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MicroRNA-7 arrests cell cycle in G1 phase by directly targeting CCNE1 in human hepatocellular carcinoma cells

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ABSTRACT

Growing evidence has demonstrated that the aberrant expression of miRNA is a hallmark of malignancies, indicating the important roles of miRNA in the development and progression of cancer. MiR-7 is considered as a tumor suppressor miRNA in multiple types of cancer. However, the role of miR-7 in human hepatocellular carcinoma (HCC) and its underlying mechanism remain elusive. In this study, we found that overexpression of miR-7 arrested cell cycle at G1 to S transition in HCC. By combinational use of bioinformatic prediction, reporter assay, quantitative real-time PCR (qRT-PCR) and Western blot, we confirmed that CCNE1, an important mediator in G1/S transition is one of new direct target genes of miR-7. Further studies revealed that silencing of CCNE1 recapitulated the effects of miR-7 overexpression, whereas enforced expression of CCNE1 reversed the suppressive effects of miR-7 in cell cycle regulation. Finally, analysis of qRT-PCR showed a reciprocal relationship between miR-7 and CCNE1 in clinical cancer tissues and multiple types of tumor cell lines. These findings indicate that miR-7 exerts tumor-suppressive effects in hepatocarcinogenesis through the suppression of oncogene CCNE1 expression and suggest a therapeutic application of miR-7 in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and ranks as the third major cause of cancer-associated mortality [1]. Although previous studies have demonstrated that various molecular alterations of well-known signaling pathways occur in the initiation and progression of HCC, the molecular pathogenesis of HCC is still complicated and poorly understood [2]. A genome-wide analysis revealed that more than half of human microRNA (miRNA) is located in the chromosomal fragile sites that are strongly associated with chromosomal alterations in malignant diseases, indicating that the roles of miRNA in cancer could be a new insight for the further understanding of the development and progression of HCC [3]. And it will be of benefits to the diagnosis and therapy in HCC.

In recent years, a large number of studies demonstrated that down-regulation of tumor suppressor miRNAs plays a critical role in development and progression of HCC, such as miR-101 [4,5], miR-139 [6,7], miR-26 [8–11], miR-124 [12], miR-199 [13] etc. Among the microRNAs that are implicated in HCC, miR-7 has recently been found to be down-regulated in HCC tissues and inhibit proliferation and metastasis in HCC cells *in vitro* and *in vivo*. Interestingly, further study showed that phosphoinositide 3-kinase catalytic subunit delta (PIK3CD), mTOR and p70S6K, all these important functional molecules in the phosphoinositide 3-kinase/Akt signaling pathway are the direct target genes of miR-7. Accordingly, the study on the mechanism revealed that miR-7 suppresses tumor growth and metastasis by targeting PI3K/AKT pathway in hepatocellular carcinoma [14]. However, the computational approaches estimate that each miRNA may have hundreds to thousands of mRNA targets [15,16]. Here, we sought to identify other potential targets of miR-7. This may help to further understand the tumor suppressive role of miR-7 in HCC.

MiRNAs are a class of small non-coding RNAs encoded by the genomes of a wide range of multicellular organisms [17]. miRNAs are initially transcribed as long primary transcripts (pri-miRNAs) that undergo sequential processing by the RNase III endonucleases Drosha and Dicer to yield the mature 20–23 nucleotide species [18]. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and then target the 3′untranslated region (3′UTR) of a specific mRNA by base pairing, leading to translational

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repression or mRNA degradation [19]. Most importantly, the ability of individual miRNAs to regulate hundreds of transcripts allows them to coordinate complex programs of gene regulation and consequently induces global changes in cellular physiology. Indeed, a large number of evidence has demonstrated that miRNAs provide functions essential for normal development and cellular homeostasis and, accordingly, dysfunction of these molecules has been linked to several human diseases, including cancer [20].

In this study, we found that exogenous expression of miR-7 arrested cell cycle in G1 phase in HCC cells. Further investigation revealed that CCNE1, an important cell cycle regulator in G1/S transition is a new target gene regulated by miR-7. Loss-of-functional study showed that silencing of CCNE1 recapitulates the effect of miR-7 on HCC cells. Moreover, the rescue experiment confirmed that CCNE1 is an important downstream gene of miR-7 in inducing cell cycle arrest in G1 phase. Additionally, we found that expression patterns of miR-7 were inversely correlated with CCNE1 in multiple tumor cell lines and HCC tissues. Our findings will help to elucidate the functions of miRNAs and their roles in hepatocarcinogenesis.

