Research Paper

MiR-125b realizes anti-tumor action via PI3K/Akt/GSK3β pathway by targeting PIK3CD in hepatocellular carcinoma

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ABSTRACT

The present study was designed to investigate the function and mechanisms of *miR-125b* in hepatocellular carcinoma (HCC). The expressions of *miR-125b* in HCC tissues and cells were detected using quantitative real-time polymerase chain reaction (qRT-PCR). Chi-square test was used to analyze the influences of *miR-125b* expression on clinical features of HCC patients. The effects of *miR-125b* expression on cell proliferation, migration and invasion were investigated using MTT and transwell assays, respectively. Protein analysis was performed by western blot. The potential target of *miR-125b* was identified via bioinformatic analysis and luciferase report assay. *MiR-125b* was significantly downregulated in HCC tissues and cells (*P*<0.01). Moreover, its down-regulation was negatively correlated with TNM stage (*P*=0.024) and lymph node metastasis (*P*=0.013). Enhanced expression of *miR-125b* could inhibit HCC cell proliferation, migration and invasion *in vitro*. PIK3CD was confirmed as a target of *miR-125b* in HCC, and its expression was negatively regulated by *miR-125b*. Moreover, PIK3CD could reverse the function of *miR-125b* in HCC. Additionally, *in vivo* experiments proved that *miR-125b* could suppress HCC cell growth. *MiR-125b* may play anti-tumor action in HCC via suppressing PI3K/Akt/GSK3β signaling pathway by targeting PIK3CD.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most serious primary cancers, and is the fifth common malignant tumor in males and the ninth in females [1, 2]. It is also a leading cause of cancer-related deaths in the world [3, 4]. Even worse, the incidence of HCC is continually increasing and nearly 62 million new HCC cases are diagnosed worldwide annually [5]. Although there are various therapeutic strategies available for HCC, including surgery resection, liver transplantation, chemotherapy and radiotherapy, the 5-year survival rate of HCC patients is still low until now, due to high recurrence and metastasis [6–8]. Therefore, it is necessary to explore molecular mechanism of HCC development and progression to form more efficient therapeutic approaches for this disease.

MicroRNAs (MiRNAs) is a class of small non-coding RNAs containing 20-25 nucleotides, and they can bind to 3' untranslation region (3'UTR) of target mRNAs to regulate gene transcription and translation [9]. They

have been proven to participate in human tumor pathogenesis, possibly being biomarkers for tumor diagnosis and prognosis [10, 11]. Growing evidences have demonstrated that miRNAs could regulate the proliferation, differentiation, apoptosis, invasion and metastasis of cancer cells, including in HCC [12, 13]. *MiR-125b* is an important member in miRNA family, and its abnormal expression is observed in various tumors [14, 15]. In HCC, the expression of *miR-125b* was down-regulated and its enhanced expression was proven to inhibit the growth, proliferation, invasion and metastasis of liver cancer cells [16, 17]. *MiR-125b* might be involved in HCC progression. However, potential mechanism of *miR-125b* influencing HCC progression is still unclear.

PI3K/AKT/GSK3β signaling pathway is reported to be involved in the development and progression of various tumors [18, 19], and its activation can upregulate the expressions of downstream oncogenes to promote the proliferation, invasion and metastasis of tumor cells [20]. It has been reported that *miR-125b* can regulate cancer progression through PI3K/AKT/GSK3β pathway by targeting multiple mRNAs [21]. However, whether *miR-125b* regulates HCC progression via PI3K/AKT/GSK3β pathway remains unknown.

In the present study, we investigated the expression of *miR-125b* in HCC tissues and cells. Cell experiments were carried out to explore the roles of *miR-125b* in HCC progression, and its influence on PI3K/AKT/GSK3β signaling pathway in the disease. In addition, bioinformatic analysis and luciferase report assay were performed to explore potential targets of *miR-125b* in HCC.

RESULTS

MiR-125b significantly down-regulated in HCC tissues and cells

QRT-PCR results showed that compared with adjacent normal tissues and L02 cells, *miR-125b* level was significantly lowered in HCC tissues and HCC cells SK-Hep-1, HepG2 and Huh7 (P<0.05, Figure 1A, 1B). In HCC cells, *miR-125b* level was highest in Huh7, followed by SK-Hep-1 cells, and lowest in HepG2 cells. So in subsequent research, HepG2 cells were selected.

