MiR-18a-5p promotes proliferation, migration and invasion of hepatocellular carcinoma by targeting CPEB3

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Research

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Abstract

Objective

The study aims to explore the mechanism of miR-18a-5p targeting CPEB3 gene in regulating the occurrence and development of hepatocellular carcinoma (HCC).

Methods

Differential and survival analyses were conducted on HCC expression profiles from TCGA database to screen out target miRNAs on which targeted prediction was conducted. qRT-PCR was used to detect the expressions of miR-18a-5p and CPEB3. MTT assay examined the proliferation activity, wound healing assay analyzed the migration ability and Transwell assay detected the invasion ability of HCC cells after overexpressing miR-18a-5p.Dual luciferase assay verified the targeting relationship between miR-18a-5p and CPEB3. Meanwhile, MTT, wound healing and Transwellassays determined whether the overexpression of CPEB3 reversed the promoting effect of miR-18a-5p on HCC cells.

Results

Bioinformatic analysis showed that miR-18a-5p was significantly highly expressed in HCC tissues and its target binding site was found in CPEB3 genewith low expression.The qRT-PCR found that high miR-18a-5p expression was observed in HCC cells, and the expression of CPEB3 was significantly low. Overexpression of miR-18a-5p promoted proliferation, migration and invasion of HCC cells. Dual luciferase assay observed that miR-18a-5p inhibited the expression of CPEB3 while overexpression of CPEB3 reversed the promoting effect of miR-18a-5p on the growth of HCCcells.

Conclusion

miR-18a-5p promoted the proliferation and migration of HCC cells by inhibiting the expression of CPEB3. The role of miR-18a-5p /CPEB3 in HCCfound in this study provided a new potential target for the prognostic treatment of HCC patients.

Background

HCC is one of the most common malignant tumors in the world[1, 2] with the highest morbidity in east Asia and Africa[3]. According to statistics, China accounts for about 55% of globalHCC patients[4].At present, early diagnosis and chemotherapy, surgical resection and gene therapy are the main treatments for HCC[5, 6]. However, due to limited efficacy, morbidity and mortality remain high, and HCC remains the second most common cause of cancer-related deaths[7, 8].Although the diagnosis and treatment of HCC has been

well developed, the cure rate is still very low.Therefore, it is necessary to study the molecular mechanism of hepatocellular carcinoma to explore new therapeutic methods and improve life quality of HCC patients.

In recent years, gene therapy has become a research hotspot. MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that negatively regulate the expression of target genes at the post-transcriptional level and participate in the various biological processes[9–11]. MiR-18a has been reported to have a disorder of expression in human cancers and is associated with tumor development. Studies have shown that the functions of miR-18a vary in different tumors, and it can act as a tumor suppressor or anoncogene. MiR-18a-5p is a main mature body of miR-18a, and its role in tumors has been widely studied[12–14].For example, miR-18a-5p inhibits CDC42 expression in colorectal cancer cells, thereby inhibiting the occurrence of cancer[12].Other studies have shown that miR-18a-5p is highly expressed in gastric cancer, prostate cancer, HCC, non-small cell lung adenocarcinoma and other tumors, and promotes tumorigenesis by regulating cell proliferation, apoptosis and invasion[13–16].For example, miR-18a-5p promotes the occurrence of HCC by targeting IRF2[17]. Lu *et al.* proposed that miR-18a-5p promoted the metastasis of osteosarcoma by targeting IRF2[18]. Zhou *et al.* found that miR-18a-5p promoted proliferation, migration and invasion of renal cell carcinoma and inhibited apoptosis[19]. Zhang *et al.*demonstrated that miR-18a-5p promoted proliferation of HCC by targeting IRF2 and CBX7[11].Although the function of miR-18a-5p in HCC has been reported in the literature, the mechanism remains to be further explored.

Cytoplasmic polyadenylation element binding protein 3(CPEB3) regulates the translation processby regulating cytosolic polyadenylation[1]. Some studies have indicated that CPEB3 protein is abnormally expressed in some tumors. For example, Hansen *et al.* found that CPEB3 was down-regulated in cervical cancer[20]. Microarray analysis by D'ambrogio*et al.*exhibited that CPEB3 expression was decreased in HCC tissues compared to normal adjacent tissues[21].In addition, Zou *et al.* demonstrated that miR-107 could promote the development of HCC by targeting CPEB3/EGFR axis.Tang *et al*explored that miR-452-3p promoted proliferation and migration of HCC cells by targeting CPEB3/EGFR axis[1]. Although a lot of studies have investigated the function of CPEB3, the role of CPEB3 in HCCis rarely studied.

Therefore, this study aims to clarify the influence of miR-18a-5p on the development of HCC and its potential mechanism, so as to better understand the tumorigenesis mechanism of HCC and provide new candidates for the treatment of HCC.

