miRNA-518a-3p inhibits hepatocarcinogenesis by suppressing ZNF281

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Research Article

Keywords: hepatocarcinogenesis (HCC), miRNA-518a-3p, Zinc finger protein281(ZNF281)

Posted Date: March 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1027296/v1

Abstract

Background: Emerging evidences have indicated that microRNAs (miRNAs) play a significant role in multiple biological processes, including the control of the cell cycle, apoptosis, autophagy and metabolic reprogramming. Among them, miRNA-518a-3p has been revealed to display a notably key function during the development of many types of cancer, containing colorectal cancer, breast cancer and squamous cell carcinoma. However, its functions and the molecular mechanisms during HCC progression still remain unclear.

Methods: miRNA-518a-3p expression level was detected by RT-qPCR in HCC tumor tissues and four HCC cell lines, and its related prognostic effects were also investigated. Furthermore, its functions during HCC progression were investigated by CCK-8 experiment, transwell assay in two HCC cell lines. Moreover, its potential mechanisms were investigated by bioinformatic analysis, RT-qPCR, western blotting, luciferase reporter assay and rescue experiments.

Results: Downregulation of miRNA-518a-3p was found in HCC tumor tissues and correlated with a more awful overall survival. Gain and loss of function analyses displayed miRNA-518a-3p repressed HCC cells proliferation, migration and invasion. Then, bioinformatic analysis and luciferase reporter assay have suggested that ZNF281, upregulated in HCC tumor tissues, was negatively regulated by miRNA-518a-3p. Moreover, rescue experiments demonstrated that the suppression of HCC progression by miRNA-518a-3p was mediated by ZNF281.

Conclusion: Our studies indicated that miRNA-518a-3p inhibited hepatocarcinogenesis through suppression of ZNF281 expression, indicating that miRNA-518a-3p is a promising therapeutic approach targeting for the treatment of HCC.

1. Background

Hepatocellular carcinoma (HCC), one of the most prevalent and lethal malignant tumors, rank the third in cancer-related deaths[1]. Moreover, due to unhealthy diet and lacking of exercise, the incidence of HCC has been steadily increasing over the decades. Although effective surgery and advanced medicine treatment contribute to improved survival rate of HCC patients in the early stage, treatment efficiency for patients with advanced HCC still remains unsatisfactory[2, 3]. As a result, it is extremely crucial to investigate the underlying mechanism for HCC progression, thus, providing new therapeutic approaches for HCC treatment.

MicroRNAs, a class of non-coding RNAs which are 18-25 nt in length, could regulate the expression of different genes via interaction with 3' untranslated regions (3' UTRs)[4]. Therefore, miRNAs have been demonstrated to involve in various biological processes, such as the control of the cell cycle, apoptosis, autophagy and metabolic reprogramming. Moreover, miRNAs also display a key role in cancer cell proliferation, migration and invasion[5, 6].

According to previous studies, miR-518a-3p has shown to play remarkably key roles in various cancers development, including colorectal cancer, triple-negative breast cancer (TNBC) and squamous cell carcinoma, via regulation of various genes and multiple signal transduction pathways[7–9]. Nevertheless, the roles of miR-518a-3p during HCC development and metastasis remains to be further illustrated.

In our project, the expression of miR-518a-3p in HCC cancer tissues and cells as well as its clinical signification were investigated. Downregulation of miR-518a-3p in HCC tissues and cells was found, which was extremely correlated with lower overall survival. Then, the functions of miR-518a-3p in HCC cell proliferation, migration and invasion were detected. We found upregulation of miRNA-518a-3p resulted in repressed HCC progression, and versa vice. Moreover, the underlying mechanism revealed that miR-518a-3p suppressed the expression of Zinc finger protein 281 (ZNF281). Thus, its function in mediating the repression of HCC progression was accomplished by repressing ZNF281 expression.

Taken together, we indicated that miR-518a-3p inhibited HCC progression via suppression of ZNF281 expression, demonstrating miR-518a-3p could be a new therapeutic approach with regards to HCC targeted therapy.

2. Methods

2.1 Tissues collection

In our study, forty pairs of HCC tumor tissues and adjacent normal tissues were employed, stored at −80℃ until use, were obtained from China Three Gorges University. Written informed consent was collected from all of patients. And the study was approved by ethics committee of China Three Gorges University. The effect of treatment was assessed by referring to the criteria of Response Evaluation Criteria in Solid Tumors (RECIST)[10].

2.2 Cell culture and cell transfections

HCC cells including HepG2, Hep3b, Li-7 and SNU-387 and human normal liver epithelial cells HL-7702 were obtained from the ATCC Cell Biology Collection (Maryland, USA) and cultured in the DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), in an incubator at 37°C. The miR-518a-3p mimic, mimic-Con, miR-518a-3p inhibitor, inhibitor-Con, luciferase reporter plasmids containing wild or mutant ZNF281 were conducted by GenePharma (Suzhou, China). Lipofectamine 2000 Transfection Reagent (Invitrogen) was employed to conduct cell transfection according to the manufacturer's instructions.

