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GAMT facilitates tumor progression via inhibiting p53 in clear cell renal cell carcinoma

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Abstract

Background Clear cell renal cell carcinoma (ccRCC) is the most common type of RCC. Even though the targeted drugs for the treatment of ccRCC have a certain therapeutic effect, due to the problem of drug resistance, the search for new targets for targeted therapy of ccRCC remains urgent. GAMT is an enzyme involved in creatine metabolism. However, the precise biological roles and molecular mechanisms of GAMT in ccRCC are not fully understood.

Results Here, we found that GAMT was upregulated in ccRCC cells and tissues and associated with poor prognosis. Further, GAMT has pro-oncogenic abilities in promoting ccRCC development and progression. Intriguingly, GAMT exerted biological functions independent of its role in catalyzing creatine synthesis. Mechanistically, GAMT overexpression contributes to the development and progression of ccRCC by inhibiting tumor suppressor p53. Finally, we identified fisetin as a novel GAMT inhibitor and validated its role in suppressing ccRCC progression and sensitizing ccRCC cells to targeted drug axitinib via in vivo and in vitro assays.

Conclusions This study reveals that GAMT has pro-oncogenic abilities in promoting ccRCC development and progression. GAMT exerted its non-enzymatic functions possibly by regulating the expression of p53. Fisetin, the novel GAMT inhibitor identified herein, may serve as a new antitumor drug for ccRCC treatment.

Keywords Clear cell renal cell carcinoma, GAMT, Fisetin, p53

Background

Clear cell renal cell carcinoma (ccRCC), a lethal urological malignancy, is the most common type of kidney cancer [1]. Nearly one third of ccRCC patients present with metastatic disease, and 20% of patients experience relapse in distant sites, despite undergoing radical surgery [2]. Currently, vascular endothelial growth factor (VEGF)-targeted tyrosine kinase inhibitors (TKIs) is established as first-line therapy in advanced ccRCC. Although TKIs, such as sunitinib, sorafenib and axitinib, improved ccRCC prognosis, many patients still cannot achieve complete remission. Meanwhile, primary or acquired resistance after long-term use of TKIs has become an obstacle for improving survival time of ccRCC patients [2, 3]. Much effort has been spent on elucidating

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the pathogenesis of ccRCC occurrence and progression, but much remains unclear. Therefore, further study is needed to clarify the molecular mechanism of ccRCC and identify new targets for ccRCC treatment.

ccRCC involves metabolic reprogramming, which is essential for tumor progression and survival. Our previous study showed that creatine inhibited the progression of ccRCC [4]. Many studies showed that enzymes involved in creatine metabolism could regulate tumor progression. For example, glycine amidinotransferase (GATM), the rate-limiting enzyme in creatine synthesis, promotes tumor metastasis by increasing Snail and Slug expression [5]. Additionally, creatine kinase B (CKB) non-metabolically inhibits ferroptosis in hepatocellular carcinoma [6], and creatine transporter solute carrier family 6 member 8 (SLC6A8) inhibits apoptosis and enhances survival in breast cancer cells [6]. Guanidinoacetate methyltransferase (GAMT) is involved in the second process of creatine biosynthesis [7]. In gastric cancer and colon cancer, high-expressed GAMT is associated with poor prognosis [8, 9]. GAMT is low-expressed in pancreatic cancer, and serves as a downstream molecular to induce apoptosis [10]. However, whether and how GAMT is involved in the development and progression is unknown.

Once thought to be limited to cellular metabolism, metabolic enzymes are now known for their various functions in tumors [11]. These enzymes, such as HK, ACLY and ACSS2, which are traditionally associated with glycolysis, TCA cycle, and lipid biosynthesis, are now increasingly acknowledged for their non-metabolic roles in tumor progression, metastasis, and therapy resistance. These moonlighting functions transcend their conventional roles in metabolic pathways, participating in diverse cellular processes [12–16]. Elucidating these non-canonical roles can provide new insights into cancer pathogenesis, revealing novel avenues for therapeutic interventions. However, whether GAMT has non-canonical roles is still unknown.

Here, using targeted metabolomic analysis, we found that creatine metabolism was reprogrammed in ccRCC. We examined the expression of genes involved in creatine metabolism and found that GAMT was the only enzyme that overexpressed in ccRCC. Also, elevated GAMT expression was associated with an unfavorable prognosis. In vivo and in vitro assays indicated that GAMT promoted ccRCC proliferation, migration and invasion. Intriguingly, the oncogenic role of GAMT was independent of its metabolic function. Mechanically, up-regulated GAMT could inhibit the tumor suppressor p53 in ccRCC. Importantly, we identified fisetin as a novel GAMT inhibitor via high-throughput virtual screening and validated its role in suppressing ccRCC progression by using cell experiments, xenograft mouse model, orthotopic tumor models and ccRCC organoids. The combination of fisetin also sensitized ccRCC cells to axitinib, a first-line therapy in RCC [17], in vivo. Our study reveals a novel function of GAMT in ccRCC, offering a potential therapeutic target for ccRCC. Fisetin, the novel GAMT inhibitor identified herein, offers the potential as a drug for ccRCC treatment and for addressing TKI resistance.

Methods

Human tissue samples and cell culture

Tumor cell lines (769-P, A498, Renca, SN12) [18] and 293T cell line were sourced from the ATCC and cultured at 37℃ in 5% CO2 in RPMI-1640, MEM, or high glucose Dulbecco's Modified Eagle's medium (Pricella, China), all supplemented with 10% FBS (Vazyme, China). Sixty participants were recruited for targeted metabolomic analysis at the Chinese PLA General Hospital (Beijing, China) between January 2020 and May 2021. The Chinese PLA General Hospital approved the study protocol, and all participants provided written informed consent.

Patient-derived organoid culture and drug sensitivity test

Histologically diagnosed ccRCC tissues were obtained from fresh specimens during surgery at our center. Tissue processing and organoid culture were performed as described previously [19]. For the drug sensitivity test, 50 µl of organoid suspension were harvested in 384-well plates (Corning). Then, six concentrations of fisetin, as well as the DMSO control, were added in triplicate. CellTiter-Glo 3D Reagent (Promega) was used. The luminescence was measured with a PerkinElmer microplate reader.

Antibodies and reagents

Antibodies used in this study included anti-GAMT (10880-1-AP), anti-p53 (10442-1-AP) and anti-β-Tubulin (10094-1-AP), all obtained from Proteintech (Wuhan, China). JetPRIME (Illkirch, France) was used for transfection.

CCK-8 assay and colony formation assay

Cells were plated at a density of 1,000 cells/well in a 96-well plate, which was maintained at 37 °C. CCK-8 was added to each well over 4 days. Cells were incubated at 37 °C for an additional 3 h, and absorbance was then measured at 450 nm. For the plate colony formation assay, RCC cells were seeded at 500 cells/well in six-well plates and cultured for 7 days. The cells were then fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution for 10 min.

Wound healing assay

ccRCC cell lines were seeded in six-well plates and allowed to grow until they reached 100% confluency. Subsequently, the cell layer was gently scratched using a sterile plastic tip. Cell motility was measured at 0 and 24 h.

Cell migration and invasion assays

In vitro cell migration and invasion assays were performed using Transwell migration and invasion assays. Ten thousand cells were placed in the upper chamber with serum-free medium, whereas the lower chamber contained complete medium. After 24 h, the migrated cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution for 10 min. For the invasion assays, Matrigel (BD Biosciences) was used to coat the Transwell chambers.

LC-MS/MS analysis

Normal and tumor tissues were collected from patients undergoing radical nephrectomy and immediately frozen in liquid nitrogen for further experiments. Following metabolite extraction from the tissue samples, all samples were re-dissolved and centrifuged, and the supernatant was collected for LC–MS analysis, which follows the methodology outlined in a previous study [20]. Metabolomics was conducted as previously described [21].

