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IMMT promotes hepatocellular carcinoma formation via PI3K/AKT/mTOR pathway

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Abstract

Objectives: Hepatocellular carcinoma (HCC) remains a global challenge. Finding new specific targets has significant clinical value for the treatment of hepatocellular carcinoma. In this study, we discovered a new biomarker targeting HCC. At present, the role of Inner Membrane Mitochondrial Protein (IMMT) in various malignant tumors is receiving increasing attention, but its molecular mechanism of action in the malignant process of HCC has not been fully elucidated. This study aims to investigate the key regulatory mechanisms of IMMT in the formation of HCC.

Methods: Using TCGA data and clinical HCC samples, we first studied the expression of IMMT in HCC tissues and its correlation with malignant prognosis of patients. The regulatory effect of IMMT on HCC was studied by lentivirus infection. *In vitro*, the effects of IMMT on the proliferation, migration and apoptosis of cells were investigated by CCK8, colony formation assay, transwell and flow cytometry. Consistently, *in vivo* experiments, the regulation of tumor growth by IMMT was studied by constructing subcutaneous transplanted tumor and liver carcinoma *in situ*. In terms of mechanism, we predicted and verified the downstream genes of IMMT with the help of string database.

Results: We found that IMMT was significantly up-regulated in HCC tissues and was significantly positively correlated with poor prognosis of patients. Functionally, we demonstrated

that IMMT knockdown significantly inhibited HCC cell proliferation, migration and promoted cell apoptosis *in vitro*. Similarly, the knockdown of IMMT also significantly weakened the progression of tumors *in vivo*. In terms of mechanism, we demonstrate for the first time that IMMT can regulate the progression of HCC by influencing the activation of PI3K-AKT-mTOR pathway.

Conclusions: Collectively, our research findings elucidate the hitherto unexplored important role of the IMMT/PI3K/AKT/mTOR axis in the formation of HCC, and provide a new biomarker for clinical diagnosis and treatment of HCC.

Keywords: hepatocellular carcinoma; inner membrane mitochondrial protein; PI3K/AKT/mTOR pathway; novel target; systematic treatment

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor in the world, the overall burden of HCC is still relatively severe worldwide [1]. In fact, according to SEER's (Surveillance, Epidemiology, and End Results) report, HCC has been the fastest-growing cause of cancer-related deaths in the United States since the early 21st century [2]. If this trend continues, HCC is expected to become the third leading cause of cancer-related death by 2030 [3]. Although our understanding of the pathophysiological drivers of HCC has improved, these breakthroughs in basic scientific research have not yet been translated into clinical practice, and their impact on improving patients' survival and profitability is still limited. Currently, systematic therapies including immune checkpoint inhibitors (ICIs), tyrosine kinase inhibitors (TKIs), and monoclonal antibodies pose challenges to traditional therapies for HCC such as surgical excision and transcatheter arterial chemoembolization (TACE) [4]. Therefore, in-depth exploration of new molecular biological targets is still promising to improve the predicament of HCC treatment.

Inner Membrane Mitochondrial Protein (IMMT) is a specific protein coding gene, mainly located in the inner mitochondrial membrane. IMMT belongs to the mitochondrial contact site and cristae organizing system (MICOS) complex subunit Mic60 family [5]. In terms of biological function, IMMT mainly participates in the formation of

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cristae [6, 7], enables RNA binding activity [8], and promotes the biogenesis and maintenance of organelles. As understanding of this gene gradually deepens, people realized that this gene may play a key role in various tumors formation [9]. It has been confirmed that IMMT is a new prognostic indicator and therapeutic target for human lung adenocarcinoma [10]. In addition, Pei-Yi Chu et al. revealed the malignant role of IMMT in the development of breast cancer and renal clear cell carcinoma by means of a combination of multiomics and experimental analysis [11, 12]. However, there is currently relatively little research on the role of IMMT in the occurrence and development of HCC. Given the malignant significance of IMMT in various tumors, we have reason to speculate that it is involved in the formation and progression of HCC.

The photostatidylinositol-3-kinase (PI3K)/AKT signaling pathway is one of the most common dysregulated signaling pathways observed in cancer patients, playing a crucial role in promoting tumorigenesis, progression, and therapeutic response [13, 14]. This is mainly because the PI3K/AKT signaling pathway plays an essential role in many basic physiological activities, such as cell proliferation, migration, and apoptosis [15].

In this study, we aim to explore the functions and mechanisms of IMMT in the progression of HCC. First, using online database such as Gene Expression Profiling Interactive Analysis (GEPIA) [16], we confirmed the malignant significance of IMMT in HCC patients, and verified its high expression status in HCC tissues at the protein and mRNA levels. Secondly, corresponding *in vivo* and *in vitro* phenotypic functional experiments were conducted after over-expressing or downregulating IMMT, respectively. Finally, it was demonstrated that IMMT has a molecular regulatory effect on the PI3K/AKT/mTOR signaling pathway. Our study for the first time reported the key role of IMMT-PI3K-AKT-mTOR signaling pathway in HCC formation. The development of targeted drugs for this signaling axis in the future may provide more efficient treatment options for HCC patients.

Materials and methods

Bioinformatics analysis

Based on the TCGA database, we utilized the Gene Expression Profiling Interactive Analysis (GEPIA) online software (<http://gepia.cancer-pku.cn/index.html>) to analyze the expression level of IMMT in HCC tissues and the correlation between the expression status of IMMT and the overall survival of HCC patients. A total of 369 HCC tumor samples and 50 normal liver tissue samples were selected for analysis. In addition, we

explored the mechanism with the help of String online database software (<https://cn.string-db.org/>).

HCC tissue specimens

The HCC tissues and its paired adjacent normal liver tissue samples (16 pairs of paraffin-embedded tissue samples) used in this study were collected from the Department of Hepatobiliary Surgery, the First Affiliated Hospital of the University of Science and Technology of China (Hefei, Anhui, China) from October 2022 to March 2023. The detailed information of clinical samples is shown in Table 1. This study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of University of Science and Technology of China (Issuing no. 2022-ky297). Prior to the initiation of this research project, written informed consent was obtained from all participants.

Cell lines and cell culture

The human HCC cell lines Hep3B (#C6344) was purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China) in January 2023. HEK-293T cells were collected and frozen by our research group before. All of the above cell lines have undergone Short Tandem Repeat (STR) analysis and mycoplasma testing to ensure the accuracy and contamination-free of the used cell lines. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco™, C11995500BT, New York, USA) high glucose medium containing 10% fetal bovine serum (Beyotime, #C0226, Shanghai, China) and 1% penicillin-streptomycin (Beyotime, #C0222, Shanghai, China). Meanwhile, the cells were cultured in a moist constant temperature cell incubator containing 5% carbon dioxide at 37 °C.

