miRNA-210 promotes the progression of hepatitis B cirrhosis to liver cancer via targeting inhibition of EGR3

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Abstract

Background: This paper was aimed to research the mechanism of miRNA-210 in the progression of hepatitis B cirrhosis to liver cancer.

Methods: Health examiners liver tissues (Control group), liver tissues of patients with hepatitis B cirrhosis (Cirrhosis group) and liver cancer tissues of patients induced by hepatitis B virus infection (Liver cancer group) were collected. HL-7702, HepG2 and HepG2.2.15 cells were cultured. HepG2 and HepG2.2.15 cells were transfected by miRNA-210 inhibitor (miRNA-210 Inhibitor group) and its negative control (miRNA-210-NC group). Normal HepG2 and HepG2.2.15 cells were named Blank group. Cells proliferation and apoptosis was analyzed. qRT-PCR technology, Western blot analysis and dual luciferase reporter gene assay were performed.

Results: Tissues of Liver cancer group had higher miRNA-210 and lower EGR3 expression than Cirrhosis group (P < 0.05). Increased miRNA-210 and decreased EGR3 expression in HepG2.2.15 cells was presented when compared with those in HepG2 cells (P < 0.05). Compared to Blank group and miRNA-210-NC group, HepG2 and HepG2.2.15 cells of miRNA-210 Inhibitor group had lower A590 value, higher apoptosis rate and EGR3 expression (P < 0.05). EGR3 was directly inhibited by miRNA-210.

Conclusions: miRNA-210 might promote the progression of hepatitis B cirrhosis to liver cancer via targeting inhibition of EGR3.

Backgrounds

Liver cancer had become a prevalent malignant tumor with a sharp increase in mortality all over the world. Especially in China, hepatitis B virus (HBV) infection was one of the fatal causes of liver cancer [1]. An report in 2013 indicated that, approximately 2 billion people were infected with HBV and about 93 million people were Chinese per 350 million HBV carriers in the world [2]. HBV infection would result in damage to liver tissues and eventually lead to chronic hepatitis B as well as cirrhosis and even liver cancer [3]. It was reported that about 1 million patients were died because of liver failure, liver cirrhosis or liver cancer induced by HBV infection every year [4]. In most instances, cirrhosis caused by HBV infection would further develop into liver cancer, which was a manifestation of serious poor prognosis in patients with HBV infection [5].

MicroRNAs (miRNAs), known as a class of noncoding small single-stranded RNAs consisting of 18−25 nucleotides, were proved to participate in majority of cellular biological functions, such as differentiation, apoptosis, proliferation, and even tumorigenesis, by targeting the regulation of other specific coding genes expression [6−8]. A recent study researched that miR−122−5p, miR−199a−5p, miR−486−5p, miR−193b−5p, miR−206, miR−192−5p, miR−141−3p and miR−26a−5p were differential expressed in cirrhosis as well as liver cancer, which were involved in the conversion of cirrhosis to hepatitis B virus-associated liver cancer [9]. Riazalhosseini et al. [10] demonstrated that miRNA−196 and miRNA−146 were

involved in the development of liver cirrhosis infected by HBV to liver cancer. Xie *et al.* [11] proved that serum miR−101 level could be used as a candidate biomarker to distinguish between HBV-infected liver cancer and HBV-infected cirrhosis. miRNA−210 had been shown to be participated in tumorigenesis and progression of several malignant tumors, including liver cancer [12,13]. However, there was no research to prove whether miRNA−210 was participated in the progression of cirrhosis to liver cancer.

Therefore, in this paper, we investigated the expression of miRNA−210 in cirrhosis and liver cancer infected with HBV by in vitro studies in order to explore whether miRNA−210 affected the progression of liver cirrhosis to liver cancer. This study would provide a novel candidate therapeutic target for blocking the development of liver cirrhosis to liver cancer.

Methods

Liver tissue samples

Liver tissues from patients undergoing liver cancer resection and outpatient liver biopsy from September 2015 to August 2018 were collected. Normal liver tissues of health examiners (Control group, n = 25, 14 males and 11 females, average age of 48.8 ± 1.2 years), liver tissues of cirrhosis patients caused by chronic hepatitis B (Cirrhosis group, n = 25, 12 males and 13 females, average age of 48.6 ± 1.3 years) and liver cancer tissues of patients induced by hepatitis B virus infection (Liver cancer group, n = 25, 13 males and 12 females, average age of 49.3 ± 1.1 years) were screened for study. No statistically significant difference was observed in age and gender among the above three groups. In this study, all participants were first diagnosed with hepatitis B cirrhosis or hepatitis B virus-associated liver cancer, and all patients did not have other diseases or metastasis.

