MicroRNA-584 prohibits hepatocellular carcinoma cell proliferation and invasion by directly targeting BDNF

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Abstract. In recent decades, an increasing number of studies have demonstrated that numerous microRNAs (miRNAs) are dysregulated in hepatocellular carcinoma (HCC); these aberrantly expressed miRNAs are contributing regulators of HCC formation and progression. Thus, revealing the biological roles of miRNAs in HCC may provide novel information on the identification of effective therapeutic targets and valuable diagnosis methods. Herein, reverse transcription-quantitative PCR was performed to determine the expression profile of miRNA-584 (miR-584) in HCC tissues and cell lines. Cell Counting Kit-8 and cell invasion assays were utilized to evaluate the influence of miR-584 overexpression on HCC cell proliferation and invasion, respectively. The present study demonstrated that miR-584 expression was reduced in HCC tissues and cell lines compared with normal controls. Clinical analysis indicated that decreased miR-584 expression was significantly associated with tumor size, TNM stage and lymph node metastasis of patients with HCC. Additionally, resumption of miR-584 expression inhibited proliferation and invasion of HCC cells. Mechanistically, it was demonstrated that miR-584 can directly interact with the 3'-untranslated regions of brain-derived neurotrophic factor (BDNF) mRNA, and reduce its mRNA and protein levels in HCC cells. Furthermore, BDNF was upregulated in HCC tissues, and its level was inversely correlated with miR-584 expression. Notably, restored BDNF expression antagonized the inhibitory effects of miR-584 overexpression on HCC cells. In conclusion, miR-584 may serve tumor-suppressive roles in HCC by directly targeting BDNF, thus suggesting that miR-584 may serve as a potential candidate for treatment of patients with this disease.

Introduction

Hepatocellular carcinoma (HCC) ranks as the sixth most prevalent malignancy and the third most common cause of cancer-related mortality globally (1). Over 780,000 newly diagnosed HCC cases and ~745,000 HCC-related deaths are estimated to occur annually worldwide (2,3). In past decades, HCC morbidity has significantly increased, particularly the phenotype caused by hepatitis B or C virus infection (4). Currently, the major therapeutic interventions for patients with HCC include surgical resection, radiotherapy, chemotherapy and molecular targeted therapy (5). Despite the significant advancements in diagnosis and therapy, treatment outcomes of patients with HCC remain unsatisfactory, with a 5-year survival rate of <5% (6). Hidden lesions, high recurrence rate and metastasis are predominantly responsible for the unfavorable prognosis of patients with HCC (7). Therefore, in-depth understanding of the detailed mechanisms responsible for the occurrence and development of HCC may facilitate identification of novel effective treatment strategies.

MicroRNAs (miRNAs) are an abundant group of noncoding and short RNA molecules that span 18-23 nucleotides in length (8). miRNAs affect gene regulation by binding directly to specific elements in the 3'-untranslated regions (3'-UTRs) of target genes, and further reducing mRNAs expression or blocking protein translation of such genes (9). To date, >1,000 mature miRNAs have been identified in the human genome, and these miRNAs are estimated to regulate approximately two-thirds of all human protein-coding genes (10). An increasing number of studies have reported that a variety of miRNAs are deregulated in HCC and serve crucial roles in HCC oncogenesis by affecting multiple pathological processes, including cell proliferation, cycle, differentiation, apoptosis, metastasis and epithelial-mesenchymal transition (11-13). The expression of certain miRNAs is significantly reduced in HCC and these miRNAs may function as tumor suppressors (14-16); other miRNAs are overexpressed in HCC and serve oncogenic roles in HCC onset and development (17,18). Therefore, further investigation of miRNAs in HCC may provide valuable insights into the molecular pathways of pathogenesis in HCC and therapeutic targets for treatment of patients with this malignancy.

