miR-145-5p reduces proliferation and migration of hepatocellular carcinoma by targeting KLF5

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Abstract. MicroRNAs (miRs) are important in hepatocellular carcinoma (HCC) progression. miR-145-5p acts as a tumor suppressor in certain malignancies, however, its role in HCC remains unclear. The present study aimed to perform a functional analysis of miR-145-5p in HCC in order to elucidate its role in the pathogenesis of HCC. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to analyze tissue and cellular expression of miR-145-5p in HCC. Following miRNA mimics transfection, cell viability, apoptosis and cells migration were determined by Cell Counting kit-8, Annexin V-FITC/propidium iodide staining and Transwell analyses. The target of miR-145-5p was analyzed by luciferase reporter assay and western blot analysis. It was observed that miR-145-5p was significantly decreased in HCC tissues and cell lines. Overexpression of miR-145-5p significantly increased apoptosis, reduced cell proliferation and suppressed HCC cell migration. Kruppel-like factor 5 (KLF5) is regarded as a target of miR-145-5p in HCC cells. In addition, KLF5 overexpression partially attenuated the tumor suppressive effects of miR-145-5p. KLF5 expression was negatively associated with levels of miR-145-5p in HCC tissues. The present study demonstrated that miRNA-145-5p may, by targeting KLF5, partially suppress HCC cell growth and motility. The results of the present study suggested that miRNA-145-5p alteration in HCC may serve a role in the progression of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignant tumors globally (1). In the past decade, patients with HCC have had to endure a high incidence of recurrence and metastasis; a 5-year recurrence rate of 75-100% is reported (2). Surgical resection, liver transplantation and radiofrequency ablation may provide a cure for certain early stage patients however, due to the asymptomatic nature of HCC, the majority of patients are diagnosed at an advanced stage (3). The molecular mechanisms involved in HCC remain poorly understood. The development of novel strategies to further the understanding of HCC is required. Alterations in molecular expression in HCC have been extensively investigated. Dysregulated gene expression has been observed during the development of HCC (4). Kruppel-like factor 5 (KLF5), a zinc finger protein that belongs to the KLF family, is a transcription factor that binds the epidermal growth factor response element (5). KLF5 was recently demonstrated to be upregulated in metastatic HCC (6). Additionally, increased expression of KLF5 promotes cell proliferation and inhibits apoptosis (7). However, the mechanism of aberrant KLF5 expression remains unclear.

As a family of endogenous, small non-coding RNAs (20-25 nucleotides in length), microRNAs (miRNAs) post-transcriptionally regulate the expression of complementary target mRNA in eukaryotes, which influences several biological processes, including cell infection, development, immunity and carcinogenesis (8,9). miRNA expression profiles reveal that numerous miRNA signatures exist in various different cancer types (10-13). It is indicated that miRNA signatures may be predictive for cancer prognosis, classification and response to therapy (14,15). Emerging evidence indicates that miRNAs have significant roles in the development of HCC, and certain tumor suppressive miRNAs in HCC have already been identified (16). Decreased expression of miRNA (miR)-145-5p in several cancer types, including prostate (17) and liver cancer (18), has been previously reported. The present study determined the expression of miR-145-5p in HCC and miR-145-5p overexpression, through use of miRNA mimics, was observed to inhibit the proliferation and metastasis of HCC cells. Furthermore, the present study identified KLF5 as

a target of miR-145-5p in HCC cells. The current study also demonstrated the association between miR-145-5p and KLF5.

Materials and methods

HCC tissues and cell lines. HCC tissues and paired normal liver tissues were obtained from 25 patients with primary HCC at The Second Xiangya Hospital of Central South University (Changsha, China). The Ethics Committee of The Second Xiangya Hospital of Central South University approved this study after written informed consent was obtained from each patient. Details of the patients are presented in Table I. L02 normal human liver cell line and HuH-7, HepG2 and SK-Hep-1 HCC cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) and at 37˚C in a 5% CO_2 incubator.

