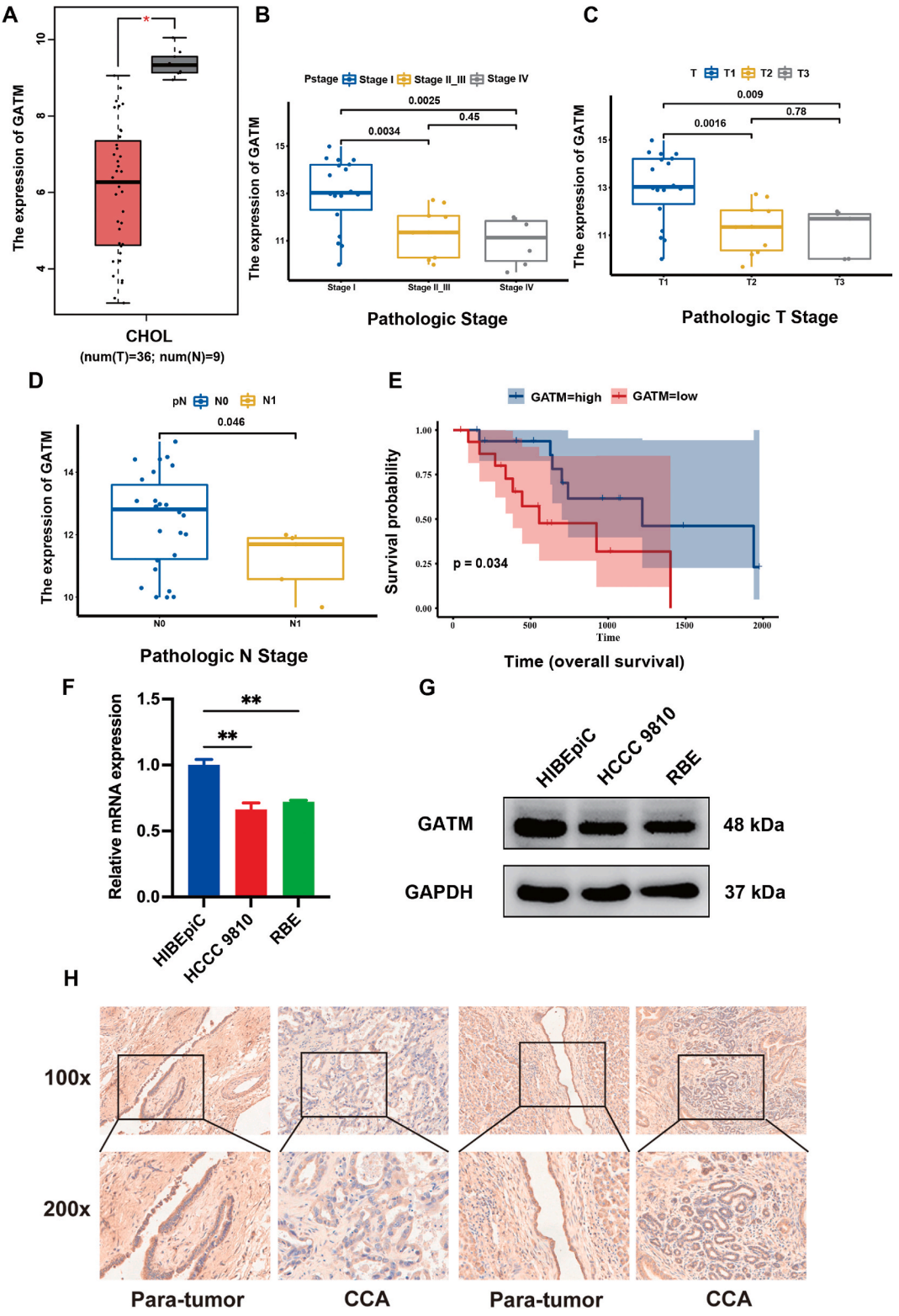

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Fig. 1. GATM is downregulated in human CCA and correlates with prognosis. A Analysis of GATM mRNA relative expression in the cholangiocarcinoma and normal tissue according to GEPIA databases. B-E Subgroup analysis of pathologic stage (B), subgroup analysis of pathologic T stage (C), subgroup analysis of lymphatic metastasis (D) and the overall survival (E) related to GATM expression based on the data of TCGA cholangiocarcinoma datasets. F-G Expression level of GATM in cholangiocarcinoma cell lines and normal HIBEpiC cells by RT-qPCR (F) and Western blotting (G). H Representative images of IHC staining of GATM in two human CCA cancer tissues and paired normal tissues. (log-rank test, $p < 0.05$).

Fig. S6C). These results suggested that GATM inhibits CCA cell proliferation and aggressiveness by regulating JNK-c-Jun signalling.

3.6. IDH1 interacts with GATM

To identify the proteins interacting with GATM in the CCA, we conducted mass spectrometry. The results showed a total of 32 expressed proteins in the GATM group, and 17 proteins were exclusively bound to GATM (Fig. 7A). IDH1 was prioritised for further experiments. The endogenous CO-IP assay indicated that GATM and IDH1 were significantly immunoprecipitated in HCCC 9810 and RBE cells (Fig. 7B). Immunofluorescence experiments revealed that GATM and IDH1 proteins were co-localized in the cytoplasm (Fig. 7C).

