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ORIGINAL ARTICLE

# Downregulated miRNA-22-3p promotes the progression and leads to poor prognosis of hepatocellular carcinoma through targeting CDKN2C

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## Summary

***Purpose:*** *CDKN2C exerts critical functions during the progression of hepatocellular carcinoma (HCC). Its dysfunction is closely linked to poor prognosis of HCC. This study aimed to uncover the underlying mechanism of CDKN2C in affecting the prognosis of HCC.*

***Methods:*** *Potential miRNAs that could regulate CDKN2c were predicted by bioinformatics, and their differential levels in HCC and normal liver tissues were detected. CDKN2C level in Huh7 and Hep3B cells influenced by the two candidate microRNAs, miRNA-22-3p and miRNA-182-5p, were examined. Correlation between miRNA-22-3p and CDKN2C in HCC was analyzed on LinkedOmics, and further confirmed by Pearson correlation test and dual-luciferase reporter gene assay. Thereafter, the prognostic potential of miRNA-22-3p in HCC was evaluated by Kaplan-Meier method. Furthermore, the regulatory effects of miRNA-22-3p/CDKN2C axis on proliferative ability and cell cycle progression of HCC were assessed.*

***Results:*** *There were five miRNAs predicted to bind to CDKN2C and among them, miRNA-22-3p and miRNA-182-5p were markedly downregulated in LIHC tissues. In Huh7 and Hep3B cells, miRNA-22-3p negatively regulated CDKN2C level, while transfection of miRNA-182-5p mimic or inhibitor did not influence CDKN2C expression. MiRNA-22-3p was closely linked to poor prognosis of HCC patients. Subsequently, dual-luciferase reporter gene assay verified the binding between miRNA-22-3p and CDKN2C.*

***Conclusions:*** *Knockdown of miRNA-22-3p suppressed proliferative ability and arrested cell cycle progression, which were reversed by overexpression of CDKN2C. MiRNA-22-3p suppresses proliferative ability and arrests cell cycle progression in HCC through targeting CDKN2C.*

***Key words:*** *hepatocellular carcinoma, CDKN2C, proliferation, cell cycle, MiRNA-22-3p*

## Introduction

Hepatocellular carcinoma (HCC) is a leading fatal malignant tumor [1]. Owing to the specific phenotypes, most of HCC patients are diagnosed in advanced stage with extremely poor prognosis. Long-term liver injury, includes hepatitis virus infection, autoimmune hepatitis, exposure to poisons, excessive drinking and genetic metabolic diseases, are risk factors for HCC [2]. So far, clinical efficacy of therapeutic strategies for LIHC has been greatly improved. Nevertheless, the 5-year survival of LIHC is as low as 7% [3]. High rates of metastasis and recurrence are the major reasons for the poor prognosis of LIHC [4]. Accurate and sensitive biomarkers for predicting the disease progression of HCC are urgently required, which could markedly elevate clinical outcomes and improve the prognosis [5].

MiRNAs are a type of non-coding, small RNAs with 22 nucleotides long. They are capable of regulating target gene expressions by targeting their 3'UTR [6]. As negative regulators, miRNAs are

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critical in various pathways [7]. In tumor diseases, miRNAs extensively influence tumor cell phenotypes, thus affecting tumor progression [8]. Increasing evidences have demonstrated the diagnostic and prognostic potentials of miRNAs in tumor diseases [9-11]. In HCC, many tumor-associated miRNAs are activated to affect tumor progression by mediating significant pathways. For instance, miR-122 is abundantly expressed in HCC, which is closely linked to HCC cell phenotypes and metabolism. Dysregulation of miRNA-122 indicates liver damage, and thus miRNA-122 could be served as a hallmark in liver [12]. It is believed that miRNAs are promising non-invasive biomarkers for predicting tumor progression.

CDKN2C (cyclin dependent kinase inhibitor 2C), also known as p18INK4C, is considered as a tumor-suppressor gene [13]. It is suggested that mutant CDKN2C predicts poor prognosis, which has been verified in human teratoma and thyroid tumor [14,15]. Similarly, dysregulated CDKN2C and its protease activity change are associated with the prognosis of HCC [16]. In this paper, we predicted candidate miRNAs that regulate CDKN2C, and their regulatory effects on HCC progression.

## Methods

*Sample collection*

Paired LIHC and adjacent normal tissues were harvested from 20 HCC patients undergoing radical resection in our hospital, including 13 males and 7 females (median age: 58 years). Tissue samples were immediately preserved at -80°C within 30 min. This study was approved by the Ethic Committee of Qingdao Municipal Hospital. Patients provided consent prior to the study entry. This study was conducted in accordance with the Declaration of Helsinki.