Material And Methods

1.1 Bioinformatic analysis

The miRNA (50 normal samples, 375 cancer samples) and mRNA (50 normal samples, 374 cancer samples) expression profiles of HCC were downloaded from The Cancer Genome Atlas (http://www.tcga.org/, TCGA) database. EdgeR package was employed to screendifferentially expressed

genes (DEGs)and |logFC|>2, padj < 0.01were set as the threshold. Survival analysis of DEmiRNA was conducted based on the clinical information of samples to determine the target miRNA. Then miDIP(http://ophid.utoronto.ca/mirDIP/index.jsp#r) and starBase (http://starbase.sysu.edu.cn/) were utilized to predict the potential targets of miRNAand the Venn diagram was plotted with down-regulated DEmRNA to find the potential targeted genes.

1.2 Cell culture

Human HCC cell lines SMMC7721 (BNCC352197), HepG2 (BNCC338070) and human normal liver cell lines HL-7702 (BNCC351907) were purchased from BeNa Culture Collection (Shanghai, China).HL-7702 and SMMC7721 were cultured in 90% RPMI-1640 with 10% FBS.HepG2 was cultured in 90% DEEM-Hcontaining10%FBS.These cells were grown at 37 °C with 5% CO_2in an incubator.

1.3 Cell transfection

MiR-18a-5p mimic (Mimic) and NC-mimic were obtained from RiboBio (Guangzhou, China), CPEB3 overexpression plasmid (oe-CPEB3) and corresponding negative control NC (oe-NC) were purchased from Genechem (Shanghai, China). Lipofectamine 2000 (Thermo Fisher Scientific, USA) was used to transfect miR-18a-5p mimic,NC-mimic,oe-CPEB3 and oe-NC vectors into SMMC7721 and HepG2 cell lines at a concentration of 50 nM according to the instructions.

1.4 qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and the RNA concentration was measured by NanoDrop 2000 system (Thermo Fisher Scientific, Inc., Waltham, MA, USA).MiRNA was transcribed into cDNA using SYBR miRNA RT-PCR kit, and mRNA was transcribed into cDNA using PrimeScript RT Master Mix (Takara, Dalian, P.R. China). The expression levels of miR-18a-5p and CPEB3 were measured by Applied Biosystems 7500 real-time PCR instrument (Thermo Fisher Scientific, Inc.). The procedureswere as follows: denaturation at 95℃ for 5 min followed by 40 cycles of denaturation at 95℃ for 10 s, annealing at 60℃ for 20 s, extension at 72℃ for 20 s. GAPDH served as an internal reference for CPEB3 and U6 served as an internal reference for miR-18a-5p.The primer sequenceswere shown in **Supplement table 1.**

1.5 Western blotting

The cell lysates were extracted with RIPA lysate buffer (Beyotime, China) and the protein concentration was determined by BCA protein assay kit (Beyotime, China). After high-temperature denaturation, proteins were separated by SDS-PAGEand transferredonto PVDF membrane (PVDF, Millipore). The membrane wasblocked with 5% skim milk for 2 hours, and incubated with primary antibody overnight at 4℃. The primary antibodies were all rabbit polyclonal antibodies, including CPEB3 (NBP1-56919, 1:1000, Novus Biologicals, USA) and GAPDH (ab9485, 1:2500, Abcam, China).Thereafter, the membrane was incubated with secondary antibody IgG (ab6721; 1:5000; Abcam) for 2 hours at room temperature. Protein signals were detected using enhanced chemiluminescence (ECL) kits (GE Healthcare, Chicago, IL, USA).

1.6 MTT assay

MTT assay was used to detect cell proliferation. Cells were seeded in 96-well plates at a density of 5×10^3. After 1, 2, 3, 4 and 5 days of cells culture, the cell viability was determined using the MTT Cell Proliferation and Toxicity Assay Kit (Beyotime, China) according to the instructions. Cells were incubated at 37℃ for 4 h with 20 μl MTT solution, then incubated with 100 μl solution buffer at 37℃ overnights. The absorbance at 570 nm was read with a spectrophotometer (Molecular Devices, USA). All experiments were repeated three times.

1.7 Wound healing assay

Wound healing assay observed the migration ability of cells. Cells were seeded in a 6-well plate and when the cell coverage reached 80%, the 200 ul pipette tip was used to gently scrape the single layer through the center of the well.The wells were washed twice briefly in the medium to remove the isolated cells.Then fresh medium was added, the cells were regrown for 48 h, and the cell migration at 0 h and 48 h was observed and photographed microscopically.

1.8 Transwell

The cells were suspended in serum-free medium, and then seeded in the upper chamber of a 24-well plateTranswellinvasion chamber (8 μm pores, BD Biosciences, USA).After incubation at 37 ° C for 24 h, the cells on the upper side were cleared away with a cotton swab and the migratory cells on the lower side were stained with 0.2% crystal violet. The number of stained cells was then counted. Each experiment was repeated three times.

1.9 Dual luciferase assay

The 3'UTR of Wild-type (Wt) or mutant (Mut) CPEB3 was cloned into pmirGLO (Promega, WI, USA) vectors to construct CPEB3-Wt and CPEB3-Mut vectors, and then the plasmid vectors and miR-18a-5p mimic or NC-mimic were co-transfected into HepG2 cell lines with empty luciferase reporter vectors.After 24 hours of transfection, the activity of firefly luciferase and Renilla luciferase in the lysed cells was determined using the dual luciferase assay system (Promega, USA) according to the manufacturer's instructions.

