EUROPEAN JOURNAL OF CANCER 46 (2010) 2828–2836

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MicroRNA-193b regulates proliferation, migration and invasion in human hepatocellular carcinoma cells

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ARTICLE INFO

Article history:
Received 13 April 2010
Received in revised form 23 June 2010
Accepted 23 June 2010
Available online 23 July 2010

Keywords:
MicroRNA
Invasion
Migration
Cell cycle

ABSTRACT

Background and Aims: Recently, some miRNAs have been reported to be connected closely with the development of human hepatocellular carcinoma. However, the functions of these miRNAs in HCC remain largely undefined.

Methods: The expression profiles of miR-193b were compared between HCC tissues and adjacent normal liver tissues using qRT-PCR method. This method was also be used to screen the potential target genes of miR-193b. A luciferase reporter assay was conducted to confirm target association. Finally, the functional effect of miR-193b in hepatoma cells was examined further.

Results: miR-193b was significantly down-regulated in most of the HCC tissues compared to the matching non-tumoural liver tissues. Furthermore, ectopic expression of miR-193b dramatically suppressed the ability of hepatoma cells to form colonies *in vitro* and to develop tumours in nude mice. CCND1 and ETS1 were revealed to be regulated by miR-193b directly. By regulating the expressions of these oncogenes, miR-193b induced cell cycle arrest and inhibited the invasion and migration of hepatoma cells.

Conclusions: miR-193b may function as a tumour suppressor in the development of HCC by acting on multiple tumourigenic pathways.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with nearly 600,000 deaths each year worldwide.[1] Despite therapeutic advances, the 5-year survival rate of HCC patients still is low and HCC remains the second most common cause of cancer mortality in China. Thus, the identification of new possible targets for the development of non-conventional treatments is urgent and will necessarily take advantage of progresses in the comprehension of the molecular pathogenesis of HCC.[2]

MicroRNAs (miRNAs) are an extensive class of small non-coding RNA molecules that are processed from much longer primary transcripts (termed pri-miRNAs) and arise from hairpin loop structures after successive enzymatic maturation steps (by Drosha in the nucleus and Dicer in the cytoplasm).[3] miRNAs can regulate gene expression both at the level of messenger RNA degradation and translation. By regulating the expression of target genes, miRNAs are involved in a variety of biological processes including cell cycle regulation, differentiation, development, metabolism, neuronal patterning and ageing.[4]

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.06.127

In recent years, many miRNAs have been shown to be aberrantly expressed in human hepatocellular carcinoma in comparison with matched non-neoplastic tissue. The study by Jiang and colleagues revealed that miR-199a, miR-21 and miR-301 were differentially expressed in the tumour compared with adjacent benign liver. On the other hand, a large number of mature and precursor miRNAs were up-regulated in the adjacent benign liver specimens.[5] Further comparison of miRNA expression profile in the HCC tumours with patient's survival time showed that a set of 19 miRNAs, involved in biological processes such as cell division, mitosis and G1-S transition, significantly correlated with the disease outcome.[5] In a similar study, a 20-miRNA metastasis signature that could significantly predict primary HCC tissues with venous metastases from metastasis-free solitary tumours was identified. Significantly, the same signature could also correlate with disease-free and overall survival. The results by Budhu and colleagues may be particularly useful to classify patients with HCC at an early stage, which may provide a more rational approach to treatment intervention.[6] Thus, either alone or in combination with other parameters, miRNA expression patterns may potentially become useful markers for HCC classification and prognostic risk stratification.

To identify the miRNAs associated with HCC, we analysed the miRNAs expression profile in a pair of HCC tissue and adjacent normal liver tissue by microarray hybridisation. A set of miRNAs, including miR-193b, were revealed to be down-regulated in HCC tissue.[7] In this study, we examined the expression level of miR-193b in human HCC specimens, screened its potential target genes and analysed its functions in hepatoma cells. Our data suggest that miR-193b may exert its tumour suppressor function via targeting different oncogenes.

2. Materials and methods

2.1. Patients and tumour characteristics

HCCs and surrounding control tissue specimens were obtained from 25 patients at General Hospital of PLA (Beijing, PR China) after surgical resection with informed consent. The tumour tissues and the adjacent normal tissues were frozen in liquid nitrogen after resection. No patient in the current study received chemotherapy or radiation therapy before the surgery. Liver samples were fully clinically characterised (Table 1). This study was performed with the approval of the Medical Ethical Committee of General Hospital of PLA.

Table 1 – Patient clinical features and miR-193b expression profile.