RNA extraction and Quantitative real-time polymerase chain reaction (qRT-PCR). Cells were lysed to harvest RNAs using TRIzol (No.1559602, Invitrogen, Carlsbad, CA, USA) method, and the extracted RNAs were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (RR037A,TaKaRa, Tokyo, Japan). RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (RR420A, TaKaRa, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references, and the relative level was calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 1.

*Cell culture and transfection*

Huh7 and Hep3B cells were purchased from the National Infrastructure of Cell Line Resource, Beijing and cultured in Dulbecco's Modified Eagle Medium (DMEM) (No.11054001, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (BSK, Gibco, Rockville, MD, USA) and 100 U/mL penicillin-streptomycin. Cell passage was limited within 30 generations. Transfection vectors were purchased from GenePharma, Shanghai, China. Cells were transfected with 100 nM vector using Lipofectamine 2000 (No.11668019, Invitrogen, Carlsbad, CA, USA), and after 24-48 h of transfection, the cells were collected for functional experiments. Transfection vector sequences are listed in Tables 2 and 3.

*Dual-luciferase reporter gene assay*

Based on the binding sequences, wild-type and mutant-type CDKN2C vectors were constructed. Cells were co-transfected with NC/miRNA-22-3p mimics and CDKN2C WT/CDKN2C MUT. Forty eight h later, luciferase activity was determined.

*Cell Counting Kit (CCK-8) assay*

Cells were seeded into 96-well plates with $1\times10^5$ cells per well. At the appointed time points, 10 μL of CCK-8 solution (CK04, Dojindo, Kumamoto, Japan) were added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

**Table 1.** qPCR primer sequences

| *Name* | *Sequence (5'-3')* |
|---|---|
| miR-22-F | AGGGTCCGAGGTATTCGCA |
| miR-22-R | AGCGAAGCTGCCAGTTGAAG |
| U6-F | CTCGCTTCGGCAGCACA |
| U6-R | AACGCTTCACGAATTTGCGT |
| GAPDH-F | CTGGGCTACACTGAGCACC |
| GAPDH-R | AAGTGGTCGTTGAGGGCAATG |
| CDKN2C-F | AGTTTGTTGCAAAATAATGTAA |
| CDKN3C-R | AGTCTGTAAAGTGTCCAGGA |

**Table 2.** Plasmid construction

| *Name* | *Sequence (5'-3')* |
|---|---|
| pGL3-CDKN2C-F | CCCTCGAGGAACTTGGCCTACGTTTCCC |
| pGL3-CDKN3C-R | CCAAGCTTTGAGGAGTGTGTGTGGAGAC |

**Table 3.** The oligo sequences of miR-22 mimics and inhibitors

| *Name* | *Sequence (5'-3')* |
|---|---|
| miR-22-mimics | AAGCUGCCAGUUGAAGAACUGU |
| | AGUUCUUCAACUGGCAGCUUUU |
| miR-22-mimics nc | UUCUCCGAACGUGUCACGUTT |
| | ACGUGACACGUUCGGAGAATT |
| miR-22-inhibitors | ACAGUUCUUCAACUGGCAGCUU |
| miR-22-inhibitors nc | CAGUACUUUUGUGUAGUACAA |
| CDKN2C-F | AGTTTGTTGCAAAATAATGTAA |
| CDKN3C-R | AGTCTGTAAAGTGTCCAGGA |

*5-Ethynyl-2'- deoxyuridine (EdU) assay*

Cells were inoculated into 96-well plates with $1\times10^5$ cells per well, and labeled with 100 μL of EdU reagent (50 μM) per well for 2 h. After washing with phosphate buffered saline (PBS), cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine and permeated with 100 μL of penetrant. After washing with PBS once, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. EdU-positive ratio was determined under a fluorescent microscope.

*Cell cycle determination*

Cells were collected and incubated in pre-cold 70% ethanol for overnight fixation. On the next day, cells were centrifuged, resuspended in 50 μL of RNaseA and subjected to 37°C water bath for 30 min. Subsequently, they were incubated with 450 μL of propidium iodide (PI) at 4°C for 30 min. Cell cycle distribution was analyzed by flow cytometry (FACSort; Becton Dickinson, USA). Cell ratio in G0/G1, S and G2/M phase was calculated and compared. Each experiment was repeated in triplicate.