2. Materials and methods

2.1. Cell culture and transfection

All the cells we used were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured under standard conditions. The different HCC cell lines were transfected similarly in 6-well plates according to the manufacturer's instructions. Briefly, 100 μM of diluted miRNA duplexes (Genepharma, China) or small interfering RNA (siRNA) against CCNE1 (Genepharma, China) per well was formulated with Lipofectamine 2000 reagent (Invitrogen, USA) in RPMI-1640 serum-free medium (Invitrogen, USA). The transfection complex was added directly to the cells and replaced with a fresh medium 6 h later. Analyses of the effects of miRNA or siRNA on recipient cells were performed 48 h after transfection.

2.2. Real-time quantitative PCR analysis

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. 1 μg of RNA was employed to synthesize cDNA using the PrimeScript RT Reagent Kit Perfect Real Time (TaKaRa, Dalian, China) or the miScript II RT Kit (Qiagen, Germany). For the detection of the CCNE1 mRNA levels, we employed the fluorescent qRT-PCR using the following primers: forward 5′-GCCAGCCTTGGGACAATAATG-3′; reverse 5′-CTTGCACGTTGAGTTTGGGT-3′. For internal control we employed GAPDH mRNA levels using the following primers: forward 5′-TCACCAGGGCTGCTTTTAAC-3′; reverse 5′-GACAAGCTTCCCGTTCTCAG-3′.

The expression level of miR-7 in different HCC cells was examined by qRT-PCR assays. All the miRNA primers (hsa-miR-7; sn-RNU6B) were obtained from AuGCT (Beijing, China) and the reactions were run in triplicates. Relative expression levels of miRNA or mRNA were analyzed using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, USA).

2.3. Luciferase report assay

HEK293A cells were seeded into 48-well plates with 50% confluence. After 24 h, cells were transfected with a mixture of 100 ng pGL3-CCNE1-3′UTR, 20 μM miR-7 mimics or negative control (NC), and 5 ng PRL-TK using Lipofectamine 2000 reagent and performed in three independent experiments. Firefly and renilla luciferase activities were examined using a dual-luciferase reporter system (Promega, USA).

2.4. Western blot analysis

Cells were trypsinized 48 h after transfection, and cells lysates were resolved by SDS–PAGE, and transferred to nitrocellulose membranes as previously described [5]. Then nitrocellulose membranes were incubated using an anti-CCNE1 rabbit polyclonal antibody (1655-1 Epitomics, Abcam, UK) or anti-beta-actin mouse monoclonal antibody (A5441 Sigma–Aldrich, St. Louis, MO, USA). Following incubation with the primary and secondary antibodies the immunoreactive bands were visualized by the Tanon5500 (Tanon, Shanghai, China).

2.5. Flow cytometry analysis

Cells were collected 48 h after transfection, and fixed into 70% ethanol at −20 °C for 24 h, then stained with 50 μg/ml propidium iodide (MP Biomedicals, USA), and examined using Epics XL-MCL (BECKMAN coulter, USA). The results were analyzed using ModFit LT V3.1 (BECKMAN coulter).

2.6. Statistical analysis

The correlation between miR-7 and CCNE1 was determined by way of Spearman correlation test in PASW18.0. All the data are expressed as the mean ± SD. of at least three independent experiments. The differences between groups were analyzed using two-sided Student's *t*-test; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Overexpression of miR-7 induces cell cycle arrest at G1 phase in HCC cells

A previous study showed that overexpression of miR-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in HCC [14]. However, considering that each miRNA can target hundreds of downstream gene, the mentioned study may not absolutely explain the mechanism of miR-7 in the inhibitory effects of HCC. Here, we focused on the mechanism of miR-7 on the control of tumor growth. Using transient transfection, we introduced miRNA mimics into three HCC cell lines, including HepG2, SMMC-7721 and BEL-7404. qRT-PCR analysis showed that miR-7 was up-regulated in all the cell lines with 50 to 150-folds (Fig. 1A). Then we sought to determine the role of miR-7 on cell cycle regulation in HCC cells. As shown in Fig. 1B, the cell subpopulation in G1 phase was obviously increased in miR-7-overexpressing cells compared with it in the control group, whereas the cell number in S phase consequently decreased. Taken together, these findings demonstrated that overexpression of miR-7 may inhibit cell proliferation through arresting cell cycle at G1 phase in HCC cells.