Patient no.	Age	Gender	Tumour size (cm × cm × cm)	Edmondson grade	HBsAg	HCV-Ab	Cirrhosis	Normalised miR-193b amount in tumour tissue relative to adjacent normal tissue $2^{-\Delta\Delta Ct}$
1	35	F	5 × 4 × 4	3	Positive	Negative	Yes	0.240
2	52	M	8.5 × 7 × 6	2	Positive	Negative	No	0.895
3	43	M	3.5 × 3 × 2	2	Positive	Negative	Yes	1.292
4	41	F	12 × 10 × 8	3	Positive	Negative	No	0.163
5	34	M	10 × 7 × 7	3	Positive	Negative	Yes	0.314
6	50	M	2.5 × 2.5 × 2	3	Positive	Negative	No	0.192
7	33	M	10 × 8 × 7	4	Positive	Negative	Yes	0.253
8	50	M	2.5 × 1.8 × 1.5	1	Negative	Negative	No	0.418
9	52	M	10.5 × 8 × 5	3	Positive	Negative	Yes	0.387
10	42	M	13 × 9 × 7	2	Positive	Negative	Yes	0.275
11	63	M	14 × 10 × 10	2	Positive	Negative	Yes	0.163
12	70	M	3.5 × 3.5 × 3	2	Positive	Negative	Yes	0.004
13	66	F	15 × 7 × 5	3	Positive	Negative	No	0.243
14	39	M	12 × 11 × 6	3	Positive	Negative	No	0.120
15	51	M	2.5 × 2 × 2	2	Positive	Negative	Yes	1.434
16	47	M	5 × 5 × 4	2	Positive	Negative	Yes	1.189
17	37	M	16 × 4 × 8	2	Positive	Negative	Yes	0.237
18	52	M	8 × 7 × 4.5	2	Positive	Negative	No	0.283
19	64	M	8 × 5 × 4	2	Negative	Positive	No	1.057
20	50	F	6 × 5.5 × 2.5	1	Negative	Negative	No	0.003
21	52	M	7 × 7 × 7	3	Positive	Negative	Yes	0.257
22	56	M	8 × 6.5 × 5	3	Positive	Negative	Yes	0.095
23	48	M	2 × 2 × 1.5	2	Positive	Negative	No	0.139
24	59	M	8.8 × 8.5 × 8	2	Negative	Negative	No	2.056
25	40	M	2.5 × 2.5 × 2	2	Positive	Negative	Yes	0.006

HBsAg indicates hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; M, male; F, female; miRNA, microRNA. Relative quantification was performed by the $2^{-\Delta\Delta Ct}$ method with adjacent normal liver tissue sample as a calibrator. Data show the means from three independent analyses. Every independent analysis was carried out after the RNA extraction step. Total RNA was poly-A tailed, reverse transcripted, and then real-time PCR tested. ΔC_T obtained from real-time PCR was subject to paired *t*-test ($\Delta C_T = C_{TmiR} - C_{TU6}$). The expression levels of miR-193b in tumour tissues were significantly lower than in adjacent normal tissues ($P < 0.01$, $t = 4.60$).

2.2. Plasmid construction

Wild-type 3′-untranslated regions (3′-UTRs) containing predicted miR-193b target sites were amplified by PCR from HepG2 cell genomic DNA. Mutant 3′-UTRs were generated by overlap-extension PCR method. Both wild-type and mutant 3′-UTR fragments were cloned downstream of firefly luciferase coding region between the *XbaI* and *NdeI* sites of a modified pGL3-control plasmid (Promega, Madison, WI), as described before.[8] DNA fragment containing primary miR-193b was amplified from HepG2 genome DNA and cloned into pcDNA3.0 expression vector (Invitrogen, Carlsbad, CA).

2.3. Cell culture and transfection

The human hepatoma cell lines HepG2 and SK-Hep-1 were grown in DMEM containing 10% FBS and 100 μg/ml penicillin/streptomycin. The miRNAs and siRNAs were designed and synthesised by GenePharma (Shanghai, China) (see in supplementary materials). siRNAs and miRNAs transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA and protein were prepared 48 h after transfection and were used for qRT-PCR or Western blot analysis. The recombinant plasmid was transfected into SK-Hep-1 cells using FuGene HD (Roche, Indianapolis, IN) according to the manufacturer's protocol.

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from the cultured cells and the HCC tissue specimens using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. qRT-PCR was used to confirm the expression levels of mRNAs and miRNAs as described before.[8,9] U6 snRNA and G3PDH mRNA levels were used for normalisation.

2.5. Western-blot analysis

Protein extracts were prepared by a modified RIPA buffer with 0.5% sodium dodecyl sulphate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini, Roche, Indianapolis, IN). Polyacrylamide gel electrophoresis, tank-based transfer to Immobilon Hybond-C membranes (Amersham Biosciences) and immunodetection were performed with standard techniques. Antibodies against ETS1 (sc-55581), β-actin (sc-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA) and CCND1 (K0062-3, MBL, Nagoya, Japan) were used in western blot analysis in accordance with the manufacturer's instruction. Signals were visualised with SuperSignal® West Pico chemoluminescent substrate (Pierce, Rockford, IL) by exposure to films.

2.6. Luciferase assay

HepG2 cells were transfected in 24-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection mixtures contained 100 ng of firefly luciferase reporter plasmid and 5 pmol of miR-193b. pRL-TK (Promega, Madison, WI) was also transfected as a normalisation control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI).

2.7. Cell cycle analysis

Cell cycle analysis was performed as described before.[8] Analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with Cell Quest Pro software. Cell-cycle modelling was performed with Modfit 3.0 software (Verity Software House, Topsham, ME).

2.8. Cell proliferation assay

Cell proliferation was monitored using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). Sk-Hep-1 cells (3000 per well) were transfected with miR-193b or siRNA against CCND1 in 96-well culture plates. 72 h later, 5 μl CCK-8 reagent was added to each well and incubated at 37 °C for 1 h. The number of viable cells was assessed by measurement of absorbance at 450 nm using Multiskan MS (Labsystems, Finland).