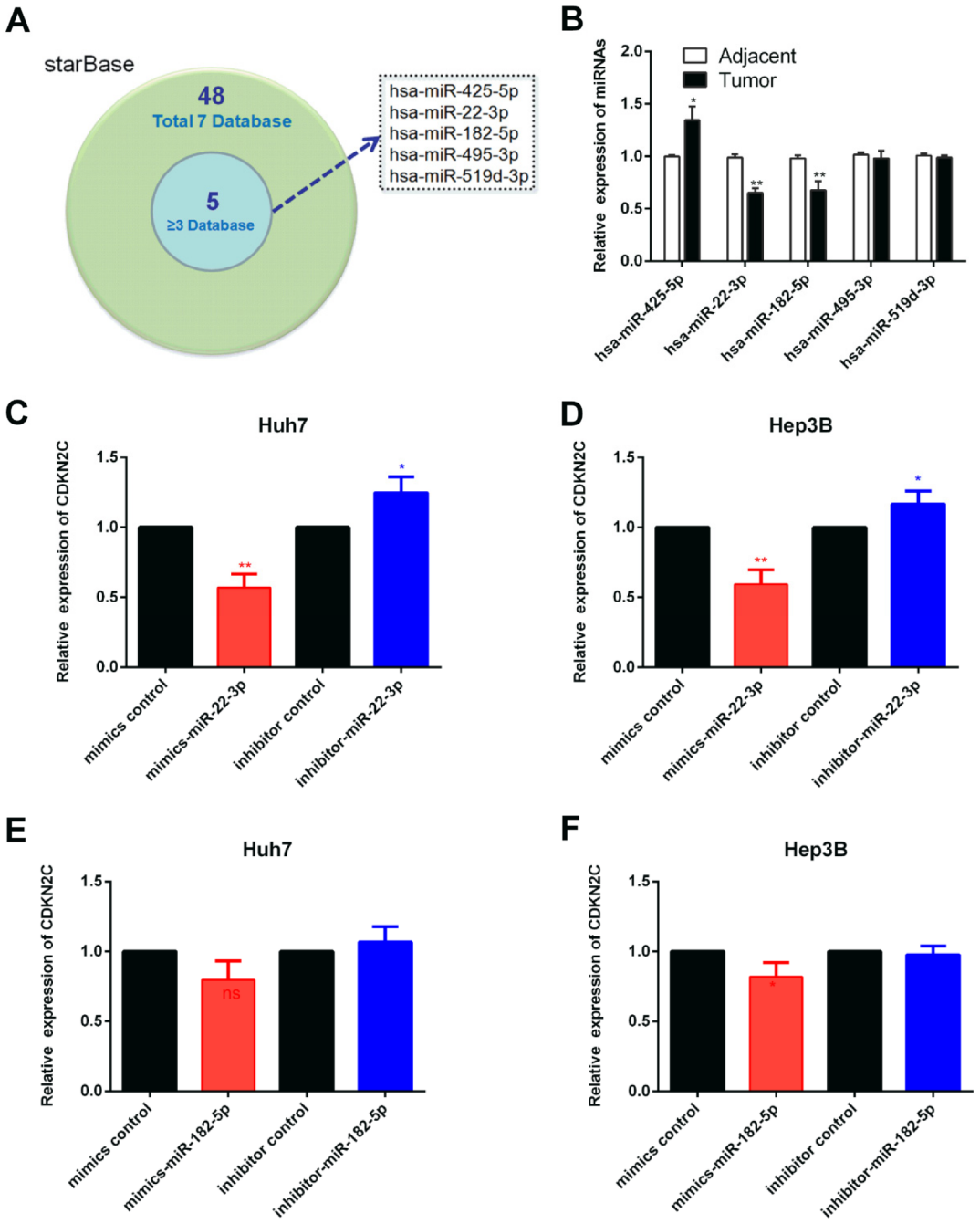


**Figure 1.** MiRNA-22-3p was predicted to be the upstream gene targeting CDKN2C. **A:** Potential miRNAs targeting CDKN2C predicted online. **B:** Relative levels of the five candidates in HCC tissues and adjacent normal tissues. **C,D:** CDKN2C level in Huh7 **C:** and Hep3B cells D: transfected with miRNA-22-3p mimics or inhibitor. **E,F:** CDKN2C level in Huh7 **(E)** and Hep3B cells **(F)** transfected with miR-182-5p mimics or inhibitor. *p<0.05, **p<0.01.

*Statistics*

SPSS 22.0 (SPSS Inc. Chicago, IL, USA) and Graphpad prism 7 (GraphPad Software Inc., La Jolla, CA, USA) were used for data analysis. Data were expressed as mean ± standard deviation. Data between two groups were compared using the t-test. Pearson correlation test was conducted to assess the relationship between miRNA-22-3p and CDKN2C. Their prognostic values were evaluated through Cox regression model. P<0.05 showed statistically significant difference.