Western Blot

Use RIPA lysate (Beyotime, #P0013B, Shanghai, China) with 1% protease inhibitor (Beyotime, #P1005, Shanghai, China) to lyse the treated cells (Hep3B) and human HCC tissue samples, and extract proteins according to the instructions. Collect the supernatant through high-speed centrifugation. Use the bicinchoninic acid assay (BCA) protein concentration detection kit (Beyotime, #P0009, Shanghai, China) to measure protein concentration for quantification and ensure consistency of internal

Table 1: Clinical and pathological characteristics of tumor samples.

Characteristic	No. of patients
Age, years	
≤60	13
>60	3
Sex	
Male	14
Female	2
Tumor size, cm	
≤5	10
>5	6
BCLC	
A-B	5
C-D	11

BCLC, Barcelona clinic liver cancer.

control proteins. Gel electrophoresis was performed on the extracted protein using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins with different molecular weights. The proteins were then transferred to the Polyvinylidene Fluoride (PVDF) membrane (Beyotime, #FFP39, Shanghai, China). Seal with 5 % skim milk at room temperature for 1 h, then rinse with phosphate buffered solution (PBST) (Solarbio, #P1033, Beijing, China) three times, each time for 10 min. The membrane was incubated with the specific primary antibody against IMMT (Wuhan Sanying, #10179-1-AP, Wuhan, China), β -actin (Cell Signaling Technology, #3700, Boston, USA), cyclinA (Cell Signaling Technology, #4656, Boston, USA), cyclinB (Cell Signaling Technology, #4138, Boston, USA), CDK1 (Cell Signaling Technology, #9116, Boston, USA), CDK2 (Cell Signaling Technology, #2546, Boston, USA), GAPDH (Cell Signaling Technology, #2118, Boston, USA), Caspase-3 (Cell Signaling Technology, #9662, Boston, USA), Cleaved Caspase-3 (Cell Signaling Technology, #9661, Boston, USA), Bcl-2 (Cell Signaling Technology, #15071, Boston, USA), PI3K (Cell Signaling Technology, #4255S, Boston, USA), P-PI3K (Cell Signaling Technology, #17366, Boston, USA), AKT (Cell Signaling Technology, #9272S, Boston, USA), mTOR (Cell Signaling Technology, #2983, Boston, USA), Phospho-mTOR (Cell Signaling Technology, #5536, Boston, USA) at 4 °C overnight. Rinse with PBST three times, each time for 10 min. Incubate the membrane with the corresponding secondary antibody (Cell Signaling Technology, #7076 or #7074, Boston, USA) at room temperature for 1 h, then rinse with PBST three times for 10 min each time. Finally, electro-chemiluminescence (ECL) reagent (Beyotime, Shanghai, China) was used for development. The results were analyzed using Image J software (v1.8.0, National Institutes of Health, Bethesda, USA).

Immunohistochemistry staining

HCC tissue samples collected from the Department of Hepatobiliary Surgery, the First Affiliated Hospital of the University of Science and Technology of China (Hefei, Anhui, China) were fixed in 10 % formalin for 24 h, embedded in paraffin, and sliced into tissue sections (0.1 mm thick). IHC staining was performed using an immunohistochemistry kit. Place the slices in a 60 °C oven for 2 h for dewaxing (first place horizontally for 30 min, then vertically for 90 min). Place the slices in three xylene tanks in sequence (10 min each) for dewaxing and hydrate with ethanol gradient (100–70 %). Incubate with antigen repair solution for antigen repair. Soak the slide in 3 % hydrogen peroxide for 30 min. Wash the slide with PBST three times for 10 min each time. 5 % goat serum (Beyotime, Shanghai, China) was incubated on a glass slide at room temperature for 1 h for sealing. Wash the slide with PBST three times for 10 min each time. Incubate overnight at 4 °C with IMMT monoclonal antibody (diluted at 1:200, Wuhan Sanying, #10179-1-AP, Wuhan, China). Wash the slide with PBST three times for 10 min each time. Incubate the slices with horseradish peroxidase (HRP) labeled secondary antibodies (Vector Laboratories, BA-1000, San Francisco, USA) for 90 min. Finally, DAB (Vector Laboratories, SK-4100, San Francisco, USA) and hematoxylin were used to stain the slides in sequence, and neutral gum was used to seal the slides. After the gum was dried, the images were observed under a microscope and photographed.

RNA isolation and quantitative real-time PCR (qRT-PCR)

According to the user manual, use the RNA extraction kit (Thermo Scientific™, K0732, Waltham, USA) to extract RNA from HCC tissues and cells. Reverse transcriptional synthesis of cDNA is performed according

Table 2: Primer sequences used for gene expression analysis.

Gene	Forward (5'-3')	Reverse (5'-3')
IMMT	CAGGCTGTCAATGCACACTCCA	CATCTACTGCCTTCTGCGTTCC
β -actin	CACCATTGGCAATGAGCGGTT	AGGTCTTGCGGATGTCACGT

IMMT, inner membrane mitochondrial protein.

to the instructions of the cDNA synthesis kit (Thermoscyacy™, A48571, Waltham, USA). Finally, qRT-PCR reaction was performed based on FastStart Universal SYBR Green master mix to detect the mRNA level of the target genes. The total system of this reaction is 20 μ L. At the same time, in order to reduce operational errors, each sample is set with three technical repeat wells, and each group of samples is set with three biological repeat wells. The process is as follows: 95 °C for 3 min, denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s. Adopting $2^{-\Delta\Delta CT}$ method to calculate gene expression differences and β -actin was used as the reference gene. The primer sequences involved in the qRT-PCR reaction are shown in Table 2.

Lentivirus construction and cell transfection

Inoculate 293T cells into a 10 cm cell culture dish (3×10^6 per dish) and cultured overnight in an incubator at 37 °C and 5 % CO₂. Then, the lentivirus packaging plasmids psPAX2, pMD2.G and the target gene plasmid containing the hairpin IMMT shRNA sequence (sh-IMMT#1: 5'-CCCGTTCTATGCTGTCAA-3'; sh-IMMT#2: 5'-GCTAAGGTGTATCTCAGTAT-3'; sh-IMMT#3: 5'-CCAAGCTTAACCGCAGCTAT-3') were used for lentivirus packaging system transfection. After 48 h, the supernatant of the culture medium containing lentivirus was collected and filtered with a 0.22 μ m filter. The collected lentivirus was packaged and stored at -80 °C. 1×10^6 Hep3B cells were inoculated into a 6-well plate. After overnight culture, Lentivirus and DMEM medium were added into the 6-well plate at a ratio of 1:1. After 24 h, replace the fresh culture medium and continue to culture for 24–48 h. Take photos, record the infection situation, and add purithromycin to screen for successfully transfected cells. Collect successfully transfected Hep3B cells and detect the expression of IMMT at the protein and mRNA levels using Western blot and qRT-PCR techniques, respectively.

