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MiR-501-5p regulates CYLD expression and promotes cell proliferation in human hepatocellular carcinoma

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Abstract

Objective: Previous studies have shown that the micro-ribonucleic acid miR-501-5p is upregulated in hepatocellular carcinoma cells and tissues with high hepatitis B virus replication, and that miR-501 overexpression significantly promotes hepatitis B virus replication. We further analysed a published microarray-based high-throughput dataset (NCBI/GEO/GSE36915) and found that miR-501-5p was upregulated in hepatocellular carcinoma tumour tissues. We therefore investigated the possible function of miR-501-5p during the development of hepatocellular carcinoma.
Methods: Expression of miR-501-5p in human hepatocellular carcinoma specimens and cell lines was assessed, using real-time polymerase chain reaction. Luciferase reporter assays were used to confirm *CYLD* as a target of miR-501-5p. The effect of miR-501-5p on cell proliferation was confirmed, using tetrazolium and colony formation assays. Gene and protein expression were examined, using real-time polymerase chain reaction and western blotting, respectively.
Results: MiR-501-5p was upregulated in hepatocellular carcinoma specimens and cell lines, and directly targeted the 3′ untranslated region of *CYLD*. MiR-501-5p upregulation corresponded with a downregulation of CYLD in the same tissues and cell lines, and overexpression of MiR-501-5p decreased CYLD expression, increased expression of cyclin D1 and c-myc and promoted the proliferation of hepatocellular carcinoma cells *in vitro*.
Conclusions: This study suggests that miR-501-5p may play an important role in the development of hepatocellular carcinoma by promoting cell proliferation, and indicates that miR-501-5p may represent a novel therapeutic target for hepatocellular carcinoma.

Key words: miR-501-5p, CYLD, proliferation, HCC

Introduction

Hepatocellular carcinoma (HCC) has become a major health burden and represented the third leading cause of cancer-related mortality worldwide in recent decades (1). In the USA, an estimated 28 720 new cases and 20 550 deaths due to HCC were expected in 2012. The incidence of HCC has been increasing sharply since the mid-1990s, with the incidence rates for males and females increasing by 3.6 and 3.0% per year, respectively, between 2004 and 2008 (2). Currently, significant progress is being made in the development of targeted therapies for HCC. The introduction of the multi-tyrosine kinase inhibitor sorafenib represented a major breakthrough in HCC therapy (3). However, at present, targeted therapies have failed to

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achieve satisfactory effects; therefore, there is a need for more effective targeted drugs for HCC.

The cylindromatosis (turban tumour syndrome; *CYLD*) gene is located at 16q12.1 and encodes a cytoplasmic protein, containing three cytoskeletal-associated protein-glycine-conserved (CAP-GLY) domains, that functions as a deubiquitinating enzyme. Mutations in *CYLD* have been associated with Brooke–Spiegler syndrome, familial cylindromatosis and multiple familial trichoepithelioma, which were originally described as distinct inherited disorders characterized by a variety of skin appendage neoplasms (4,5). Mutations in the *CYLD* gene have been demonstrated in affected families, indicating that this gene possesses the characteristic features of a tumour suppressor (6). Catalytic inactivation of CYLD in the human skin has been linked to the development of squamous cell carcinoma (7), and we previously demonstrated that CYLD regulates the progression of skin cancer via a number of mechanisms including control of tumour differentiation, angiogenesis and cell survival (8,9). CYLD has also been recognized as a negative regulator of nuclear factor-kappa B (NF-κB) activity and acts as a tumour suppressor gene in several types of cancer. Hellerbrand et al. (10) reported that transcription of *CYLD* is strongly downregulated in colorectal cancer cells compared with normal colorectal tissues, and Hayashi et al. (11) demonstrated that breast cancer tissues expressed significantly lower levels of *CYLD* mRNA than normal breast tissues. Additionally, Deng et al. (12) reported that increased expression of CYLD directly blocked NF-κB activation, and consequently increased apoptosis in lung cancer cells.