## Results

*MiRNA-22-3p was predicted to be the upstream miRNA targeting CDKN2C*

Potential miRNAs binding to CDKN2C were predicted on StarBase (http://starbase.sysu.edu.cn/) containing 7 databases. Through comprehensive screening, a total of five candidates were selected, namely hsa-miRNA-425-5p, hsa-miRNA-22-3p, hsa-miRNA-182-5p, hsa-miRNA-495-3p and hsa-miRNA-519d-3p (Figure 1A). Subsequently, their differential expressions in HCC tissues and adjacent normal tissues were determined. Among the five candidates, only miRNA-22-3p and miR-182-5p were remarkably downregulated in tumor tissues (Figure 1B). To further uncover the relationship between candidate miRNAs and CDKN2C, we detected CDKN2C level in Huh7 and Hep3B cells transfected with corresponding miRNA mimics or inhibitor which showed that miRNA-22-3p could negatively regulate CDKN2C level in HCC cells, while the other one did not affect its level (Figure 1C-1F). As a result, miRNA-22-3p was considered as the upstream gene targeting CDKN2C in HCC.

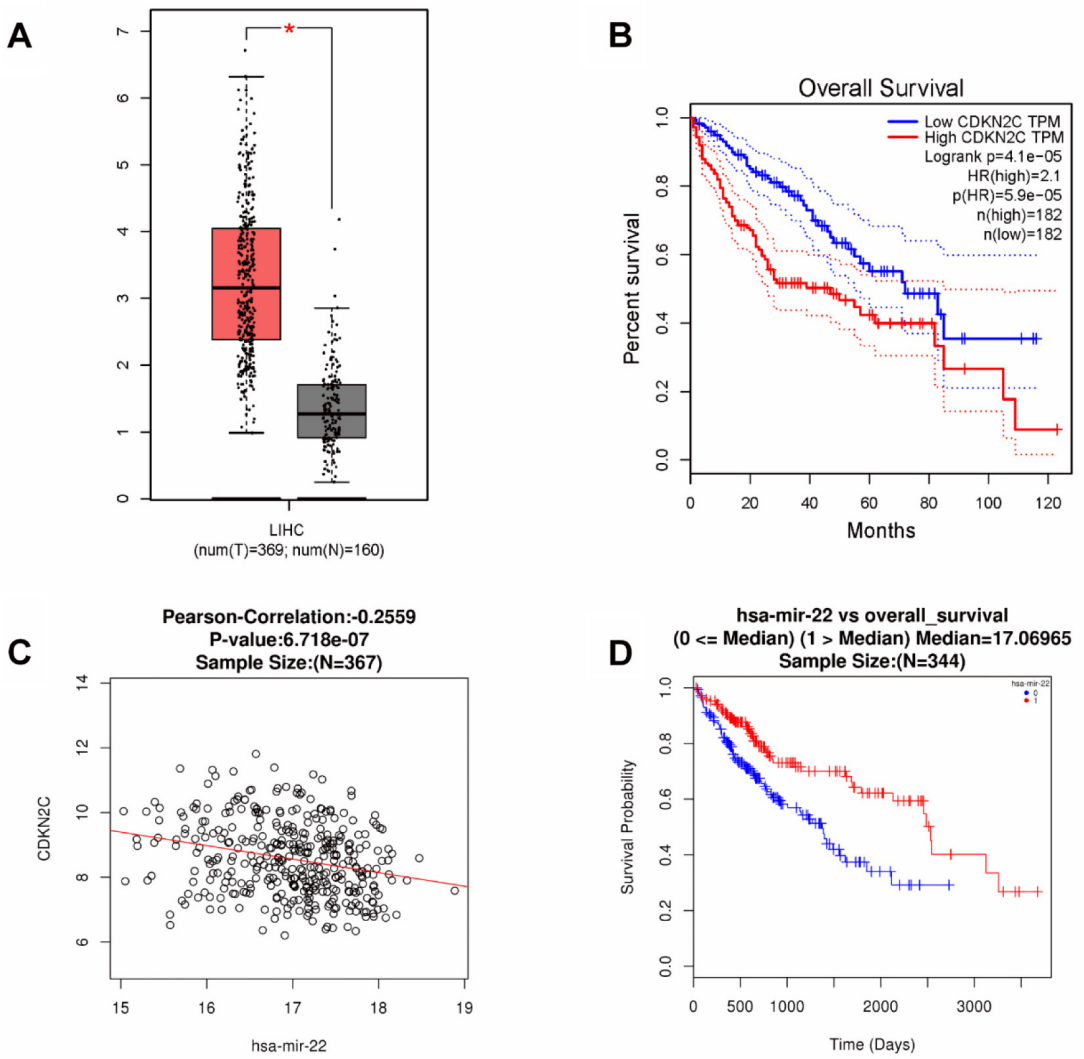


**Figure 2.** Prognostic potentials of CDKN2C and miRNA-22-3p in HCC. **A:** CDKN2C level in normal tissues (n=160) and HCC tissues (n=369) analyzed in GEPIA. **B:** Survival analysis in HCC patients based on their CDKN2C levels analyzed in GEPIA. **C:** Pearson correlation test revealed a negative correlation between miRNA-22-3p and CDKN2C level in HCC tissues (n=367). **D:** Survival analysis in HCC patients (n=344) based on their miRNA-22-3p levels analyzed in GEPIA. *p<0.05.

