

MiR-3910 Promotes the Growth and Migration of Cancer Cells in the Progression of Hepatocellular Carcinoma

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Abstract

Introduction Previous studies have reported that specific depletion of mammalian sterile-like kinase (MST1) in the mouse liver driven Hepatocellular carcinoma (HCC). However, how the expression of MST1 was regulated in the progression of HCC remains largely unknown.

Materials and methods The expression of miR-3910 in the HCC tissues and cell lines were examined using q-PCR. The functions of miR-3910 in HCC were examined using MTT assay, Boyden chamber assay and soft agar assay. The effects of miR-3910 on the metastasis of HCC cells were evaluated using the mouse model.

Results Here, we have shown that miR-3910 regulated the expression of MST1. MiR-3910 was up-regulated in HCC samples and cell lines, and the expression of miR-3910 was induced by the oncogenic RasV12. In the functional study, miR-3910 was found to promote the growth and migration of HCC cells, and knocking down miR-3910 inhibited the metastasis of HCC cells. Mechanically, it was found that miR-3910 activated YAP signaling by targeting MST1.

Conclusion Taken together, this study demonstrated that miR-3910 exerted oncogenic effects on the progression of HCC and suggested that miR-3910 might be a therapeutic target for cancer therapy.

Keywords MiR-3910 · YAP · HCC · MST1 · Cell growth and migration

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1, 2], especially in China due to the prevalence of hepatitis B virus (HBV) [3]. Although the molecular-target therapeutic strategy has been developed, the prognosis for the HCC is still very poor [1]. Recent studies have demonstrated that several key pathways, such as wnt/beta-catenin signaling [3], Ras/ERK signaling, and YAP/TEAD signaling [4, 5], were aberrantly activated in the tumorigenesis of HCC, suggesting that understanding the regulation for these pathways might provide novel insights into the treatment of HCC.

Several studies have demonstrated the oncogenic roles of YAP/TEAD signaling in the tumorigenesis of HCC [6]. YAP is the key molecule in the YAP/TEAD pathway [6, 7]. In the normal cells, the activity and location of YAP are tightly regulated by LATS1 and LATS2, which phosphorylate several residues in the YAP protein and lead it to degradation [6]. In the cancer cells, inactivation of the upstream regulator leads to the accumulation of YAP in the cytoplasm. Then, the YAP protein trans-localized to the nucleus and regulated the expression of multiple apoptosis-related and migration-related genes, such as cyclin E, Cyr61, CTGF, survivin. MST1 (mammalian sterile-like Thr/Ser kinase) phosphorylates and activates LATS1 and LATS2, which lead to the inactivation of YAP protein [8]. It has been reported that knocking out MST1 and MST2 leads to the tumorigenesis of HCC [9], highlighting its suppressive roles in the cancer.

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The activity of MST1 has been regulated at multiple levels. It has been reported that phosphorylation of MST at serine 385 in mitosis inhibited its tumor-suppressing activity [10]. In addition, heterodimerization of MST1 and MST2 abolished its tumor suppression activity [11].

In recent years, the roles of microRNA in the development of hepatocellular carcinoma have been widely accepted. A serum microRNA panel has been found as biomarkers for the diagnosis of hepatocellular carcinoma [12]. MiR-155 has been reported to target PI3K/AKT pathway [13]. Moreover, numerous microRNAs have been demonstrated to regulate the growth, apoptosis, and migration of HCC cells [14, 15]. With regard to YAP signaling, the expression of MST1 has been shown to be regulated by miR-138 and miR-199a-5p in the remodeling of vasculature and hepatocyte differentiation [16–18]. However, whether the expression of MST1 could be regulated by other microRNAs in the tumorigenesis of HCC remains to be elucidated.

In this study, we turned to the bioinformatics analysis, searched the Target Scan database, and found MST1 as a potential target for miR-3910. We have examined the expression and functions of miR-3910 in HCC and investigated the regulation of MST1 by miR-3910.

Materials and Methods

Cell Culture

The normal liver cell lines (L02 and Chang) and HCC cell lines (7404, huh-7, Hep3B, HepG2, QGY, and MHCC97) were derived from ATCC (American Typical Culture Center). Cells were cultured in DMEM containing antibiotics and 10% FBS and incubated in the atmosphere containing 5% CO₂.