3.2. CCNE1 is a new direct downstream of miR-7 in HCC cells

In the mechanistic study, we wondered to know the reason for miR-7-induced cell cycle arrest in HCC cells. We employed established bioinformatics procedures to screen and identify the potential target genes. Interestingly, the Targetscan prediction showed that the 3′UTR of CCNE1 contains a seed sequence matched with mature miR-7, and this putative binding site is highly conserved across different species (human, monkey, rat,

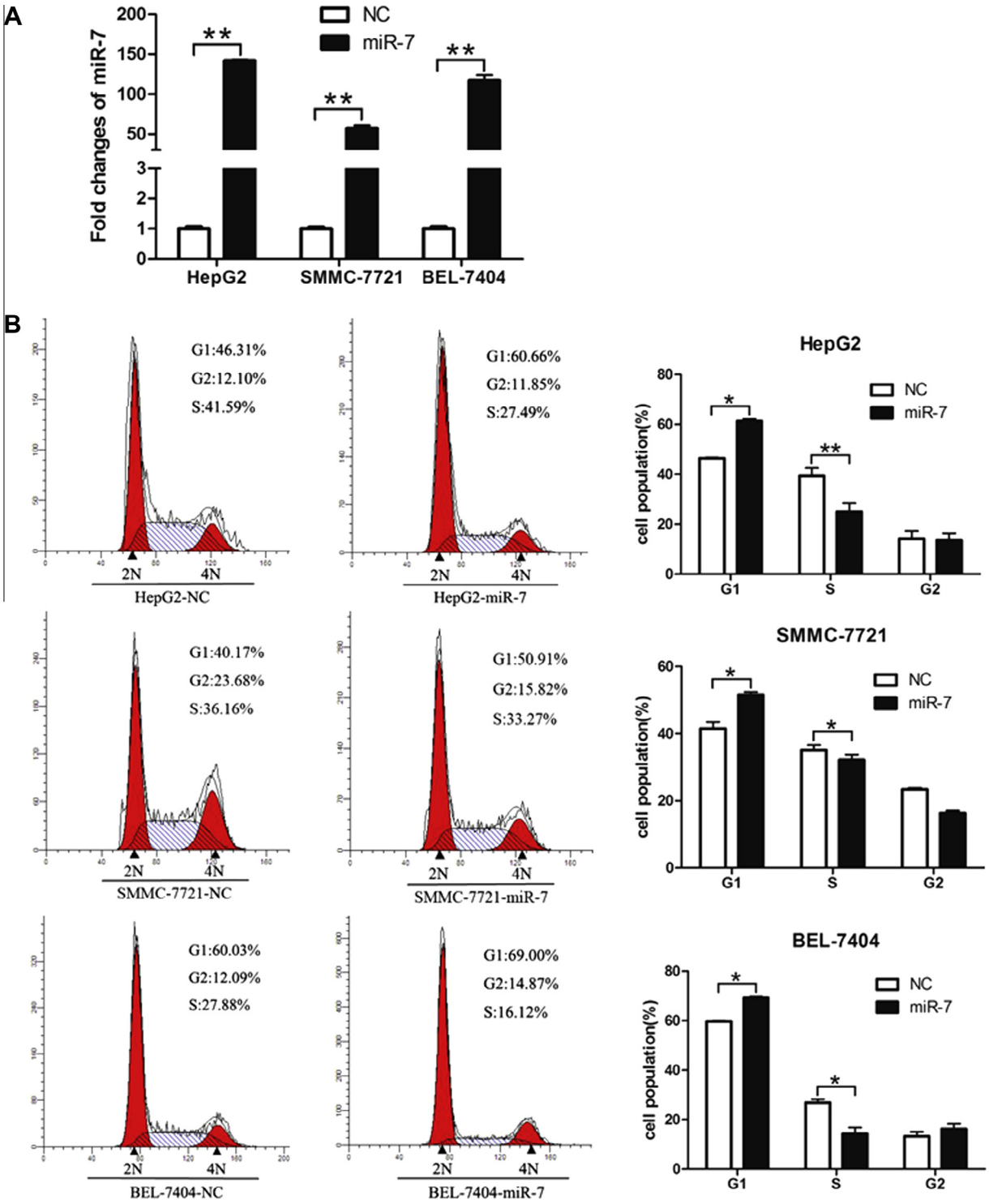

Fig. 1. miR-7 significantly induced G1 phase arrest in HCC cells. HepG2, SMMC-7721 and BEL-7404 cells were transiently transfected with miR-7 or negative control. Then the above cells were analyzed using the following experiments. (A) Quantitative Real-time PCR analyses of miR-7 expression. The expression level of miR-7 was examined 48 h after transfection and normalized to U6. (B) Flow cytometry analyses for cell cycle distribution. Cell cycle distribution was analyzed 48 h after transfection $^{*}P < 0.05$, $^{**}P < 0.01$.