Association of *miR-125b* level with clinical characteristics of HCC patients

According to their mean expression level of *miR-125b* in tumor tissues, 98 HCC patients were divided into two groups: low (n=40) and high (n=58) expression groups. Analysis results showed that *miR-125b* level was negatively associated with lymphatic metastasis (Table 1, P=0.013) and TNM stage (P=0.024). However, the expression level of *miR-125b* was not significantly correlated with age, gender, smoking status or tumor size in HCC patients (P>0.05).

miR-125b significantly inhibited the proliferation of HCC cells

Transfection efficiency was evaluated via qRT-PCR and the result showed that relative expression level of *miR-125b* was obviously higher in HepG2 cells transfected by *miR-125b* mimics than in mimic-NC group (P<0.05, Figure 2A). MTT analysis showed that proliferation was significantly decreased among HCC cells transfected by

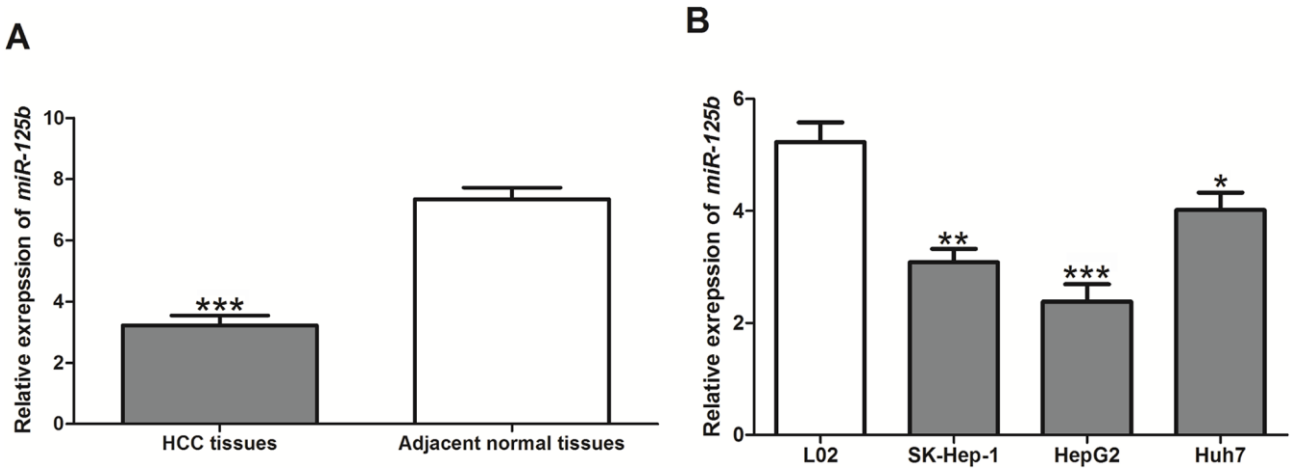

Figure 1. Relative expression level of *miR-125b* in HCC tissues and cells. (**A**) The expression level of *miR-125b* in HCC tissues and adjacent normal ones; (**B**) The expression level of *miR-125b* among HCC cells SK-Hep-1, HepG2 and Huh7 and human normal hepatocytes L02. *P<0.05, **P<0.01 and ***P<0.001 indicated significant difference between compared two groups.

Table 1. The association of *miR-125b* expression level with the clinical characteristics of HCC patients.

Characteristics	N=98	Low expression (n=40)	High expression (n=58)	*P*
Age (years)				0.360
≥60	46	21	25	
<60	52	19	33	
Gender				0.681
Men	66	26	40	
Women	32	14	18	
Smoking				0.109
Yes	56	19	37	
No	42	21	21	
Tumor size (cm)				0.987
≤3	54	22	32	
>3	44	18	26	
Lymphatic metastasis				0.013
Yes	44	24	20	
No	54	16	38	
TNM stage				0.024
I-II	62	20	42	
III-IV	36	20	16	

