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ORIGINAL RESEARCH

MiR-133a-5p Facilitates Cuproptosis in Hepatocellular Carcinoma Through Targeting of ATP7B

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Purpose: We explored the effects of miR-133a-5p and ATP7B on cuproptosis in hepatocellular carcinoma.

Methods: Initially, we assessed the impact of miR-133a-5p on hepatocellular carcinoma (HCC) using CCK-8 assays, cell scratch assays, and flow cytometry. Subsequently, we utilized elesclomol in combination with copper ions to induce cuproptosis in the HCC cell lines PLC/PRF/5 and Huh-7. We evaluated the influence of miR-133a-5p on cuproptosis using CCK-8 assays, cell scratch assays, flow cytometry, and Western blotting. To elucidate the underlying mechanisms, we employed bioinformatics to identify potential downstream target genes of miR-133a-5p and conducted dual-luciferase reporter assays to confirm the binding sites. Finally, we validated the regulatory effect of miR-133a-5p on ATP7B by modulating miR-133a-5p expression through cell transfection experiments.

Results: The results from the CCK-8 assay, cell scratch assay, and flow cytometry demonstrated that miR-133a-5p significantly inhibits the proliferation and migration of HCC cells while promoting their apoptosis. Furthermore, Elesclomol in combination with copper ions induces cuproptosis in HCC cells. Compared to the cuproptosis observed in HCC as a control, miR-133a-5p further suppresses the proliferation and migration of HCC cells, enhances their death, and increases the expression of cuproptosis-related proteins more prominently. Bioinformatics analysis suggested that ATP7B might be a downstream target gene of miR-133a-5p. This was confirmed by dual luciferase assays, which identified a binding site between miR-133a-5p and ATP7B. Additionally, the expression levels of ATP7B were found to decrease or increase in response to the regulation by miR-133a-5p.

Conclusion: MiR-133a-5p facilitates cuproptosis in hepatocellular carcinoma through targeting of ATP7B.

Keywords: cuproptosis, microRNA, ATP7B, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) ranks among the primary causes of cancer-related deaths. Early signs of HCC are typically mild, leading to delayed diagnosis and advanced-stage detection in many patients. This delay significantly restricts viable treatment options, including surgical resection, ablation, and liver transplantation, ultimately resulting in a poor prognosis.[1] Although several treatment methods and medications are currently available for HCC, such as sorafenib, which prolongs patient survival by inhibiting tumor growth and angiogenesis, the emergence of sorafenib resistance limits its long-term efficacy in advanced HCC cases.[2] Lenvatinib has shown greater therapeutic benefits compared to sorafenib, but resistance to lenvatinib is also on the rise.[3] Both drugs can curb tumor cell proliferation by inducing ferroptosis.[4] Ferroptosis, marked by the buildup of intracellular iron ions, the production of lipid peroxides, and the breakdown of the antioxidant defense system, is a key area of focus in HCC treatment.[5,6] Despite its potential, ferroptosis faces certain challenges. Studies suggest that HCC cells can evade ferroptosis induction by increasing the

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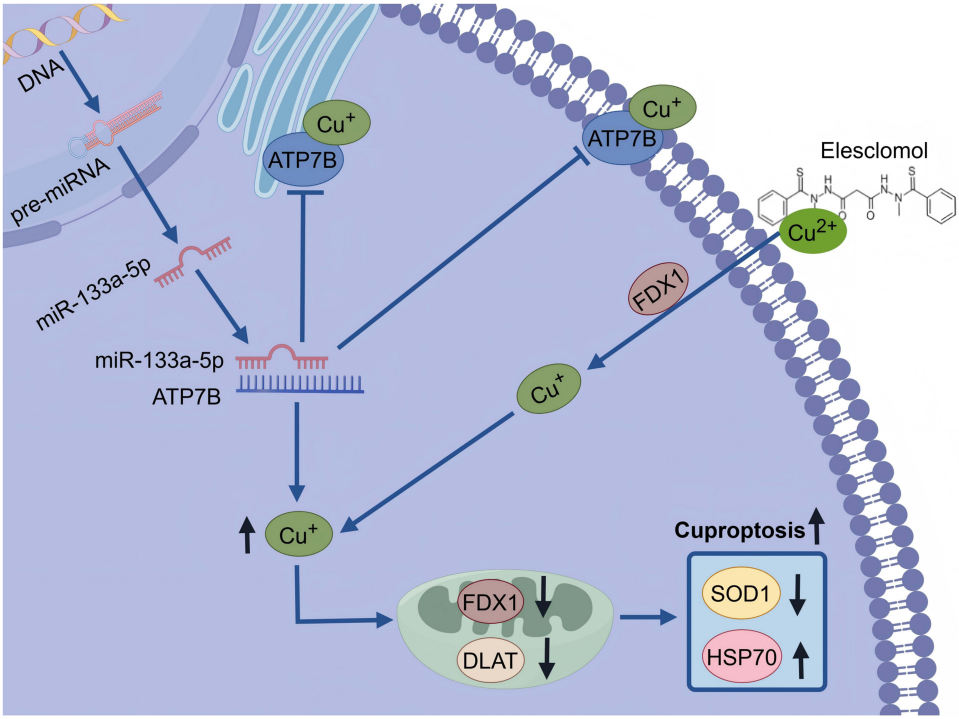
Graphical Abstract



expression of antioxidant enzymes like glutathione peroxidase, which may contribute to treatment resistance.[7] Moreover, elevated copper levels in HCC cells can hinder ferroptosis by reducing prolyl 4-hydroxylase domain (PHD) activity, thereby stabilizing HIF1α, a gene that boosts lipid metabolism.[8] Hence, there is a critical need to discover a new and more effective therapeutic approach for HCC.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, typically 21–25 nucleotides in length, that are ubiquitously present in eukaryotic cells and serve as a critical component of epigenetic regulation.[9,10] MiRNAs regulate the expression of target genes at the post-transcriptional level by binding to complementary sequences in the 3'-untranslated region (UTR) of the target mRNA, thereby inhibiting the translation process and promoting the degradation of the target mRNA, ultimately suppressing protein synthesis.[11] This regulatory mechanism plays a significant role in the pathophysiology and metastasis of tumors.[12] Recent studies have demonstrated that miRNA, acting as a tumor suppressor gene, exerts its anti-tumor effects by modulating downstream target genes in glioma, gastric cancer, and breast cancer.[13–15] In HCC, miR-133a is implicated in multiple pathogenic pathways associated with HCC development, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.[16,17] Additionally, miR-133a-3p can be secreted into exosomes upon stimulation by interleukin-6 (IL-6), contributing to tumor suppression within the tumor microenvironment.[18] Furthermore, miR-133a can promote the progression of hepatocellular carcinoma cells by targeting coronin-like actin-binding protein 1C (CORO1C) and insulin-like growth factor 1 receptor (IGF-1R).[19,20] It can also inhibit FOSL2 via the TGF-β/Smad3 signaling pathway, thereby exerting a tumor suppressor role in hepatocellular carcinoma.[21] By downregulating various target genes, miR-133a inhibits the proliferation, migration, and invasion of hepatocellular carcinoma cells, highlighting its potential as a therapeutic target for tumor treatment. Moreover, it has been noted that variations in copper ion concentrations can influence the expression of specific microRNAs (miRNAs), particularly within cardiac tissue.[22,23] Furthermore, research has demonstrated that individuals with Wilson's disease exhibit elevated levels of copper ions, which may be associated with the expression of certain miRNAs, thereby impacting the physiological state and functionality of cells.[23]

