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RESEARCH ARTICLE

miR-144 suppresses the proliferation and metastasis of hepatocellular carcinoma by targeting E2F3

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Abstract MicroRNAs (miRNAs) play essential roles in the progression of hepatocellular carcinoma (HCC). miR-144 acts as a tumor suppressor in some malignancies, while its role in HCC is unclear. Here, we found that miR-144 was significantly decreased in HCC tissues and cell lines. Forced overexpression of miR-144 remarkably reduced cell proliferation, increased apoptosis, and suppressed migration and invasion of HCC cells. E2F transcription factor 3 (E2F3) was identified as a target of miR-144 in HCC cells. Moreover, E2F3 overexpression partially attenuated the tumor suppressive effects of miR-144, and the expression of E2F3 was negatively correlated with miR-144 level in HCC tissues. Our data suggest that miR-144 might suppress the growth and motility of HCC cells partially by targeting E2F3.

Keywords miR-144 · Hepatocellular carcinoma · Growth · Growth · Migration · Invasion · E2F3

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors in Asia and Africa, especially in China [1]. During the past decade, the long-time survival of HCC patients remains poor due to a high incidence of recurrence and metastasis, with a 5-year actuarial recurrence rate of 75–100 % reported in the literature [2]. Surgical resection, radiofrequency ablation, and liver transplantation may provide cure for some early stage patients; however, most patients are diagnosed at advanced stage due to the asymptomatic nature of HCC [3]. The molecular mechanism of HCC remains poorly understood, and development of new strategies for better understanding of HCC is anticipated.

MicroRNAs (miRNAs) are a family of endogenous, small non-coding RNAs (20~25 nucleotides in length), which post-transcriptionally regulate the expression of complementary target mRNAs in eukaryotes, influencing a variety of biological processes including cell development, infection, immunity, and carcinogenesis [4, 5]. Expression profiles of miRNAs reveal that there are representative signatures for many cancers, and that these could be predictive of cancer classification, prognosis, and response to therapy [6]. Emerging evidence shows that miRNAs play essential roles in HCC development, and several tumor suppressive miRNAs have been identified in HCC [7, 8].

Several groups have reported downregulation of miR-144 in many cancers including colorectal cancer (CRC), non-small-cell lung cancer (NSCLC), osteosarcoma (OS), and HCC [9–12]. However, the effect of miR-144 is different in different types of cancer. In the present study, we report that miR-144 was downregulated in HCC and that miR-144 overexpression inhibited proliferation and metastasis in HCC cells. We further identified E2F transcription factor 3 (E2F3) as a target of miR-144 in HCC cells, and E2F3 supplement partially attenuated the tumor suppressive effects of miR-144 in HCC cells.

Materials and methods

HCC tissues and cell lines

HCC tissues and paired normal liver tissues were obtained from 33 patients with primary HCC in our department. This study was approved by the Ethics Committee of Wuhan General Hospital of Guangzhou Command, and written informed consent was obtained from each patient. HCC cell

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lines (HepG2, HuH-7, and Hep3B) and normal human liver cell line L02 were obtained from ATCC. Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FBS at 37 °C in a 5 % CO_2 incubator.

RNA isolation and real-time quantitative PCR

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) or miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), followed by reverse transcription. Real-time quantitative PCR (qPCR) detection of genes was performed using SYBR Green Master Mix (ABI, Foster City, CA, USA) by LightCycler System (Roche, Pleasanton, CA, USA). Detection of miR-144 in cell lines was performed using TaqMan microRNA assays. The relative expression level of miR-144 and E2F3 were normalized to U6 and β-actin, respectively, using $2^{-\Delta\Delta Ct}$ method.

Construction of expression vectors

miR-144 and control mimics/inhibitors were obtained from RiboBio (Guangzhou, China). The E2F3 expression vector was generated by amplification of the entire coding region of E2F3. The amplified fragment was inserted into pcDNA3.1 using NotI and EcoRI (named as pc-E2F3). For luciferase reporter, 3′-untranslated region (3′-UTR) of E2F3 was amplified and inserted into psiCHECK-2 vector using XhoI and NotI. Mutation was performed using a mutation kit (NEB, Ipswich, Canada).