Clinical HCC Samples

Human clinical HCC samples were collected from the patients who received surgery at the First Affiliated Hospital of Zhengzhou University. This study was approved by the ethics committee of Zhengzhou University, and the written informed consent was obtained from the patients. Tissues were kept at –80 °C in a freezer.

qPCR

Total RNA and miRNA were isolated using microRNA Extraction Kit (Qiagen, Hilden, Germany). After the reverse transcription following the manufacturer's instructions, qPCR was performed using a Stratagene

MNeasy Mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. Complementary DNA (cDNA) was randomly primed from 2 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription quantitative PCR (RT-PCR) was subsequently performed in triplicate with QuantiTech SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using the ΔCt (U6-miR-3910) for the quantification of the relative mRNA expression level. The forward primer for miR-3910 is 5'-ctttgctgtcagttttct-3', and the reverse primer for miR-3910 is 5'-cttctgtgtaacatcctttt-3'.

Plasmid Construction

The coding sequence of MiR-3910 was inserted into the expression vector pCDH. The sequence for anti-miR-3910 is 5'-tgtcttggtttatgccttt-3'. The coding sequence of RasV12 and MST1 was cloned into the expression vector P23-Flag. Anti-miR-3910 was purchased from GeneChem (Shanghai, China). The expression vectors were transfected into 7404 and Huh-7 cells using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Cells were selected with puromycin, and the resistant cells were pooled to confirm the expression of exogenous miR-3910 using qPCR.

Western Blot

The protein from the cell culture was extracted using RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors. Extracts were centrifuged. After being separated by 10% SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with the primary and secondary antibodies. The immunoreactive protein bands were visualized by ECL kit (Pierce). Antibody to MST1 (1:5000) was purchased from Abcam, and antibodies to GAPDH (1:5000) and Flag tag were purchased from Santa Cruz Biotechnology. Secondary antibodies, mouse IgG and rabbit IgG, were obtained from Cell Signaling Technology.

MTT Assay

MTT assay was used to examine the growth of 7404 and Huh-7 cells. Equal numbers of cells were seeded in 48-well plates containing 500 µl DMEM and cultured for various durations. Everyday, 50 µl MTT was added into the medium and incubated with cells for 4 h. Then, the supernatant was cleared and cells were resolved with 500 µl DMSO. OD540nm was measured.

