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RESEARCH ARTICLE

microRNA-4270-5p inhibits cancer cell proliferation and metastasis in hepatocellular carcinoma by targeting SATB2

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Abstract

Hepatocellular carcinoma (HCC) remains a lethal cancer type for both males and females. MicroRNAs (miRNAs) contribute to the initiation, development and metastasis of cancer. Although several miRNAs have been identified as drivers or suppressors of HCC, the molecular mechanisms of many miRNAs have not been investigated. Currently, we discovered that miR-4270-5p was a significantly downregulated miRNA in HCC. We revealed that miR-4270-5p overexpression inhibited cell proliferation and invasion of HCC cells. The data manifested that miR-4270-5p directly targeted SATB2, a key regulator of epithelial mesenchymal transition (EMT), in HCC cells and reversed the EMT process. The rescue experiments suggested that SATB2 overexpression reversed the biological function of miR-4270-5p in HCC cells. Clinical data indicated that SATB2 expression was negatively correlated with miR-4270-5p levels in HCC patients. Our findings provided potential targets for prognosis and treatment of patients with HCC.

Keywords microRNA-4270-5p · Hepatocellular carcinoma · SATB2

Introduction

In 2018, there was an estimated 841,000 newly diagnosed liver cancer cases worldwide, being the sixth leading cause of cancer related death for both sexes [1]. Hepatocellular carcinoma (HCC) is the most commonly diagnosed liver cancer type [2]. It is known that chronic infection with hepatitis B virus (HBV), alcohol intake, smoking are risk factors for HCC [3]. The convention treatment for patients with HCC included surgery removal of tumors and liver transplantation [4]. However, due to the aggressive nature of HCC cells, patients with HCC often develop recurrence and metastasis and the prognosis of these patients remains dismal [5, 6]. It is urgent to investigate the molecular mechanism of HCC to provide novel targets for diagnosis and treatment of patients.

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microRNAs (miRNAs) are short, single-stranded nucleotides with no protein coding potential [7]. Via directly binding to complementary sites in the 3′UTR of gene mRNAs, miRNAs facilitate mRNA degradation and inhibition of translation [8]. Transcriptomic studies have revealed that many miRNAs were differentially expressed in HCC compared with normal tissues [9]. Accumulating evidences suggested that miRNAs were involved in HCC progression via targeting key oncogenes and tumor suppressors [10, 11, 12]. For example, miR-142-3p was a downregulated miRNA in HCC and inhibited glycolysis and proliferation of HCC cells via targeting LDHA [13]. Microarray identified many differentially expressed miRNAs in HCC [14]. However, the function of several miRNAs has not been studied yet.

SATB2 (special AT-rich binding protein-2) is a cancer-associated transcription factor [15]. Upregulation of SATB2 was observed in several cancer types [16, 17]. Recent studies showed that SATB2 facilitated epithelial mesenchymal transition (EMT) process and enhanced metastatic ability of cancer cells [18]. SATB2 was elevated in HCC samples and its expression was associated with TNM stage and vascular invasion [19]. The regulation of SATB2 in HCC remains elusive.

In the present study, we re-analyzed the previous published data and found miR-4270-5p as a downregulated

miRNA in HCC. Our current study aimed to explore the expression and molecular mechanism of miR-4270-5p in HCC.

Materials and methods

Patient samples

HCC tumors and matched non-tumor liver tissues were collected from 50 patients with HCC during June 2014 to September 2018 in the China-Japan Union Hospital of Jilin University. These patients provided written informed consent before surgery. They received no chemotherapy or radiotherapy before the surgery. The tissue samples were confirmed by two histopathologists and staged according to 8th edition of AJCC [20]. The protocol of the study was reviewed and approved by the Ethical Committee of the China-Japan Union Hospital of Jilin University (Approval number: 2014CJUH0603). Samples were immediately stored in − 80 ℃ refrigerator.

Cell culture and reagent

The immortalized liver cell line THLE-2 and HCC cell lines (Huh7, MHCC97, Hep3B) were purchased from American Type Culture Collection (Manassas, VA). These cells were maintained in DMEM (Gibco; Thermo Fisher Scientific) with 10% FBS (Gibco; Thermo Fisher Scientific) in a 37 ℃ humid incubator with 5% CO_2. miR-NC mimic and miR-4270-5p mimic were synthesized by Ribo Bio (Guangzhou, China). miR-NC mimic or miR-4270-5p mimic was transfected into cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The cells were cultured for another 48 h and then subjected to RT-qPCR for validation of transfection efficiency.

Overexpression of SATB2

The open reading frame (ORF) of SATB2 was PCR amplified from Huh7 cDNA and ligated into expression plasmid (pcDNA3). For overexpression, pcDNA3 or pcDNA3–SATB2 was transfected into cells using Lipofectamine 3000 (Invitrogen) following manufacturer's protocol. The cells were cultured for additional 48 h. After that, cells were harvested and subjected to western blotting and RT-qPCR.

miRNA target prediction

The potential target genes of miR-4270-5p was predicted on miRDB software (https://www.mirdb.org/). It was also used to analyze the putative binding site between them.