Copper is a vital trace element necessary for human bodily functions, playing a key role in sustaining cellular operations and physiological balance. It is involved in numerous enzymatic reactions and biological processes, which are essential for overall health.[24] Nevertheless, both excessive and insufficient levels of copper can interfere with its positive impacts, potentially causing copper toxicity.[25,26] Initial studies on the mechanisms of copper toxicity have predominantly centered on various pathways, such as oxidative stress, cellular damage, mitochondrial dysfunction, and genetic disorders, all of which culminate in cellular and tissue injury.[27,28] Tsvetkov's research highlighted that the interaction between excessive copper ions and fatty acylation proteins in the tricarboxylic acid cycle results in a decline or loss of iron-sulfur cluster proteins. This, in turn, triggers mitochondrial dysfunction and disrupts protein homeostasis within cells, intensifying the cellular stress response and leading to cell death.[29]

Cuproptosis-associated protein ATP7B is a critical regulator of intracellular copper homeostasis, predominantly expressed in the liver and brain.[30] As an essential copper transporter in hepatocytes, ATP7B plays a pivotal role in the metabolic processes involving copper. When intracellular copper levels rise, ATP7B relocates to the cell membrane, facilitating copper efflux into the bile, thus promoting copper excretion and preventing its toxic accumulation. Conversely, under conditions of normal or low copper concentrations, ATP7B primarily resides within the endoplasmic reticulum, where it contributes to copper storage and utilization, which is crucial for maintaining systemic copper equilibrium.[31] In individuals with Wilson's disease, mutations in the ATP7B gene lead to copper accumulation, potentially resulting in cuproptosis, a phenomenon associated with clinical manifestations of hepatic and neurological damage.[32] Research by Tsvetkov et al has shown that in a murine model of Wilson's disease, the absence of ATP7B causes intracellular copper accumulation, cellular death, disruption of lipoacylation and iron-sulfur cluster proteins, increased Hsp70 expression, and cuproptosis.[29] Previous studies have indicated that miR-133a enhances the sensitivity of both Hep-2 cells and their drug-resistant variant, Hep-2v, to cisplatin by downregulating ATP7B.[33]

The expression of miR-133a serves as a prognostic biomarker and a potential therapeutic target for HCC patients with abnormal copper metabolism. Using the TargetScan online database, we predicted the potential binding sites of miR-133a-5p and ATP7B mRNA, and subsequently validated these binding sites through dual-luciferase assays. This study elucidates the potential mechanism by which miR-133a-5p may modulate the sensitivity of HCC to cuproptosis via the downstream ATP7B, thereby offering a novel approach for the treatment of HCC by inducing cuproptosis in the future.

Materials and Methods

Study Approval and Sample Collection

Ten human HCC samples and their corresponding normal liver tissues were obtained from the First Affiliated Hospital of Anhui Medical University (The median age of the ten patients was 54 years (interquartile range: 46.5–63), including four patients with primary liver cancer and cirrhosis, five patients with primary liver cancer alone, and one patient with liver cancer and mixed-type cirrhosis. Additionally, nine cases were pathologically classified as moderately differentiated HCC, while one case was classified as well-differentiated HCC). All patients provided written informed consent for the use of their tissues in this research. The study adhered to the ethical guidelines approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (PJ2024-03-60).

Cell Culture and Agents

Human hepatocellular carcinoma cell lines PLC/PRF/5, Huh-7, SK-Hep-1, HCCLM3, Hep3B, and HepG2 were obtained from Guangzhou Saikuk Biotechnology Co., Ltd. The cell passage number was no more than 15. The PLC/PRF/5 and HepG2 cell lines were cultured in DMEM medium, while the Huh-7, HCCLM3, and Hep3B cell lines were cultured in minimal essential medium (MEM). The SK-Hep-1 cell line was maintained in RPMI-1640 medium. All media were supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). The DMEM, MEM, and RPMI-1640 media, as well as the penicillin/streptomycin, were supplied by Procell (Wuhan, China), and the FBS was provided by WISENT (Canada). All cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Prior to any experiments, regular mycoplasma testing was conducted using the Mycoplasma Detection Kit (Beyotime, Shanghai, China).

RNA Extraction and RT-qPCR

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen™, Waltham, Massachusetts, USA), and cDNA synthesis was carried out using a reverse transcriptase kit (Yeasen, Shanghai, China). MiRNA extraction was performed using a MiRNA extraction kit (Tiangen, Sichuan, China). Real-time fluorescence quantitative PCR (RT-qPCR) analysis of ATP7B was conducted using Hieff® qPCR SYBR Green Master Mix (No Rox) (Shanghai Yeasen, China) under the following thermal cycling conditions: an initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 20 seconds, and 72 °C for 20 seconds. RT-qPCR for miR-133a-5p was executed using the miRCute Plus MicroRNA Fluorescence Quantitative Detection Kit (SYBR Green®) (Tiangen, Sichuan) with the following protocol: an initial denaturation at 95 °C for 15 minutes, followed by 45 cycles of 95 °C for 20 seconds, 60 °C for 34 seconds, 75 °C for 15 seconds, and 60 °C for 34 seconds. The forward and reverse primer sequences for ATP7B were CTGGTGGTTGCTGTGGCTGAG and AACGGTGGCTTCTGTGGCTTG, respectively. For GAPDH, the forward primer sequence was GAGTCAACGGATTGGTCGT, and the reverse primer sequence was GACAAGCTTCCCGTTCTCAG. The forward and reverse primer sequences for miR-133a-5p were AGCUGGUAAAAUGGAACCAAAU and UUGGUCCAUUUUACCAGGUUU, respectively. The primer sequences for the U6 gene were supplied by Shanghai Sangon Biotechnology Co., Ltd. All the remaining primer sequences were designed by Shanghai Sangon Bioengineering Co.,Ltd.

Cuproptosis Induction Assay

PLC/PRF/5 and Huh-7 cells that were growing well and in the logarithmic growth phase were collected. Following cell counting, 3×10^5 cells per well were seeded in 6-well plates, with each well containing 2 mL of media. The cells were cultured at 37 °C in a 5% CO_2 incubator for 24 hours, and treatments were administered when the cell density reached approximately 90%. The control group did not receive any drugs. The experimental groups were divided into the Elesclomol (MedChemExpress, Shanghai, China) group, the Cu^{2+} (Aladdin, Shanghai, China) group, and the Elesclomol+Cu^{2+} group. PLC/PRF/5 cells were incubated with a 4 μmol/L Cu^{2+} solution, an 80 nmol/L Elesclomol solution, or a combination of both solutions for 24 hours. For Huh-7 cells, a 4 μmol/L Cu^{2+} solution, a 40 nmol/L Elesclomol solution, and a combination of both solutions were used for a 24-hour incubation period. To verify the successful induction of copper death, a 30 nmol/L Ammonium tetrathiomolybdate (ATTM) (MedChemExpress, Shanghai, China) solution was added to cells incubated with copper ions and Elesclomol solution for 24 hours, and changes in cell viability were detected in subsequent experiments.

Cell Proliferation Assay

Well-grown PLC/PRF/5 and Huh-7 cells in the logarithmic growth phase were plated at a density of 6,000 cells per well in a 96-well plate and incubated for 24 hours at 37 °C with 5% CO_2. Following this, 10 μL of CCK-8 solution (Biosharp, China) was introduced into each well, and the absorbance (OD) at 450 nm was determined using a microplate reader (BioTek, USA).

