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METTL14-mediated miR-122-5p maturation stimulated tumor progression by targeting KAT2A in hepatocellular carcinoma

Xiangyu Fan✉, Wenjuan Qiao, Xu Guo, Jiaqi Wang & Lisong Zhao

m6A modifications are involved in regulating microRNA (miRNA) processing and maturation, and are associated with tumor development. Therefore, this study was aimed to explore the mechanism of miR-122-5p in regulating hepatocellular carcinoma (HCC) progression. mRNA expression and transfection efficiency were detected by RT-qPCR. Western blot was employed to measure protein level. Cell functions were evaluated through CCK-8 and transwell, respectively. Intracellular m6A levels were analyzed by MeRIP. Dual luciferase reporter gene, RIP and co-IP were applied to verify the binding relationship. Xenograft tumor model was carried out for in vivo validation of miR-122-5p function. We reported that miR-122-5p was clearly lessened in HCC. Functionally, miR-122-5p introduction inhibited the malignant progression of HCC. Mechanistically, METTL14 insertion promoted miR-122-5p maturation by labeling pri-miR-122 with m6A. In addition, miR-122-5p exerted suppressor effects by targeting Lysine acetyltransferase 2 A(KAT2A). Moreover, we also found that KAT2A overexpression limited β-catenin expression through succinylation modification. Finally, animal data also illustrated that miR-122-5p introduction could hinder the growth of HCC tumors in vivo. We revealed the existence of a METTL14/miR-122-5p/KAT2A/β-catenin mechanistic axis in HCC, which has not been reported in the literature. This newly discovered mechanistic axis may provide new ideas for HCC therapy.

Keywords HCC, m6A, METTL14, miR-122-5p, KAT2A

Abbreviations

miRNA	microRNA
HCC	Hepatocellular carcinoma
CRC	Colorectal cancer
BC	Breast cancer
ncRNA	Non-coding RNAs
WT	Wild-type
MUT	Mutant
KAT2A	Lysine acetyltransferase 2 A
EMT	Epithelial-mesenchymal transition

Liver cancer is a difficult disease to cure with high morbidity and mortality. About 90% patients are hepatocellular carcinoma (HCC). There were approximately 865,269 new cases and 757,948 deaths globally in 2022 [1]. And by 2030, there will be more than 1 million deaths case of liver cancer[2]. The preferred treatment options for patients with early-stage HCC are resection, transplantation, and local ablation, while patients with intermediate-stage disease may choose to receive hepatic artery chemoembolization. Unfortunately, advanced disease needs to receive systemic therapy[3]. Due to the insidious onset and high degree of malignancy of HCC, most late stage patients missed the optimal treatment period and only have a very low 5-year survival rate[4]. Although there were lots of progress of HCC therapy, about 70% of HCC cases still have poor prognosis because of it recur[5]. Therefore, in order to improve the prognosis and survival time, there is an urgent need to find out the new biomarkers and potential therapeutic targets for HCC patients.

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Carcinogenesis is a multi-stage disease that is progressively triggered by mutations and epigenetic alterations, ultimately leading to the development of malignant tumors[6]. As a widely known modification, m6A has received much attention in tumor development[7]. There are many potential links between cancer and m6A modifications,[8]. For example, in colorectal cancer (CRC), YTHDF1 was augmented and regulated the ability of spheroids formation in CRC cells by targeting Wnt/β-catenin pathway[9]. In hypoxia breast cancer (BC) cells, HIF1A and HIF2A stimulated ALKBH5 expression, leading to increase of NANOG m6A demethylation and expression, which induced BC stem cell phenotype[10]. In HCC, upregulation of METTL3 increased the levels of tumor suppressor SOCS2 by m6A modification, which accelerated SOCS2 degradation and altered HCC cell proliferation[11]. In glioblastoma, METTL3 or METTL14 silencing limited m6A level and enhanced stem cell growth[12]. Studies of m6A will help to understand the broader tumor biology. Therefore, the benefits and drawbacks of m6A-mediated modifications need more studies to elucidate on HCC progression.

Recent advances in the analysis of the human transcriptome have shown that about 90% of genes are transcribed, yet only about 2% of the sequences encode proteins. Of these, the vast majority are various non-coding RNAs (ncRNA)[13]. NcRNAs play a role in various areas of life and contribute to the organization and stability of the genome. As a small ncRNA, microRNAs (miRNAs) are about 17–25 nucleotides in size[14]. microRNAs have been implicated in development and disease, and control gene expression by sequence complementary sites of mRNAs[14]. miRNAs are also key regulators in various biological processes[15]. Increasing studies have pointed out that miRNA dysregulation is associated with tumorigenesis and progression. Among them, miR-122-5p has been evidenced to serve as oncogenic or suppressive factors in multiple malignancies. The upregulation of miR-122-5p contributed to lung cancer and breast cancer progression[16,17], while it served as a tumor-suppressing factor in gastric cancer, nasopharyngeal carcinoma, intrahepatic cholangiocarcinoma, pancreatic cancer[18-21]. In HCC, miR-122-5p expression was apparently downregulated compared with normal liver tissues, its overexpression could restrain the proliferation and metastasis of HCC cells[22]. Importantly, compared with other dysregulated miRNAs, miR-122-5p has high tissue specificity and clinical association. The expression changes of miR-122-5p in tissues are associated with tumor metastasis and vascular invasion of HCC patients, making it potentially a prognostic marker[23]. The latest mechanism research confirmed that miR-122-5p was involved in HCC progression via targeting multiple oncogenic genes (FOXK2, PKM2, and LDHA)[24-26] and the signal axis (TGFBR2-SAMD2-SMAD3)[27]. Therefore, miR-122-5p can be one of the core molecules affecting the disease process of HCC. In this study, we further clarified its upstream and downstream regulatory relationship in HCC tumorigenesis and expanded the molecular mechanism network of miR-122-5p in HCC.

Of interest, m6A modification plays a vital role in regulating miRNA expression[28]. Specifically, m6A methylation-associated enzymes can be involved in tumor advancement by modulating production process and expression of miRNAs. For example, ALKBH5 silencing enhanced mature miR-124-3p levels, which subsequently attenuated the invasiveness of glioblastoma[29]; METTL3-induced maturation of miR-589-5p contributed to the malignant characterization of HCC[30]. However, although abnormal levels of m6A modification and miRNA expression have been observed in samples of HCC patients, it is difficult to determine the effect of m6A modification on miRNA processing and cannot provide a clear targeted basis for clinical treatment. Meanwhile, current studies in HCC mostly focus on the regulation of mRNA by m6A modification, and the research on whether the processing of specific miRNA is affected by m6A modification is relatively limited. Thus, this study explored the regulatory mechanism between m6A modification and miR-122-5p in HCC.

KAT2A, a succinyltransferase, is widely involved in gene transcription, cell cycle, and metabolic reprogramming in pan-cancer[31,32]. Notably, KATA2 was identified to be aberrantly up-regulated in multiple tumors including colorectal cancer, diffuse large B cell lymphoma, prostate cancer, and promotes metastasis, proliferation, and acquired drug resistance of tumor cells[33-35]. In HCC, KATA2 also functioned as an oncogenic factor, its overexpression enhanced tumor cell growth and glycolysis[36]. Moreover, KATA2 can mediate tumor advancement by modulating succinylation level of substrate proteins. For instance, KATA2 facilitated HCC cell growth through promoting succinylation of PGAM1 and Sp1[36, 37], providing novel insights into tumor pathogenesis. Here, KATA2 was identified as a downstream target of miR-122-5p, and we further explored its molecular network in HCC from the perspective of succinylation modification.

Our research would take the regulation of miR-122-5p on HCC as entry point, exploring the upstream m6A regulatory mechanism mediating the processing and maturation of miR-122-5p at epigenetic level, and probing the succinylation mechanism mediated by the downstream target gene KAT2A of miR-122-5p at protein post-translational modification level. This study will broaden our understanding of the HCC development mechanisms and may provide a theoretical basis for targeted miRNA-based therapy.

Methods

Tissue collection and cell culture

The study was authorized and approved by The Fourth Hospital of Harbin Medical University Ethics Committee. This study conforms to the principles of the Declaration of Helsinki, and informed consent was obtained from all patients. Tissues contain HCC tumor and paired adjacent normal were acquired from 45 HCC patients who underwent resection. Forty-five patients were pathologically diagnosed with HCC and were free of metastasis or other tumors. In addition, all patients did not receive any treatment prior to surgery. Patients who agreed to the study signed an informed consent form before the collection of their tissues.

THLE3, Hep3B, SNU449, Huh7, Focus, HA22T were purchased from the American Culture Collection. All cells were cultured with 10% FBS-DMEM (Gibco) medium at 37 °C, 5% CO_2.