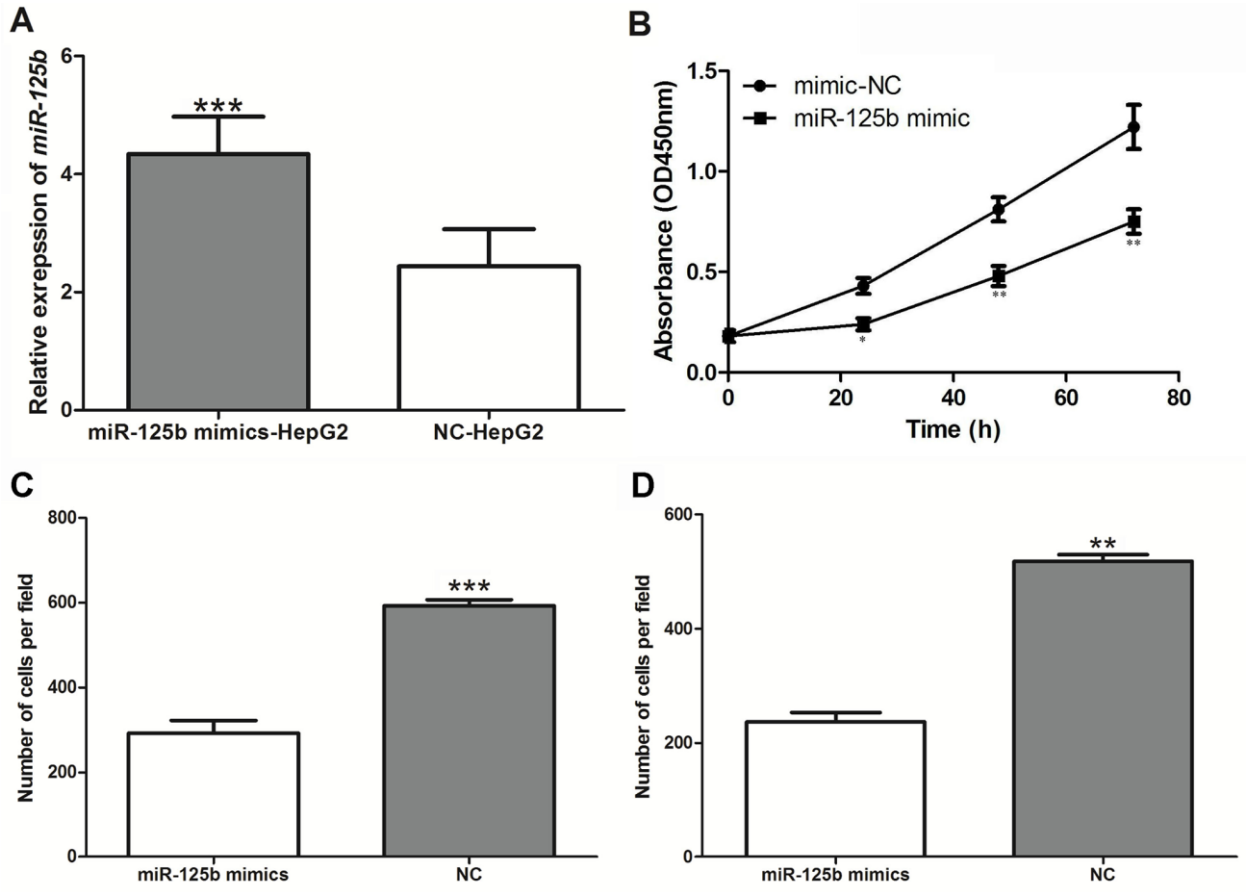

Figure 2. The effects of *miR-125b* expression on biological behaviour of HepG2 cells. (**A**) Relative expression level of *miR-125b* in HepG2 cells transfected by *miR-125b* mimics and NC. (**B**) The effects of *miR-125b* expression level on the proliferation of HepG2 cells. (**C**) The influences of *miR-125b* mimics on the migration of HCC cells. (**D**) The influences of *miR-125b* mimics on the invasion of HCC cells. **P*<0.05, ***P*<0.01 and ****P*<0.001 indicated significant difference between compared two groups.

miR-125b mimics, compared with NC group (P<0.05, Figure 2B).

MiR-125b influenced the migration and invasion of HCC cells

Compared with NC group, the number of migrating cells was significantly lowered in *miR-125b* mimics transfection group (P<0.001, Figure 2C). Likewise, the number of invasive cells in *miR-125b* mimics transfection group was also significantly lower than that in NC group (P<0.01, Figure 2D).

MiR-12b targeted PIK3CD in HCC cells

Bioinformatic analysis demonstrated that *miR-125b* might bind to the 3'UTR of PIK3CD (Figure 3A). PIK3CD might be a potential target of *miR-125b* in HCC

