MicroRNA-98 suppresses cell proliferation, migration and invasion by targeting collagen triple helix repeat containing 1 in hepatocellular carcinoma

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Abstract. MicroRNAs (miRNAs) are emerging as critical regulators in carcinogenesis and tumor progression. miR-98 has previously been verified to be important in tumor progression, however, its function in hepatocellular carcinoma (HCC) remains to be elucidated. The expression levels of miR-98 in HCC tissues and cell lines were determined by reverse transcription quantitative polymerase chain reaction. Subsequently, the effect of miR-98 on cell proliferation, migration and invasion was evaluated by MTT assay, transwell migration assay and transwell invasion assay. Furthermore, a luciferase reporter assay was conducted to confirm the action of miR-98 on downstream target genes, including collagen triple helix repeat containing 1 (CTHRC1). In the present study, it was confirmed that miR-98 was significantly downregulated in HCC tissues and cell lines. Overexpression of miR-98 inhibited HCC cell proliferation, migration and invasion *in vitro*. In addition, at the molecular level, the tumor oncogene CTHRC1 was identified to be the direct target of miR-98. Our findings suggested that miR-98 was significantly downregulated in HCC and suppressed HCC cell proliferation, migration and invasion partially via the downregulation of CTHRC1. Thus, these data demonstrated that miR-98 could be a potential therapeutic target in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer worldwide and is also the third leading cause of cancer-associated mortality (1). Currently, surgical resection or liver transplantation remains the main and effective treatment throughout the world (2). However, the recurrence rate within 2 years in patients who have undergone tumor resection remains >50% (3). Uncontrolled tumor metastasis, frequent intrahepatic spread and extrahepatic metastasis are the primary causes for the poor prognosis of HCC (4). Therefore, investigation of the molecular mechanism of metastasis and recurrence may improve the prognosis of patients with HCC.

MicroRNAs (miRNAs) are 21-24 nucleotide, small, non-coding RNAs that regulate gene expression by base pairing with target mRNAs in the 3'-untranslated region (3'-UTR), leading to mRNA cleavage or translational repression (5). Growing evidence suggests that miRNAs are important in various biological processes, including cell proliferation, development and differentiation (6-8). Furthermore, an increasing number of miRNAs have been observed in various types of cancer and may be involved in modulating cancer cell behavior. For example, Li *et al* demonstrated that downregulated miR-646 in clear cell renal carcinoma correlated with tumor metastasis by targeting the nin one binding protein (9). Li *et al* found that miR-21 overexpression was associated with acquired resistance to the epidermal growth factor receptor tyrosine kinase inhibitor in non-small cell lung cancer (10). Wang *et al* indicated that miRNA-497 could inhibit ovarian cancer cell migration and invasion through targeting SMAD specific E3 ubiquitin protein ligase (11). These data emphasized the importance of miRNAs in cancer development and provide new insights into understanding the molecular mechanism of tumorigenesis. However, the expression profile and biological role of miR-98 in HCC remains to be elucidated.

In the present study, the potential involvement of miR-98 in HCC was investigated. The expression level of miR-98 in HCC tissues and cell lines was confirmed and its effects on HCC cell proliferation, migration and invasion were assessed. In addition, the underlying mechanism of the function of miR-98 in HCC was examined.

Materials and methods

HCC samples and cell lines. Paired HCC and adjacent non-tumor tissues were obtained from 30 patients were recruited into a clinical trial at the Department of General Surgery, Huaihe Hospital of Henan University (Kaifeng, China) between 2012 and 2013. The present study was approved by the Ethics Committee of Henan University. Tissues were

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immediately snap frozen and stored at -80˚C. Three HCC cell lines (HepG2, HuH-7 and Hep3B) and the normal human liver cell line L02 were obtained from the American Type Culture Collection (Manassas, VA, USA), cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated in a humid atmosphere with 5% CO_2 at 37˚C.