Micro-ribonucleic acids (miRNAs) are a class of small endogenous non-coding RNAs involved in multiple biological processes. MiRNAs bind to the 3′ untranslated region (UTR) of specific target genes to negatively regulate post-transcriptional gene expression, and may act as tumour suppressors or oncogenes (13–15). Increasing evidence indicates that miRNAs are involved in the development of human cancer, and miRNAs have been widely proposed as potential targets for anti-cancer therapies (16–18). A previous study showed that miR-501-5p was upregulated in HepG2 cells and tissues with high HBV replication, and that miR-501 overexpression could significantly promote HBV replication (19). By analysing a published microarray-based high-throughput dataset (NCBI/GEO/GSE36915), we found that miR-501-5p was significantly upregulated in HCC. These studies indicated that miR-501-5p is an important role in modulating tumour progression, and may represent a promising therapeutic target in HCC.

In the current study, we compared the expression of miR-501-5p in HCC cells and normal hepatic cells, and examined the effect of overexpressing miR-501-5p on the proliferation of HCC cells *in vitro*. We demonstrate that miR-501-5p may promote proliferation in HCC by directly targeting the 3′ UTR of *CYLD* mRNA, which consequently upregulates the cell-cycle regulator cyclin D1 and c-myc. This study suggests that miR-501-5p may play an important role in the development and progression of HCC.

Patients and methods

Cell culture

Human HCC cells, QGY-7703, BEL-7402, MHCC97H, Huh7, HepG2, MHC97L, HCCC-9810 and Hep3B, and non-cancer hepatocyte cells Lo2, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), at 37°C in a 5% CO_2 atmosphere in a humidified incubator.

Tissue specimens

For the use of the clinical materials for research purposes, prior patient consent and approval were obtained from the Institutional Research Ethics Committee. This study was conducted on eight pairs of snap-frozen HCC tumours and matched non-cancerous tissues from adjacent regions, which were diagnosed histopathologically at our hospital. The eight HCC tissues and the matched adjacent non-cancerous tissues were frozen and stored in liquid nitrogen until further use.

Plasmids and transfection

The 100-900-bp region of the human CYLD 3′ UTR was PCR-amplified from genomic DNA from HepG2 or BEL-7402 cells and cloned into pGL3 vectors (Promega, Madison, WI, USA). 2′-*O*-methyl-modified RNA duplexes corresponding to a miR-501-5p mimic, a miR-501-5p inhibitor and a negative control (NC) RNA sequence were purchased from RiboBio (Guangzhou, China). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

RNA extraction and real-time quantitative PCR

Real-time quantitative RT-PCR was performed using SYBR Green I (Invitrogen) with an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Gene expression data were normalized to the geometric mean of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene to control for variability in expression levels and calculated as $2^{-[(\mathrm{Ct\ of\ gene})-(\mathrm{Ct\ of\ }GAPDH)]}$, where Ct represents the threshold cycle for each transcript. Quantitation of miRNA was based on the Ct, and relative expression was calculated as $2^{-[(\mathrm{Ct\ of\ miR\text{-}501\text{-}5p})-(\mathrm{Ct\ of\ U6})]}$ after normalization to U6 small nuclear RNA. The extracted RNA was pretreated with RNase-free DNase, and 500 ng of RNA from each sample was used for cDNA synthesis primed with the specific microRNA RT-primer purchased from RiboBio (RiboBio, Guangzhou, China). For PCR amplification of cDNA, the primers of miR-501-5p and U6 were purchased from RiboBio, and an initial amplification using primers was performed with a denaturation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing at 60°C for 20 s and primer extension at 70°C for 5 s. The following primers were used: GAPDH forward: 5′-CTGACCTGCCGTCTAGAAA-3′, GAPDH reverse:

5′-GTGGTGTGACTTAGAGGGG-3′; CyclinD1 forward: 5′-CTC CTGGTGAACAAGCTCAAGT-3′, CyclinD1 reverse: 5′-GCGGTAGT AGGACAGGAAGTTG-3′; c-myc forward: 5′-GATTCTCTGCTCTC CTCGAC-3′, c-myc reverse: 5′-TCCAGACTCTGACCTTTTGC-3′.