Bioinformatics analysis

We downloaded the microRNA expression dataset GSE158523 and identified differentially expressed miRNAs (DE-miRNAs) between individuals with hepatocellular carcinoma and healthy individuals using the "limma"

package in R software. First, we performed quantile normalization and log2 transformation to minimize technical variations and ensure comparability across samples. We used DESeq2 and edgeR package for the differential expression analysis. Then, we selected miRNAs with an FDR (False Discovery Rate) < 0.05 as the significance threshold. Additionally, we considered the magnitude of expression changes, selecting only those miRNAs with |log2(FC)| > [specific fold change value, e.g., 1 indicating a 2-fold change], ensuring biological relevance in expression differences. After filtering all differentially expressed miRNAs based on the above criteria, we ranked them by statistical significance (typically sorted by P-value or FDR; if these were identical, further sorted by absolute fold change). The top 10 miRNAs were then selected as the focus of our study. The heatmap was generated using the pheatmap package in R, with hierarchical clustering performed on both rows (miRNAs) and columns (samples) using Euclidean distance and complete linkage. The color scale represents the z-scores, with red indicating higher expression and blue indicating lower expression. Moreover, the miRmap (probability exact > 90), Targetscan (top 100 binding site scores & Site type:8mer), and Starbase databases were used to predict potential downstream mRNAs of miR-122-5p. The intersection of the three databases was analyzed by Venn diagram, and a total of 9 genes were obtained (including KAT2A, HNRNPU, SLC7A1, MASP1, etc.). Subsequently, Starbase was used to analyze the correlation between miR-122-5p and these genes in liver cancer. Besides, UALCAN database was applied to identify KAT2A expression and prognostic value in liver cancer.

RT-qPCR

RNeasy Mini Kit (Qiagen) was employed to isolate total RNA according to a standardized procedure. OD260/280 values of RNA between 1.8 and 2 were allowed to continue with subsequent experiments. Then, PrimeScript RT Master Mix kit (Takara) was conducted to reversed transcribe. PCR operations were carried out using the SYBR Premix Kit (Takara). Relative expression of genes was analyzed using the $2^{-\Delta\Delta CT}$ method. and normalized to GAPDH (for mRNA) or U6 (for miRNA). Primer sequences were listed in Table 1. GAPDH were employed for mRNA normalizing while U6 for miRNA normalizing. The experiments were repeated 3 times independently.

Cell viability

Cells were collected (2×10^4 cells/ml) and inoculated into 96-well plates and incubated for 48 h. Subsequently, cells were reacted with 10 µl CCK-8 reagent (Dojindo) for 2 h. Finally, the OD_{450} value was examined by microplate reader (Bio-Rad Laboratories) to assess cell viability. The experiments were repeated 3 times independently.

Cell transfection

The mimic (miR-122-5p and nc) were acquired from GenePharma (Shanghai). Vectors for gene overexpression and the corresponding blank control were acquired from Ribobio (Guangzhou). 6-well plates were performed to inoculate cells. Until cell fusion reached 60–80%, Hep3B and Huh7 cells were transfected with mimic sequences (50nM) or vectors by Lipofectamine 3000 (Invitrogen) according to the standard procedure. After 48 h, RT-qPCR was employed to evaluate transfection efficiency. The experiments were repeated 3 times independently.

Transwell assay

Cells were inoculated in 24-well plates, which fitted with 8 µm chambers (Corning). 200 µl cell suspension (containing 2×10^5 cells) with serum-free was supplemented to the upper chamber, and the lower chamber was supplemented with 500 µl of medium containing 10% FBS. After 24 h of incubation, cells in the lower chamber were fixed with 4% paraformaldehyde (Solarbio) for 15 min, and then stained with 0.1% crystalline violet (Solarbio) for 10 min. After the excess dye was washed away with PBS, five randomly selected fields were observed under a microscope, and the number of migrated cells was recorded. Transwells for invasion

Gene	Primers sequences (5'-3')
miR-122-5p	F: TGGAGTGTGACAATGGGACC R: CCAGTGCAGGGTCCGAGGT
METTL3	F: CATTGCCCACTGATGCTGTG R: AGGCTTTCTACCCCATCTTGA
METTL14	F: GAACACAGAGCTTAAATCCCCA R: TGTCAGCTAAACCTACATCCCTG
WTAP	F: ACTGGCCTAAGAGAGTCTGAAG R: GTTGCTAGTCGCATTACAAGGA
KIAA1429	F: ATACTGATGGTCTGGTGCTAAGA R: TGGAGGGCTTCCATTAAACTGAT
FTO	F: GCTGCTTATTTCGGGACCTG R: AGCCTGGATTACCAATGAGGA
ALKBH5	F: AGTTCCAGTTCAAGCCTATTCG R: TGAGCACAGTCACGCTTCC
KAT2A	F: CAGGGTGTGCTGAACTTTGTG R: TCCAGTAGTTAAGGCAGAGCAA
GAPDH	F: ACAACTTTGGTATCGTGGAAGG R: GCCATCACGCCACAGTTTC
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT

Table 1. Primers sequences of PCR.

experiments were performed using Matrigel gel (BD Biosciences) lined with 40 μl/well at the bottom, and other steps were the same as those for migration experiments. The experiments were repeated 3 times independently.

MeRIP assay

MeRIP was carried out by Magna MeRIP m6A kit (Millipore). Total RNA was extracted with Trizol reagent and then incubated with DNase R (Sigma-Aldrich). RNA fragmentation (~ 100 nucleotides) was then conducted by RNA fragmentation buffer. Subsequently, RNA fragments were reacted with magnetic beads coupled with m6A antibody or IgG antibody (Synaptic Systems) overnight in MeRIP immunoprecipitation buffer at 4 °C. Next, after the magnetic beads were washed five times with MeRIP elution buffer, the beads were reacted with IP buffer containing RNase inhibitor and protease inhibitor for 1 h at 4 °C. Finally, miR-122-5p enrichment was analyzed via RT-qPCR. The experiments were repeated 3 times independently.

RIP assay

Magna RIP Kit (Millipore) was employed to conduct RIP analysis according to the instructions. Cells were lysed in RIP lysis buffer. Then, magnetic beads coupled with Ago2 antibody, KAT2A, METTL14 antibody, DGCR8, or IgG antibody (Abcam) was employed to incubate cell lysates at 4 °C overnight. Subsequently, the complexes were eluted via wash buffer containing proteinase K. Finally, extracted and purified RNA was performed for RT-qPCR analysis. The experiments were repeated 3 times independently.

Co-IP

Immunoprecipitation was conducted by the PierceTM Immunoprecipitation Kit (ThermoFisher Scientific). In short, IP lysis buffer containing a protease/phosphatase inhibitor mixture was carried out to lysis cell. METTL14 or DGCR8 proteins were precipitated using anti-METTL14 antibody or DGCR8 antibody (Abcam) by overnight incubation with cell lysates at 4 °C. Immunoprecipitation complexes were treated with RNase A (20 μg/ml) or RNase inhibitor (200 U/ml) at 37 °C for 5 min. Western blot analysis was then analyzed using anti-DGCR8 or METTL14 antibodies. The experiments were repeated 3 times independently.

Western blot

Cells total protein were isolated via lysis buffer (Beyotime) and quantified through the BCA Protein Assay Kit (Solarbio). Subsequently, total protein (30 μg/lane) was separated using 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was then blocked by TBST with 5% BSA (Sigma-Aldrich). After 1 h of sealing, the membrane was incubated overnight with the primary antibody dilution at 4 °C. After recovery of the primary antibody, secondary antibody was then continued incubated for 1 h. Finally, the membrane was visualized using the Enhanced Chemiluminescent Protein Detection Kit (Bio-Rad Laboratories) for visualization and imaged under the Las-4000 imaging system. Relative quantification of proteins was analyzed using Image J software with GAPDH as an internal reference. Primary antibodies used were anti-METTL14, anti-DGCR8, anti-β-catenin, and anti-GAPDH. Secondary antibodies were goat anti-rabbit antibodies. All antibodies were obtained from Abcam. The experiments were repeated 3 times independently.

Dual luciferase reporter gene

Based on the binding site sequences of KAT2A mRNA and miR-122-5p, KAT2A-3′UTR wild-type (WT) and mutant (MUT) sequences were designed and constructed into pGL3 luciferase vector (Promega), respectively. 2×10^5 cells were then inoculated into 6-well plates. After cell attachment, cells were transfected with the above vectors and miRNA NC or miR-122-5p mimics using Lipofectamine 3000. After 48 h transfection, changes of luciferase activity were detected through a dual luciferase assay kit (Promega). The experiments were repeated 3 times independently.