chimpanzee, and goat) (Fig. 2A and B). Therefore, we constructed reporter plasmids containing wild-type or mutant 3′UTR of CCNE1 (Fig. 2A). The wild-type or mutant reporter plasmid was co-transfected into HEK-293A cells with miR-7 mimics or negative control. The transfection efficiency was normalized by co-transfection with renilla reporter vector. As shown in Fig. 2C, overexpression of miR-7 significantly decreased the relative luciferase activity of wild-type CCNE1 3′UTR but had minimal effect on the mutant CCNE1 3′-UTR. This suggested that miR-7 may directly bind to the 3′-UTR of CCNE1.

We next attempted to validate whether miR-7 could down-regulate the expression level of CCNE1 in HCC cells. Using qRT-PCR analysis, we found that the mRNA level of CCNE1 was significantly decreased with the overexpression of miR-7 in HCC cells (Fig. 2D).

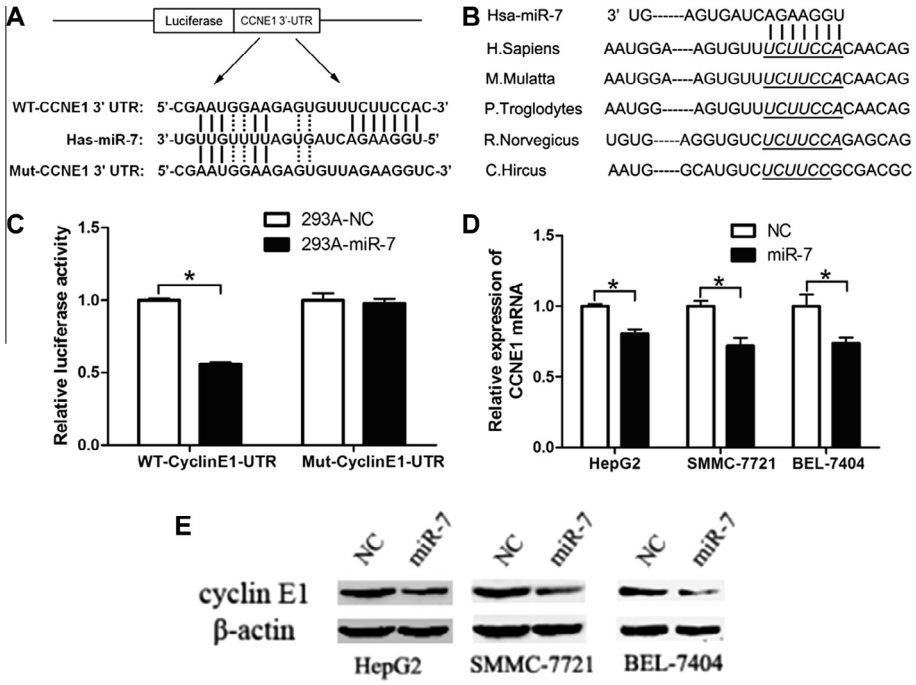

Fig. 2. CCNE1 is a direct downstream target of miR-7 in HCC cells. (A) miR-7 and its potential binding sequence in the 3′UTR of human CCNE1. The wild-type and mutant CCNE1 3′-UTR were constructed into pGL3 vectors. (B) Putative binding site of miR-7 on CCNE1 3′-UTR in different species. The binding sequence is highly conserved across different species. (C) Relative luciferase activity analyses. The luciferase activity was examined 48 h after transfection. Data represents relative firefly luciferase units from three separate experiments, and normalized to that of renilla luciferase. (D) qRT-PCR analyses of CCNE1 mRNA. The mRNA level of CCNE1 was examined 48 h after treatment with miR-7 and normalized to GAPDH. (E) Western blotting analyses of CCNE1 expression. HCC cells were transfected with miR-7 or NC, and the CCNE1 level was measured 48 h later $^{*}P < 0.05$.