Analysis of cell viability and apoptosis

Cell proliferation rates were measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan); 0.5×10^4 cells were seeded in 96-well plate and incubated for 24, 48, 72, and 96 h, respectively. Then, 10 μl CCK-8 reagent was added to each well. The OD450 nm value was determined by a microplate reader.

For apoptosis analysis, cells were harvested, washed in ice-cold PBS, and fixed in ice-cold ethanol. Cells were incubated with 20 μg/mL PI (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature, and the cells were analyzed using FACS (BD, Franklin Lakes, NJ, USA).

Analysis of cell invasion and migration

Cell migration and invasion assays were evaluated using a Transwell chamber (Millipore, Billerica, MA, USA). Filters for invasion assays were precoated with Matrigel (BD, Franklin Lakes, NJ, USA) in the upper compartment before cell seeding. In the upper chamber, 5×10^4 cells were seeded, and the lower chamber was filled with medium supplemented with 10 % FBS. Migratory and invasive cells on the bottom surface were fixed with 4 % paraformaldehyde solution, stained with 0.1 % crystal violet. Four random fields from each membrane were counted, and experiments were performed in triplicate.

Western blot analysis

Protein lysates were subjected to SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Target proteins were detected with primary antibodies recognizing E2F3 and β-actin, respectively. After incubation with HRP-conjugated secondary antibodies, protein bands were visualized using ECL system (Pierce, Rockford, IL, USA).

Luciferase reporter assay

HEK-293 cells were cultured in 24-well plate and incubated for 24 h, and then co-transfected with 100 nM of miR-144 or control mimics and wild type (WT) or Mutant (Mut) 3′-UTR of E2F3 using Lipofectamine 2000 reagent. Forty-eight hours after transfection, HEK-293 cells were harvested, and the relative luciferase activity was evaluated using Dual-Luciferase Reporter System (Promega, Wisconsin, WI, USA).

Statistical analysis

Data are shown as mean ± SD and analyzed by using SPSS 12.0. Quantitative variables were analyzed using Student's *t* test or one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

miR-144 was decreased in HCC

Expression of miR-144 in 33 HCC and matched non-tumoral tissues were determined by qRT-PCR. miR-144 was significantly decreased in HCC tissues compared with that of non-tumor tissues (Fig. 1a). Expression of miR-144 in three HCC cell lines, HepG2, HuH-7, and Hep3B, was significantly decreased compared with that of the normal human liver cell line L02 (Fig. 1b).

miR-144 suppressed the proliferation of HCC cells

To investigate the biological effect of miR-144 on HCC progression, HepG2 cells were transfected with miR-144 or control mimics. CCK-8 assay showed that miR-144 overexpression significantly suppressed the rate of cell proliferation (Fig. 2a). Flow cytometry revealed a dramatic increase in the percentage of apoptosis cells in miR-144-overexpressing HepG2 cells as compared with control HepG2 cells

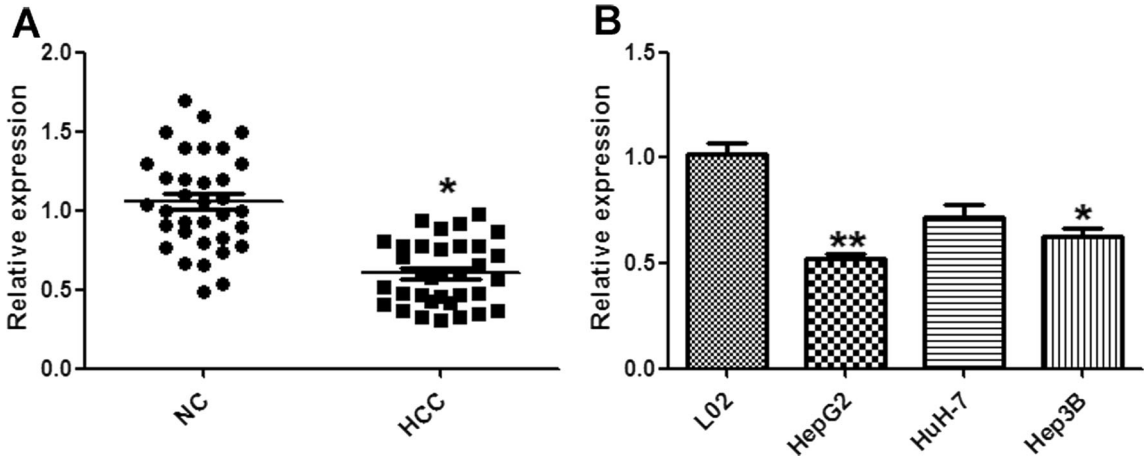

Fig. 1 miR-144 was decreased in HCC. The expression of miR-144 was measured by qPCR in 33 paired HCC and non-tumor tissues (**a**) and three HCC cell lines and L02 cells (**b**). *$P<0.05$, **$P<0.01$ compared with control