With the approval of the ethics committee of our hospital, all participants had been informed.

Cell culture and transfection

In this research, all cells (including human normal liver HL−7702 cells, hepatoma cells HepG2, and HepG2.2.15 cells stably expressing HBV) were available from the Cell Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. HL−7702 cells and HepG2 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS), while HepG2.2.15 cells were maintained in RPMI1640 medium containing 10% FBS and 380 μg/mL G418 (Sigma, Dorset, UK). All cells were placed in a 5% CO_2 incubator at 37° C, 95% humidity.

HepG2 and HepG2.2.15 cells were digested with 0.25% trypsin and were dispersed in RPMI1640 medium (without FBS) with a density of 1.3 * 10^5 cells/mL. Six-well plates containing 1 mL of cell suspensions per well was placed in the 5% CO_2 incubator at 37° C, 95% humidity. One day later, residual liquid in each well was discarded, and HepG2 and HepG2.2.15 cells were divided into 3 groups: Blank group (cells were

recultured by 1 mL serum free RPMI1640 medium), miRNA−210 Inhibitor group (cells were recultured by 1 mL serum free RPMI1640 medium containing 100 nmol/L miRNA−210 inhibitor and 2 μL DharmaFECT 4) and miRNA−210-NC group (cells were recultured by 1 mL serum free RPMI1640 medium containing 100 nmol/L miRNA−210 inhibitor negative control and 2 μL DharmaFECT 4). All plates were returned to the 5% CO_2 incubator for 8 h incubation at 37°C, 95% humidity. Then residual liquid in each well was replaced by 1 mL RPMI1640 medium (10% FBS) and cells were cultured for 2 days at the same conditions. In this study, miRNA−210 inhibitor and miRNA−210 inhibitor negative control were both provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipfectamine 2000 transfection kit, purchased from Thermo Fisher Scientific, Waltham, MA, USA, was used to perform transfection according to the instructions.

MTT assay for proliferation

HepG2 and HepG2.2.15 cells of Blank group, miRNA−210 Inhibitor group and miRNA−210-NC group were dispersed in RPMI1640 medium (10% FBS) and inoculated in 96-well plates ($1*10^4$ cell per well) for 0, 1, 2, 3, 4, 5, 6 and 7 days culturing. On each day, cells of each group were removed from the incubator and MTT solution (20 μL, 5mg/mL) was added into each well for 4 h incubation at 37°C. Dimethyl sulfoxide (DMSO, 150 μL) was used to replace the residual liquid in each well. After the crystals were sufficiently dissolved, the absorbance value of each well was detected at a wavelength of 590 nm by using a microplate reader.

Apoptosis rate detection by Annexin V-FITC and propidium iodide (PI) double staining

HepG2 and HepG2.2.15 cells of Blank group, miRNA−210 Inhibitor group and miRNA−210-NC group were cultured for 72 h and then prepared into cell suspensions with PBS. All cell suspensions were transferred to centrifuge tubes respectively, and subjected to centrifugation for 5 min at 1500 r/min, 4°C. Cells at the bottom of tubes were resuspended by 1 × Binding Buffer to cell suspensions of $(0.5−1) × 10^6$ cells/mL. These cell suspensions with a volume of 100 μL were transferred to a 5 mL flow tube, and incubated with FITC-labeled annexin V (5 μL) and PI (5 μL) at room temperature in the dark for 15 min. A total of 400 μL 1×Binding Buffer was added into cells and apoptosis was detected using flow cytometry.

qRT-PCR detection of miRNA−210 and EGR3 mRNA expression

Total RNA in tissues and cells were routinely acquired by TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA). A certain amount of RNA samples (5 μg) were underwent reverse transcription on