Corresponding to the reduction in the mRNA level, Western blot analysis showed that enforced expression of miR-7 also remarkably reduced the protein level of CCNE1 (Fig. 2E). Taken together, these results indicated that miR-7 negatively regulates CCNE1 in a posttranscriptional manner in HCC cells.

3.3. Silencing of CCNE1 recapitulates the phenotype of HCC cells in cell cycle

To explore the role of CCNE1 in HCC cells, we silenced the expression of endogenous CCNE1 in HCC cells. As shown in Fig. 3A, the CCNE1-targeting siRNA efficiently inhibited the expression level of CCNE1 compared with negative control-transfected group. Furthermore, we performed flow cytometry analysis to investigate the effect of the CCNE1-targeting siRNA on cell cycle progression. The results showed an inhibitory effect on G1/S transition and retained the cell cycle at G1 phase in the CCNE1 siRNA-transfected cells compared with it in the control groups (Fig. 3B). The inhibition of cell cycle progression induced by si-CCNE1 mimicked the phenotype induced by overexpression of miR-7 in HCC cells. These results demonstrated that reduction of CCNE1 by siRNA had similar effects on HCC cells induced by miR-7, indicating that CCNE1 may serve as a downstream functional target of miR-7.

3.4. CCNE1 is an important functional molecule in miR-7-induced G1 phase arrest

If CCNE1 indeed acts as a functional mediator of miR-7, reintroduction of CCNE1 into miR-7-overexpressing cells should be able to abrogate the effects of miR-7. To test the assumption, we constructed a vector expressing CCNE1 without the 3′UTR and co-transfected it with miR-7 mimics into HCC cells. As shown in Fig. 4B, the level of CCNE1 protein was recovered after treatment with CCNE1 (miR-7 + CCNE1 group) compared with it in only miR-7-transfected cells. Furthermore, cell cycle distribution assay showed that the exogenous expression of CCNE1 rescued the G1 phase arrest induced by miR-7 in HCC cells (Fig. 4A). Taken together, these findings demonstrated that enforced expression of CCNE1 could antagonize miR-7-induced cell cycle arrest, and further confirmed that CCNE1 is a crucial functional mediator of miR-7 in HCC cells.

3.5. CCNE1 expression is inversely correlated with miR-7 in cell lines and clinical samples

We initially speculated that the CCNE1 expression may have an inverse relationship with miR-7 expression levels. Then we employed qRT-PCR assay to measure the endogenous expression levels of miR-7 and CCNE1 in seven cell lines, including HCC cells (HepG2, SMMC-7721, BEL-7404), prostate cancer cells (PC-3, DU 145, and 22RV1), and a normal prostate cell line (WPMY-1). As shown in Fig. 4C, the expression level of CCNE1 is inversely correlated with miR-7 in these cell lines. Furthermore, to investigate whether this correlation also existed in human tumor tissues, we analyzed one published human tumor tissue expression data set including both miRNA and mRNA expression data [21]. Using this data set which consisted of only prostate tumors, we found that the expression of CCNE1 was reversely associated with the expression of miR-7 (Fig. 4D). Altogether, these data showed that miR-7 is inversely associated with CCNE1 in both cancer cell lines and carcinoma tissues.

4. Discussion

In the past 10 years, an increasing number of evidence suggests that the aberrant miRNA expression signature is a hallmark of malignancies, including HCC [3,22,23]. Although functional studies demonstrate that the dysregulated miRNAs in cancer can be classified as oncomiRs or tumor suppressor miRNAs, a global reduction of miRNA abundance appears a general feature of human cancers