Transfection Cell Lines

The PLC/PRF/5 and Huh-7 cell lines were seeded in six-well plates and allowed to reach a cell confluence of approximately 60% before transfection. For transfection, an RNAimax-based system (including NC, in_miR-133a-5p, and oe_miR-133a-5p) was utilized, with the transfection reagent diluted in serum-free culture medium. After a 6-hour incubation at 37 °C in a 5% CO_2 incubator, the transfection medium was replaced with DMEM containing 10% FBS. The expression efficiency of the green fluorescent protein (GFP) was evaluated using fluorescence microscopy 48 hours after transfection. The transfection efficiency of miR-133a-5p was measured by RT-qPCR, and subsequent experiments were performed after confirming successful transfection.

Western Blot and Agents

PLC/PRF/5 and Huh-7 cells, which were in excellent condition and actively proliferating during the logarithmic growth phase, were collected. After counting, 4×10^5 cells were seeded into each well of 6-well plates and incubated at 37 °C

with 5% CO_2 for 24 hours. Protein concentration was measured using the BCA method to prepare a 30 μg/μL protein sample for SDS-PAGE analysis. Equal amounts of protein were separated by polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride(PVDF) membrane. The membranes were blocked with 5% skim milk for 2h at room temperature. The samples were incubated in primary antibody for 24 h overnight and secondary antibody for 1 h at room temperature. The antibodies used were: FDX1 (1:1000,T510671, Abmart, China), GAPDH (1:10,000,10,494-1-AP, Proteintech, China), SOD1 (1:1000,T56666, Abmart, China), HSP70 (1:500,#4873, Cell Signaling Technology, USA), ATP7B (1:1000,#PA5-102826, ThermoFisher, USA), and DLAT (1:1000,T58125, Abmart, China). Finally, the cells were observed using an enhanced chemiluminescence (ECL) assay kit (Biosharp, Hefei, China).

Wound Healing Test

The PLC/PRF/5 and Huh-7 cell lines were seeded in 6-well plates at a density of 5×10^5 cells per well. The cells were cultured in a humidified incubator with 5% CO_2 at 37 °C until they reached 90% confluence. A sterile 200 μL pipette tip was used to create a uniform scratch in the cell monolayer. The wells were then gently rinsed 2–3 times with PBS to remove any dislodged cells and debris. The width of the scratch was measured at 0 hours and 24 hours post-scratch, and images of the scratched regions were taken using a microscope. The scratch closure was quantitatively evaluated using ImageJ software, and the 24 hours results were compared to the 0 hours baseline to determine the effects of different treatments on cell migration.

Flow Cytometry

PLC/PRF/5 and Huh-7 cells, which were in optimal condition and in the logarithmic growth phase, were harvested. Following enumeration, 3×10^5 cells per well were seeded into 6-well plates and cultured at 37 °C in a 5% CO_2 incubator for 24 hours. The cells were then trypsinized, and the resulting supernatant and cell pellet were transferred to a flow tube. This mixture was centrifuged at 800 rpm for 5 minutes, following which the supernatant was discarded. Lastly, 10 μL of 7-AAD (Yeasen, China) stain and 200 μL of PBS buffer were added to the tube for flow cytometric analysis.

Bioinformatics Analyses

The potential downstream target genes of miR-133a-5p were retrieved from the miRDB, miRTarBase, and TargetScan databases. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of these potential downstream targets were performed using the Wei Sheng Xin (www.bioinformatics.com.cn) online platform. The biological functions and potential signaling pathways related to these targets were elucidated. By integrating information from the three aforementioned databases, a Venn diagram was constructed, and the genes located at the intersection were chosen as candidate key genes for this study.

Immunohistochemistry

The paraffin-embedded samples were deparaffinized and immersed in sodium citrate buffer (pH 6.0; Servicebio, China) for antigen retrieval. The tissue sections were then incubated with the primary antibody against ATP7B overnight at 4 °C. After the overnight incubation, the sections were treated with the secondary antibody and stained with DAB (Solebo, China). Subsequently, the sections were counterstained with hematoxylin (Servicebio, China), mounted, and examined under a microscope. Images were captured and analyzed.

Dual Luciferase Reporter Gene Assay

293T cells were plated in 96-well plates at a concentration of 3×10^4 cells per well. When the cells reached about 60% confluence on the transfection day, transfection was performed. A solution containing 10 μL DMEM, 0.1 μg WT/MUT ATP7B plasmid, and 5 pmol microRNA mimic or Negative Control (NC) was prepared. In addition, 10 μL DMEM was mixed with 0.2 μL Lipo3000 transfection reagent. These solutions were subsequently added to the 293T cells. Six hours after transfection, the medium was changed to DMEM containing 10% fetal bovine serum. Forty-eight hours later, the Firefly luciferase activity was measured using the Promega Dual-Luciferase Reporter Assay System, and the results were recorded. The Renilla luciferase activity, used as the internal control, was also quantified and documented.

Statistics

Data analysis was performed using GraphPad Prism 8.0 software, with each experimental group being independently replicated at least three times. Quantitative data are expressed as mean ± standard deviation (x±s). Thank you very much for your professional guidance. According to your suggestion, for all statistical methods in the manuscript, we compared data from more than two groups by using the Bonferroni test method in one-way analysis of variance (ANOVA) to avoid false positive results of the experiment. Statistically significant difference was $P<0.05$.

Results

Elevated Levels of miR-133a-5p Inhibited the Proliferation and Migration of Hepatocellular Carcinoma Cells

To explore the expression and regulatory mechanisms of miR-133a-5p in HCC, we first evaluated the expression levels of miR-133a-5p in 10 paired samples of hepatocellular carcinoma and adjacent non-tumor tissues. The results showed a significant reduction in miR-133a-5p expression in hepatocellular carcinoma tissues compared to the adjacent tissues (Figure 1A). The expression levels of miR-133a-5p in five hepatocellular carcinoma cell lines were detected, and PLC/PRF/5 and Huh-7 with low expression levels were selected for subsequent experiments (Figure 1B). To clarify the regulatory role of miR-133a-5p in hepatocellular carcinoma, we conducted knockdown and overexpression studies in these two cell lines (Figure 1C). The findings indicated that increased expression of miR-133a-5p inhibited both the proliferation and migration of hepatocellular carcinoma cells, while decreased expression of miR-133a-5p resulted in enhanced proliferation and migration abilities (Figure 1D–H). Therefore, miR-133a-5p may serve as a potential therapeutic biomarker in hepatocellular carcinoma.

miR-133a-5p Suppresses the Development of Hepatocellular Carcinoma Through Promoting Cell Death

To elucidate the mechanism through which miR-133a-5p inhibits the proliferation and migration of HCC cells, we induced the overexpression of miR-133a-5p in PLC/PRF/5 and Huh-7 cell lines. Morphological assessments revealed a reduction in cell size, the appearance of vesicles on the cell membrane, and a significant decrease in cell count compared to the control group (Figure 2A). Furthermore, flow cytometry analysis using 7-AAD staining indicated an increased rate of cell death following the overexpression of miR-133a-5p compared to the control (Figure 2B). These findings suggest that the overexpression of miR-133a-5p promotes the death of HCC cells.

Excessive Copper Ions Induce Cuproptosis in Hepatocellular Carcinoma Cells

We investigated the effects of miR-133a-5p overexpression on cuproptosis in HCC cells. Our results indicated that exposing HCC cells to copper ions or elesclomol (ES) alone for 24 hours had little impact on cell proliferation. In contrast, the combined treatment of both agents significantly reduced HCC cell proliferation (Figure 3A and B). When the copper chelator ammonium tetrathiomolybdate VI(ATTM) was added, partial protection from cell death and recovery of proliferation capacity were observed (Figure 3C and D). Furthermore, the combination of the two drugs also decreased the migratory ability of HCC cells, an effect that was alleviated by ATTM (Figure 3E and F), the statistical results are meaningful (Figure 3G and H). As a result, excessive copper ions triggered cell death in PLC/PRF/5 and Huh-7 cells.