Cell proliferation assay

Resuspension the stable transfected Hep3B cell line in the logarithmic growth phase, adjust the cell suspension to contain 2000 cells per 100 μ L after cell counting, inoculate the cells into a 96-well plate with 100 μ L cell suspension per well, and set up three multiple wells to reduce errors. The 96-well plate was incubated in a constant temperature incubator containing 5 % CO₂ at 37 °C. Cell Counting Kit 8 (CCK8) reagent (Dojindo, CK04, Kyushu Island, Japan) was added on the first day, the second day, the third day, the fourth day, and the fifth day, respectively, and then incubated for 2 h. The absorbance was measured at 450 nm with an automatic microplate reader (SPARK, Tecan Austria GmbH, Switzerland).

Colony formation assay

Stable IMMT knockout or overexpression Hep3B cells were seeded into a 6-well plate with a density of 1000 cells per well. Each group was set with

three duplicate wells, shaken evenly, and cultured in the cell incubator for about two weeks. When obvious colonies formed, the cells were fixed with 4 % paraformaldehyde for 15 min, and then treated with crystal violet (Beyotime, C0121, Shanghai, China) staining for 30 min. Finally, use the developer and ImageJ software for photography and quantitative analysis.

Transwell migration assays

The cells in the logarithmic growth phase were digested with trypsin, and the concentration of cells was adjusted to 1×10^4 cells/mL using serum-free DMEM medium. 200 μ L cell suspension was added to the upper chamber of transwell chamber, and DMEM culture medium containing 10 % fetal bovine serum (FBS) was added to the lower chamber. After 24 h, take out the chamber, fix it with 4 % Paraformaldehyde for 15 min, then stain it with 0.5 % Crystal violet for 15 min, wipe the upper cells of the chamber with a cotton swab, seal it with neutral gum, take photos under the microscope (OLYMPUS, #BX63, Tokyo, Japan), and randomly select three visual fields for counting.

Cell cycle and apoptosis analysis

Cell cycle: In this study, the effects of IMMT knockdown on cell cycle of Hep3B were detected by flow cytometry. Inoculate stable IMMT knockdown Hep3B cells in logarithmic growth phase with appropriate density into a 6-well plate, culture for 48 h, collect cells, and fix them with 75 % ethanol for 2 h. The fixed cells were suspended with the mixture of propidium iodide and RNase A (Beyotime, C1052, Shanghai, China) and incubated for 40 min away from light. The proportion of cells in each stage was analyzed by FlowJo v10.8.1 software (San Francisco, USA).

Apoptosis: According to the instructions of Annexin V/PI apoptosis detection kit (Biolegend, 640922, California, USA), the impact of knockdown IMMT on Hep3B cell apoptosis was detected using flow cytometry. Inoculate stable IMMT knockdown Hep3B cells in logarithmic growth phase with appropriate density into a 6-well plate, culture for 48 h, collect cells. Add 5 μ L Annexin V-FITC and 5 μ L propidium iodide (PI), incubate at room temperature in dark for 5 min. The specific operating steps are consistent with the method for detecting cell cycle (note that cells do not need to be fixed with ethanol at this time).

Animal experiments

The effect of IMMT knockdown on tumor formation was verified by constructing subcutaneous tumor transplantation in nude mice. The constructed stable IMMT knockdown Hep3B cells were implanted subcutaneously into the armpit of the forearm of nude mice. Starting from the seventh day, measure the length and width of subcutaneous tumors every 3 days. The subcutaneous graft volume was calculated according to the following formula: $V=0.52 \times L \times W^2$ (V: tumor volume; L: tumor diameter; W: tumor width). By means of high-pressure tail vein injection, PX330 vector, px330-sg-p53, CMV-SB13 and sgIMMT plasmids were injected into C57BL/6 mice to construct liver carcinoma *in situ* model. All animals were raised in an specific pathogen free (SPF) environment, and all animal experiments were approved by The First Affiliated Hospital of University of Science and Technology of China

(Anhui Provincial Hospital) Experimental Animal Ethics Committee (Issuing no. USTCACUC24120122047).

Statistical analysis

Charting and statistical analysis with Prism 9.0 (GraphPad, San Diego, USA). Experimental results are expressed as mean \pm standard deviation. Differences between the groups were analyzed using Student's *t*-tests, and $p<0.05$ means a significant difference and has statistical significance.

Results

IMMT is elevated in HCC tissues and correlated with the malignant prognosis of HCC patients

Use the GEPIA online website (<http://gepia.cancer-pku.cn/index.html>) to tally the information in the TCGA database. We found that compared with normal liver tissues, the expression of IMMT was significantly upregulated in HCC tissues and significantly correlated with shorter overall survival in HCC patients (Figure 1A and F). In order to fully confirm the expression of IMMT in HCC, we conducted studies from the two levels of RNA and protein. The qRT-PCR experiment confirmed the high expression level of IMMT in HCC at the RNA transcription level (Figure 1B). WB and IHC experiments further support our conclusion at the protein translation level (Figure 1C–E). In order to deeply evaluate the potential clinical value of high expression status of IMMT in HCC tissues, we analyzed the correlation between high expression level of IMMT and overall survival of HCC patients using GEPIA. The results showed that patients with higher expression of IMMT exhibited shorter overall survival, and there was a significant correlation between the two indicators (Figure 1F). The above phenomena suggest that the upregulation of IMMT may be related to the formation of HCC, which has the potential as a new therapeutic target for HCC.

IMMT acted as an oncogene in the progression of HCC

A series of *in vitro* experiments further confirmed the carcinogenic effect of IMMT in the formation of HCC. We detected the expression of IMMT in various human liver cancer cell lines through WB experiment, and found that the expression level of IMMT was higher in Hep3B cells (Supplementary Figure 1A). Therefore, this cell was selected for