2.3 RT-qPCR

Total RNAs from cells and tissues were extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNAs were prepared by using HiScript® II Reverse Transcriptase Kit (Vazyme). AceQ Universal SYBR qPCR Master Mix (Vazyme) was used for RT-qPCR detection. U6 and GAPDH were used for normalization. Primers for RT-qPCR are listed in Table S1.

2.4 Cell proliferation assay

The Cell Counting Kit-8 (CCK8) experiment was employed to access the growth of HCC cell lines according to the manufacturer's instructions. Briefly, about $1\in10^4$ cells were seeded into each well of 96-well plates, followed by incubation with CCK-8 solutions for 2 hours at 1, 2 and 3 day. The OD value was measured at 450 nm with an automatic microplate reader[11].

2.5 Migration and invasion assays

The transwell chambers were employed to perform the experiment. At first, cells were transfected according to previous described method, following by trypsinizing and resuspending in the serum-free medium. Next, about $2\in10^4$ cells were seeded into each chamber and cultured for 24hrs. Cells in the upper chamber were gently wiped with a cotton ball after washing cells by PBS. Then, the upper chamber was stained with 0.1% crystal violet solution for 20 mins with methanol fixation for 30 mins. Finally, brightfield microscope was employed to count the number of cells. As to the invasion assays, the filters should be coated with Matrigel. Then, almost similar method was conducted.

2.6 Target prediction

The potential targets of miR-518a-3p were predicted by an online database miRDB (http://mirdb.org/).

2.7 The luciferase reporter assay

The luciferase reporter vectors containing wild-type or mutant ZNF281 with either miR-518a-3p mimic or mimic-Con were transfected into the SNU-387 and Li-7 cells. The assay was detected by Dual Luciferase Reporter Assay system (Promega). The final activity was measured as firefly luciferase activity normalized by renilla luciferase activity.

2.8 Western blot assay

After transfection, cells were lysed in the RIPA buffer (Beyotime, China) supplemented with protease inhibitor cocktail (PIC) for 30 mins on ice, following by centrifugation for 15 minutes at 12,000g to get proteins. The concentration of proteins was measured with a BCA protein assay kit (Beyotime, China). Then, proteins were loaded into SDS-PAGE gels for separation, following by transferring onto the polyvinylidene fluoride (PVDF) membranes. The membranes were washed by 1xTBST buffer. Next, 5% fat-free milk was employed to block. Afterward, indicated primary antibodies were employed to incubate these membranes overnight at 4℃, following by washing these membranes for 3 times with 1xTBST buffer. Finally, the membranes were incubated with HRP-conjugated secondary antibodies for 1h at room temperature, following by washing these membranes for 3 times with 1xTBST buffer. The enhanced chemiluminescence reagent (Beyotime, China) was employed to detect proteins. Primary antibodies and secondary antibody were used as followed: rabbit anti- ZNF281 (1:1000, Abcam, ab101318), rabbit anti-GAPDH (1:5000, Abcam, ab181602) and goat anti-rabbit IgG H&L (HRP) (1:1500, Abcam, ab205718).

2.9 Statistical analysis

Data were analyzed by using GraphPad Prism (version 8; GraphPad Software) and were shown as mean ± SD. The statistical significance was assessed by the Student's two-tailed paired and unpaired t-test. (*P < 0.05).

3. Results

3.1 miR-518a-3p was downregulated in HCC tumor tissues and correlated with prognosis of HCC patients

According to previous studies, a reduced expression of miR-518a-3p has been shown in CRC cells as well as CRC tissues [7, 12]. Thus, in order to illustrate the correlation between miR-518a-3p expression and HCC, its expression level was detected by RT-qPCR analysis. Compared with adjacent normal tissues, decreased expression level of miR-518a-3p was found (Figure 1A). Besides, patients were divided into higher and lower group by medium cutoff of miR-518a-3p expression in cancer samples. In accordance with the previous study, Kaplan-Meier survival curve also suggested that patients with higher expression of miR-518a-3p displayed improved overall survival than that of lower expression (Figure 1B). However, there was no significant difference in gender, age, tumor staging, lymph node metastasis and types of cancer between the higher and lower expression group. Furthermore, consistent with that in cancer tissues, its expression was also notably decreased in four HCC cell lines compared to normal liver cell lines (Figure 1C). All these data have indicated that miR-518a-3p may play a vital role in the development of HCC.