RNA-seq and data analysis

Cellular RNA was prepared and sent to GENE DENOVO (Guangzhou, China) for sequencing. Gene expression levels were calculated using TPM. The KEGG analysis was used to analyze the potential pathways. A $P < 0.05$ was used to identify statistically KEGG pathways. RNA-seq data were deposited in SRA database (SUB14882267).

Immunohistochemical staining

The tissue microarrays included human ccRCC tissues and paired adjacent tissues collected from our center. Anti-GAMT and anti-p53 antibodies were used at a ratio of 1:200. Two independent investigators conducted a blinded review of the stained sections and scored them based on the intensity and percentage of positively stained cells. Immunohistochemical scores were assessed on a scale of 0–12. Tissues with a score < 6 were considered to have low expression, while those with scores > 6 were considered to have high expression.

Animal experiments

Cells (1×10^6) suspended in Matrigel (Corning) were subcutaneously injected into 4–6 weeks female BALB/c-nu/nu mice (Gem Pharmatech) or injected into right subrenal capsule of 4–6 weeks female BALB/c mice to construct orthotopic tumor models. Each group consisted of a minimum of four mice, which were sacrificed at 4 weeks post-injection. Luminescence imaging and image analysis was performed using indiGo software. Starting at 1 weeks, mice were treated with the following therapeutic regimens: Axitinib (MCE, USA) was dosed at 50 mg/kg via an oral gavage twice daily. Fisetin (MCE, USA) was dosed at 100 mg/kg once daily by intraperitoneal injection. All animal experimental procedures were approved by the Animal Care and Use Committee.

High-throughput virtual screening and cellular thermal shift assay

High-throughput virtual screening (HTVS) was performed using the MCE compound database. A cellular thermal shift assay (CETSA) was performed as previously described [22]. Briefly, 769-P cells were exposed to DMSO or fisetin and incubated at 37 °C for 2 h. Identical cell suspension volumes were then heated for 3 min at temperatures ranging from 38 to 58 °C, increasing by 2 °C increments between each pair. After heating, the cells were incubated at room temperature for 3 min before being placed on ice. Soluble fractions were isolated via centrifugation and subsequently analyzed via western blotting. Melting curves were generated by plotting fold changes in immunoblot band densities.

Statistical analysis

Differences in mRNA or protein levels between two groups were analyzed using an unpaired Student's t-test. One-way or two-way analysis of variance was conducted for comparisons involving three or more groups. Asterisks in the graphs denote significantly contrasting test results. All primers sequences were provided in Table S1-3. All statistical analyses were performed using GraphPad Prism 6.0. A P-value < 0.05 was deemed statistically significant.

Results

GAMT is highly expressed in ccRCC and correlated with poor prognosis

First, we explored the metabolic alterations in ccRCC. We performed targeted metabolomic analysis on 30 patients with early-stage ccRCC and 30 patients with advanced-stage ccRCC, along with 30 samples of adjacent normal tissue (Fig. 1A). Then, pathway enrichment was assessed based on the top 10 metabolites. We found that arginine and proline metabolism was enriched significantly. Among this pathway, metabolites from creatine metabolism, including creatine and creatinine, were selected from the top 10 metabolites (Fig. 1B). Since these metabolites are all molecules in creatine metabolism pathway, we analyzed the gene expression levels related to this pathway using the TCGA-KIRC database.

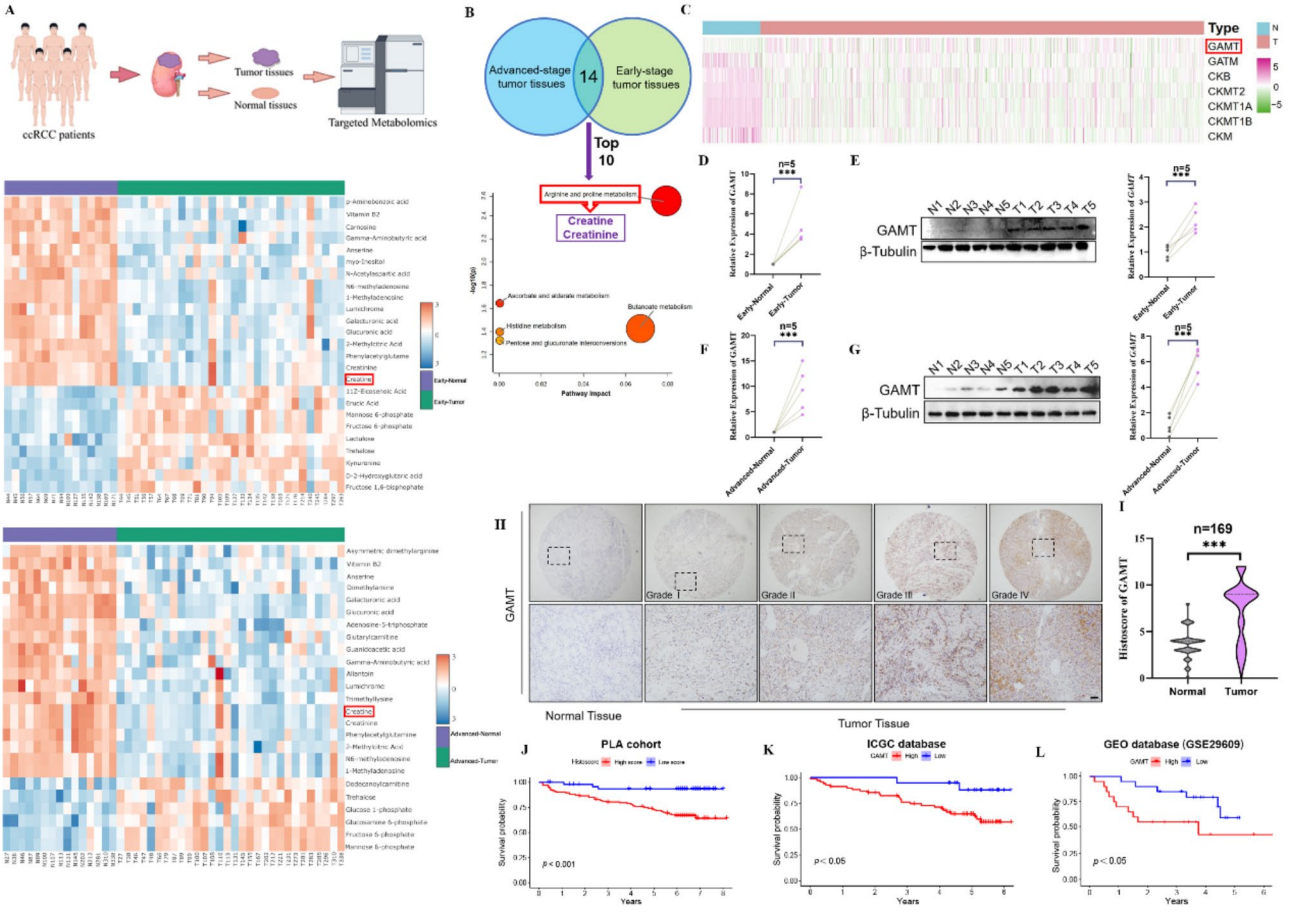

Fig. 1 GAMT is highly expressed in ccRCC and correlated with poor prognosis. (**A**) Metabolomics flow chart and heatmap of the differential metabolites at the early (upper) and advanced tumor stages (lower). (**B**) Venn diagram shows the significantly enriched pathways. (**C**) Heatmap overview of differentially expressed genes in creatine metabolism. **D**, **E.** qPCR (**D**) and Western blot (**E**) analyses of GAMT in early-stage tissues. **F**, **G.** qPCR (**F**) and Western blot (**G**) analyses of GAMT in advanced-stage tissues. **H**, **I.** Representative IHC images of GAMT in normal and ccRCC tissues from clinical tissue microarray (**H**); Comparation of histoscore between normal and tumor tissues (**I**). **J**. **K**-**M** curves for overall survival of ccRCC patients from high histoscore group and low histoscore group. **K**, **L.** Kaplan–Meier curves for overall survival of patients with ccRCC from the ICGC (left) and GEO (right) databases. *** $p < 0.001$

GAMT was uniquely identified as a highly expressed gene in the creatine metabolism pathway (Fig. 1C).