Western blotting analysis revealed that the protein level of IDH1 was not significantly altered by GATM knockdown or overexpression (Fig. 7D). In contrast, GATM protein expression negatively correlated with changes in IDH1 protein levels (Fig. 7E). We then administered a protein synthesis inhibitor (cycloheximide, CHX) and a proteasome inhibitor (MG132) to CCA cells. The results showed that after interfering with the expression of IDH1, the degradation rate of the GATM protein was attenuated, and the half-life was significantly prolonged (Fig. 7F). Following treatment with MG132 in IDH1 upregulated CCA cells, the inhibitory effect of IDH1 on GATM protein expression weakened (Fig. 7G). Therefore, we hypothesised that IDH1 promotes the degradation of GATM protein in CCA cells via the proteasome pathway, thereby affecting GATM protein levels.

3.7. IDH1 was involved in GATM-mediated JNK/c-Jun signalling inactivation and CCA suppression

To investigate whether IDH1 affects the inhibitory function of GATM, we concurrently overexpressed GATM and IDH1 in CCA cell. Western blotting results showed that IDH1 upregulation reversed the inactivation of JNK/c-Jun signalling phosphorylation by GATM (Fig. 8A). Colony formation, CCK-8 and EDU experiments indicated that the growth inhibition of CCA cells by GATM could be partially reversed after IDH1 overexpression (Fig. 8B–D, Figure S7A-7B). In the Transwell assay, the migratory and invasive tendencies of CCA cells were largely restored after the simultaneous upregulation of IDH1 (Fig. 8E, Fig. S7C). These findings confirm that IDH1 counteracts the inhibitory effect of GATM on CCA.

4. Discussion

In the present study, we demonstrated that GATM expression was downregulated in both CCA tissues and cell lines, which was consistent with the results obtained from the GEPIA database analysis. Moreover, TCGA data analysis reported that GATM downregulation significantly correlated with poor OS and aggressive pathological features. GATM knockdown promoted the proliferation, invasiveness, and tumourigenicity of CCA cells, whereas GATM upregulation yielded the opposite effect both *in vitro* and *in vivo*. Immunohistochemistry of subcutaneous tumours indicated that GATM may inhibit EMT, which induces tumour progression and treatment resistance [41]. Importantly, our results confirmed that GATM modulates the phosphorylation and activity of JNK/c-Jun signalling to suppress CCA progression, which could be reversed by the interacting protein, IDH1. Collectively, GATM may serve as a potential antitumorigenic and prognostic marker for CCA.

Recent reports have highlighted the significance of GATM in various tumour types, such as breast cancer, colorectal cancer, renal cell carcinoma, and hepatocellular carcinoma, emphasising its dual role in either promoting or inhibiting tumourigenesis. The direction of its impact typically depends on expression levels and downstream regulatory molecules. GATM, which encodes glycine amidinotransferase, is a mitochondrial enzyme that facilitates the transfer of the key substrate, guanidinoacetic acid, in creatine synthesis [11]. Studies have implicated creatine in diverse roles, contributing to the progression of breast cancer [42] and metastasis of colorectal cancer [18]. Additionally, creatine can inhibit tumour cell growth and modulate CD8 T cell antitumor immunity [43,44]. Notably, our results align with research on clear cell renal cell carcinoma, in which low GATM expression was found to be correlated with poor survival in patients treated with immune checkpoint inhibitors [19]. GATM is reportedly expressed at low levels in hepatocellular carcinoma (HCC) tissues, and GATM knockdown promotes the proliferation and migration of HCC cells [45]. However, the role of GATM in CCA development and growth has not been explored. Through screening differentially expressed genes related to OS between CCA and normal tissues in TCGA databases, we identified GATM as a focal point for further investigation. Our findings underscore the inhibitory effects of GATM and suggest its potential as a novel therapeutic target in CCA.

Next, we conducted RNA-seq analysis to identify the downstream mechanisms of GATM in CCA. These results revealed that the MAPK pathway participates in CCA progression. MAPK signalling is frequently overactivated in melanoma, colorectal cancer, lung cancer, and CCA [46–48]. The MAPK protein family regulates both physiological and pathological processes, and abnormal MAPK signal transduction is associated with the occurrence and progression of cancer [46]. JNK is a member of the MAPK protein family that regulates cell proliferation, migration, and cancer progression [49]. The JNK pathway is often hyperactivated in various leukaemias and solid tumours, including cancers of the skin, lung, colon, breast, and liver [48,50,51], implicating JNKs as a tumour promoter. The