Cell proliferation assay

The proliferation ability of cells was determined with a CCK-8 kit (DoJinDo Biotech, Shiga, Japan). The treated cells were maintained in wells of 96-well plates for 0, 24, 48, 72 and 96 h. 10 µL CCK-8 was added to the cells and incubated for an additional 2 h. The absorbance of culture medium at 450 nM was recorded using a Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell invasion assay

The invasion ability of cells was determined with the Transwell chambers (pore size of 8 µm) (Corning, Corning, NY, USA). Before the experiment, the membrane was pre-coated with Matrigel (200 ng/ml) (Invitrogen). 1×10^4 cells were suspended in DMEM medium with no FBS and seeded in the upper chamber. The lower chamber was added with DMEM with 10% FBS. After cultured for 48 h, the chamber was removed from the 24-well plates and the cells attached to the upper side of chamber was removed. The cells attached to the lower side of chamber was fixed with 4% paraformaldehyde (PFA) at room temperature for 30 min. The chamber was further stained in 1% crystal violet at room temperature for 30 min. Three random fields of each group were selected and observed by a microscope (Olympus Corporation). The cell number was counted and calculated.

Protein extraction and western blotting

Total proteins of cells were extracted with RIPA lysis buffer (Thermo Fisher Scientific). The lysates were quantified with a BCA Protein Assay kit (Thermo Fisher Scientific). 20 µg lysates were separated on an 8% SDS-PAGE gel and transferred to PVDF membranes. The membrane was blocked in 5% non-fat milk at room temperature for 30 min and then incubated in SATB2, E-cadherin (AB40680, 1:2000, AbSci, College Park, MD), Vimentin (AB41533, 1:2000, AbSci), MMP19 (ab53146, 1:1000, Abcam, Cambridge, UK) and GAPDH (ab8245, 1:5000, Abcam) antibodies for at room temperature 2 h. After that, membrane was incubated in HRP-conjugated antibodies against mouse (ab97040, 1:10,000, Abcam) and rabbit (ab7090, 1:10,000, Abcam) at room temperature for 2 h. The membrane was then developed with the ECL Western Blotting Substrate (Thermo Fisher Scientific). The immunoreactive bands were quantified with Image J software 1.5.1 (National Institute of Health) and normalized to GAPDH.

RNA extraction and RT-qPCR

Total RNA of tissues and cells were extracted using TRIzol reagent (Invitrogen). RNA was reverse transcribed to

cDNA with M-MLV reverse transcriptase (Invitrogen). For semi-quantification of miR-4270-5p, the RT-qPCR was carried out with miRcute qPCR detection kit (Tiangen, Tianjin, China) and the miR-4270-5p levels were normalized to U6 levels using the $2^{-\Delta\Delta Cq}$ method [21]. For detection of mRNA levels, RT-qPCR was performed with TB Green™ Prime-Script PLUS RT-PCR Kit (TaKaRa Bio, Tokyo, Japan) and the mRNA expression of SATB2, E-cadherin, N-cadherin, Vimentin, Fibronectin were normalized to GAPDH expression using the $2^{-\Delta\Delta Cq}$ method [21]. The primer sequences are as follows: miR-4270-5p-forward:5′-GCCGAGTCA GGGAGTCAGGG-3′; miR-4270-5p-reverse:5′-CTCAAC TGGTGTCGTGGA-3′; U6-forward:5′-CTCGCTTCG GCAGCACA-3′; U6-reverse:5′-AACGCTTCACGAATT TGCGT-3′; SATB2-forward:5′-GCAGTTGGACGGCTC TCTT-3′; SATB2-reverse:5′-CACCTTCCCAGCTTGATT ATTCC-3′; E-cadherin-forward:5′-AAAGGCCCATTT CCTAAAAACCT-3′; E-cadherin:5′-TGCGTTCTCTAT CCAGAGGCT-3′; N-cadherin-forward:5′-AGCCAACCT TAACTGAGGAGT-3′; N-cadherin-reverse:5′-GGCAAG TTGATTGGAGGGATG-3′; Vimentin-forward:5′-TGC CGTTGAAGCTGCTAACTA-3′; Vimentin-reverse:5′-CCAGAGGGAGTGAATCCAGATTA-3′; Fibronectin-forward:5′- AGGAAGCCGAGGTTTTAACTG-3′; Fibronectin-reverse:5′- AGGACGCTCATAAGTGTCACC-3′. MMP19-forward:5′-GCTTCCTACTCCCCATGACAG-3′; MMP19-reverse:5′- CCCATATTGTGACAGGTAGTCCA-3′. GAPDH-forward:5′-CTGGGCTACACTGAGCACC-3′; GAPDH-reverse:5′- AAGTGGTCGTTGAGGGCAATG-3′.

Dual luciferase reporter assay

The 3′UTR of SATB2 containing predicted binding site for miR-4270-5p was amplified from Huh7 cDNA and cloned into pmirGLO plasmid. A mutant form of SATB2 3′UTR was constructed using the QuikChange Site-directed Mutagenesis kit (Agilent Technology, Santa Clara, CA). SATB2 3′UTR-WT or 3′UTR-Mut was co-transfected with miR-NC mimic or miR-4270-5p mimic into cells with Lipofectamine 3000. Following 48 h transfection, the relative luciferase activity of each group was detected with a Dual Luciferase Reporter System kit (Promega. Corporation, Madison, WI, USA).

Statistical analysis

The data were analyzed with GraphPad Prism 6 and p value less than 0.05 was statistically significant. Every experiment was repeated three times and the data were presented as mean ± SD. A two-tailed Student's t test was used to analyze two groups. One-way ANOVA was employed to analyze three groups followed by a Newman–Keuls post-hoc test.