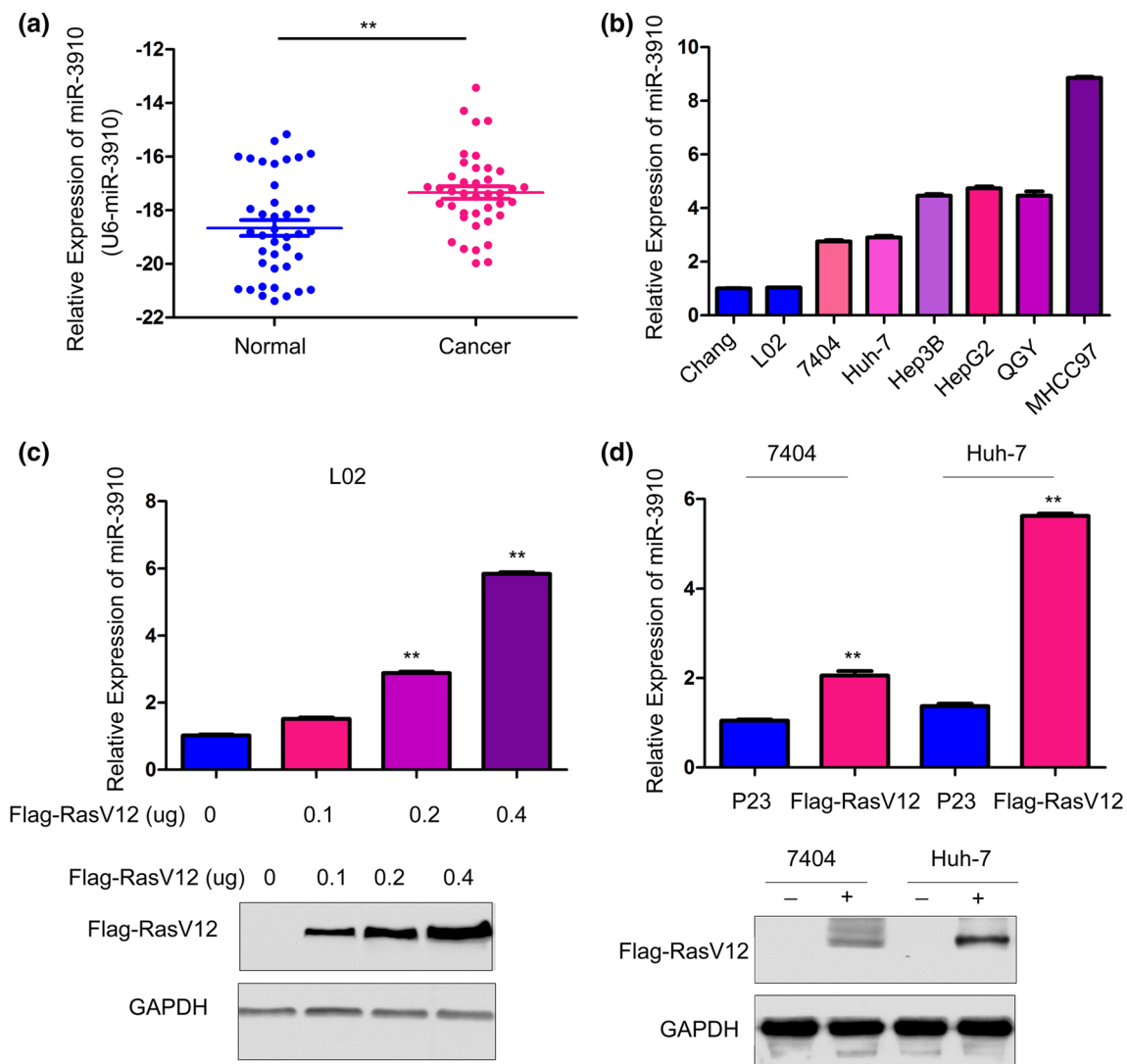


Fig. 1 Expression of miR-3910 was elevated in human HCC. **a** The mRNA level of miR-3910 in 40 human HCC tissues and paired non-cancerous tissues was determined using qPCR. U6 was used as an internal control. **b** The mRNA level of miR-3910 in human normal liver cells (Chang and L02) and HCC cells (Hep3B, Huh-7, HepG2, 7404, QGY, and MHCC97) was determined using qPCR. **c** Oncogenic RasV12 induced the expression of miR-3910 in L02 cells. L02 cells

were transfected with a dosage of RasV12 expression vector, and the mRNA level of miR-3910 was examined using qPCR. **d** Oncogenic RasV12 induced the expression of miR-3910 in 7404 and Huh-7 cells. 7404 and Huh-7 cells were transfected with RasV12 expression vector, and the mRNA level of miR-3910 was examined using qPCR. ** $P < 0.01$

Cell Migration Assay

Cell migration was evaluated using Boyden chamber. In the upper chamber, cells (2×10^5) were suspended in 0.05 ml medium containing 1% FBS and placed. The lower chamber was loaded with 0.152 ml medium containing 10% FBS. Eight hours later, cells that migrated to the lower surface of filters were detected with traditional H&E staining. The migrated cells were counted under the inverted microscope. The experiments were repeated for three times.

Cell Apoptosis Assay

Cells were fixed with 75% ethanol solution at 4 °C over night. After centrifugation, cells were treated with RNase and stained with annexin V and PI for 1 h. The cells were examined using BD Caliber (BD Bioscience).

Animal Experiment

Orthotopic tumors were established by the direct intra-hepatic injection of 7404/miR-con cells or 7404/anti-