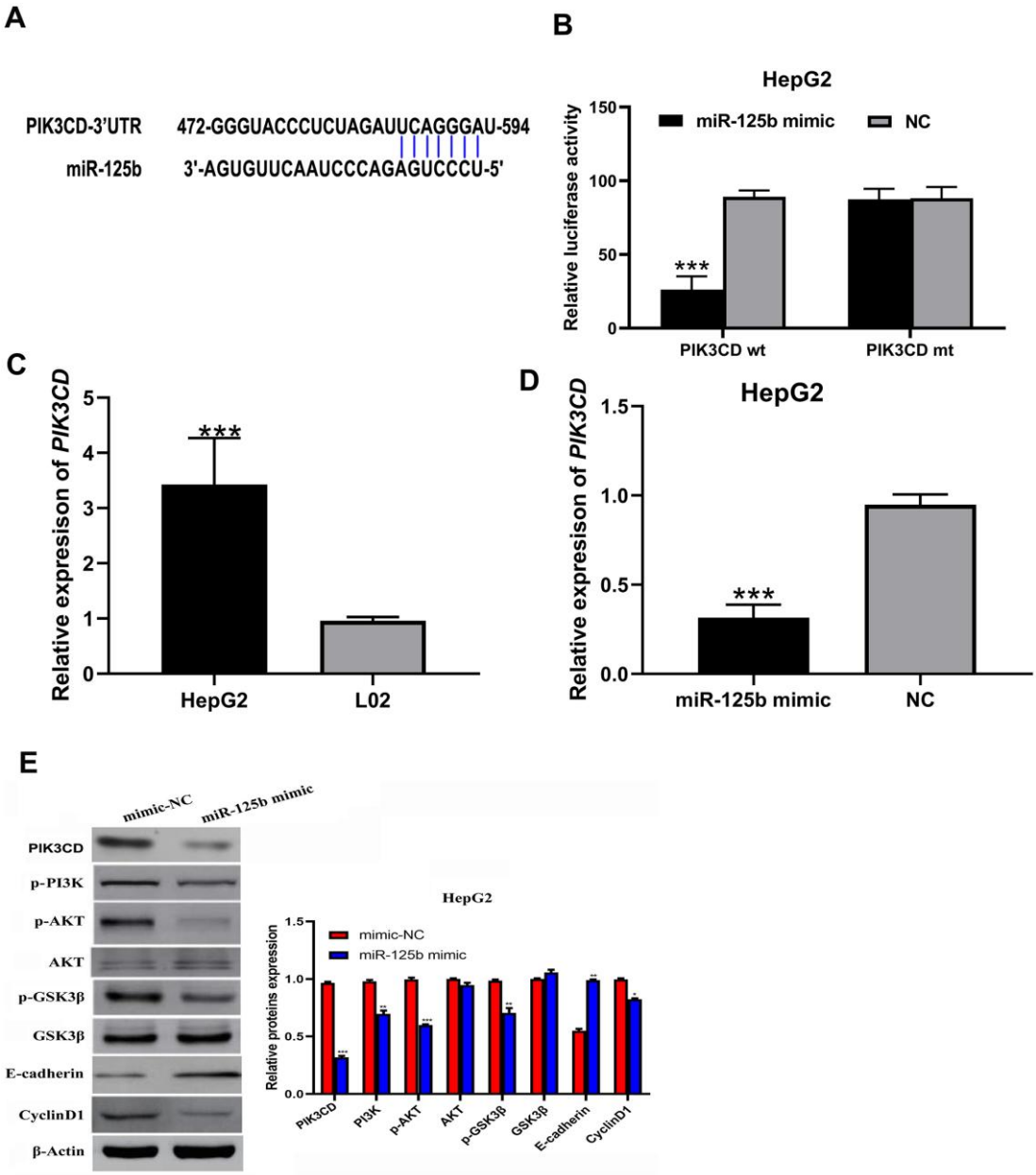

Figure 3. *MiR-125b* regulated PI3K/AKT/GSK3β pathway through targeting PIK3CD. (**A**) Bioinformatic analysis showed that *miR-125b* could bind to the 3'UTR of PIK3CD. (**B**) Dual luciferase assay revealed a targeted relationship between *miR-125b* and PIK3CD. (**C**) The expression of PIK3CD mRNA was significantly up-regulated in HepG2 cells compared to normal liver cell line L02. (**D**) Moreover, enhanced expression of *miR-125b* led to the downregulation of PIK3CD. (**E**) The expressions of PI3K/AKT/GSK3β signaling pathway-related proteins PI3K, AKT, p-AKT, p-GSK3β, GSK3β and CyclinD1 in HepG2 cells.* P<0.01 indicated significant difference between compared two groups.

cells. Dual luciferase assay was performed to investigate the targeted relationship. As shown in Figure 3B, co-transfection by *miR-125b* mimic and PIK3CD mt could significantly suppress luciferase activity (P<0.001), revealing the targeted association of *miR-125b* with PIK3CD (Figure 3B). QRT-PCR analysis suggested that the expression of PIK3CD mRNA was significantly increased in HepG2 cells, compared to normal liver cell line L02 (Figure 3C). Moreover, enhanced expression of *miR-125b* in HepG2 cells resulted in the down-regulation of PIK3CD (Figure 3D). All the results revealed that PIK3CD might be a potential target of *miR-125b* in HCC.

MiR-125b regulated PI3K/AKT/GSK3β signaling pathway in HCC cells

Western bolt showed that PIK3CD, p-PI3K, p-AKT, p-GSK3β and CyclinD1 expression levels were significantly down-regulated in HCC cells transfected by *miR-125b* mimics, compared with NC group. On the contrary, E-cadherin protein level was significantly increased (P<0.01, Figure 3E).