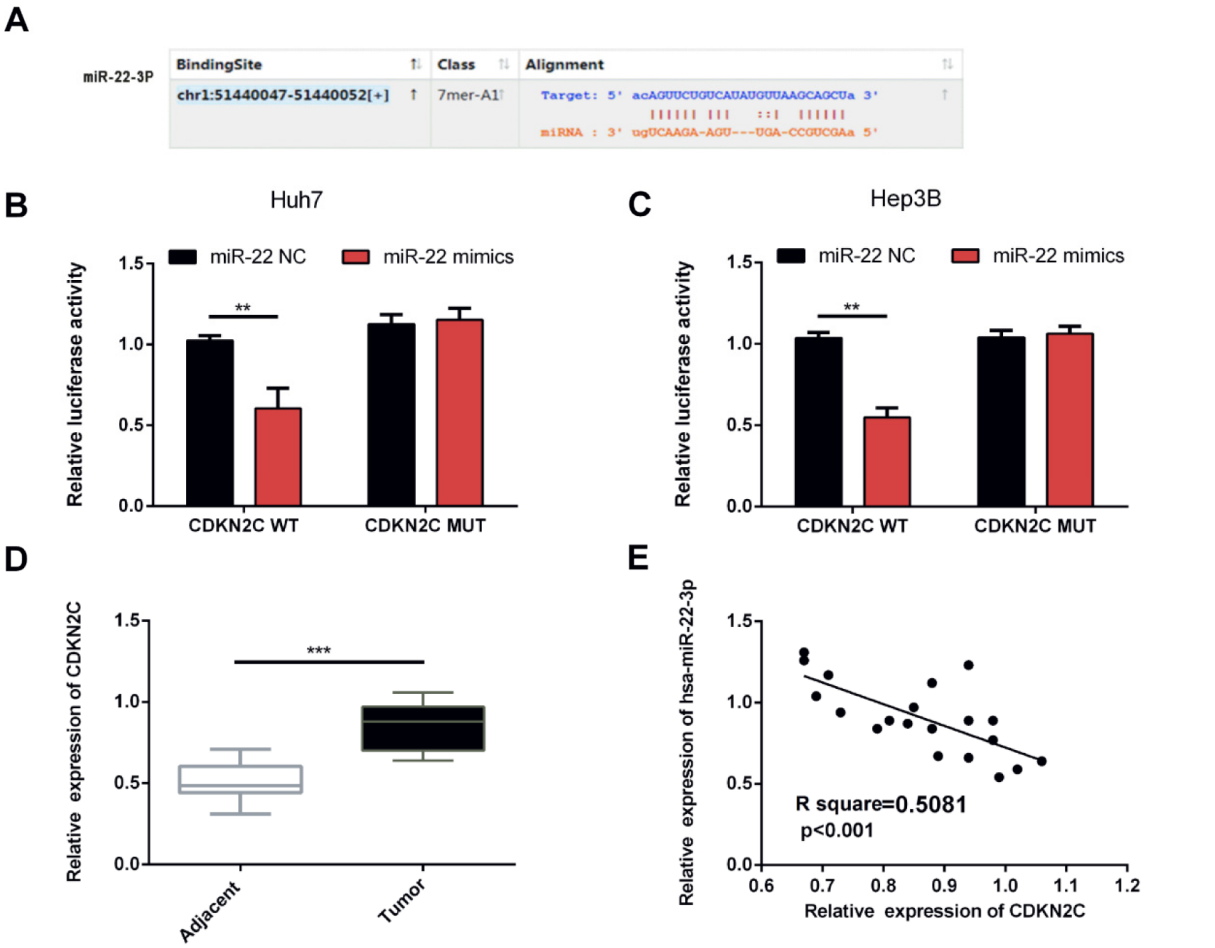


**Figure 3.** Binding relationship between miRNA-22-3p and CDKN2C. **A:** Binding sequences between CDKN2C and miRNA-22-3p. **B,C:** Luciferase activity in Huh7 **(B)** and Hep3B cells **(C)** co-transfected with NC/miRNA-22-3p mimics and CDKN2C WT/CDKN2C MUT, respectively. **D:** CDKN2C levels in 20 paired HCC tissues and adjacent normal ones. **E:** A negative correlation between miRNA-22-3p and CDKN2C level in 20 LIHC tissues.**p<0.01, ***p<0.001.