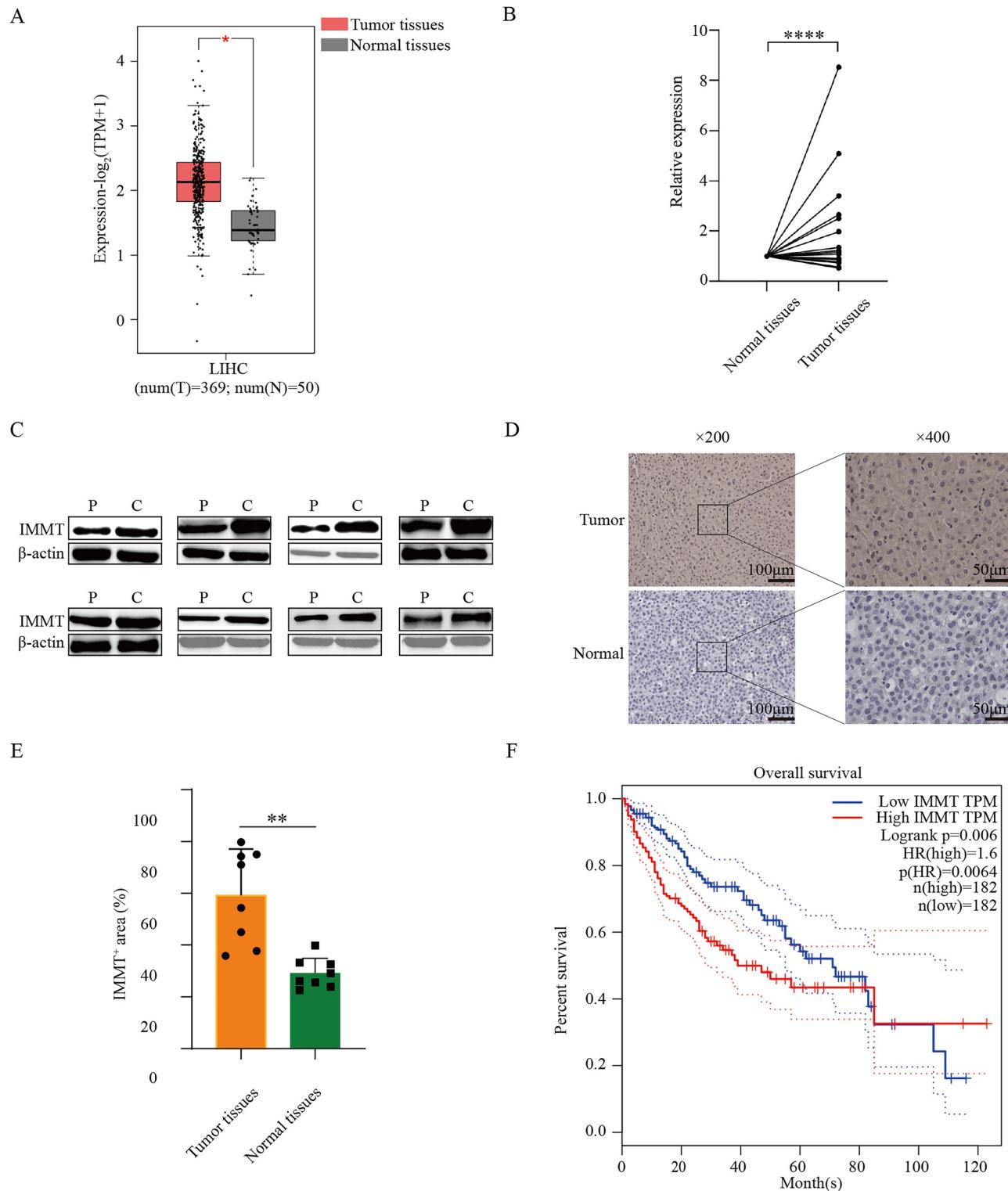


Figure 1: IMMT is highly expressed in HCC and associated with poor prognosis in HCC patients. (A) The boxplot shows the relative expression levels of IMMT in HCC tumor tissues and normal liver tissues based on the GEPPIA database. Non-paired *t*-test. (B) IMMT mRNA expression level analysis in HCC and adjacent normal liver tissues. Paired *t*-test. (C) Detection of IMMT protein expression in HCC and adjacent normal liver tissues. (D) The representative immunohistochemical (IHC) staining images in archival paraffin sections of patient-matched HCC tissues vs. normal tissues (n=8). (E) Quantification of IMMT immunostaining. Paired *t*-test. (F) The correlation of IMMT expression and OS in HCC patients. Log-rank test. Data are expressed as the mean \pm SD ns, not significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$. IMMT, inner membrane mitochondrial protein; HCC, hepatocellular carcinoma; P, paracancer tissue; C, cancer tissue; OS, overall survival.

exploratory experiments. The knockdown of IMMT is achieved by constructing short hairpin RNA. We designed three sh-IMMT sequences, but after testing, it was found that only sh-IMMT #1 had the best specificity knockdown result (Supplementary Figure 1B). Therefore, subsequent knockdown experiments of IMMT were conducted using this sequence. The mRNA and protein expression levels of IMMT in Hep3B cells transfected with IMMT shRNA and overexpression lentivirus were detected by WB and qRT-PCR to determine the knockdown and overexpression efficiency of IMMT (Figure 2A and B). The CCK8 experiment was conducted using stable IMMT knockdown and overexpression of Hep3B cells. The results showed that knockdown of IMMT significantly inhibited the proliferation of Hep3B cells (Figure 2C), while overexpression of IMMT showed the opposite effect (Figure 2D). Similarly, the colony formation experiment further indicates that knocking down IMMT weakens the proliferation ability of Hep3B cells (Figure 2E and F). What's more, the transwell migration experiment supports the carcinogenic effect of IMMT from another perspective. Knocking down IMMT would strongly inhibit the migration of Hep3B cells (Figure 2G and H). In summary, the above *in vitro* functional experimental results indicate that IMMT plays a certain role in the formation of HCC through an uncharted mechanism, which is worth further exploration.

IMMT has regulatory effects on cell cycle and apoptosis

By flow cytometry, we found that knocking down IMMT significantly increased the proportion of G0/G1 phase cells in a state of quiescent division, while the proportion of S phase and G2 phase cells in a state of active division decreased relatively, especially in the S+G2 phase (Figure 3A and B). The detection of proteins involved in cell cycle showed that the expression of protein (cyclinA, cyclinB, CDK1, CDK2) conducive to cell division in shIMMT group were significantly down-regulated compared with the NTC group (Figure 3C). All these results revealed that IMMT knockdown could hinder the mitosis process of Hep3B cells, reduce the cell proliferation rate, and make more Hep3B cells in a relatively static state. In addition, Annexin V-FITC/PI apoptosis double staining results confirmed that IMMT knockdown could induce apoptosis (Figure 3D and E), which was further supported by Western Blot. Compared with the NTC group, we noticed that the knockdown of IMMT could

upregulate the expression of Cleaved Caspase-3 and inhibit the expression of anti-apoptotic protein Bcl-2 (Figure 3F). These results further explained the regulatory mechanism of IMMT on a series of functional phenotypes of HCC cell line Hep3B, suggesting that IMMT could regulate the occurrence and development of HCC by affecting cell cycle and apoptosis.

IMMT promotes the formation of HCC *in vivo*

The above *in vitro* functional experiments support our further understanding of the carcinogenic effect of IMMT *in vivo*. Therefore, we investigated in nude mice with immune deficiency and C57BL6 mice with complete immune function. Firstly, by constructing subcutaneous transplanted tumors in nude mice, we found that knocking out IMMT can inhibit tumor growth (Figure 4A–C). At the same time, by means of high-pressure tail vein injection, we injected IMMT knockout plasmid into C57BL6 mice together with PX330 vector, px330-sg-p53, CMV-SB13 three plasmid vector system to construct liver carcinoma *in situ*. The results showed that compared with the control group, the volume of liver carcinoma *in situ* in IMMT knockout mice was smaller and the survival period of mice could be significantly prolonged (Figure 4D and E). The results of the above *in vivo* experiments strongly support the malignant effect of IMMT on HCC formation and inspire us to further explore the specific molecular mechanisms of IMMT regulating HCC progression.