Results

miR-4270-5p was a downregulated miRNA in HCC

Previous microarray analysis suggested that miR-4270-5p was one of most significantly downregulated miRNA in HCC [22]. To verify the finding, we collected 50 pairs of tumors and non-tumor liver tissues from patients with HCC. Consistently, the RT-qPCR data indicated that miR-4270-5p was significantly decreased in HCC tumors compared with non-tumor liver tissues (Fig. 1a). Furthermore, we found that miR-4270-5p was remarkably decreased in HCC samples of advanced stage (III–IV) compared with those of early stage (I–II) (Fig. 1b). The miR-4270-5p expression was also lower in tumors with venous infiltration compared with their counterparts (Fig. 1c). Expression of miR-4270-5p was not associated with age, sex, HBsAG status and cirrhosis status of patients (Table 1). The expression of miR-4270-5p in THLE-2, an immortalized liver cell lines, is significantly higher in comparison with a panel of HCC cell lines (Huh7, MHCC97, Hep3B) (Fig. 1d). Thus, miR-4270-5p was indeed a downregulated miRNA in HCC and its expression was associated with tumor progression and metastasis.

miR-4270-5p overexpression inhibited HCC cell proliferation and invasion

To study the function of miR-4270-5p, we transfected miR-4270-5p mimic into HCC cells. As detected by RT-qPCR, miR-4270-5p mimic greatly increased miR-4270-5p levels in Huh7 and MHCC97 cells (Fig. 2a). In the cell proliferation assay, we found that miR-4270-5p overexpression repressed cell proliferation in Huh7 and MHCC97 cells (Fig. 2b, c). In the cell invasion assay, it was further found that miR-4270-5p overexpression repressed cell invasion in Huh7 and MHCC97 cells (Fig. 2d, e). The data showed that miR-4270-5p was involved in HCC cell proliferation and invasion.

miR-4270-5p controlled the expression of key genes in epithelial mesenchymal transition (EMT)

EMT process was essential for high invasive capability of HCC cells [23]. Since we found an association between miR-4270-5p expression and metastasis of HCC cells, we next detected the expression of EMT markers in HCC cells treated with miR-4270-5p mimic or miR-NC mimic. Interestingly, RT-qPCR showed that miR-4270-5p overexpression increased epithelial gene expression (E-cadherin) and decreased mesenchymal gene expression (N-cadherin, Vimentin, Fibronectin) in Huh7 and MHCC97 cells (Fig. 3a,