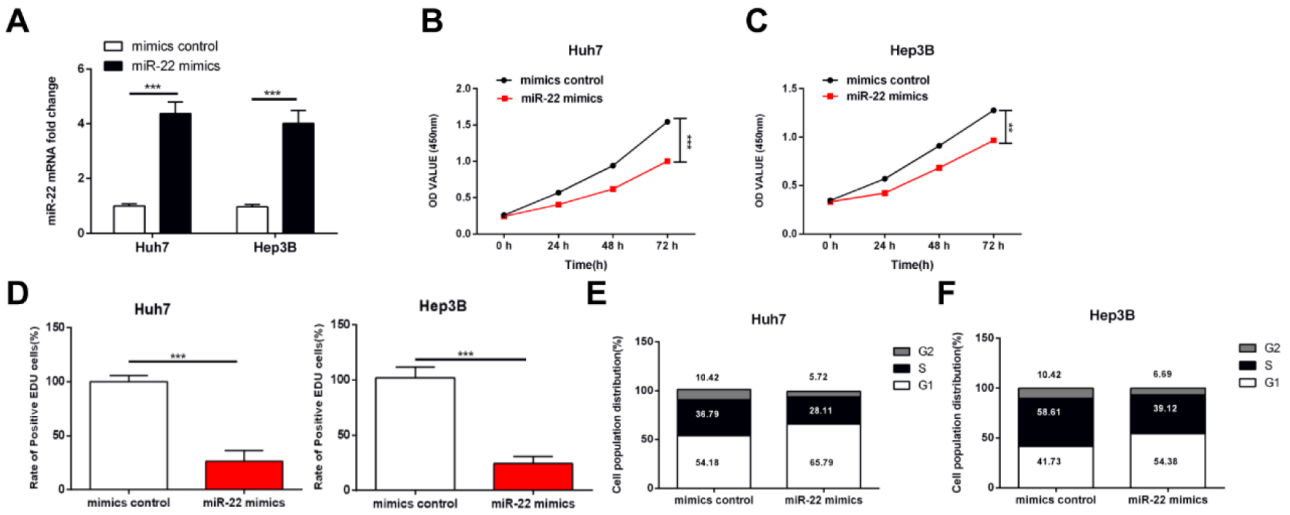


**Figure 4.** MiRNA-22-3p regulated proliferative ability and cell cycle progression in HCC. **A:** Transfection efficacy of miRNA-22-3p mimics in Huh7 and Hep3B cells. **B,C:** Viability in Huh7 **(B)** and Hep3B cells **(C)** transfected with NC or miRNA-22-3p mimics. **D:** Clonality in Huh7 and Hep3B cells transfected with NC or miRNA-22-3p mimics. **E,F:** Cell cycle distribution in Huh7 and Hep3B cells transfected with NC or miRNA-22-3p mimics. **p<0.01, ***p<0.001.

*Prognostic potentials of CDKN2C and miRNA-22-3p in HCC*

Expression pattern and prognostic potential of CDKN2C in HCC were assessed in the GEPIA database (http://GEPIA.cancer-pku.cn/index.html) [17]. In the meantime, relationship between expression levels of miRNA-22-3p and CDKN2C, and their influences on survival of HCC patients were determined on LinkedOmics (http://www.linkedomics.org) [18]. Compared with normal tissues, CDKN2C was abnormally upregulated in HCC tissues (Figure 2A). Survival analysis revealed that HCC patients expressing high level of CDKN2C suffered worse prognosis (Figure 2B). Pearson correlation test illustrated a close correlation between miRNA-22-3p and CDKN2C levels in HCC patients (Figure 2C). Besides, worse prognosis in HCC was seen in those patients expressing low level of miRNA-22-3p (Figure 2D).

*Binding relationship between miRNA-22-3p and CDKN2C*

Potential binding sequences between miRNA-22-3p and CDKN2C were predicted (Figure 3A). Based on their binding sequences, dual-luciferase