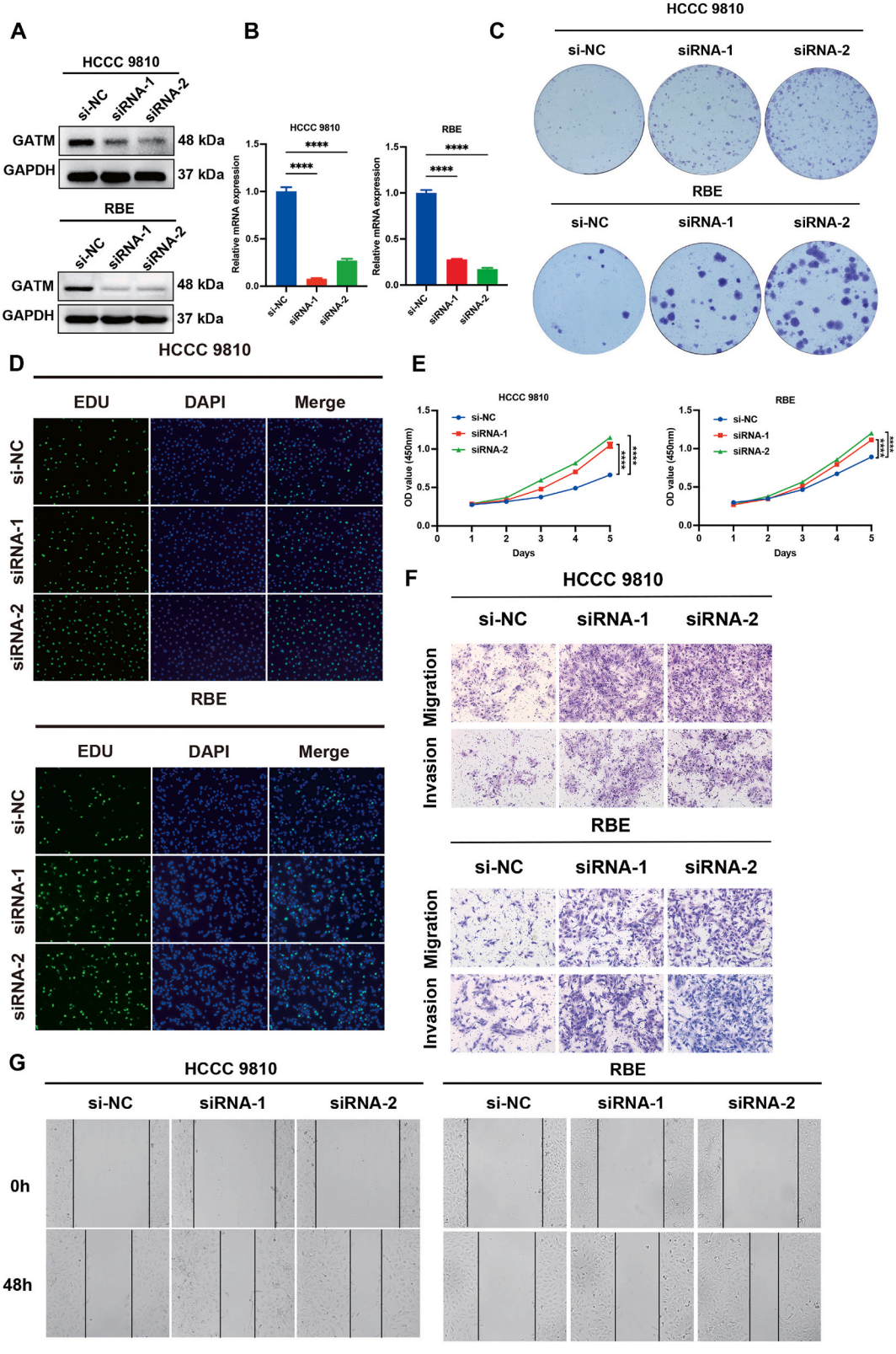

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Fig. 2. Downregulation of GATM promotes proliferation, migration and invasion of CCA *in vitro*. A-B HCCC 9810 and RBE cells were transfected with GATM siRNA, and the efficiency was detected by Western blotting (A) and qRT-PCR (B). C-E Clone formation (C), EdU assays (D), CCK-8 (E) assay were performed to identify the proliferation ability after GATM knockdown. F-G Transwell assays (F) and wound healing assay (G) were performed to identify the migration and invasion ability after GATM knockdown. One-way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