CHX treatment

Cells were reacted with cycloheximide (100 $\mu g \cdot mL^{-1}$, Sigma-Aldrich) for 0, 6, 12, 24 h, respectively. Subsequently, protein was extracted using RIPA buffer at each specified time point. Then, the protein expression of β-catenin was evaluated through a standard western blotting process. The experiments were repeated 3 times independently.

Animal experiments

All animal handling procedures were approved by The Fourth Hospital of Harbin Medical University Hospital Ethics Committee. All animal experimental operations followed the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Twelve male BALB/c nude mice (6 weeks old; 19–22 g) were purchased from Beijing Laboratory Animal Center and kept in an experimental animal barrier environment for one-week adaptive feeding. Then these mice were maintained under specific-pathogen-free conditions with 12 h light/dark cycle and free access to food and water. Huh7 cells stably transfected with agomiR-122-5p and control agomiR-NC were subcutaneously inoculated in the right axilla at a density of 2×10^6 cells per mouse (6 mice per group). The nude mice were randomly divided into control groups (agomiR-NC) and experimental groups (agomiR-122-5p) by the random number method. Also, researchers involved in the daily breeding, observation and data collection of animals were not aware of the grouping of the animals. The size of the formed tumors was recorded once a week and assessed using the following formula: tumor volume (mm^3) = length (L) × width (W) ^{2}/2. 5 weeks later, all mice were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg), and the tumors were removed and weighed. IHC was applied to examine the expression of KAT2A and β-catenin in tumor tissues of nude mice.

Statistical analysis

GraphPad Prism software (version 8.0; GraphPad Software) was carried out to analyze the difference of data. Mean ± standard deviation was employed to display experiment data. Student's t-test was carried out to analyze the differences of data which obtained from two different groups, while analysis of variance (ANOVA) test was used for the comparison of more than two groups. Statistical results were significant when $P < 0.05$ and were indicated by "*". Additionally, details for the statistical analysis, including effect sizes and confidence intervals are presented in Supplementary Table 1 .

Results

miR-122-5p is significantly diminished in HCC

In recent years, many aberrantly expressed miRNAs were identified as oncogenes or suppressor in tumors. To explore differentially expressed miRNAs in HCC, we analyzed the GEO data (GSE158523). By plotting heat map, we filtered out the top 10 differentially expressed miRNAs, including miR-122-5p, miR-376b-3p, miR-379-3p, miR-136-5p, miR-1185-1-3p, miR-337-5p, miR-412-5p, miR-154-5p, miR-323a-3p, miR-1197 (Fig. 1A). Subsequently, we further validated their expression in clinical tissue samples obtained from 45 HCC patients. As shown in the experimental data, we found that only three miRNAs, miR-122-5p (0.54 ± 0.32, $P < 0.001$), miR-379-3p (0.81 ± 0.33, $P < 0.05$) and miR-136-5p (0.85 ± 0.27, $P < 0.05$) was abnormally reduced in HCC. While miR-1197 (1.15 ± 0.28, $P < 0.05$) was significantly increased (Fig. 1B). Hence, we chose miR-122-5p with the most pronounced difference for the follow-up study. Meanwhile, a similar trend was presented in HCC cell lines. miR-122-5p were remarkably weakened in all five HCC cell lines compared with normal hepatocytes (Fig. 1C). Specifically, we chose Hep3B (0.37 ± 0.07) and Huh7 (0.32 ± 0.10) cells with the lowest expression levels to continue the subsequent studies. The data revealed that miR-122-5p may regulated HCC progression as a tumor suppressor.

miR-122-5p insertion inhibits malignant progression of HCC cells

Next, we further explored the function of miR-122-5p in HCC. miR-122-5p overexpression cell model was constructed. We observed that miR-122-5p was notably elevated in Hep3B (3.77 ± 0.49) and Huh7 (4.24 ± 0.59)

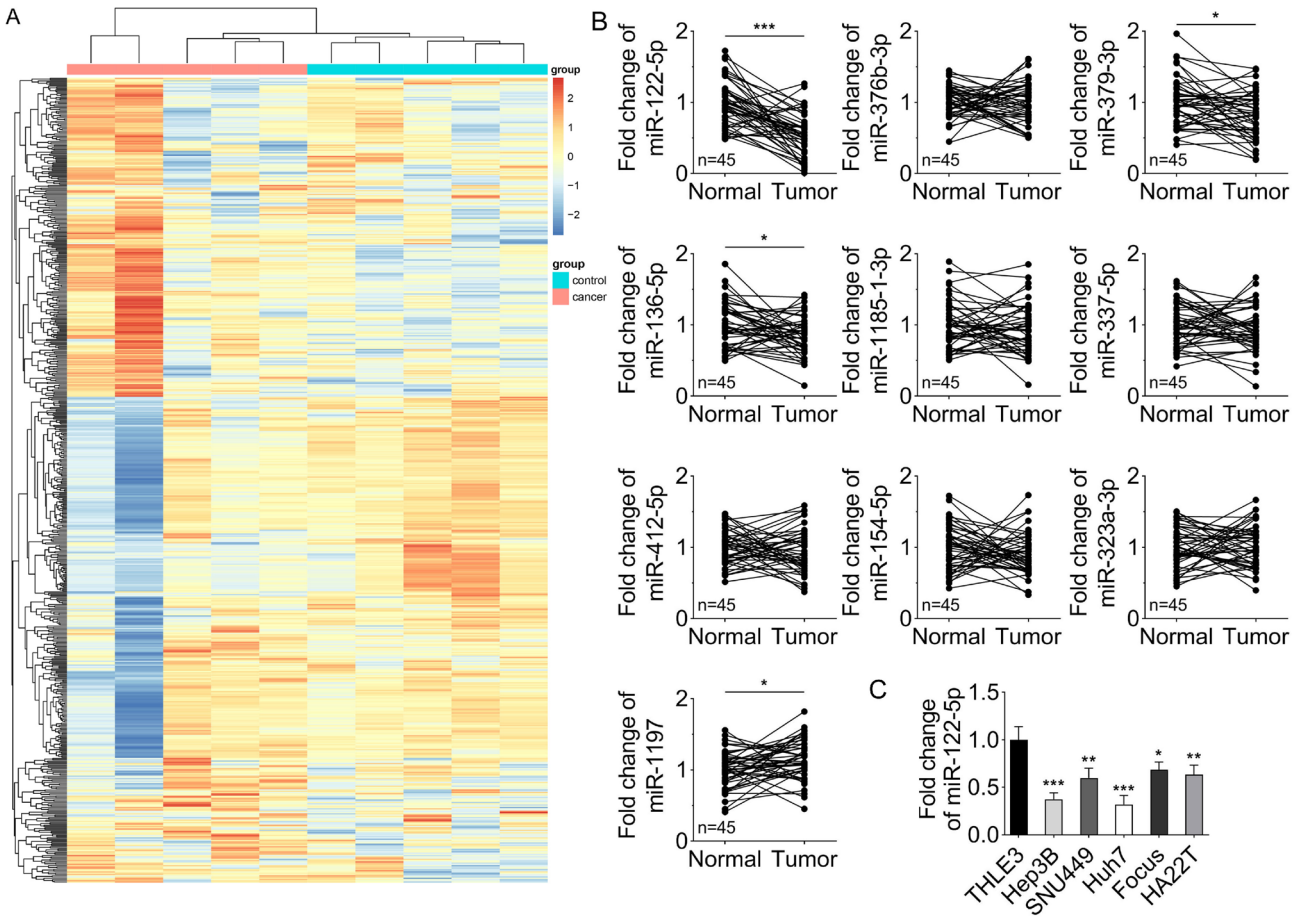

Fig. 1. miR-122-5p was lowly expressed in HCC. (**A**) Heat map of differentially expressed miRNA in HCC from GSE158523 dataset. (**B**) Expression of top 10 differential miRNAs in HCC tissue samples ($n = 45$) was detected by RT-qPCR. (**C**) Expression of miR-122-5p in HCC cells was detected by RT-qPCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cells after miR-122-5p mimic transfection (Fig. 2A). Next, we evaluated the viability of Hep3B and Huh7 cells after transfection by CCK-8. The results displayed that miR-122-5p upregulation obviously restrained the cell viability of Hep3B (40.60 ± 6.84) and Huh7 (44.23 ± 4.97) cells (Fig. 2B). Subsequently, transwell analysis revealed that miR-122-5p insertion remarkably diminished the number of migrating Hep3B (54.00 ± 12.53) and Huh7