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Key words: hepatocellular carcinoma, microRNA-584, brain-derived neurotrophic factor, proliferation, invasion
miRNA-584 (miR-584) deregulation has been reported in multiple human cancer types, including glioma (19), neuroblastoma (20) and gastric cancer (21). However, the expression and clinical value of miR-584, and its role in addition to related mechanisms remain unclear in HCC. In the present study, aberrant downregulation of miR-584 was observed in HCC tissues and cell lines. Decreased miR-584 level was markedly correlated with aggressive clinical characteristics of patients with HCC. miR-584 attenuated HCC cell proliferation and invasion by directly targeting brain-derived neurotrophic factor (BDNF). The results of the present study may promote the identification of novel valuable prognosis markers and therapeutic targets for patients with HCC.

Materials and methods

Clinical samples. A total of 56 pairs of HCC and adjacent non-cancerous tissues were obtained from patients that had undergone surgical resection at The Seventh People’s Hospital, Shanghai University of Traditional Chinese Medicine between March 2014 and December 2016. All 56 patients enrolled in the present study had received chemotherapy, radiotherapy or other treatments prior to surgery. All tissues were immediately snap-frozen in liquid nitrogen following isolation and stored at -80˚C. All patients provided written informed consent. The ethics committee of The Seventh People’s Hospital, Shanghai University of Traditional Chinese Medicine approved this research (approval no. 20140312).

Cell culture. An immortalized normal human liver epithelial cell (L-O2) and two human HCC cell lines (Hep3B and Huh7) were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured DMEM supplemented with 10% FBS, 100 µg/ml streptomycin (both from Sigma-Aldrich; Merck KGaA). All cells were cultured at 37˚C in a humidified incubator with 5% CO2 and 100 µg/ml penicillin and 100 µg/ml streptomycin (both from Sigma-Aldrich; Merck KGaA). All cells were cultured at 37˚C in a humidified incubator with 5% CO2.

Transfection. miR-584 mimics and negative control miRNA mimics (miR-NC) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-584 mimics sequence was 5'-UAUUGGUUUCGCCGGAGACG-3' and the miR-NC sequence was 5'-UUCCGGAACGUGUCGUGTT-3'. To restore BDNF expression, BDNF overexpression plasmid pCDNA3.1-BDNF (Genecopoeia, Inc.) was introduced into cells, with an empty pCDNA3.1 plasmid as a control. Cells were inoculated into 6-well plates with a density of 8x10^5 cells/well, and transfected with miR-584 mimics (100 pmol), miR-NC (100 pmol), pCDNA3.1-BDNF (4 µg) or pCDNA3.1 (4 µg) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Transfected cells were subsequently maintained in a humidified incubator containing 5% CO2 at 37˚C used for further experiments. After 48 h incubation, reverse transcription-quantitative PCR (RT-qPCR) was performed to determine miR-584 and BDNF expression. Cell Counting Kit-8 (CCK-8) and cell invasion assays were conducted at 24 and 48 h post-transfection.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from tissues or cells was isolated using TRIzol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. To determine miR-584 expression, total RNA was converted into cDNA by using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), followed by qPCR with a TaqMan MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions for RT were as follows: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. The cycling conditions for qPCR were as follows: 50˚C for 2 min, 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec; and annealing/extension at 60˚C for 60 sec. To quantify BDNF mRNA expression, RT was performed to produce cDNA using a PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The cycling conditions for RT was as follows: 37˚C for 15 min and 85˚C for 5 sec. Subsequently, qPCR was carried out on an ABI 7900 thermocycler (Applied Biosystems; Thermo Fisher Scientific Inc.) by using a SYBR Premix Ex Taq™ Kit (Takara Biotechnology Co., Ltd.). The cycling conditions for qPCR were as follows: 5 min at 95˚C, followed by 40 cycles of 95˚C for 30 sec and 65˚C for 45 sec. U6 small nuclear RNA and GAPDH were used as internal controls for miR-584 and BDNF, respectively. Relative gene expression was analyzed using the 2-△△Cq method (22). The primers were designed as follows: miR-584, 5'-TGAATGTGTGTTGATGCCA-3' (forward) and 5'-ATCATTTGTCCTTGGATGTT-3' (reverse); U6 snRNA, 5'-GCTTGGCAGACACATATAACTAATT-3' (forward) and 5'-GGTTTACGAAATTGCCGTT-3' (reverse); BDNF, 5'-TGCGTGACGTTATTAGCGAGTTGTT-3' (forward) and 5'-CGATTGCTGATTTGCATTT-3' (reverse); and GAPDH, 5'-TCCATGACACTTTTGCGATT-3' (forward) and 5'-GTGGCTGTGTAAGTGCAGGAGAC-3' (reverse).