Transfection. miR-98 mimics and the miRNA mimic negative control (miR-NC) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Small interfering RNA against collagen triple helix repeat containing 1 (si-CTHRC1) and the negative control (si-NC) were designed by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of the si-CTHRC1 and si-NC were as follows: si-CTHRC1, 5'-ACAUUCAGCUCCAUUAAAGdTdT-3' and si-NC, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. Transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The final concentration was 200 nm for miR-98 mimics or miR-NC and 100 nm for si-CTHRC1 or si-NC.

Cell proliferation assay. The cell proliferation assay was performed by 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well plates at $5x10^3$ per well 24 h after transfection and cultured for 24, 48 and 72 h, after which 20 μl MTT was added to each well. Subsequently, the cells were incubated for 4 h prior to adding 150 μl dimethyl sulfoxide. Once the insoluble crystals were completely dissolved, the absorbance values at 570 nm were measured using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Cell migration and invasion assay. Cell migration and invasive ability was examined using a 24-well transwell plate with 8 mm pore polycarbonate membrane inserts, according to the manufacturer's protocol (Corning Inc., Corning, NY, USA). The Matrigel (Sigma-Aldrich, St. Louis, MO, USA) employed for the invasion assays was applied to the upper surface of the membranes. A total of $5x10^4$ cells per well were seeded into the top chamber in serum-free media 24 h after transfection and this was replaced with complete growth media for 12 h. Cells that migrated or invaded through the surface of the membrane were fixed with methanol and stained with hematoxylin (Sigma-Aldrich). Migrating or invasive cells from three random microscope fields per filter were selected for cell counting under a microscope (Olympus CKX41; Olympus Corporation, Tokyo, Japan).

Luciferase reporter assay. The 3'-UTR of CTHRC1 containing the predicted miR-98 binding site was constructed by Guangzhou RiboBio Co., Ltd. The mutant CTHRC1 3'-UTR was created by mutating multiple nucleotides complementary to the miR-98 seed region. HEK 293T cells were cultured in 96-well plates with 50-70% confluence 24 h prior to transfection. A mixture of 100 ng pmiR-RB-Report™ h-CTHRC1 wild type (Wt) or mutant (Mut) reporter plasmid vector together with 50 nM miR-98 mimics or miR-NC (Guangzhou RiboBio Co., Ltd.) were co-transfected. The luciferase activity was measured 48 h post-transfection using a Dual-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA) with *Renilla* luciferase activity as the reporter gene and firefly luciferase as the reference gene.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers instructions. To quantitate miR-98 expression, total RNA was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). To measure the mRNA levels of CTHRC1, total RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan). qPCR was performed using SYBR Premix Ex Taq II (Takara Bio Inc.) in an Agilent Mx3005P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) with an initial denaturation at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min.. The following primers were used: miR-98, forward 5'-CGG CTGAGGTAGTAGATTGT-3' and reverse 5'-GTCGTATCC AGTGCAGGGTCC-3'; CTHRC1, forward 5'-TGGACACCC AACTACAAGCA-3' and reverse 5'-GAACAAGTGCCAACC CAGAT-3'. GAPDH was used as an internal control with the following primers: GAPDH forward, 5'-GAAGGTGAAGGT CGGAGTC-3', and reverse 5'-GAAGATGGTGATGGGATT TC-3'. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Cq}$) (12).

Western blot analysis. Cultured cells were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) with 1% phenylmethanesulfonyl fluoride. Protein was loaded and separated by 10% SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The blots were probed with the following primary antibodies at 4˚C overnight: Rabbit anti-CTHRC1 (1:1,000; ab85739; Abcam, Cambridge, MA, USA), and rabbit anti-GAPDH (1:5,000; cat. no. ab9485; Abcam). The blots were subsequently incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (1:3000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Signals were visualized using ECL substrates (Pierce Biotechnology Inc., Rockford, IL, USA). GAPDH was used as an endogenous control.