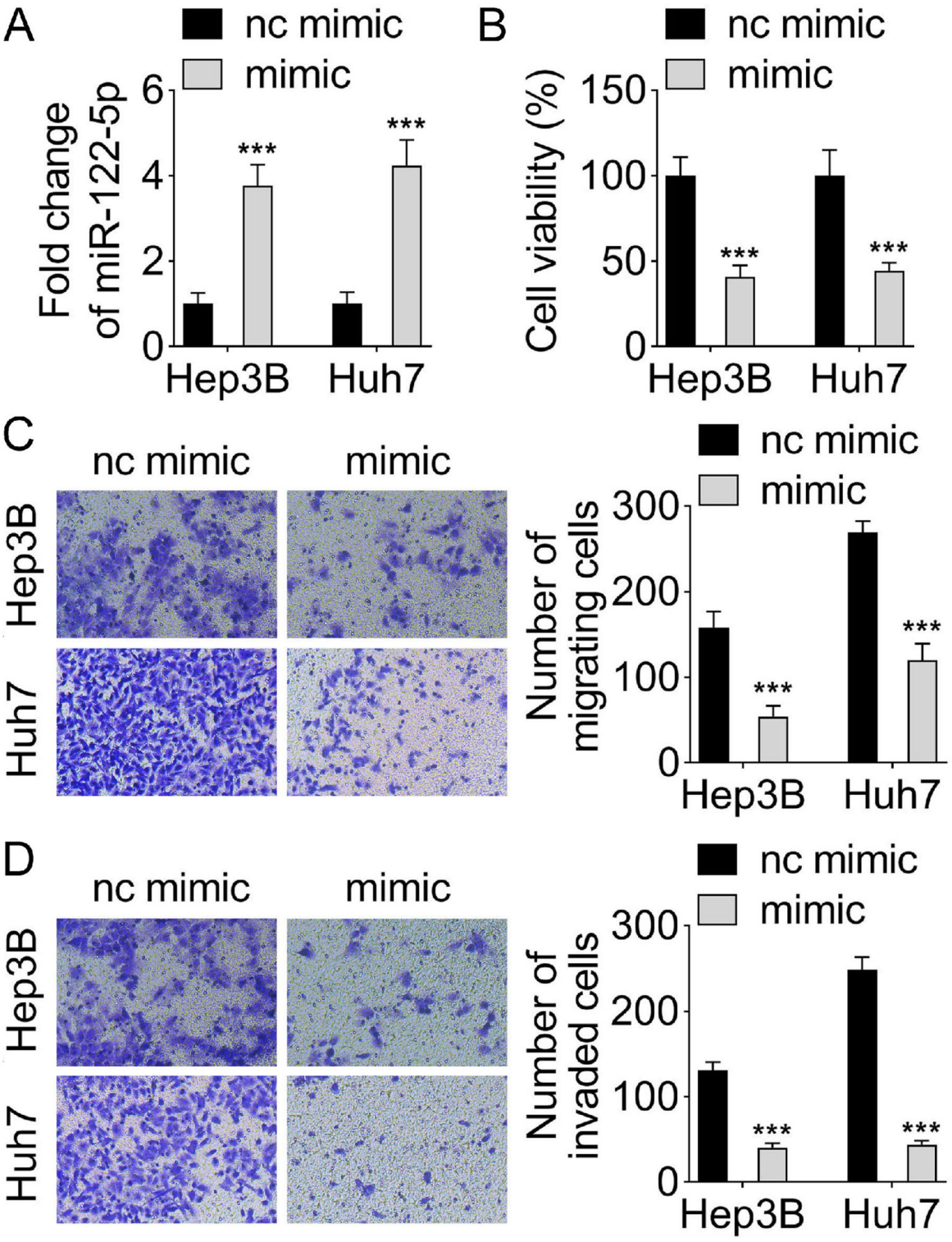

Fig. 2. miR-122-5p was involved in the malignant progression of HCC cells. (**A**) Overexpression transfection efficiency of miR-122-5p was detected in HCC (Hep3B and Huh7) cells by RT-qPCR. (**B**) Changes of cell viability were detected by CCK-8. (**C**) Changes of cell migration were examined by transwell. (**D**) Changes of cell invasion were measured by transwell. ***, $P < 0.001$.

(119.67 ± 19.66) cells (Fig. 2C), and invaded Hep3B (40.00 ± 5.57) and Huh7 (43.67 ± 4.73) cells (Fig. 2D). These data confirmed that miR-122-5p weakened the malignant phenotype of HCC cells by acting as a suppressor.

METTL14 regulates miR-122-5p maturation in an m6A-dependent manner

m6A modifications is reported related to maturation of miRNA. To clarify the regulation of m6A key genes in miR-122-5p processing, we successfully overexpressed m6A 'writers' METTL3 (5.27 ± 0.05), METTL14 (6.15 ± 0.26), WTAP (3.68 ± 0.17), KIAA1429 (6.87 ± 0.27), and 'easer' FTO (2.68 ± 0.36) and ALKBH5 (5.83 ± 0.29) expression (Fig. 3A). Subsequently, the changes of miR-122-5p expression after m6A regulators insertion was continued analyzed. Notably, miR-122-5p (2.73 ± 0.24) was remarkably augmented only in METTL14 overexpressed cells (Fig. 3B). This illustrated that METTL14 was essential for maintaining miR-122-5p levels. Next, we examined the effect of METTL14 overexpression on pri-miR-122 m6A levels by MeRIP. We found that m6A level of pri-miR-122 (0.65 ± 0.05) was notably heightened after insertion of METTL14 (Fig. 3C). In addition, we also verify the effect of METTL3 introduction on miR-122 various stages, including pre-miR-122, mature miR-122-5p, and pri-miR-122. we found that METTL14 insertion distinctly strengthened pre-miR-122 (2.52 ± 0.16) and miR-122-5p (2.07 ± 0.16) expression. In contrast, excessive METTL14 suppressed the pri-miR-122 (0.46 ± 0.04) expression (Fig. 3D). This proved that METTL14 upregulation was able to tag pri-miR-122 via m6A methylation thereby promoting the maturation of miR-122-5p. Pri-miRNA transcripts are cleaved by DGCR8 to form precursor miRNAs (pre-miRNAs)[38]. Therefore, we next proceeded to investigate whether METTL14 affects the maturation of miR-122-5p by binding to pri-miR-122, and DGCR8 served as a positive control. RIP results initially confirmed that like DGCR8 (0.56 ± 0.09), METTL14 (0.50 ± 0.07) was clearly recognized and bound to pri-miR-122 (Fig. 3E). In addition, we also further verified by co-IP that METTL14 and DGCR8 have a binding relationship (Fig. 3F). Besides, siRNA targeting METTL14 was used to successfully knock down the expression level of METTL14 in HCC cells (Figure S2A), and si-METTL14#1 (0.31 ± 0.08, 0.33 ± 0.06) was employed for following tests. MeRIP assay revealed that the enrichment level of m6A on pri-miR-122 (0.20 ± 0.03, 0.25 ± 0.05) was decreased after METTL14 inhibition (Figure S2B). Moreover, the miR-122-5p expression (0.45 ± 0.06, 0.40 ± 0.09) was declined after METTL14 silencing, while pri-miR-122 expression (1.81 ± 0.15, 1.68 ± 0.10) was elevated (Figure S2C). Taken together, our research further confirmed that METTL14 regulated miR-122-5p maturation by altering the m6A modification of pri-miR-122.

miR-122-5p targets binding to KAT2A

Next, to further explore the downstream mechanism of miR-122-5p, we predicted the possible targets of miR-122-5p by bioinformatics online tools (Figure S1). We obtained that KAT2A had a binding site with miR-122-5p (Fig. 4A). Subsequently, a reduce of dual luciferase activity (0.36 ± 0.06) in cells co-transfected with miR-122-5p and wt-KAT2A confirmed the binding relationship between KAT2A and miR-122-5p (Fig. 4B). Notably, we further confirmed the binding relationship between KAT2A and miR-122-5p by RIP assay. We found that KAT2A (6.48 ± 0.85) was obviously enriched in anti-Ago2 group (Fig. 4C). And miR-122-5p (0.52 ± 0.05) was also clearly enriched by KAT2A (Fig. 4D). In addition, we analyzed the effect of miR-122-5p on KAT2A expression. We found that miR-122-5p overexpression remarkably reduced KAT2A expression (0.45 ± 0.07), indicating a negative regulatory relationship between miR-122-5p and KAT2A (Fig. 4E). Finally, we examined KAT2A level in HCC clinical tissue samples and cells. Undoubtedly, KAT2A were distinctly elevated in both HCC tissues (2.02 ± 0.31) and Hep3B (2.41 ± 0.37) and Huh7 (2.85 ± 0.44) cells (Fig. 4F-G). These data evidenced that KAT2A was a regulatory target of miR-122-5p in HCC.