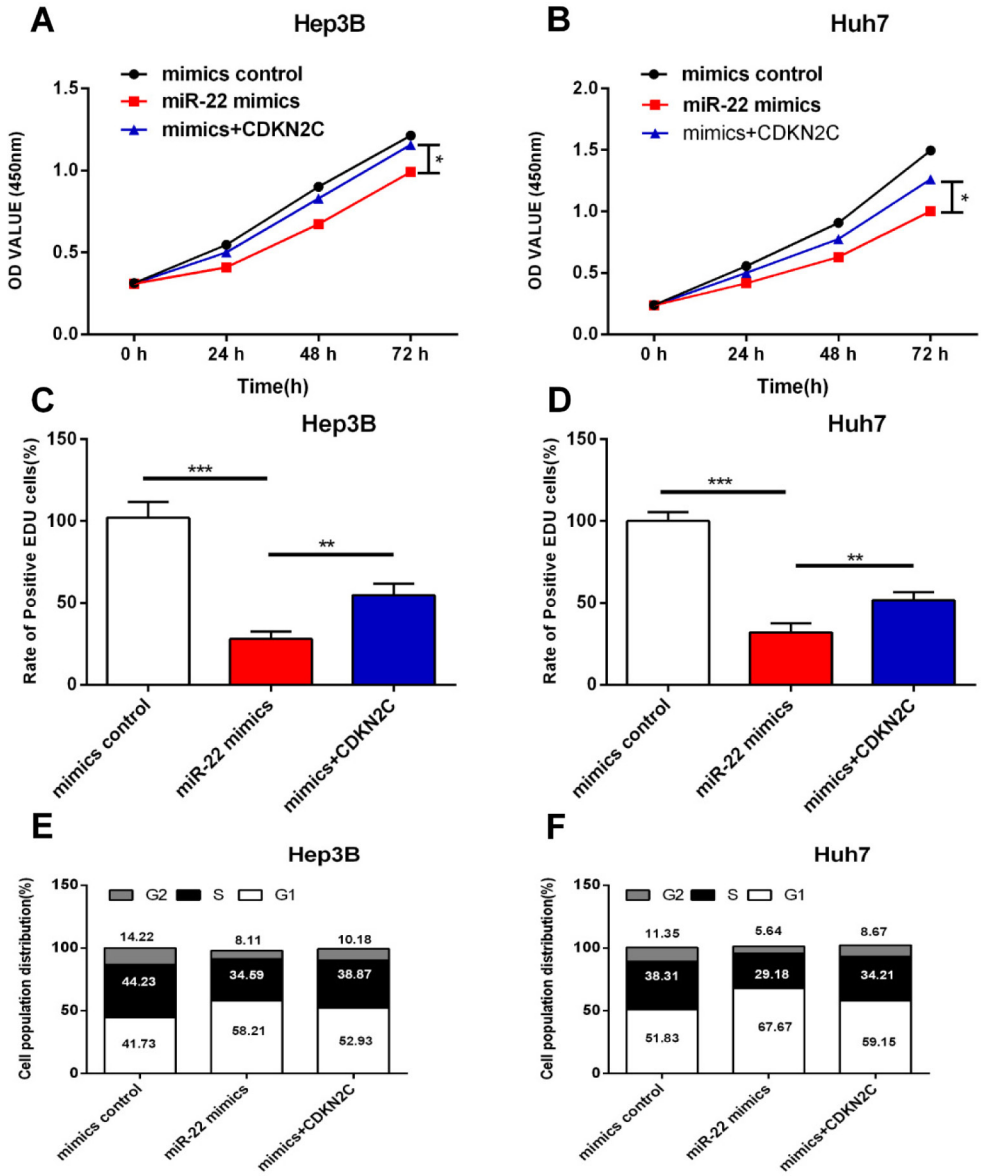


**Figure 5.** CDKN2C was responsible for miRNA-22-3p-regulated phenotypes of HCC. **A,B:** Viability in Huh7 **(A)** and Hep3B cells **(B)** transfected with NC, miRNA-22-3p mimics or miRNA-22-3p mimics+pGL3-CDKN2C. **C,D:** Clonality in Huh7 **(C)** and Hep3B cells **(D)** transfected with NC, miRNA-22-3p mimics or miRNA-22-3p mimics+pGL3-CDKN2C. **E,F:** Cell cycle distribution in Huh7 **(E)** and Hep3B cells **(F)** transfected with NC, miRNA-22-3p mimics or miRNA-22-3p mimics+pGL3-CDKN2C. *p<0.05, **p<0.01, ***p<0.001.

reporter gene assay was conducted to identify their binding relationship. Declined luciferase activity in Huh7 and Hep3B cells co-transfected with miRNA-22-3p mimics and CDKN2C WT vector confirmed their binding relationship (Figure 3B, 3C). In tissue samples collected in our hospital, CDKN2C was markedly upregulated in HCC tissues (Figure 3D). Moreover, miRNA-22-3p level was negatively correlated to that of CDKN2C in HCC tissues (Figure 3E).

*MiRNA-22-3p regulated proliferative ability and cell cycle progression in HCC*

Subsequently, regulatory effects of miRNA-22-3p on proliferation and cell cycle progression of HCC cells were assessed. Firstly, transfection of miRNA-22-3p markedly upregulated *in vitro* level of miRNA-22-3p in Huh7 and Hep3B cells (Figure 4A). As CCK-8 assay revealed, overexpression of miRNA-22-3p remarkably decreased the viability in HCC cells (Figure 4B, 4C). Similarly, clonality was attenuated in HCC cells overexpressing miRNA-22-3p, indicating the suppressed proliferative ability (Figure 4D). In addition, the ratio of proliferating cells was reduced after transfection of miRNA-22-3p mimics (Figure 4E, 4F). It is believed that miRNA-22-3p exerted anti-tumor effect through suppressing tumor cell proliferation.