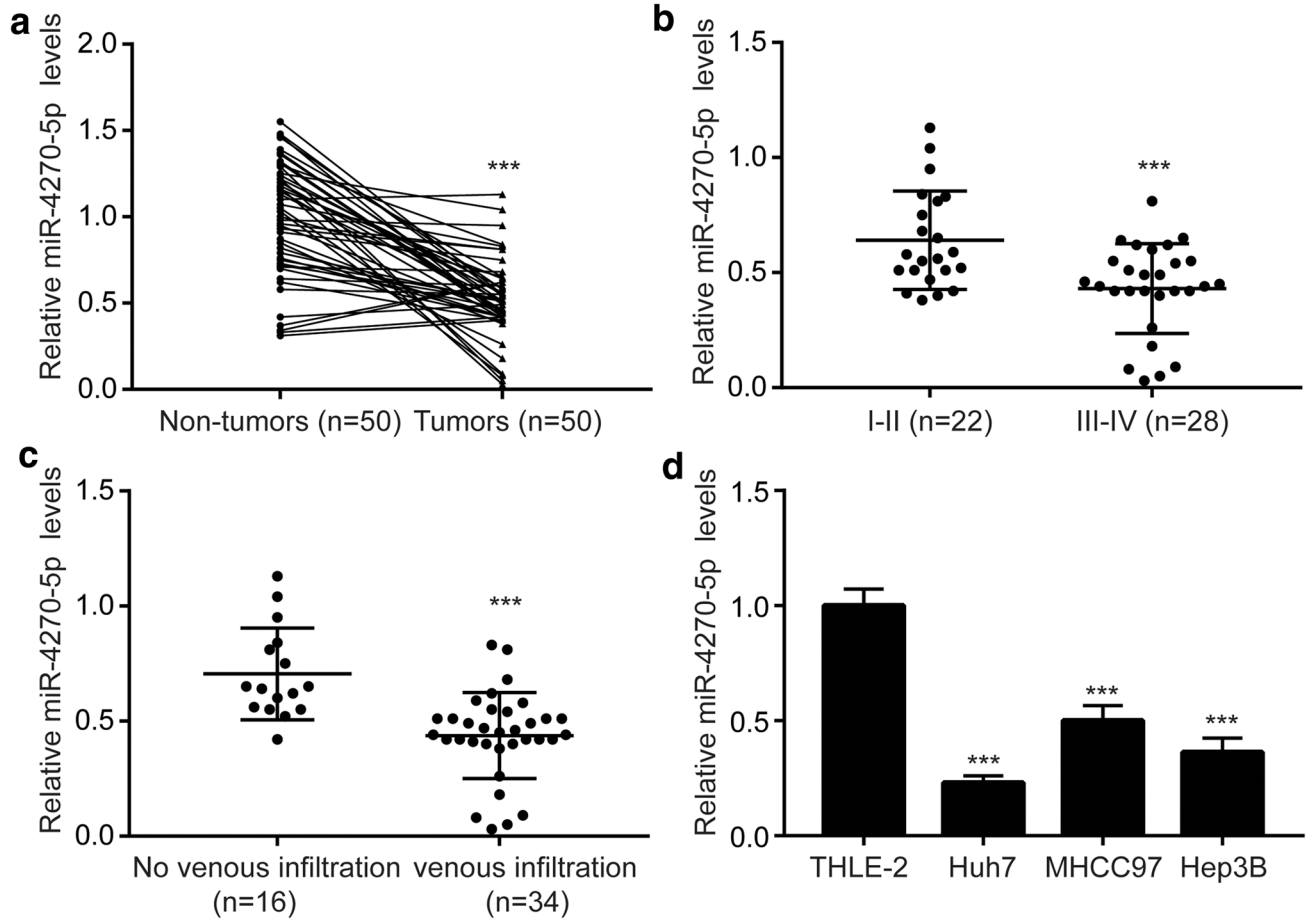

Fig. 1 miR-4270-5p was downregulated in hepatocellular carcinoma (HCC). **a** RT-qPCR was performed to detect miR-4270-5p expression in 50 pairs of tumors and matched non-tumor liver tissues from patients with HCC. **b** Comparison of miR-4270-5p levels in HCC samples of early stage ($n=22$) and those of advanced stage ($n=28$). **c** Comparison of miR-4270-5p levels in HCC samples without venous infiltration ($n=16$) and those with venous infiltration ($n=34$). **d** RT-qPCR was performed to detect miR-4270-5p expression in the immortal liver cells (THLE-2) and HCC cells (Huh7, MHCC97, Hep3B). *** $p<0.001$

b). Western blotting further confirmed that E-cadherin protein expression was increased and Vimentin protein expression was decreased in Huh7 and MHCC97 cells transfected with miR-4270-5p mimic (Fig. 3c, d).

SATB2 was a target gene of miR-4270-5p

In lung cancer, miR-4270-5p targeted MMP19, a pro-metastasis gene, to suppress cancer cell migration, invasion, colony forming and proliferation [24]. In HCC cells, we found that miR-4270-5p mimic did not decrease MMP19 in both cell lines (Fig. 4a, b), indicating MMP19 might not be the major target of miR-4270-5p in HCC. We then used miRDB software to predict potential target genes of miR-4270-5p. Among the predicted genes, SATB2 was an oncogene in HCC and promoted cell proliferation and invasion via mediating EMT process. There was a putative binding site for miR-4270-5p in the 3′UTR of SATB2 mRNA (Fig. 4c). The RT-qPCR results suggested that

Table 1 Relationship between miR-4270-5p expression and their clinic–pathological characteristics of 50 patients with HCC

Characteristics	Number of cases	miR-4270-5p		*p* value
		High	Low	
Age (years)				0.387
<55	20	12	8	
≥55	30	13	17	
Sex				0.345
Male	36	16	20	
Female	14	9	5	
HBsAG				0.244
Positive	31	18	13	
Negative	19	7	12	
Cirrhosis				0.391
Positive	28	16	12	
Negative	22	9	13	

Samples with miR-4270-5p expression higher or lower than median expression are considered as high or low expression group