Statistical analysis. All statistical analyses were performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Statistical differences were determined by analysis of variance or Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-98 expression in HCC tissues and cell lines. RT-qPCR analysis was performed to examine the expression level of miR-98 in HCC tissues and cell lines. In 30 cases of HCC tissues and adjacent non-tumor tissues, the data revealed that miR-98 was downregulated in HCC tissues when compared

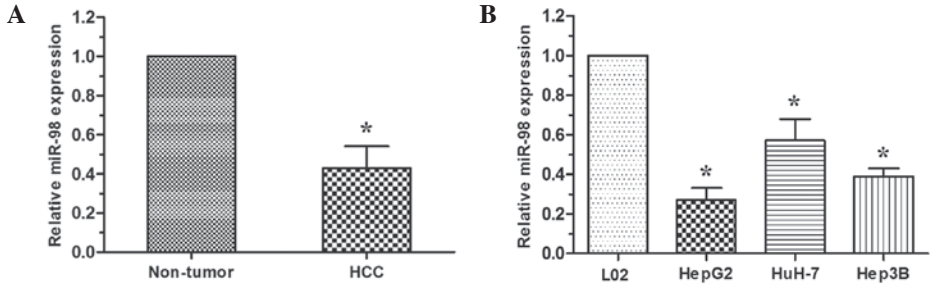

Figure 1. miR-98 is downregulated in human HCC tissues and cell lines. (A) The relative expression of miR-98 was examined by RT-qPCR in 30 pairs of HCC tissues and adjacent non-tumor tissues. (B) RT-qPCR analysis of miR-98 expression in three HCC cell lines (HepG2, HuH-7 and Hep3B) and the normal human liver cell line L02. Each sample was analyzed in triplicate and normalized to GAPDH. *P<0.05. HCC, hepatocellular carcinoma; miR-98, microRNA-98; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

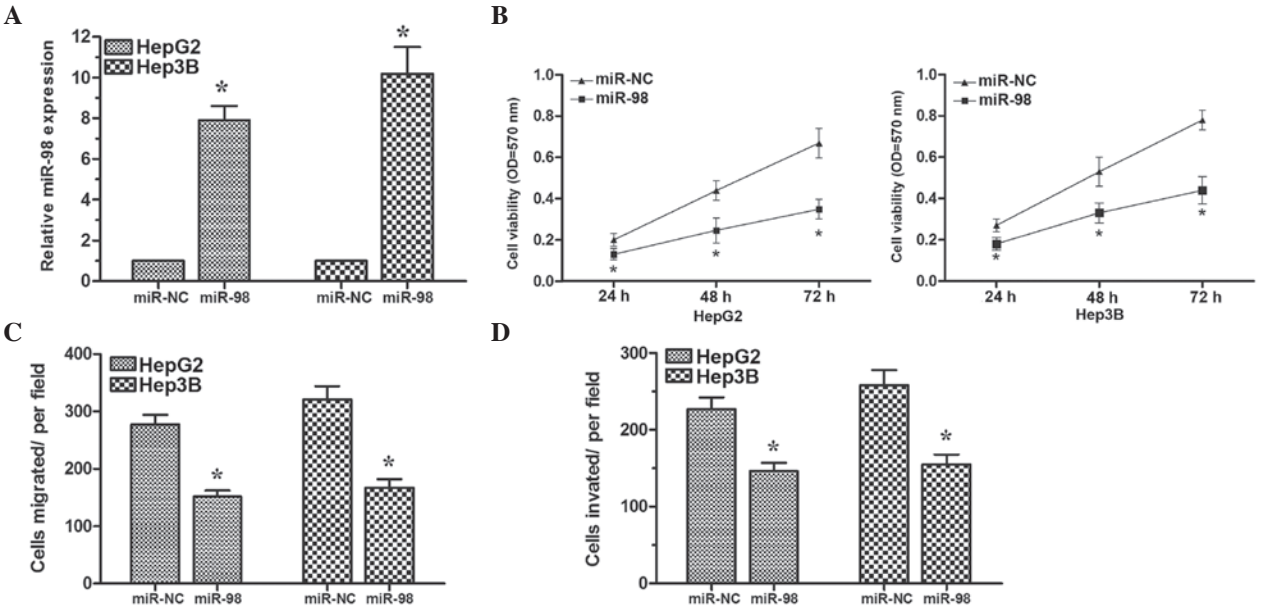

Figure 2. miR-98 suppresses HCC cell proliferation, migration and invasion. (A) Successful overexpression of miR-98 was confirmed by reverse transcription quantitative polymerase chain reaction following transfection with the miR-98 mimics or miR-NC. (B) Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide assay at different time points. (C and D) Cell migration and invasion were measured by transwell migration and invasion assays. *P<0.05, compared with cells transfected with miR-NC. HCC, hepatocellular carcinoma; miR-98, microRNA-98; NC, negative control; OD, optical density.