1.10 Statistical analysis

Data were processed by SPSS 21 (IBM Corp., Armonk, NY, USA) and exhibited as Mean ± standard deviation (SD). Differences between two groups were compared using *t*-test, and differences among the groups were evaluated by one-way analysis of variance (ANOVA).$P < 0.05$ was considered statistically significant.

Results

2.1 MiR-18a-5p binds to CPEB3 in HCC with negative correlation.

A total of 126 DEmiRNAs and 1981 DEmRNAs were obtained by edgeR differential analysis (Fig. 1A-B), in which the expression of miR-18a-5p was significantly up-regulated in HCC tissues (Fig. 1C).Survival analysis showed that the miR-18a-5p expression was inversely correlated with the prognosis of HCC

(Fig. 1D).It has been reported that miR-18a-5p can promote the proliferation, migration and invasion of a variety of cancers and serve as a prognostic factor[22–25]. The target gene of miR-18a-5p was predicted by miDIP and StarBase database.Thepotential genes(CPEB3, CYP39A1, ESR1, GPM6A)were obtained by intersection with the down-regulated DEmRNA. (Fig. 1-E)Pearson correlation analysis showed that miR-18a-5p was negatively correlated with CPEB3 and ESR1 (Fig. 1F).The relationship between miR-18a-5p and ESR1 in HCC has been clearly reported[22]. The expression of CPEB3 was significantly lowly expressed in HCC patients, and the overall survival time of those patients was lower (Fig. 1G-H). MiR-18a-5p and CPEB3 in HCC patients were found to be highly correlated (Fig. 1I). The above results suggested that miR-18a-5p was likely to regulate the occurrence and development of HCC by targeting CPEB3.

2.2 MiR-18a-5p isup-regulated and CPEB3 isdown-regulated in HCC cells

To further explore the relationship between miR-18a-5p and CPEB3 in HCC cells, we analyzed the expression of miR-18a-5p as well as the expression of CPEB3 mRNA and proteins. The result of qRT-PCR showed that miR-18a-5p in SMMC7721 and HepG2 cells was significantly up-regulated compared with normal HL-7702 cells (Fig. 2A).While the mRNA expression and the protein levels of CPEB3 in HCC cells was significantly down-regulatedin HCC cell lines compared with normal cell line (Fig. 2B-D). Combined with the correlation between miR-18a-5p and CPEB3 in patients (Fig. 1H), we speculated that miR-18a-5p was involved in the regulation of HCC through negative regulating CPEB3 expression.

2.3 Overexpression of miR-18a-5p promotesproliferation, migration and invasion of HCC cells

In order to detect the effect of miR-18a-5p on the biological function of HCC, miR-18a-5p was overexpressed in SMMC7721 and HepG2 to study its rolein the proliferation and metastasis of HCC. First, the results of qRT-PCRindicated that the expression of miR-18a-5p was significantly up-regulated (Fig. 3A). The proliferation, migration and invasion abilities of cells were significantly up-regulated compared with the controlafter overexpressing miR-18a-5p in SMMC7721 and HepG2 cell lines(Fig. 3B-F). The above results indicated that miR-18a-5p promoted the proliferation, migration and invasion of HCC cells.

2.4 MiR-18a-5p inhibits the expression of CPEB3 inHCC cells

In order to explore the regulatory mechanism of miR-18a-5p inHCC, we conducted predictive analysis of its downstream target genesand found that CPEB3 might be the target gene. Then, the binding site sequences of miR-18a-5p and CPEB3 were predicted bystarBase database (Fig. 4A). The results of dual luciferase assay showed that overexpression of miR-18a-5p significantly inhibited luciferase activity in the wild group but had no effect on the mutant group (Fig. 4B). Subsequently, the mRNA and protein

expression levelsof CPEB3 in SMMC7721 and HepG2 after overexpressing miR-18a-5p was significantly down-regulated compared with the control (Fig. 4C-D). These results indicated that miR-18a-5p in HCC cells could directly target CPEB3 and inhibiteditsexpression.

2.5 MiR-18a-5p promoted proliferation, migration and invasion of HCC cells by targeting CPEB3

Next, we overexpressed miR-18a-5p and CPEB3 in SMMC7721 and HepG2 cell lines to analyze whether miR-18a-5p promoted proliferation and metastasis of HCCcells by inhibiting CPEB3 expression. The mRNA expression and protein levels of CPEB3 mRNA was greatlydown-regulated after overexpression of miR-18a-5p, while there was no significantly difference from thoseof the control groupwhenoverexpressingCPEB3and miR-18a-5p at the same time(Fig. 5A-B). We further tested cell proliferation (Fig. 5C), migration (Fig. 5D-E) and invasion abilities(Fig. 5F), finding that the proliferation, migration and invasion abilitieswere significantly up-regulated after the overexpression of miR-18a-5p, but when miR-18a-5p and CPEB3 were overexpressed at the same time, the above abilities of SMMC7721 and HepG2 cell lines recovered to the level of the control group with no difference.These results indicated that miR-18a-5p could promote the proliferation, migration and invasion of HCC cells by inhibiting the expression of CPEB3.