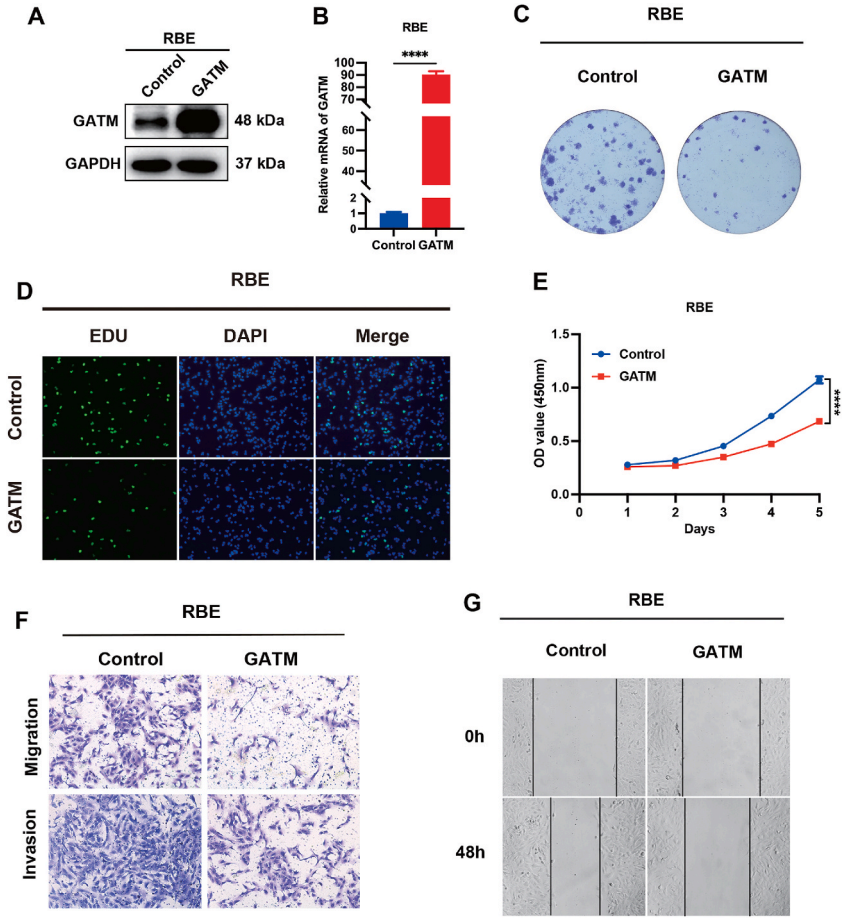

Fig. 3. GATM overexpression inhibits proliferation, migration and invasion of CCA *in vitro*. A-B RBE cells were transfected with GATM expression plasmid, and the efficiency was detected by Western blotting (A) and qRT-PCR (B). C-E Clone formation (C), EdU assays (D), CCK-8 (E) assay were performed to identify the effect of overexpression of GATM in proliferation ability. F-G Transwell assays (F) and wound healing assay (G) were performed to evaluate the migration and invasion ability upon GATM upregulation. Student's *t*-test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

c-Jun is activated by phosphorylation of serines 63 and 73, which leads to activation of many transcription factors, such as ETS, NF-κB, and AP-1. These transcription factors induce a variety of critical cell-proliferating and growth-regulating factors, such as c-Myc, cyclin D1, and c-Fos [47]. The JNK/c-Jun signalling plays favourable roles in cholangiocellular tumorigenesis, proliferation, and differentiation [39,52]. In our study, the expression of p-JNK and p-c-Jun increased in CCA cells with GATM knockdown and decreased in the GATM overexpression group. We also demonstrated that hyperactivity of JNK/c-Jun signalling induced by an activator could counteract the inhibitory effect of GATM upregulation on the proliferation, migration, and invasion of CCA cells.

We performed protein mass spectrometry to screen for GATM-interacting proteins, and IDH1 was of particular interest. Overexpression of the wild-type IDH1 reportedly promotes NSCLC and pancreatic cancer progression [26,27]. IDH1 increases the expression of acetaldehyde dehydrogenase 1 and promotes the proliferation, migration, and invasion of CCA cells [28]. We demonstrated the protein interaction between GATM and IDH1 in CCA cells via endogenous immunoprecipitation and immunofluorescence assays. CHX and MG132 experiments revealed that IDH1 promoted the degradation of GATM protein to negatively regulate GATM protein levels. We further investigated whether IDH1 affects the regulatory effect of GATM on the JNK/c-Jun signalling pathway and biological behaviour of CCA. These results suggested that the inhibition of JNK/c-Jun pathway phosphorylation caused by GATM can be largely restored by IDH1. Simultaneous overexpression of GATM and IDH1 partially reversed the inhibition of CCA cell