Cell Counting Kit-8 (CCK-8) assay. At 24 h post-transfection, transfected cells were collected and were inoculated into 96-well plates with a density of 3,000 cells per well. The cells were then incubated at 37˚C in a humidified incubator with 5% CO2, for four time points: 0, 1, 2 and 3 days. At each time point, cells were incubated at 37˚C with 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.) for 2 h, and then the absorbance at 450 nm detected with a Spectramax M5 microplate reader (Molecular Devices, LLC).

Cell invasion assay. Transfected cells were harvested at 48 h post-transfection and digested into single cell suspensions. A total of 1x10^5 cells in 200 µl FBS-free DMEM were seeded into the upper compartment of 24-well Transwell Boyden chambers that were precoated with Matrigel (BD Biosciences). The lower compartments were covered with 600 µl DMEM containing 20% FBS. Following culture for 24 h at 37˚C, the non-invaded cells were gently removed using a cotton swab. The invaded cells were fixed with 100% methanol at 37˚C for 30 min, stained with 0.1% crystal violet at 37˚C for 30 min, washed thrice with PBS and dried in air. Finally, the number of invasive cells was counted under an inverted microscope (magnification, x200; Olympus Corporation, Tokyo, Japan) in five randomly selected fields per chamber.
Bioinformatics analysis and luciferase reporter assay. TargetScan version 7.1 (targetscan.org/) and PicTar (Last update: March 26, 2007; pictar.mdc-berlin.de) were used to predict the potential targets of miR-584. BDNF was predicted as a candidate target of miR-584. Wild-type (Wt) 3′-UTR of BDNF containing the predicted miR-584 binding sites and mutant (Mut) 3′-UTR of BDNF (from accaua to UGGUAU) lacking complementarity with miR-584 designed and produced by Shanghai GenePharma co., ltd. were cloned into the pGL3 plasmid (Ambion; Thermo Fisher Scientific, inc.) and designated as pGL3-BdnF-Wt-3′-UTR and pGL3-BdnF-Mut-3′-UTR, respectively. Cells were plated into 24-well plates at a density of 1.5x10^5 cells per well. Following overnight incubation, miR-584 mimics or miR-NC, together with pGL3-BDNF-Wt-3′-UTR or pGL3-BDNF-Mut-3′-UTR, were transfected into cells using Lipofectamine® 2000 according to manufacturer’s protocol. Transfected cells were harvested 48 h after transfection, and luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis. Cells or tissues were homogenized using a radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc.), and total protein concentration was detected using a BCA assay kit (Beyotime Institute of Biotechnology). Equal amounts of proteins (30 µg) were separated by SDS-PAGE on a 10% gel. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology), blocked in 5% skimmed milk at room temperature for 2 h and incubated overnight at 4°C with primary antibodies. The primary antibodies used in this study included mouse anti-human monoclonal BDNF antibody (cat. no. ab205067; 1:1,000; Abcam) and mouse anti-human monoclonal GAPDH antibody (cat. no. ab125247; 1:1,000; Abcam). The membranes were washed in Tris-buffered saline containing 0.1% Tween-20 three times, then were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (cat. no. ab205719; 1:5,000; Abcam) at room temperature for 2 h. Protein signals were visualized using Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as the loading control. Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for analysis of band density.