(Fig. 2b). The effect of miR-144 mimic was confirmed by qRT-PCR (Fig. 2c).

miR-144 inhibited migration and invasion of HCC cells

To study the effect of miR-144 on motility ability of HCC cells, miR-144 or control mimics were transfected into HepG2 cells, migration and invasion assays were performed. miR-144 overexpression substantially inhibited the migration and invasion capabilities of HepG2 cells (Fig. 3a, b).

E2F3 was a direct target of miR-144

To explore the target of miR-144 in HCC, TargetScan 6.2 was used. E2F3 was predicted to be a potential target of miR-144 (Fig. 4a). Luciferase activity assay found that miR-144 overexpression only significantly suppressed the luciferase activity of the WT 3′-UTR of E2F3 in HEK-293 cells (Fig. 4b). Moreover, overexpression of miR-144 remarkably inhibited E2F3 expression, while inhibition of miR-144 significantly increased E2F3 expression (Fig. 4c).

E2F3 overexpression partially attenuated the tumor suppressive effects of miR-144

CCK-8 assay (Fig. 5a), in vitro migration and invasion (Fig. 5b, c) assays, all showed that the supplement of E2F3 by pc-E2F3 significantly attenuated the tumor suppressive effect of miR-144 in HepG2 cells. The effect of pc-E2F3 was validated by qRT-PCR (Fig. 5d).

miR-144 was inversely correlated with E2F3 in HCC

Expression of E2F3 in 33 HCC and non-tumor tissues was measured by qRT-PCR. Results showed that E2F3 mRNA level was significantly increased in HCC tissues compared with that of the non-tumor tissues (Fig. 6a). Moreover, E2F3 was found to be inversely correlated with miR-144 level in HCC tissues (Fig. 6b).

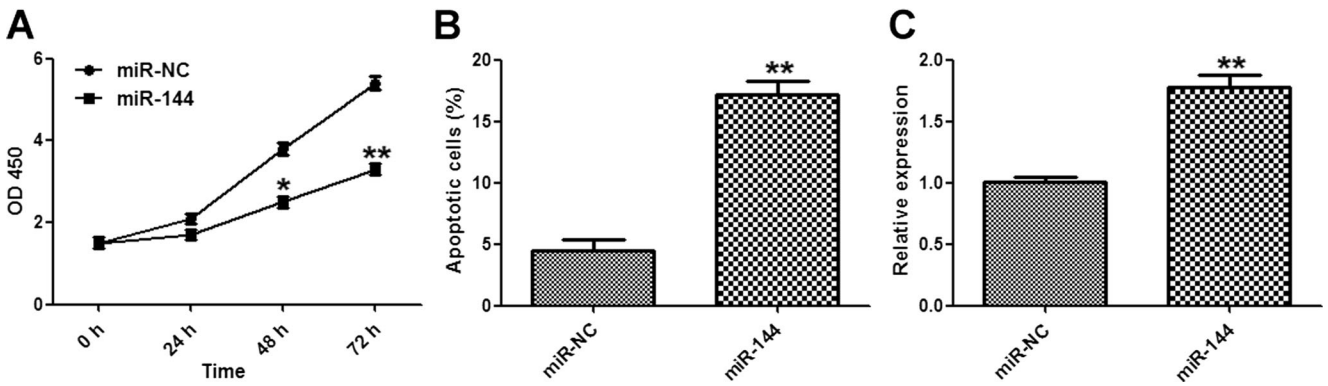

Fig. 2 miR-144 suppressed the proliferation of HCC cells. HepG2 cells were transfected with miR-144 or control mimic (miR-NC). **a** CCK-8 assay was performed to examine proliferation. **b** Quantitative analysis of apoptotic cell death in HepG2 cells. C, qRT-PCR was used to determine the expression of miR-144. Data were obtained in three independent experiments. *$P<0.05$, **$P<0.01$ compared with control