Next, to examine the expression levels of GAMT, we selected five pairs of samples from both early-stage and advanced ccRCC cohort that had undergone targeted metabolomic sequencing. The results indicated that GAMT mRNA and protein levels were higher in tumor tissues than in normal tissues (Fig. 1D-G). In addition, we also collected six paired ccRCC tissue samples from our center and found that GAMT was overexpressed in these samples (Fig. **S1** A, B). Then, we used a tissue microarray at our center to evaluate the clinical relevance of GAMT. Our analysis revealed elevated GAMT expression along with the ascending tumor grade (Fig. 1H, I). Kaplan–Meier analysis indicated that elevated GAMT expression was associated with an unfavorable prognosis in our database, ICGC and GSE29609 RCC databases (Fig. 1J-L). Collectively, these findings suggest a potential oncogenic role for GAMT in ccRCC, which prompted us to further explore its function in the development of ccRCC.

GAMT promotes ccRCC proliferation, migration and invasion

We further investigated whether GAMT affects the occurrence and development of ccRCC both in vitro and in vivo. First, GAMT expression was detected in tumor cell lines. Strong GAMT expression was observed in most tumor cells (Fig. S1 C, D). Then, we selected 769-P and SN12 cells with relatively high GAMT levels to knockdown their expression, and selected A498 and Renca cells to overexpress GAMT (Fig. **S1** E–H). GAMT knockdown markedly reduced 769-P cell proliferation and colony formation, as evidenced by the CCK-8 and colony formation assay results. However, Overexpression of GAMT increased the proliferation of A498 cells (Fig. 2A-C).

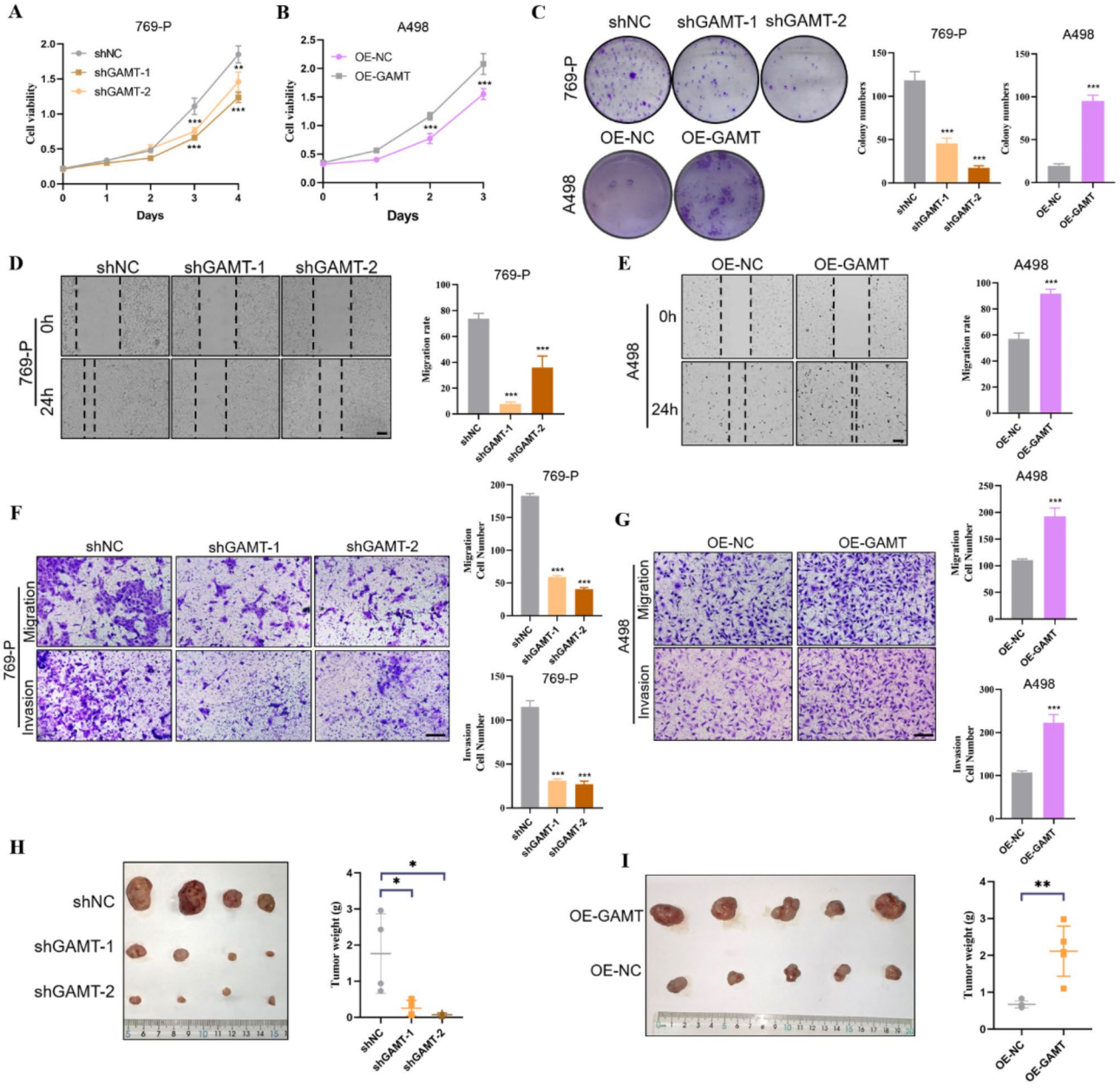

Fig. 2 GAMT induces ccRCC proliferation, migration, and invasion. **A**-**C** Effect of GAMT knockdown and overexpression on ccRCC cell proliferation was assessed via CCK8 (**A**, **B**) and plate colony formation assays (**C**). Quantification of colony numbers (right). **D**, **E**. Effect of GAMT knockdown (**D**) and overexpression (**E**) on ccRCC cell migration and invasion was measured via wound healing. **F**, **G.** Effect of GAMT knockdown (**F**) and overexpression (**G**) on ccRCC cell migration and invasion was measured using Transwell assays. Quantification of colony numbers (right). **H**, **I.** Image (left) and tumor weight (right) of xenograft tumors from mice with GAMT knockdown SN12 cells and GAMT overexpression Renca cells. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Further, GAMT knockdown significantly impaired the migration and invasion capabilities of 769-P cells, as determined through the Transwell and wound healing analyses. In contrast, GAMT overexpression in A498 cells increased their migration and invasion capabilities (Fig. 2D-G). In vivo experiments also showed GAMT knockdown reduced xenograft tumor growth, whereas GAMT overexpression promoted xenograft tumor growth (Fig. 2H, I). Our findings indicate that GAMT is necessary for ccRCC proliferation, migration, and invasion both in vitro and in vivo.

GAMT influences ccRCC independent of its enzyme activity

GAMT is an enzyme that involved in creatine biosynthesis pathway. However, our study has showed that creatine could inhibit ccRCC cell progression [4]. Several pervious studies have reported that proteins can function via non-enzymatic activities [23]. This prompted us to investigate

whether the unusually high expression of GAMT occurs via nonenzymatic mechanisms. Next, we determined whether the enzymatic activity of GAMT affected ccRCC development. We found no differences in creatine concentrations between GAMT knockdown and GAMT overexpression ccRCC cells (Fig. 3A, B). We treated high GAMT expression and GAMT overexpression ccRCC cells with cyclocreatine, an analog of creatine and an inhibitor of the creatine biosynthesis pathway [24]. We found that cyclocreatine (3 mM) had a minimal effect on creatine concentrations (Fig. 3C).