Western blotting

Western blotting was performed according to standard methods. The membranes were probed with polyclonal rabbit antibodies, anti-CYLD (1:1000; Abcam, Cambridge, MA, USA), anti-CyclinD1 and anti-c-myc (1:1000; Cell Signaling, Danvers, MA, USA). The membranes were then stripped and re-probed with an anti-α-tubulin mouse monoclonal antibody (1:1000; Cell Signaling) as a loading control.

Luciferase assay

Cells were seeded in triplicate in 24-well plate and allowed to settle for 24 h. One hundred nanograms of pGL3-CYLD-luciferase plasmid (or Mut) was transfected into HCC cells using the Lipofectamine 2000 reagent, according to the manufacturer's instruction. Luciferase and control signals were measured at 48 h after transfection, using the Dual Luciferase Reporter Assay Kit (Promega), according to a

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protocol provided by the manufacturer. Three independent experiments were performed and the data were presented as the mean ± SD.

The MTT assay

Cells were seeded on 96-well plates and stained at indicated time points with 100 μl sterile 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) dye (0.5 mg/ml, Invitrogen) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μl of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). The absorbance was measured at 450 nm. All experiments were performed in triplicate.

Colony formation assay

Cells were seeded on a 6-well plate (1×10^3 cells per well) and cultured for 10 days. The colonies were stained with 1.0% crystal violet for 5 min after fixation with 10% formaldehyde for 15 min. All experiments were performed in triplicate.

Anchorage-independent growth ability assay

A total of 500 cells were trypsinized and suspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar–cell mixture was plated on top of a bottom layer with 1% complete medium agar mixture. After 10 days, viable colonies that were larger than 0.1 mm were counted. Colony size was measured with an ocular micrometer and colonies >0.1 mm in diameter were counted. The experiment was performed for three independently times for each cell line.

Software and statistical analysis

The expression of miR-501-5p in the matched tumour tissues and non-cancer liver tissues of 89 patients with HCC was analysed, using a published microarray-based high-throughput dataset (NCBI/GEO/GSE36915). Student's *t*-test was used to evaluate the significant difference between two groups of data in all the pertinent experiments. Data were represented as the mean ± SEM. *P* value <0.05 (using a two-tailed paired *t*-test) was considered statistically significant.

Results

MiR-501-5p is upregulated and CYLD is downregulated in human HCC tissues and cell lines

To investigate the function of miR-501-5p in the development of human HCC, we analysed the expression of miR-501-5p in the matched tumour tissues and non-cancer liver tissues of 89 patients with HCC, utilizing a published microarray-based dataset (NCBI/GEO/GSE36915) (20). As shown in Fig. 1A, miR-501-5p was significantly upregulated in HCC ($P < 0.001$). Subsequently, real-time PCR demonstrated that miR-501-5p was overexpressed in eight HCC cell lines compared with the non-cancer hepatocyte cell line Lo2 (Fig. 1B; $P < 0.05$). MiR-501-5p was also significantly upregulated in eight HCC tissue specimens compared with the paired adjacent non-cancerous liver tissues (Fig. 1C; $P < 0.05$). Western blotting for CYLD revealed that CYLD was significantly downregulated in the same cell lines and tissues (Supplementary data, Fig. S1). Collectively, these results indicate that miR-501-5p is overexpressed and CYLD is downregulated in HCC cell lines and tissues.

Overexpression of MiR-501-5p promotes the proliferation of HCC cells

HepG2 cells were selected for experiments to examine the function of miR-501-5p in HCC. To investigate whether miR-501-5p plays a role in the development and progression of HCC, HepG2 cells were transfected with miR-501-5p mimic, miR-501-5p inhibitor or NC RNA and then subjected to cell proliferation assays.

As shown in Figure 2A, HepG2 cells were successfully transfected with miR-501-5p. The MTT assay demonstrated that ectopic expression of miR-501-5p significantly increased the cellular growth of HepG2 cells (Fig. 2B). Furthermore, overexpression of miR-501-5p promoted HepG2 cell proliferation in the colony formation assay and anchorage-independent growth ability assay (Fig. 2C, D). MiR-501-5p also promoted cell proliferation in BEL-7402 cells transfected with the miR-501-5p mimic (Supplementary data, Fig. S2). These results suggest that overexpression of miR-501-5p increased the proliferation of HCC cells *in vitro*.