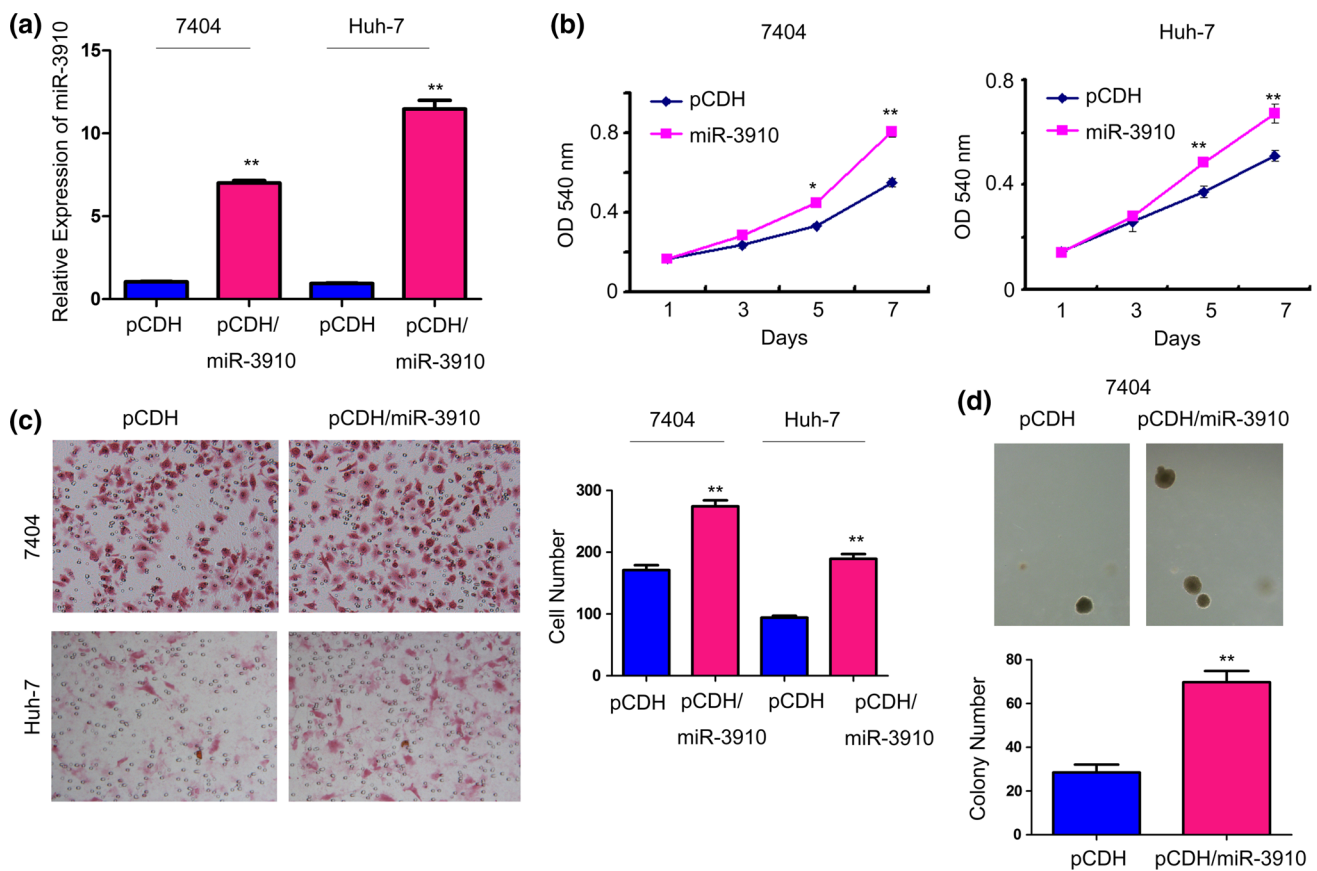


Fig. 2 MiR-3910 promoted the growth and migration of the HCC cells. **a** Overexpression of miR-3910 mimics in 7404 and Huh-7 cells. qPCR analysis was performed to determine the expression of miR-3910 in 7404 and Huh-7 cells after the stable transfection. **b** MTT assay to examine the effects of miR-3910 on the growth of 7404 and

Huh-7 cells. **c** Overexpression of miR-3910 promoted the migration of 7404 and Huh-7 cells in Boyden chamber assay. **d** Forced expression of miR-3910 enhanced the anchorage-independent growth of 7404 cells. * $P < 0.05$; ** $P < 0.01$

miR-3910 cells. Briefly, the left hepatic lobe of each mouse under isoflurane anesthesia was exposed through a small (8–10 mm) transverse incision made in the left cranial abdomen beginning 2–3 mm below the xyphoid process and extending to the left. One million 7404/miR-con cells or 7404/anti-miR-3910 cells, in a total volume of 0.02 ml of a serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA), were slowly injected into the liver through the diaphragmatic surface with a 28-gauge needle. The needle was inserted at a shallow angle so that a gentle injection produced a visible, translucent, subcapsular bleb on the liver surface. After injection, a sterile cotton swab was placed on the needle insertion site as the needle was withdrawn, and gentle pressure was applied for 1 min to ensure hemostasis. The abdominal musculature and skin were then closed with the absorbable suture material and sterile surgical clips, respectively. Six weeks later, the mice were killed and tumor number was counted.

Statistics Analysis

Each experiment was repeated at least three times. The data presented as mean \pm SD and statistical comparisons were made using Student's *t* test. Significance was presented as ** $P < 0.01$ or * $P < 0.05$.