GAMT deficiency is a rare, inherited metabolic disorder resulting from mutations in the GAMT gene. The 327 G > A mutation is a common mutation that leads

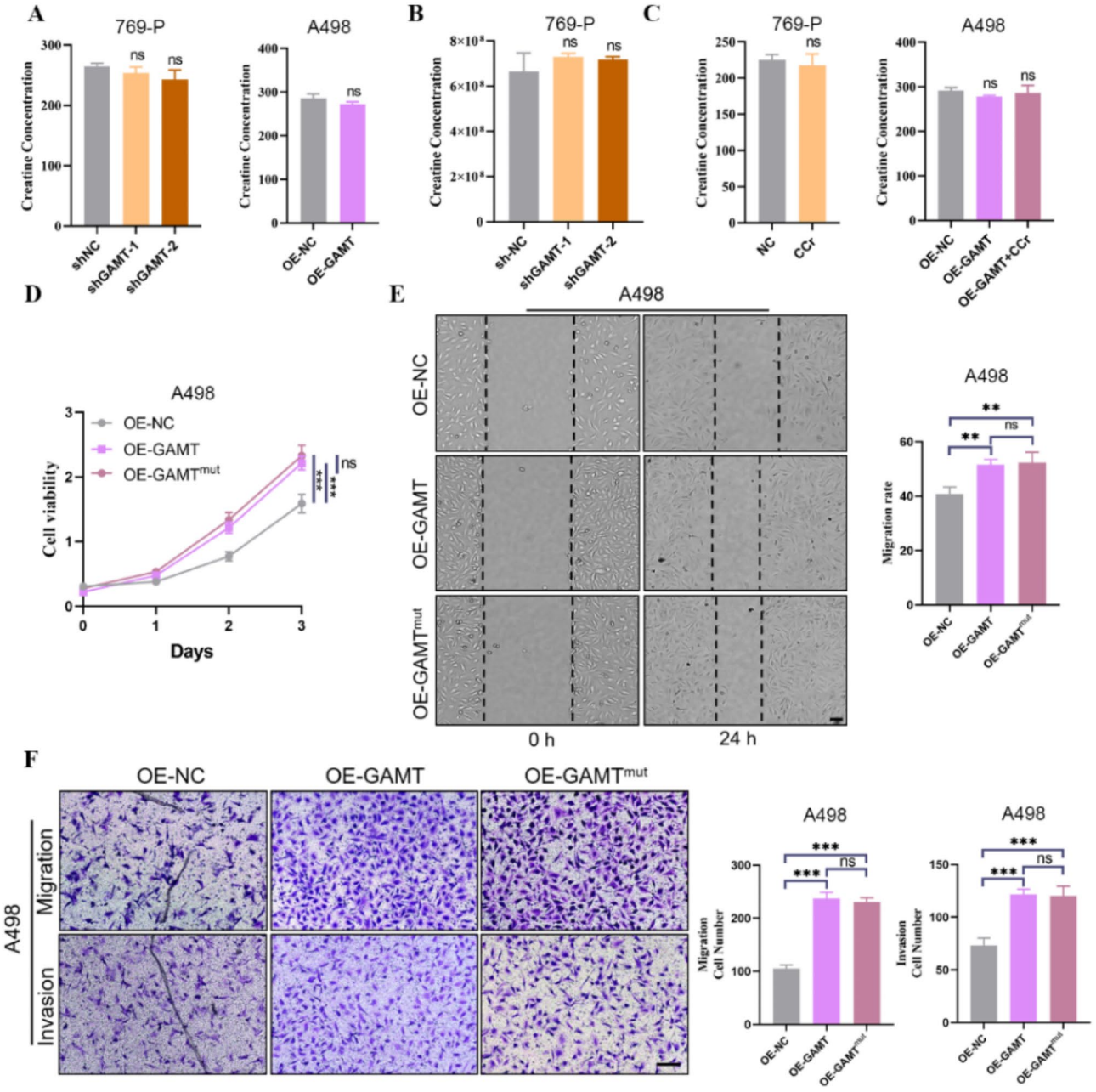

Fig. 3 GAMT regulates ccRCC independent of its enzyme activity. **A**, **B**. Creatine levels in RCC cells with GAMT knockdown or overexpression were validated using ELISA (**A**) and LC–MS (**B**). **C.** Effect of cyclocreatine (CCr) on creatine levels in GAMT high-expression ccRCC cells and GAMT overexpression ccRCC cells validated using ELISA. **D.** CCK-8 assay was performed on ccRCC cells stably expressing mutated or wild-type GAMT. **E.** Representative images of migrated ccRCC cells overexpressing GAMT and GAMT overexpression ccRCC cells stably expressing mutant GAMT via wound healing. **F.** Representative images of migrated or invaded ccRCC cells overexpressing GAMT and GAMT overexpression ccRCC cells stably expressing mutant GAMT via Transwell assays. ** $p < 0.01$, *** $p < 0.001$

to the loss of functional GAMT proteins [25]. We constructed a 327 G > A mutant plasmid and transfected it into A498 cells. Similarly, we found no significant differences in ccRCC proliferation, migration and invasion between GAMT-overexpressing and GAMT-mutated ccRCC cells (Fig. 3D-F). These data suggest that GAMT promotes ccRCC development independent of its enzymatic activity.

GAMT promotes ccRCC progression by targeting tumor suppressor protein p53

To explore how GAMT promotes ccRCC development and progression, we conducted RNA sequencing assay to compare the transcriptome of control and GAMT knockdown ccRCC cells. According to the KEGG pathway analysis of the RNA sequencing results, the p53 signaling pathway was significantly identified (Fig. 4A). Knowing that GAMT acts through p53 signaling pathway, we examined the expression of p53 and its downstream targets p21. Subsequently, GAMT knockdown and overexpression did not change p53 mRNA levels but significantly affected the protein levels (Fig. 4B, C, Fig. S2A, B). Also, p53 and p21 expression levels increased after GAMT knockdown, but decreased after GAMT overexpression (Fig. 4B, C).

Next, we determined if p53 is required for GAMT-mediated oncogenesis. We introduced si-p53 and p53 overexpression vector into GAMT knockdown or GAMT overexpression cells, separately. The results indicated that GAMT knockdown-induced cell proliferation, migration and invasion were partial rescued by p53 knockdown (Fig. 4D, E, H, J). Moreover, p53 overexpression abrogated cell proliferation, migration and invasion induced by GAMT overexpression (Fig. 4F, G, I, K). The correlation analysis based on our ccRCC cohort also showed significant negative correlations between GAMT and p53 (Fig. 4O). Taken together, our results suggest that GAMT-induced oncogenesis is p53-dependent in ccRCC.

GAMT inhibitor Fisetin exhibits strong inhibitory effects on ccRCC

Currently, there are no commercially available GAMT inhibitors for clinical applications. Therefore, we sought to identify potential GAMT inhibitors by performing molecular docking-based HTVS of 85,000 compounds across three databases. We identified four potential compounds that have been used in clinical experiments and selected fisetin (exhibiting the best docking score) for further in vitro and in vivo validation (Fig. 5A, B). After evaluating the IC_{50} value of fisetin, we found that fisetin significantly inhibited GAMT expression and promoted p53 expression in a dose-dependent manner (Fig. 5C, Fig. S3). Then, we used CETSA to verify the binding of fisetin to GAMT. The results showed that fisetin treatment elevated the melting temperature, implying a physical interaction between GAMT and fisetin (Fig. 5D).