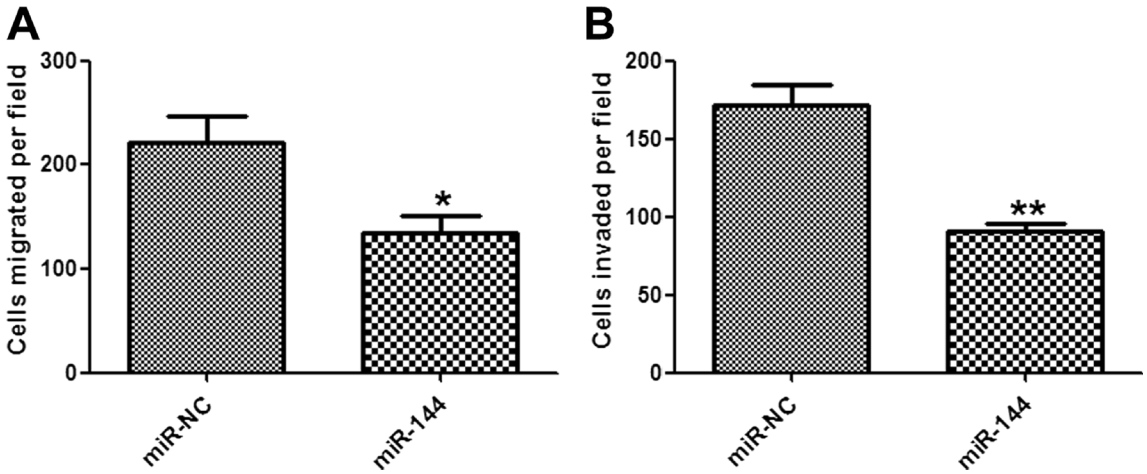

Fig. 3 miR-144 inhibited migration and invasion of HCC cells. **a** In vitro migration assay of HepG2 cells transfected with miR-144 or miR-NC. **b** In vitro invasion assay of HepG2 cells transfected with miR-144 or miR-NC. Data were obtained in three independent experiments. *$P<0.05$ compared with control

Discussion

Until now, accumulating studies have demonstrated that a variety of miRNAs are involved in HCC development and progression, and aberrant expression of some miRNAs has been used as prognostic indicators for HCC [13]. Recently, there is an increasing research interest on the role of miR-144 in carcinogenesis and cancer treatment. Several groups have reported downregulation of miR-144 in various types of cancer including CRC, NSCLC, and OS that implied miR-144 as a potential tumor suppressor. Akiyoshi et al. reported that miR-144 level was inversely correlated with gastric cancer development [14]. Sureban et al. also showed that knockdown of doublecortin and CaM kinase-like-1 (DCAMKL-1) increased miR-144 expression, which in turn inhibited epithelial-mesenchymal transition (EMT) of pancreatic cancer [15]. However, there are also contradictive reports. Zhang et al. reported that miR-144 promoted proliferation, migration, and invasion of nasopharyngeal carcinoma through repression of phosphatase and tensin homolog (PTEN) [16]. The