KAT2A overexpression inhibits the anti-tumor effect of miR-122-5p overexpression

Then, we tested whether miR-122-5p exerted its effects by regulating KAT2A expression in HCC. First, we tested the transfection efficiency of KAT2A overexpression vector. A significant increase of KAT2A in Hep3B (5.71 ± 0.38) and Huh7 (6.41 ± 0.54) cells transfected with the overexpression vector was observed by RT-qPCR assay (Fig. 5A). Subsequently, CCK-8 and transwell were carried out to assay cell viability, migration and invasion. The results showed that KAT2A overexpression clearly limited the tumor suppressor effect of miR-122-5p overexpression. Specifically, cell viability of Hep3B (85.97 ± 6.65) and Huh7 (87.23 ± 5.49) cells, cell migration of Hep3B (104.67 ± 6.66) and Huh7 (221.00 ± 24.25) cells, and cell invasion of Hep3B (104.33 ± 14.01) and Huh7 (207.67 ± 19.04) cells restrained by miR-122-5p overexpression were reactivated by KAT2A (Fig. 5B-E). These data revealed that miR-122-5p overexpression inhibited tumor progression through targeting KAT2A.

KAT2A regulates β-catenin protein stability through succinylation modification

KAT2A is a succinylation modification regulator. Therefore, we proceeded to investigate the effect of KAT2A on succinylation levels in HCC cells. We found that KAT2A overexpression increased overall cellular succinylation levels of Hep3B (2.38 ± 0.19) and Huh7 (1.77 ± 0.07) cells. In addition, KAT2A insertion also increased the succinylation level of β-catenin in Hep3B (1.68 ± 0.18) and Huh7 (1.62 ± 0.16) cells. Meanwhile, the expression level of β-catenin in Hep3B (0.57 ± 0.09) and Huh7 (0.62 ± 0.11) cells was limited by KAT2A (Fig. 6A). Finally, we analyzed the effect of KAT2A on β-catenin protein stability. We found that KAT2A overexpression remarkably weakened the stability of β-catenin protein of Hep3B (37.03 ± 5.75) and Huh7 (38.43 ± 5.53) cells at 24 h (Fig. 6B-C). These data further confirmed that KAT2A was involved in tumor progression by regulating the succinylation level of β-catenin in HCC.

miR-122-5p overexpression limits HCC tumor growth in vivo

The function of miR-122-5p in HCC tumorigenicity in vivo was determined by a mouse xenograft model. As shown in Fig. 7A-C, the xenograft tumor weight (273.70 ± 49.89) and volume (288.67 ± 34.67) in the agomiR-122-5p group were smaller than those in the agomiR-NC group. In addition, miR-122-5p overexpression also

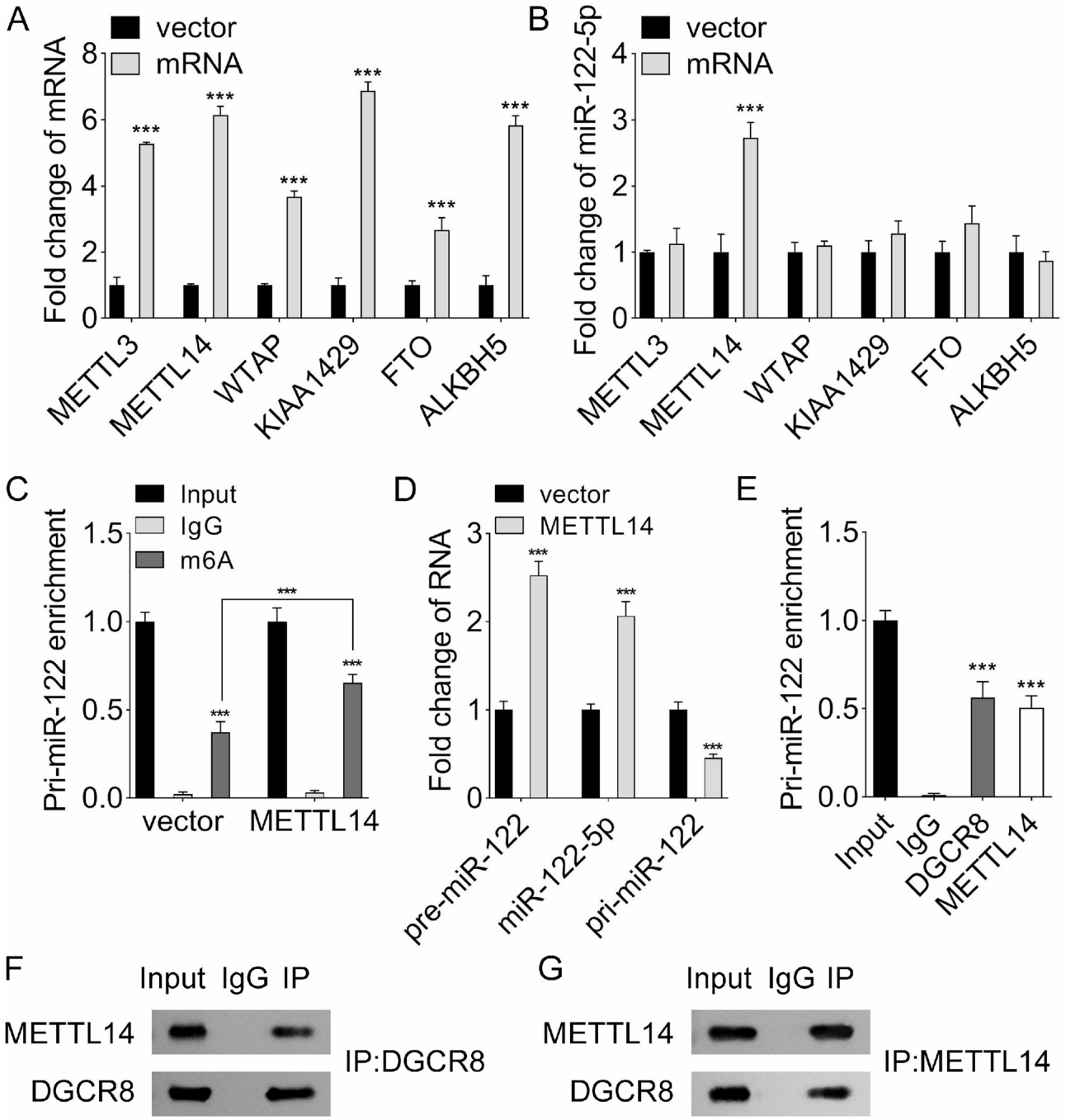

Fig. 3. METTL14 regulated miR-122-5p maturation in an m6A-dependent manner. (**A**) Overexpression transfection efficiency of methylation-related enzymes in Hep3B cells was detected by RT-qPCR. (**B**) Effect of methylation-related enzyme overexpression on miR-122-5p expression in Hep3B cells was analyzed by RT-qPCR. (**C**) Changes of cellular m6A levels in Hep3B cells were detected by MeRIP. (**D**) The effect of METTL14 overexpression on miR-122 expression in Hep3B cells at various stages was analyzed by RT-qPCR. (**E**) RIP assay was conducted to verify the interaction between METTL14 and pri-miR-122 in Hep3B cells. (**F**-**G**) Verification of the binding of METTL14 and DGCR8 by CO-IP assay in Hep3B cells. ***, $P < 0.001$.

distinctly restrained KAT2A expression (Fig. 7D), while β-catenin expression was boosted (Fig. 7E). In summary, miR-122-5p introduction could hinder the growth of HCC tumors in vivo.