MiR-125b regulated the proliferation, migration and invasion of HCC cells through PI3K/AKT/GSK3β pathway mediated by PIK3CD

To explore the mechanism of *miR-125b* functioning in HCC progression, HepG2 cells were co-transfected by *miR-125b* mimic and PIK3CD over-expression vector. According to Western blot analysis, the over-expression of PIK3CD could enhance the expressions of p-AKT and p-GSK3β and reduce E-cadherin expression, revealing the activation of PI3K/AKT/GSK3β pathway in HepG2 cells (Figure 4A). Moreover, the proliferation, migration and invasion of HepG2 cells transfected by *miR-125b* mimic were distinctly promoted by

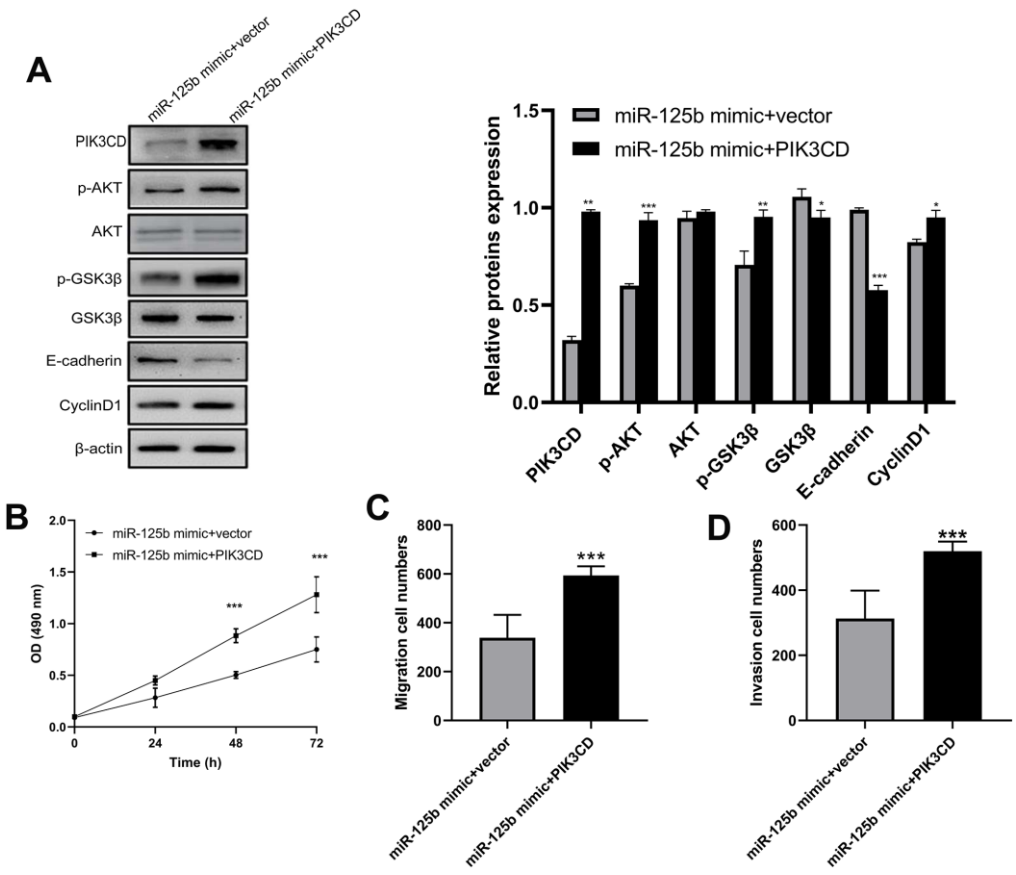

Figure 4. *MiR-125b* regulated the proliferation, migration and invasion of HCC cells through PI3K/AKT/GSK3β pathway mediated by PIK3CD. (**A**) Expression levels of PI3K/AKT/GSK3β pathway-related proteins AKT, p-AKT, p-GSK3β, GSK3β and CyclinD1 in HepG2 cells transfected by *miR-125b* mimic+vector and *miR-125b* mimic+PIK3CD. (**B**) The proliferation of HepG2 cells, inhibitory effect of *miR-125b* mimics on cell proliferation was significantly neutralized by overexpressed PIK3CD. (**C**) The migration of HepG2 cells, inhibitory effect of *miR-125b* mimics on cell proliferation was significantly neutralized by overexpressed PIK3CD. (**D**) The invasion of HepG2 cells, inhibitory effect of *miR-125b* mimics on cell proliferation was significantly neutralized by overexpressed PIK3CD. *P<0.05, **P<0.01 and ***P<0.001 indicated significant difference between compared two groups.

overexpressed PIK3CD (Figure 4B–4D). All the data suggested that PIK3CD could neutralize anti-tumor action of *miR-125b* in HCC.

miR-125b mimics inhibited the progression of HCC cells *in vivo*

In *In vivo* experiment, *miR-125b* expression level was significantly upregulated in nude mice after *miR-125b* mimic injection ($P<0.01$, Figure 5A). Moreover, enhanced expression of *miR-125b* could significantly reduce tumor volume and weight ($P<0.05$), compared with mimic-NC group (Figure 5B, 5C). Similar tumor volume curves between these two groups might be caused by small sample size of the mice.