Results

Expression of miR-3910 Was Elevated in the Hepatocellular Carcinoma (HCC)

The expression of miR-3910 in cancer has never been addressed before. To study the expression pattern of miR-3910 in the HCC, we first expressed the mRNA level of miR-3910 in 40 clinical HCC tissues and the paired non-cancerous tissues by qPCR. As shown in Fig. 1a, the mRNA level of miR-3910 was significantly higher in HCC

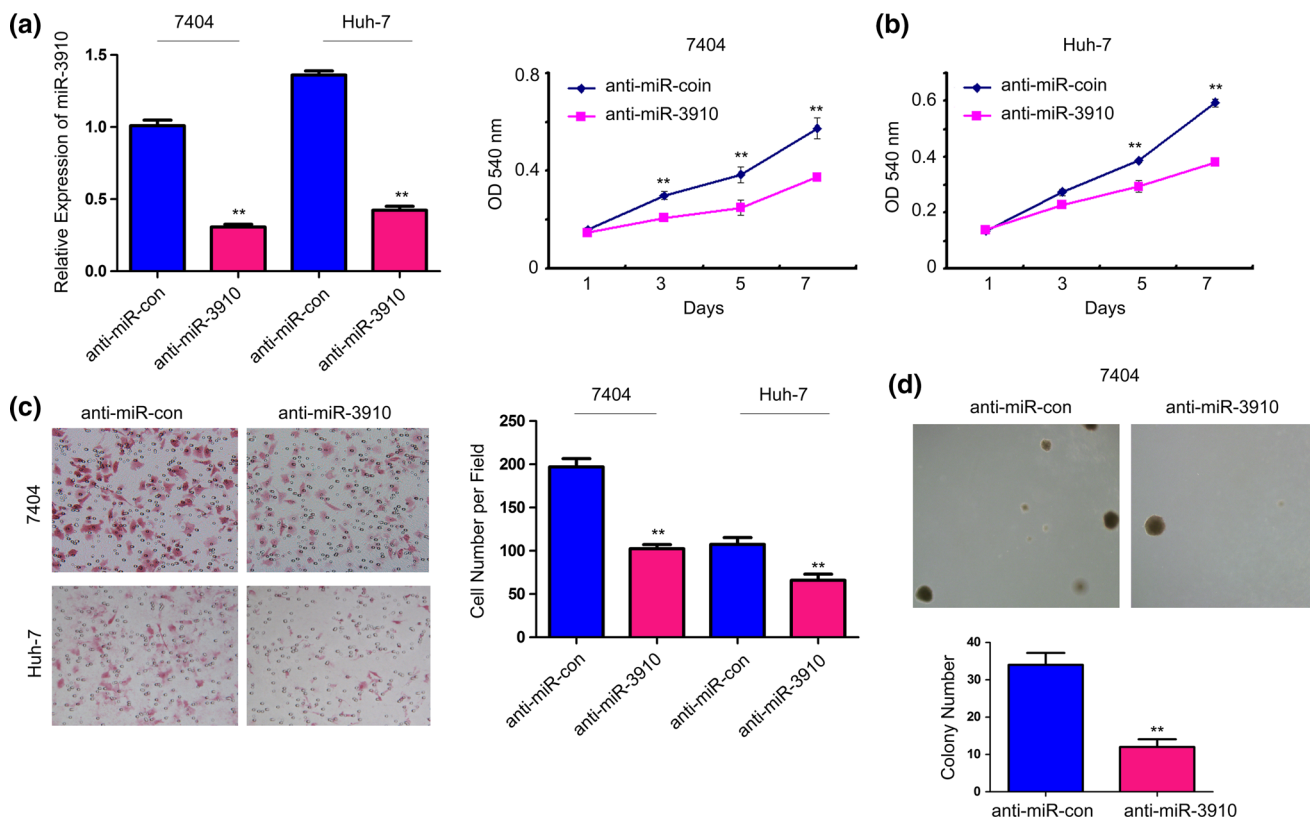


Fig. 3 Inhibition of miR-3910 impaired the growth and migration of 7404 and Huh-7 cells. **a** qPCR analysis was performed to determine the expression of miR-3910 in 7404 and Huh-7 cells after the transfection of anti-miR-3910. **b** Inhibiting the function of miR-3910 impaired the growth of 7404 and Huh-7 cells by MTT assay.

tissues (Fig. 1a). In addition, the mRNA level in normal liver cells and HCC cells was examined. Low expression of miR-3910 was observed in normal liver cell line (Chang and L02), and high miR-3910 was found in the HCC cell lines (7404, Huh-7, Hep3B, HepG2, QGY, and MHCC97) (Fig. 1b).

Activation of Ras-ERK signaling is frequently observed in HCC. Therefore, we next examined whether Ras regulated the expression of miR-3910 in HCC cells. As shown in Fig. 1c, overexpression of RasV12 in normal liver cell line L02 induced the expression of miR-3910 in a dose-dependent manner (Fig. 1c). Consistently, up-regulation of RasV12 in HCC cell lines 7404 and Huh-7 also elevated the expression of miR-3910 (Fig. 1d). Taken together, these observations suggested the expression of miR-3910 in HCC possibly by the overactivation of Ras signaling.