Downregulation of miR-501-5p inhibits the proliferation of HCC cells

Figure 3A shows that HepG2 cells were successfully transfected with the miR-501-5p inhibitor. The MTT assay demonstrated that inhibition of miR-501-5p significantly reduced the cellular growth of HepG2 cells (Fig. 3B). Furthermore, downregulation of miR-501-5p inhibited HepG2 cell proliferation in the colony formation assay and anchorage-independent growth ability assay (Fig. 3C and D). Moreover, as shown in Supplementary data, Figure S2, we also

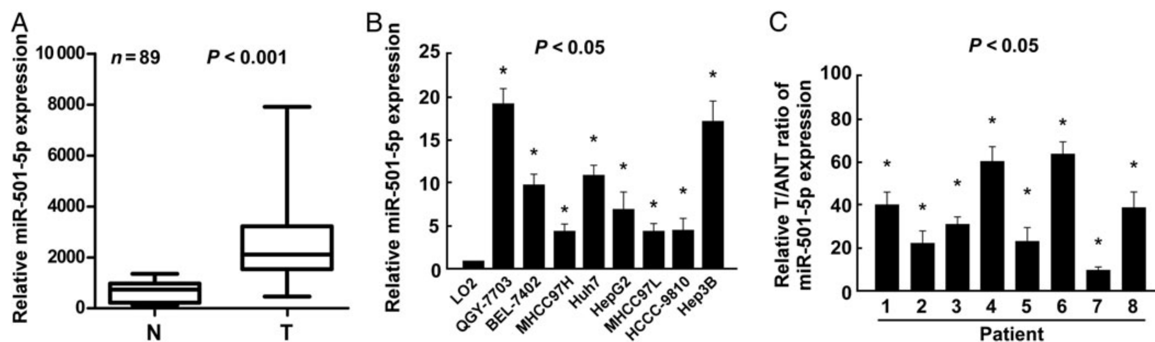

Figure 1. Expression of miR-501-5p is upregulated in hepatocellular carcinoma (HCC). (A) The expression of miR-501-5p in the tumour tissues and matched non-cancerous liver tissues of 89 patients with HCC was analysed using miRNAMap-2.0. (B) Real-time PCR analysis of miR-501-5p expression in eight HCC cell lines and the non-cancer hepatocyte cell line Lo2. (C) Real-time PCR analysis of the expression of miR-501-5p in eight HCC tissues (T) and the paired adjacent non-cancerous liver tissues (ANT). Average miR-501-5p expression was normalized to U6 expression. Each bar represents the mean ± SD of three independent experiments; *$P < 0.05$.