3.2 Upregulation of miR-518a-3p repressed the malignant phenotype of HCC cell lines

To further explain the function of miR-518a-3p during the development and metastasis, Li-7 cells and SNU-387 cells were transfected with mimic-Con or miR-518a-3p mimic. The overexpression efficiency in both HCC cell lines (Li-7 and SNU-387) was confirmed by RT-qPCR analysis (Figure 2A). Then, CCK-8 experiment was employed to detect cell growth. A notably slower rate of cell growth was found in the miR-518a-3p mimic treated groups compared to that in mimic-Con groups in Li-7 and SUN-387 cells (Figure 2B and 2C). Moreover, the transwell analysis was used to investigate the changes of migration and invasion after miR-518a-3p overexpression. The overexpression of miR-518a-3p gave rise to a decreased number of cells that permeate through the membrane with or without matrigel, thus, demonstrating that miR-518a-3p suppressed the ability of migration & invasion (Figure 2D and 2E). All in all, the results indicated miR-518a-3p may serve as a tumor suppressor gene during HCC progression.

3.3 miR-518a-3p silencing promoted HCC progression in vitro

Li-7 cells and SNU-387 cells were transfected with NC or miR-518a-3p inhibitor. The knockdown efficiency in both HCC cell lines was confirmed by RT-qPCR analysis (Figure 3A). Subsequently, to further determine the function of miR-518a-3p in HCC cell growth, CCK-8 analysis was performed. A relatively higher growth rate was found in miR-518a-3p inhibitor-treated group, indicated that miR-518a-3p negatively regulates HCC cells proliferation (Figure 3B and 3C). Also, the transwell assay was used to investigate the ability of migration as well as invasion. Consistent with previous studies, miR-518a-3p silencing remarkably increased the number of cells that permeate through the membrane with or without Matrigel. As a conclusion, miR-518a-3p suppressed HCC progression in vitro (Figure 3D, 3E and 3F).

3.4 miR-518a-3p suppressed HCC progression via regulating ZNF281

An online database for miRNA target prediction (http://mirdb.org/) was employed to elucidate the mechanism of miR-518a-3p suppression for the development and metastasis of HCC. From bioinformatic analysis, ZNF281 was predicted as a potential target for miR-518a-3p. Moreover, in order to confirm whether ZNF281 was a putative target of miR-518a-3p, plasmids with wild binding site or mutant binding site of miR-518a-3p and luciferase reporter gene were constructed (Figure 4A). Thus, miR-518a-3p mimic and mimic-Con were separately co-transfected with ZNF281 WT or ZNF281 mutant luciferase reporter plasmid into Li-7 and SNU-387 cells. Luciferase assay has shown that the relative luciferase activity of the Li-7 cells with ZNF281 WT luciferase reporter plasmid was remarkably decreased after miR-518a-3p overexpression. However, there was no obviously difference within mutant group (Figure 4B). Also, similar results were found in the SNU-387 cells (Figure 4C). As a result, it indicated that miR-518a-3p suppressed HCC progression via modulating ZNF281. Next, the expression of ZNF281 was measured in HCC cancer samples to further unfold the mechanism. A relatively higher expression level of ZNF281 was found in HCC tissues in comparison with adjacent normal tissues (Figure 4D). Additionally, ZNF281 expression was negatively correlated with the miR-518a-3p expression in the HCC tissues (Figure 4E). All in all, miR-518a-3p suppressed HCC cell proliferation, migration and invasion via regulating ZNF281.

3.5 Knockdown of ZNF281 eliminated the effects of miR-518a-3p suppression in HCC progression

In order to further confirm whether ZNF281 is a functional downstream target of miR-518a-3p, knockdown of ZNF281 or miR-518a-3p by siRNA or miRNA inhibitor, separately or together, were performed in both HCC cell lines. It has shown that knockdown of miR-518a-3p induced the expression level of ZNF281, whereas knockdown of miR-518a-3p in ZNF281 silencing group led to no obvious change on the expression level of ZNF281, indicated that the function of miR-518a-3p was mediated by ZNF281 during CRC development (Figure 5A and 5B). Thus, miR-518a-3p, a rate-limiting binding factor for ZNF281, function as a suppressor to reduce the activity of ZNF281. Besides, the changes of migration and invasion were observed. Knockdown of ZNF281 inhibited the increased migration and invasion which were induced by miR-518a-3p knockdown in both Li-7 cells and SNU-387 cells, thus, suggested that

the function of miR-518a-3p in mediating the repression of HCC progression was achieved by modulating ZNF281 (Figure 5C, 5D and 5E).

4. Discussion

HCC, one of the most common causes for cancer-related deaths, is responsible for among 830,000 cases death in China in 2020. Although great advances in screening and prevention techniques contribute to both treatment and diagnosis of HCC patients, the patient survival with HCC still remains poor[3, 13]. As a result, urgent needs are required to further investigate the underlying mechanism for HCC progression to support the therapeutic development. MicroRNAs have been reported to play a key role in multiple biological processes and its abnormal expression has been confirmed to associate with development of various types of cancers[14]. Moreover, miR-518a-3p has been illustrated to play an essential role in the development of many kinds of cancers, including colorectal cancer, triple-negative breast cancer and squamous cell carcinoma[7–9]. Qu et al's research has revealed that its expression level was downregulation in CRC cells in comparison with normal colonic cell line. Also, its expression level was reduced in CRC tissues. Further mechanism studies demonstrated that miR-518a-3p repressed cell growth and promoted cell apoptosis by regulating the NIK-dependent NF-κB pathway[7]. As yet, little is well-known about roles of miR-518a-3p and its underlying mechanism during the development and metastasis of HCC.