IMMT promotes HCC progression via PI3K/AKT/mTOR signalling pathway

The functional phenotype experiments *in vitro* and *in vivo* provided us with confidence and motivation to further understand the specific molecular mechanisms of IMMT carcinogenesis. Therefore, with the help of the String molecular interaction website platform, we found that molecules with reliable interactions with IMMT, apart from the genes involved in maintaining mitochondrial function, only the hit gene PI3K has a strong correlation with it (Figure 5A). So, we validated the relationship between IMMT and PI3K. The results demonstrated the molecular regulatory effect of IMMT on the PI3K/AKT/mTOR signaling pathway (Figure 5B). To further illustrate the issue, we designed a feedback experiment. *In vitro*, CCK8 and colony

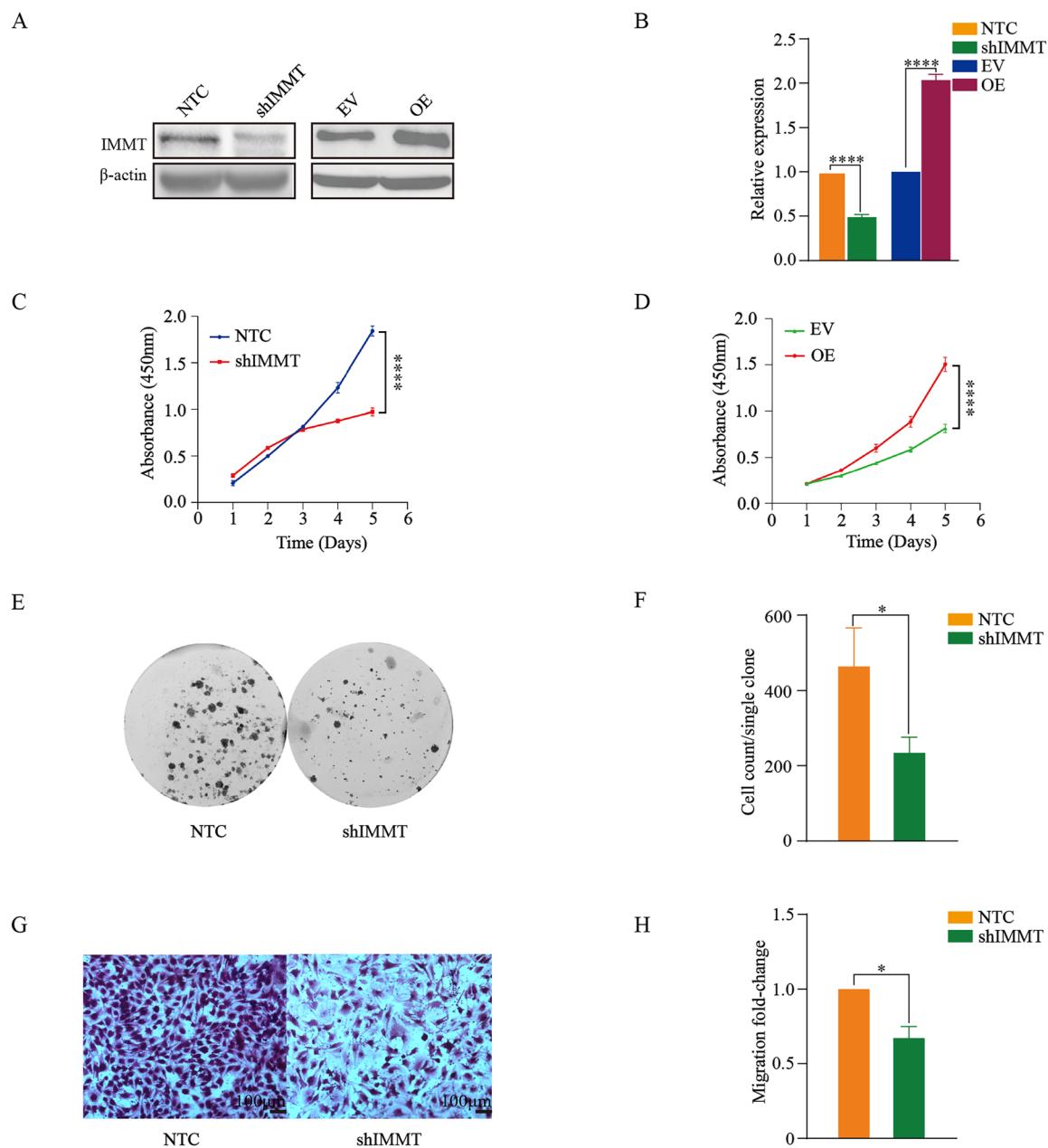


Figure 2: IMMT can promote the proliferation and migration of HCC cells *in vitro*. (A) Protein detection to analyse the knockdown and overexpression efficiency of IMMT. (B) mRNA analysis to determine the knockdown and overexpression efficiency of IMMT. Student's *t*-test. (C and D) CCK8 was performed to identify changes in proliferation ability after IMMT knockdown and overexpression. Student's *t*-test. (E and F) The colony-forming ability was determined after IMMT knockdown and overexpression in the Hep3B. Student's *t*-test. (G and H) Transwell assay was performed to detect the influence of IMMT changes on cell migration. Student's *t*-test. Data are expressed as the mean \pm SD ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

formation experiments have confirmed that supplementing with PI3K activator (740Y-P, 20 μ M) could significantly rescue the inhibitory effect of knocking out IMMT on cell proliferation (Figure 5C–E). The above results revealed the regulatory effect of IMMT on HCC formation from the perspective of mechanism, and a series of *in vitro* rescue experiments further supported our conclusion.