DISCUSSION

HCC is a malignant tumor with high morbidity and mortality, and seriously threatens human healthy worldwide [4]. Despite great progressions in treatments, clinical outcomes of HCC patients are still unsatisfactory, due to metastasis and relapse [22, 23]. To improve the prognosis of HCC patients, various researches have been devoted to explore molecular etiology of the disease, which may contribute to target therapy. Growing evidence has demonstrated that miRNAs play important roles in both physiological and pathological conditions [24]. Given their regulatory effects on gene expression, miRNAs are involved in various human cancers, such as HCC [25]. In the current study, we investigated functional roles of *miR-125b* in HCC progression, as well as relevant molecular mechanisms. We found that the down-regulation of *miR-125b* could promote the progression of HCC through PI3K/AKT/GSK3β signaling pathway.

MiR-125b belongs to miRNA-125 family, a highly conserved group throughout evolution [26]. Alterations in *miR-125b* expression are significantly correlated with tumorigenesis and tumor progression. For example, Jin et al. reported that the up-regulation of *miR-125b* showed

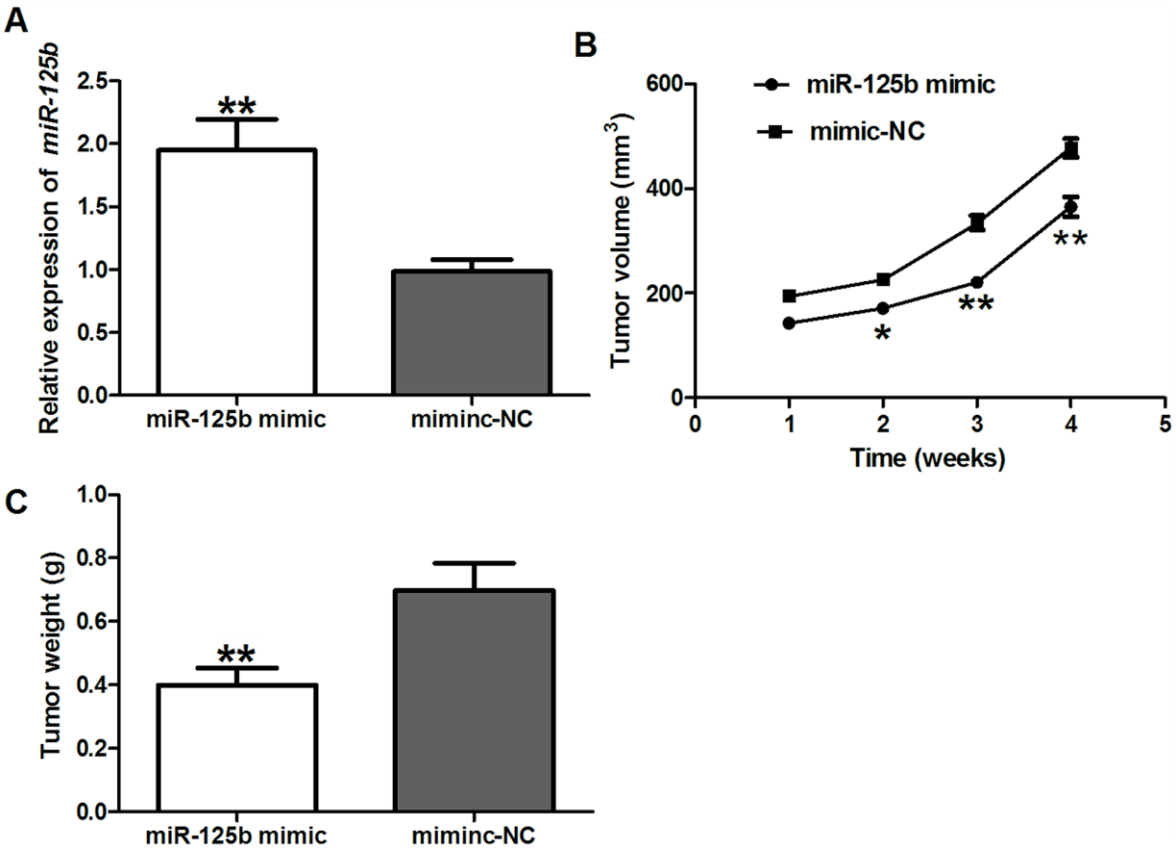

Figure 5. *miR-125b* mimics inhibited the progression of HCC cells *in vivo*. (**A**) In nude mice, the expression level of *miR-125b* was significantly upregulated in *miR-125b* mimic group, compared with mimic-NC group; (**B**) Tumor volume was significantly decreased in nude mice with *miR-125b* mimics. Initially, tumor volumes in *miR-125b* mimic and mimic-NC groups were 187.5±7.6 mm^3 and 194.6±8.4 mm^3, respectively.; (**C**) Tumor weight was also obviously lower in *miR-125b* mimic group than in mimic-NC group. **P*<0.05, ***P*<0.01 and ****P*<0.001 indicated significant difference between compared two groups.