RNA isolation & reverse transcription-quantitative polymerase chain reaction (RT-qPCR). By using TRIzol or miRNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), RNA was extracted, followed by reverse transcription using cDNA Reverse Transcription kit (Qiagen, Inc.). RT-qPCR was performed using SYBR-Green Master Mix (cat. no. RR420A; Takara Biotechnology Co., Ltd., Dalian, China) on LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany). miR-145-5p primers were purchased from Sigma-Aldrich (Merck KGgA, Darmstadt, Germany; cat. no. MIRAP00180). The reaction system included 2.5 μl cDNA (1:20 dilution by double-distilled water), 10 μl reaction mixture (Qiagen, Inc.), 2.0 mM forward primer, and 2.0 mM reverse primer, respectively. The experiment was repeated three times. The primers of KLF5 were as follows: 5'-CTTCCACAACAG GCCACTTACTT-3' (forward) and 5'-AGAAGCAATTGT AGCAGCATAGGA-3' (reverse). GAPDH primers were as follows: 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). The cycling conditions were as follows: Initial denaturation step at 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 sec, and 60˚C for 30 sec. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method and levels were normalized to the reference gene, GAPDH.

miRNA mimics and construction of expression vectors. miR-145-5p mimics (cat. no. miR30000157-1-2), which mimic endogenous miRNA, and inhibitors (cat. no. miR20000437-1-2) were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The control group was transfected with an empty pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The entire coding region of KLF5 was amplified and restriction enzymes, *Not*I and *Eco*RI (New England BioLabs, Inc., Ipswich, MA, USA), were employed to insert the KLF5 fragment into the mammalian expression vector, pcDNA3.1. The primers were as follows: 5'-GGGCGGCCATGGCTACAAGGGTGCTGAG CATGAG-3' (forward); 5'-GGGAATTCTCAGTTCTGG TGCCTCTTCATATGCAGGGCC-3' (reverse). The control involved empty vectors without inserted fragments. For the luciferase reporter, Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was used and the 3'-untranslated region (3'-UTR) target sequence from KLF5 was amplified and subsequently inserted into psiCHECK-2 vector (Promega Corporation).

Cell viability and apoptosis analysis. Using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA), cell proliferation rates were measured. Cells ($1x10^5$ cells/well) were seeded before incubation with mimics/inhibitors (50 nM mimics and 200 nM inhibitors). The transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mimics and inhibitors were combined with 10 μl Lipofectamine® 2000 diluted in 250 μl Opti-MEM I (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 30 min. The mixture was then added to cells which attained 90% confluence in the 96-well plates. After 24, 48, and 72 h transfection, 10 μl CCK-8 reagent was added to each well. The optical density at 450 nm was determined using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.). In order to analyze apoptosis, cells were harvested and washed in ice-cold PBS and were then fixed in 70% ice-cold ethanol at 4˚C for 1 h. Cells were incubated with 20 μg/ml propidium iodide and Annexin V-APC (Sigma-Aldrich; Merck KGgA) for a period of 20 min at room temperature, and were then analyzed using Fluorescence Activated Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA). The cells were analyzed by CellQuest software (version 3; BD Biosciences).

Cells migration analysis. A total of 48 h following transfection, cell migration was evaluated using a Transwell chamber (EMD Millipore, Billerica, MA, USA). Transwell inserts were pre-coated with Matrigel (BD Biosciences) and were placed in the upper compartment prior to cell seeding. Cells ($5x10^4$) were seeded in the upper chamber without FBS. The lower chamber was then filled with medium that was supplemented with 10% FBS (FBS; Gibco; Thermo Fisher Scientific, Inc.). Migratory cells on the bottom surface were then fixed by 4% paraformaldehyde solution for 20 min at room temperature, and stained with 0.1% crystal violet for 15 min at room temperature. From each membrane, the number of migrated cells in four random fields were counted by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Experiments were conducted in triplicate.

Western blot analysis. HCC and control tissues were lysed using radio immunoprecipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China) 48 h following transfection. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Then, for each sample, 20 μg extracted protein was separated on a 10% SDS-PAGE gel (cat. no. P0012A, Beyotime Institute of Biotechnology) at 90 V for 25 min followed by 120 V for 1.5 h and transferred to polyvinylidene difluoride membranes (EMD Millipore). The transfer was performed using a Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories, Inc.) at 4˚C for 1.5 h at a constant

Table I. Characteristics patients with hepatocellular carcinoma (n=25).