In this study, we first found that the expression level of miR-518a-3p was downregulated in HCC tissues in comparison with adjacent normal tissues. Besides, the survival rate of patients with a higher expression of miR-518a-3p was more awful than that of a lower expression. Consistent with above mentioned findings, its expression level was also reduced in HCC cells, suggesting miR-518a-3p may play critical roles in HCC progression. Next, in order to further elucidate its function in HCC progression, HCC cells were transfected to knockdown or overexpress miR-518a-3p. From our data, cell growth, migration and invasion were induced after miR-518a-3p silencing. Similarly, its upregulation led to suppression of cell growth, migration and invasion, suggesting that miR-518a-3p served as a vital tumor suppressor gene during HCC progression.

ZNF281, a zinc-finger transcription factor, has been reported to act as a key regulator in tissue development and cellular stemness[15, 16]. Notably, recent studies have indicated that ZNF281 also served as a novel oncogene, which has a high expression level in many kinds of tumors, including colorectal cancer, breast cancer and pancreatic carcinomas[17–19]. Moreover, recent study has demonstrated that ZNF281 expression level was higher in HCC cells in comparison with immortalized hepatocytes. Furthermore, ZNF281 silencing led to reduced proliferation, DNA synthesis and anchorage-independent growth of HCC cells[20]. Nevertheless, it still remains unknown whether the suppression of HCC progression by miR-518a-3p was mediated by ZNF281. Thus, in order to confirm the hypothesis, the luciferase assay was employed. We found that overexpression of miR-518a-3p gave rise to a reduced level of luciferase activity in WT group, whereas the relative luciferase activity of cells in MUT group

displayed no significant change. What's more, ZNF281 expression was upregulated in HCC tumor samples, thus, revealing miR-518a-3p suppressed HCC progression by regulating ZNF281. Finally, in order to confirm that the inhibition of HCC progression by miR-518a-3p was mediated by ZNF281, silencing of ZNF281 or miR-518a-3p by siRNA or miRNA inhibitor, separately or together, was performed. Our data have shown that the increased migration and invasion induced via knockdown of miR-518a-3p were diminished when ZNF281 silencing.

5. Conclusion

In summary, we documented the downregulation of miR-518a-3p in HCC tumor samples and cells. Also, the overall survival rate of HCC patients was higher in the group with lower miR-518a-3p expression. Moreover, miR-518a-3p was demonstrated to play significant roles in suppressing the development and metastasis of HCC by regulating ZNF281.

Abbreviations

microRNAs (miRNAs), hepatocellular carcinoma (HCC), Cell Counting Kit-8 (*CCK-8*) , 3' untranslated regions (3' UTRs), triple-negative breast cancer (TNBC), Response Evaluation Criteria in Solid Tumors (RECIST), fetal bovine serum (FBS), penicillin/streptomycin (P/S), protease inhibitor cocktail (PIC), polyvinylidene fluoride (PVDF), Zinc finger protein 281 (ZNF281)

Declarations

Competing interest

The authors declare no conflict of interest.

Ethics approval

The study was approved by ethics committee of China Three Gorges University (LL201911WK7).

Funding

We got research fund from Research Project of China Three Gorges University. China Three Gorges University provided venues and equipment for this work. It is a non-profit organization.

Author contributions

YH designed the research. DC and KS did the experiment and analyze the data. KS wrote the manuscript. All authors read and approved the final manuscript.

Data Statement

All data are sourced from our laboratory and presented in the article. No additional data are available.

Acknowledgements

We would like to thank all colleagues from clinical and research department in Yichang Central People`s Hospital and Renhe Hospital.

Consent for publication

All authors declare that this research does not involve personal data. We have obscured the personal information that may be involved before analysis process. All patients have signed an informed consent form.

Availability of Data and Materials

Our experimental data comes from our laboratory. The consumables used have been marked in the manuscript. All data generated or analyzed during this study are included in this published article. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