Discussion

HCC is a common malignant tumor, with approximately 745,000 deaths each year [26]. The 5-year overall survival rate for HCC patients is about 30% [11, 26]. Therefore, improving the survival rate remains to be a major challenge. We found that miRNAs often had expression dysregulation in tumors[6]. This also suggests that miRNA may become a potential target for the development of chemotherapy drugs in the future. Abnormal expression ofmiRNAs such as miR-486-5p, miR-106b, miR-372 and miR-452-3p has been reported in HCC[26-29]. Studies have shown that miR-18a is involved in the regulating the occurrence of various cancers, including proliferation and metastasis of colorectal cancer [12], gastric cancer [15], lung cancer [14] and HCC[13]. Although it has been proposed that miR-18a promotes the proliferation and metastasis of HCC by inhibiting target genes [6, 11], its mechanism in HCC has not been fully studied. In this study, we first analyzed the expression of miR-18a-5p in HCCcells and the role of miR-18a-5p. The results showed that the expression of miR-18a-5p was up-regulated in HCCcells, and its overexpressionsignificantly promoted the cellproliferation, migration and invasion. The above results are consistent with previous researches, further proving the promoting role of miR-18a-5p in HCC.

In this study, we found that the expression of CPEB3 in HCC cells was significantly down-regulated, whichwascontrary to and negatively correlated with the expression of miR-18a-5p. Bioinformatics analysis showed that miR-18a-5p could specifically bind to CPEB3. However, the expression of CPEB3 was significantly down-regulated after overexpression of miR-18a-5p. Further, luciferase assay proved that miR-18a-5p could regulate CPEB3. We also overexpressed miR-18a-5p and CPEB3 atthesametimeto

detect the biological function of the cells, and found that overexpression of CPEB3 significantly reduced the promoting effect of miR-18a-5p on HCCcells. The above results suggested that miR-18a-5p promoted proliferation, migration and invasion of HCC cells through negatively regulating CPEB3.

In conclusion, this study proved that miR-18a-5p can promote the proliferation, migration and invasion of HCC cells by down-regulating CPEB3. This study revealed the importance of the miR-18a-5p/CPEB3 axis in the proliferation and metastasis of HCC.Theresulthelps people tobetterunderstand the mechanism of miR-18a-5p in HCC, and also finds an entry point for finding new targeted therapeutic approaches for HCC.

Supplemental Table

Supplement table 1

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC
CPEB3	GAGCTGTTGAACTGGCAATG	ACTGCAGACAGGTGACGTTG
miR-18a-5p	GATAGCAGCACAGAAATATTGG C	GTGCAGGGTCCGAGGT
U6	CGCGCTTCGGCAGCACATAT ACT	ACGCTTCACGAATTTGCG TGTC

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Helsinki Declaration II and was approved by the Institutional Review Boards of Tangshan Gongren Hospital and Shanghai Engineering Research Center of Pharmaceutical Translation.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials in the current study are available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare no potential conflicts of interest.

Authors' contributions

Dr JX Z and DY W contributed to the study design. JX Z conducted the literature search. XG L, LB W, FZ Q and DM C acquired the data. JX Z and DY W wrote the article. JX Z performed data analysis. DY W drafted. XT W, YB W and L C revised the article and gave the final approval of the version to be submitted. All authors read and approved the final manuscript.

Availability of data and materials

The data and materials in the current study are available from the corresponding author on reasonable request.