Statistical analysis. Data are presented as the mean ± standard deviation, and analyzed with SPSS version 18 (SPSS, Inc.). Student’s t-test was used to evaluate the differences between two groups; while the differences between three groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls test. The association between expression levels of miR-584 and various clinical characteristics of HCC was determined using χ² test. Spearman's correlation analysis was applied to investigate the association between miR-584 and BDNF mRNA level in HCC tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-584 is downregulated in primary HCC tissues and cell lines. To identify the expression pattern of miR-584 in HCC, miR-584 expression in 56 pairs of HCC tissues and adjacent non-cancerous tissues was investigated. P<0.05 vs. non-cancerous tissues.

Table I. Association between miR-584 expression and clinical characteristics of hepatocellular carcinoma patients.

<table>
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<th>Clinical characteristics</th>
<th>miR-584 expression</th>
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<td>≥5 cm</td>
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miR, microRNA.
SonG et al: mir-584 IN HCC

Subsequently evaluated. All patients with HCC were divided into the low-miR-584- (n=28) or high-miR-584-expression groups (n=28) based on the cut-off value, which was defined as the median expression level of miR-584. Low expression level of miR-584 was significantly associated with tumor size (P=0.032), advanced tumor node metastasis (TNM) stage (P=0.014) and lymph node metastasis (P=0.007; Table I). Furthermore, miR-584 expression in two human HCC cell lines (Hep3B and Huh7) and an immortalized normal human liver epithelial cell (L-O2) was determined using real-time quantitative polymerase chain reaction (RT-qPCR). miR-584 expression was lower in the two examined HCC cell lines than in L-O2 (P<0.05; Fig. 1B). These findings demonstrated that miR-584 downregulation may be associated with HCC progression.

miR-584 upregulation restricts cell proliferation and invasion in HCC. To examine the detailed roles of miR-584 in HCC, Hep3B and Huh7 cells were transfected with miR-584 mimics or miR-NC. RT-qPCR analysis data confirmed that miR-584 was significantly overexpressed in Hep3B and Huh7 cells transfected with miR-584 mimics (P<0.05; Fig. 2A). Afterwards, CCK-8 assay was conducted to detect proliferative abilities of Hep3B and Huh7 cells following transfection with miR-584 mimics or miR-NC. As illustrated in Fig. 2B, miR-584 upregulation resulted in a significant reduction of the proliferation abilities of Hep3B and Huh7 cells compared with cells transfected with miR-NC (P<0.05). Furthermore, whether miR-584 overexpression affected HCC cell invasion ability was evaluated using a cell invasion assay. It was demonstrated that transfection with miR-584 mimics decreased invasion capacities of Hep3B and Huh7 cells compared with the miR-NC groups (P<0.05; Fig. 2C and D). Collectively, these results suggested that miR-584 may serve a tumor-suppressive role in HCC.

BDNF is a direct target of miR-584 in HCC. To clarify the mechanisms underlying the inhibitory effects of miR-584 in HCC progression, potential miR-584 targets were predicted using bioinformatics. BDNF was predicted to feature a potential binding site in its 3′-UTR (Fig. 3A) and was selected for further investigation, as BDNF has crucial roles in HCC tumorigenesis and tumor development (23-26). Then, a luciferase reporter assay was used to determine whether the 3′-UTR of BDNF could be directly targeted by miR-584 in HCC. The results demonstrated that miR-584 overexpression significantly reduced luciferase activities of the pGL3-BDNF-Wt-3′-UTR group in Hep3B and Huh7 cells (P<0.05). However, no significant change in luciferase activities was observed in the pGL3-BDNF-Mut-3′-UTR group (Fig. 3B). Additionally, it was demonstrated that the BDNF expression was notably decreased at the mRNA (P<0.05; Fig. 3C) and protein (P<0.05; Fig. 3D) levels when miR-584 was overexpressed in Hep3B and Huh7 cells. These results demonstrated that BDNF is a direct target of miR-584 in HCC.