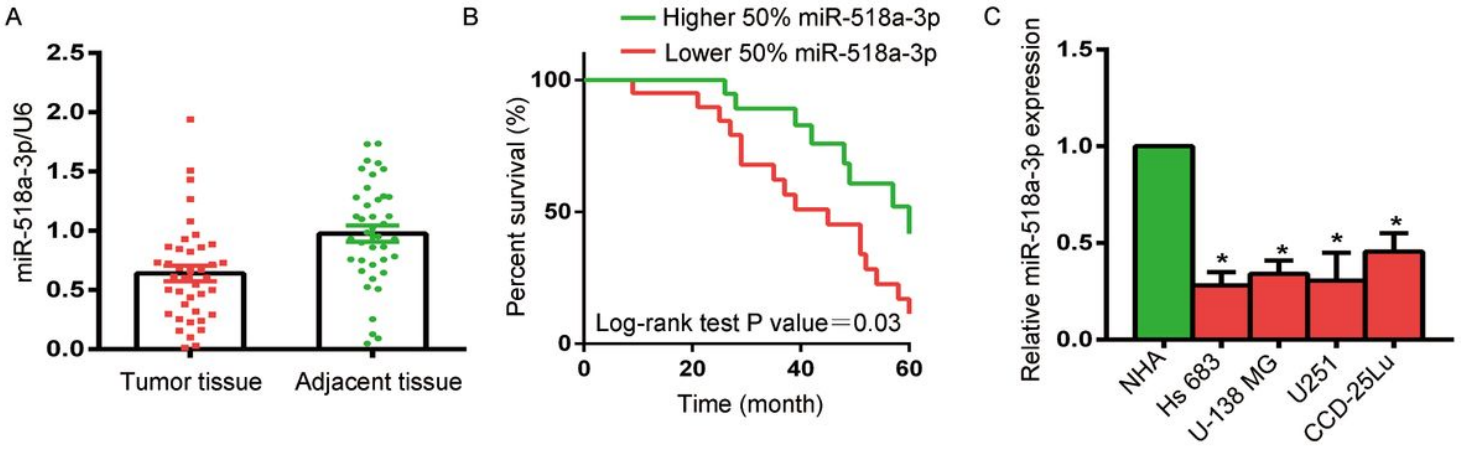

Figure 1

miR-518a-3p expression was reduced in HCC tumor samples and cells. (A) RT-qPCR experiments have shown that the expression of miR-518a-3p was decreased in HCC tissues compared with adjacent normal tissues (n=40). (B) Kaplan–Meier analysis of overall survival of HCC patients. Patients with the higher miR-518a-3p level displayed a relatively bad prognosis (p=0.03). (C) RT-qPCR experiment demonstrated a downregulation of miR-518a-3p in HepG2, Hep3b, Li-7 and SNU-387 cells compared with normal liver cell lines. Student t-test were used for statistics. *P<0.05.