Discussion

Indeed, as endogenous non-coding RNAs, miRNAs have been shown to be widely dysregulated in disease, including cancers. Many dysregulated miRNAs involved in cancer development, progression, and metastasis by acting as oncogenes (called "oncomiRs") or tumor suppressor genes ("oncosuppressor miRs")[39,40]. For instance,

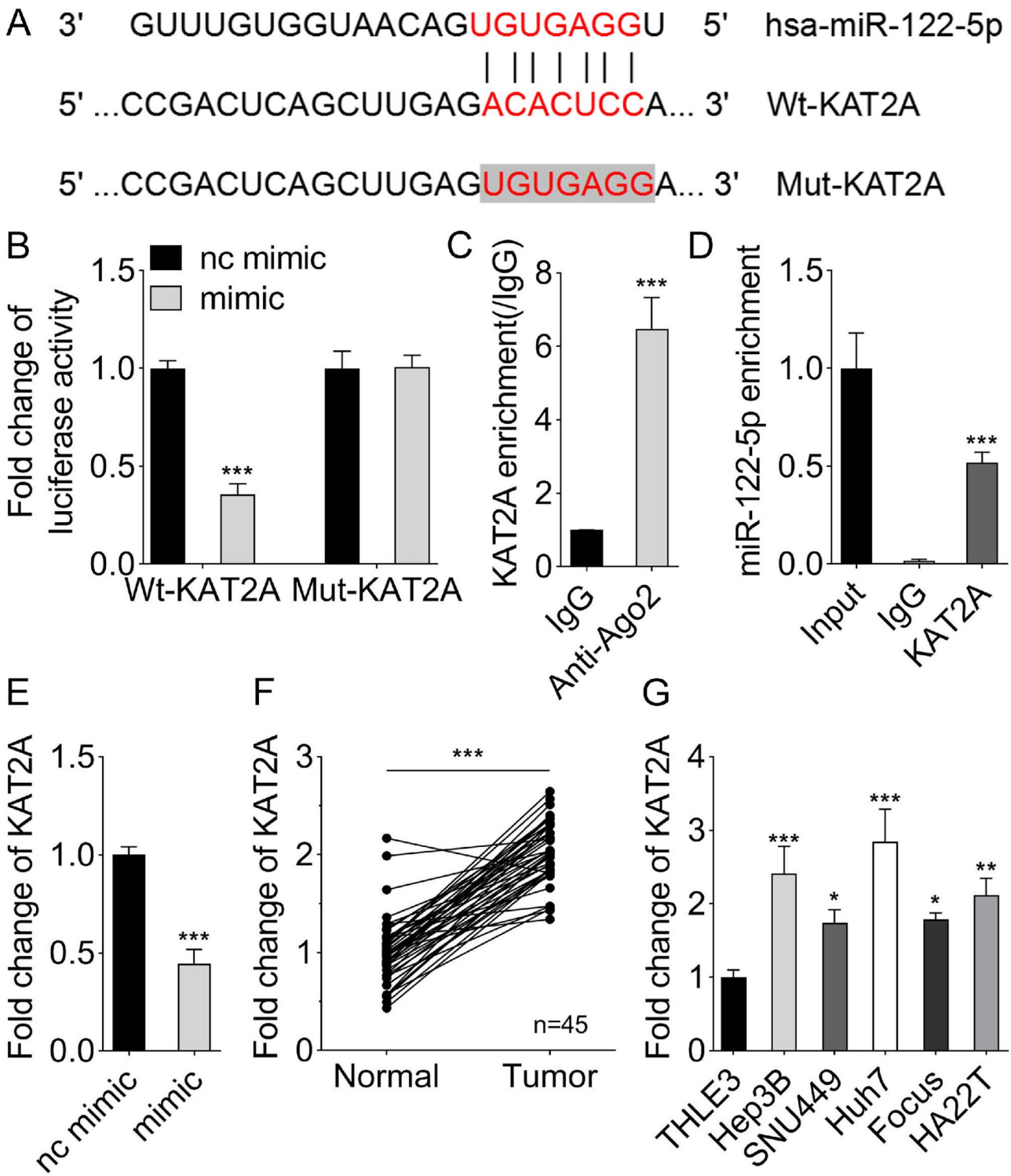

Fig. 4. KAT2A was a downstream target of miR-122-5p. (**A**) Binding site of miR-122-5p and KAT2A. (**B**) The binding relationship between miR-122-5p and KAT2A in Hep3B cells was confirmed using luciferase reporter vector assay. (**C**-**D**) RIP was performed to verify the binding of miR-122-5p to KAT2A in Hep3B cells. (**E**) RT-qPCR was performed to detect the effect of miR-122-5p on KAT2A expression in Hep3B cells. (**F**) RT-qPCR was conducted to detect the expression level of KAT2A in clinical samples ($n = 45$). (**G**) RT-qPCR was applied to evaluate the expression level of KAT2A in HCC cell lines. ***, $P < 0.001$.

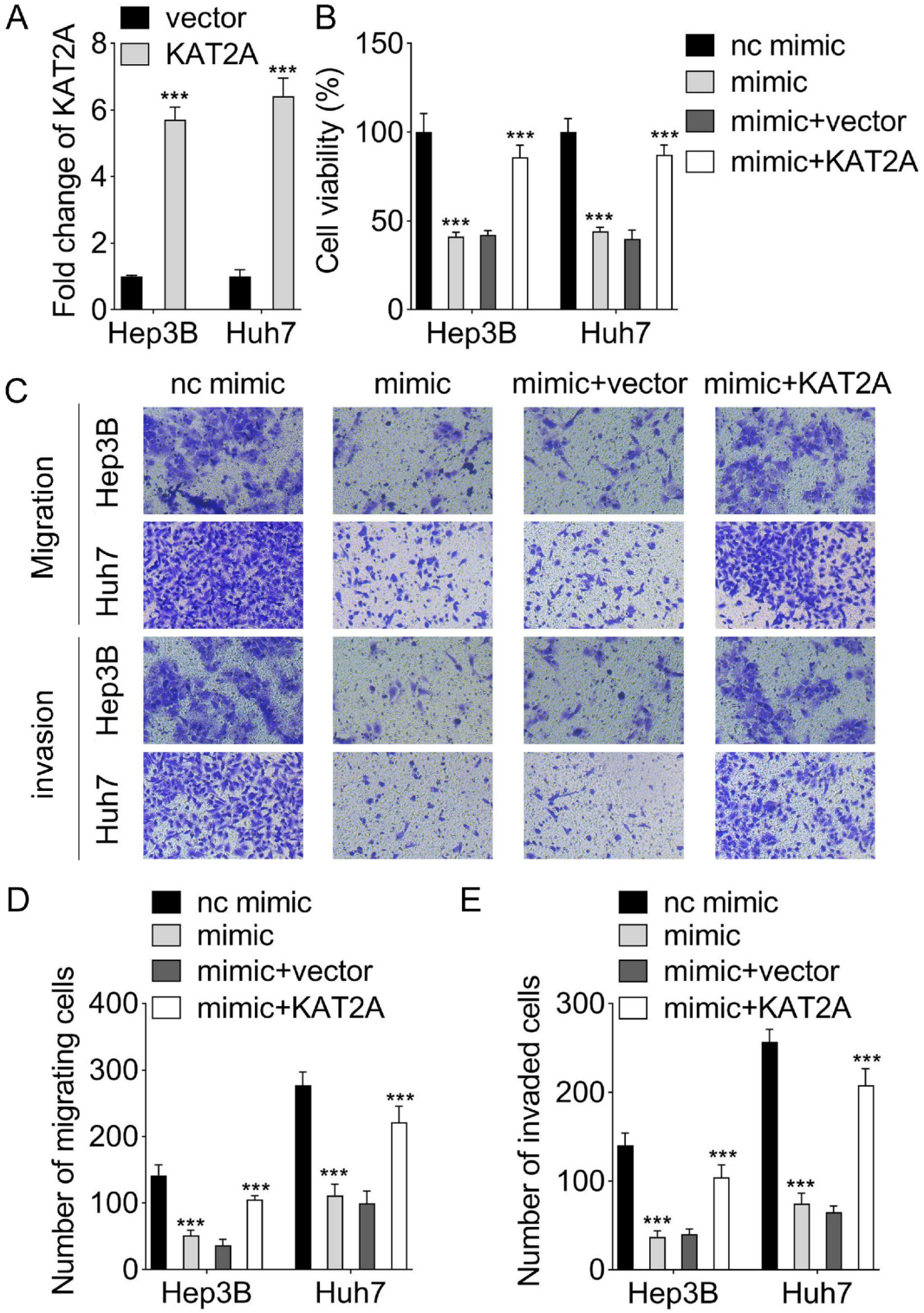

Fig. 5. miR-122-5p/KAT2A axis regulated malignant progression of HCC. (**A**) Overexpression transfection efficiency of KAT2A in HCC (Hep3B and Huh7) cells was detected by RT-qPCR. (**B**) Changes of cell viability were detected by CCK-8 after indicated transfection. (**C**-**E**) Changes of cell migration and invasion were detected by transwell assays after indicated transfection. ***, $P < 0.001$.

let-7 was down-regulated in multiple cancers, which had been shown to be a tumor suppressor miRNA that inhibited tumor development by suppressing oncogenes such as RAS or MYC[41]. miR-320b in esophageal cancer cells was associated with proliferation, migration, invasion and metastasis[42]. microRNA-370-3p limited tumor progression in HCC by suppressing the IL-8/STAT3 signaling pathway through interaction with Twist1