MiR-3910 Promoted the Growth, Migration, and Anchorage-Independent Growth of HCC Cells

To elucidate the biological roles of miR-3910 in the progression of HCC, we first introduced the expression of miR-3910 mimics in 7404 and Huh-7 cells (Fig. 2a). The

c Inhibiting the function of miR-3910 impaired the migration of 7404 and Huh-7 cells. **d** Inhibiting the function of miR-3910 repressed the anchorage-independent growth of 7404 cells in the soft agar assay. * $P < 0.05$; ** $P < 0.01$

effects of miR-3910 on the growth, migration, and anchorage-independent growth of HCC cells were evaluated by MTT assay, Boyden chamber assay, and soft agar assay. As shown in Fig. 2, overexpression of miR-3910 promoted the proliferation, migration, and anchorage-independent growth of 7404 and Huh-7 cells (Fig. 2b–d), suggesting the oncogenic roles of miR-3910 in HCC.

Next, we knocked down the expression of endogenous miR-3910 by anti-miR-3910 (Fig. 3a). Treating HCC cells with anti-miR-3910 inhibited the growth of 7404 and Huh-7 cells in the MTT assay (Fig. 3b). Moreover, knocking down the expression of miR-3910 impaired the migration of 7404 and Huh-7 cells (Fig. 3c). In addition, down-regulation of miR-3910 attenuated the anchorage-independent growth of 7404 cells (Fig. 3d). Taken together, these data demonstrated that MiR-3910 promoted the growth, migration, and anchorage-independent growth of HCC cells.

MiR-3910 Activated Yap Signaling by Targeting MST1

To explore the underlying molecular mechanism through which miR-3910 promoted the growth and migration of