Verify the regulatory effect of IMMT on PI3K/AKT/mTOR signaling pathway *in vivo*

In order to exclude the influence of *in vitro* environment and further confirm our conclusion, we designed to conduct another verification *in vivo* environment of animals. Subcutaneous tumor transplantation experiments

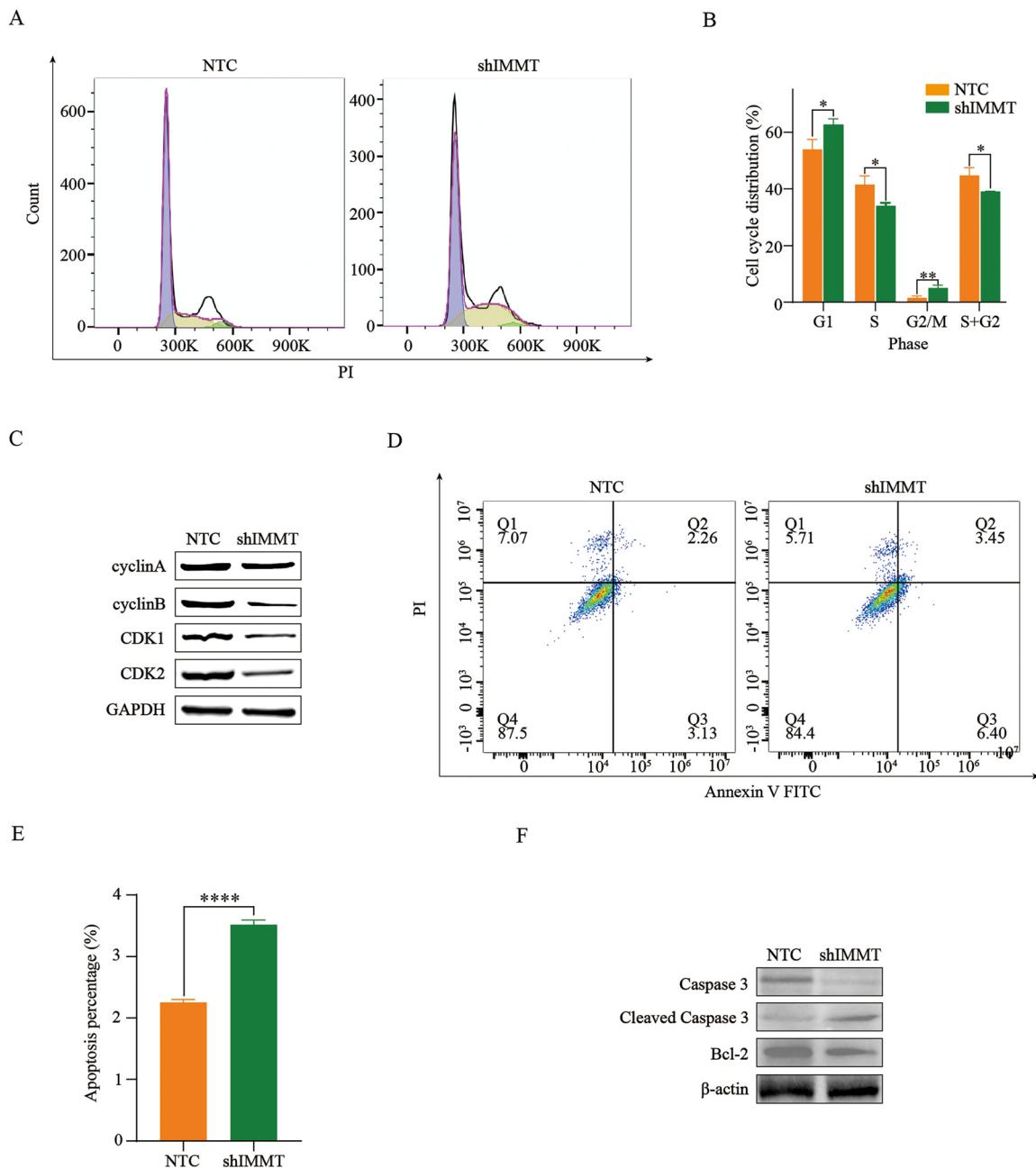


Figure 3: IMMT is associated with cell cycle progression and apoptosis. (A and B) cell-cycle distribution was measured by PI staining in Hep3B cells transfected with shIMMT and the control, followed by flow cytometric analysis. Student's *t*-test. (C) Detection of proteins involved in cell cycle. (D and E) cell apoptosis was measured by FITC-Annexin V and PI staining in Hep3B cells transfected with shIMMT and the control, followed by flow cytometric analysis. Student's *t*-test. (F) The protein expression of Caspase-3, cleaved Caspase-3 and Bcl-2 were analyzed in Hep3B cells transfected with shIMMT and the control. Data are expressed as the mean \pm SD ns, not significant; * p <0.05, ** p <0.01, *** p <0.001 and **** p <0.0001. PI, propidium iodide.

in vivo further supported the influence of this molecular mechanism on the formation of HCC. We found that while knocking out IMMT, the use of PI3K activator (740Y-P; intraperitoneal injection; 15 mg/kg; 4 weeks) significantly weakened the inhibitory effect of IMMT on tumor growth (Figure 6A–C). Similarly, the model of carcinoma *in situ* of

the liver was established by high-pressure tail vein injection. Compared with the sgIMMT group, the tumor inhibitory effect caused by knocking out IMMT was reversed in the sgIMMT+740Y-P group (Figure 6D and E). The application of two different animal models *in vivo* greatly enriched our conclusions and improved the correctness of IMMT/