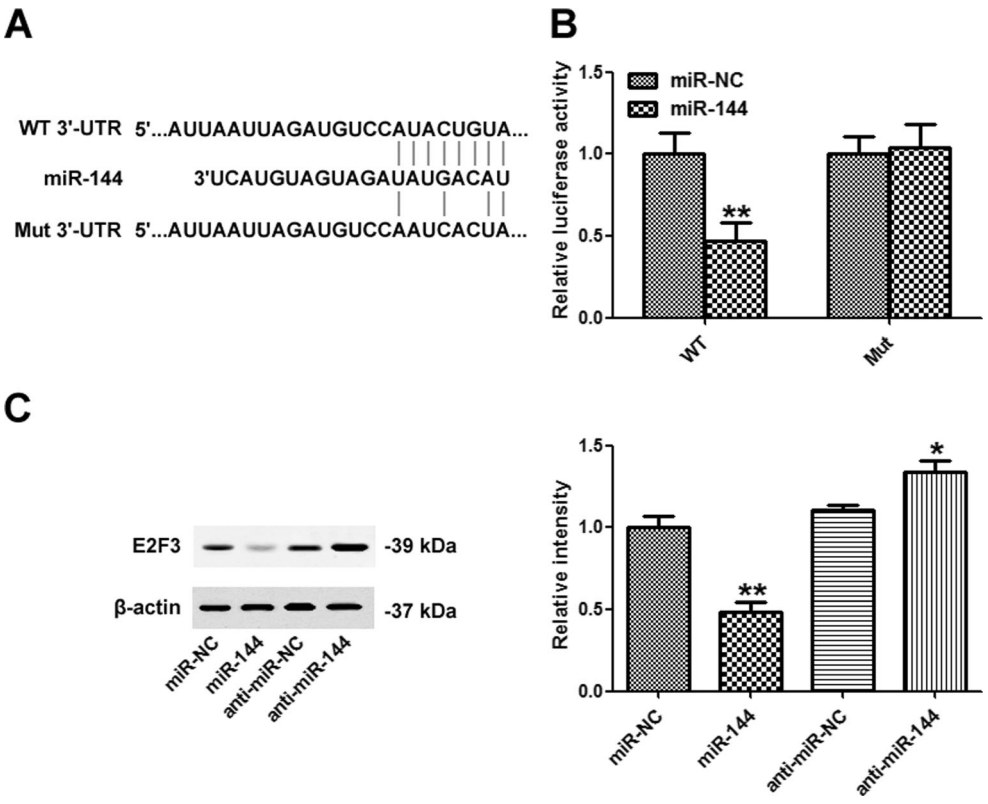

Fig. 4 E2F3 was a direct target of miR-144. **a** The wild type or the mutated sequences of the E2F3 mRNA 3′-UTR. **b** The luciferase activity of the wild type E2F3 3′-UTR (WT) and mutant E2F3 3′-UTR (Mut) co-transfected with miR-144 or miR-NC was measured. **c** The protein level of E2F3 in HepG2 cells transfected with miR-144 or miR-NC, miR-14 inhibitor (anti-miR-144), or control inhibitor (anti-NC) was determined by Western blot. Data were obtained in three independent experiments. *$P<0.05$; **$P<0.01$ compared with control

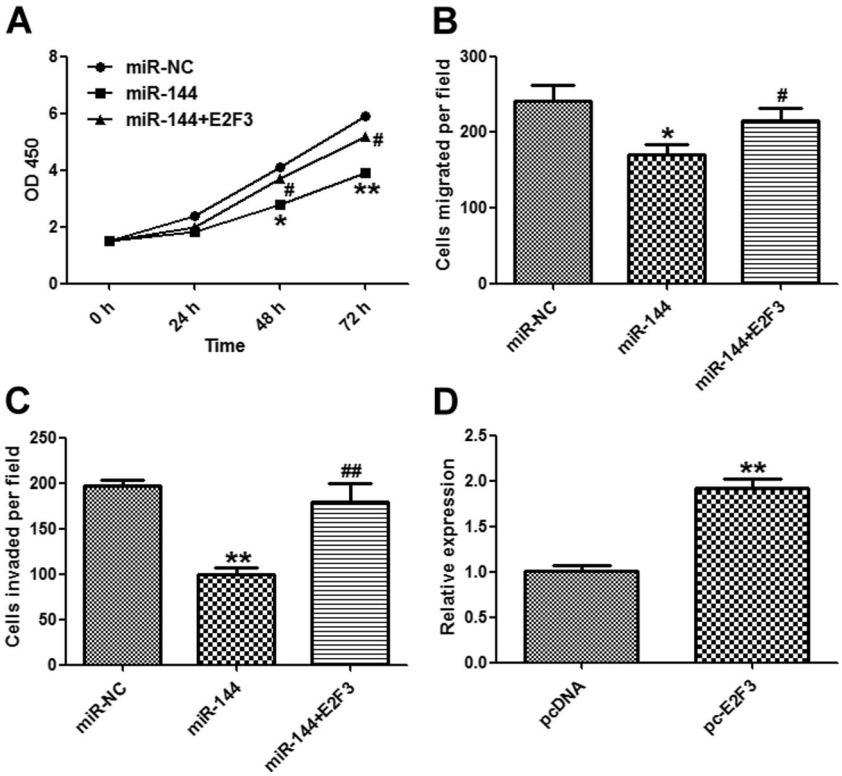

Fig. 5 E2F3 overexpression partially attenuated the tumor suppressive effects of miR-144. **a** HepG2 cells were transfected with miR-144 with/without E2F3 overexpression plasmid (pc-E2F3), and CCK-8 assay was performed. In vitro migration (**b**) and invasion (**c**) assays of HepG2 cells transfected with miR-144 with/without pc-E2F3. **d** qRT-PCR was used to detect the mRNA level of E2F3 in cells transfected with pc-E2F3 or the control. Data were obtained in three independent experiments. *$P<0.05$; **$P<0.01$ compared with control; #$P<0.05$ compared with miR-144 transfected group