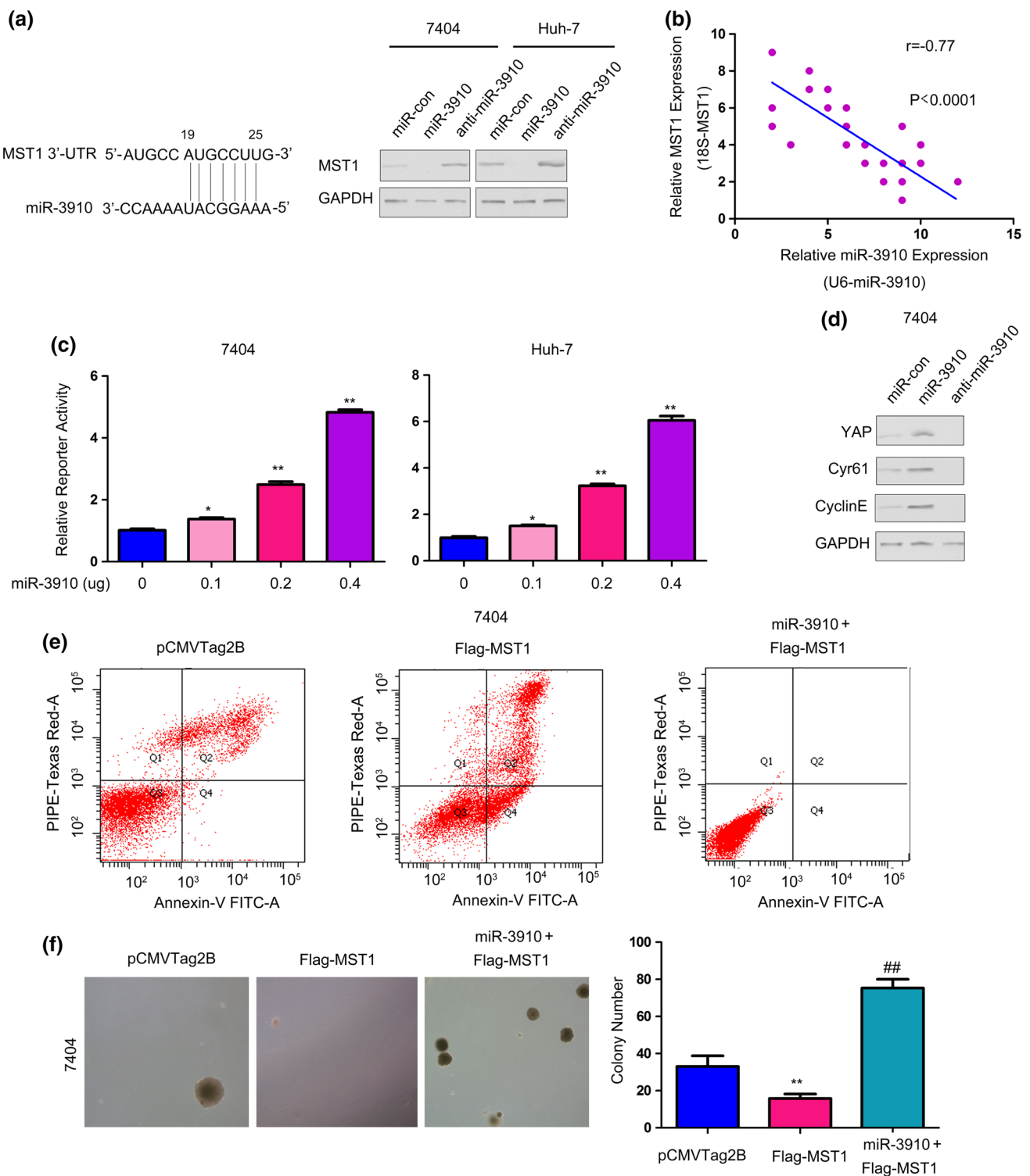
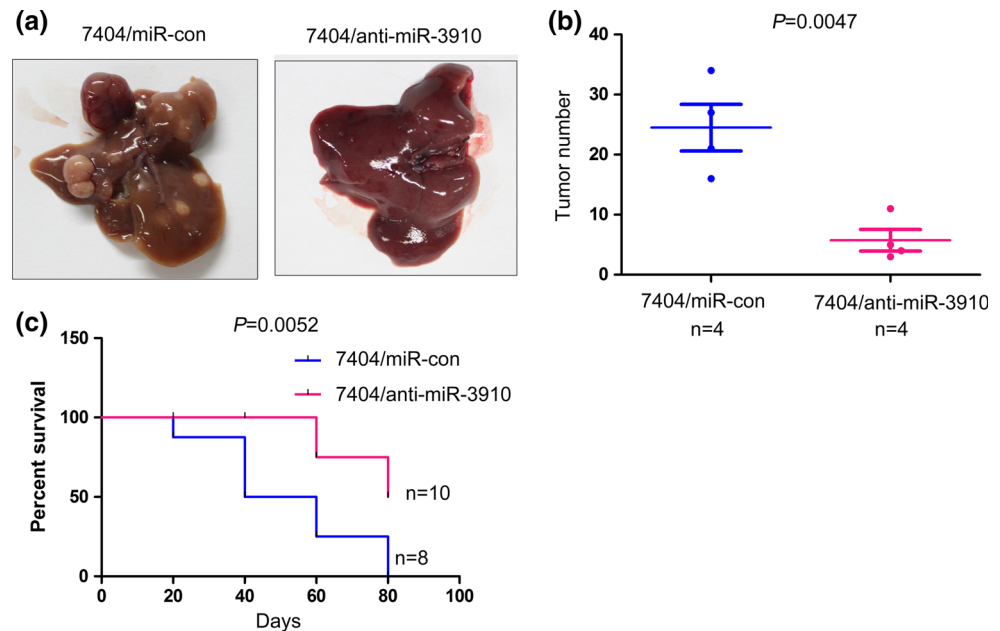


Fig. 4 MiR-3910 targeted MST1 to activate YAP signaling in HCC. **a** A schematic illustration for the binding between miR-3910 and MST1 3'-UTR. Western blot analysis shows the protein level of MST1 in 7404 and huh-7 cells treated with miR-3910 or anti-miR-3910. **b** The mRNA level of MST1 inversely correlated with miR-3910 in HCC samples. **c** MiR-3910 activated the reporter for YAP

signaling in the luciferase assay. **d** MiR-3910 and anti-miR-3910 regulated the expression of target gene downstream of YAP signaling. **e** MiR-3910 rescued cell apoptosis induced by the overexpression of MST1. **f** MiR-3910 rescued the anchorage-independent growth of 7404 cells induced by the overexpression of MST1. * $P < 0.05$; ** $P < 0.01$

Fig. 5 Knocking down the expression of miR-3910 inhibited the intrahepatic metastasis of HCC. **a, b** The intrahepatic metastasis was performed to examine the roles of miR-3910 in the metastasis of HCC cells. The morphology of livers is shown in **a**, and the tumor number is quantified in **b**. **c** Survival curve of the mice in the intrahepatic metastasis assay. * $P < 0.05$; ** $P < 0.01$



HCC cells, we turned to the bioinformatics analysis. Searching the Target Scan database revealed that MST1, the regulator for the Yap signaling, was a potential target for miR-3910. To verify this hypothesis, we first examined whether miR-3910 mimics or anti-miR-3910 modulated the expression of MST1. As shown in Fig. 4a, treating 7404 and Huh-7 cells with miR-3910 significantly down-regulated the expression of MST1, while anti-miR-3910 transfection slightly up-regulated the expression of MST1 (Fig. 4a). Moreover, in the HCC tissues, the mRNA level of MST1 was negatively correlated with the expression of miR-3910 (Fig. 4b). These results suggested the regulation of MST1 by miR-3910.