Characteristic	Number of patients
Age (years)	
<50	10
≥50	15
Sex	
Male	17
Female	8
Hepatitis history	
Yes	11
No	14
Liver cirrhosis	
Yes	13
No	12
Tumor diameter (cm)	
<5	17
≥5	8
Tumor differentiation grade	
I+II	18
III+IV	7

voltage setting of 100 V. The membranes were blocked using 4% non-fat dry milk in tris-buffered saline (TBS) with Tween-20 (Sigma-Aldrich; Merck KGgA) at room temperature for 30 min. Membranes were then incubated with rabbit anti-human KLF5 polyclonal antibody (1:500; cat. no. ab24331) and rabbit anti-human GAPDH polyclonal antibody (1:1,000; cat. no. ab9485; both Abcam, Cambridge, MA, USA) overnight at 4˚C. Subsequently, horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab7090; Abcam) was incubated at 37˚C for 1 h with the membranes and protein bands were visualized with the enhanced chemiluminescence system (EMD Millipore). Experiments were conducted in triplicate. The relative expression of protein was calculated by densitometric analysis using ImageJ software version 2.0.0 (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter assay. HepG2 cells ($1x10^5$ cells/well) were cultured in a 24-well plate and incubated for 24 h. The cells were divided into four groups: i) miRNA negative control (cat. no. B04001; Shanghai GenePharma Co., Ltd., Shanghai, China) and wild-type 3'UTR of KLF5 group; ii) miR-145-5p mimic and wild-type 3'UTR of KLF5 group; iii) miRNA negative control and mutant 3'UTR of KLF5 group; and iv) miR-145-5p mimic and mutant 3'UTR of KLF5 group. HepG2 cells were harvested 48 h after transfection. Using the Dual-Luciferase Reporter System (Promega Corporation), relative luciferase activity was evaluated and normalized to the activity of Renilla luciferase.

Statistical analysis. Data are presented as the mean ± standard deviation, and were analyzed with SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). Using Student's t-test or one-way analysis of variance followed Bonferroni's post-hoc test, quantitative variables were analyzed. Pearson's correlation coefficient was used to determine the correlation between KLF5 mRNA expression and miR-145-5p levels in HCC tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-145-5p expression is decreased in HCC. RT-qPCR was used to determine the expression of miR-145-5p in 25 HCC and matched non-tumor tissues. Compared with non-tumor tissues, the present study observed significantly decreased miR-145-5p level in HCC tissues (P<0.05; Fig. 1A). The expression of miR-145-5p was significantly reduced in all three HCC cell lines compared with L02 normal human liver cells (P<0.05; Fig. 1B).

miR-145-5p inhibits HCC cell proliferation and migration. Additionally, the present study observed that miR-145-5p suppressed the proliferation of HCC cells. RT-qPCR confirmed that transfection with the miR-145-5p mimic increased the expression of miR-145-5p (P<0.05; Fig. 2A). Following transfection with miR-145-5p or control mimics in HepG2 cells, the biological effect of miR-145-5p on the progression of HCC was determined by CCK-8 assay. The CCK-8 assay indicated that overexpression of miR-145-5p, induced by miR-145-5p mimics, significantly suppressed the cell proliferation rate after 48 h (P<0.05; Fig. 2B). A significant increase in the percentage of apoptotic cells in miR-145-5p-overexpressing HepG2 cells was observed when compared with control HepG2 cells (P<0.05; Fig. 2C). In addition, miR-145-5p or control mimics were transfected into HepG2 cells to determine the effect of miR-145-5p on motility of HCC cells, and migration assays were conducted. Overexpression of miR-145-5p significantly inhibited the migratory capabilities of HepG2 cells (P<0.05; Fig. 3A and B).

KLF5 is a direct target of miR-145-5p. In order to determine the target of miR-145-5p in HCC, TargetScan 7.0 (www.targetscan.org) was used. It was demonstrated that KLF5 is a potential target of miR-145-5p (Fig. 4A). Results of the luciferase activity assay suggested that miR-145-5p overexpression significantly inhibited the luciferase activity of the WT 3'-UTR of KLF5 in HepG2 cells compared with the control (P<0.05; Fig. 4B). In addition, miR-145-5p overexpression significantly suppressed KLF5 protein expression (P<0.05), while miR-145-5p inhibition did not change KLF5 protein expression compared with the control (Fig. 4C and D).

