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ORIGINAL ARTICLE

MiR-145 suppresses cell proliferation and motility by inhibiting ROCK1 in hepatocellular carcinoma

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Abstract MicroRNAs (miRNAs) play key roles in cancer development and progression. In the present study, we investigated the role of miR-145 in the progression of hepatocellular carcinoma (HCC). Ten HCC cell lines and samples from 96 patients with HCC were analyzed for the expression of miR-145 by quantitative real-time polymerase chain reaction (qRT-PCR). Overexpression of miR-145 was established by transfecting mimics into HepG2 and QGY-7703 cells. Cell proliferation and cell migration were assessed by cell viability assay and transwell assay. Western blot was to verify ROCK1 as a novel target gene of miR-145. Our results showed that miR-145 was frequently downregulated in HCC tumors and cell lines. Overexpression of miR-145 in HCC cell lines significantly inhibited cell proliferation, migration, and invasion in vitro. ROCK1 was identified as a target of miR-145, and ectopic expression of miR-145 downregulated ROCK1. Together, these findings indicate that miR-145 acts as a tumor suppressor and its downregulation in tumor tissues may contribute to the progression and metastasis of HCC through a mechanism involving ROCK1, suggesting miR-145 as a potential new diagnostic and therapeutic target for the treatment of HCC.

Keyword MiR-145 · Hepatocellular carcinoma · ROCK1 · Proliferation · Migration

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and the second in China [1, 2]. Despite advancements in early diagnosis and surgical treatment over the last few decades, the prognosis for HCC patients remains pessimistic. The aggressive nature of HCC is resistance to apoptosis and vascular invasion [3]; however, genetic and molecular events contributing to the initiation and progression of HCC are still unclear. Thus, it is important to explore the key molecules in tumor progression for effective treatment of HCC.

MicroRNAs (miRNAs) are endogenous small ncRNAs that participate in posttranscriptional gene regulation [4]. Recent studies have demonstrated that miRNAs play critical roles in the coordination of a wide variety of biological processes, including differentiation, proliferation, angiogenesis, and metabolism [5–8]. As miRNAs may act as tumor oncogenes or tumor suppressors, they are potential cancer biomarkers. Previously, miR-145 was identified as a tumor-suppressive miRNA in multiple types of cancers, including renal, prostate, bladder, lung, and colon cancer, as well as B cell malignancies. MiR-145 has been reported to be frequently downregulated in various tumors, and it inhibits cell proliferation and cell invasion in various tumors [9–12]. However, the functions of miR-145 in modulating the progression of HCC have not been investigated. The purpose of this study was to investigate the biological functions and molecular mechanisms of miR145 in human HCC and to identify the target genes regulated by the miR-145.

Materials and methods

Clinical specimen collection

Samples from 96 patients with HCC who underwent hepatic resection at our hospital (Kunming General Hospital, Chinese People's Liberation Army) between 2004 and 2014 were collected in this study. Adjacent non-tumoral liver tissues from these patients (5 cm to tumor area) served as normal controls. Consent was obtained from all patients, and the experimental protocols were approved by the local ethics committee. Patient charts were reviewed to obtain clinical data regarding age, gender, tumor size, TNM stage (according to the American Joint Committee on Cancer), and tumor differentiation, and death or time of last follow-up. Patient survival was calculated from the day of surgery until death, in months.

Cell culture

The immortalized normal liver epithelial cells, THLE-3, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured under the conditions stated by the manufacturer. The HCC cell lines, HepG2, Hep3B, and PLC/PRF/5, were purchased from ATCC, and MHCC97H, MHCC97L, BEL-7402, Huh7, SMMC-7721, and QGY-7703 were purchased from Shanghai Yansheng industrial Co. (Shanghai, China). The HCC cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco-BRL) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL), at 37 °C in a 5 % CO_2 atmosphere in a humidified incubator.