Further, we assessed the effects of fisetin on the tumor phenotype both in vitro and in vivo. First, we examined the therapeutic effect of fisetin in ccRCC cells and three ccRCC-derived organoid models. Fisetin markedly suppressed cell proliferation, migration, and invasion in 769-P cells with high GAMT expression (Fig. 5E-G). Similarly, fisetin reduced both number and size of three organoids (Fig. 5H, I). We also treated GAMT overexpression A498 cells with fisetin, which indicates the similar tumor-suppressive effect on ccRCC cells (Fig. S4 A-D). Next, we validated its efficacy in vivo. Fisetin suppressed the growth of SN12 subcutaneous and Renca orthotopic xenograft growth in mice (Fig. 5J, K). Moreover, live imaging also demonstrated that fisetin inhibited the growth of tumor cells with the gradually increased duration of treatment (Fig. 5L). Since resistance is common in RCC patients undergoing TKI treatment, we performed combined axitinib plus fisetin treatment. Our results showed the combination suppressed tumor growth (Fig. 5M). Together, our data suggest that fisetin, a GAMT-targeted inhibitor, is a potential therapeutic strategy for ccRCC.

Discussion

ccRCC is the most common histologic subtype among kidney cancer, which does not have notable clinical signs, leading to diagnostic difficulties and delay in treatment. Although treatments, such as targeted therapy and immune checkpoint inhibitors, have significantly progressed, they are still insufficient for ccRCC patients due to drug resistance [26]. Therefore, there is an urgent need to find new therapeutic targets for ccRCC.

At present, multiple studies have shown that ccRCC is fundamentally a metabolic disorder characterized by the reprogramming of energy metabolism, which allows the tumor cells to survive in conditions of energy deprivation and hypoxia [27–30]. The metabolic reprogram of ccRCC covers many processes, such as aerobic glycolysis and fatty acids metabolism. Notably, the metabolism involving glycolytic flux is partitioned [31–33], concomitant with impaired mitochondrial bioenergetics and oxidative phosphorylation (OxPhos) machinery. Furthermore, dysregulation of fatty acid oxidation has been observed in different grade of ccRCC as well [31, 34–37]. These results suggest that targeting metabolic abnormalities are potential therapeutic strategies of ccRCC. Here, using targeted metabolomic analysis of clinical ccRCC tissue samples and TCGA database, we found GAMT, as one of enzymes among creatine metabolism, is significantly changed in ccRCC.

Currently, there is little known about the potential role of GAMT in cancer development and progression. In

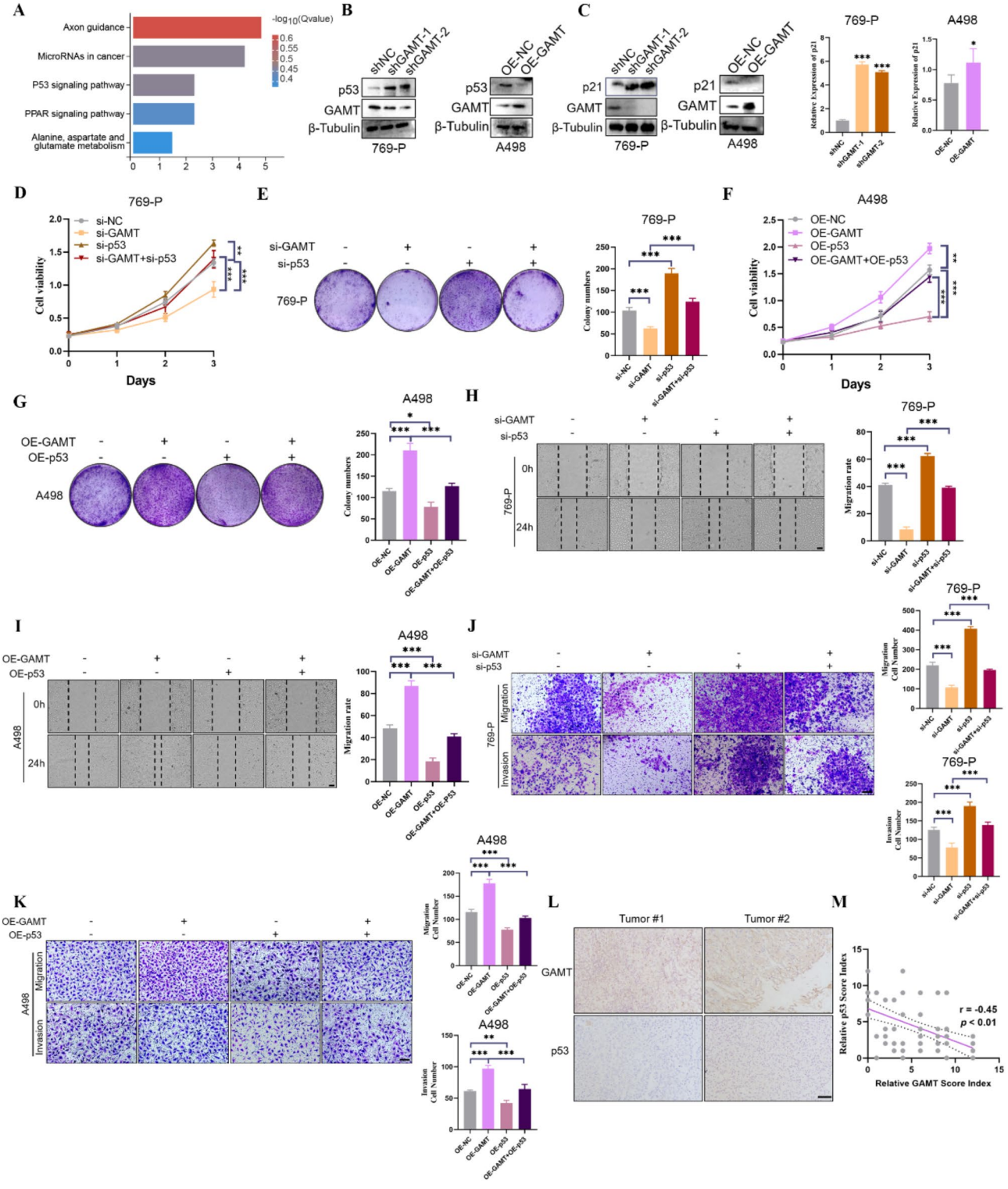

Fig. 4 GAMT could regulate p53 expression and promotes ccRCC progression via p53. (**A**) KEGG pathway analysis identified p53 signaling pathway. (**B**) Western blot validated the expression of p53 in GAMT knockdown and overexpression ccRCC cells. (**C**) Western blot (left) and qPCR (right) analysis validated the expression of p21 in GAMT knockdown and overexpression ccRCC cells. **D**, **E.** p53 can partially rescue the viability and proliferation of ccRCC cell caused by GAMT knockdown. **F**, **G.** p53 can partially rescue the viability and proliferation of ccRCC cell caused by GAMT overexpression. **H**, **I.** p53 can partially rescue the migration and invasion of ccRCC cell caused by GAMT knockdown. **J**, **K.** p53 can partially rescue the migration and invasion of ccRCC cell caused by GAMT overexpression. **L**, **M.** Representative immunohistochemistry staining of GAMT and p53 protein expression in RCC tissues (**L**). Correlation analysis of GAMT and p53 expression levels in 63 ccRCC patients (**M**). * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$