To further validate the occurrence of cuproptosis in HCC, we examined the expression levels of cuproptosis proteins. The results indicated a reduction in the expression of these proteins, which was effectively reversed by ATTM (Figure 3J and K), the statistical results are meaningful (Figure 3I and L).

ATP7B May Serve as a Potential Downstream Target of miR-133a-5p

To better understand the role of miR-133a-5p in cuproptosis in HCC, we employed the miRDB, miRTarBase, and TargetScan databases to identify potential downstream target genes of miR-133a-5p. We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses on the identified targets. Our results indicated that the predicted target gene ATP7B was significantly enriched in the NF-κB signaling pathway (Figure 4A), a key transcription factor involved in immune responses, inflammation, cell proliferation, and survival.[34]

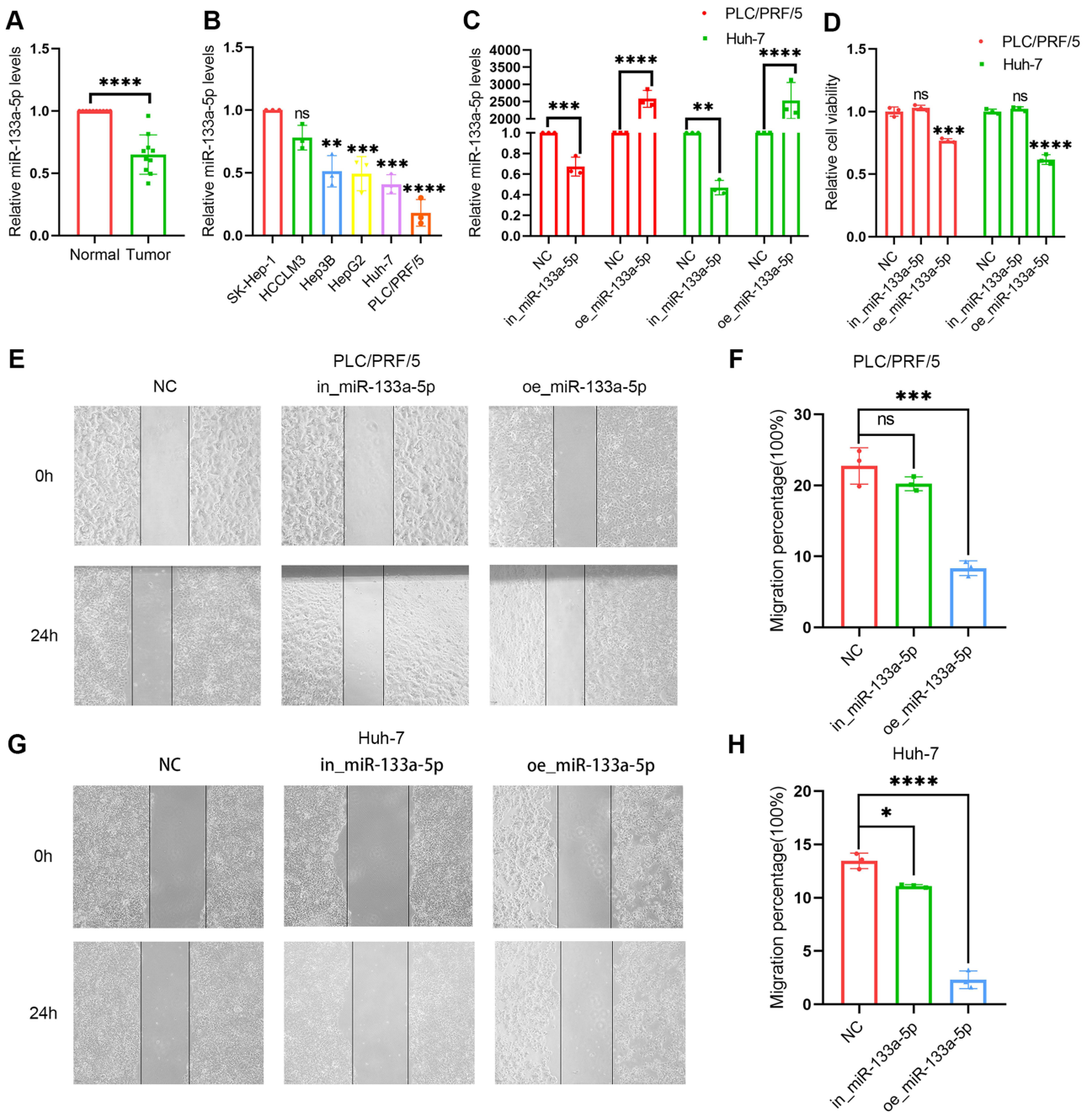


Figure 1 Effect of miR-133a-5p on hepatocellular carcinoma.(**A**) In human HCC tissues, the expression level of miR-133a-5p was markedly lower compared to that in adjacent non-tumor tissues(n=10). (**B**) The expression levels of miR-133a-5p were quantified in six distinct HCC cell lines. (**C**) The changes in miR-133a-5p expression after knockdown or overexpression in PLC/PRF/5 and Huh-7 cells were analyzed. (**D**) The influence of miR-133a-5p knockdown and overexpression on the viability of PLC/PRF/5 and Huh-7 cells was investigated. (**E** and **G**) The impact of miR-133a-5p knockdown and overexpression on the migratory ability of PLC/PRF/5 and Huh-7 cells was assessed. (**F** and **H**) Statistical analyses were conducted to evaluate the effects of miR-133a-5p knockdown and overexpression on the migratory capacity of PLC/PRF/5 and Huh-7 cells. (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, NC were transfection reagent group).

Studies have shown that ATP7B expression can be altered under inflammatory conditions, leading to disrupted copper metabolism, which subsequently affects NF-κB activity. This pathway plays a vital role in the development and progression of hepatocellular carcinoma.[35] Moreover, copper acts as a cofactor for various enzymes essential for lipid metabolism and antioxidant processes.[36,37] By regulating copper metabolism, ATP7B influences the activities of these enzymes, potentially impacting lipid metabolism, which is linked to insulin resistance[38] (Figure 4B).

We proposed a potential binding interaction between miR-133a-5p and ATP7B by utilizing the miRDB, miRTarBase, and TargetScan databases (Figure 4C). To test this hypothesis, we first evaluated the expression levels of ATP7B in HCC tissues and