qRT-PCR

Tissues were immersed in RNA later (Ambion, Austin, TX, USA) and stored at 20 °C until RNA extraction. Total RNA, including miRNA, was extracted using the mirVana™ miRNA isolation kit (Ambion) following the manufacturer's protocol. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Science, Waltham, MA, USA). Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara) on Light Cycler 480 (Roche, Switzerland). The ROCK1-specific primer sequences were as follows: 5′-GGTGGTCGGT GGGGTATTTT-3′ (forward) and 5′-CGCCCTAAC CTCACTTCCC-3′ (reverse). β-Actin was used as an endogenous control, and its primer sequences were as follows: 5′-CTCCATCCTGGCCTCGCTGT-3′ (forward) and 5′-GCTGTCACCTTCACCGTTCC-3′ (reverse). For the miRNA expression assay, miRNAs were isolated by the miRNeasy mini kit. Following this, the TaqMan MicroRNA Assays kit was used to determine the miRNA expression on a 7500 Fast Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The universal small nuclear RNA, U6, was used as an endogenous control. For each sample, independent experiments were repeated three times. The relative expression levels of messenger RNA (mRNA) and miRNA were analyzed by use of the $2^{-\Delta\Delta Ct}$ method.

Western blot

Soluble proteins were collected and stored at −80 °C after a centrifugation at 12,000*g* for 15 min. The protein amount was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). For testing, after denaturation, the proteins were separated with gel electrophoresis using 10 % SDS-PAGE and then wet transferred to a PVDF membrane for 2 h of blocking in 5 % skim milk. The membrane was washed once with TBST and then incubated for overnight at 4 °C with relevant antibodies (1:1000). The membrane was again washed three times with TBST and then incubated with secondary antibody (goat anti-rabbit/mouse IgG 1:2000) for 2 h at room temperature. The membrane was washed a third time with TBST, and then ECL liquid was added and placed in a darkroom. Actin was used to ensure equivalent protein loading.

Transfection

Mature miR-145 mimics, the NC, and the luciferase reporter plasmid were designed and synthesized by GenePharma (Shanghai, China). The sequence of miR-145 mimics was 5′-GUCCAGUUUUCC CAGGAAUCCCU-3′. The sequence of NC mimics was 5′-UUCUCCGAACGUGUCACGUTT-3′. A total of 1×10^4 cells/well was seeded into six well plates, and then, cells were transfected in a solution with 75-nM miR-145 mimics or miR-NC (Yingrun Biotechnology, Inc.) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA and protein were extracted at 48 h posttransfection and used for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis.

siRNA and transfection

For depletion of ROCK1, the small interfering RNAs (siRNAs) were synthesized and purified by RiboBio. ROCK1 siRNA and control siRNA were used in the loss-of-function experiments. Transfection of oligonucleotides and siRNAs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

Cell proliferation assay

A cell proliferation assay was performed with MTT (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1×10^4 cells/well were plated in a 96-well plate. The plates were incubated for 24 h in a humidified atmosphere containing 5 % CO_2 at 37 °C. Following this, 50 μl MTT (5 mg/ml) in PBS was added and incubated for 4 h in a humidified atmosphere containing 5 % CO_2 at 37 °C. Next, 150 μl DMSO was added following removal of the supernatant. A microplate reader (Bio-Rad) was used to determine the absorbance at 570 nm. Each assay was performed in triplicate wells and repeated three times.

Cell migration assay

The 24-well Boyden chamber with 8-μm pore size polycarbonate membrane (Corning, NY) was used for evaluating the cell motility. Cells were transfected with miR-145 mimic or control RNA. Matrigel was used to pre-coat the membrane to simulate a matrix barrier for invasion assay. Four thousands of cells were seeded on the upper chamber with 200-μl serum-free medium after transfected with RNA duplex for 48 h. Six hundred-microliter medium with 20 % serum, served as a chemoattractant, was added to the lower chamber. After 24-h incubation, the membranes were fixed with methanol and stained with 0.1 % crystal violet. Five visual fields (×200) were randomly selected from each membrane, and the cell numbers were counted via a light microscope. All experiments were performed in triplicate.

Statistical analysis

All values are presented as means+SEM. Statistical significance was determined using the Student's *t* test. *P* values lower than 0.05 were considered statistically significant.