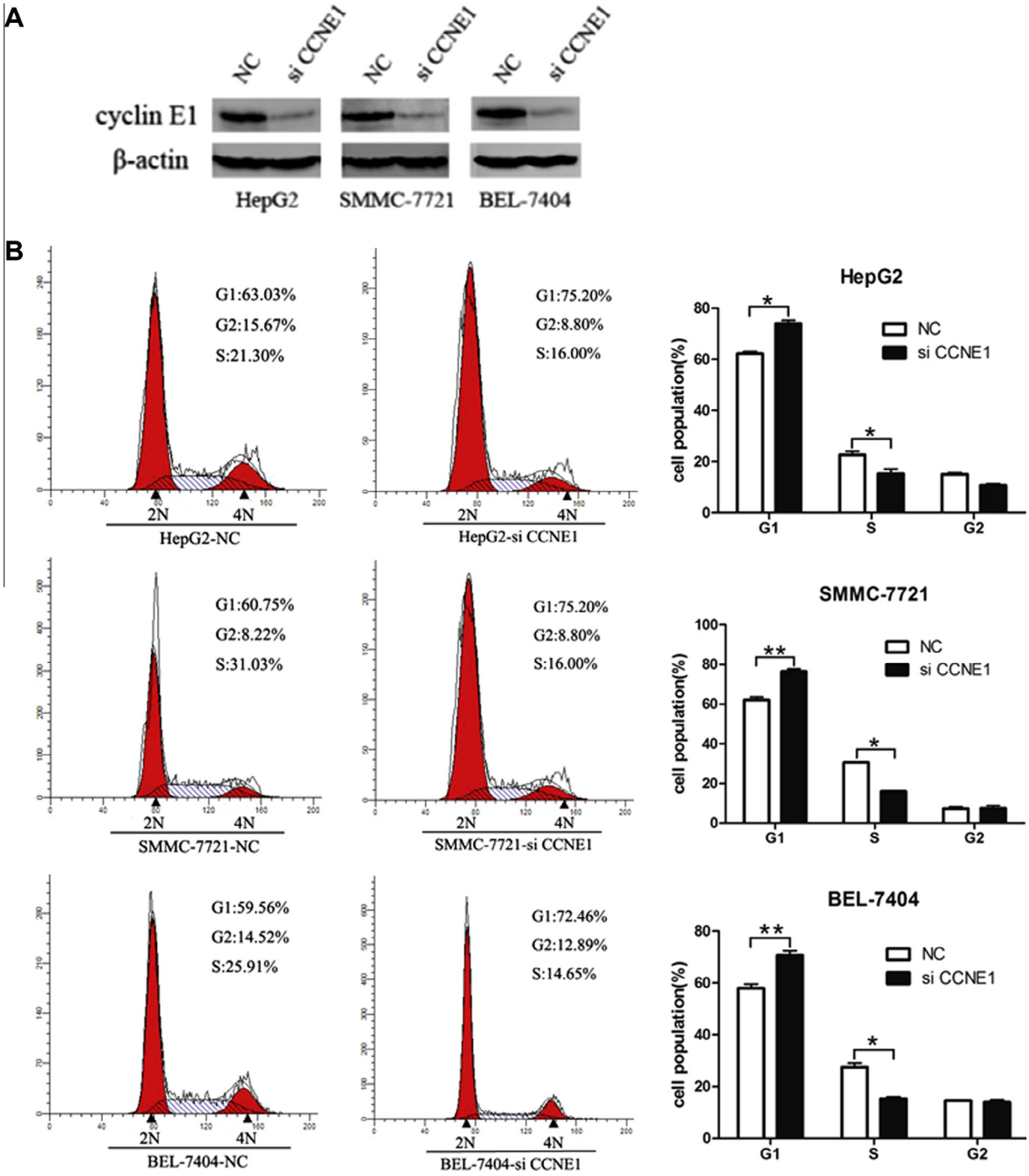

Fig. 3. CCNE1 silencing recapitulated the effects of miR-7 on HCC cells. (A) Western blotting analyses of CCNE1 expression. The protein level of CCNE1 was examined 48 h after transfection of si-CCNE1 or NC. (B) Flow cytometry analyses for cell cycle distribution. Cell cycle distribution was analyzed 48 h after treatment with si-CCNE1 or NC $^{*}P < 0.05$, $^{**}P < 0.01$.

and plays a causal role in the process of malignant transformation [24,25]. MiR-7 was first identified as a tumor suppressor miRNA in malignant glioblastoma (GBM) [26]. Mechanistic studies reveal that overexpression of miR-7 inhibits tumor growth and invasion in GBM cells by targeting PAK1 and EGFR signaling pathways [26,27]. Furthermore, recent studies from other types of cancer reported that miR-7 is frequently down-regulated in breast, tongue, gastric, lung, liver, and Schwannoma tumor tissues. And consequently, many new targets of miR-7, such as FAK, KLF4, IGF1R, PA28γ, PIK3CD, mTOR, p70S6K and Ack1 are identified in a series of tumor cells [14,28–33]. We can infer that, to date, the mechanism by which miRNA exerts its function is still a topic of great interest in cancer biology. Although many studies have reported the role of miR-7, much remains to be illuminated to supplement the network of its interactions. Here, by combinational use of bioinformatic prediction, reporter assay, qRT-PCR and Western blot, we validated that CCNE1, an important mediator in G1/S transition is one of new direct target genes of miR-7 in tumor cells. And our further study in clinical cancer samples showed that the expression patterns of miR-7 and CCNE1 are inversely correlated in tumor tissues. Herein, our findings suggest that CCNE1 could be a new target gene of miR-7 in cancer.