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Figures

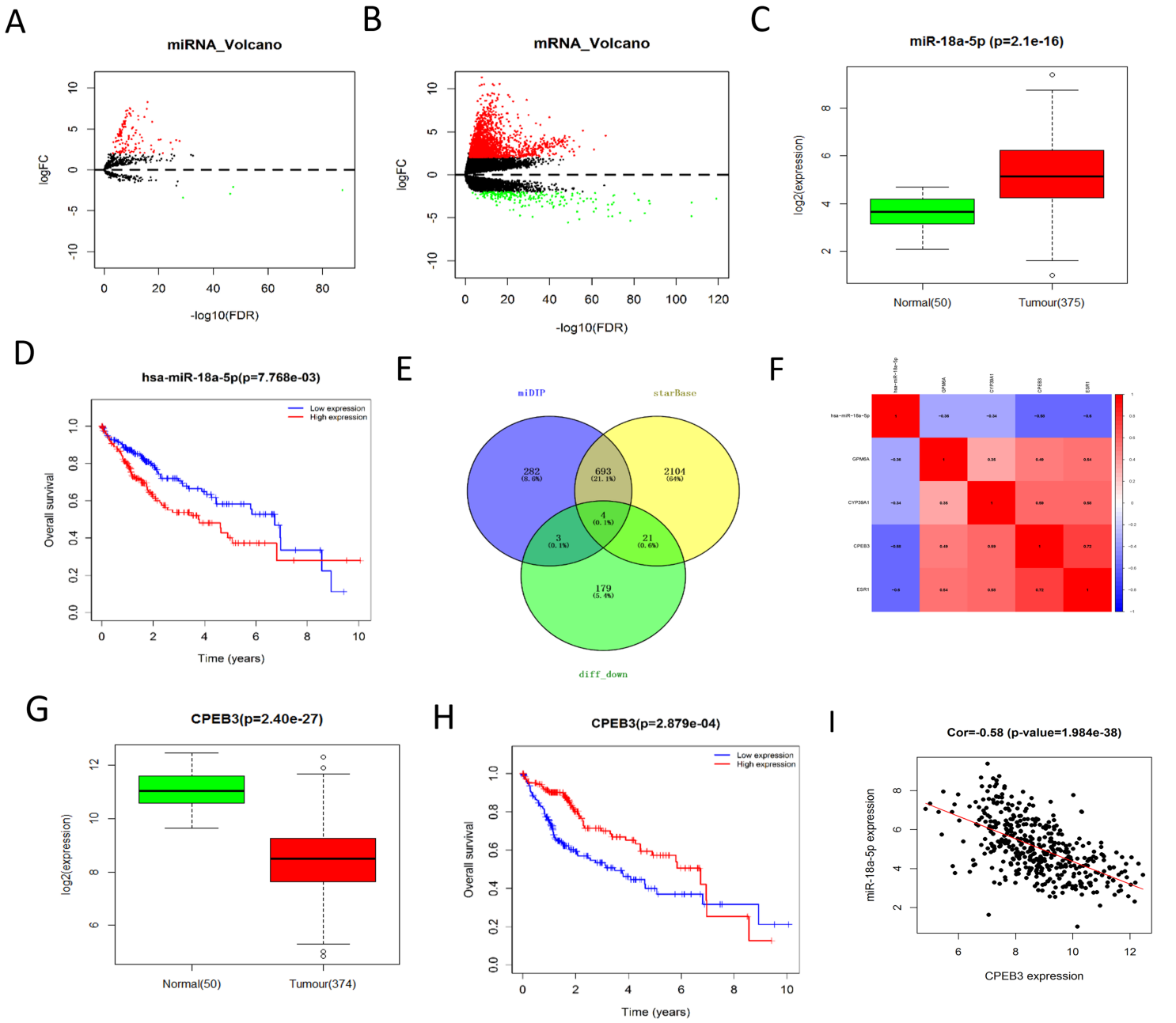

Figure 1

The targeting relationship between miR-18a-5p and CPEB3 A-B: Differential gene volcanic map of miRNA and mRNA in HCC from TCGA database, green represents down-regulated miRNA and mRNA while red represents up-regulated miRNA and mRNA; C: The boxplot of miR-18a-5p expression with green for the normal group and red for the patient group; D: The survival curves of miR-18a-5p, the abscissa represents time (in years), the ordinate represents survival rate, the red curve represents the high-expression group, and the blue curve represents low-expression group; E:Venny diagram of the DEmRNA and predicted target genes of miR-18a-5p; F: The correlation between miR-18a-5p and its predicted target genes; G: Boxplot of CPEB3 expression, green for normal group and red for patient group; H: The survival curves of CPEB3, the abscissa represents time (in years), the ordinate represents survival rate, the red curve

represents the high-expression group, and the blue curve represents the low-expression group; I: Pearson correlation diagram of miR-18a-5p and CPEB3.