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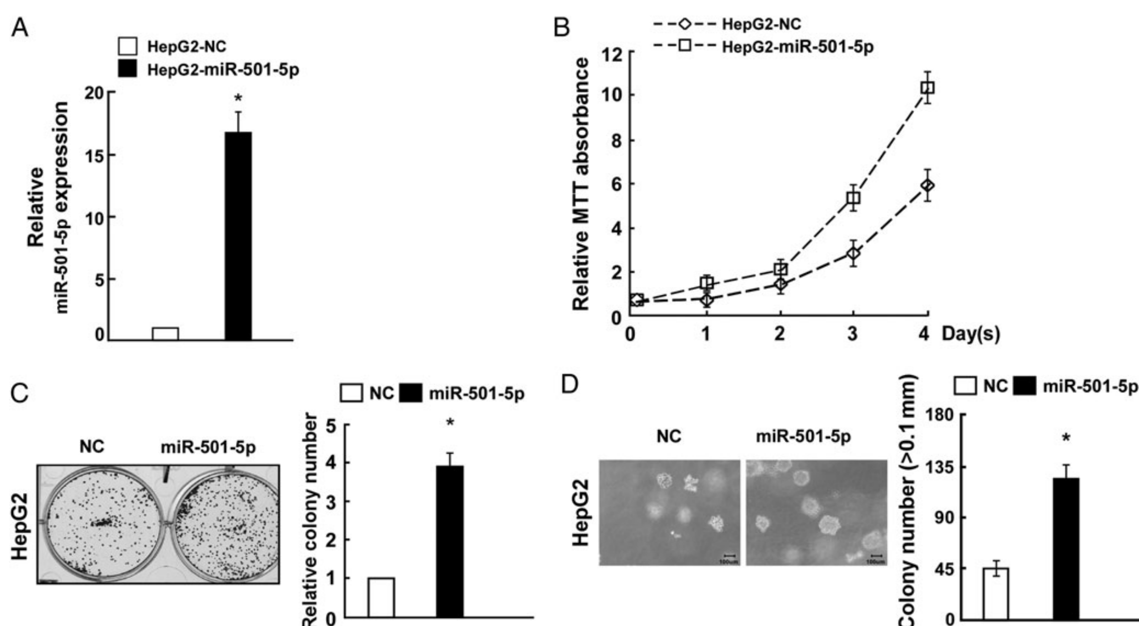

Figure 2. Ectopic overexpression of miR-501-5p promotes the proliferation of HepG2 cells. (A) Real-time PCR analysis confirmed that HepG2 cells were successfully transfected with the miR-501-5p mimic or negative control (NC) RNA. (B) The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay demonstrated that ectopic overexpression of miR-501-5p promoted the proliferation of HepG2 cells. (C) Ectopic overexpression of miR-501-5p promoted the colony-forming ability of HepG2 cells. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies at 10 days after transfection. (D) The anchorage-independent growth assay demonstrated that ectopic overexpression of miR-501-5p promoted the tumourigenicity of HepG2 cells. Representative micrographs (left) and quantification of colonies larger than 0.1 mm (right) are shown. The scale bar represents 100 μm. Each bar represents the mean ± SD of three independent experiments; $^*P < 0.05$.