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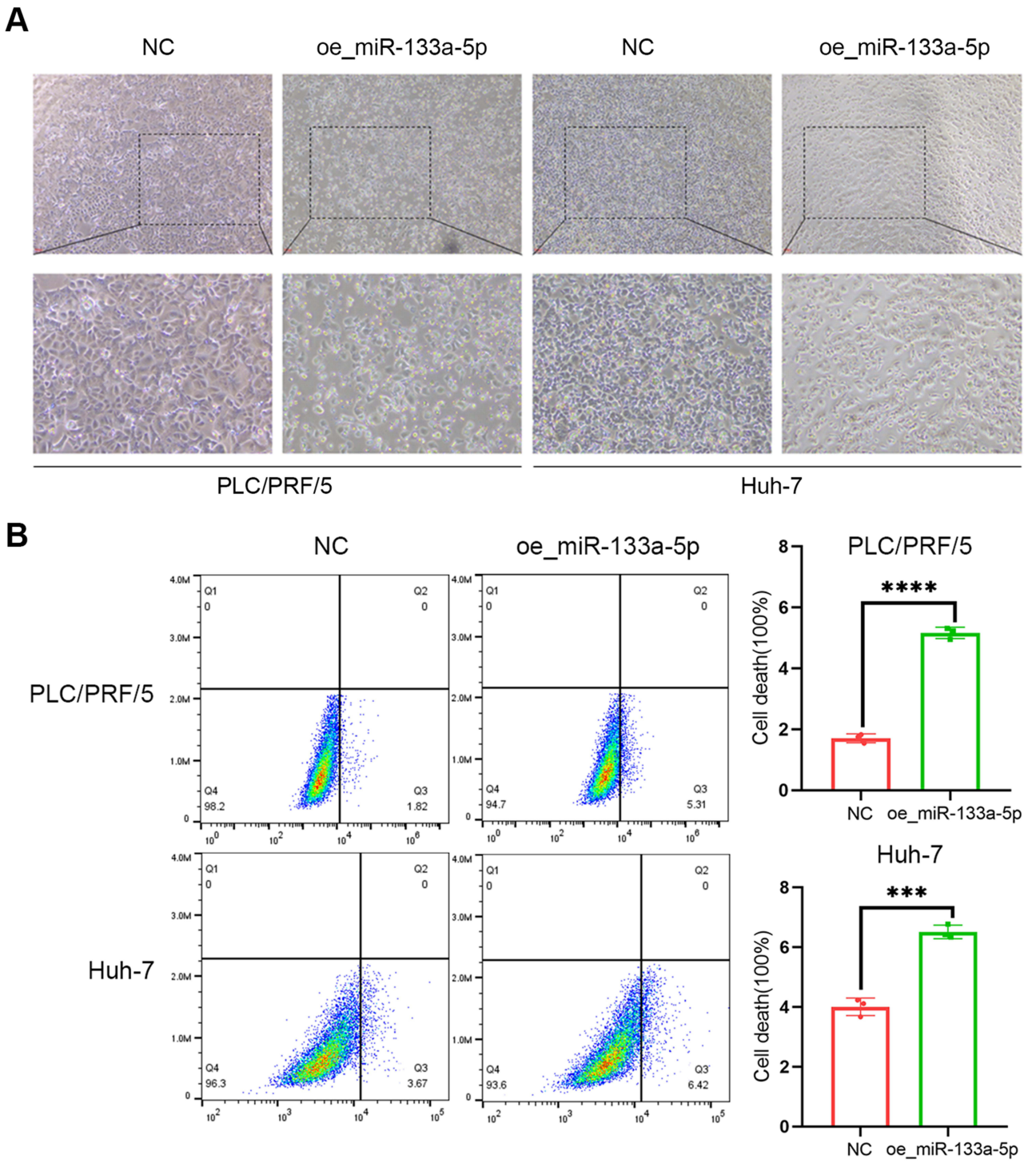

Figure 2 Effect of overexpression of miR-133a-5p on hepatocellular carcinoma.(**A**) Effect of miR-133a-5p overexpression on cell morphology in PLC/PRF/5 and Huh-7. (**B**) Effect on cell death following overexpression of miR-133a-5p in PLC/PRF/5 and Huh-7(7-AAD). (****P* < 0.001, *****P* < 0.0001, NC were transfection reagent group).

cell lines. Immunohistochemical and Western blot analyses demonstrated a notable increase in ATP7B expression in HCC tissues compared to adjacent non-tumor tissues (Figure 4D–F). Following the overexpression or knockdown of miR-133a-5p in the two cell lines, we observed a corresponding reduction or enhancement in ATP7B expression (Figure 4G and H). The statistical results of ATP7B expression in six cell lines were meaningful (Figure 4I). And the statistical of ATP7B expression after overexpression and knockdown of miR-133a-5p were meaningful (Figure 4L). Additionally, the binding sites between miR-133a-5p and ATP7B

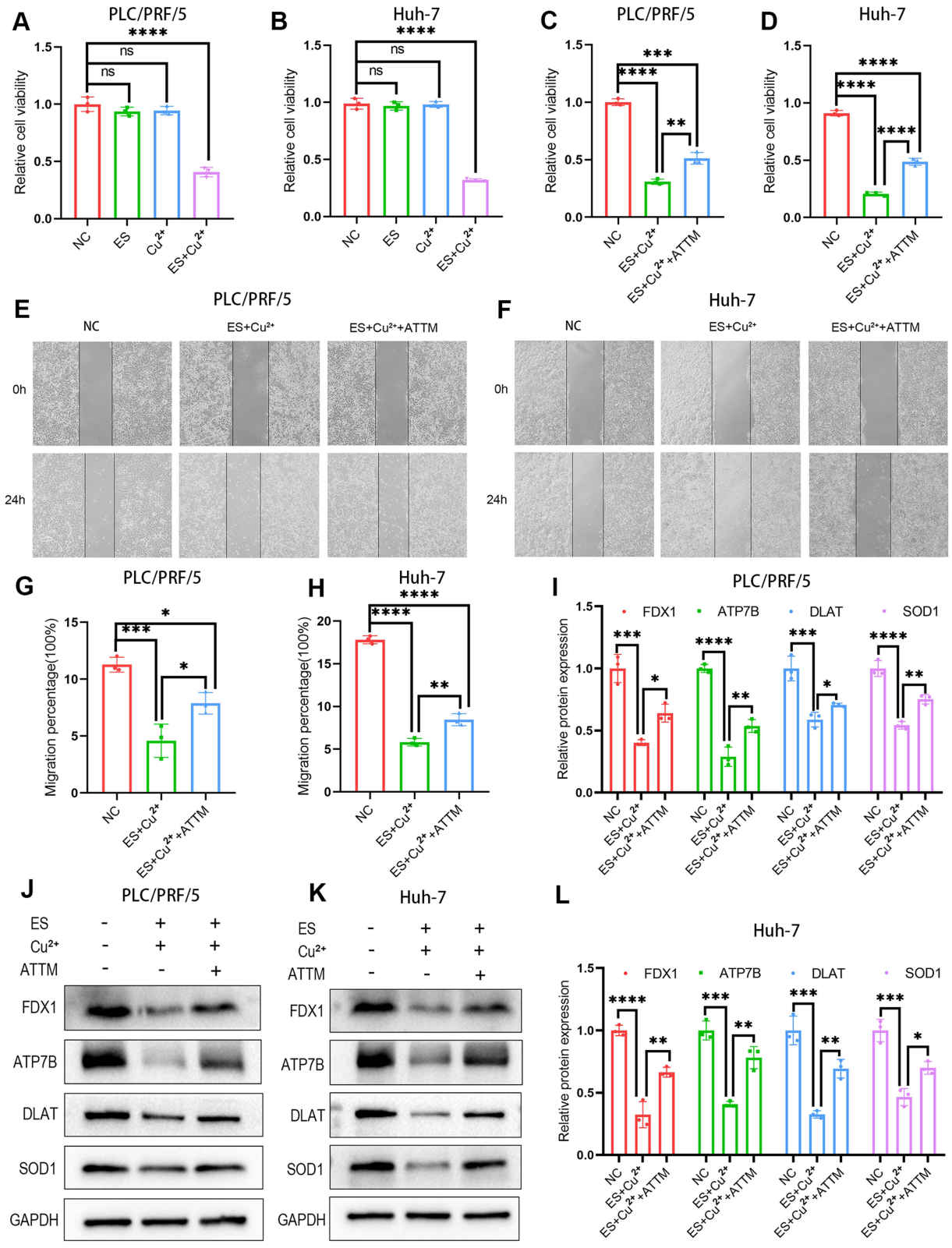

Figure 3 Hepatocellular carcinoma is capable of cuproptosis. (**A**) Effect of elesclomol, Cu^{2+},elesclomol+Cu^{2+} incubation for 24h on the viability of PLC/PRF/5. (**B**) Effect of elesclomol, Cu^{2+}, elesclomol+Cu^{2+} incubation for 24h on the viability of Huh-7.(C and D) The copper chelator ATTM partially rescued the reduced viability of PLC/PRF/5 and Huh-7 cells induced by elesclomol+Cu^{2+}. (**E** and **F**) Copper chelator ATTM could partially rescue the reduced migration ability of PLC/PRF/5 and Huh-7 cells induced by elesclomol+Cu^{2+}.(**G–I** and **L**) Statistics of cell migration ability and protein expression.(**J** and **K**) Changes in the expression of cuproptosis-related proteins in PLC/PRF/5 and Huh-7 cells incubated with elesclomol+Cu^{2+} and elesclomol+Cu^{2+}+ATTM, respectively. (*P<0.05,**P<0.01,***P<0.001,****P<0.0001, NC were hepatocellular carcinoma control groups).