Results

MiR-145 expression is downregulated in HCC

The expression of miR-145 was examined in a series of HCC tissue samples using qRT-PCR. As shown in Fig. 1a, the expression of miR-145 was significantly downregulated in HCC tissues compared with paired adjacent normal tissues ($P<0.001$), indicating that miR-145 could act as a tumor suppressor in HCC. Furthermore, the miR-145 expression was investigated in different HCC cell lines by qRT-PCR, and results showed that miR-145 expression was significantly decreased in HCC cell lines including HepG2, Hep3B, MHCC97H, MHCC97L, BEL-7402, Huh7, SMMC-7721, PLC/PRF/5, and QGY-7703, compared with that in the immortalized normal liver epithelial cells THLE3, indicating that miR-145 may have a suppressive role in the development of HCC in vitro (Fig. 1b).

Ectopic expression of miR-145 inhibits HCC cells proliferation

To determine the function of miR-145 on HCC progression, we transfected HepG2 and QGY-7703 cells with miR-145 mimics or negative control. We evaluated the effect of miR-145 on cell proliferation using MTT assay. As shown in Fig. 2, overexpression of miR-145 significantly inhibited cell proliferation. The MTT value of cells transfected with miR-145 mimics was significantly lower than that of cells transfected with miR-NC at 48 h posttransfection ($P<0.05$; Fig. 2a, b).

MiR-145 inhibits cell migration and invasion in HCC cell lines

We measured the effect of miR-145 on tumor cell migration and invasion by transwell assay (Fig. 3). The transfected cells (miR-145 mimics and NC mimics) growing in the log phase were collected and cultured on transwell apparatus. Following 24-h incubation, cell migration was significantly decreased in

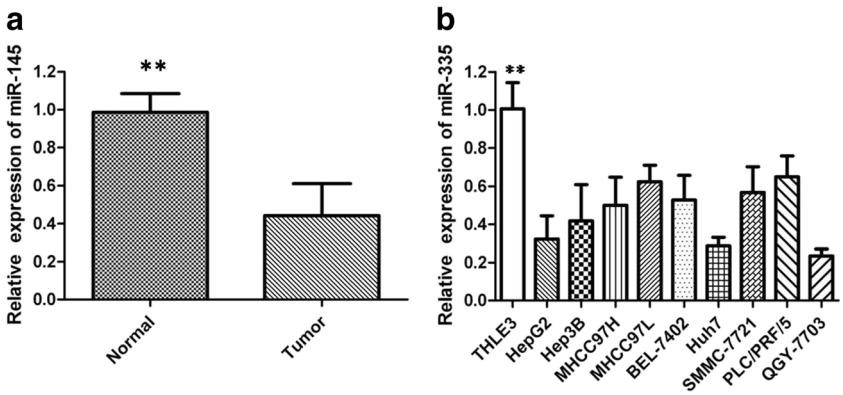

Fig. 1 **a** The miR-145 relative expression levels were determined by qRT-PCR in 96 pairs of HCC tissues and the adjacent non-neoplastic tissues. **b** The relative expression levels of miR-145 in human HCC cells in comparison with normal liver epithelial cells THLE3

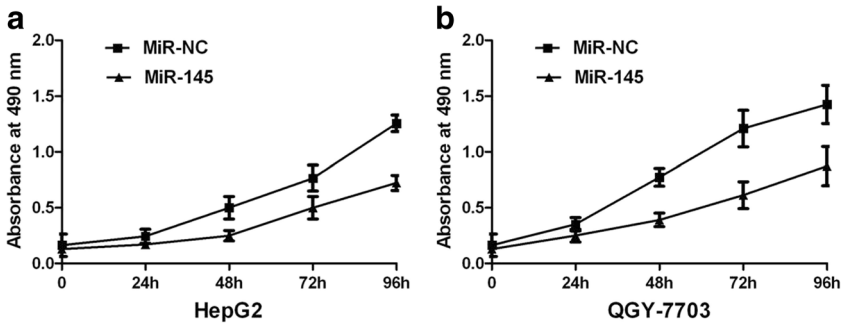

Fig. 2 **a** The MTT assay revealed significant cell proliferation inhibition in miR-145 transfectant compared with the control from HepG2 cell line. **b** The MTT assay revealed significant cell proliferation inhibition in miR-145 transfectant compared with the control from QGY-7703 cell line

the miR-145 groups compared with the control group ($P<0.01$). Using transwell apparatus pre-coated with Matrigel, the effects of miR-145 on cell invasiveness were examined. Following 24-h incubation, miR-145 transfected cells demonstrated significantly decreased invasiveness compared with the control cells ($P<0.01$). These results indicated that miR-145 inhibited cell migration and invasion in HCC cell lines.