Gene Amp PCR System 9700 (Applied Biosystems,Foster City, CA) to synthesize cDNA by applying RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Sydney, Australia) was applied to perform qRT-PCR amplification reaction with 1 μL cDNA template. miRNA−210 amplification reaction was circulated 40 times under the following conditions: 95°C for 10 min; 95°C for 15 s; 60°C for 60 s, and EGR3 mRNA amplification reaction conditions were 35 cycles of 94°C for 30 s; 53°C for 45 s and 72°C for 45 s. U6 was used as an internal reference for miRNA−210, while GAPDH was set as an internal reference for EGR3 mRNA. Primers sequences were shown as follows: miRNA−210-F, 5'-GTGCAGGGTCCGAGGT−3', miRNA−210-R, 5'-TATCTGTGCGTGTGACAGCGGCT−3'. U6-F, 5'-CTCGCTTCGGCAGCAC−3', U6-R, 5'-AACGCTTCACGAATTTGCG−3'. EGR3-F, 5'-TACAATCAGATGGCTACAGAGAAT−3', EGR3-R, 5'-TTCCCAAGTAGGTCACGGTC−3'. GAPDH-F, 5'-TCGGAGTCAACGGATTTGGTC−3', GAPDH-R, 5'-GCCATGGGTGGAATCATATTGG−3'. Data was corrected using 2-$\Delta\Delta$Ct methods to reduce RNA concentration quantification error and RNA reverse transcription efficiency error.

Western blot detection of EGR3 protein expression

Total proteins from tissues and cells were routinely acquired by using RIPA cell lysis buffer, and were quantified with Bradford protein assay kit (Beyotime, Shanghai, China). Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to polyvinylidene fluoride (PVDF) membrane for 2 h blocking with 5% skim milk powder. Mouse anti-human EGR3 monoclonal antibody (1:1000, Abcam,UK) was used to incubate the PVDF membrane for 12 h at 4°C. The membrane was then washed with TBS buffer 3 times, and subsequently being incubated with horseradish peroxidase-labeled goat anti-mouse IgG (l:2000, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 1 h at room temperature. The membrane was washed with TBS buffer 3 times again. ECL kit was used to visualize blots and image was processed by gel imaging analysis system. GAPDH was considered as the internal reference and EGR3 protein relative expression was expressed as the integral optical density ratio of target strip to internal reference strip.

Dual luciferase reporter gene assay

EGR3 was predicted to be a target gene for miRNA−210 via TargetScan. Thus, Dual luciferase reporter gene assay was performed to further verify the regulatory relationship between EGR3 and miRNA−210. Briefly, HepG2 cells seeded in 24-well plates were transfected at 80−90% confluence. EGR3 wild-type plasmid vectors combined with miRNA−210 mimics or miRNA−210 negative control was used to co-transfect HepG2 cells. HepG2 cells were also co-transfected by EGR3 mutant plasmid vectors and miRNA−210 mimics or miRNA−210 negative control. In this study, all plasmid vectors were provided from Shanghai GenePharma Co., Ltd. (Shanghai, China). Transfected HepG2 cells were named as mimic + wild group, mimic + mutation group, NC + wild group and NC + mutation group depending on the difference of transfection vectors. HepG2 cells of each group were subjected to dual luciferase activity assay after 48 h

culturing. The relative activity of luciferase was presented as the ratio of Fireny Luciferase activity to Renilla Luciferase activity.

Statistical analysis

Data were exhibited in the form of mean ± standard deviation (SD). Statistical analysis was conducted by applying two-tailed Student t-test or one-way ANOVA with $P < 0.05$ as the threshold.

Results

miRNA−210 was overexpressed in chronic hepatitis B cirrhosis and hepatitis B virus infected liver cancer

miRNA−210 relative expression was determined by qRT-PCR (shown in Figure 1). When compared with relative miRNA−210 expression in liver tissues of Control group, it was significantly overexpressed in Cirrhosis group and Liver cancer group ($P < 0.05$). Tissues of Liver cancer group had much higher miRNA−210 relative expression when compared with Cirrhosis group ($P < 0.05$) (Figure 1A). In addition, HepG2 and HepG2.2.15 cells showed much higher relative miRNA−210 expression than that of HL−7702 cells ($P < 0.05$). However, markedly increased relative miRNA−210 expression in HepG2.2.15 cells was presented when compared with HepG2 cells ($P < 0.05$) (Figure 1B). miRNA−210 expression was significantly increased with the development of chronic hepatitis B cirrhosis to liver cancer induced by hepatitis B virus infection.

miRNA−210 inhibitor transfection successfully inhibited miRNA−210 expression in HepG2 and HepG2.2.15 cells

For HepG2 and HepG2.2.15 cells of Blank group and miRNA−210-NC group, no significant difference was found in miRNA−210 relative expression between the two groups. However, dramatically lower miRNA−210 relative expression in HepG2 and HepG2.2.15 cells of miRNA−210 Inhibitor group was exhibited when compared with Blank group and miRNA−210-NC group ($P < 0.01$) (Figure 2AB), suggesting that HepG2 and HepG2.2.15 cells was successfully transfected by miRNA−210 inhibitor.