2.9. Transwell cell invasion and migration assay

For invasion assay, HepG2 transfectants were serum starved for 24 h in DMEM containing 0.1% FBS. Serum-starved cells were trypsinised and resuspended in DMEM containing 0.1% FBS and 2×10^5 cells were added to the upper chamber of each well (6.5 mm in diameter, 8-μm pore size; Corning, NY, USA) coated with 30 mg/cm^2 Matrigel (ECM gel, Sigma–Aldrich, St. Louis, MO). Medium containing 0.1% FBS and supplemented with HGF (20 ng/ml) was placed in the lower compartment of the chamber. After 24 h at 37 °C, cells on the upper membrane surface were removed by careful wiping with a cotton swab and the filters were fixed by treatment with 95% ethanol for 30 min and stained with 0.2% crystal violet solution for 30 min. Invasive cells adhering to the undersurface of the filter were then counted (five high-power fields/chamber) using an inverted microscope. The migration assay is the same with invasion assay excepting no matrigel was used and the permeating time for cells was 12 h.

2.10. Colony formation assay

Twenty-four hours after transfection, 200 transfected cells were placed in a fresh six-well plate and maintained in DMEM containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min.

2.11. Tumourigenicity assays in nude mice

NC RNA and miR-193b-transfected SK-Hep-1 cells (8×10^5) were suspended in 100 μl PBS and then injected subcutaneously into either side of the posterior flank of the same female BALB/c athymic nude mouse at 4–6 week of age. Tumour growth was examined daily for at least 5 weeks. Tumour volume (V) was monitored by measuring the length (L) and width (W) with calipers and calculated with the formula $(L \times W^2) \times 0.5$.

2.12. Statistical analysis of data

Unless stressed, all results are expressed as means ±SD. Differences were assessed by two tailed Student *t*-test using Excel software. $P < 0.05$ was considered to be statistically significant. GenEX software (TATAA Biocenter, Munich, Germany) was employed to analyse real time PCR data. Paired student's *t*-test was performed to determine the difference of miRNA's expression levels observed between non-cancerous and cancer tissues.

3. Results

3.1. miR-193b is down-regulated in Human Hepatocellular Carcinoma (HCC)

miR-193b was revealed to be down-regulated in one HCC tissue specimen relative to the matching adjacent normal liver tissue.[7] Expressions of miR-193b were examined further in an independent series of primary HCC tumours and adjacent non-tumoural livers (The clinical parameter of the HCC patients is shown in Table 1) by using quantitative Real-time PCR method. The results showed that miR-193b was significantly repressed (2–330 fold) in 76% of tumours (19 of 25 patients) compared to the matching adjacent non-tumoural liver tissues (Table 1). These results suggest that down-regulation of miR-193b may be involved in most of human HCC development. miR-193b is an intergenic microRNA located at 16p13.12. A CpG island is found at the predicted 5′end of pri-miRNA transcripts of miR-193b (Fig. S2A). Moreover, miR-193b was reported to be epigenetically silenced in prostate cancer.[10] To analyse if miR-193b is an epigenetically silenced miRNA in hepatocellular carcinoma, hepatoma cells were treated with 5azadC (5-aza-2′-deoxycytidine) and TSA (trichostatin). However, the results of qRT-PCR revealed that 5azadC+TSA treatment did not increase the expression of miR-193b in HepG2 and SK-Hep-1 cells (Fig. S1). Next, methylation analysis of miR-193b by genomic bisulphite sequencing was carried out at a dense CpG island located ~1 kb upstream of mature miR-193b locus (Fig. S2A). No methylation was found in any of the five HCC samples (#20-#23, and #25) studied, the result of sample #20 is shown in Fig. S2B. The data mentioned above suggest that miR-193b is not epigenetically silenced in hepatocellular carcinoma. The repression of miR-193b in HCC could be regulated by certain transcription factors.

3.2. miR-193b Suppresses colony formation in vitro and tumourigenicity in vivo

To examine the potential role of miR-193b in tumourigenesis, the capacity of colony formation was evaluated on HCC cell line (SK-Hep-1 and HepG2) that was transfected with miR-193b, or negative control RNA duplex. Notably, miR-193b-transfected cells displayed obviously fewer and smaller colonies compared with NC RNA-transfectants (Fig. 1A). These results indicate a growth-inhibitory role of miR-193b on hepatoma cell lines.

To further confirm the antitumour effects of miR-193b, NC RNA and miR-193b–transfected SK-Hep-1 cells were injected into six nude mice as described. Five weeks after injection, tumours appeared in the sites injected with NC RNA-transfected SK-Hep-1 cells (5/6). The tumour became palpable 20–24 days after injection and grew to 137–672 mm^3 at the end of observation (5 weeks). However, no tumours were observed in the flanks injected with miR-193b-transfected SK-Hep-1 cells (Fig. 1B). The results mentioned above indicate that over-expression of miR-193b significantly inhibits tumourigenicity of SK-Hep-1 cells in nude mouse xenograft model.