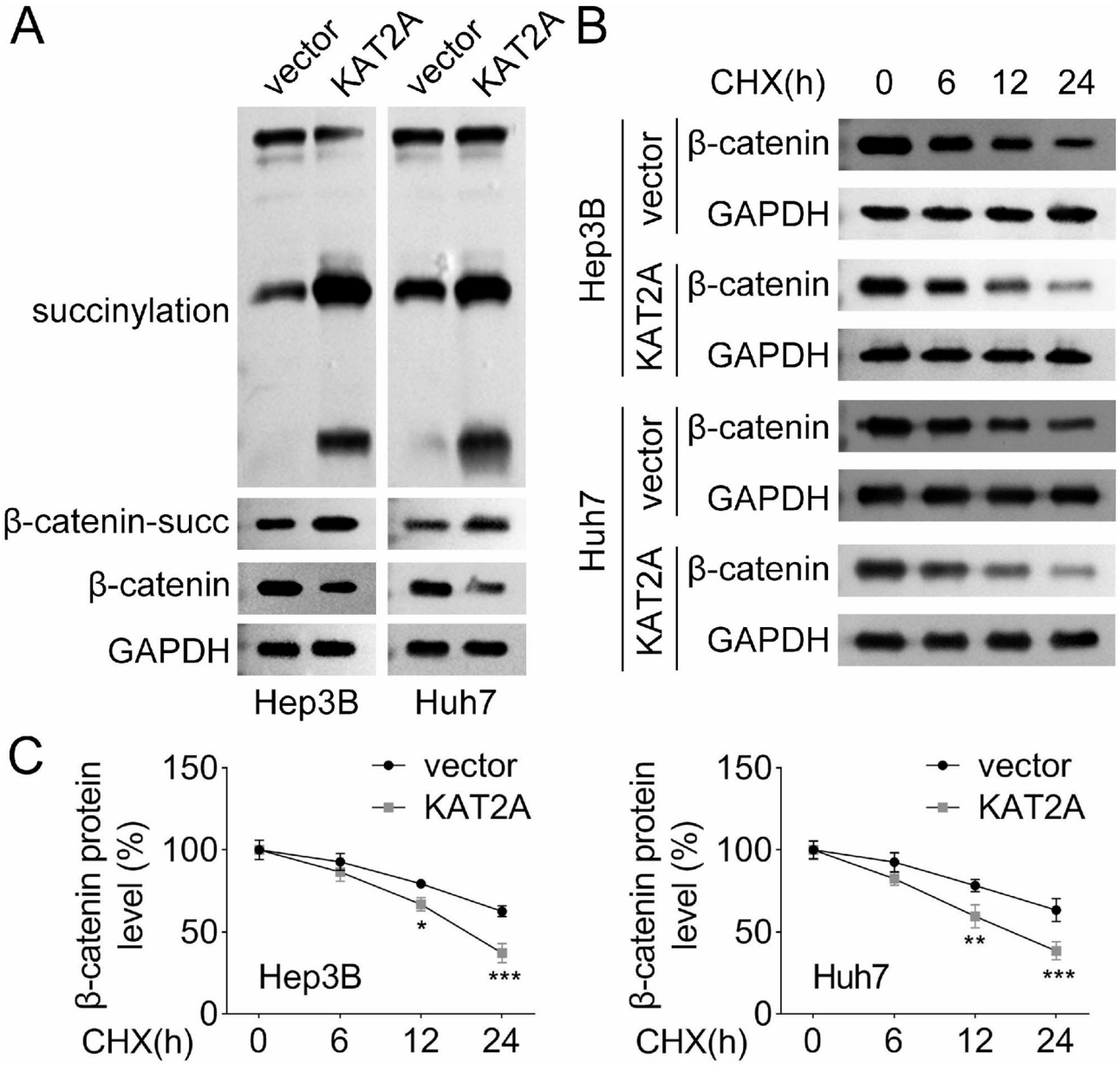

Fig. 6. KAT2A regulated the expression of β-catenin. (**A**) Overall cellular succinylation level, β-catenin succinylation level and β-catenin protein expression level after overexpression of KAT2A were detected by western blot. (**B**-**C**) β-catenin protein stability after overexpression of KAT2A were detected by western blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and Snail mRNA[43]. However, aberrant miRNAs regulating the malignant progression of HCC still need to be investigated for identification, which may provide more effective therapeutic approaches for HCC patients. In our study, the presence of abnormally downregulated miR-122-5p was screened by GSE158523 dataset. This low expression level was subsequently further validated in HCC clinical samples and cells, which is consistent with past reports[24,25]. Besides, the expression and signal transduction of miR-122-5p were also stimulated by external factors in HCC. Exogenous treatment with licorice roots extract and taurine contributed to the upregulation of miR-122-5p, which subsequently hindered the growth and metabolic activity of HCC[44,45]. Our cellular functional studies revealed that miR-122-5p insertion significantly limited the malignant progression of HCC cells, such as cell viability, migration, and invasion. Animal data also illustrated that miR-122-5p introduction could hinder the growth of HCC tumors in vivo. All these findings indicated that targeting miR-122-5p promises to be a valuable therapeutic strategy for HCC.

Nowadays, dysregulation of miRNA processing is linked to enhanced tumorigenesis[46]. In the typical biosynthetic pathway of miRNAs, primary miRNAs (pri-miRNAs) are transcribed, then pri-miRNAs were recognized by DROSHA and DGCR8 and cleaved into precursor miRNAs (pre-miRNAs). The pre-miRNA is then output to the cytoplasm for further cleavage by the DICER enzyme and cofactors[47]. Misregulation of

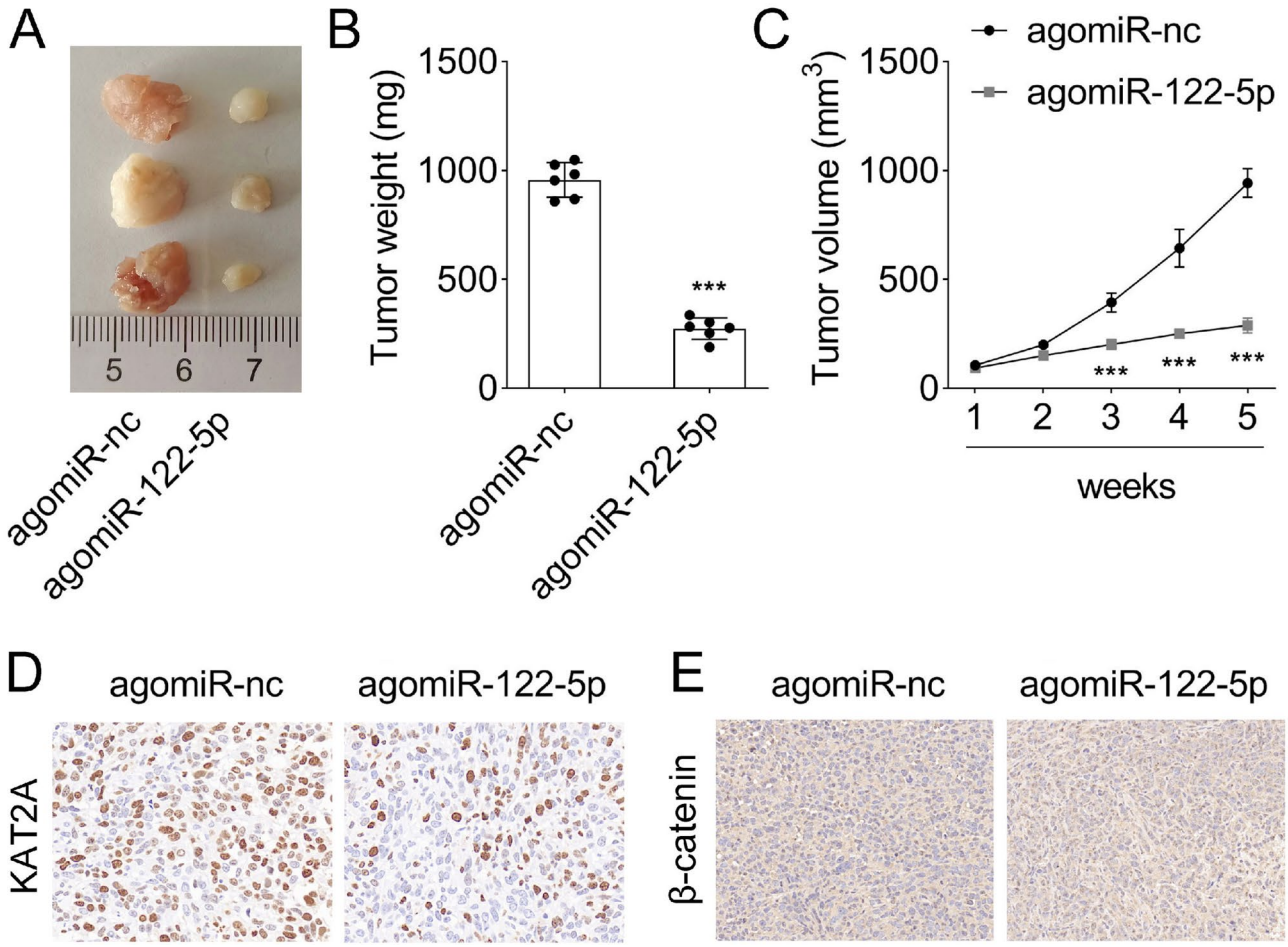

Fig. 7. miR-122-5p overexpression limited HCC tumor growth in vivo. (**A**) Representative tumor images of nude mice xenograft model. (**B**) xenograft tumor weight. (**C**) xenograft tumor volume. (**D**) KAT2A expression was examined in nude mice xenograft. (**E**) β-catenin expression was analyzed in nude mice xenograft. ***, $P < 0.001$.