with that in the paired adjacent non-tumor tissues (P<0.05; Fig. 1A). The expression level of miR-98 was then detected in HCC cell lines (HepG2, HuH-7 and Hep3B) and the normal human liver cell line L02. The expression of miR-98 was decreased in HCC cell lines compared with the L02 cell line (P<0.05; Fig. 1B). These results indicated that reduced expression of miR-98 may be critical in HCC progression and development.

Effect of miR-98 on HCC cell proliferation, migration and invasion. To assess the biological role of miR-98 in HCC, HepG2 and Hep3B cells were transfected with the miR-98 mimics or miR-NC. Transfection efficiency was confirmed by RT-qPCR (Fig. 2A). MTT assay revealed that cell proliferation was significantly inhibited in HepG2 and Hep3B cells transfected with miR-98 mimics compared with cells transfected with miR-NC (Fig. 2B). Furthermore, transwell assays demonstrated that overexpression of miR-98 could inhibit the migratory and invasive abilities of HepG2 and Hep3B cells (Fig. 2C and D). Taken together, these results demonstrated that miR-98 could suppress cell proliferation, migration and invasion in HCC cells.

CTHRC1 is a target of miR-98 in HCC cells. To examine the molecular mechanism by which miR-98 suppresses the proliferation and invasion of HCC cells, TargetScan (http://www.targetscan.org/) was adopted and CTHRC1 was found to be the putative target of miR-98 (Fig. 3A). In order to confirm that CTHRC1 is a direct target of miR-98, luciferase reporter constructs containing the wild-type (Wt) or mutant (Mut) 3'-UTR of the CTHRC1 gene were engineered. The luciferase reporter assay demonstrated that miR-98 significantly inhibited the Wt but not Mut luciferase activity in HEK 293T cells (Fig. 3B). In addition, western blot analyses demonstrated that overexpression of miR-98 significantly inhibited CTHRC1 expression in HepG2 and

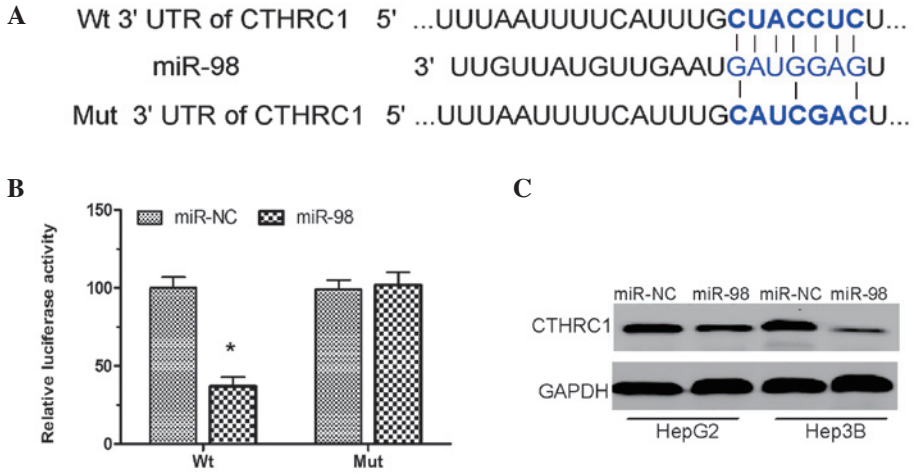

Figure 3. CTHRC1 is a direct target of miR-98. (A) WT and Mut sequences of the putative miR-98 target sequences of CTHRC1 3'-UTR. (B) A luciferase reporter assay demonstrated the inhibitory effect of miR-98 on CTHRC1 3'-UTR luciferase activity in HEK 293T cells. (C) The expression of CTHRC1 in HepG2 and Hep3B cells transfected with the miR-98 mimics or miR-NC was analyzed by western blot analysis. *P<0.05. miR-98, microRNA-98; 3'-UTR, 3'-untranslated region; NC, negative control; WT, wild-type; Mut, mutant; CTHRC1, collagen triple helix repeat containing 1.