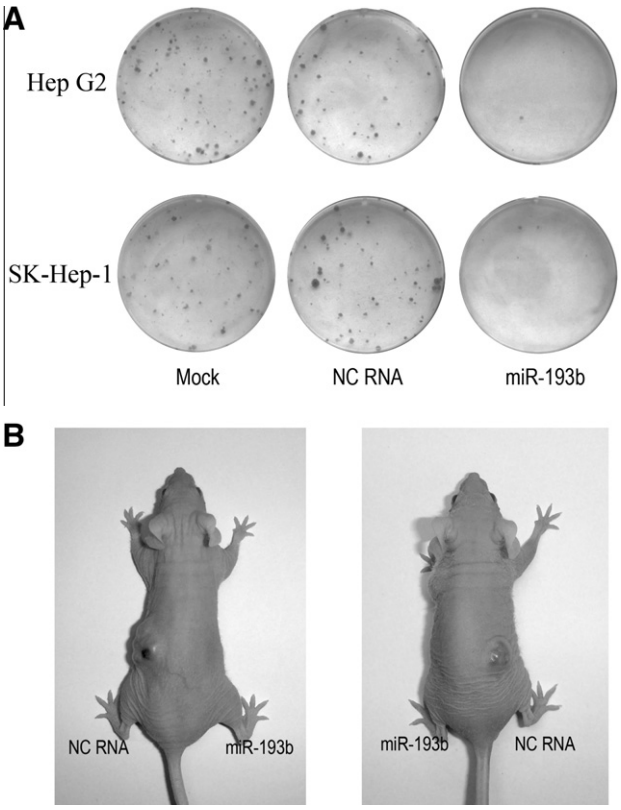

Fig. 1 – Effect of miR-193b on hepatoma cell growth *in vitro* and *in vivo*. (A) Effect of miR-193b on colony formation of hepatoma cell lines. HepG2 and SK-Hep-1 cells were transfected with miR-193b or negative control RNA. 24 h after transfection, 200 transfected cells/well were seeded in a fresh six-well plate and colonies were stained with 0.1% crystal violet after two weeks. The results were reproducible in three independent experiments. (B) Effect of miR-193b on tumour formation in nude mouse xenograft model. NC RNA and miR-193b in Fig. 1B indicate the flanks injected with NC RNA-transfected and miR-193b-transfected SK-Hep-1 cells, respectively. Photographs illustrate representative features of tumour growth 5 wk after inoculation.

3.3. miR-193b Directly represses the expressions of CCND1 and ETS1 through their 3′-UTRs

On the basis of miRNA target prediction, over a hundred genes are the putative target genes of miR-193b.[11,12] Over-expression of miR-193b significantly inhibited the tumourigenicity of SK-Hep-1 cells in nude mouse xenograft model (Fig. 1B). For this reason, the target genes regulated by miR-

193b should function as oncogenes involved in cell proliferation, apoptosis, cell invasion or angiogenesis. According to this criterion, a set of oncogenes were picked out from all putative target genes (Table S1); these genes were most likely regulated by miR-193b.

To identify the genuine targets, synthetic miR-193b and negative control RNA were transfected into HepG2 cells. qRT-PCR was used to detect the expression levels of those putative target genes (Table S1). According to the results of qRT-PCR, several putative target genes were not detected in HepG2 cells, and the expression levels of 7 putative target genes had no remarkable changes in HepG2 cells transfected with miR-193b (Fig. S3). Whereas, the mRNA expressions of CCND1 (Cyclin D1) and ETS1 were repressed by miR-193b over-expression (Fig. 2A), suggesting that they were regulated by miR-193b.

To test whether regulation was direct, 3′-UTRs of human ETS1 and CCND1 were cloned into the modified pGL3-control vector, placing the 3′-UTRs with putative miR-193b binding sites downstream of the coding sequence of luciferase. These constructs were co-transfected into HepG2 cells with miR-193b. miR-193b but not negative control RNA specifically decreased the luciferase levels from each reporter (Fig. 2B and C). The analogous reporters with point substitutions disrupting the target sites were also cotransfected with miR-193b. There was no significant decrease in the relative luciferase activity compared with negative control RNA (Fig. 2B and C). These data suggest that miR-193b may repress the mRNA expressions of ETS1 and CCND1 at posttranscriptional level by directly targeting their 3′-UTRs.

Western blot analysis showed that miR-193b markedly reduced protein expression levels of ETS1 and CCND1 compared