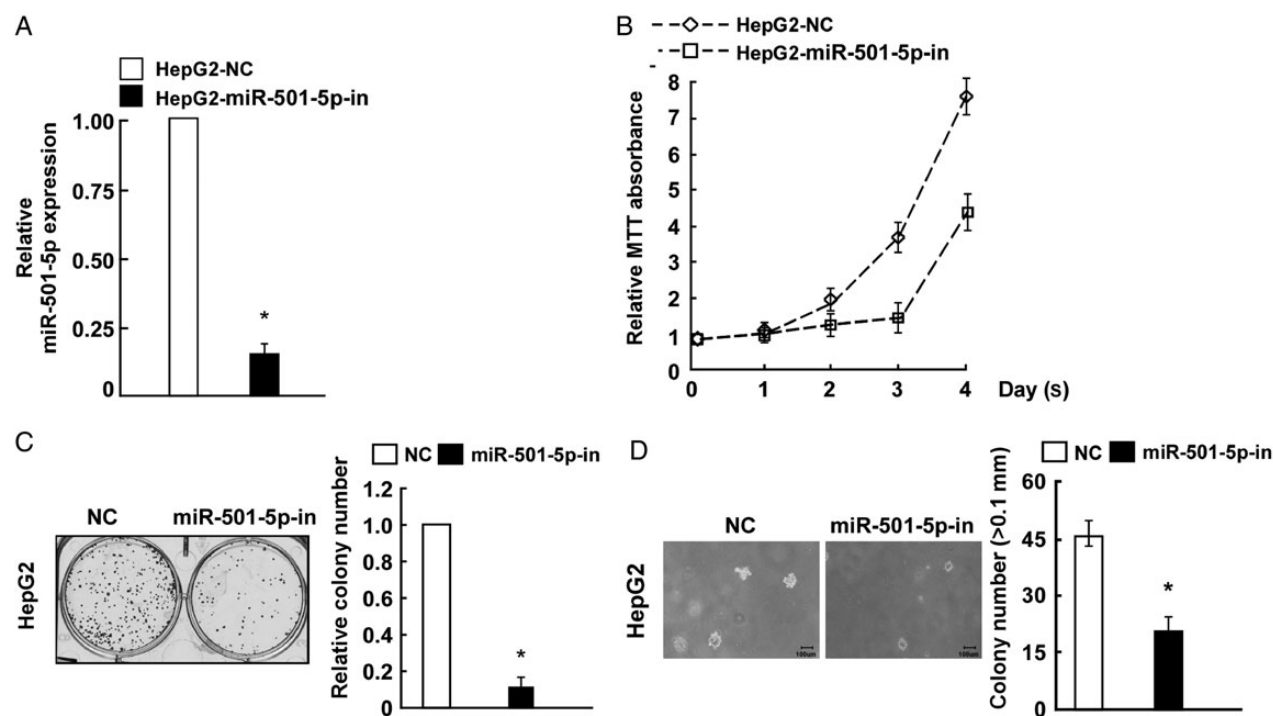

Figure 3. Downregulation of miR-501-5p inhibits the proliferation of HepG2 cells. (A) Real-time PCR analysis confirmed that HepG2 cells were successfully transfected with the miR-501-5p inhibitor or NC RNA. (B) The MTT assay demonstrated that downregulation of miR-501-5p reduced the proliferation of HepG2 cells. (C) Downregulation of miR-501-5p reduced the colony-forming ability of HepG2 cells. Representative micrographs (left) and quantification (right) of the crystal violet-stained cell colonies at 10 days after transfection are shown. (D) The anchorage-independent growth assay demonstrated that downregulation of miR-501-5p reduced the tumourigenicity of HepG2 cells. Representative micrographs (left) and quantification of colonies larger than 0.1 mm (right) are shown. The scale bar represents 100 μm. Each bar represents the mean ± SD of three independent experiments; $^*P < 0.05$.

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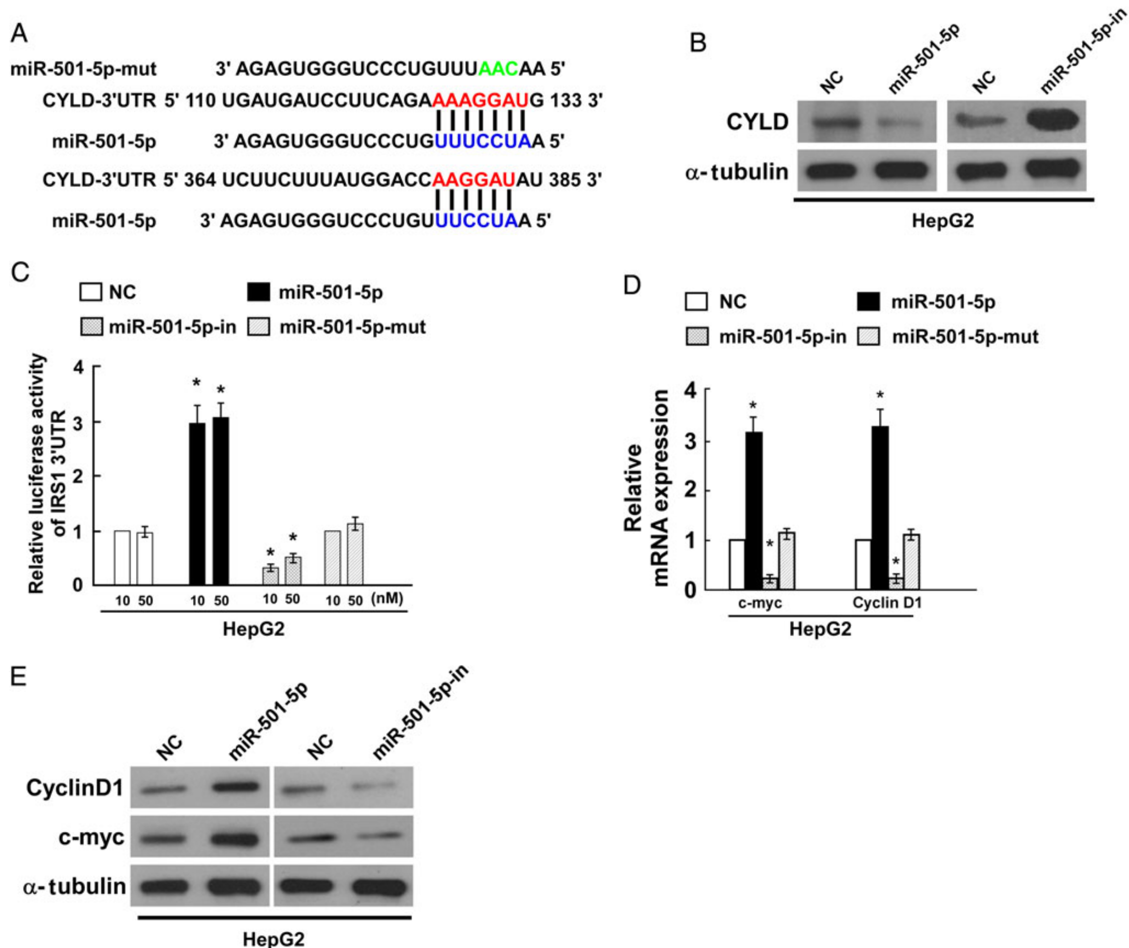