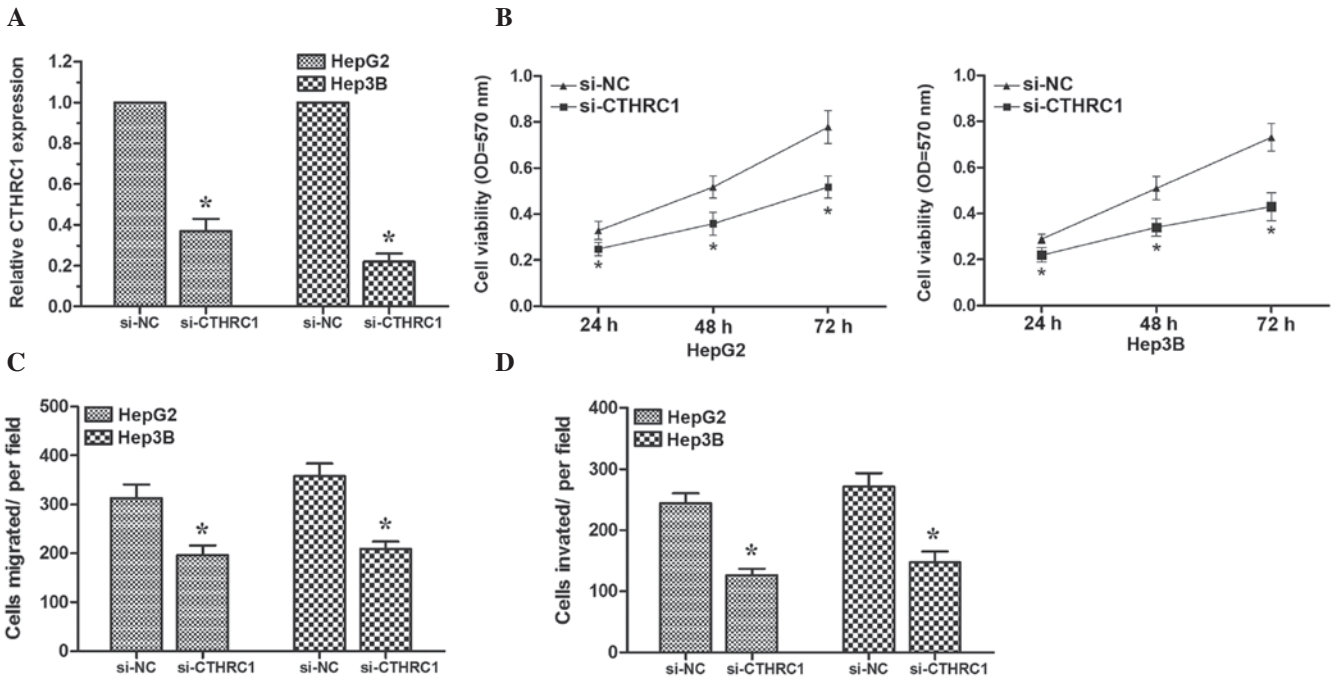

Figure 4. Decreased expression of CTHRC1 inhibits HCC cell proliferation, migration and invasion. (A) Reverse transcription quantitative polymerase chain reaction was performed to detect the expression level of CTHRC1 following transfection of si-CTHRC1 or si-NC into HepG2 and Hep3B cells. (B-D) Cell proliferation, migration and invasion assays were performed following transfection with si-CTHRC1 or si-NC into HepG2 and Hep3B cells. *P<0.05, compared with cells transfected with si-NC. HCC, hepatocellular carcinoma; miR-98, microRNA-98; CTHRC1 collagen triple helix repeat containing 1; NC, negative control; si, small interfering RNA.

Hep3B cells (Fig. 3C). These results indicated that miR-98 directly modulates CTHRC1 expression through binding to the 3'UTR of CTHRC1 mRNA.