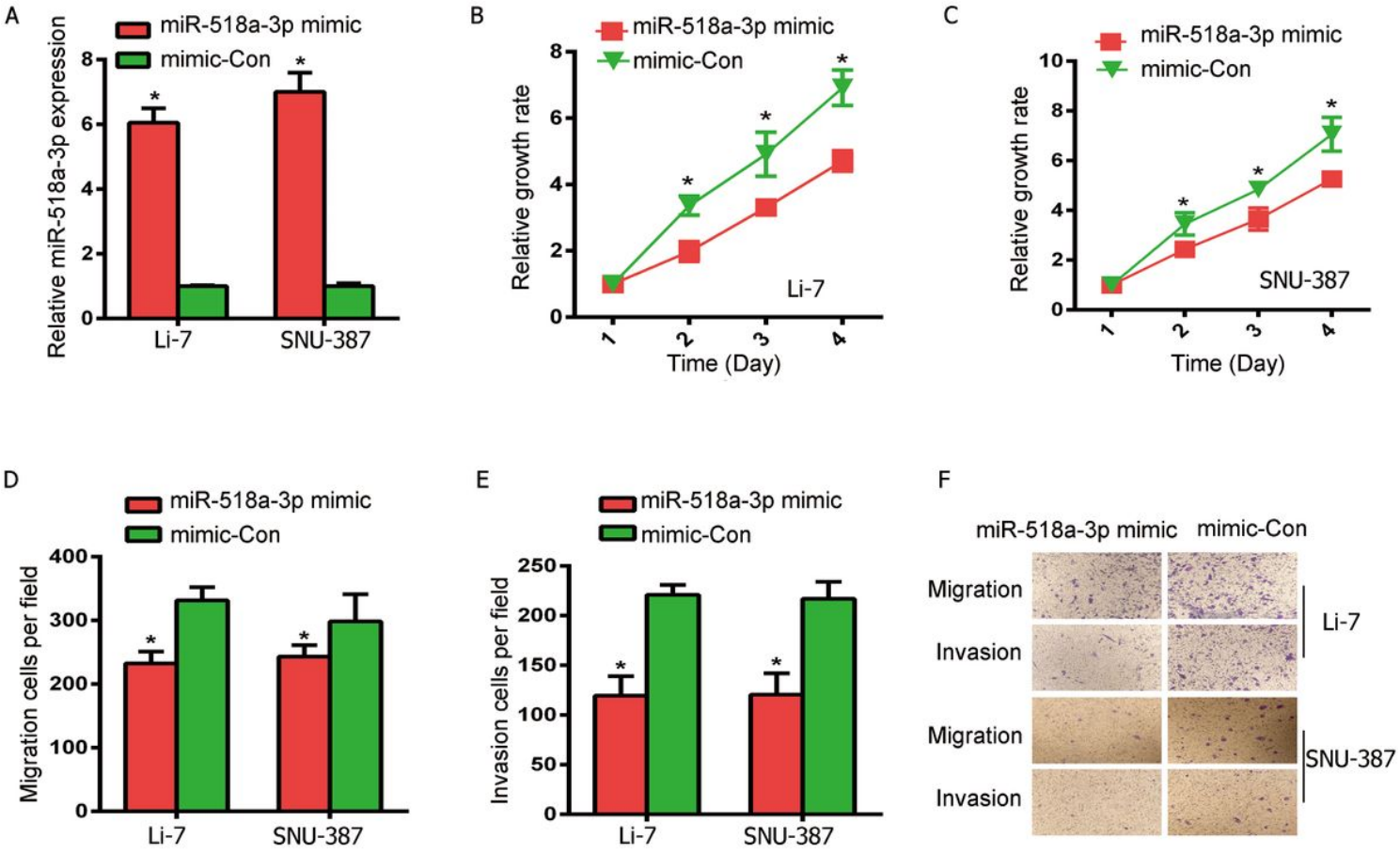

Figure 2

miR-518a-3p suppressed HCC cell growth, migration and invasion. (A) RT-qPCR experiment confirmed the overexpression efficiency in Li-7 and SNU-387 cells. Growth curve of Li-7 (B) and SNU-387 (C) cells with miR-518a-3p mimic transfection or NC was measured by CCK-8 analysis. Migration (D and F) and invasion (E and F) experiments were used to detect the function of miR-518a-3p for metastasis of HCC. Student t-test were used for statistics. *P<0.05.