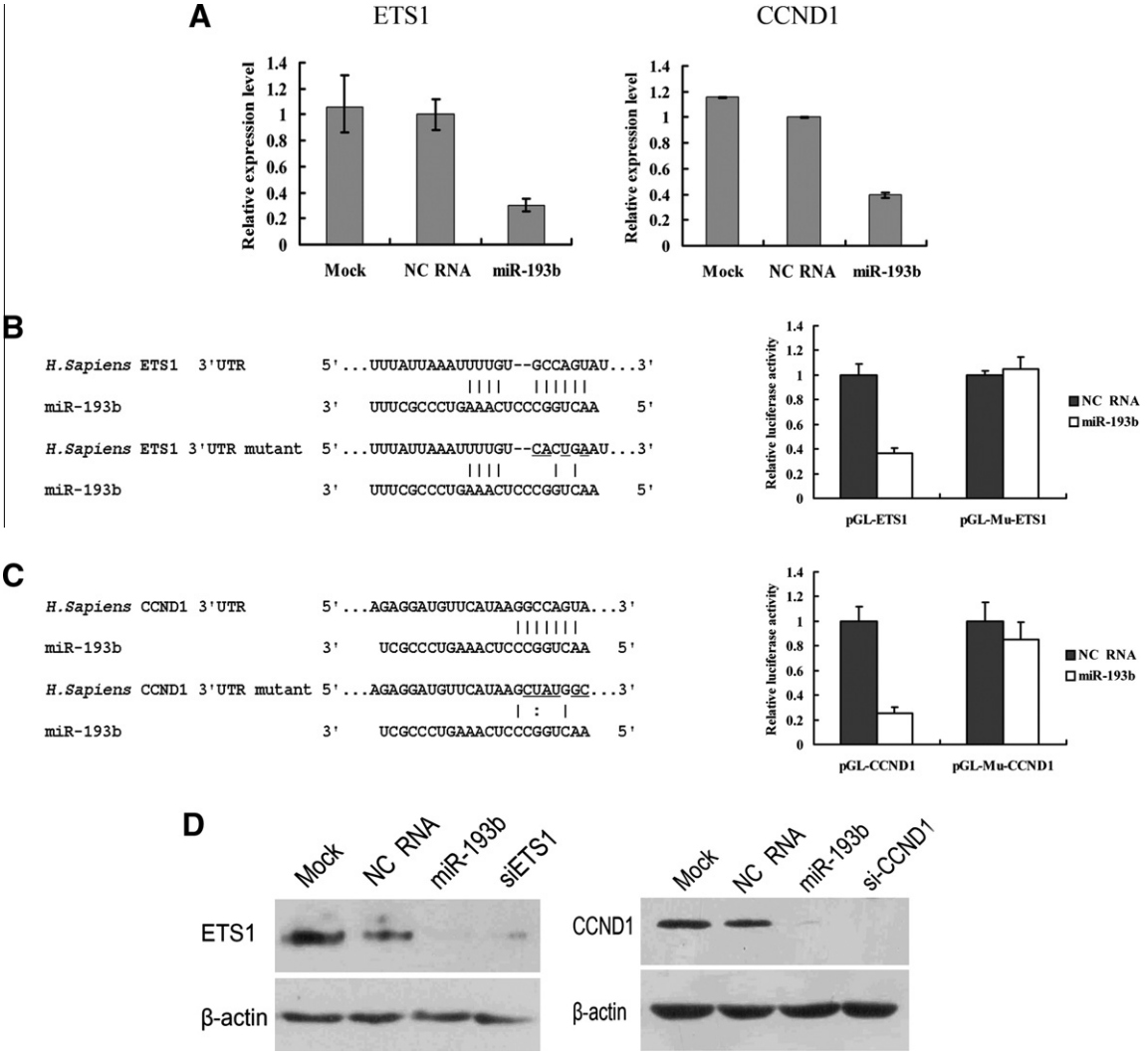

Fig. 2 – miR-193b down-regulates the expressions of ETS1 and CCND1 directly. (A) HepG2 cells transfected with miR-193b duplexes and NC RNA as described, and the expression levels of ETS1 and CCND1 were analysed by qRT-PCR. The G3PDH mRNA served as an internal control. (B and C) Luciferase assays indicated that miR-193b down-regulated the expression of ETS1 (B), and CCND1 (C) by binding with their 3́-UTRs. (D) HepG2 cells were transfected with miR-193b, siRNA and NC RNA as described, and were subsequently processed for Western blot analysis by hybridisation with antibodies against ETS1, CCND1 and β-actin.

with negative control RNA and mock-transfected cells (Fig. 2D). In addition, two siRNAs against these target genes could also inhibit their expressions, respectively. All these results reveal that ETS1 and CCND1 are the genuine targets of miR-193b.

To determine the clinical significance of miR-193b target genes, we examined the mRNA levels of CCND1 and ETS1 in 25 pairs of matched HCC specimens by qRT-PCR. As expected in Table S2, the mRNA expression levels of all these target genes were negatively correlated with miR-193b. For example, amongst the total 19 pairs of HCC tissues with miR-193b under-expression, CCND1 and ETS1 were up-regulated in 14 and 15 samples, respectively. However, we also observed that miR-193b dysregulation in some other HCC tissues could not account for the expression levels of CCND1 and ETS1. These data suggest that miR-193b might play a critical role on the regulation of these target genes in most but not all of the HCC patients. These might also represent the complexity of gene regulation especially in HCC. We speculate that other factors (either protein or RNA) might antagonise or interfere the effect of miR-193b on these targets, which needs further investigations in the future.