positive correlation with malignant behaviors of renal cell carcinoma cells *in vitro* [27]. In chondrosarcoma, *miR-125b* might serve as a tumor suppressor to enhance chemotherapy sensitivity [28]. Such evidence reveals distinct roles of *miR-125b* in different cancers. In the current study, we found that the expression of *miR-125b* exhibited a decreasing trend in HCC tissues and cell lines. Moreover, the down-regulation of *miR-125b* was obviously correlated with positive lymph node metastasis and advanced TNM stage. *miR-125b* mimics transfection could significantly inhibit HCC cell proliferation, migration and invasion. All these data suggested that *miR-125b* might be a tumor suppressor against HCC, repressing malignant development and progression. Moreover, *in vivo* experiments demonstrated that enhanced expression of *miR-125b* could obviously suppress tumor growth in HCC. The conclusions were in consistent with those from published articles. Xu et al. reported that the expression of *miR-125b* was obviously decreased in HCC mouse model [29]. Furthermore, the down-regulation of *miR-125b* might contribute to tumor progression and poor survival [30, 31]. In a word, *miR-125b* exerts an anti-tumor effect against HCC progression.

In addition, we also investigated molecular mechanisms of *miR-125b* influencing the progression of HCC. Bioinformatic analysis and luciferase report assay confirmed that PIK3CD might be a potential target of *miR-125b* in HCC. Moreover, the expression of PIK3CD was negatively regulated by *miR-125b*. Anti-tumor action induced by *miR-125b* in HCC could be neutralized by PIK3CD. All the results suggested a targeted relationship between *miR-125b* and PIK3CD in HCC. In addition to HCC, targeted relationship between *miR-125b* and PIK3CD was also reported in anaplastic thyroid cancer [32], cervical cancer [33] and Ewing's sarcoma [34]. We found that expression levels of p-PI3K, p-AKT, p-GSK3β and CyclinD1 proteins in PI3K/AKT/GSK3β pathway were distinctly reduced in *miR-125b* mimics group compared to NC group, while E-cadherin protein was up-regulated. All the results suggested that *miR-125b* could regulate PI3K/AKT/GSK3β pathway through targeting PIK3CD. Other studies also showed similar results. Xiao et al. found that PI3K/AKT/GSK3β pathway was significantly associated with the development of HCC, and that CyclinD1, p-AKT and p-GSK3β were significantly reduced by miR-218 over-expression in HCC [35]. Besides, Li and colleagues indicated that overexpressed *miR-125b* could significantly restrain p-AKT level [36]. However, Wang and coworkers suggested that down-regulated *miR-125b* inhibited the expression of p-Akt in non-small cell lung cancer (NSCLC) [21]. Such difference might be caused by different mechanisms across tumors.

Several limitations in the current study should be stated. Firstly, the sample size was relatively small that might reduce statistical power of our findings. Secondly, only one HCC cell line was used in *in vitro* experiments. Due to heterogeneity between HCC cell lines, our findings required further verification in other HCC cell lines. Thirdly, in addition to PIK3CD, several specific targets have been proposed for *miR-125b*, such as SIRT6 [37], and SIRT6 could also regulate PI3K signaling pathway [38]. Whether *miR-125b* influences the progression of HCC through PI3K/AKT mediated by SIRT6 should be explored in further studies. Moreover, in *in vivo* experiment, we only explored the influence of *miR-125b* expression level on growing ability of HCC, but detailed molecular mechanism was not discussed. Additionally, HCC is a complex disease regulated by multiple factors, but the interaction of *miR-125b* with other genes or signaling pathways was not investigated in the current study. Further well-designed studies should be carried out to verify and improve our conclusions.

In conclusion, *miR-125b* is significantly decreased in HCC tissues and cells, and may inhibit the proliferation, migration and invasion of HCC cells via suppressing PI3K/AKT/GSK3β signaling pathway by targeting PIK3CD.

MATERIALS AND METHODS

Tissue samples

A total of 98 HCC patients were recruited form The Fifth Medical Center of PLA General Hospital. Tumor tissues and corresponding adjacent normal ones (4cm away from tumor tissue) were collected from the patients in surgery. Tissue samples were immediately put into liquid nitrogen, and then stored in a -80° C refrigerator. Patients did not receive radiotherapy or chemotherapy before surgery. This study was supported by the Ethics Committee of The Fifth Medical Center of PLA General Hospital. All subjects signed written informed consents before tissue collection. Meanwhile, clinical information of HCC patients was also collected from their medical records.

Cell culture

HCC cell lines SK-Hep-1, HepG2 and Huh7, and human fetal liver cell line L02 were purchased from the Institute of Biochemistry and Cell Biology; Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (GE Healthcare Life Sciences, Little Chalfont, UK) or DMEM (Invitrogen, Carlsbad, CA) media supplemented with 10% fetal bovine serum (FBS). The culture was conducted in a humid atmosphere

containing 5% CO_2 at 37° C for passage. Nutrient solution was replaced every three days.