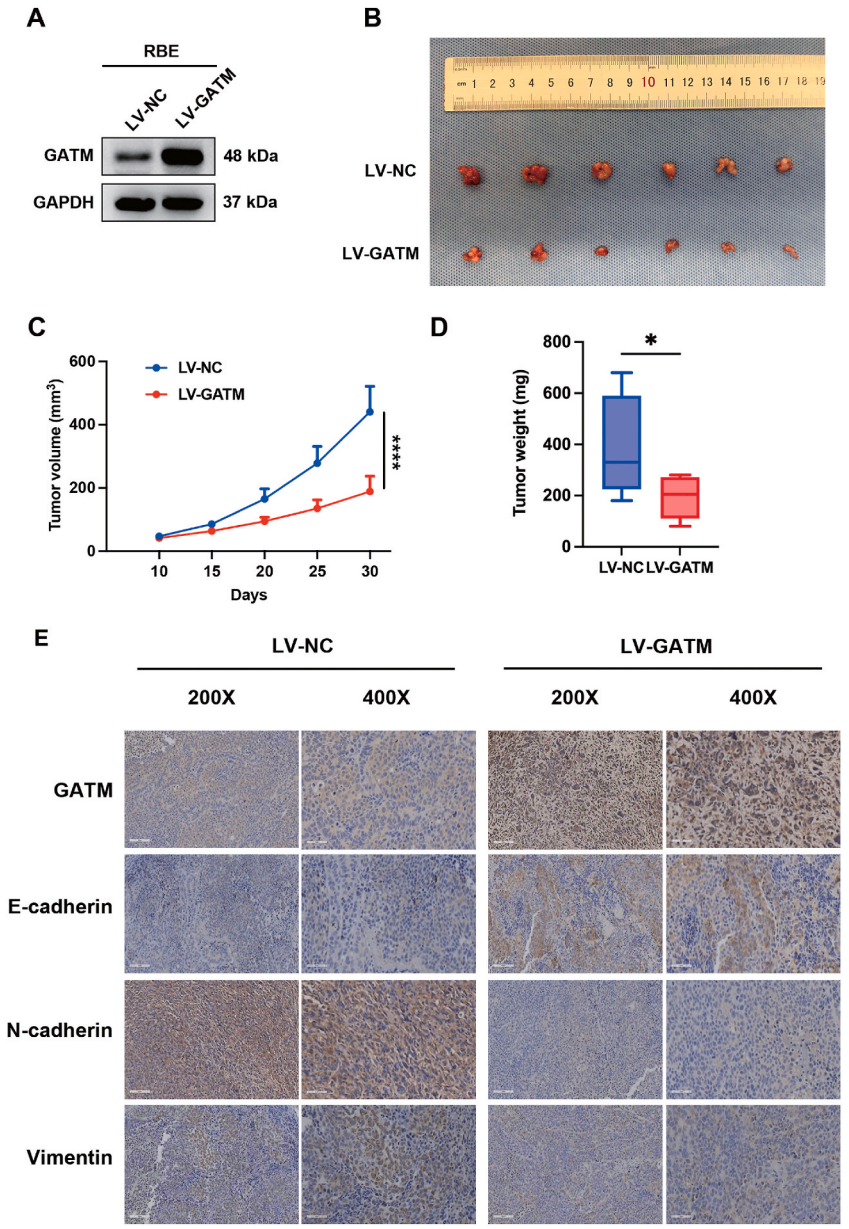

Fig. 4. GATM overexpression promotes the growth of CCA *in vivo* A The upregulated efficiency was detected by Western blotting in RBE cells infected with LV-NC or LV-GATM. B The images of tumours were generated in nude mice using control and GATM overexpression RBE cells, n = 6, respectively. C Volume change in nude mice after tumor implantation. Two-way ANOVA: ****p < 0.0001. D The upregulated GATM were able to suppress tumorigenicity resulting in an obvious reduction in tumor weight compared to the control group (N = 6). E IHC staining images of subcutaneous tumours. Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001.

proliferation, migration, and invasion by GATM. Taken together, our data indicated that GATM functions as a negative regulator of the JNK/c-Jun signalling pathway, exerting inhibitory effects on CCA. IDH1 interacts with GATM and promotes the degradation of GATM protein. Overexpression of IDH1 restores the biological function of CCA by reversing the inhibition of JNK/c-Jun pathway phosphorylation by GATM. Nevertheless, further studies are required to explore the upstream molecular mechanisms of the GATM-regulated MAPK cascade in CCA.

5. Conclusion

Our study is the first to investigate the role of GATM in the proliferation and metastasis of CCA cells. Our findings revealed that GATM is downregulated in CCA tumour tissues and cells, as well as functions as a tumour suppressor in human CCA. Moreover, we