Figure 4. MiR-501-5p directly targets the 3′ UTR of *CYLD* mRNA. Schematic representation of the mature miR-501-5p sequence and miR-501-5p target site in the 3′ UTR of *CYLD* mRNA. (A) The mutated *CYLD* 3′ UTR mRNA was created by mutating three nucleotides in the putative miR-501-5p target site (CYLD-3′UTR-mut). (B) Western blot analysis of CYLD expression in HepG2 cells overexpressing miR-501-5p or transfected with the miR-501-5p inhibitor, compared with control cells at 48 h after transfection. (C) Luciferase assay of the pGL3-CYLD-3′UTR or pGL3-CYLD-3′UTR-mut reporter genes cotransfected into with HepG2 cells with 10 nM or 50 nM of miR-501-5p mimic or miR-501-5p inhibitor. (D) Real-time PCR analysis of Cyclin D1 mRNA and *c-myc* expression in HepG2 cells transfected with 10 nM or 50 nM of the miR-501-5p mimic or miR-501-5p inhibitor. (E) Western blotting of Cyclin D1 and *c-myc* expression in HepG2 cells transfected with 10 nM or 50 nM of the miR-501-5p mimic or miR-501-5p inhibitor. Each bar represents the mean ± SD of three independent experiments; *$P < 0.05$.

found that inhibition of miR-501-5p decreased the proliferative ability of BEL-7402 cells. These results suggest that downregulation of miR-501-5p reduced the proliferation of HCC cells *in vitro*.

MiR-501-5p directly targets CYLD mRNA in HCC cells

To explore the molecular mechanism of the function of miR-501-5p in HCC cells, publicly available algorithms (TargetScan, PicTar, miRanda) were used to predict human miR-501-5p target gene(s). *CYLD* was identified as a potential target of miR-501-5p (Fig. 4A), and CYLD protein expression was found to be significantly decreased in HCC cell lines and tissues (Supplementary data, Fig. S1). To examine the effect of miR-501-5p on *CYLD*, we tested the expression of CYLD protein in HepG2 cells transfected with the miR-501-5p mimic or miR-501-5p inhibitor, respectively. The results of western blotting showed that CYLD expression was downregulated in HepG2 cells overexpressing miR-501-5p and upregulated in HepG2 cells transfected with the miR-501-5p inhibitor (Fig. 4B). To examine whether miR-501-5p–mediated downregulation of CYLD occurred via miR-501-5p targeting the *CYLD* 3′ UTR, we subcloned a fragment of the *CYLD* 3′ UTR that contains a miR-501-5p binding site into the luciferase reporter vector pGL3. Overexpression of miR-501-5p consistently and dose-dependently reduced the luciferase activity of the *CYLD* 3′ UTR reporter gene; inhibition of miR-501-5p had the opposite effect. Moreover, the ability of miR-501-5p to regulate the CYLD 3′ UTR reporter gene was abolished by introducing point mutations in the miR-501-5p–binding seed region (Fig. 4C). These results suggest that *CYLD* is a direct target of miR-501-5p.