We next examined the regulation of YAP signaling by miR-3910. MiR-3910 activated the reporter for YAP/TEAD signaling in a dose-dependent manner (Fig. 4c). Consistent with this finding, the expression of miR-3910 up-regulated the expression of YAP and several target genes downstream of YAP signaling, such as Cyr61 and cyclin E (Fig. 4d), while anti-miR-3910 down-regulated the expression of YAP, Cyr61, and cyclin E. These findings suggested that miR-3910 activated the YAP/TEAD signaling. Moreover, the apoptosis and the repression of anchorage-independent growth of 7404 cells induced by overexpression of MST1 could be rescued by miR-3910 (Fig. 4e, f). Collectively, these observations indicated that miR-3910 promoted the growth and migration of HCC cells possibly by targeting MST1.

Knocking Down MiR-3910 Inhibited the Tumorigenesis of HCC in the Intrahepatic Metastasis Mouse Model

To explore the function of miR-3910 in vivo, we turned to the intrahepatic metastasis mouse model. 7404 cells over-expressing anti-miR-3910 (7404/anti-miR-3910) were injected into the one of the liver lobes. As the gross morphology shown in Fig. 5A, injection of 7404 cells into one of the liver lobes led to significant intrahepatic metastasis. However, knocking down the expression of miR-3910 using anti-miR-3910 inhibited the tumor formation in other lobes (Fig. 5a, b). Consistently, knocking down the expression of miR-3910 prolonged the survival of the nude mice (Fig. 5c). Taken together, these studies demonstrated that knocking down miR-3910 inhibited the tumorigenesis of HCC.

Discussion

The roles of microRNA in the tumorigenesis of HCC have been well documented [19–21]. Numerous studies have shown that dys-regulation of microRNA promoted the growth, migration, and metastasis of HCC cells [22]. In this study, we have shown that the expression of miR-3910 was elevated in the HCC clinical samples and cell lines. MiR-3910 promoted the growth of HCC cells both in the liquid

culture and on the soft agar. Moreover, miR-3910 was a positive regulator for cell motility, which was demonstrated by Boyden chamber assay and intrahepatic metastasis assay. These observations clearly demonstrated the oncogenic roles of miR-3910 in the tumorigenesis of HCC.

Another important finding of this study is that oncogenic RasV12 signaling regulated the expression of miR-3910. Overactivation of Ras signaling was observed in about 90% HCC clinical samples [4]. However, targeting Ras signaling is unsuccessful due to the lack of effective inhibitors. Therefore, identifying the effectors downstream Ras would benefit the therapy for HCC. In this study, we have shown that RasV12 induced the expression of miR-3910 in both the normal liver cells and HCC cells, suggesting the universe regulation of miR-3910 by RasV12. As a result, miR-3910 might be a target for the treatment of HCC with Ras overactivation.

Activation of YAP signaling in the tumorigenesis of HCC has been recognized recently [23, 24]. Knocking out MST1, MST2, and SAV induced the tumor formation in the mouse liver [9], emphasizing the pivotal function of YAP signaling in the initiation of HCC. Therefore, understanding the regulation of YAP signaling would be helpful for the cancer therapy. In this study, we have found that miR-3910 regulated the expression of MST1 and rescued the apoptosis induced by MST1, which confirmed that miR-3910 exerted its oncogenic roles by targeting MST1.

The expression of MST has been reported to be regulated by MiR-199 [16]. Consistent with our observations, miR-199 has been shown to promote the liver fibrosis [25]. Moreover, the potential roles of miR-199 in the diagnosis of hepatocellular carcinoma [26]. Also, miR-199 has been to regulate the progression of HCC by targeting ROCK1 [27]. These data further supported the notion that targeting the expression of MST promoted HCC.

In summary, although our results are very indicative, further studies using miR-3910 knocking out mice would provide more insights into the roles of miR-3910.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Ethical approval research involving animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research involving human participants All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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