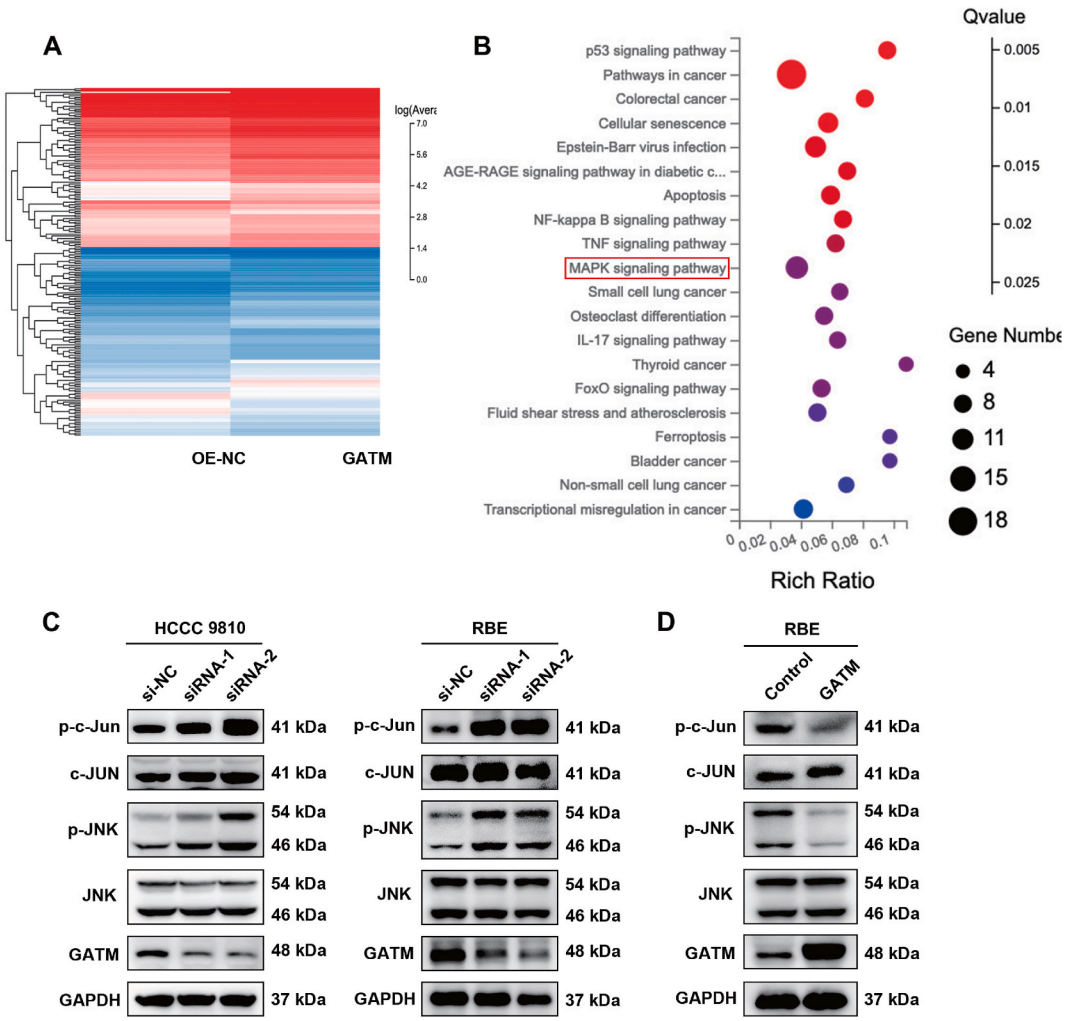

Fig. 5. GATM regulated the expression of the JNK-c-Jun signaling. A Hot map of 201 differential genes in the RNA-seq. B Top 20 signaling pathway maps enriched in the RNA Sequence. C Western blotting analysis of p-c-Jun, c-Jun, p-JNK, JNK expression after GATM downregulation. D Western blotting analysis of p-c-Jun, c-Jun, p-JNK, JNK expression after GATM upregulation. One-way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

established that GATM exerts its inhibitory effect on CCA progression by modulating the JNK/c-Jun signalling pathway. IDH1 interacts with GATM and promotes the degradation of GATM protein. IDH1 restores the biological function of CCA by weakening the GATM-mediated inhibition of JNK/c-Jun phosphorylation. Our findings suggest a potential therapeutic strategy for patients with CCA.

Ethics statement

This study gained approval by the Ethics Committee of the First Affiliated Hospital of Nanchang University with the approval number: (2023)CDYFYYLK(02-010), dated February 1st, 2023. Informed consent was waived in the study. All animal experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and approved by the Animal Center of The First Affiliated Hospital of Nanchang University with the approval number: CDYFY-IACUC-202303QR007), dated March 14, 2023.

Consent to participate

Not applicable.

Consent for publication

The authors declare that they agree to submit the article for publication.