3.4. *miR-193b Represses cell proliferation by inducing a G1 arrest in SK-Hep-1 cells*

Repression of CCND1 prevents cells from entering the S phase, causing an accumulation of cells in G0/G1.[13] To verify whether miR-193b can trigger G1 arrest in hepatoma cell lines, miR-193b and siRNA against CCND1 were transfected into SK-Hep-1 cells. Mock- and NC RNA-transfected SK-Hep-1 cells showed a normal cell cycle distribution. In contrast, cell cultures transfected with miR-193b or siCCND1 had increased numbers of cells in G0/G1 and corresponding de-

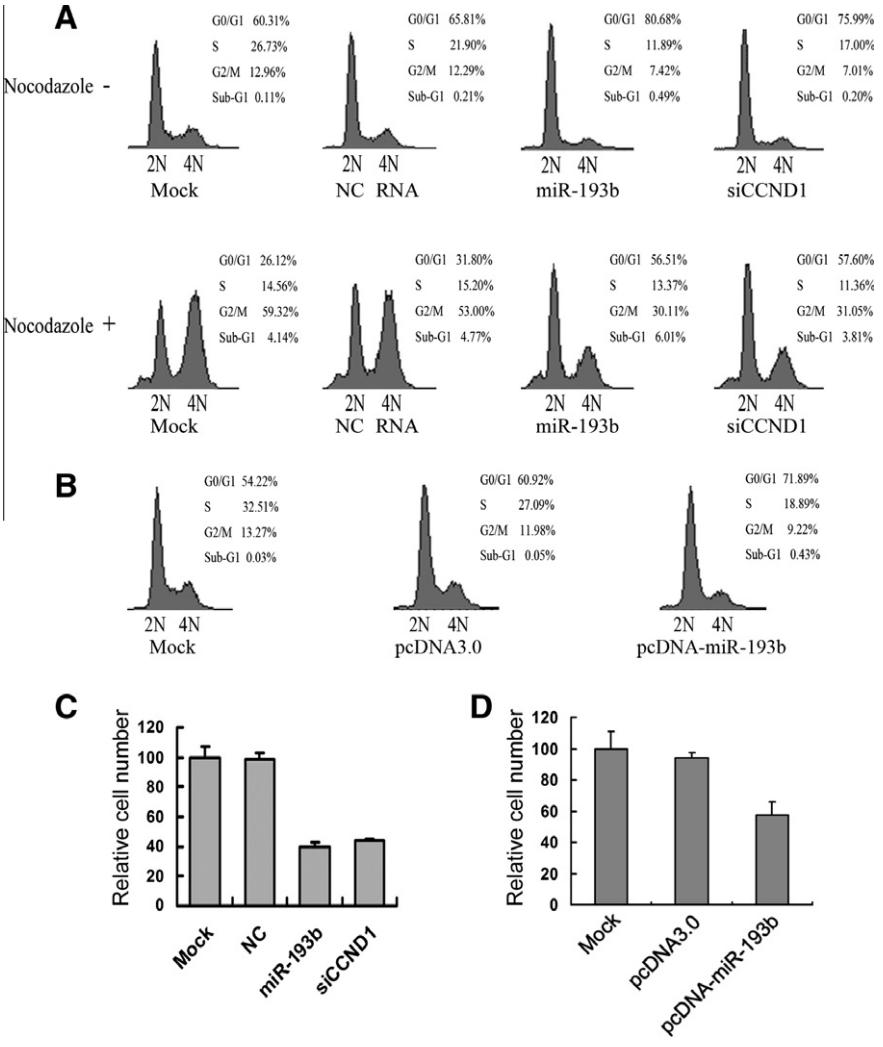

Fig. 3 – miR-193b induces G1 arrest and inhibits growth in SK-Hep-1 cells. (A) miR-193b and siRNA against CCND1 triggered G1 arrest in SK-Hep-1 cells. SK-Hep-1 cells were treated with nocodazole 24 h posttransfection and cell cycle distribution was detected 20 h later. 2N, cells having diploid DNA content; 4N, cells having tetraploid DNA content. (B) G1 arrest could be induced by miR-193b expressed from genomic fragment. SK-Hep-1 cells were transfected with pcDNA3.0 and pcDNA-miR-193b. The cells were collected and used for cell cycle analysis 48 h posttransfection. (C–D) miR-193b inhibited growth in SK-Hep-1 cells. The viable cells 72 h after transfection were detected by CCK-8 assay as described. Data are means of three separated experiments ± SD.

creases in the numbers of cells in S and G2/M (Fig. 3A). This finding suggests that miR-193b can induce G1 arrest in SK-Hep-1 cell line. The G0/G1 accumulation phenotype became clearer when the microtubule-depolymerising drug nocodazole was added 24 h posttransfection to block cells from reentering the cell cycle after mitosis. This treatment caused most of the NC RNA-transfected cells to accumulate in G2/M, whereas a large fraction of miR-193b or siCCND1-transfected cells remained in G0/G1 (Fig. 3A). To confirm that the cellular phenotype induced by miR-193b is not artificial, we determined whether G0/G1 cell accumulation phenotype could be induced by miR-193b expressed from genomic fragment. SK-Hep-1 cells were transfected with the plasmids expressing primary forms of miR-193b. Flow cytometry analysis revealed that vector-expressed miR-193b could also induce G0/G1 cell accumulation phenotype in SK-Hep-1 cells (Fig. 3B). But the effect of vector-expressed miR-193b on cell cycle arrest was weaker than that of synthetic mature miRNA, possibly due to relatively low transient transfection efficiency.

We next evaluated the effect of miR-193b on the growth of HCC cells. The results of cell proliferation assay showed that ectopic expression of miR-193b led to significant inhibition of cell proliferation compared to control cells (Fig. 3C and D). These results indicate a growth-inhibitory role of miR-193b on hepatoma cell line SK-Hep-1.