*CDKN2C was responsible for miRNA-22-3p-regulated phenotypes of LIHC*

Rescue experiments were performed to identify the involvement of CDKN2C in HCC progression regulated by miRNA-22-3p. It was shown that the decreased viability in HCC cells overexpressing miRNA-22-3p was partially reversed by co-transfection of pGL3-CDKN2C (Figure 5A, 5B). Consistently, reduced EdU-positive ratio after overexpression of miRNA-22-3p was partially enhanced because of co-overexpression of CDKN2C (Figure 5C, 5D). Co-transfection of miRNA-22-3p mimics and pGL3-CDKN2C could block the inhibitory effect of miRNA-22-3p on proliferating HCC cells (Figure 5E, 5F). Hence, miRNA-22-3p/CDKN2C regulatory loop was responsible for influencing the progression of HCC.

## Discussion

CDKN2C (p18) belongs to the INK4 family alongside p15, p16 and p19. The INK4 family members exert an inhibitory effect on tumor cell growth, and are considered as tumor-suppressor genes [19]. Their critical regulatory functions have been discovered in targeting iPSCs (induced pluripotent stem cells). CDKN2C aims to accelerate the reprogramming of iPSCs [20]. P18 deficiency leads to functional decline of iPSCs. During tumor progression, iPSCs characteristics are closely correlated with tumor resistance and poor prognosis [21]. A previous study has reported that CDKN2C mutation is abundantly occurred in parathyroid carcinoma. Determination of CDKN2C level helps monitor the prognosis of parathyroid carcinoma [22]. The tumorigenesis ability of mutant CDKN2C has been illustrated in a nude mice model [23]. Collectively, mutation of CDKN2C provides a favorable environment for tumorigenesis [24].

So far, a total of 2657 mature miRNAs have been discovered in humans (miRBase, release 22, 2018, http://www.mirbase.org). MiRNAs are responsible for various aspects of tumor cell behaviors [25]. Extracellular matrix-containing exosomes have been well concerned nowadays. Specifically, miRNAs expressed in exosomes contribute to tumor diagnosis and determination of tumor subtypes. Therefore, miRNAs possess great value in tumor therapy [26]. Researches on miRNAs rely on high-throughput sequencing and bioinformatics technology [27,28]. In this article, potential miRNAs binding to CDKN2C were predicted in different online websites, and the intersection was further analyzed. At least, two candidates were selected. However, only miRNA-22-3p exerted regulatory effects on CDKN2C level in HCC cells. Previous studies have shown that miRNA-22-3p stimulates cancer cell apoptosis and inhibits proliferative ability, serving as a tumor-suppressor gene [29,30]. In addition, miRNA-22-3p displays its biological functions through activating the classic signaling AKT or targeting the downstream DDIT4 and HMGB1 [30-32]. The role of miRNA-22-3p in HCC, however, remains largely unclear. Our experimental data first proved that miRNA-22-3p served as a tumor-suppressor gene involved in HCC progression. It could regulate *in vitro* proliferation and apoptosis of LIHC cells. To further explore the specific molecular mechanism, dual-luciferase reporter gene assay and Pearson correlation test further confirmed their negative correlation in HCC. Subsequently, functional experiments demonstrated that miRNA-22-3p markedly attenuated the proliferative ability and arrested cell cycle in HCC. Rescue experiments verified the important function of miRNA-22-3p/CDKN2C regulatory loop in influencing the progression of HCC. Notably, a relevant study reported that miRNA-22-3p level in the body fluid is able to predict the occurrence of some diseases, indicating that miRNA-22-3p may be a predictive indicator for human diseases [33]. Detecting pathological biomarkers in the body fluid is convenient and precise. Further experiments are expected to validate the potential of miRNA-22-3p in the screening of HCC.

In this article, we first predicted the potential miRNA targeting CDKN2C by bioinformatics analyses. Through *in vitro* experiments, the regulatory effects of miRNA-22-3p and CDKN2C on the malignant phenotypes of HCC cells were assessed. Nevertheless, we failed to uncover downstream genes of CDKN2C and relative pathways involved in HCC, which are required to be further analyzed.

## Conclusions

MiRNA-22-3p suppresses proliferative ability and arrests cell cycle progression in HCC through targeting CDKN2C.

## Conflict of interests

The authors declare no conflict of interests.

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