Overexpression of KLF5 reduces the tumor suppressive effects of miR-145-5p. Transfection of the KLF5 vector increased KLF5 expression significantly (P<0.05; Fig. 5A). Furthermore, KLF5 overexpression weakened the tumor suppressive effect of miR-145-5p in HepG2 cells (Fig. 5B). Additionally, the migration of HepG2 cells was significantly reduced by miR-145-5p compared with the control (P<0.05), while KLF5 overexpression reversed these suppressive effects (Fig. 5C and D).

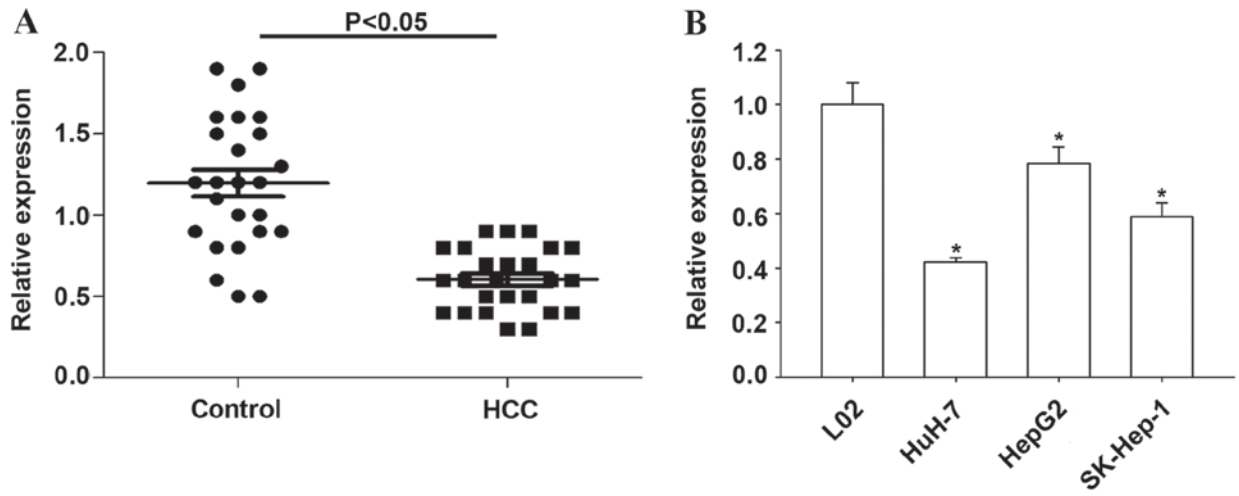

Figure 1. Relative miR-145-5p expression in HCC and controls. (A) Expression of miR-145-5p was measured by reverse transcription-quantitative polymerase chain reaction in HCC and normal liver tissue from 25 patients with primary HCC. (B) Reduced expression of miR-145-5p was observed in HuH-7, HepG2 and SK-Hep-1 HCC cell lines, compared with L02 normal human liver cell line. *P<0.05 vs. L02 cell line. HCC, hepatocellular carcinoma; miR, microRNA.