MiR-145 plays its suppressor role in HCC through targeting ROCK1

Several predicative software, including TargetScan (http://www.targetscan.org/), demonstrated that ROCK1 is a potential target based on putative target sequences at the 3′-UTR position of ROCK1. Since ROCK1 has been demonstrated to be involved in HCC cell proliferation, migration, and invasion, it was hypothesized that ROCK1 may also be involved in miR-145-mediated biological processes in HCC cells. The ROCK1 levels were measured in HCC tissues and paired normal adjacent liver tissues. As shown in Fig. 4a, b, the average level of ROCK1 expression was significantly higher in HCC tissues as compared with paired normal adjacent liver tissues. In addition, qRT-PCR and Western blot analyses revealed that overexpression of miR-145 significantly decreased the expression of ROCK1 compared with control at the mRNA and protein levels in HepG2 and QGY-7703 cells ($P<0.01$, Fig. 4c, d).

The above results prompted us to examine whether the suppressive effect of miR-145 is mediated by repression of ROCK1 in HCC cells. Therefore, HepG2 cells were transfected with siRNA against ROCK1 or the negative control, and ROCK1 expression was significantly decreased (Fig. 5a, b). ROCK1 silencing significantly inhibited cell proliferation and suppressed migration and invasion (Fig. 5c, d), similar to those induced by miR-145. Taken together, these findings indicated that ROCK1 is a functionally important target of miR-145 that is involved in the proliferation, migration, and invasion of HCC cells.

Discussion

Growing evidence indicates that miRNAs, a type of endogenous, small noncoding RNAs, participate in diverse cellular processes. Through specifically binding and cleaving mRNAs or inhibiting their translation, miRNAs function as either oncogenes or tumor suppressors [13]. Previous studies reported that miR-145 acts as a tumor suppressor in various tumors, and overexpression of miR-145 was linked to better disease-free survival in cancer patients [14]. However, the function and mechanisms of miR-145 in HCC tumorigenesis are far from being illustrated.

In this study, we firstly explored the role of miR-145 in HCC. To the best of our knowledge, when compared with its normal adjacent tissue, we initially found a significantly

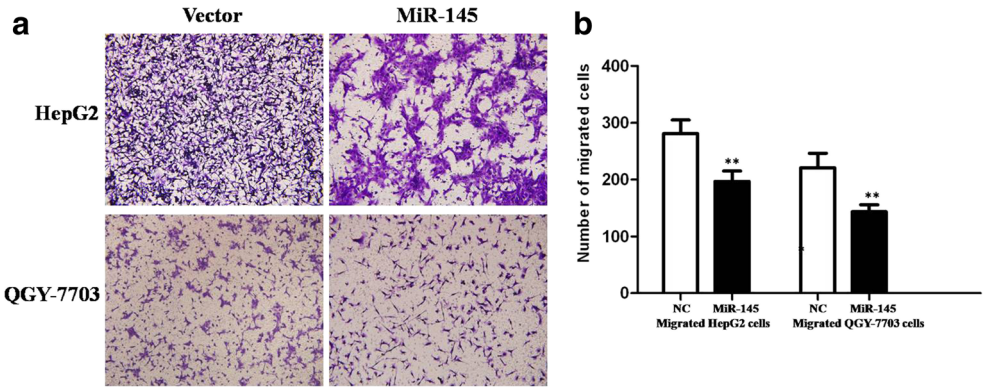

Fig. 3 The transwell assay also showed significant cell migration inhibition in miR-145 transfectant, compared with cell lines transfected with NC