Subsequently, real-time PCR demonstrated that overexpression of miR-501-5p significantly increased the expression of *Cyclin D1* mRNA and *c-myc* in HepG2 cells (Fig. 4D). Additionally, the results of western blotting were consistent with the real-time PCR data (Fig. 4E). These findings indicate that miR-501-5p may play an important role in regulating the proliferation of HCC cells.

Discussion

MicroRNAs, a class of small regulatory RNA molecules that negatively regulate their mRNA targets in a sequence-specific manner, have been demonstrated to play important roles in multiple biological processes such as cellular differentiation, proliferation, oncogenesis, angiogenesis, invasion and metastasis, and may function as either

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tumour suppressors or oncogenes (21–23). In the current study, we found that miR-501-5p was significantly overexpressed in HCC cell lines compared with the non-cancer hepatocyte cell line Lo2. Additionally, miR-501-5p was upregulated in HCC tissues in comparison with the matched adjacent non-cancerous tissues. Furthermore, ectopic expression of miR-501-5p significantly increased the growth of HepG2 and BEL-7402 cells, while inhibition of miR-501-5p reduced cell proliferation and the colony-forming ability of the cells on soft agar. These results indicate that upregulation of miR-501-5p may correlate with the clinical progression of HCC and that miR-501-5p may function as an oncomiRNA in HCC.

CYLD has been recognized to regulate a variety of signalling pathways including transforming growth factor-β signalling, Wnt/β-catenin signalling and NF-κB signalling by deubiquitinating upstream regulatory factors. Brummelkamp et al. (24) found that CYLD specifically deconjugated K63-linked polyubiquitin chains and could negatively regulate the NF-kappa B pathway and also promote apoptosis in cylindromatosis tumours. Dysregulation of components of the ubiquitin system can alter cell survival or cell proliferation, and has been linked to a variety of types of cancer. As a tumour suppressor gene, reduced expression of *CYLD* has been observed in a variety of types of human cancer (25,26). Recent studies demonstrated that CYLD acts as an important regulator of hepatocyte homeostasis, and could protect cells from spontaneous apoptosis by preventing uncontrolled activation of TGF-β activated kinase 1 (TAK1) and c-Jun N-terminal kinase (JNK) (27,28).

In this study, we found that the tumour suppressor CYLD was significantly downregulated in HCC cell lines and tissues. Furthermore, bioinformatic analysis revealed that *CYLD* was a potential target gene of miR-501-5p. We used three different methods to confirm that *CYLD* is a bona fide target of miR-501-5p. Western blotting analysis showed that overexpression of miR-501-5p downregulated CYLD protein expression, and also upregulated expression of the cell cycle regulator cyclin D1 and c-myc. Luciferase activity assays and point mutation analysis demonstrated that downregulation of *CYLD* was mediated by miR-501-5p specifically targeting the *CYLD* 3′ UTR. The biological ability of miR-501-5p to protect against apoptosis and cell survival, with respect to the function of CYLD, is currently under investigation in our laboratory.

Taken together, this study demonstrates that miR-501-5p is markedly upregulated in HCC cells and clinical tissues compared with the matched adjacent non-cancerous tissues from the same patients. Furthermore, *CYLD* is a direct target gene of miR-501-5p and overexpression of miR-501-5p reduced the expression of CYLD and inhibited the proliferation of HCC cells *in vitro*, whereas downregulation of miR-501-5p had the opposite effects. Further investigation is required to fully characterize the biological function of miR-501-5p and its clinical relevance in the development of HCC. Although the precise mechanisms are not yet fully understood, this study suggests that miR-501-5p may play an important role in regulating the proliferation of HCC cells, and indicates that miR-501-5p may represent a potential therapeutic target for HCC.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement

The authors declare that they have no competing interest.

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