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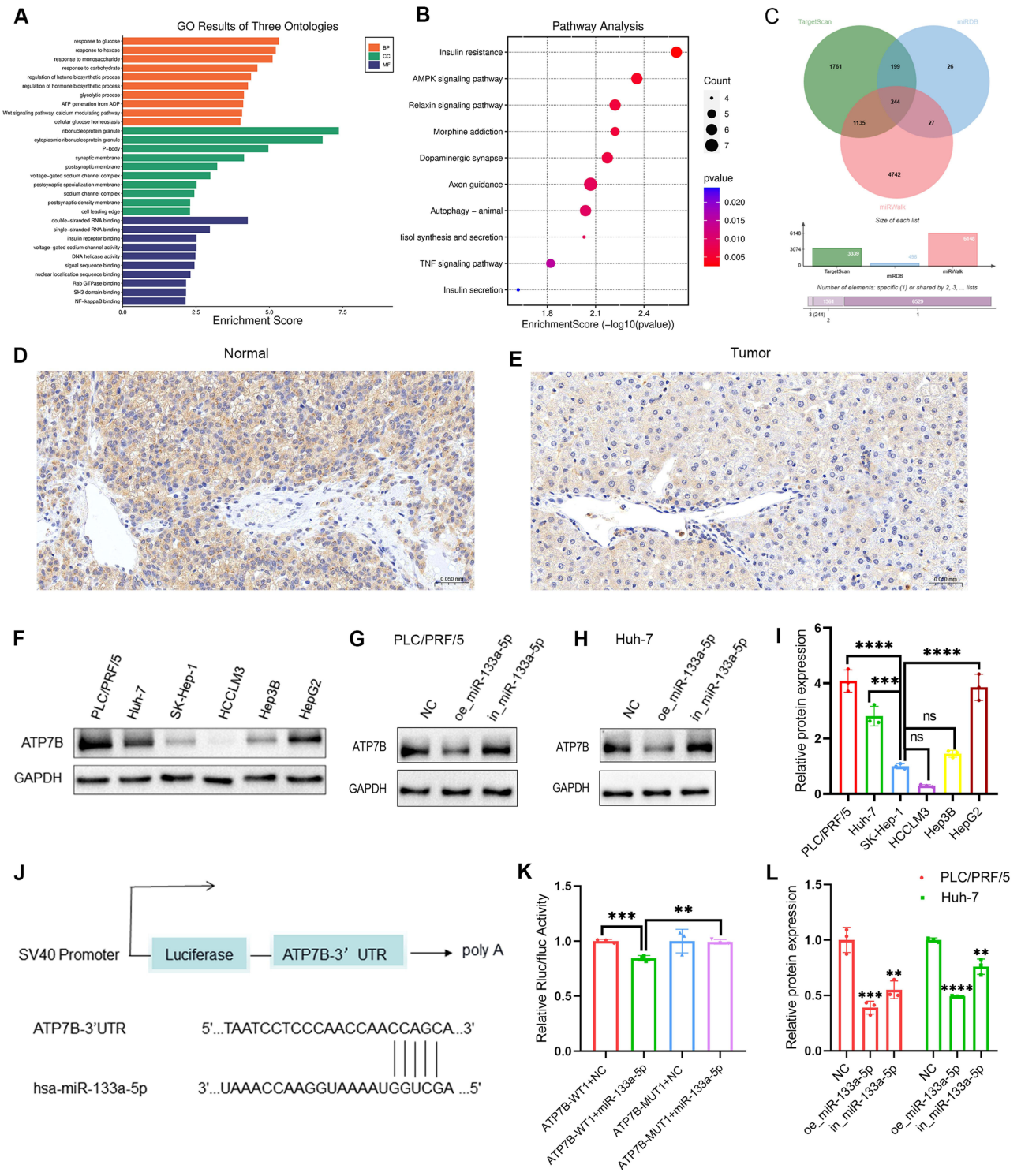

Figure 4 MiR-133a-5p regulates cuproptosis in hepatocellular carcinoma through ATP7B.(**A** and **B**) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of potential downstream target genes of miR-133a-5p.(**C**) miR-133a-5p and ATP7B mRNA were identified as potential targets at the intersection of the miRDB, miRTarBase, and TargetScan databases.(**D** and **E**) Immunohistochemical results of ATP7B in HCC and paracancerous tissues. (**F**) Expression of ATP7B in six hepatocellular carcinoma cell lines.(**G** and **H**) The expression of ATP7B protein after overexpression and knockdown of miR-133a-5p (NC were transfection reagent groups.(**I** and **L**) Statistics of protein expression results in (**F**–**H**). (**J**) Dual luciferase assay confirmed that ATP7B and miR-133a-5p had binding sites. (**K**) Statistics of dual luciferase assay results. (***P*<0.01, ****P*<0.001,*****P*<0.0001).

were verified through a dual-luciferase reporter assay (Figure 4J), the statistical results are meaningful (Figure 4K). Taken together, these results suggest that ATP7B acts as a potential downstream target of miR-133a-5p, indicating that miR-133a-5p might affect the cuproptosis of HCC by regulating ATP7B.

MiR-133a-5p Promotes Cuproptosis in Hepatocellular Carcinoma Cells

To elucidate the mechanism by which miR-133a-5p influences cuproptosis in HCC, we induced cuproptosis in HCC cells overexpressing miR-133a-5p. The findings revealed a significant reduction in the proliferative and migratory capabilities of HCC cells compared to those subjected to cuproptosis alone (Figure 5A, B, G and H), the statistical results are meaningful (Figure 5E and F). Additionally, the rate of cell death was elevated (Figure 5I), the statistical results are