miR-584 is negatively correlated with BDNF expression in HCC tissues. As BDNF was identified as a direct target of miR-584 in HCC, the association between miR-584 and BDNF in HCC was investigated further. RT-qPCR and western blot analysis were used to detect BDNF mRNA and protein expression in HCC tissues and adjacent non-cancerous tissues. The mRNA (P<0.05; Fig. 4A) and protein (P<0.05; Fig. 4B and C) levels of BDNF were higher in HCC tissues compared with adjacent non-cancerous tissues. Spearman's correlation analysis indicated an inverse association between miR-584 and BDNF mRNA in HCC tissues (r=-0.5433, P<0.0001; Fig. 4D). These results suggested that BDNF upregulation in HCC may be partly caused by miR-584 downregulation.
Restored BDNF expression counteracts the suppressive effects of miR-584 overexpression in HCC cells. Rescue experiments were conducted to explore whether BDNF mediates the suppressive roles of miR-584 in HCC. Hep3B and Huh7 cells were cotransfected with miR-584 mimics and pcDNA3.1 or pcDNA3.1-BDNF. Following transfection, western blot analysis revealed that BDNF protein expression was restored in miR-584 mimic-transfected Hep3B and Huh7 cells following cotransfection with pcDNA3.1-BDNF (P<0.05; Fig. 5A). BDNF restoration eliminated the effects of miR-584 overexpression on proliferation (P<0.05; Fig. 5B) and invasion (P<0.05; Fig. 5C and D) of Hep3B and Huh7 cells. Thus, these results suggested that miR-584 prohibits HCC development via direct inhibition of BDNF expression.

Discussion

In recent decades, an increasing number of studies have demonstrated that numerous miRNAs are dysregulated in HCC; these aberrantly expressed miRNAs are contributing regulators of HCC formation and progression (27-29); thus, revealing the biological roles of miRNAs in HCC may identify new therapeutic targets and diagnosis methods. In the present study, it was observed that miR-584 expression was significantly reduced in HCC tissues and cell lines. Reduced miR-584 expression was associated with tumor size, TNM stage and lymph node metastasis of patients with HCC. Ectopic expression of miR-584 inhibited the proliferative and invasion abilities of HCC cells. BDNF was demonstrated to be a direct target of miR-584 in HCC. BDNF was also upregulated in HCC tissues, and this upregulation was negatively correlated with miR-584 expression. Rescue experiments indicated that recovery of BDNF expression rescued the inhibitory effects of miR-584 overexpression in HCC cells. These results indicated that upregulating miR-584 expression may be effective in treating patients with HCC.

miR-584 dysregulation has been observed in multiple types of human cancer. For example, miR-584 is underexpressed in glioma tissues and cell lines (30). Glioma patients with low miR-584 level exhibit shorter postoperative survival time compared with those with high miR-584 level (19).
Figure 4. Negative correlation between miR-584 and BDNF mRNA expression in HCC tissues. (A) BDNF mRNA expression in 56 pairs of HCC tissues and adjacent non-cancerous tissues was analyzed using reverse transcription-quantitative PCR. *P<0.05 vs. non-cancerous tissues. (B) Representative western blot analysis and (C) semi-quantification demonstrated BDNF protein expression in HCC tissues and adjacent non-cancerous tissues. *P<0.05 vs. non-cancerous tissues. (D) Spearman's correlation analysis was employed to evaluate the correlation between miR-584 and BDNF mRNA in HCC tissues. r=−0.5433, P<0.0001. BDNF, brain-derived neurotrophic factor; HCC, hepatocellular carcinoma; N, non-cancerous tissue; T, tumor; miR, microRNA.