multiple miRNAs allows for altered expression in multiple cancers, thereby affecting cancer progression[48]. For example, selective blockade of miR-145 processing by CCAT2 leads to reduced miR-145 and regulates CRC stem cell proliferation[49]. Recently, a wide range of epigenetic variants have been the focus of renewed exploration of the regulatory mechanisms underlying altered miRNA biogenesis[50]. Therefore, a detailed understanding for regulating mechanisms of miRNAs is very essential in cancer.

In recent years, there has been increasing evidence of epigenetic interactions between m6A methylation modifications and miRNA expression in cancer[51,52]. m6A labeling serves as a key pathway to regulate miRNA biogenesis. Specifically, m6A modifications on pri-miRNAs can promote or inhibit the miRNA maturation process, which subsequently affects tumor progression by altering miRNA expression levels[28]. It has been found that METTL3 induced m6A methylation of pri-miRNA, which is then specifically recognized by DGCR8 and promoted miRNA maturation[28]. In addition, Cigarette smoke also promoted PC progression by inducing miR-25-3p processing via m6A labeling[53]. METTL3 strengthened miR221/222 maturation by binding to DGCR8 in bladder cancer[54]. METTL3 knockdown was shown to reduce miR-221-3p expression by decreasing pri-miR-221-3p m6A level, which induces apoptosis through the HIPK2/Che-1 axis in drug-resistant cells[55]. These studies confirm that m6A regulators are capable of modulating miRNA processing in cancer and are associated with cancer progression. In HCC, previous studies disclosed that METTL3 was responsible for the pri-miR-589 m6A modification, and aggravated HCC metastasis by upregulating miR-589-5p maturation[30]. METTL14 positively enhanced miR-126 maturation through interacting with DGCR8, thereby inhibiting metastasis of HCC cells[56]. Currently, the research on m6A-mediated miRNA modification in HCC is still in its infancy, it is unclear whether m6A methylation modification also affects miR-122-5p maturation and expression. In our study, we first screened potential m6A regulators by evaluating the influence of m6A enzymes on miR-122-5p expression. Among m6A "writers" (METTL3, METTL14, WTAP, and KIAA1429) and "eraser " (FTO and ALKBH5), we found that miR-122-5p was only affected by METTL14, and there was a significant positive correlation between them. Moreover, we verified that METTL14 insertion promoted miR-122-5p maturation in an m6A-dependent manner by binding DGCR8 recognition of pri-miR-122. Collectively, we revealed the upstream m6A regulatory mechanism of miR-122-5p from an epigenetic perspective. Notably, this was similar to the role of m6A "writer"

METTL3 in HCC reports, which also promoted the maturation of oncogene miR-589-5p in an m6A-dependent manner and subsequently accelerated HCC progression[30]. Meanwhile, the latest reports confirmed that m6A "writers" in other tumors were also generally involved in promoting the processing of miRNA, including oral squamous cell carcinoma[57], lung cancer[58], and prostate cancer[59], etc. The difference is that m6A "erasers" usually serve the role of hindering miRNA processing in tumors. For example, FTO and ALKBH5 could respectively inhibit the processing of miR-17-5p and miR-124-3p, and then regulated the metastasis of breast cancer and glioblastoma[29,60]. However, the mechanism of miRNA processing by these m6A demethylases in HCC has been insufficiently reported.

Furthermore, we went on to explore the downstream targets of miR-122-5p. Our data confirmed that KAT2A was a regulatory target of miR-122-5p in HCC. KAT2A has been widely reported as a carcinogenic factor in previous research[31], and down-regulation of KAT2A could decrease the growth activity of cancer cells[37]. Here, our study also validated the carcinogenic effect of KAT2A in HCC, and KAT2A overexpression markedly reversed the inhibition of miR-122-5p on HCC cell proliferation and metastasis. Also, recent reports disclosed that KAT2A interacted with ALDOB and participated in immune escape of HCC[61]. Moreover, KAT2A is also a vital succinyltransferase that dynamically regulates succinylation modification. For instance, KAT2A catalyzed the succinylation of HBV covalently closed-loop DNA at H3K79 (H3K79succ) via its succinyltransferase activity, thereby promoting its transcription[62]. In human pancreatic ductal adenocarcinoma, KAT2A was evidenced to promote the malignant progression, such as proliferation, migration and invasion through the upregulating succinyltransferase activity of 14-3-3ζ[63]. At present, the research on the mechanism of KAT2A-mediated succinylation in oncology is relatively insufficient, the succinylation modifications regulated by KAT2A in HCC and its target of action still need to be further investigated. In our study, we found that KAT2A increased the overall intracellular succinylation level and the succinylation level of β-catenin. At the same time, KAT2A overexpression suppressed the expression and stability of β-catenin. Given that HCC tumor progression is a complex process and related to multiple factors, more in-depth study of the KAT2A/β-catenin axis is needed in the future.

Taken together, we have revealed a novel METTL14/miR-122-5p/KAT2A/β-catenin regulation axis in HCC at the epigenetic level. This regulatory axis connects RNA methylation (METTL14), microRNA regulation (miR-122-5p), succinylation (KAT2A), and the classical signaling pathway (β-catenin), providing multi-level intervention opportunities for HCC. Targeting this axis may simultaneously affect epigenetic regulation and key signaling pathways, and it is expected to overcome the limitations of single-target therapy. Specifically, the combined intervention of METTL14 activator, miR-122-5p mimic and KAT2A inhibitor may produce a synergistic effect to inhibit the progression of HCC. However, the present study still has some limitations. In the clinical aspect, there is a lack of more sample sizes to verify the correlation of this axis molecule. Moreover, in the future, combining existing standard therapies for HCC (such as sorafenib, immunotherapy, etc.) or optimizing drug delivery systems specifically targeting this axis is expected to further promote the transformation of this mechanism into clinical applications. During the mechanism exploration process, we only conducted functional experiments on miR-122-5p overexpression in Hep3B and Huh7 cells with relatively low expression of miR-122-5p. If miR-122-5p inhibition tests can be taken in other HCC cells with relatively high expression, this will help better verify the biological function of miR-122-5p in HCC. Furthermore, in the future, using patient-derived xenotransplantation (PDX) models to verify the molecular mechanism of this study can provide reliable data for subsequent drug development (such as miRNA therapy or combination therapy regimens).

Conclusion

In conclusion, our results suggested that miR-122-5p was significantly reduced in HCC and overexpression of miR-122-5p inhibited malignant progression of HCC. Mechanistically, overexpression of METTL14 promoted miR-122-5p maturation by tagging pri-miR-122 with m6A. In addition, miR-122-5p exerted suppressor effects by targeting KAT2A. Finally, we also found that KAT2A regulated β-catenin expression through succinylation modification. Our discovery of the METTL14/miR-122-5p/KAT2A/β-catenin axis may not only deepen our understanding of the mechanism of HCC development, but also facilitate the development of miRNA-targeted therapy based on miRNA.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Besides, the dataset analyzed during the current study are available in Gene Expression Omnibus (GEO) database (GSE158523), https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE158523.

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Author contributions

X.F. designed the study, wrote, reviewed, and revised the paper. W.Q. collected data, processed statistical data, and performed the experiments. X.G. and J.W. analyzed and interpreted the data. L.Z. partly contributed to the experiments and data analysis. All authors reviewed the results and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The Fourth Hospital of Harbin Medical University. All participants were provided with written informed consent at the time of recruitment, and all experiments involving human tissue specimens comply with the Declaration of Helsinki. Animal studies were performed in compliance with the ARRIVE guidelines.

Additional information

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