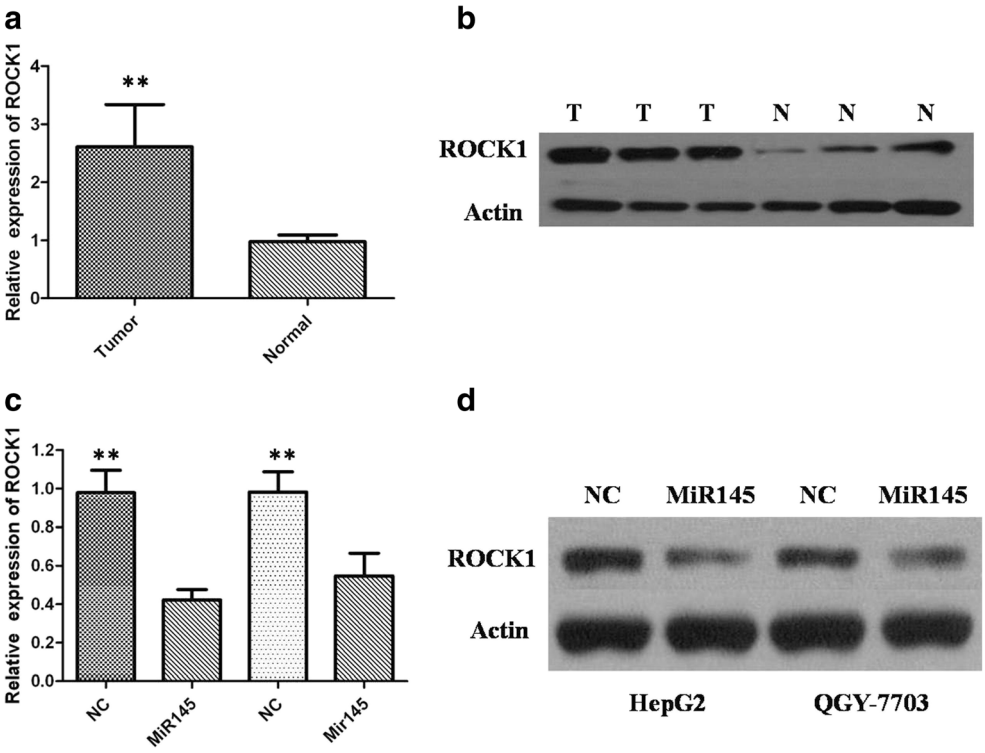

Fig. 4 **a** The relative ROCK1 mRNA was significantly increased in HCC tissues. **b** The relative ROCK1 protein was significantly increased in HCC tissues. **c** MiR-145 negatively regulates ROCK1 mRNA expression. **d** MiR-145 inhibited the expression of ROCK1 at the protein level in HCC cells

decreased expression pattern of miR-145 in HCC tissue. Similar result was detected in ten HCC cell lines compared with normal epithelial cell line, which further strengthened the conclusion that miR-145 was frequently downregulated in HCC.