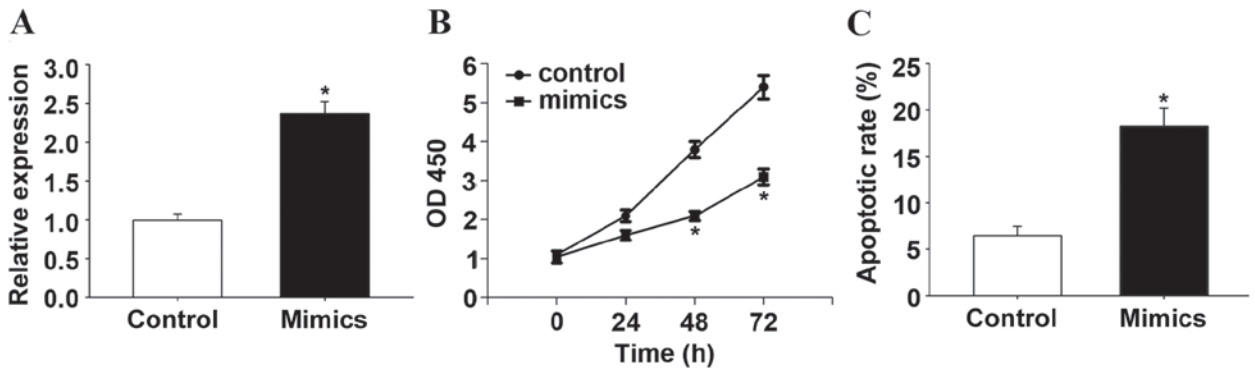

Figure 2. miR-145-5p reduces proliferation of hepatocellular carcinoma cells. (A) Reverse transcription-quantitative polymerase chain reaction indicated that mimic transfection led to miR-145-5p overexpression compared with control. (B) CCK-8 assay determined that mimic transfection reduced cell proliferation. (C) Levels of cellular apoptosis increased following mimic transfection compared with the control. *P<0.05 vs. control. miR, microRNA.

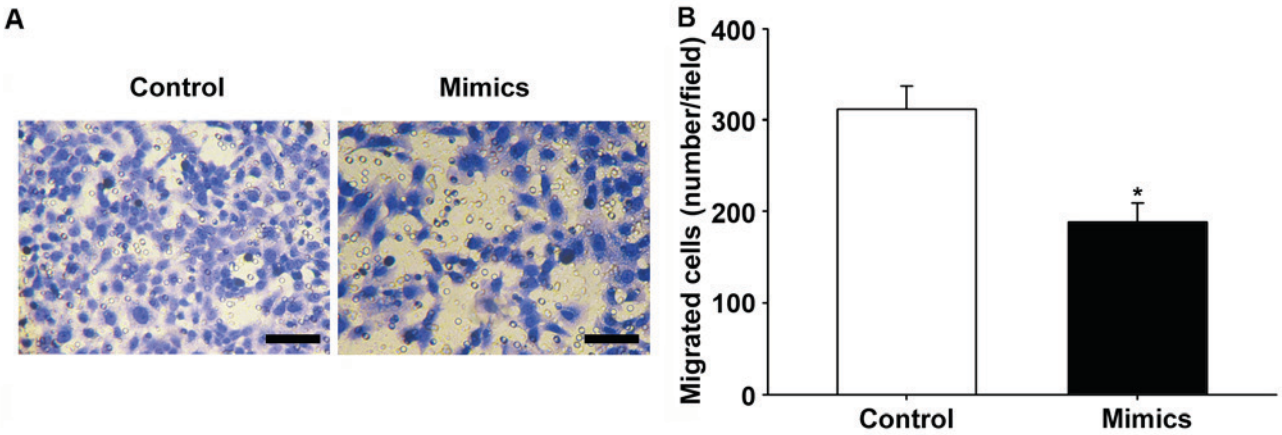

Figure 3. miR-145-5p inhibits migration of HCC cells. (A) Transwell assay of HepG2 cells with control or miR-145-5p transfection; scale bar, 50 μm. (B) *In vitro* migration of HCC cells with control or miR-145-5p transfection. *P<0.05 vs. control. miR, microRNA; HCC, hepatocellular carcinoma.

miR-145-5p and KLF5 are negatively correlated in HCC. The relative expression of KLF5 was detected in 25 non-tumor tissues and HCC tissues. The results indicated that, in HCC tissues, KLF5 mRNA levels were significantly increased compared with non-tumor tissues (P<0.05; Fig. 6A). In addition, the present study observed that KLF5 mRNA expression was inversely correlated with miR-145-5p levels in HCC tissues (r=-0.45) (Fig. 6B).