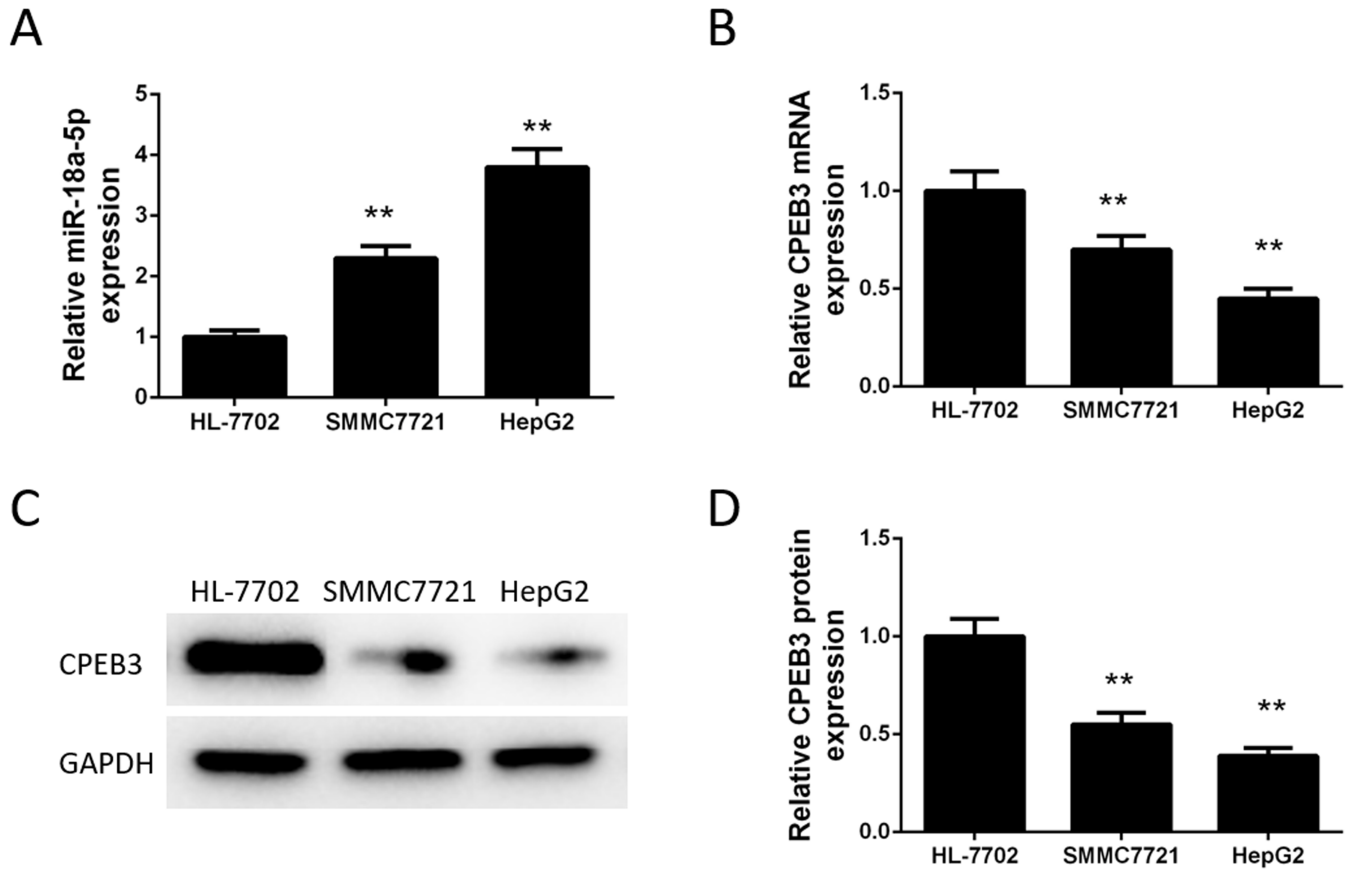

Figure 2

The expression of miR-18a-5p and CPEB3 in HCC cells A-B: The relative expression of miR-18a-5p and CPEB3 mRNA in HL-7702, SMMC7721 and HepG2 cells was detected by qRT-PCR; C-D: The protein expression levels of CPEB3 in SMMC7721, HepG2 and HL-7702 cells was detected by Western blot; ** P<0.01.