Down-regulation of miRNA−210 inhibited HepG2 and HepG2.2.15 cells proliferation

Two days after transfection, the A_{590} value of HepG2 cells in miRNA−210 Inhibitor group was much lower than that in Blank group and miRNA−210-NC group ($P < 0.05$), whereas no obvious difference was found in A_{590} value between Blank group and miRNA−210-NC group at the same time point ($P < 0.05$) (Figure 3A). Similar result was also occurred in HepG2.2.15 cells (Figure 3B). Low miRNA−210 expression declined proliferation of HepG2 and HepG2.2.15 cells.

Down-regulation of miRNA−210 promoted HepG2 and HepG2.2.15 cells apoptosis

Cells apoptosis was investigated by Annexin V-FITC/PI double staining in this article. When compared with Blank group, the apoptosis rate of HepG2 and HepG2.2.15 cells in miRNA−210-NC group was not obviously changed. However, HepG2 and HepG2.2.15 cells of miRNA−210 Inhibitor group exhibited remarkably higher apoptosis rate than that of Blank group and miRNA−210-NC group ($P < 0.05$) (Figure 4AB), which indicated that declining miRNA−210 expression promoted HepG2 and HepG2.2.15 cells apoptosis.

EGR3 was decreased in chronic hepatitis B cirrhosis and hepatitis B virus infected liver cancer

As shown in Figure 5A, B and C, relative EGR3 mRNA and protein expression in liver tissues of Cirrhosis group and Liver cancer group was both dramatically declined when compared with Control group ($P < 0.05$). Meanwhile, liver tissues of Liver cancer group showed much lower relative EGR3 mRNA and protein expression than those of Cirrhosis group ($P < 0.05$). Furthermore, much lower relative EGR3 mRNA and protein expression was observed in HepG2 and HepG2.2.15 cells when compared with that in HL−7702 cells ($P < 0.05$), and significantly lower relative EGR3 mRNA and protein expression was discovered in HepG2.2.15 cells when compared with HepG2 cells ($P < 0.05$) (Figure 5D, E and F). All of the above results revealed that EGR3 was decreased in chronic hepatitis B cirrhosis and hepatitis B virus infected liver cancer.

EGR3 was directly inhibited by miRNA−210

Target Scan prediction indicated that EGR3 bound to miRNA−210 in the 3'-UTR region (Figure 6A). EGR3 mRNA and protein was lowly expressed in HepG2 and HepG2.2.15 cells of Blank group. However, after miRNA−210 expression was inhibited, HepG2 and HepG2.2.15 cells of miRNA−210 Inhibitor group presented significantly increased EGR3 mRNA and protein expression, which was dramatically higher than that Blank group and miRNA−210-NC group ($P < 0.05$) (Figure 6B, C and D). According to dual luciferase reporter gene activity assay, there was no obvious difference in relative fluorescence unit among mimic + mutation group, NC + wild group and NC + mutation group. However, the relative fluorescence unit of mimic + wild group was much lower than that of the other three groups ($P < 0.05$)

(Figure 6E). All of these results illustrated that miRNA−210 was able to directly suppress EGR3 expression by binding EGR3 at 3'-UTR region.

Discussion

Liver cancer ranked the third leading cause of cancer-related death worldwide, with cirrhosis being the main cause. The evolutionary feature of liver cancer was from intermediate stage progressed to advanced stage and even death in most cases [14]. Preventing the development of liver cirrhosis to liver cancer was an urgent problem to be solved clinically, which could extremely improve the prognosis of patients. In this article, we discovered that miRNA−210 was extremely highly expressed in HBV-infected liver cancer than that in liver cirrhosis caused by HBV infection, and its down-regulation inhibited proliferation and promoted apoptosis of HepG2 and HepG2.2.15 cells via interfering with EGR3 expression targetedly.