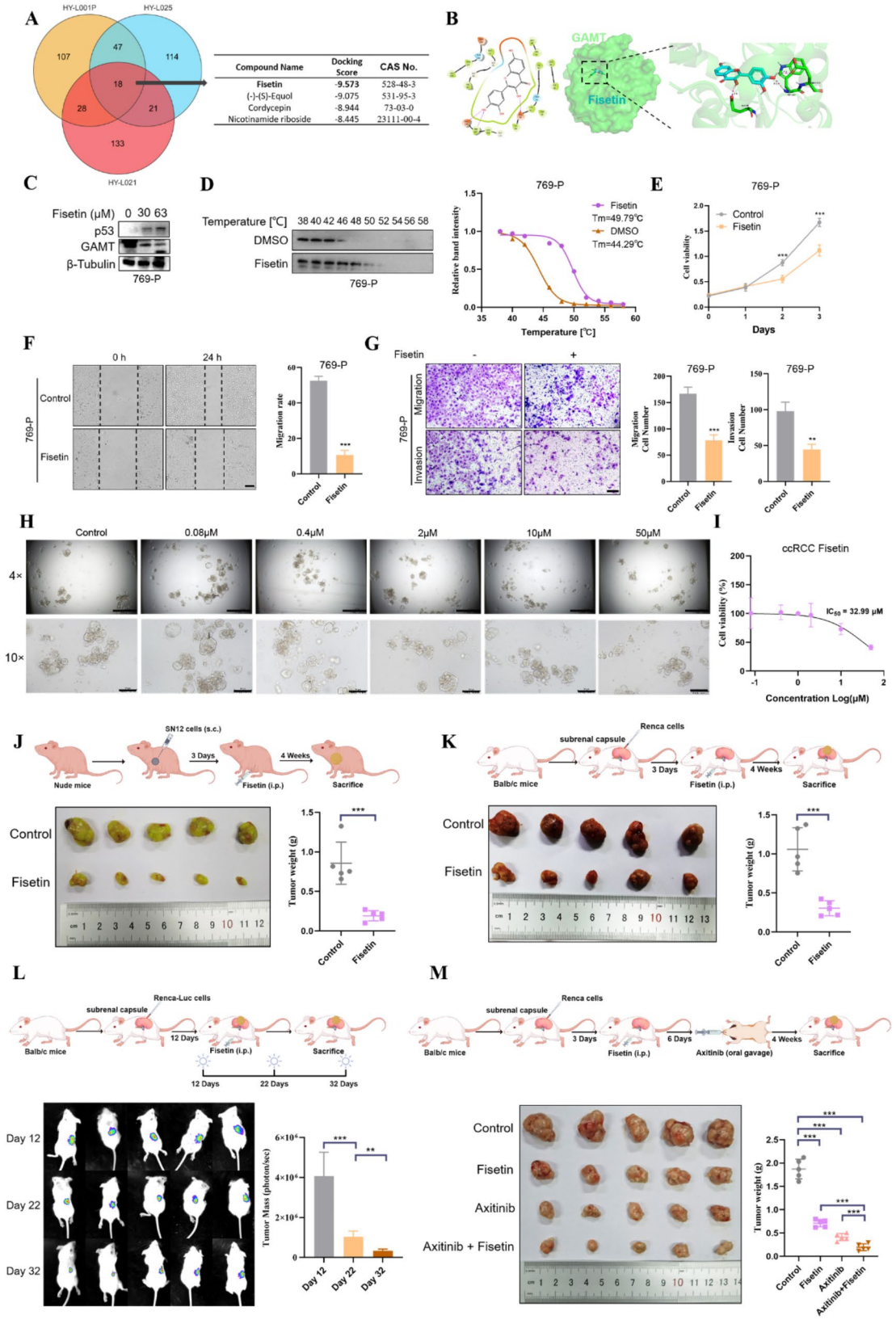

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Fig. 5 GAMT inhibitor fisetin shows inhibitory effect on ccRCC and decreases TKI resistance. (**A**) Venn diagram and docking scores of the top four GAMT inhibitor candidates identified via HTVS. (**B**) Chemical structure and in silico docking of fisetin into the active pocket of the human GAMT protein. (**C**) Western blot analysis of GAMT and p53 expression in ccRCC cells treated with fisetin after 48 h. (**D**) Western blot validated the direct binding between fisetin and GAMT via CETSA assay in 769-P cells. CETSA curves showing GAMT protein stability in the presence of DMSO or fisetin in both cell lines. **E-G.** Effect of GAMT knockdown on ccRCC cell proliferation, migration and invasion was assessed via CCK8 (**E**), wound healing (**GF**) and Transwell assays (**G**). **H.** Representative images showed the number and size of ccRCC organoid treated by fisetin. **I.** Drug IC_{50} values of ccRCC organoids. **J**, **K.** Image and tumor weight of subcutaneous (**K**) and orthotopic (**L**) xenograft tumors from mice treated with fisetin. **L.** Representative bioluminescent images of balb/c mice undergone orthotopic implantation with Luc-labeling Renca cells treated with fisetin. **M.** Mice were administered with fisetin and axitinib alone or in combination. Image and tumor weight of subcutaneous xenograft tumors from mice. The mice were treated with fisetin or axitinib or the combination for 23 days. ** $p < 0.01$, *** $p < 0.001$

pancreatic cancer, GAMT is low-expressed and could be promoted by transcription factor RUNX2, and further induced cell apoptosis [10]. An investigation of the clinical implications of GAMT showed that it may associate with the prognosis of patient with gastric cancer and colon cancer [8, 9, 38]. However, the effect of GAMT on ccRCC remains poorly understood. In this study, we found that GAMT exhibits high expression levels in ccRCC tissues and is inversely associated with poor prognosis in the ICGC, GEO ccRCC, and our ccRCC databases. Both in vitro and in vivo experiments demonstrated that GAMT enhances ccRCC cell proliferation, migration, invasion and tumor growth. These results suggest that *GAMT* is an oncogene that promotes ccRCC progression.

Our previous study revealed that creatine could inhibit ccRCC cell progression [4]. However, considering the elevated expression of creatine synthase GAMT, we speculated that GAMT may play a non-canonical role in ccRCC. Next, we detected the creatine concentration in GAMT knockdown and overexpression ccRCC cells. Unexpectedly, creatine levels did not significantly change upon overexpression of GAMT and treatment with cyclocreatine, a creatine biosynthesis inhibitor. Further, no significant differences in cell proliferation, migration, or invasion were observed after mutating the active site of GAMT. These findings These results confirm our conjecture that elevated GAMT has functions beyond its enzymatic activity in ccRCC. Notably, numerous metabolic enzymes, traditionally associated with metabolic functions, also play roles in tumor regulation independent of their enzymatic activities [39]. Enzymes involved in sugar metabolism, such as PKM2 and HK, which are typically associated with glycolysis, could exhibit protein kinase activities crucial for tumorigenesis [40–42]. Moreover, other enzymes involved in lipid metabolism and TCA cycle, such as ACSS2, ACLY, and α-KGDH could form complexes with chromatin modulators to regulate gene expression [14, 16, 43, 44]. These results extend our knowledge regarding the non-canonical functions of GAMT in tumor.

We then investigated the mechanism of GAMT in promoting ccRCC progression. Using RNA sequencing, we found that GAMT knockdown could regulate ccRCC progression via the p53 signaling pathway. Specifically, GAMT could inhibit the protein expression of p53. p53 is an essential tumor suppressor that regulates vast range of processes, including the cell cycle, apoptosis, proliferation and tumor progression [45]. Meanwhile, the p53 protein also undergoes many post-translational modifications, such as ubiquitination, phosphorylation and acetylation, which influences p53 protein level, cellular localization, target selectivity [46]. The C-terminal lysine residues of p53 is the most common ubiquitinated site. Moreover, the ubiquitination of p53 is regulated by diverse regulators, including MDM2, MDMX, CHIP, COP1 and Pirh2 [47]. However, whether the regulatory mechanism by which GAMT influences p53 protein expression is mediated through the ubiquitination pathway remains to be further investigated. Previous study indicated that some molecular could activate and increase the affinity of protein for substrates, which contributes to the proximity to the ubiquitin docking site and then allows efficient ubiquitin binding [48]. Whether GAMT have the same function still needs further study. Then, to undermine the essential role of p53 in GAMT-mediated oncogenesis, we performed rescue experiments and found that GAMT induced cell proliferation, migration and invasion were rescued by p53. These results further indicate the downstream regulatory mechanism involved in GAMT, which help us to better understand the role of GAMT play in ccRCC.