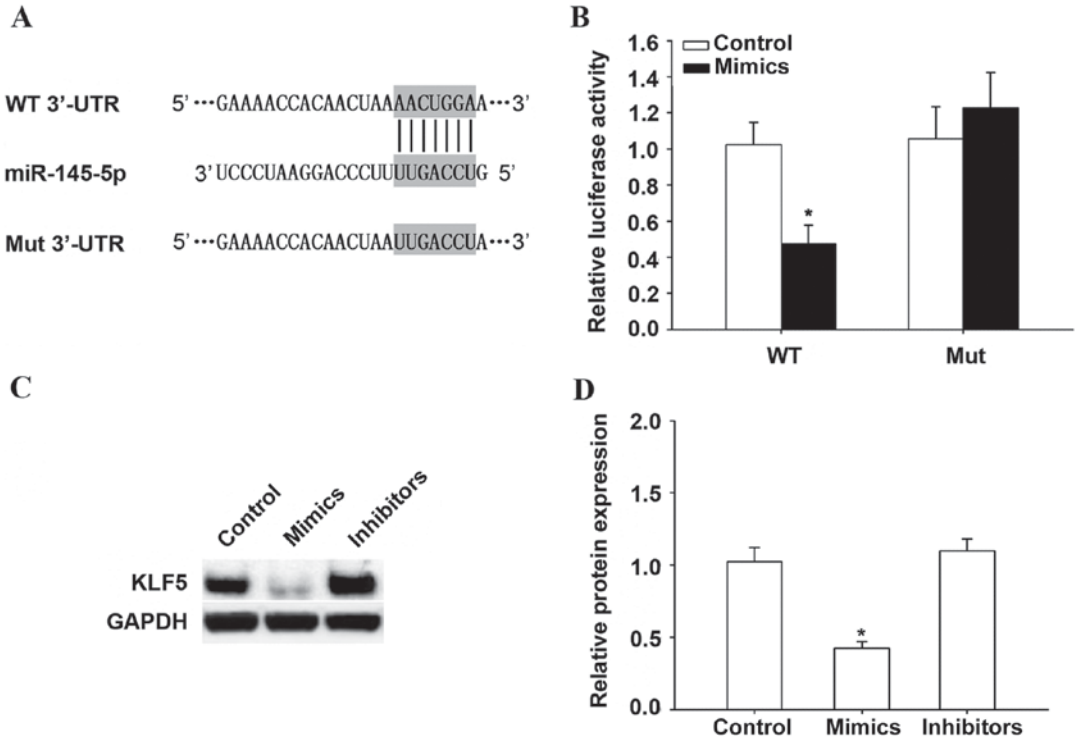

Figure 4. Target confirmation of miR-145-5p. (A) Target prediction by TargetScan 7.0 and the WT or Mut sequences of 3'-UTR from KLF5 mRNA. (B) Luciferase activity of WT or Mut co-transfected with control or miR-145-5p mimics. (C) Protein expression levels of KLF5 in HepG2 cells following transfection with miR-145-5p mimics or inhibitors, or control, by western blot. (D) Relative protein expression levels of KLF5 in HepG2 cells following transfection with miR-145-5p mimics or inhibitors, or control, calculated by grey mean value of binding using ImageJ software. *P<0.05 vs. control. miR, microRNA; WT, wild-type; Mut, mutant; UTR, untranslated region; KLF5, Kruppel-like factor 5.

Discussion

HCC has one of the highest mortality rates despite significant improvements in diagnosis and treatment in recent years (19). Although the mechanisms involved in HCC have been previously demonstrated, the involvement of epigenetic regulation remains predominantly unknown (20). As miRNAs are the most promising components of the epigenetic pathway in terms of targets for the development of novel therapeutic approaches, understanding the role of miRNA in HCC is essential. It is currently known that multiple miRNAs are involved in HCC development and progression. It has previously been observed that several miRNAs have aberrant expression in HCC and it is thought that these miRNAs may be used as prognostic indicators in HCC (21). miR-145-5p is a novel miRNA that is suggested to be implicated in cancer treatment and carcinogenesis. Downregulation of miR-145-5p has been reported in prostate cancer (17). Additionally, miR-145-5p was downregulated in prostate cancer (22), lung cancer (23) and colorectal cancer (24), indicating that miR-145-5p may have tumor suppressive effects. Expression levels of miR-145-5p were inversely associated with the proliferation of prostate cancer cells. Furthermore, in embryonic stem cells, by targeting the 3'UTR of SRY-box 2 mRNA, miR-145-5p suppresses self-renewal of human embryonic stem cells (17). Thus, investigation of miR-145-5p provides novel insight into the molecular mechanisms of cancer progression and carcinogenesis. The present study aimed to analyze the effects of miR-145-5p on HCC and to identify the potential target genes of miR-145-5p in order to study the molecular mechanism behind its role in HCC oncogenesis. Expression of endogenous miR-145-5p in 25 HCC tissues was significantly downregulated. Similarly, miR-145-5p was downregulated in HCC cell lines, including HuH-7, HepG2 and SK-Hep-1, compared with the L02 normal liver cell line. Additionally, overexpression of miR-145-5p decreased proliferation rate and induced apoptosis in HCC cells. Transwell analysis indicated that miR-145-5p inhibited migration of HCC cells. This evidence suggested that downregulation of miR-145-5p may lead to increases in the proliferation, migration and aggression of HCC. The current study was extended to identify the potential targets of miR-145-5p in HCC cells. *In silico* bioinformatics analyses demonstrated that the 3'UTR of KLF5 is one potential target of miR-145-5p. *In vivo* and *in vitro* results demonstrated that KLF5 expression levels were significantly decreased in HCC cells overexpressing miR-145-5p. It is regarded that KLF5 is highly expressed in HCC tissues compared with healthy control tissues. The involvement of KLF5 in tumor progression has also been demonstrated in breast (25), intestinal (26), esophageal (27) and gastric cancer (28). The current study proposes that miR-145-5p is an important regulator of KLF5 since miR-145-5p overexpression in HCC cells reduced KLF5 expression. Decreased levels of miR-145-5p may be a key step in the pathogenesis of HCC. It is possible that miRNAs regulate several genes, by targeting the 3'UTR of mRNA, and the subsequent changes in the expression of these target genes may participate in specific tissue development or cancer progression. The present study has identified that KLF5, a target of miR-145-5p, is a key marker gene in cancer. miR-145-5p may also contribute