Cell transfection

miR-125b mimics, negative control (NC), overexpressed vector (specific to PIK3CD) and empty vector were designed for cell experiments. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Culture was proceeded at 37° C with 5% CO_2 for 48h. Then *miR-125b* expression in transfected cells was detected through quantitative real-time polymerase chain reaction (qRT-PCR).

RNA extraction and qRT-PCR

Total RNA in tissues and cells was extracted via Trizol method (TaKaRa, Japan) and reversely transcribed into the first chain of cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's instruction. Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) was used for PCR amplification and the reaction was performed in Applied Biosystems AB7500 Real Time PCR system (Applied, Biosystems). Relative expression of *miR-125b* was analyzed using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$), and *U6* was used as an internal normalized reference. Tests were repeated three times.

MTT proliferation assay

Cell proliferation was measured through MTT method following standardized protocol (Roche). After transfection, HCC cells were inoculated in 96-well plates and their density was adjusted to 2×10^4/well. All groups were incubated for 24, 48 and 72h. 50μl MTT reagent (5mg/mL) was added into every well, and then underwent 37° C incubation for 4h. Proliferation ability of cells was determined using MTT cell proliferation kit (Cayman Chemical) following relevant instruction. MTT enzyme-linked immunometric meter was used to measure OD value (450nm).

Migration and invasion detection

The migration and invasion of HCC cells were detected employing Transwell trial. Transwell chambers were pre-coated by matrigel (BD Biosciences), and 50μl serum-free medium with BSA (bull serum albumin) was added to the upper compartment for 2h of dehydration at 37° C. Then 200μl cell suspension was added to the upper chamber while 500μl DMEM media containing 10% FBS was added to the lower chamber. Cells were incubated for 24h at 37° C with 5% CO_2. Invasive cells were stained with 0.1% crystal violet for 30 min. Under a microscope the cells in ten random sights were counted.

Uncoated matrigel Transwell trial was used to test the migration of HCC cells.

Western blot analysis

Transfected cells were lysed using RIPA buffer (Thermo Scientific, Belmont, MA, USA) for 30 min at 4° C. Protein in cells was extracted and separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a PVDF membrane (Roche) through electroblotting. PVDF membrane was blocked by nonfat milk at room temperature for 24h or at 4° C overnight. It was incubated at 4° C overnight by primary antibodies (1:1000), including anti-PIK3CD, anti-PI3K, anti-p-PI3K, anti-Akt, anti-p-Akt, anti-GSK3β, anti-E-cadherin, anti-Cyclin D1 and anti-β-actin. Then the membrane was incubated for 1.5h at room temperature with secondary antibody (1:2000, Abcam, China). The target band of protein was shown using ECL Western blotting kit (Millipore, Boston, MA, USA).

Dual Luciferase assay

For luciferase report assay, mutant PIK3CD 3'UTR (mt) and wide PIK3CD 3'UTR (wt) were amplified and cloned to luciferase expression vector pGL-3-ector (Promega, San Luis, CA, USA). Cells were seeded on 6-well plates (1×10^5 cells per well), and then co-transfected by either wt or mt, in combination with *miR-125b* mimic or NC using Lipofectamine 2000. The transfected cells were incubated at 37° C with 5% CO_2 for 48 hours. Then, the cells were harvested, and their firefly and renilla luciferase activities were estimated adopting Dual-Luciferase Reporter Assay (Promega) kit according to the guidance of the manufacturer. Relative luciferase activity was expressed by the ratio of Firely/Renilla. Each test was repeated three times.

***In vivo* experiments**

In this study, Balb/c nude mice were used for *in vivo* experiment. 20 Bal b/c nude mice (5-8 weeks, 18-20g) were purchased from SLRC Laboratory Animal Center (Shanghai, China), and HepG2 cells (3×10^6/200μl) were inoculated at the upper part of the groin of the mice. The mice were randomly divided into experimental (*miR-125b* mimic) and control (mimic-NC) groups. The major axis (a) and minor axis (b) of subcutaneous tumors were measured once a week, and formula calculating tumor volume was as follows: $V=1/2\times a\times b^2$. After 4 weeks, nude mice were sacrificed for the measuring of their tumor weights.

Statistics analysis

In this study, SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad, San Diego, CA, USA) were used for statistics analysis and graph construction, respectively. All experiments were repeated in triplicate and continuous data were shown in mean±SD (standard deviation). Difference between groups was compared through two-tailed Student's t-test (for continuous variables) or χ^2 test (for categorical variables). $P<0.05$ was considered to be significant threshold.

AUTHOR CONTRIBUTIONS

H.C., X.J., L.M., H.L., X.Z. conceived and designed the experiments, analyzed the data, and wrote the paper. YY.Z., X.N., Y.Z., Y.G., Z.Z. performed the experiments. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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