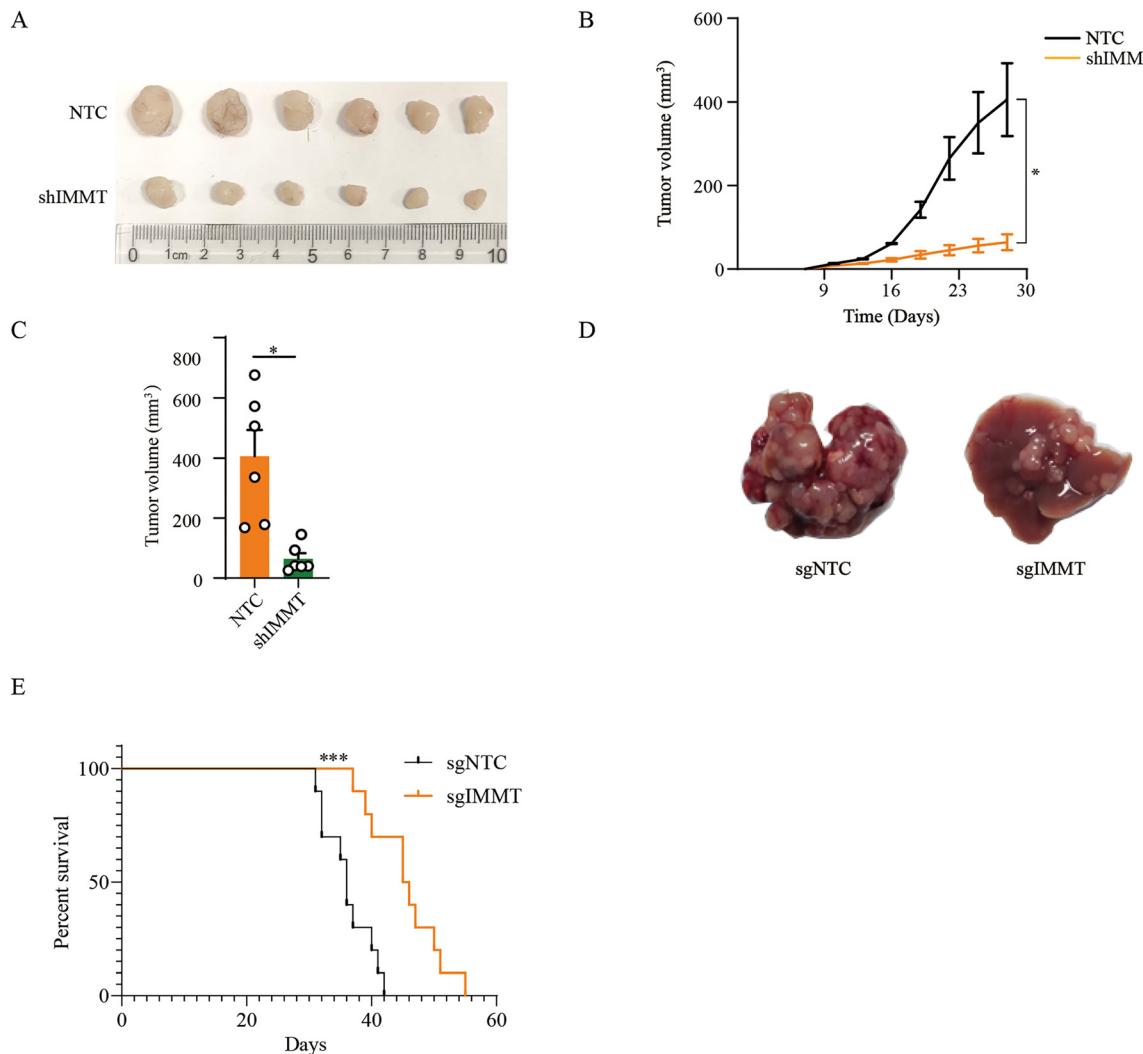


Figure 4: IMMT has carcinogenic effect *in vivo*. (A–C) 1×10^6 shNT or shIMMT Hep3B cells were subcutaneously injected into nude mice ($n=6$). Photos of tumors (A), tumor growth curves (B) and final volume (C) were presented. Student's *t*-test. (D) Liver carcinoma *in situ* images from sgNTC and sgIMMT groups. (E) Survival curves of C57BL6 mice in sgNTC and sgIMMT groups. Log-rank test. Data are expressed as the mean \pm SD ns, not significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

PI3K/AKT/mTOR signal axis. In the future, clinical transformation research on the IMMT/PI3K/AKT signal axis may bring new insights into the diagnosis and treatment of HCC.

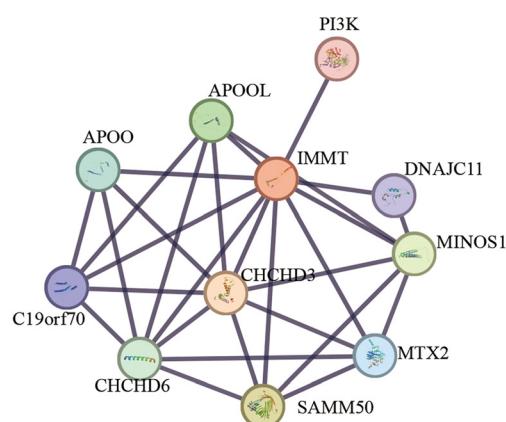
Discussion

Hepatocellular carcinoma (HCC) is a disease that seriously affects human health and has attracted worldwide attention for many years [17]. Due to the insidious onset of liver cancer, many diagnosed patients are in the terminal stage of the disease, and conventional techniques such as surgery, radiotherapy, and chemotherapy are difficult to achieve good treatment results [18, 19]. Systematic anticancer treatment strategies play an important role in the treatment of advanced

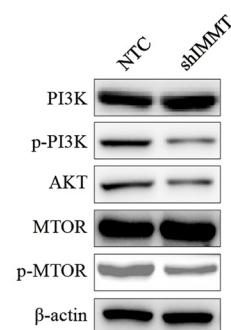
liver cancer, which can prolong the survival time of patients [20]. Therefore, the comprehensive application of various important biomarkers and effective intervention targets will be the key to further improving the specific prevention and control strategies for HCC based on the idea of cancer evolution and development, through integrated analysis of relevant mechanisms and molecular epidemiological studies.

As the inner mitochondrial membrane protein, IMMT performs complex functions in many physiological activities, including transcriptional activation of mitochondrial biogenesis and organelle biogenesis and maintenance [6, 21]. In terms of tumorigenesis, Katsuhiko Naoki et al. confirmed the positive correlation between the high expression of IMMT and the poor prognosis of lung adenocarcinoma patients [10]. Pei Yi Chu et al. revealed the function of IMMT

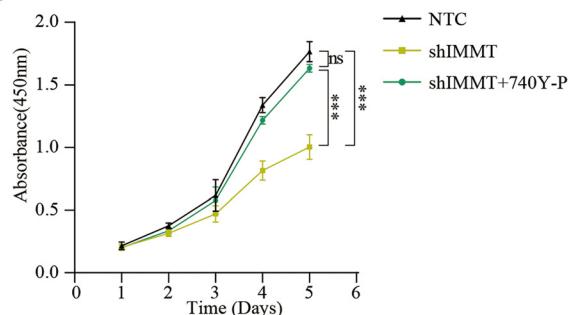
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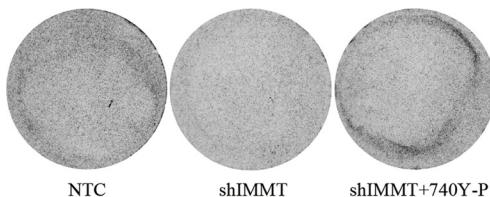
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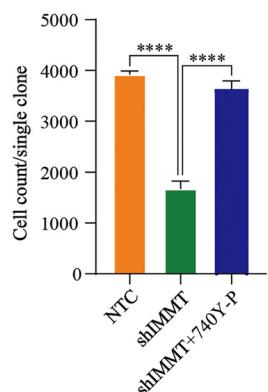
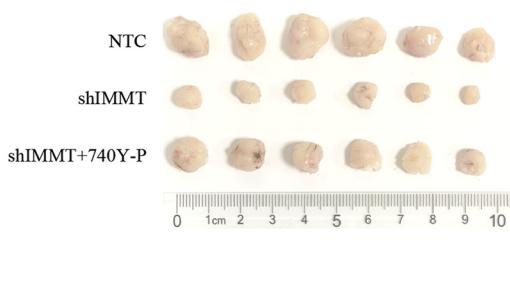


Figure 5: IMMT affects the formation of HCC by regulating the PI3K/AKT signaling pathway. (A) Multiple genes interact with IMMT. (B) IMMT has a regulatory effect on the PI3K/AKT/mTOR signaling pathway. (C) CCK8 experiment verified the regulation of PI3K/AKT/mTOR by IMMT. Student's *t*-test. (D and E) Colony formation assay supported the regulation of PI3K/AKT/mTOR by IMMT. Student's *t*-test. Data are expressed as the mean \pm SD ns, not significant; * p <0.05, ** p <0.01, *** p <0.001 and **** p <0.0001.