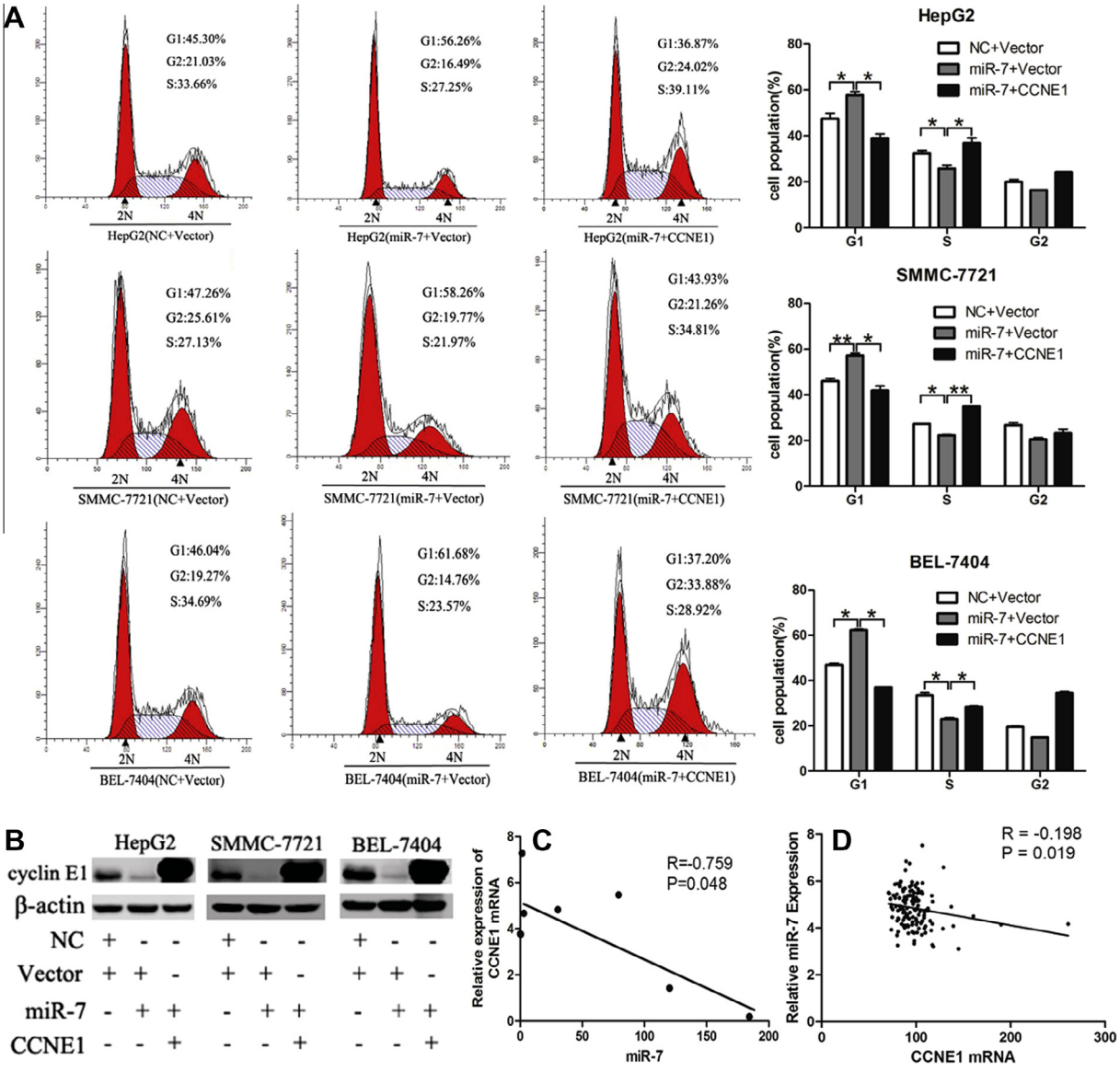

Fig. 4. CCNE1 treatment abrogated miR-7-induced G1 phase arrest of HCC cells. HepG2, SMMC-7721 and BEL-7404 cells were transiently transfected with miR-7 or NC for 12 h before CCNE1 or vector control treatment for another 48 h. The above cells were analyzed using the following experiments. (A) Flow cytometry analyses for cell cycle distribution. (B) Western blotting analyses of CCNE1 expression. The correlation between miR-7 and CCNE1 expression was assessed by linear correlation in (C) seven cancer cell lines, (D) prostatic carcinoma tissues. The endogenous expression levels of miR-7 and CCNE1 were measured by qRT-PCR, and normalized to that of U6 and GAPDH $^{*}P < 0.05$, $^{**}P < 0.01$.