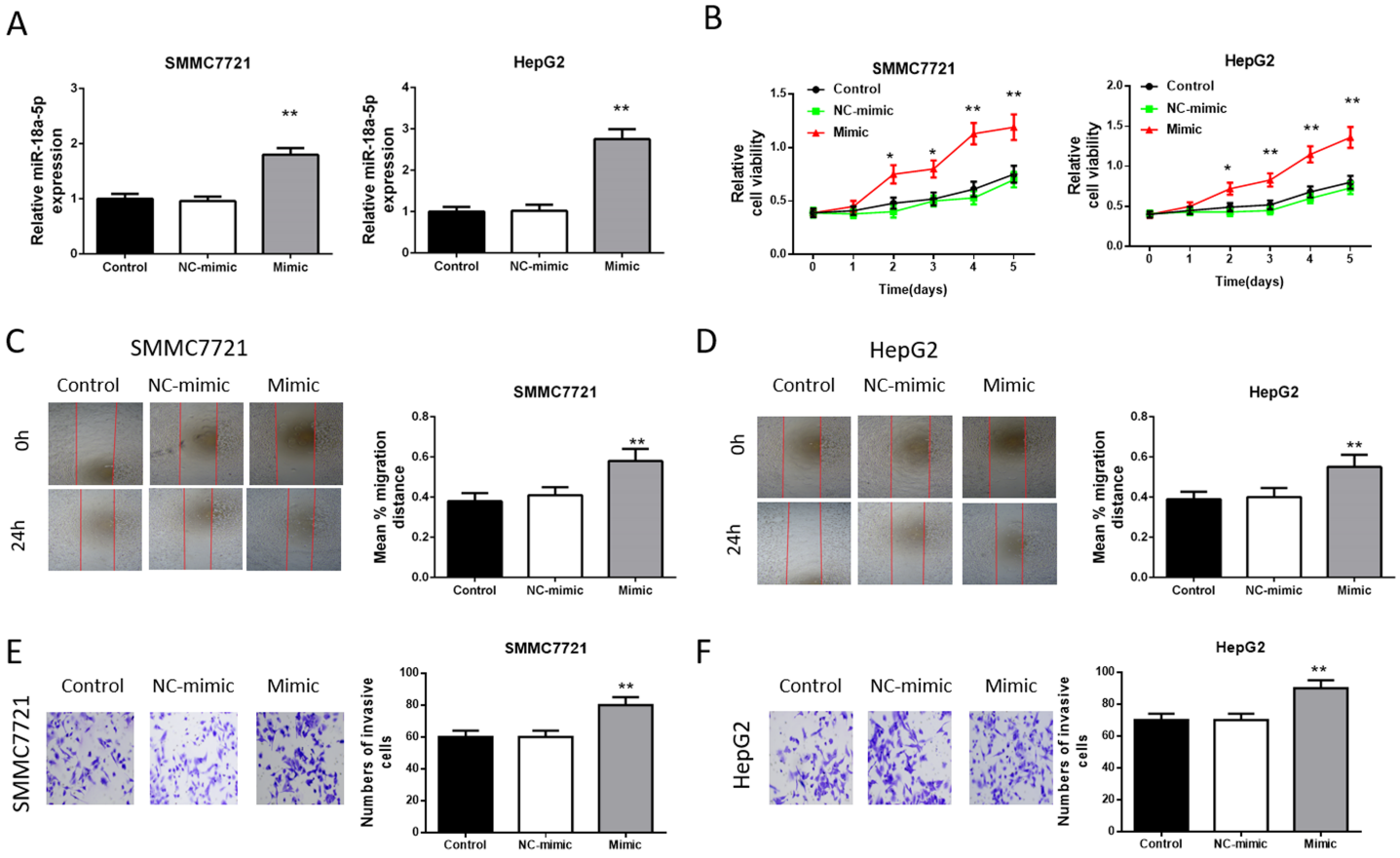

Figure 3

Overexpression of miR-18a-5p promoted proliferation, migration and invasion of HCC cells A: The expression of miR-18a-5p was detected by qRT-PCR; B: The proliferation capacity of SMMC7721 and HepG2 cells with overexpression of miR-18a-5p was detected by MTT assay; C-D: The migration capacity of SMMC7721 and HepG2 cells with overexpression of miR-18a-5p was detected using wound healing assay; E-F: Transwell assay detected the invasion capacity of SMMC7721 and HepG2 cells after overexpression of miR-18a-5p; * P<0.05, ** P<0.01.

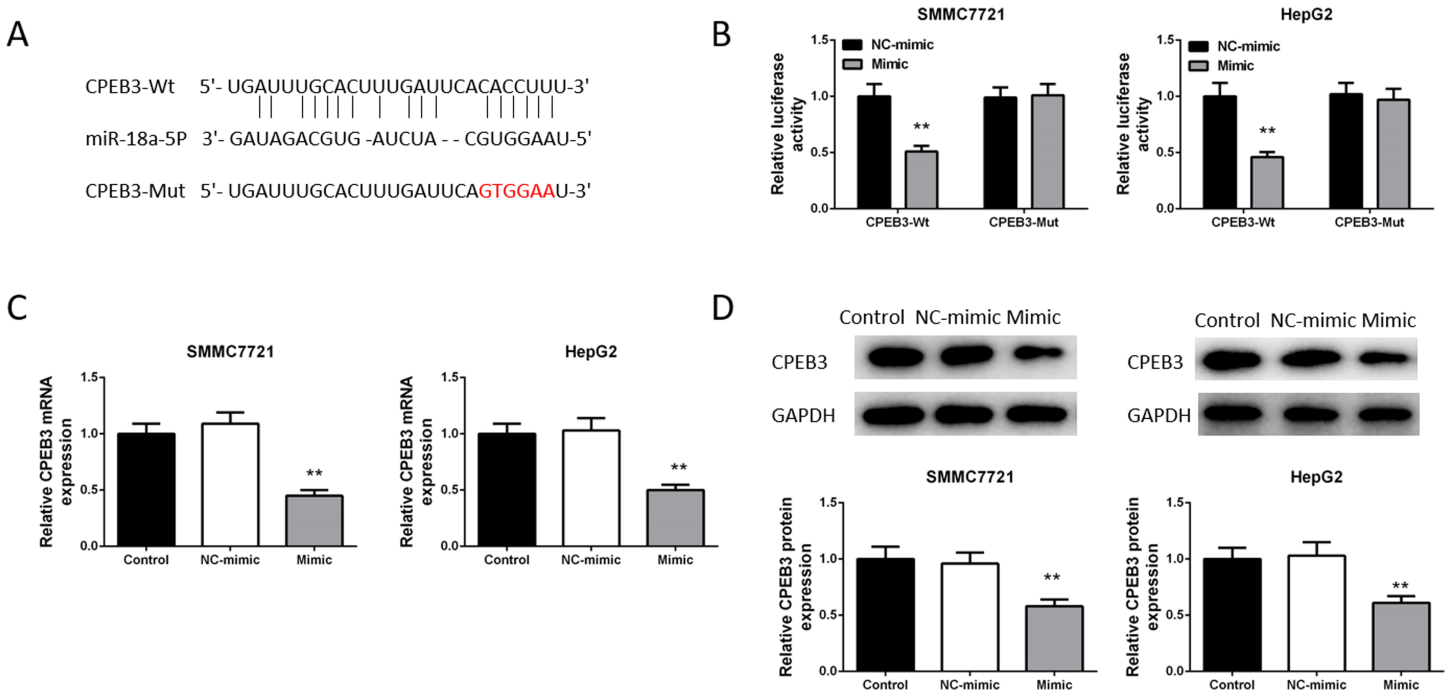

Figure 4

MiR-18a-5p inhibits the expression of CPEB3 A:Target binding sites of miR-18a-5p and CPEB3; B: Luciferase activity in different treatment groups after overexpression of miR-18a-5p; C: The expression of CPEB3 mRNA in SMMC7721 and HepG2 cells after miR-18a-5p overexpression was detected by qRT-PCR; D: The protein expression of CPEB3 in SMMC7721 and HepG2 cells was detected by western blot after miR-18a-5p overexpression; ** P<0.01.