Surgery is the primary clinical treatment for ccRCC. Despite the use of targeted therapy for advanced ccRCC, resistance to these drugs eventually develops in some patients with ccRCC [49, 50]. GAMT is highly expressed in ccRCC. However, small molecule targeting GAMT has not been reported. Here, through virtual screening, we discovered that fisetin acts as a potential GAMT inhibitor agonist with antitumor activity in ccRCC cells. Fisetin, a naturally occurring flavonoid currently in clinical trials, has shown promising tumor-suppressive effects by regulating VEGF, MAPK signaling pathway, apoptosis and angiogenesis processes [51, 52]. In this study, fisetin decreased GAMT expression, increased p53 expression and suppressed the malignant behavior in ccRCC cells, ccRCC organoids, xenograft mouse model and orthotopic tumor models. Axitinib is a first line treatment for

advanced ccRCC patients. However, TKI resistance often results in the recurrence of tumors, leading to treatment failure [53]. Importantly, in this study, we found that fisetin could enhance the efficacy of axitinib for ccRCC treatment. However, the mechanism in detail for how GAMT was involved in this process still needs be further explored. Therefore, this GAMT-based study reveals a potential therapeutic candidate and suggests its application in ccRCC treatment.

Conclusions

Together, this study expands on earlier research by demonstrating GAMT's nonenzymatic role in ccRCC and reveals that GAMT could inhibit p53 protein expression in ccRCC. Also, fisetin, a potential GAMT inhibitor, may act as an important drug for ccRCC therapy in the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-025-00641-y.

Supplementary Material 1: **Supplementary Fig. S1**. GAMT knockdown and overexpression efficiencies in CcRCC cells. A, B qPCR (A) and Western blot (B) analyses of GAMT in ccRCC tissues. C, D. qPCR (B) and western blot (C) analyses of GAMT in ccRCC cell lines and tissues. E–H. qPCR and western bloting were used to detect the knockdown and overexpression efficiency of GAMT in RCC cell lines. ** $p < 0.01$, *** $p < 0.001$. **Supplementary Fig. S2**. GAMT knockdown and overexpression does not affect p53 mRNA level. A RNA sequencing validated the expression of p53 in GAMT knockdown ccRCC cells. B. qPCR analysis validated the expression of p53 in GAMT knockdown and overexpression ccRCC cells. **Supplementary Fig. S3**. The IC_{50} values of Fisetin in 769-P cells were measured. **Supplementary Fig. S4**. GAMT inhibitor Fisetin shows inhibitory effect on GAMT overexpression CcRCC cells. A Effect of GAMT knockdown on ccRCC cell proliferation was assessed via CCK8. B, C. Effect of GAMT knockdown on ccRCC cell migration and invasion was assessed via wound healing (B) and Transwell invasion assays (C). D. Image and tumor weight of subcutaneous xenograft tumors from mice injected with GAMT overexpression Renca cells and then treated with fisetin.

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Author contributions

B.Z., K.L and J.F.: Conceptualization, Writing– original draft and Writing– review & editing. B.Z. and YQ.O.: Investigation, Methodology. Y.H.W and T.Y.J.: Software and Methodology. S.T. and X.R.C.: Data curation and Resources. W.L.Q and T.W.C.: Formal analysis and visualization. X.Z., X.B.L. and X.M.: Supervision and Project administration.

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Data availability

The datasets generated and/or analysed during the current study are available in the SRA repository (SUB14882267). The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All studies were approved by the Ethics Committee of the third medical center of PLA General Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Young M, et al. Renal cell carcinoma. Lancet (London England). 2024;404:476–91. https://doi.org/10.1016/s0140-6736(24)00917-6
2. Powles T, et al. Renal cell carcinoma: ESMO clinical practice guideline for diagnosis, treatment and follow-up. Annals Oncology: Official J Eur Soc Med Oncol. 2024;35:692–706. https://doi.org/10.1016/j.annonc.2024.05.537
3. Zisman A, et al. Risk group assessment and clinical outcome algorithm to predict the natural history of patients with surgically resected renal cell carcinoma. J Clin Oncology: Official J Am Soc Clin Oncol. 2002;20:4559–66. https://doi.org/10.1200/jco.2002.05.111
4. Zheng B, et al. Identification of metabolic dysregulation and biomarkers for clear cell renal cell carcinoma. Clin Translational Med. 2024;14(e70142). https://doi.org/10.1002/ctm2.70142
5. Zhang L, et al. Creatine promotes cancer metastasis through activation of Smad2/3. Cell Metabol. 2021;33:1111–23..e1114.
6. Kurth I, et al. Therapeutic targeting of SLC6A8 creatine transporter suppresses colon cancer progression and modulates human creatine levels. Sci Adv. 2021;7:eabi7511. https://doi.org/10.1126/sciadv.abi7511
7. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80:1107–213. https://doi.org/10.1152/physrev.2000.80.3.1107
8. Gong C, et al. Elastic net-based identification of GAMT as potential diagnostic marker for early-stage gastric cancer. Biochem Biophys Res Commun. 2022;591:7–12. https://doi.org/10.1016/j.bbrc.2021.12.055
9. Cui Y, et al. Identification of Metabolic-Associated genes for the prediction of colon and rectal adenocarcinoma. OncoTargets Therapy. 2021;14:2259–77. https://doi.org/10.2147/ott.S297134
10. Yuan H, et al. Role of a novel circRNA-CGNL1 in regulating pancreatic cancer progression via NUDT4-HDAC4-RUNX2-GAMT-mediated apoptosis. Mol Cancer. 2024;23:27. https://doi.org/10.1186/s12943-023-01923-7
11. Bian X, et al. Regulation of gene expression by glycolytic and gluconeogenic enzymes. Trends Cell Biol. 2022;32:786–99. https://doi.org/10.1016/j.tcb.2022.02.003
12. Xu D, et al. The evolving landscape of noncanonical functions of metabolic enzymes in cancer and other pathologies. Cell Metabol. 2021;33:33–50. https://doi.org/10.1016/j.cmet.2020.12.015
13. Li X, Egervari G, Wang Y, Berger SL, Lu Z. Regulation of chromatin and gene expression by metabolic enzymes and metabolites. Nat Rev Mol Cell Biol. 2018;19:563–78. https://doi.org/10.1038/s41580-018-0029-7
14. Sutendra G, et al. A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation. Cell. 2014;158:84–97. https://doi.org/10.1016/j.cell.2014.04.046
15. Wang Z, et al. Methionine is a metabolic dependency of tumor-initiating cells. Nat Med. 2019;25:825–37. https://doi.org/10.1038/s41591-019-0423-5
16. Li X, et al. Nucleus-Translocated ACSS2 promotes gene transcription for lysosomal biogenesis and autophagy. Mol Cell. 2017;66:684–e697689. https://doi.org/10.1016/j.molcel.2017.04.026
17. Yan XQ, et al. Toripalimab plus axitinib versus Sunitinib as first-line treatment for advanced renal cell carcinoma: RENOTORCH, a randomized, open-label, phase III study. Annals Oncology: Official J Eur Soc Med Oncol. 2024;35:190–9. https://doi.org/10.1016/j.annonc.2023.09.3108