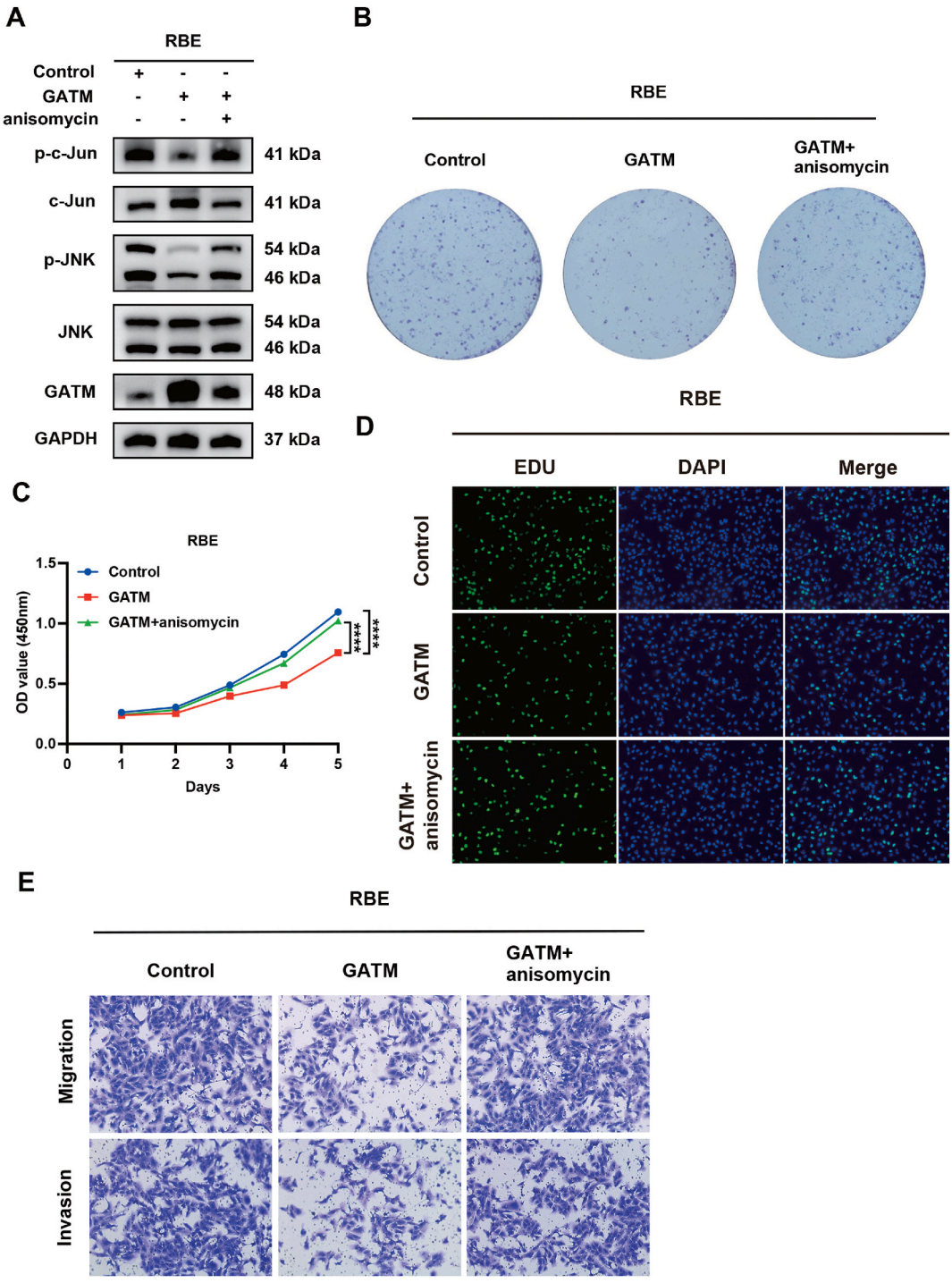

Fig. 6. GATM regulated the proliferation and invasiveness of CCA cells via inhibiting JNK/c- Jun signaling pathways. A The activation of JNK-c-Jun pathway was detected by Western blot. B-D Clone formation(B), CCK8 assays(C) and EdU assays(D) showed anisomycin reverses the inhibiting effect of GATM overexpression on the proliferation of CCA cells. E Transwell assays showed anisomycin reverses the inhibiting effect of GATM overexpression on the migration and invasion of CCA cells. One-way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Data availability statement

Publicly available datasets were analyzed in this study. TCGA database: https://tcga-data.nci.nih.gov/. Original data was available as supplementary file 2.

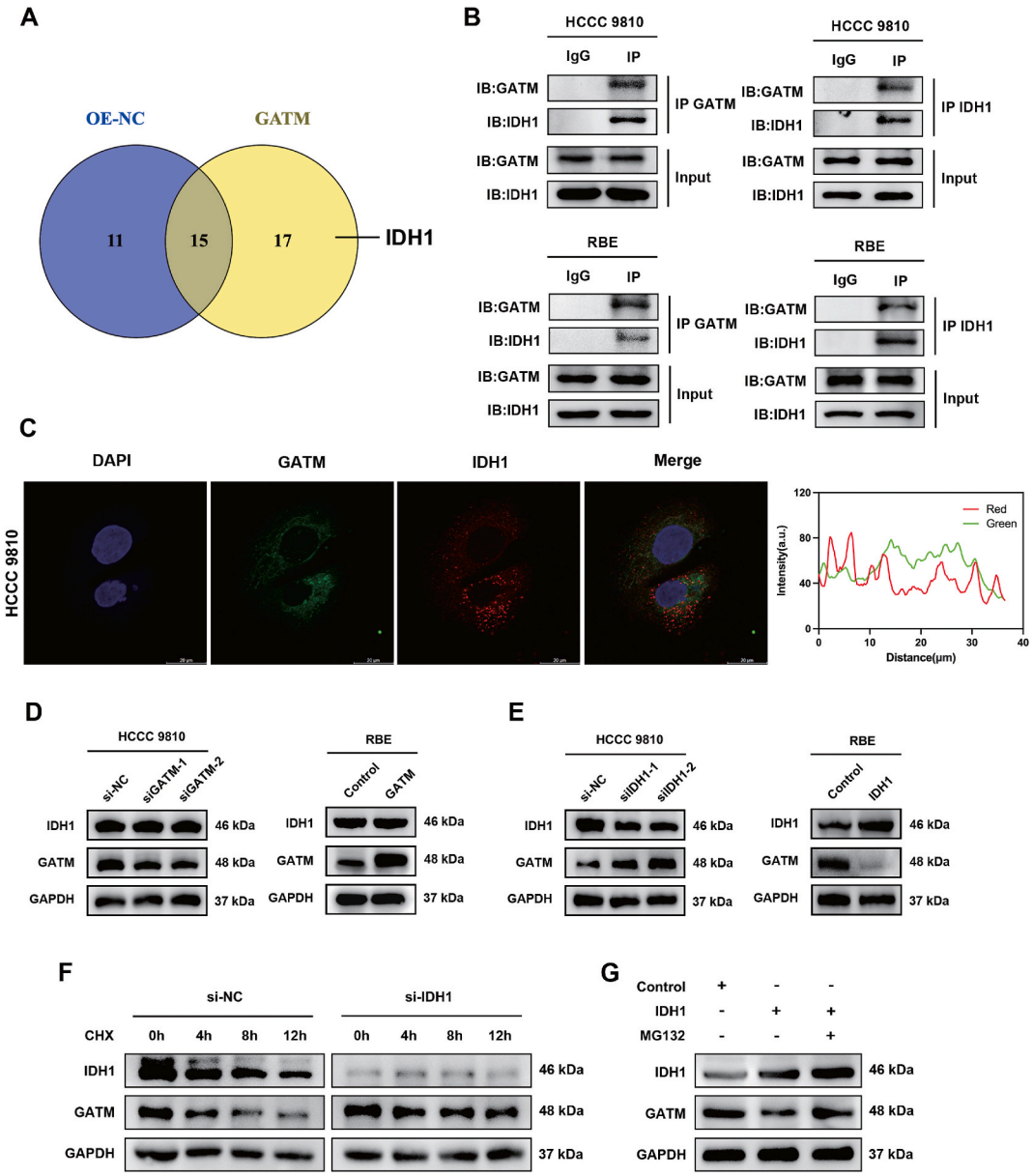

Fig. 7. GATM interacts with IDH1 protein. A Venn diagram of proteins by protein mass spectrometry. B Endogenous co-immunoprecipitation of GATM and IDH1 in HCCC 9810 and RBE cells. C Cell immunofluorescence assays exhibited the locations of GATM and IDH1 in HCCC 9810 cells. D Western blotting evaluated the protein level of IDH1 in CCA cells with GATM knockdown and upregulated. E Western blotting evaluated the protein level of GATM in CCA cells with IDH1 knockdown and upregulated. F Protein stability was detected in the control and IDH1 knockdown group, which was treated with cycloheximide for the indicated time. G Western blotting assessed the protein level of GATM in CCA cells with IDH1 overexpression treated with MG132 for 4 h.