Accumulating data had proved that miRNA−210 was participated in the regulation of multiple tumors. In pancreatic cancer, Amponsah *et al.* [15] illustrated that greatly overexpressed miRNA−210 was played an inhibitory effect on tumor cells growth, which could also reverse the resistance of tumor cells on gemcitabine. A study of miR−210 in regulating the progression of prostate cancer showed that miR−210 level was remarkably elevated in metastatic prostate cancer, revealing that high expression of miR−210 had an adverse effect on prostate prognosis [16]. miR−210 was also reported to be abnormally overexpressed in upper tract urothelial carcinoma, which was participated in promoting the progression of this tumors [17]. Meanwhile, miR−210 was also closely related to the poor survival of patients with soft-tissue sarcoma [18]. In the current literature, a small number of studies have also found that miR−210 had an important effect on the occurrence and development of liver cancer. Zhan *et al.* [19] revealed that increased miRNA−210 serum level might be a effective biomarker for the treatment and prognosis of liver cancer patients, which attenuated the sensitivity of patients with liver cancer subjecting to transarterial chemoembolization. For patients with liver cancer, up-regulated miRNA−210 expression resulted in poor prognosis and promoted angiogenesis [12]. In this article, we proved that miRNA−210 exhibited aberrantly up-regulation in liver cirrhosis and liver cancer induced by HBV infection, and down-regulation of miRNA−210 could inhibit HepG2 and HepG2.2.15 cells proliferation and promoted these cells apoptosis. More importantly, miRNA−210 expression was much higher in liver cancer tissues and HepG2.2.15 cells when compared with that in liver cirrhosis tissues and HepG2 cells respectively. Thus, we speculated for the first time that overexpressed miRNA−210 promoted the conversion of cirrhosis to liver cancer.

EGR3, one of the important members of EGR family, was a well-known suppressor of tumor initiation and progression in certain cancer events. In head and neck cancer, EGR3 level was significantly reduced when compared with that in adjacent normal tissues, and meanwhile, overexpression of EGR3 in head and neck cancer cells significantly impaired these cells colony forming ability in vitro [20]. Pio *et al.* [21] declared that EGR3 expression level was closely associated with the recurrence of prostate cancer. Patients with non-recuttent prostate cancer had relatively higher EGR3 expression, whereas lower EGR3 expression was

found in patients with recurrent prostate cancer. Disorder expression of EGR3 expression was also involved in the progression of liver cancer. It was found to be exerted an inhibitor influence in the growth of liver cancer by elevating Fas ligand, and lower EGR3 expression was associated with enhanced proliferation ability and reduced apoptotic ability of liver cancer cells [22]. In fact, little work had been done in researching the link between EGR3 and liver cancer. In this study, we observed that EGR3 was lowly expressed in liver cirrhosis and liver cancer induced by HBV infection. Its expression was also declined in HepG2 and HepG2.2.15 cells, with HepG2.2.15 cells exhibiting much higher EGR3 expression. Our further data also suggested for the first time that EGR3 expression was directly inhibited by miRNA−210. Therefore, we speculated that miRNA−210 might promote the deterioration of liver cirrhosis to liver cancer by inhibiting the expression EGR3 protein. This mechanism proposed from this article might propose a reasonable theoretical basis for the targeted therapy of liver cancer clinically.

Conclusions

In conclusion, we investigated the mechanism of miRNA−210 in influencing liver cancer induced by HBV-infected liver cirrhosis. It could be discovered that miRNA−210 might promote the progression of HBV-infected liver cirrhosis to liver cancer by targeting inhibition of EGR3 protein expression. Thus, detection of miRNA−210 in liver tissues might be served as a biochemical indicator for diagnosis and treatment of HBV-infected liver cancer.

Abbreviations

hepatitis B virus HBV

MicroRNAs miRNAs

Declarations

Ethics approval and consent to participate: The ethics committee of Qingdao Sixth People's Hospital approved the study. The study was written informed consent from the patients.

Consent for publication: Not applicable.

Availability of data and material: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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

Funding: Not applicable.

Author's Contribution:

XJL: conception, design and analysis of data, performed the data analyses and wrote the manuscript