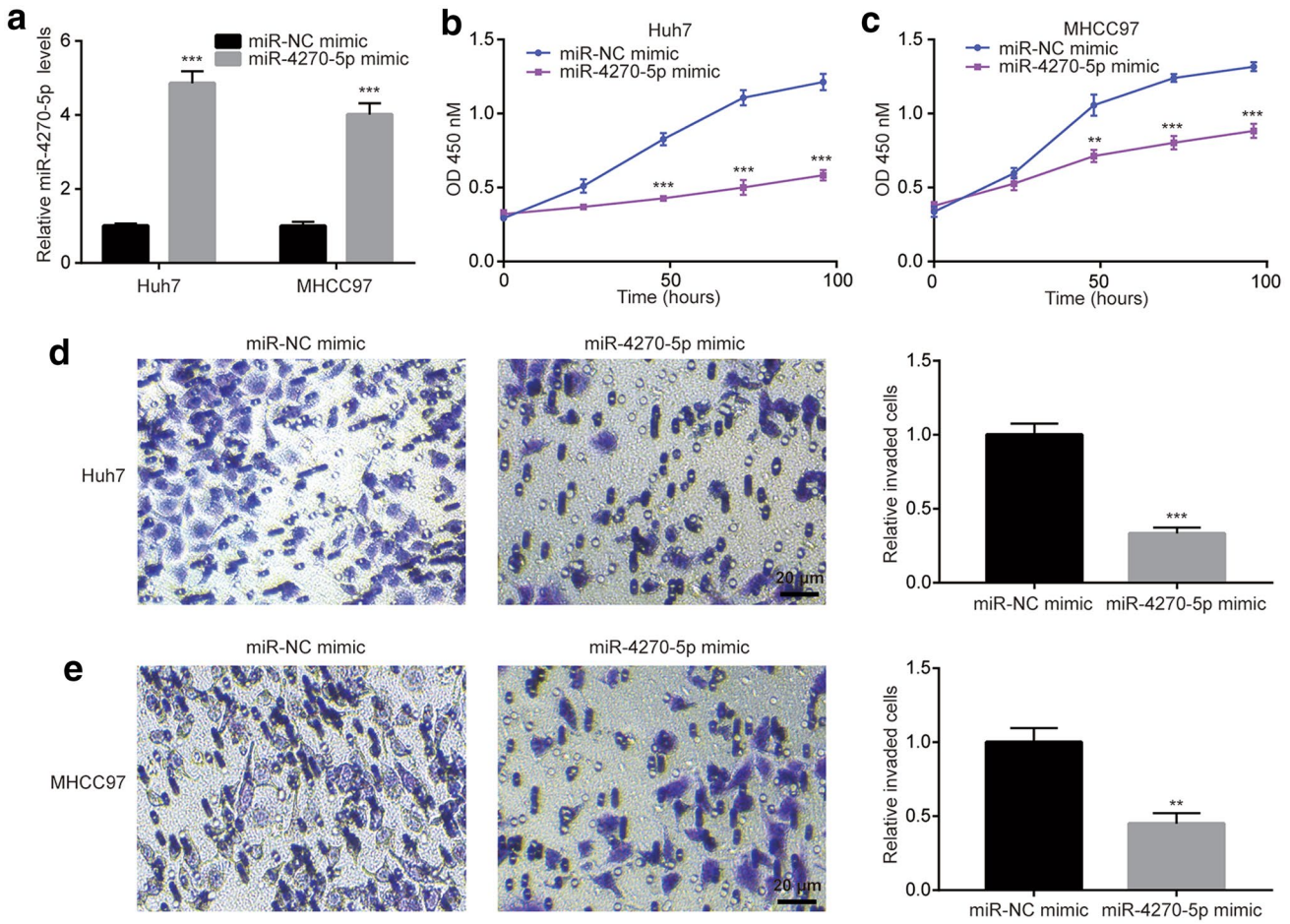

Fig. 2 miR-4270-5p inhibited HCC cell proliferation and invasion. **a** The expression of miR-4270-5p in Huh7 and MHCC97 cells transfected with miR-NC mimic or miR-4270-5p mimic was detected with RT-qPCR. **b**, **c** The CCK-8 assay was carried out to detect the proliferation ability of Huh7 (**b**) and MHCC97 (**c**) cells transfected with miR-NC mimic or miR-4270-5p mimic. **d**, **e** The Transwell assay was carried out to detect the invasion ability of Huh7 (**d**) and MHCC97 (**e**) cells transfected with miR-NC mimic or miR-4270-5p mimic. ** $p < 0.01$, *** $p < 0.001$

miR-4270-5p mimic decreased SATB2 mRNA expression in Huh7 and MHCC97 cells (Fig. 4d). Western blotting further showed that miR-4270-5p mimic decreased SATB2 protein expression in Huh7 and MHCC97 cells (Fig. 4e). To confirm their direct association, dual luciferase reporter assay was performed. As we expected, miR-4270-5p mimic repressed relative luciferase activity of SATB2 3′UTR-WT in Huh7 and MHCC97 cells (Fig. 4f, g). For further evaluation of their association in clinical samples, we detected SATB2 expression in 50 pairs of tumors and non-tumor liver tissues. Consistent with previous report, SATB2 mRNA was increased in HCC samples compared with non-tumor liver tissues (Fig. 5a). Additionally, there was a strong negative correlation between expression ratio of SATB2 (tumors/non-tumors) and miR-4270-5p (tumors/non-tumors) in HCC samples (Fig. 5b).

miR-4270-5p inhibited HCC cell proliferation and invasion through repression of SATB2

To examine whether SATB2 was critical for biological function of miR-4270-5p, we constructed recombinant SATB2. Transection of pcDNA3–SATB2 increased SATB2 mRNA expression in Huh7 and MHCC97 cells as detected by RT-qPCR (Fig. 6a). Western blotting showed that pcDNA3–SATB2 also increased SATB2 protein expression in Huh7 and MHCC97 cells (Fig. 6b). The Transwell assay indicated that SATB2 overexpression attenuated the inhibitory effect of miR-4270-5p on cell invasion in Huh7 and MHCC97 cells (Fig. 6c, d). Additionally, the proliferation assay indicated that SATB2 overexpression attenuated the inhibitory effect of miR-4270-5p on cell proliferation in Huh7 and MHCC97 cells (Fig. 6e, f). These results manifested that miR-4270-5p mainly relied on regulation of SATB2 to control HCC cell proliferation and invasion.