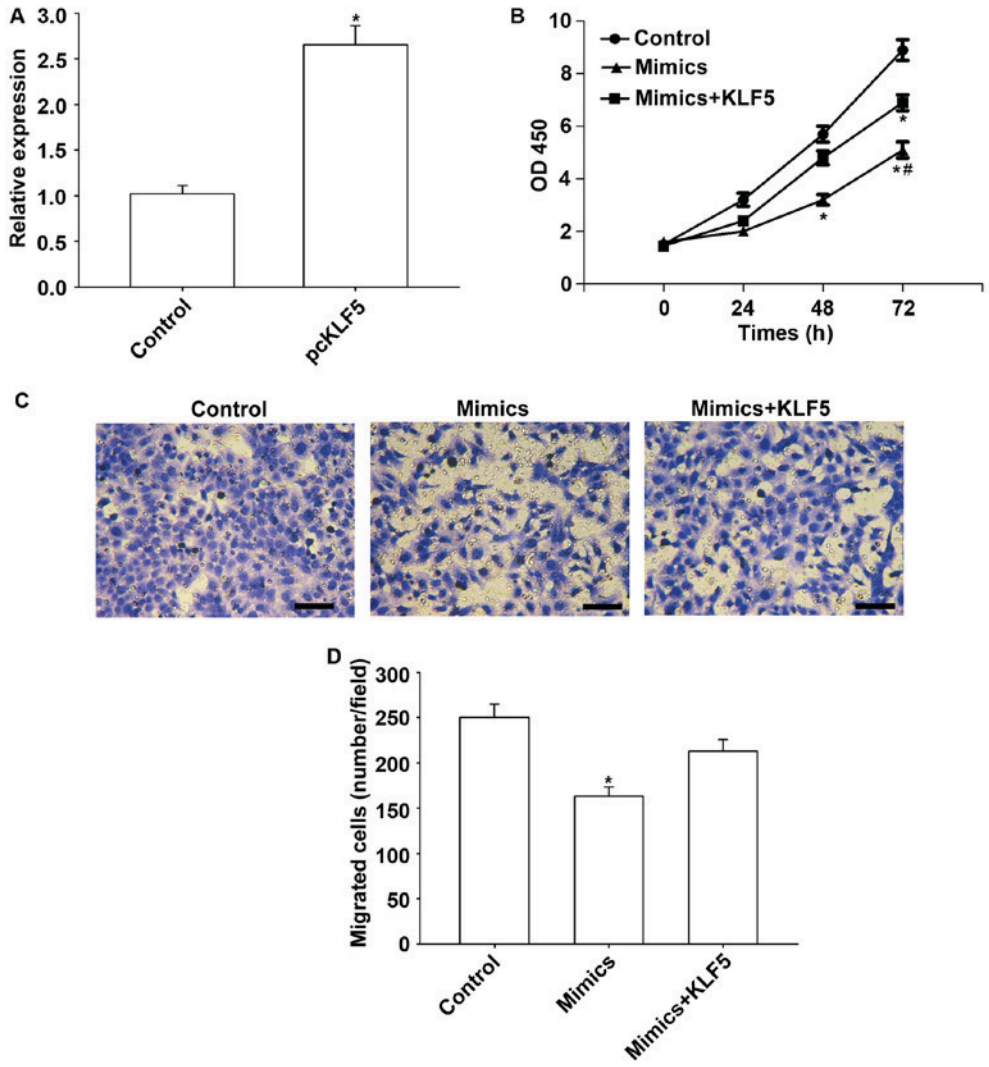

Figure 5. KLF5 overexpression reduces the tumor suppressive effects of miR-145-5p in HepG2 cells. (A) Expression of KLF5 after transfection with control or pc-KLF5. (B) Cell proliferation in cells transfected with control and miR-145-5p mimics with/without pc-KLF5 transfection. (C) Transwell assay of HepG2 cells transfected with control and miR-145-5p mimics with/without pc-KLF5 transfection; scale bar, 50 μm. (D) *In vitro* migration of hepatocellular carcinoma cells transfected with control and miR-145-5p with/without pc-KLF5 transfection; number of migrated cells per field. *P<0.05 vs. control; #P<0.05 vs. mimcs+KLF5. KLF5, Kruppel like factor 5; miR, microRNA.

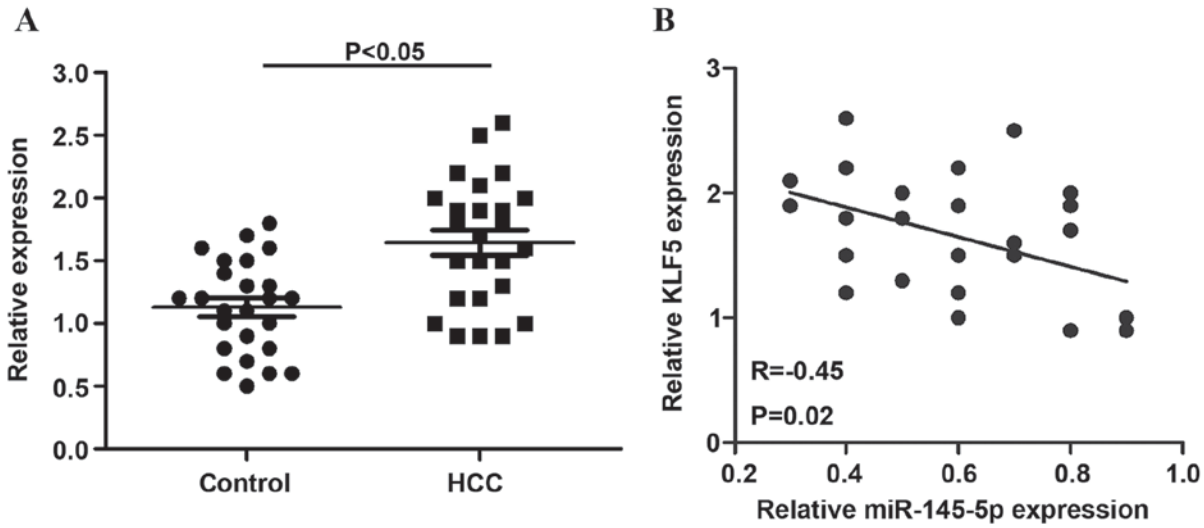

Figure 6. miR-145-5p is negatively correlated with KLF5 in HCC. (A) Higher relative expression of KLF5 was observed in HCC compared with normal control. (B) Correlation between relative expression of miR-145-5p and KLF5 in HCC as determined by reverse transcription-quantitative polymerase chain reaction. KLF5, Kruppel-like factor 5; miR, microRNA.

to the dysregulation of other functional genes during tumor development. Thus, further studies should focus on the gene network of miR-145-5p in HCC and the miRNA profiles of circulating HCC tumor cells, which may demonstrate the roles of miRNA and improve the understanding of its function in HCC pathogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL and HS performed the experiments and wrote the paper. JY and CY designed the study and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The Ethics Committee of The Second Xiangya Hospital of Central South University approved this study and written informed consent was obtained from each patient.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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