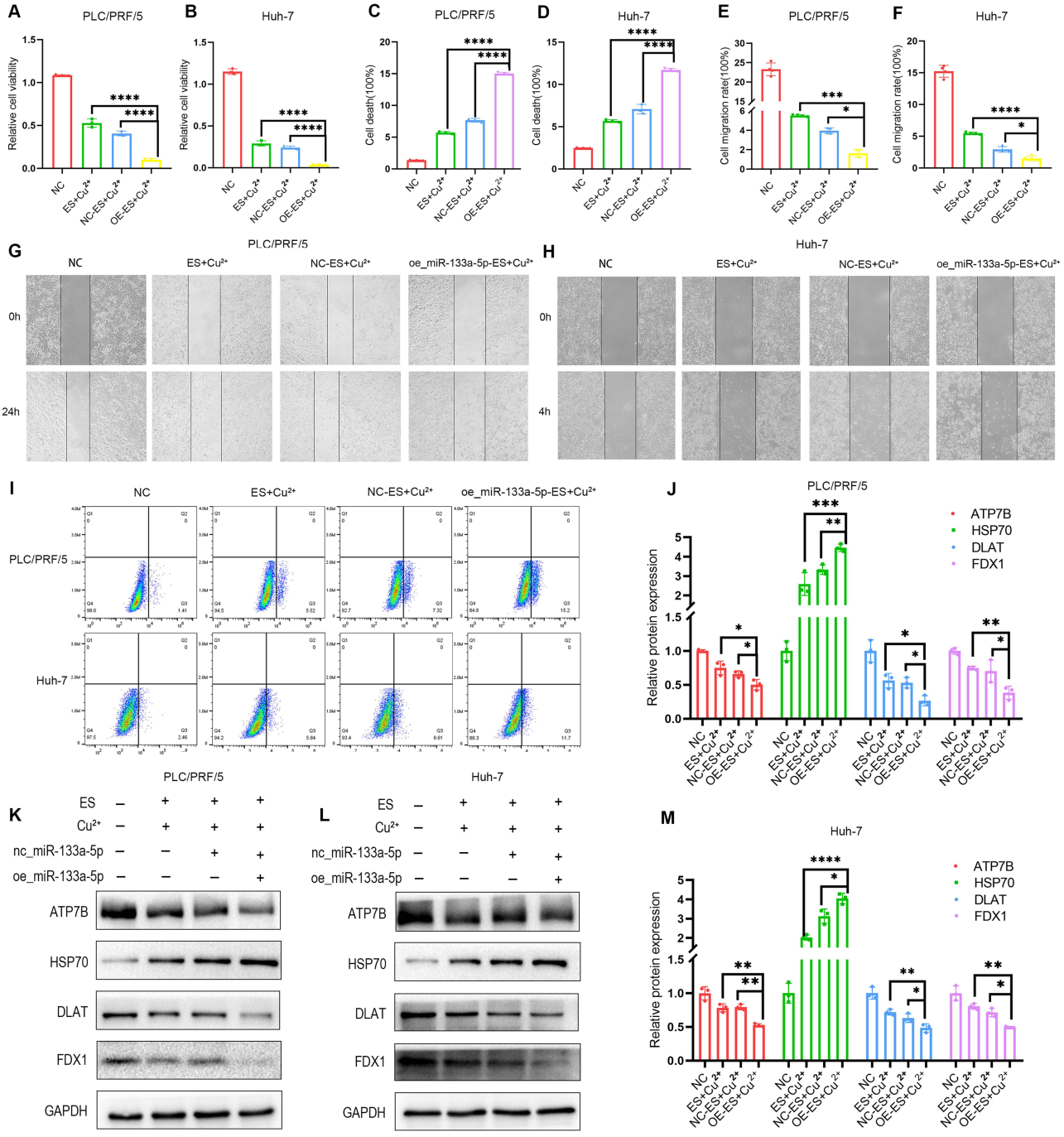

Figure 5 Effect of miR-133a-5p on cuproptosis in hepatocellular carcinoma.(**A** and **B**) The effect of elesclomol+Cu^{2+} and elesclomol+Cu^{2+}+ oe_miR-133a-5p incubation for 24h on the viability of PLC/PRF/5 and Huh-7 cells (NC as hepatocellular carcinoma control group).(**C–F**, **J** and **M**) Statistical analysis of cell death, migration and protein expression (NC were HCC control group, NC-ES+Cu^{2+} were transfection reagent group).(**G** and **H**) The impact of elesclomol+Cu^{2+} and elesclomol+Cu^{2+}+oe_miR-133a-5p incubation for 24h on the migratory ability.(**I**) Flow cytometry was used to detect the effect of elesclomol+Cu^{2+} and elesclomol+Cu^{2+}+oe_miR-133a-5p incubation for 24h on cell death. (**K** and **L**) Western blot was used to detect the expression of cuproptosis-related proteins in elesclomol+Cu^{2+} and elesclomol+Cu^{2+}+oe_miR-133a-5p incubation for 24h.(*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, NC were hepatocellular carcinoma control groups, NC-ES+Cu^{2+} were transfection reagent group).

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meaningful (Figure 5C and D). And the expression levels of proteins associated with cuproptosis were markedly diminished, including ATP7B protein (Figure 5K and L), the statistical results are meaningful (Figure 5J and M). Collectively, these results indicate that the upregulation of miR-133a-5p in PLC/PRF/5 and Huh-7 cell lines suppresses HCC cell proliferation and migration while promoting HCC cell death. These results fully indicate that miR-133a-5p promotes cuproptosis in HCC by regulating ATP7B.

Discussion

We have provided evidence demonstrating that copper overload can induce cell death in PLC/PRF/5 and Huh-7 cells. Furthermore, miR-133a-5p and ATP7B play a critical role in the development of HCC linked to copper metabolism disorders. Specifically, First, our results demonstrate that the expression level of miR-133a-5p in HCC tissues is significantly lower than in the surrounding non-cancerous tissues. This finding is consistent with previous studies on different types of cancer.[13,39] Our research also revealed that ATP7B expression levels were notably increased in hepatocellular carcinoma tissues when compared to adjacent non-tumor tissues. Consequently, we selected the PLC/PRF/5 and Huh-7 cell lines, characterized by relatively lower expression of miR-133a-5p, based on the detection of miR-133a-5p and ATP7B in six different hepatocellular carcinoma cell lines. Cells with comparatively higher ATP7B expression were utilized for further experimentation. Previous studies have shown that ATP7B mRNA encodes a crucial copper transporter primarily expressed in the cytoplasm and cell membrane of hepatocytes, playing a vital role in the regulation of copper metabolism and homeostasis within the body.[40] Deficiencies in ATP7B disrupt this homeostasis, leading to impaired copper excretion and pathological accumulation of copper in the liver and brain, ultimately contributing to the development of Wilson's disease.[41] ATP7B exhibits high expression levels in the liver, resulting in a significant amount of research dedicated to this protein. Most studies have concentrated on the link between ATP7B and Wilson's disease, whereas the association between ATP7B and cuproptosis has been less explored.

In our research, the upregulation of miR-133a-5p in the hepatocellular carcinoma cell lines PLC/PRF/5 and Huh-7 significantly decreased cell viability and migration ability. In contrast, downregulating miR-133a-5p restored these cellular functions compared to the overexpression scenario. These results indicate that miR-133a-5p has a suppressive impact on the growth and movement of HCC cells. Multiple studies have shown that microRNAs (miRNAs) act as tumor suppressors, playing a crucial role in inhibiting cancer progression in various cancer cell types, consistent with our findings.[42–44] Considering that a single miRNA can regulate the expression of multiple downstream mRNAs, and a single mRNA can be controlled by multiple miRNAs, it is reasonable to propose that miR-133a-5p affects the proliferation and migration of HCC cells by modulating several downstream target genes.