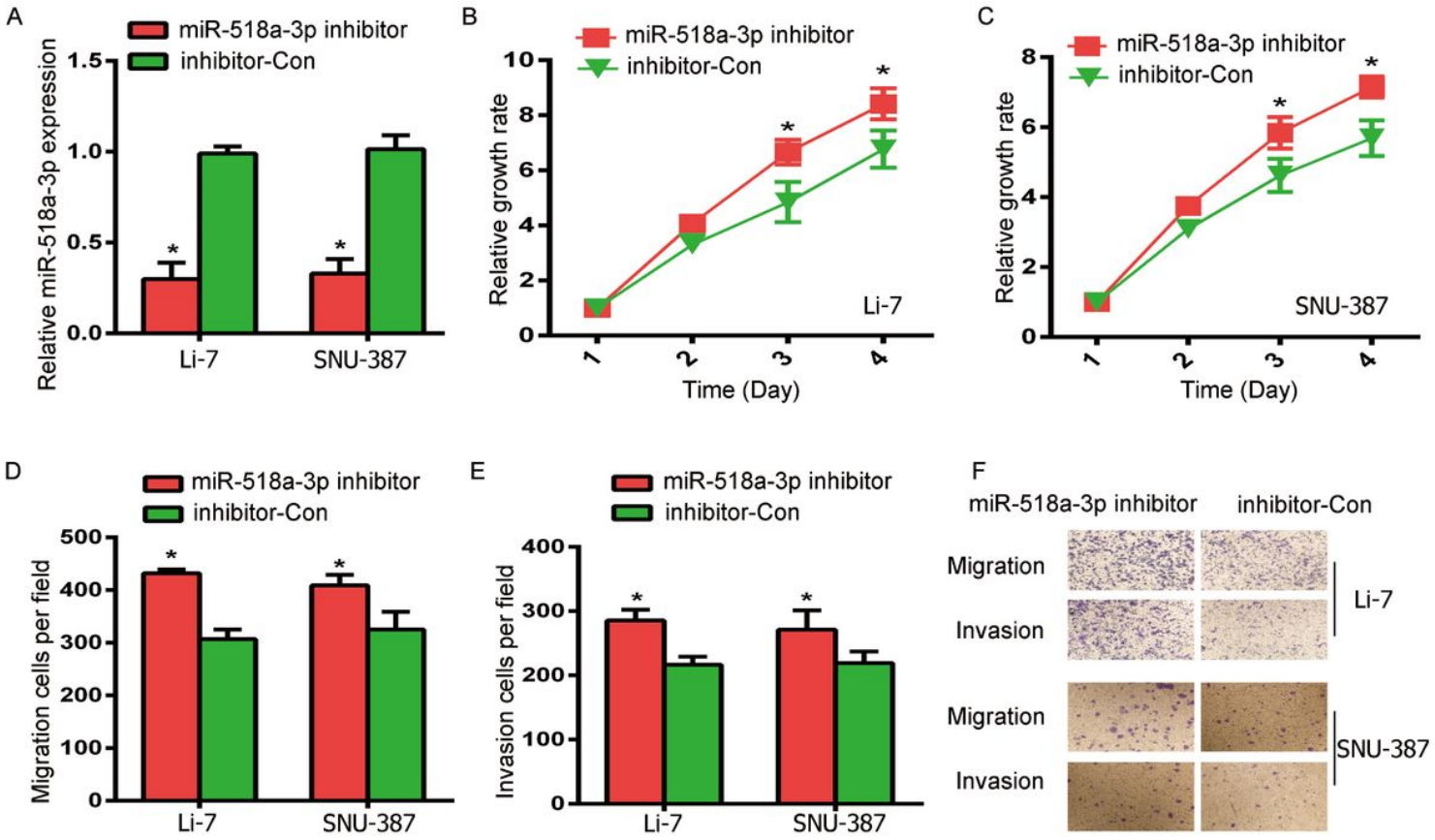

Figure 3

miR-518a-3p served as a potential tumor suppressor gene during the development of HCC. (A) RT-qPCR experiment confirmed the knockdown efficiency of miR-518a-3p in Li-7 and SNU-387 cells. Growth curve of Li-7 (B) and SNU-387 (C) cells with miR-518a-3p inhibitor or NC was detected by CCK-8 experiment. Migration (D and F) and invasion (E and F) assay were employed to detect the role of miR-518a-3p for metastasis of HCC. Student t-test were used for statistics. *P<0.05.

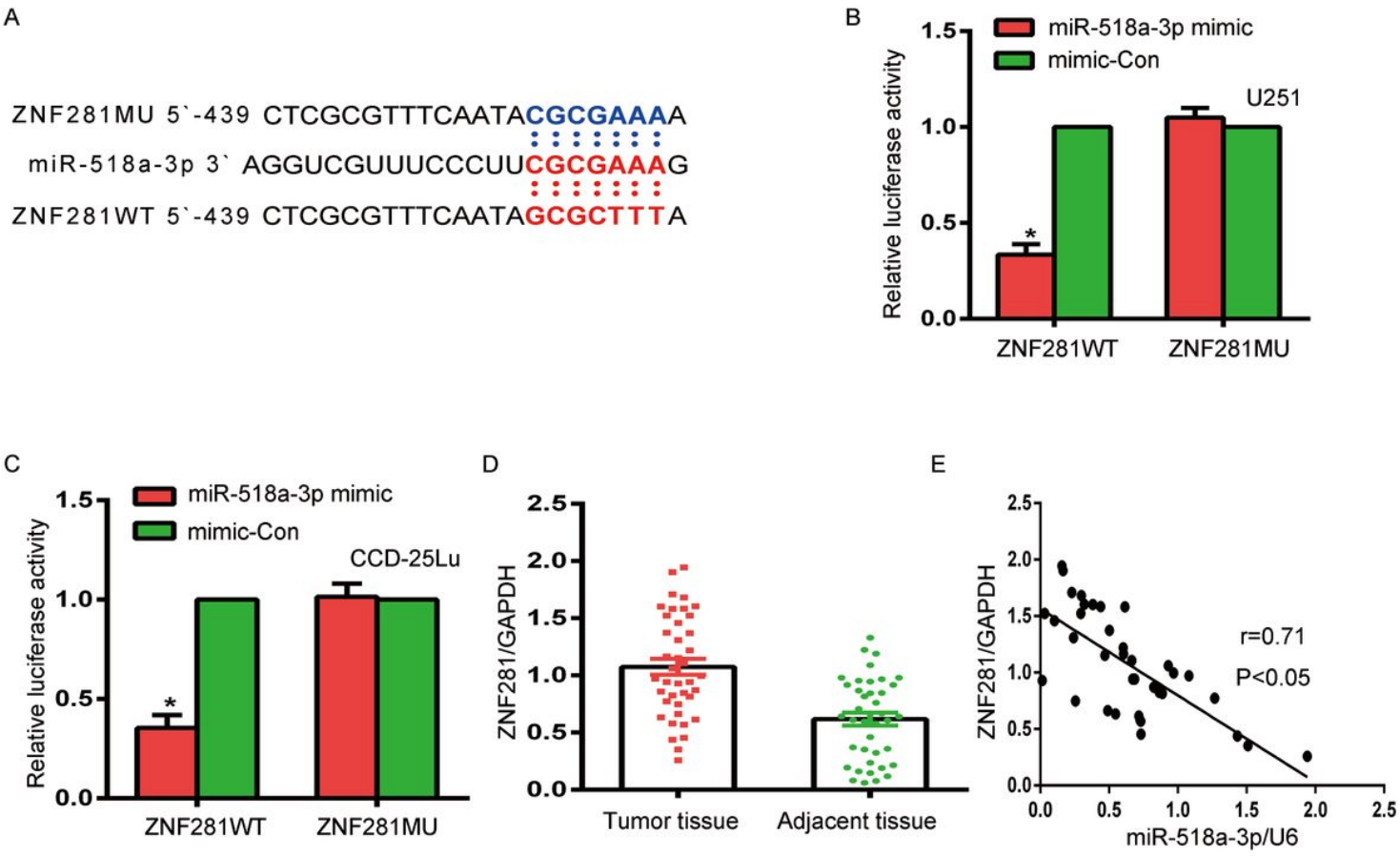

Figure 4

miR-518a-3p regulated HCC progression by targeting ZNF281. (A) The image to depict the luciferase reporter plasmid containing wild or mutant ZNF281. The relative luciferase activity of Li-7 cells (B) and SNU-387 cells (C) with ZNF281 wild or mutant after transfected with miR-518a-3p mimic. A reduced level of luciferase activity in wild group was found after miR-518a-3p overexpression. However, the relative luciferase activity of cells in mutant group displayed no significant change. (D) RT-qPCR experiments have shown that the expression of ZNF281 was increased in HCC tissues compared with adjacent normal tissues. (E) ZNF281 expression was negatively correlated with miR-518a-3p expression in HCC tissues. *P<0.05.