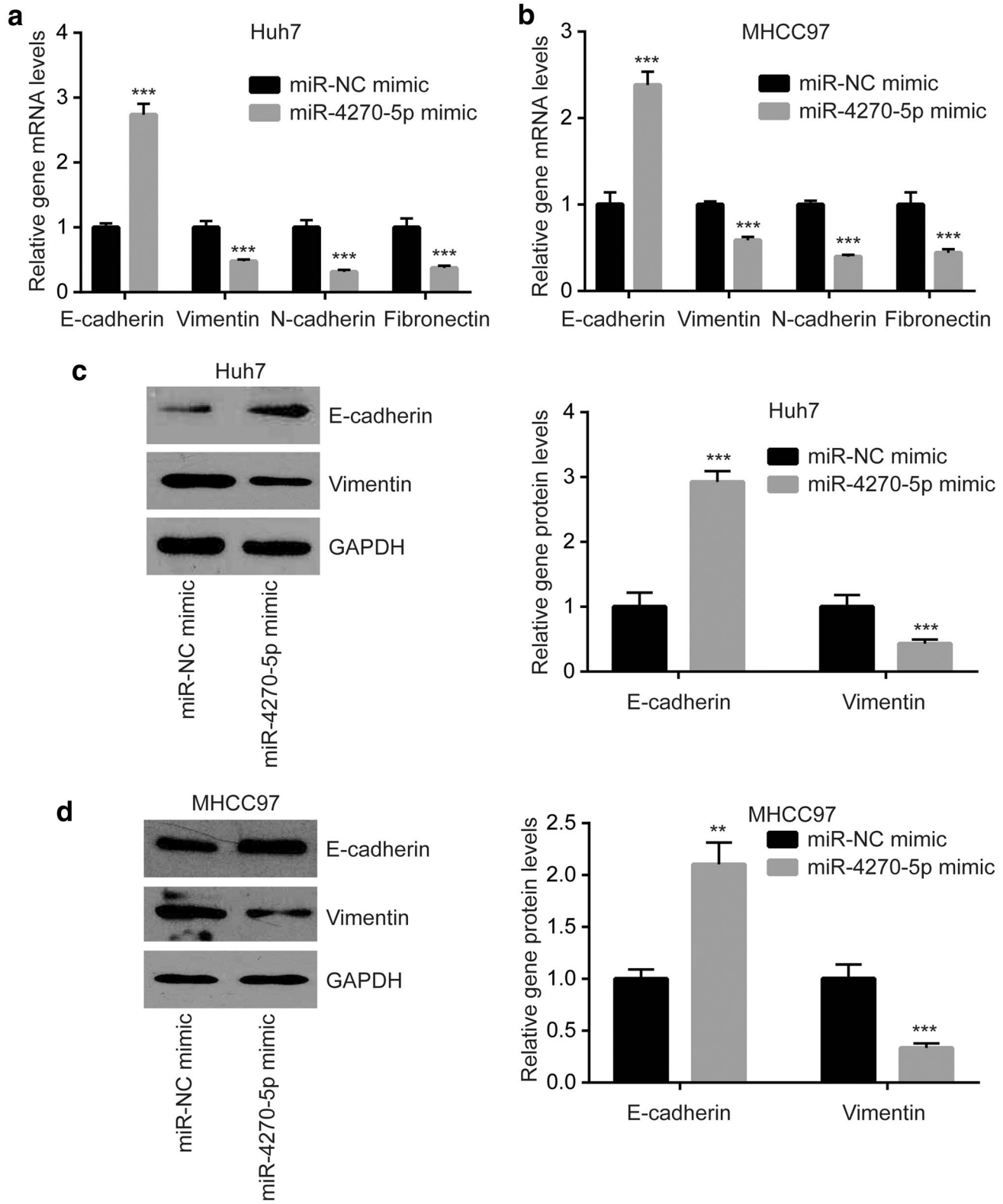

Fig. 3 miR-4270-5p regulated epithelial mesenchymal transition in HCC cells. **a**, **b** RT-qPCR was applied to detect the mRNA expression of epithelial marker (E-cadherin) and mesenchymal markers (N-cadherin, Vimentin, Fibronectin) in Huh7 (**a**) and MHCC97 (**b**) cells transfected with miR-NC mimic or miR-4270-5p mimic. **c**, **d** Western blotting was applied to detect the protein expression of epithelial marker (E-cadherin) and mesenchymal marker (Vimentin) in Huh7 (**c**) and MHCC97 (**d**) cells transfected with miR-NC mimic or miR-4270-5p mimic. ** $p < 0.01$, *** $p < 0.001$