Furthermore, our findings indicate that ATP7B expression levels are elevated in HCC tissues relative to adjacent non-tumor tissues. Given its role as a critical regulator of copper homeostasis, mutations in the ATP7B gene can lead to Wilson's disease and disturbances in copper metabolism. Consequently, ATP7B may play a pivotal role in mediating cuproptosis in hepatocellular carcinoma cells. Elemental analysis confirmed that the copper concentration in HCC tissues is higher than that in healthy liver tissues,[45] suggesting a greater likelihood of cuproptosis in HCC. Cuproptosis is a newly identified form of cell demise distinct from apoptosis, necrosis, and ferroptosis.[29] This process primarily involves FDX1-mediated fatty acylation of mitochondrial proteins and subsequent aggregation of these fatty acylated proteins due to copper ions. In this study, we have pioneered the use of elesclomol in combination with copper ions to induce cuproptosis in HCC cells. Our research indicated that the concurrent administration of elesclomol and copper ions led to a reduction in cell viability and migration ability. However, these effects were reversed with the addition of the copper chelator ATTM, thus confirming the influence of the elesclomol-copper ion combination on HCC cells. Ferredoxin 1 (FDX1), an Fe-S cluster protein, is essential in the process of cuproptosis by transforming intracellular Cu(II) ions into the more toxic Cu(I) form.[46,47] FDX1 functions as a critical electron donor in various cellular metabolic processes and participates in the biosynthesis of steroids, heme, and Fe-S clusters within the human body.[29,48,49] Studies have shown that FDX1 can induce oxidative stress in hepatocellular carcinoma cells by activating the PI3K/AKT signaling pathway and mitophagy, thereby promoting the progression of hepatocellular carcinoma.[47] Additionally, some studies have revealed that FDX1 can directly interact with lipoyl synthase (LIAS) to enhance its lipoacylation function in cellular proteins and facilitate the functional association between LIAS and the lipoyl protein carrier GCSH, thus influencing cellular metabolism.[46] Simultaneously, the downregulation of FDX1 expression has been found to regulate copper homeostasis, highlighting the pivotal

role of FDX1 in the process of cuproptosis.[50] In the context of cuproptosis, FDX1 expression decreases. Additionally, dihydrolipoamide S-acetyltransferase (DLAT), a part of the pyruvate dehydrogenase complex in the tricarboxylic acid cycle, accumulates due to lipoacylation, resulting in increased oligomeric protein levels and decreased DLAT expression in HCC cells experiencing cuproptosis.[51,52] DLAT can facilitate the accumulation of leucine in hepatocellular carcinoma and mediate GLUT1 to activate MET, thereby promoting the metastasis of hepatocellular carcinoma.[53,54] These mechanisms collectively contribute to the poor prognosis observed in patients with hepatocellular carcinoma. HSP70 is a class of molecular chaperones that are highly upregulated under cellular stress conditions. Their primary functions encompass assisting in protein folding, inhibiting the aggregation of misfolded proteins, and facilitating the degradation of damaged proteins.[55] HSP70 plays a pivotal role in maintaining cellular homeostasis and responding to environmental stressors. In hepatocellular carcinoma, HSP70 has been shown to enhance cell invasion and metastasis capabilities, thereby promoting tumor progression. Superoxide dismutase 1 (SOD1) is a critical antioxidant enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide and molecular oxygen, thus safeguarding cells against oxidative stress-induced damage. SOD1 also serves as a key regulator in maintaining intracellular redox equilibrium.[56] When cuproptosis begins in hepatocellular carcinoma cells, the cells initiate a stress response to heavy metal toxicity, marked by an upregulation of heat shock protein 70 (HSP70) and a downregulation of superoxide dismutase 1 (SOD1), an antioxidant enzyme, due to oxidative stress.[57,58] ATP7B is a P-type ATPase predominantly responsible for intracellular copper transport and excretion. It is highly expressed in the liver, where it facilitates copper transport to bile for excretion and contributes to ceruloplasmin synthesis.[59] By modulating copper transport, ATP7B may influence the redox state and angiogenesis within the tumor microenvironment, thereby potentially affecting tumor growth and metastasis.[60,61] Upon the occurrence of cuproptosis in HCC cells, the expression of ATP7B, a crucial protein responsible for copper transport, is significantly reduced due to the aberrant accumulation of intracellular copper ions. Our findings are consistent with the observed changes in the expression levels of these proteins, while an opposite trend was noted upon the application of the copper chelator ATTM. These observations support our induction of cuproptosis in HCC cells, thereby allowing us to successfully establish a cuproptosis model in HCC cells. Collectively, these results provide strong evidence that miR-133a-5p exacerbates cuproptosis in HCC cells.

MiR-133a-5p has been shown to suppress the proliferation and migration of hepatocellular carcinoma cells, particularly in the PLC/PRF/5 and Huh-7 cell lines. However, when miR-133a-5p is overexpressed in hepatocellular carcinoma cells with impaired copper metabolism, a markedly higher rate of cell death is observed compared to the situation where miR-133a-5p is highly expressed in typical hepatocellular carcinoma cells. This enhanced inhibition of proliferation and migration, coupled with the significant anti-tumor effect, suggests that miR-133a-5p may serve as a more effective therapeutic target for HCC patients with copper metabolism disorders. Furthermore, through bioinformatics analysis, we identified ATP7B as a potential downstream target of miR-133a-5p. Therefore, it is hypothesized that miR-133a-5p might exert its inhibitory effects on the proliferation and migration of hepatocellular carcinoma cells and promote their apoptosis by modulating the expression of ATP7B. The dual-luciferase reporter assay confirmed that ATP7B interacts with miR-133a-5p, indicating that ATP7B is among the downstream targets regulated by miR-133a-5p. To substantiate the regulatory effect of miR-133a-5p on hepatocellular carcinoma cells through its interaction with ATP7B, Western blot (WB) analysis was conducted. The results showed that overexpression of miR-133a-5p in hepatocellular carcinoma cell lines PLC/PRF/5 and Huh-7 led to a significant reduction in ATP7B protein levels. In contrast, inhibition of miR-133a-5p expression resulted in an increase in ATP7B protein levels compared to the overexpression condition. However, it is important to acknowledge certain limitations of our study. Specifically, the expression levels of miR-133a-5p and ATP7B were not evaluated using primary hepatocellular carcinoma cells as a control to validate the consistency of the observed effects. Additionally, the functional implications of miR-133a-5p and ATP7B in vivo, such as within animal models, remain unexplored.

Given that microRNAs (miRNAs) play a pivotal role in gene regulation by inhibiting downstream target genes, they present promising targets for cancer therapy.[62–64] This study's findings indicate that miR-133a-5p significantly enhances cuproptosis in HCC cells by regulating the expression of the downstream ATP7B mRNA. As a result, the overexpression of miR-133a-5p promotes cuproptosis in HCC, suggesting that miR-133a-5p expression could serve as a prognostic biomarker and a potential therapeutic target for HCC patients.

Conclusion

In the hepatocellular carcinoma cell lines PLC/PRF/5 and Huh-7, the expression of miR-133a-5p is downregulated, whereas the expression of ATP7B is upregulated. Both elesclomol and copper ions can induce cuproptosis in these hepatocellular carcinoma cells. Our results suggest that miR-133a-5p promotes cuproptosis in PLC/PRF/5 and Huh-7 cells by regulating ATP7B mRNA, which in turn inhibits the proliferation and migration of hepatocellular carcinoma cells. Therefore, miR-133a-5p may act as a tumor suppressor and could be a potential therapeutic target for HCC patients. Furthermore, it may provide improved therapeutic benefits for patients with cancers related to copper metabolism disorders.

Data Sharing Statement

All data is contained in the manuscript.

Ethics Approval and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 helsinki Declaration and its later amendments or comparable ethical standards. The study adhered to the ethical guidelines approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (PJ2024-03-60).

Consent for Publication

Informed consent was obtained from all patients.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas, and took part in drafting, revising or critically reviewing the article. Additionally all authors gave final approval of the version to be published, agreed on the journal to which the article has been submitted, and also agreed to take responsibility and be accountable for the contents of the article.

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Disclosure

The authors report no conflicts of interest in this work.

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