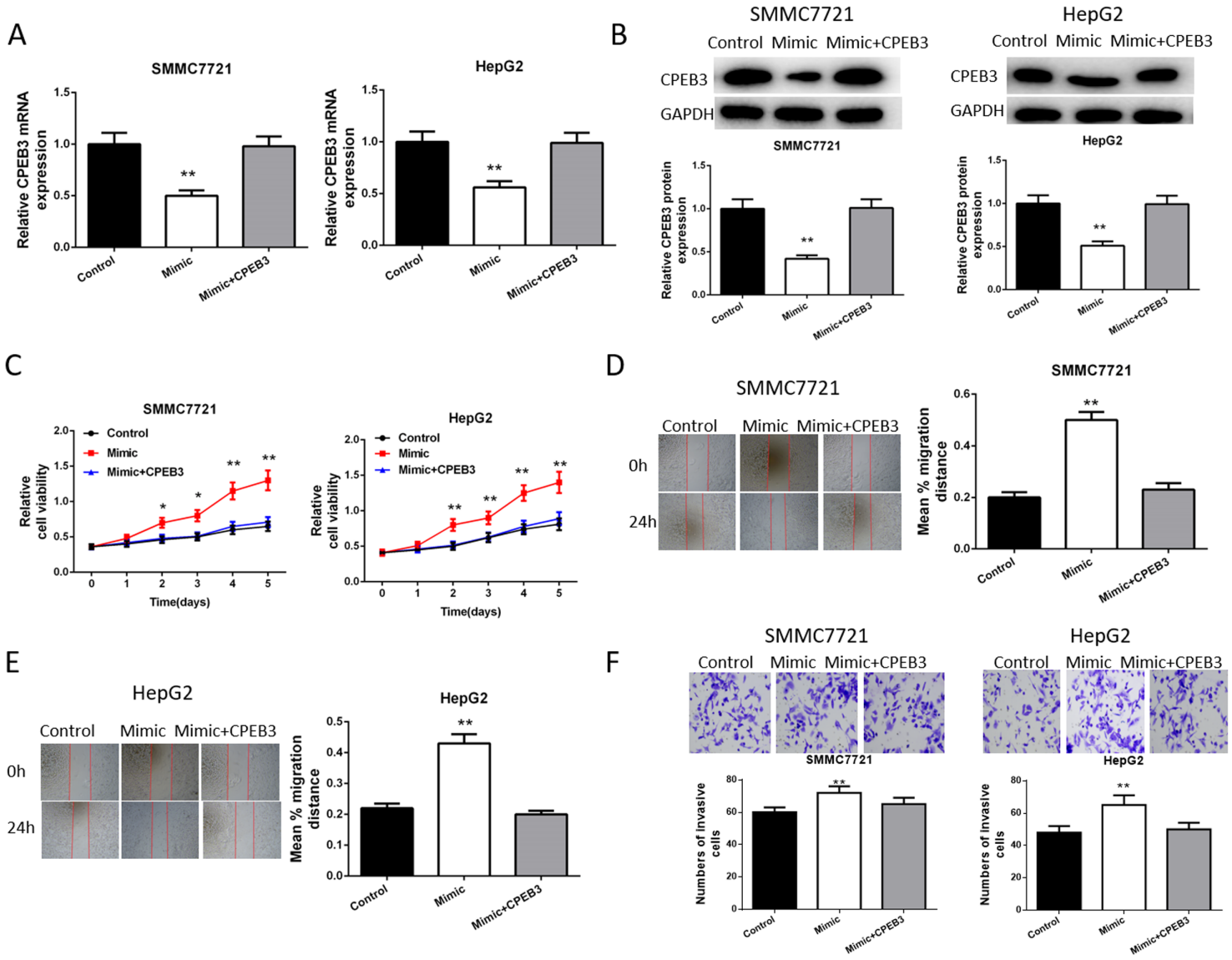

Figure 5

Mir-18a-5p inhibited the expression of CPEB3 and promoted the proliferation and metastasis of HCC cells A: The expression of CPEB3 mRNA in Control, Mimic and Mimic+CPEB3 cells was detected by qRT-PCR; B: CPEB3 protein expression in different treatment groups was analyzed by western blot; C: The proliferation capacity of SMMC7721 and HepG2 cells in different treatment groups was detected by MTT assay; D-E: The migration ability of SMMC7721 and HepG2 cells in different treatment groups was detected by wound healing assay; F: The invasion ability of SMMC7721 and HepG2 cells in different treatment groups was determined by Transwell assay; * P<0.05 and **P<0.01.

Supplementary Files

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