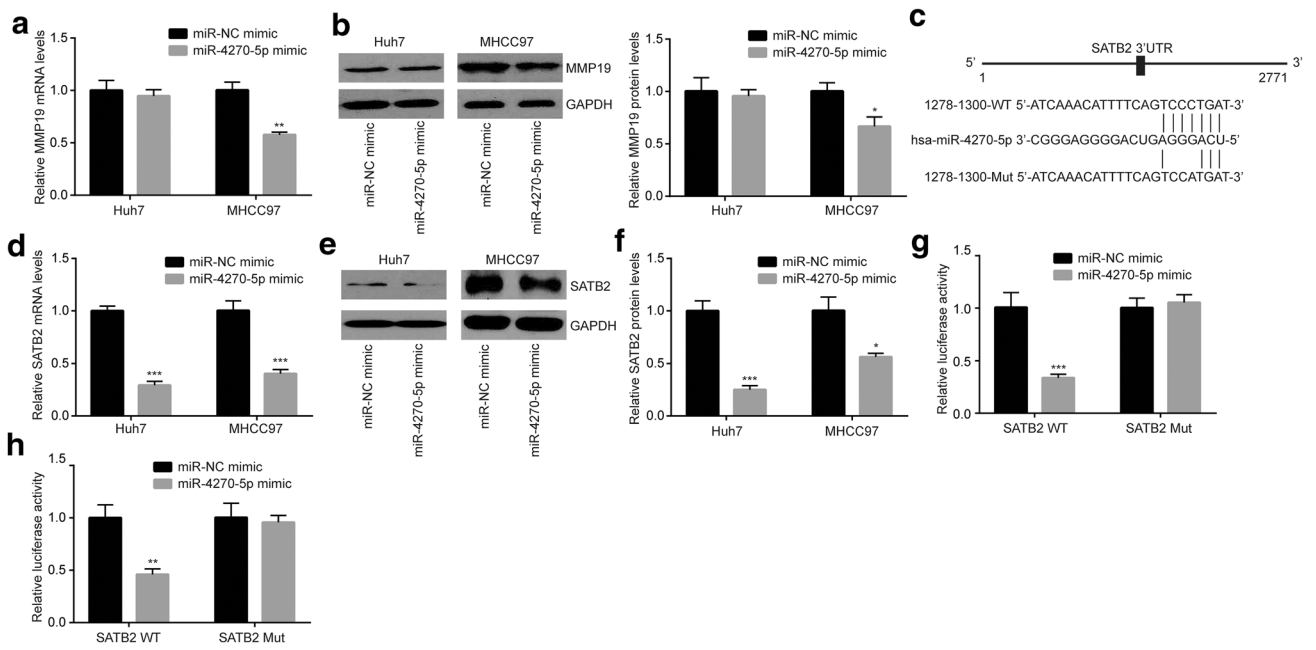

Fig. 4 SATB2 was targeted by miR-4270-5p. **a** The expression of MMP19 mRNA in Huh7 and MHCC97 cells transfected with miR-NC mimic or miR-4270-5p mimic was detected with RT-qPCR. **b** The expression of MMP19 protein in Huh7 and MHCC97 cells transfected with miR-NC mimic or miR-4270-5p mimic was detected with western blotting. **c** The 3′UTR of SATB2 mRNA contained complementary binding sites for miR-4270-5p. **d** The expression of SATB2 mRNA in Huh7 and MHCC97 cells transfected with miR-NC mimic or miR-4270-5p mimic was detected with RT-qPCR. **e** The expression of SATB2 protein in Huh7 and MHCC97 cells transfected with miR-NC mimic or miR-4270-5p mimic was detected with western blotting. **f**, **g** The dual luciferase reporter assay was performed to detect the relative luciferase activity of SATB2 3′UTR-WT and 3′UTR-Mut in Huh7 (**f**) and MHCC97 (**g**) cells transfected with miR-NC mimic or miR-4270-5p mimic. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

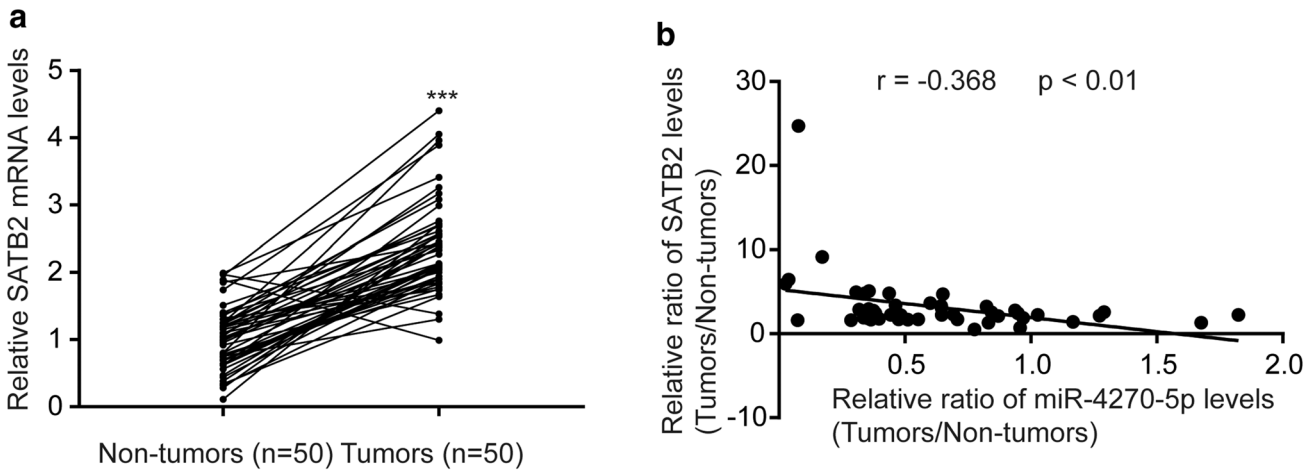

Fig. 5 The expression of SATB2 was associated with miR-4270-5p levels in HCC samples. **a** RT-qPCR was performed to detect SATB2 expression in 50 pairs of tumors and matched non-tumor liver tissues from patients with HCC. **b** Pearson correlation analysis was used to investigate the association between expression ratio of SATB2 (tumors/non-tumors) and miR-4270-5p (tumors/non-tumors) expression in 50 HCC samples. *** $p<0.001$