In the present study, we found that overexpression of miR-7 induces cell cycle arrest at the G1/S transition in HCC cells, suggesting that down-regulation of miR-7 in HCC may facilitate tumor cells to divide and grow more quickly. In the mechanistic study on miR-7's role in cell cycle regulation, we identified that CCNE1 may be a new target gene of miR-7 in tumor cells. CCNE1 forms a complex with and functions as a regulatory subunit of CDK2, being required for G1/S transition [34]. Excessive activity of the CCNE1-CDK2 complex drives cells to copy their DNA prematurely, resulting in genome instability [35], and thus may contribute to tumorigenesis by accelerating cell division [36]. In addition, genome-wide surveys showed that the overexpression of CCNE1 is a common phenomenon in multiple tumors, including HCC [37,38]. As for HCC, overexpression of CCNE1 was found in 70% of HCC patients, which correlated with the poor prognosis of those patients [39].

Overexpression of oncogene has been implicated in the development and progression of a variety of human cancers and, therefore, provides a potential target for cancer gene therapy. When we silenced CCNE1 expression in HCC cell lines, we observed a significant up-regulation of G1 phase in all the tested cell lines. Meanwhile, consistent with our observations, Li et al. reported that knockdown of CCNE1 can markedly suppress cancer cell proliferation by inducing G1 phase arrest in other two HCC cell lines [40]. All these recapitulated the phenotype of exogenous expression of miR-7 in HCC cells, suggesting that the exploration of miR-7's target genes led to the identification of CCNE1 as a direct target of miR-7. And our rescue experiment for reintroduction of exogenous CCNE1 obviously inhibited miR-7-induced G1 phase arrest, further confirmed our finding that CCNE1 is a functional downstream mediator for miR-7 in HCC. The information from our bioinformatic prediction showed that the binding site of miR-7 on the

3′UTR of CCNE1 is conserved across various species and our analysis of qRT-PCR also showed an obviously inverse correlation in tumor cell lines, including liver and prostate cancer cell lines. All these suggest that the interaction between miR-7 and CCNE1 may have an important function during evolution and multiple malignant diseases.

In recent years, tumor suppressor miRNA-based therapeutics has been developed in the field of cancer gene therapy [41–43]. Comparing with single oncogene-targeting RNA interference (RNAi) technology, overexpression of one tumor suppressor miRNA can function by targeting multiple oncogenic activities. Furthermore, as a natural substance, it has no off-target side effect like artificial short hairpin RNA (shRNA) [43]. Mendell's group for the first time demonstrated that miR-26a delivery confers dramatic tumor regression in a c-Myc-induced tumor model. However, in mechanistic study, they only showed that CCND2 and CCNE2 are the target genes in the miR-26a-induced cell cycle arrest and cancer cell death [8]. Recently, two reports from one group demonstrated that miR-26a can target IL-6 and HGF, and consequently down-regulate IL-6-STAT3 and HGF-MET, two important oncogenic signaling pathways. These findings may further explain the potential effect of miR-26a in anti-HCC [10,11]. And it is likely that many equally or more effective tumor suppressor miRNAs with therapeutic potential remain to be functionally characterized. For example, miR-124 and miR-199a, two important tumor suppressor miRNAs in HCC were systematically administrated into mouse model using viral or non-viral delivery system and its potential anti-tumor activity was also validated *in vitro* and *in vivo* [12,13]. And in our recent study, we have demonstrated that overexpression of miR-101 controls HCC by targeting multiple oncogenic activities [5]. The combinational analysis of a previous report and our present study shows that the tumor suppressive functions of miR-7 are also mediated by multiple targeting genes, including CCNE1 [14]. These findings facilitate a better understanding of the molecular pathogenesis of HCC and suggest that miR-7 might be a candidate for the treatment of HCC.

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