18. Sinha R, et al. Analysis of renal cancer cell lines from two major resources enables genomics-guided cell line selection. Nat Commun. 2017;8:15165. https://doi.org/10.1038/ncomms15165
19. Liu XY, et al. SYTL4 downregulates microtubule stability and confers Paclitaxel resistance in triple-negative breast cancer. Theranostics. 2020;10:10940–56. https://doi.org/10.7150/thno.45207
20. Gerriets VA, et al. Foxp3 and Toll-like receptor signaling balance T(reg) cell anabolic metabolism for suppression. Nat Immunol. 2016;17:1459–66. https://doi.org/10.1038/ni.3577
21. Liao C, et al. Integrated metabolic profiling and transcriptional analysis reveals therapeutic modalities for targeting rapidly proliferating breast cancers. Cancer Res. 2022;82:665–80. https://doi.org/10.1158/0008-5472.Can-21-2745
22. Martinez Molina D, et al. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. Sci (New York N Y). 2013;341:84–7. https://doi.org/10.1126/science.1233606
23. Zhang M, et al. USP18 recruits USP20 to promote innate antiviral response through deubiquitinating STING/MITA. Cell Res. 2016;26:1302–19. https://doi.org/10.1038/cr.2016.125
24. Patel R, et al. Cyclocreatine suppresses creatine metabolism and impairs prostate cancer progression. Cancer Res. 2022;82:2565–75. https://doi.org/10.1158/0008-5472.Can-21-1301
25. Mercimek-Mahmutoglu S, et al. A pilot study to estimate incidence of Guanidinoacetate methyltransferase deficiency in newborns by direct sequencing of the GAMT gene. Gene. 2016;575:127–31. https://doi.org/10.1016/j.gene.2015.08.045
26. Escudier B, et al. Renal cell carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Annals Oncology: Official J Eur Soc Med Oncol. 2016;27:v58–68. https://doi.org/10.1093/annonc/mdw328
27. di Meo NA, et al. The dark side of lipid metabolism in prostate and renal carcinoma: novel insights into molecular diagnostic and biomarker discovery. Expert Rev Mol Diagn. 2023;23:297–313. https://doi.org/10.1080/14737159.2023.2195553
28. Lucarelli G, et al. Metabolomic insights into pathophysiological mechanisms and biomarker discovery in clear cell renal cell carcinoma. Expert Rev Mol Diagn. 2019;19:397–407. https://doi.org/10.1080/14737159.2019.1607729
29. di Meo NA, et al. Renal cell carcinoma as a metabolic disease: an update on main pathways, potential biomarkers, and therapeutic targets. Int J Mol Sci. 2022;23. https://doi.org/10.3390/ijms232214360
30. De Marco S, et al. The cross-talk between Abl2 tyrosine kinase and TGFβ1 signalling modulates the invasion of clear cell renal cell carcinoma cells. FEBS Lett. 2023;597:1098–113. https://doi.org/10.1002/1873-3468.14531
31. Bianchi C, et al. The glucose and lipid metabolism reprogramming is grade-dependent in clear cell renal cell carcinoma primary cultures and is targetable to modulate cell viability and proliferation. Oncotarget. 2017;8:113502–15. https://doi.org/10.18632/oncotarget.23056
32. Ragone R, et al. Renal cell carcinoma: A study through NMR-Based metabolomics combined with transcriptomics. Dis (Basel Switzerland). 2016;4. https://doi.org/10.3390/diseases4010007
33. Lucarelli G, et al. Metabolomic profile of Glycolysis and the Pentose phosphate pathway identifies the central role of glucose-6-phosphate dehydrogenase in clear cell-renal cell carcinoma. Oncotarget. 2015;6:13371–86. https://doi.org/10.18632/oncotarget.3823
34. Lucarelli G, et al. Integrated multi-omics characterization reveals a distinctive metabolic signature and the role of NDUFA4L2 in promoting angiogenesis, chemoresistance, and mitochondrial dysfunction in clear cell renal cell carcinoma. Aging. 2018;10:3957–85. https://doi.org/10.18632/aging.101685
35. Bombelli S, et al. 36-kDa Annexin A3 isoform negatively modulates lipid storage in clear cell renal cell carcinoma cells. Am J Pathol. 2020;190:2317–26. https://doi.org/10.1016/j.ajpath.2020.08.008
36. Lucarelli G, et al. MUC1 tissue expression and its soluble form CA15-3 identify a clear cell renal cell carcinoma with distinct metabolic profile and poor clinical outcome. Int J Mol Sci. 2022;23. https://doi.org/10.3390/ijms232213968
37. Milella M, et al. The role of MUC1 in renal cell carcinoma. Biomolecules. 2024;14. https://doi.org/10.3390/biom14030315
38. Liu JB, et al. Chemo-resistant gastric cancer associated gene expression signature: bioinformatics analysis based on gene expression omnibus. Anticancer Res. 2019;39:1689–98. https://doi.org/10.21873/anticanres.13274
39. Lu S, Wang Y. Nonmetabolic functions of metabolic enzymes in cancer development. Cancer Commun (London England). 2018;38:63. https://doi.org/10.1186/s40880-018-0336-6
40. Mellati AA, Yücel M, Altinörs N, Gündüz U. Regulation of M2-type pyruvate kinase from human meningioma by allosteric effectors Fructose 1,6 diphosphate and L-alanine. Cancer Biochem Biophys. 1992;13:33–41.
41. Christofk HR, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. 2008;452:230–3. https://doi.org/10.1038/nature06734
42. Li X, et al. A splicing switch from ketohexokinase-C to ketohexokinase-A drives hepatocellular carcinoma formation. Nat Cell Biol. 2016;18:561–71. https://doi.org/10.1038/ncb3338
43. Sivanand S, et al. Nuclear Acetyl-CoA production by ACLY promotes homologous recombination. Mol Cell. 2017;67:252–65..e256.
44. Wang Y, et al. KAT2A coupled with the α-KGDH complex acts as a histone H3 succinyltransferase. Nature. 2017;552:273–7. https://doi.org/10.1038/nature25003
45. Ventura A, et al. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007;445:661–5. https://doi.org/10.1038/nature05541
46. Liu Y, Su Z, Tavana O, Gu W. Understanding the complexity of p53 in a new era of tumor suppression. Cancer Cell. 2024;42:946–67. https://doi.org/10.1016/j.ccell.2024.04.009
47. Liu Y, Tavana O, Gu W. p53 modifications: exquisite decorations of the powerful guardian. J Mol Cell Biol. 2019;11:564–77. https://doi.org/10.1093/jmcb/mjz060
48. Sahtoe DD, et al. Mechanism of UCH-L5 activation and Inhibition by DEUBAD domains in RPN13 and INO80G. Mol Cell. 2015;57:887–900. https://doi.org/10.1016/j.molcel.2014.12.039
49. Barata PC, Rini BI. Treatment of renal cell carcinoma: current status and future directions. Cancer J Clin. 2017;67:507–24. https://doi.org/10.3322/caac.21411
50. Stewart GD, et al. The multispeciality approach to the management of localised kidney cancer. Lancet (London England). 2022;400:523–34. https://doi.org/10.1016/s0140-6736(22)01059-5
51. Zhou C, et al. Biological effects and mechanisms of Fisetin in cancer: a promising anti-cancer agent. Eur J Med Res. 2023;28. https://doi.org/10.1186/s40001-023-01271-8
52. Rahmani AH, Almatroudi A, Allemailem KS, Khan AA, Almatroodi SA. The potential role of Fisetin, a flavonoid in cancer prevention and treatment. Molecules. 2022;27. https://doi.org/10.3390/molecules27249009
53. Motzer RJ, et al. Axitinib versus Sorafenib as second-line treatment for advanced renal cell carcinoma: overall survival analysis and updated results from a randomised phase 3 trial. Lancet Oncol. 2013;14:552–62. https://doi.org/10.1016/s1470-2045(13)70093-7

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