Figure 5. Upregulated BDNF antagonizes the inhibitory effects of miR-584 in hepatocellular carcinoma cell proliferation and invasion. (A) Downregulation of BDNF protein expression in Hep3B and Huh7 cells caused by miR-584 overexpression was recovered by cotransfection with pcDNA3.1-BDNF. *P<0.05 vs. miR-NC. *P<0.05 vs. miR-584 mimics + pcDNA3.1-BDNF. (B) Cell proliferation of Hep3B and Huh7 cells, and invasion (×200 magnification) of (C) Hep3B and (D) Huh7 cells were assessed following transfection with miR-NC, miR-584 mimics + pcDNA3.1 or miR-584 mimics + pcDNA3.1-BDNF. *P<0.05 vs. miR-NC. *P<0.05 vs. miR-584 mimics + pcDNA3.1-BDNF. miR, microRNA; NC, negative control; BDNF, brain-derived neurotrophic factor.
and cell lines. Low miR-584 level is closely associated with poor differentiation and lower probability of patients with neuroblastoma compared with those with high miR-584 level (20). In gastric cancer, decreased miR-584 expression has been validated as an independent prognostic factor for unfavorable outcomes (21). miR-584 is also weakly expressed in clear-cell renal cell (31) and thyroid carcinoma (32). These findings suggest that miR-584 is frequently downregulated in human cancers and may be identified as a biomarker for the diagnosis of specific human cancer types.

Deregulated miR-584 contributes to the carcinogenesis and progression of several human cancer types. For instance, miR-584 overexpression suppresses cell proliferation, migration, invasion and vasculogenic mimicry of glioma by directly targeting pituitary tumor-transforming gene 1 protein-interacting protein and Rho-associated protein kinase 1 (ROCK1) (19,30,33). Xiang et al (20) reported that miR-584 directly targets matrix metalloproteinase 14 (MMP14) and inhibits the growth, metastasis and angiogenesis of neuroblastoma cells in vitro and in vivo. Zheng et al (21) and Li et al (34) demonstrated that miR-584 upregulation restricts gastric cancer cell growth, metastasis and angiogenesis and increases cell apoptosis in vitro and in vivo by blocking WWP1 and MMP14. Ueno et al (31) demonstrated that restoration of miR-584 expression leads to a significant reduction in cell migration and invasion of clear-cell renal cell carcinoma by inhibiting ROCK1 expression. Studies conducted by Xiang et al (32) and Orlandella et al (35) revealed that miR-584 re-expression represses cell motility and promotes apoptosis in thyroid cancer by regulating ROCK1 and tumor suppressor candidate 2. These findings suggest that miR-584 may serve as a promising therapeutic target for treatment of specific types of cancer.

miRNAs serve regulatory roles in the initiation and progression of human cancers by directly interacting with the 3'-UTRs of their target genes and regulating gene expression (36). Therefore, studies should identify the targets of miR-584 in HCC. The present study demonstrated that BDNF is a direct target gene of miR-584 in HCC. BDNF, which is located at the short arm of chromosome 1, is a member of the neurotrophin family (37). It is overexpressed in a variety of human malignancies, including gastric (38), ovarian (39) and breast (40) cancers. Aberrant expression of BDNF is closely associated with oncogenesis and development of human cancers through regulation of various biological behaviors (41-43). A previous study also reported the upregulation of BDNF in HCC and that BDNF upregulation may serve key roles in the genesis and progression of HCC (23). BDNF downregulation suppresses cell growth, angiogenesis and metastasis, and induces apoptosis in HCC (24-26). Therefore, reducing BDNF expression may be a new therapeutic strategy for treating patients with HCC.

In conclusion, miR-584 is underexpressed in HCC tissues and cell lines. Low expression level of miR-584 is significantly correlated with large tumor size, advanced TNM stage and lymph node metastasis of patients with HCC. miR-584 overexpression reduces HCC cell proliferation and invasion by directly targeting BDNF. BDNF inhibition is required for tumor-suppressing roles of miR-584 in HCC cells. Thus, miR-584 may be a novel target for treatment of patients with HCC in the future.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
YS and GW carried out RT-qPCR and western blot analysis. JZ performed the CCK-8 assay. Cell invasion and luciferase reporter assays were conducted by JN and SZ. YY and WX performed the statistical analysis and designed the study, and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the research ethics committee of The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China), and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine. Written informed consent was obtained from all patients for the use of their clinical tissues.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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