3.5. *miR-193b Negatively regulates cell migration and Invasion in vitro*

Transcription factor ETS1 is over-expressed and plays a crucial role in the invasive property in hepatoma cell lines.[14,15] HGF, also known as scatter factor, can induce the expression of ETS1 in hepatoma cells by binding with hepatocyte growth factor receptor (MET).[15] Then, we examined the effect of miR-193b on HGF-induced migration of HepG2 cells using the transwell migration assay. The migration of HepG2 cells was significantly induced by HGF, and miR-193b could reduce the migration induced by HGF (Fig. 4A and B). Next, the effects of miR-193b on the invasion of HepG2 cells were determined by Matrigel invasion assay system. In line with the results from the above-mentioned migration assays, miR-193b also inhibited the invasion of HepG2 cells (Fig. 4C and D). To rule out other targets effect of miR-193b on cell migration and invasion, we used ETS1 siRNA to downregulate the ETS1 gene expression. siETS1 could also inhibit the migration and invasion induced by HGF (Fig. 4A–D). In addition, our results suggested that the reduction of the number of migrative/invasive cells is not due to apoptosis and inhibition of cell proliferation (not shown). Similar results were observed in SK-Hep-1 cell lines (not shown). Taken together, the results mentioned above suggest that miR-193b is a potent suppressor of hepatoma cell migration and invasion by repressing the expression of transcription factor ETS1, at least in partial.

4. Discussion

In this study, we found that miR-193b was frequently down-regulated in HCC tissues. Ectopic expression of miR-193b dramatically suppressed the ability of hepatoma cells to form colonies *in vitro* and to develop tumours in nude mice. Furthermore, two oncogenes, which play crucial roles in the development of hepatocellular carcinoma, were identified as target genes of miR-193b. By regulating the expressions of these oncogenes, miR-193b suppressed cell proliferation and colony formation and inhibited the invasion and migration of hepatoma cells.

Hepatocarcinogenesis is a complex process associated with accumulation of genetic and epigenetic changes that occur during initiation, promotion and progression of the disease. Initial steps involve the disruption of a set of interdependent pathways controlling the homeostasis between cell growth and apoptosis. At later stages, cells may acquire angiogenic, invasive and metastatic properties.[16] Like protein-coding genes, some microRNAs also function as oncogenes or tumour suppressors in human hepatoma. Various studies have reported the molecular functional links between miRNA abnormal expression and the hallmarks of malignant transformation: aberrant cell growth, cell death, differentiation, angiogenesis, invasion and metastasis.[4] Notably, some miRNAs can simultaneously regulate the expression of multiple target genes. Here, we found that miR-193b, down-regulated in hepatocellular carcinoma, could repress the expression of two oncogenes (ETS1 and CCND1) involved in the regulation of tumour cell migration and invasion and proliferation.

Abnormal cell cycle control is not only a critical step in HCC maintenance but also appears to be a critical early event. Indeed, an increased proliferation rate and a proliferating cell fraction characterise liver cirrhosis too, and correlate with a higher propensity to develop HCC.[16] In recent years, a series of miRNAs involved in cell cycle control were reported. miR-221, emerged as a significantly up-regulated miRNA in hepatocellular carcinoma, could promote cell cycle progression by modulating the expression of the cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57.[17] As previously mentioned, miR-122, down-regulated in more than 70% of HCCs, can regulate cyclin G1 expression directly. The up-regulation of CCNG1 consequent to miR-122 down-regulation in human HCC may thus promote tumourigenesis.[18] miR-195, another miRNA down-regulated in HCCs, can block the G(1)/S transition by repressing Rb-E2F signalling through targeting multiple molecules, including cyclin D1, cyclin D3, cyclin E1, CDK6 and E2F3.[8,19] Whilst we were preparing this manuscript, Chen and colleagues reported that miR-193b could repress cell proliferation and regulate CCND1 expression in melanoma cells.[20] Here, we found that miR-193b not only triggered a G1 arrest in hepatoma cells by regulating the expression of CCND1 but also inhibited the tumourigenicity of hepatoma cells in nude mouse xenograft model (Fig. 2B and Fig. 3A).

This study has also shed light on the potential role of miR-193b in hepatoma metastasis. Tsai and colleagues have reported that miR-122, the most abundant miRNA in the liver, can affect hepatocellular carcinoma intrahepatic metastasis via regulation of ADAM17.[21] miR-21 is highly over-expressed in HCC tumours and cell lines. Moreover, aberrant expression of miR-21 can contribute to HCC growth and metastasis by modulating PTEN expression.[22] In our previous studies, we have found that miR-34 and miR-101, down-regulated in hepatocellular carcinoma, inhibited the invasion and migra-