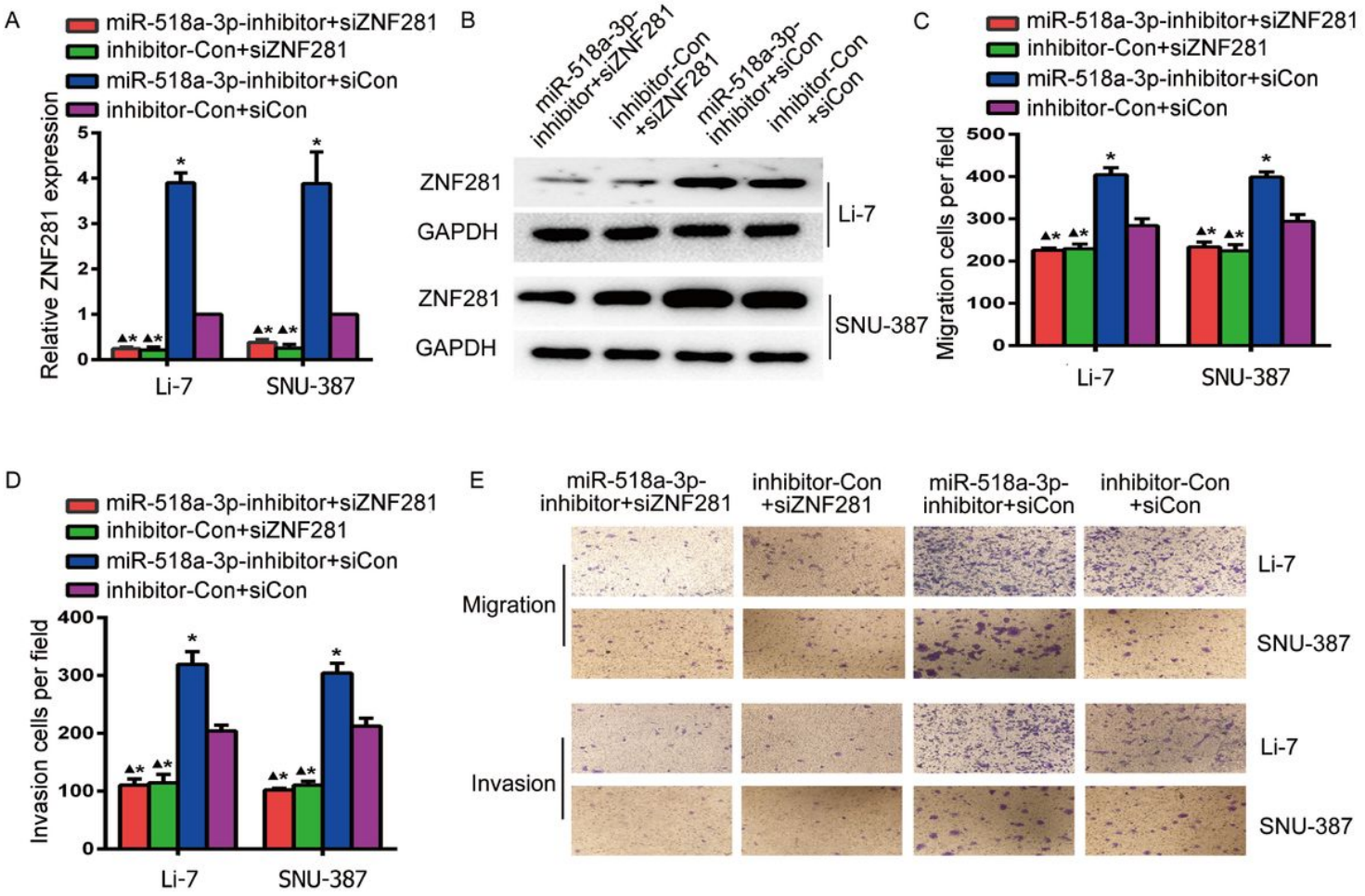

Figure 5

The function of miR-518a-3p in mediating the repression of HCC progression was accomplished by repressing ZNF281 expression. (A) RNA expression level and (B) protein expression level of ZNF281 were measured for the cells which miR-518a-3p or ZNF281 were silenced, separately or together. Migration (C and E) and invasion (D and E) experiment to investigate the effect of ZNF281 silencing via co-depletion of both miR-518a-3p and ZNF281 or separate depletion of miR-518a-3p or ZNF281. The statistics data were represented. *P<0.05.

Supplementary Files

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