CRediT authorship contribution statement

Yi Yu: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Wei Gan:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Jianping Xiong:** Visualization, Supervision, Funding acquisition. **Junhe Li:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

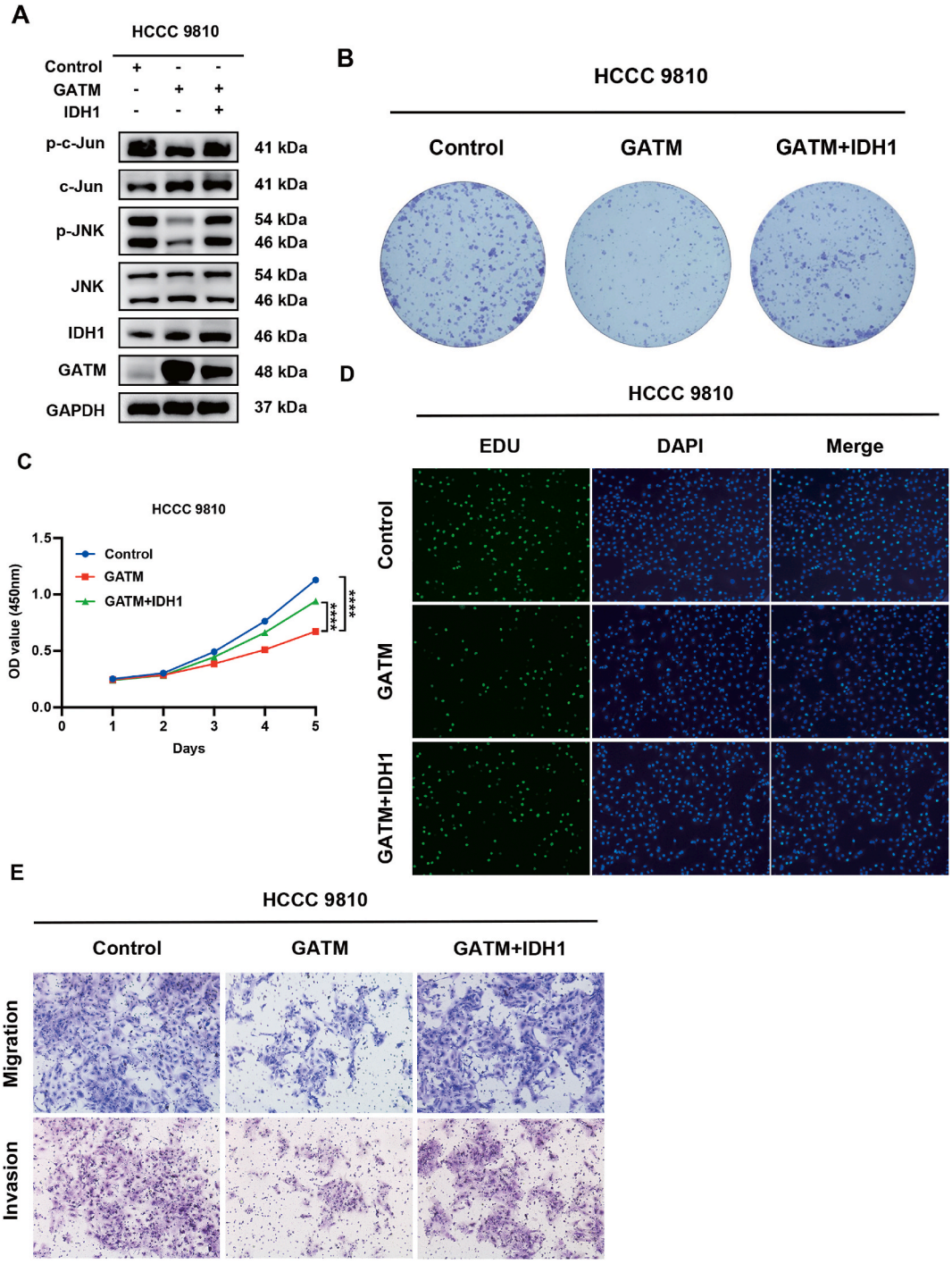

Fig. 8. IDH1 involved in GATM-mediated JNK/c-Jun signaling inactivation and CCA suppression. A Western blotting analyzed protein expression of JNK-c-Jun pathway with GATM and IDH1 overexpression. B-D Clone formation(B), CCK8 assays(C) and EdU assays(D) showed the proliferation of CCA cells with GATM and IDH1 overexpression. E Transwell assays showed migration and invasion of CCA cells with GATM and IDH1 overexpression. One-way ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37344.

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