function of miR-144 in carcinogenesis and cancer progression seems to be complicated and highly tissue-specific.

In this study, we detected the miR-144 expression in the 33 HCC samples. Forced overexpression of miR-144 remarkably reduced cell proliferation, increased apoptosis, and suppressed migration and invasion of HCC cells. Luciferase activity combined with Western blot confirmed that E2F3 was a direct target of miR-144 in HCC cells. Moreover, E2F3 overexpression partially attenuated the tumor suppressive effects of miR-144, and the expression of E2F3 was negatively correlated with miR-144 level in HCC tissues. E2F3, a member of E2F transcription factor family involved in the regulation of cell proliferation, is an oncogene located at 6p22 with strong proliferative potential [17]. E2F3 is involved in a variety of processes and plays critical roles in the development of several cancers, including prostate cancer, bladder cancer, and breast cancer [18–20]. E2F3 has been reported to be regulated by several miRNAs. Huang et al. reported that miR-125b suppressed colony formation of bladder cancer cells and tumor development in nude mice via inhibiting E2F3 [21]. Zhang et al. found that miR-195 inhibited proliferation and invasion of human glioblastoma cells by targeting E2F3 [22]. In HCC, Xiao et al. found that miR-503 suppressed HCC cell proliferation by inhibiting E2F3 [17]. Moreover, Zeng et al. also

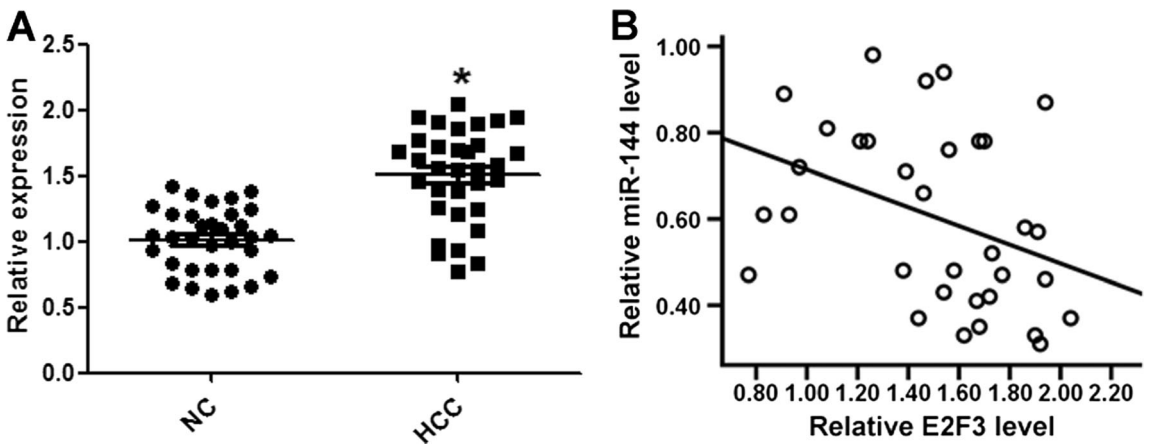

Fig. 6 miR-144 was inversely correlated with E2F3 in HCC. **a** E2F3 mRNA levels in HCC tissues were analyzed by qPCR. **b** Correlation of miR-144 expression and E2F3 mRNA level was analyzed ($P=0.014$, $r=-0.423$). *$P<0.05$ compared with control

showed that upregulation of E2F3 was associated with poor prognosis in HCC [23]. Our work expanded the role of miR-144 and E2F3 in HCC.

In conclusion, our study demonstrated the biological functions of miR-144, with the ability to suppress growth, migration, and invasion of HCC cells by targeting E2F3. This result suggested that miR-144 might be a helpful therapeutic strategy for HCC treatment in the future.

Conflicts of interest None

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