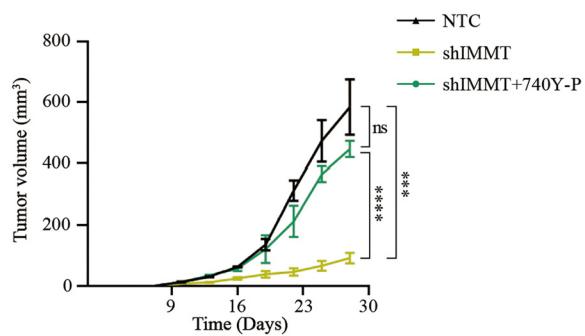
in the malignant progression of breast cancer and renal clear cell carcinoma [11, 12]. In addition, according to John et al.'s report, down-regulating the expression of IMMT in Hela cells can inhibit the growth rate of the tumor cells and induce cell apoptosis, greatly increasing the apoptosis rate [22]. These research results in other tumor types have led us

to expect the malignant function of IMMT in the development of HCC. To date, no work has been carried out or completed on the relationship between IMMT and HCC. In this study, we first confirmed the key role of IMMT in the formation and progression of HCC, and it is a new target for the diagnosis of HCC patients.

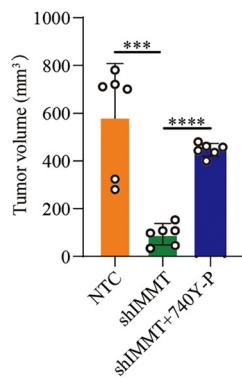
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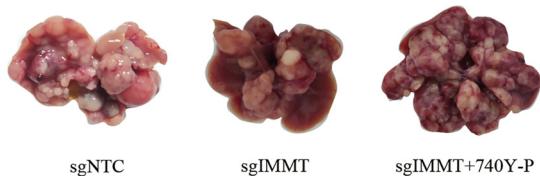
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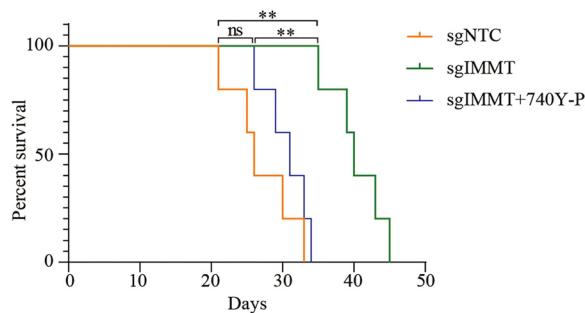


Figure 6: The IMMT/PI3K/AKT/mTOR signal axis is related to the formation of HCC *in vivo*. (A–C) 1×10^6 shNT or shIMMT Hep3B cells were subcutaneously injected into nude mice with PI3K activator (740Y-P) (n=6). Photos of tumors (A), tumor growth curves (B) and final volume (C) were presented. Student's *t*-test. (D) Liver carcinoma *in situ* images from sgNTC, sgIMMT and sgIMMT+ 740Y-P groups. (E) Survival curves of C57BL6 mice in sgNTC, sgIMMT and sgIMMT+ 740Y-P groups. Log-rank test. Data are expressed as the mean \pm SD ns, not significant; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

In this study, through the analysis of a large number of HCC samples in the TCGA database, it was found that high expression of IMMT was associated with poor prognosis of HCC and was an independent prognostic indicator for HCC patients. In addition, using clinically collected HCC tissue samples, we further verified the high expression of IMMT in HCC tissues at protein and RNA levels. Functionally, by means of lentiviral infection specific to IMMT, stable IMMT knockdown and overexpressed cell lines were constructed.

In vitro cell proliferation experiments such as CCK8 and colony formation, we found that knocking down IMMT could greatly reduce cell proliferation ability, while transwell cell migration experiment proved that IMMT had the function of regulating cell migration. In addition, using flow cytometry, we investigated the regulatory effects of IMMT on cell cycle and apoptosis. In the animal experiment section, the construction of subcutaneous transplanted tumor fully supported the results of our *in vitro* functional experiment.

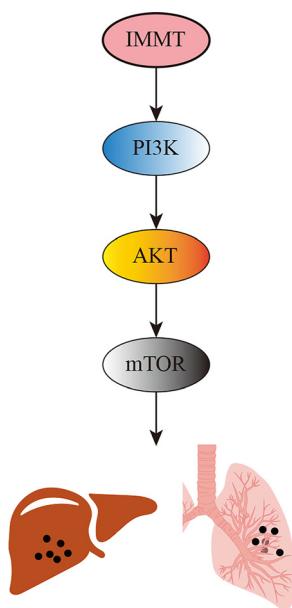


Figure 7: IMMT promotes tumor progression and metastasis.

Finally, we discovered the regulatory effect of IMMT on the PI3K/AKT signaling pathway, which initially confirmed the potential function of IMMT/PI3K/AKT signaling axis in the development of HCC.

This study provides a new biological target for the clinical treatment of HCC, and further translational research on this target may bring new insights into the diagnosis and treatment of HCC in clinical practice (Figure 7). However, tumor is a very complex ecological niche, and it is too simple to explain the malignant effect of IMMT on HCC from the perspective of molecular mechanism. Tumor growth involves various aspects of the body, such as tumor immune microenvironment, circulation, metabolism, nutrition, etc. Further in-depth research on the impact of IMMT on HCC in multiple aspects is necessary.

Conclusions

At present, the treatment of HCC has encountered a bottleneck, molecular targeted therapy has brought new hope to patients with HCC. Our study has found and identified a new therapeutic target for HCC, and the future development of targeted drugs targeting IMMT and its downstream molecules may open a new chapter for the treatment of HCC, which will be a meaningful work.

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Research ethics: This study has been approved by the Medical Research Ethics Committee of the First Affiliated Hospital of University of Science and Technology of China (Issuing no. 2022-ky297), and all patients involved in this study have provided them with written informed consent and obtained their consent. All animal experiments were approved by the The First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital) Experimental Animal Ethics Committee (Issuing no. USTCACUC24120122047).

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: Yao Liu designed the study. Jiabei Wang, Yunguang Zhang and Linmao Sun performed the experiments. Jiabei Wang and Yunguang Zhang wrote the manuscript. Yao Liu analyzed and verified the integrity of the data. All authors contributed to the article and approved the submitted version. All authors approved the manuscript and agreed to be accountable for all aspects of the research.

Competing interests: The authors declare that they have no conflicts of interest to report regarding the present study.

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