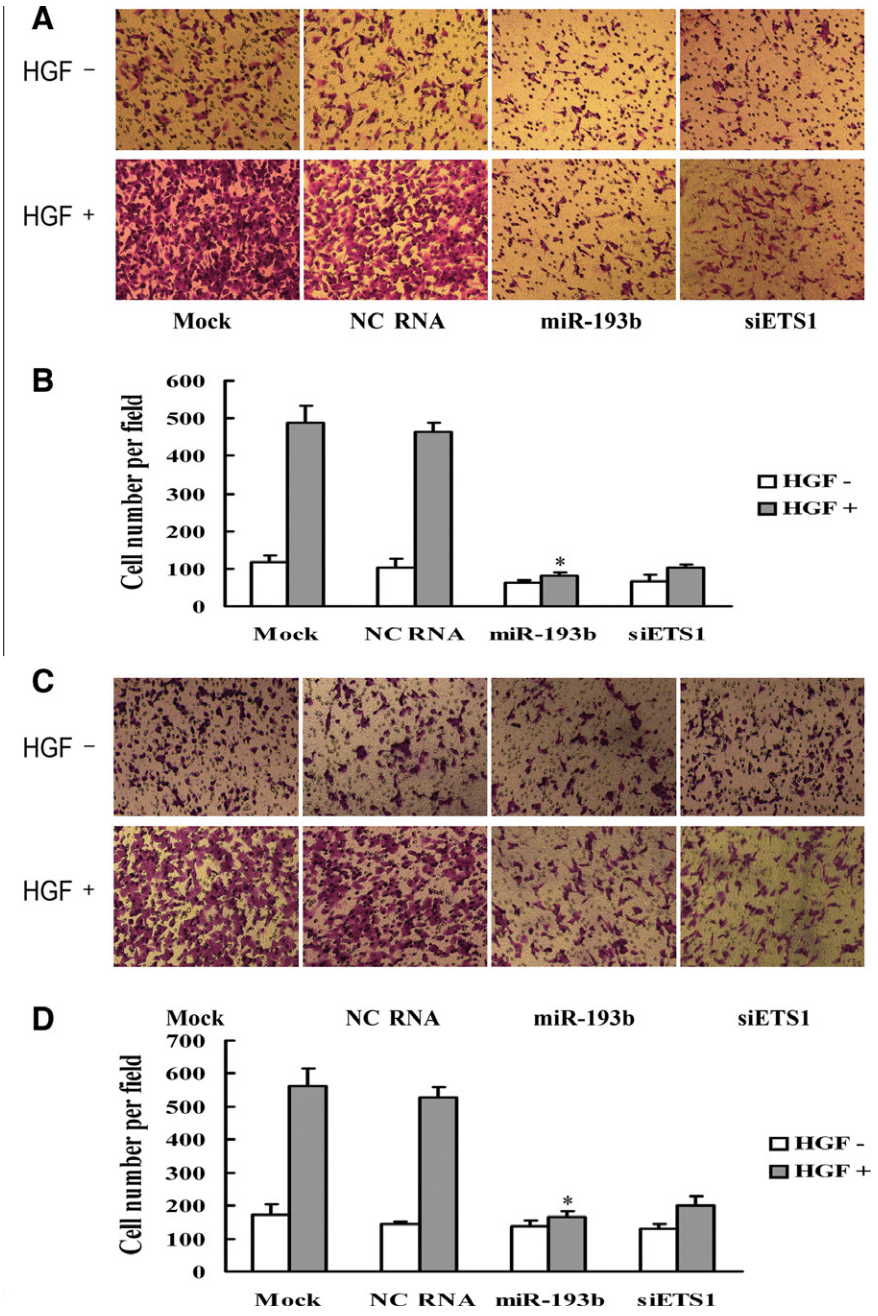

Fig. 4 – miR-193b suppresses migration and invasion of HepG2 cells by repressing the expression of ETS1. (A) Cell migration assay. HepG2 cells were transfected with miR-193b, NC RNA or ETS1 siRNA and then subjected to transwell migration assays, as described. After 12 h, migration cells were counted after staining with crystal violet. (B) Migration cell numbers are the average count of three random microscopic fields. Each bar represents the mean ± SD of the counts from a single representative experiment. *P < 0.01. (C) Cell invasion assay. Cells transfected with control RNA, miR-193b or ETS1 siRNA were subjected to transwell invasion assays, as described. After incubation for 24 h cells that invaded through the pores to the under surface of the membrane were fixed, stained and counted. (D) Invading cell numbers are the average count of three random microscopic fields. Each bar represents the mean ± SD of the counts from a single representative experiment. *P < 0.01.

tion of cultured HCC cells by regulating the expressions of MET and FOS, respectively.[7,23] Here, we showed that miR-193b repressed the expression of the transcription factor ETS1. ETS1 oncogene is up-regulated and involved in the over-expression of MMP-1, -3 and -7 in human HCC and may contribute to the metastasis of HCC.[14,15] Furthermore, our results revealed that ectopic expression of miR-193b reduced cell migration and invasion of HepG2 cells, just like siRNA against ETS1. miR-193b has been reported to repress uPA expression and inhibited cell invasion in MDA-MB-231 and MDA-MB-435 cells.[24] But we detected no significant change of uPA protein in HepG2 cells transfected with miR-193b.

Therefore, down-regulation of ETS1 could be a possible mechanism by which miR-193b regulates migration and invasion potential of HCC cells.

In summary, we detected the expression levels of miR-193b in HCCs and investigated the potential role of miR-193b in hepatocarcinogenesis. We found that miR-193b could simultaneously affect cell proliferation, migration and invasion by acting on different targets. These results outline an important role of miR-193b in the development of HCC and implicate its potential application in cancer therapy.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported partially by Chinese State Key Projects for Basic Research (2010CB912801, 2007CB914601, 2006CB910407), Important National Science and Technology Specific Projects (2008ZX10002-016) and Chinese National Natural Science Foundation Projects (30873008, 30870529).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.06.127.

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