Knockdown of CTHRC1 suppresses HCC cell proliferation, migration and invasion. To determine whether CTHRC1 could also inhibit HCC cell proliferation, migration and invasion, targeted knockdown of CTHRC1 expression using RNA interference was performed in HepG2 and Hep3B cells. The expression levels of CTHRC1 were determined by RT-qPCR (Fig. 4A). MTT assay revealed that HCC cells transfected with si-CTHRC1 exhibited a significantly reduced proliferation ability compared with cells transfected with si-NC (Fig. 4B). Subsequently, transwell migration and invasion assays were performed. The results demonstrated that knock down of CTHRC1 could inhibit HCC cell migration and invasion (Fig. 4C and D). These data suggested that decreased expression of CTHRC1 could inhibit HCC cell proliferation, migration and invasion, demonstrating that CTHRC1 may act as a direct target gene of miR-98 in HCC.

Discussion

miRNAs serve as crucial regulators of gene expression, regulating cellular physiology and development (13). Increasing evidence suggests that dysregulated expression of miRNAs is associated with tumorigenesis and the progression of various types of human cancer, including HCC (14). For example, Xu *et al* found that miRNA-195 was significantly reduced in HCC tissues and upregulation of miR-195 suppressed tumorigenicity and regulated G1/S transition of HCC cells (15). Wang *et al* demonstrated that miR-302b was decreased in HCC and suppressed HCC cell proliferation and G1-S transition via targeting AKT2 expression (16). Thus, identifying and elucidating the exact roles and underlying mechanisms of miRNAs in HCC may aid our understanding of the pathogenesis of HCC. The present study focused on the potential tumor suppressor miR-98.

In the current study, it was confirmed that miR-98 was downregulated in HCC tissues and cell lines. In addition, overexpression of miR-98 suppressed HCC cell proliferation, migration and invasion. CTHRC1 was identified as a target of miR-98 in HCC cells. Knock down of CTHRC1 expression inhibited HCC cell proliferation, migration and invasion, phenocopying the overexpression of miR-98 in HCC cells. These data demonstrated that miR-98 acts as a tumor suppressor in HCC via downregulating CTHRC1 expression.

miR-98 has been demonstrated to be decreased in various types of cancer and functions as a tumor suppressor. For example, Chen *et al* found that the expression of miR-98 was decreased in glioma tissues and overexpression of miR-98 inhibited glioma cell invasion (17). Siragam *et al* demonstrated that overexpression of miR-98 could inhibit breast cancer cell proliferation, survival, tumor growth, invasion and angiogenesis (18). Furthermore, Huang *et al* found that miR-98 was reduced in esophageal squamous cell carcinoma (ESCC) and overexpression of miR-98 significantly inhibited the migration and invasion of ESCC cells, which was reversed by transfection of EZH2 (19). The present data expanded our understanding of the tumor suppressive role of miR-98 in HCC. Overexpression of miR-98 inhibited HCC cell proliferation, migration and invasion.

CTHRC1 is a 30-kDa glycosylated secreted protein containing a short collagen-like motif with 12 Gly-X-Y repeats similar to the collagen domains present in the C1q/tumor necrosis factor α-related protein (20). Accumulating evidence suggested that CTHRC1 was increased in various types of tumor. For example, Ke *et al* revealed that overexpression of CTHRC1 was associated with tumor aggressiveness and poor prognosis in human non-small cell lung cancer (21). Ma *et al* suggested that CTHRC1 acted as a prognostic factor and promoted invasiveness of gastrointestinal stromal tumors by activating Wnt/PCP-Rho signaling (22). Kim *et al* found that CTHRC1 was highly expressed in colorectal cancer and CTHRC1 acted via extracellular-signal-regulated kinase-dependent induction of matrix metalloproteinase-9 to promote invasion of colorectal cancer cells (23). In the present study, miR-98 suppressed the proliferation, migration and invasion of HCC cells by targeting CTHRC1, elucidating the role of CTHRC1 in HCC development.

In conclusion, the current study demonstrated that miR-98 could act as a tumor suppressor in HCC cells, which was mediated through inhibiting CTHRC1 expression. These findings suggest that miR-98 could serve as a therapeutic agent in HCC treatment.

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