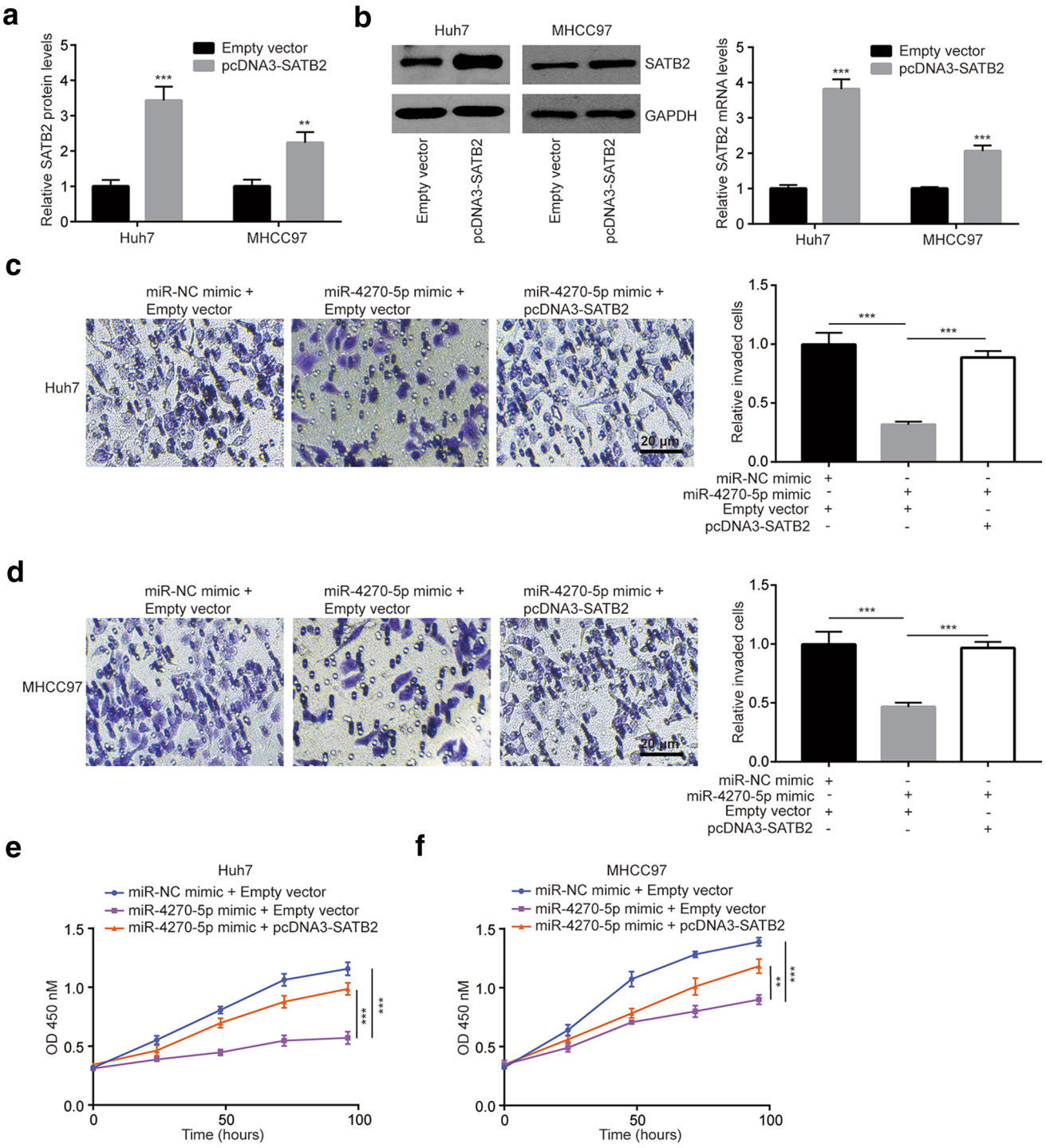

Fig. 6 miR-4270-5p inhibited cell proliferation and invasion via targeting SATB2. **a** The expression of SATB2 in Huh7 and MHCC97 cells transfected with pcDNA3 or pcDNA3–SATB2 was detected with RT-qPCR. **b**, **c** The Transwell assay was carried out to detect the invasion ability of Huh7 (**b**) and MHCC97 (**c**) cells transfected with miR-NC mimic or miR-4270-5p mimic in combination with pcDNA3 or pcDNA3–SATB2. **d**, **e** The CCK-8 assay was carried out to detect the proliferation ability of Huh7 (**d**) and MHCC97 (**e**) cells transfected with miR-NC mimic or miR-4270-5p mimic in combination with pcDNA3 or pcDNA3–SATB2. ** $p < 0.01$, *** $p < 0.001$

Discussion

Dysregulation of miRNAs contributed to HCC development via activating sustained cell growth signaling and promoting the expression of pro-metastasis genes [6, 9, 25]. Little is known about the function of miR-4270-5p in cancer. Most recently, one study has discovered that miR-4270-5p was aberrant expressed in lung cancer due to decreased expression of LINC00472 [26]. Via analyzing previous published data, it was found that miR-4270-5p was one of most significantly downregulated miRNAs in HCC [22]. We experimentally confirmed that miR-4270-5p was significantly decreased in HCC samples and its expression was associated with tumor stage and venous infiltration. Sun et al. found that miR-4270-5p was significantly decreased in lung cancer with brain metastasis compared with those with no brain metastasis and could inhibit brain metastasis via targeting MMP19 [24]. Their findings and our observation of clinical samples implied that miR-4270-5p might contribute to HCC cell proliferation and metastasis. Indeed, in function assays, we found miR-4270-5p inhibited HCC cell proliferation and invasion. Our data revealed a critical role of miR-4270-5p downregulation during HCC development.

Epithelial mesenchymal transition (EMT) is a well-characterized biological process by which the gene expression is reprogramed to express mesenchymal genes and acquire ability of migration and invasion [27, 28]. In HCC, during EMT, the expression of epithelial genes (such as E-cadherin) were decreased and the expression of mesenchymal genes (such as N-cadherin, Vimentin, Fibronectin) were increased [29]. In HCC cells, we observed that miR-4270-5p promoted epithelial gene expression and inhibited mesenchymal gene expression, indicating miR-4270-5p might attenuated EMT process in HCC. SATB2 was a transcription factor implicated in EMT process of cancer cells [19]. SATB2 could be an oncogene or tumor suppressor in different cancer types [18, 30]. It was reported that decreased SATB2 promoted lung cancer cell invasion via regulation of G9a [30]. In HCC, SATB2 was overexpressed and promoted cancer cell proliferation and invasion via mediating EMT [19]. We also found that SATB2 was increased in our collected tumor samples. The abnormal expression of SATB2 was the consequence of regulation by several miRNAs [31]. miR-34b/c directly regulated SATB2 to inhibit osteoblast proliferation and differentiation [32]. Importantly, SATB2 was a target gene of miR-211 in HCC [33]. In the present study, we discovered that SATB2 was also a target of miR-4270-5p. The association of miR-4270-5p and SATB2 was further confirmed in clinical samples. These results suggested that miR-4270 might control EMT process via repression of SATB2 in HCC.

In conclusion, the current study defined a novel tumor suppressive role of miR-4270-5p in HCC. Mechanistically, miR-4270-5p directly repressed SATB2 expression and inhibited the EMT process. The study on miR-4270-5p will provide novel insights for understanding the molecular mechanisms of HCC.

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Data availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no competing interests of any type in the current study.

Ethical standards The study was performed in accordance with the Declaration of Helsinki and obtained the approval from the Ethics Committee of the China-Japan Union Hospital of Jilin University.

Informed consent Not applicable.

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