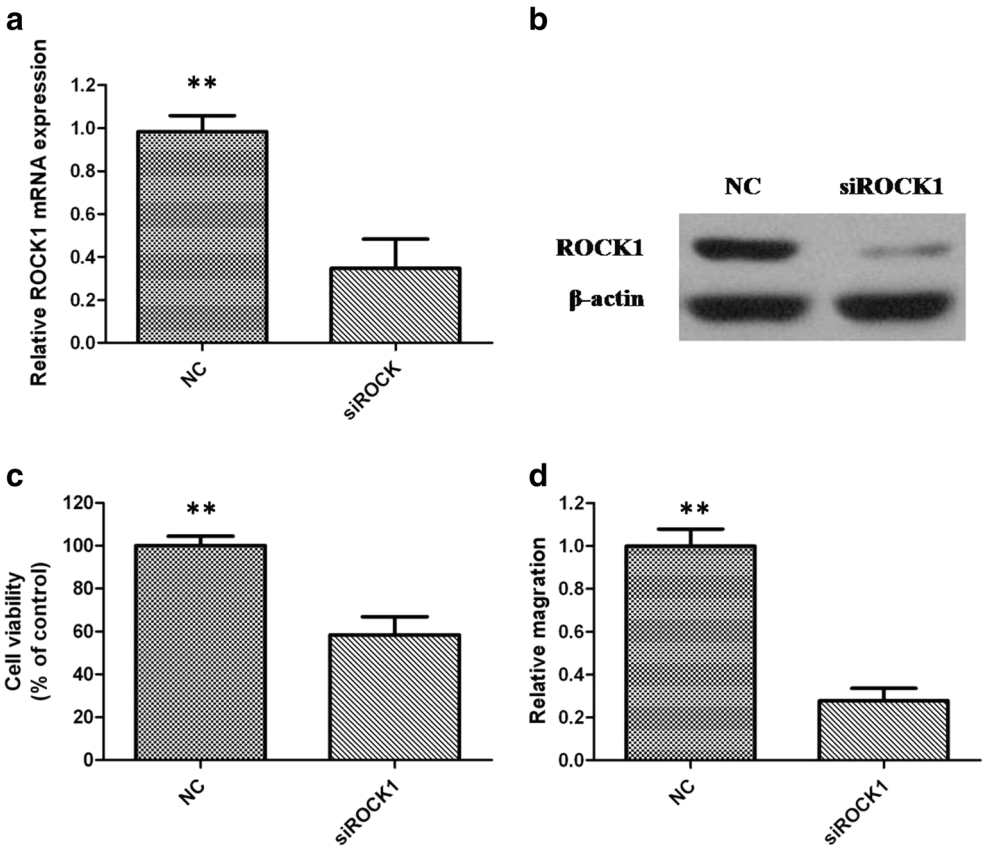

Fig. 5 **a**, **b** The relative ROCK1 mRNA and protein expression level were significantly decreased in siRNA-transfected cells. **c** Knockdown of ROCK1 inhibited cell growth in HCC cells. **d** Knockdown of ROCK1 by siRNA inhibited migration of HCC cells

In addition, a close association between downregulated miR-145 and cancer cell proliferation and metastasis has been observed. We hypothesized that overexpression of miR-145 could decrease the metastatic potential of HCC. Subsequently, the biological function of miR-145 in HCC cells was examined. A gain-of-function study was further conducted in HCC cell lines. Our results demonstrated that restoration of miR-145 in HCC cells can significantly inhibit cell proliferation, invasion, and migration, confirming previous studies suggesting a tumor-suppressive role for miR-145. Thus, downregulation of miR-145 might have a critical function in HCC development.

To further dissect the mechanism by which miR-145 functioned as a tumor suppressor in HCC, we analyzed the relationship of miR-145 and ROCK1 in HCC. In the present study, we identified ROCK1 as a target of miR-145 and showed that miR-145 overexpression is correlated with ROCK1 downregulation leading to the inhibition of cell proliferation, migration, and invasion. ROCK1 plays important roles in regulating cell polarity and migration by enhancing actomyosin contraction and focal adhesions, thus increasing cellular contraction, migration, and chemotaxis [15]. Several studies have demonstrated that ROCK1 is associated with cancer progression and ROCK1 expression is elevated in several cancers [16]. The MTT assay showed significant inhibition of cell proliferation in the si-ROCK1-transfected cells compared with control group. The transwell assay demonstrated significant cell migration inhibitions in the si-ROCK1 transfectants compared with the counterparts, suggesting that elevated ROCK1, induced by suppressed miR-145, participates in progression of HCC.

In conclusion, the present study showed that miR-145 was downregulated in HCC cell lines and tissues, and its ectopic expression inhibited cell proliferation and migration/invasion. The tumor-suppressor function of miR-145 was mediated by the downregulation of its downstream target gene ROCK1. These results indicate that miR-145 may be a potential therapeutic target for the treatment of HCC.

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Compliance with ethical standards

Conflicts of interest None

Informed consents All specimens were collected in accordance with informed consents of patients, and all procedures complied with the protocol approved by the Ethical Committee of Kunming General Hospital.

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