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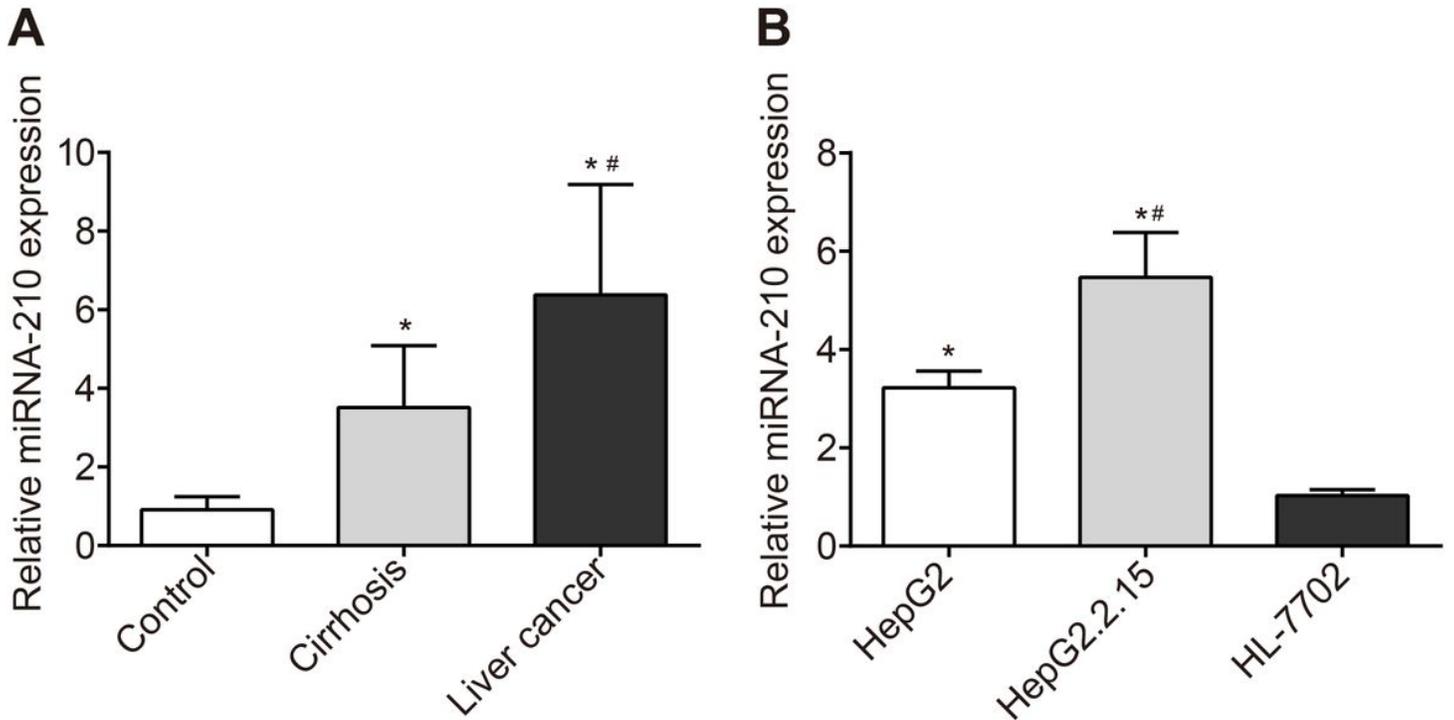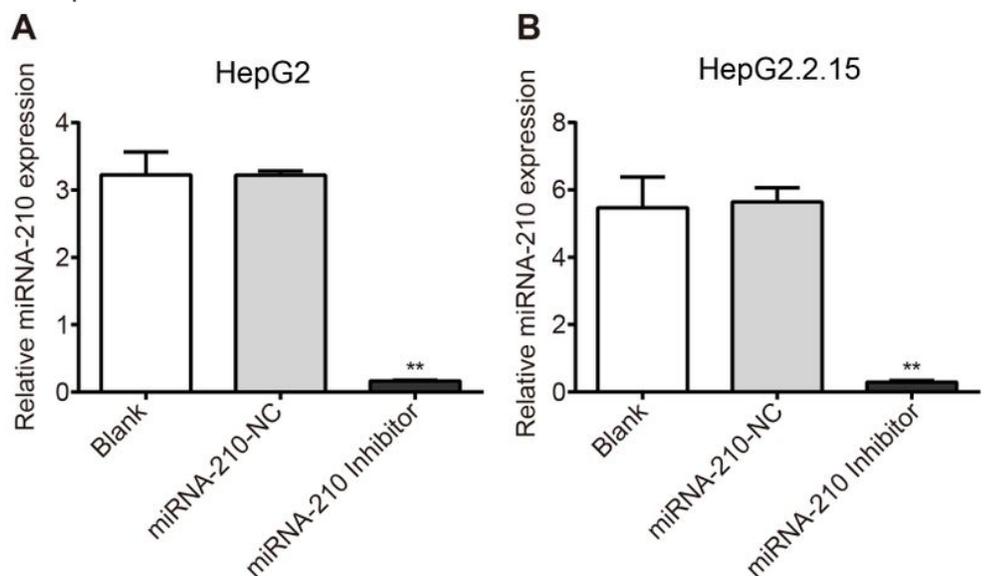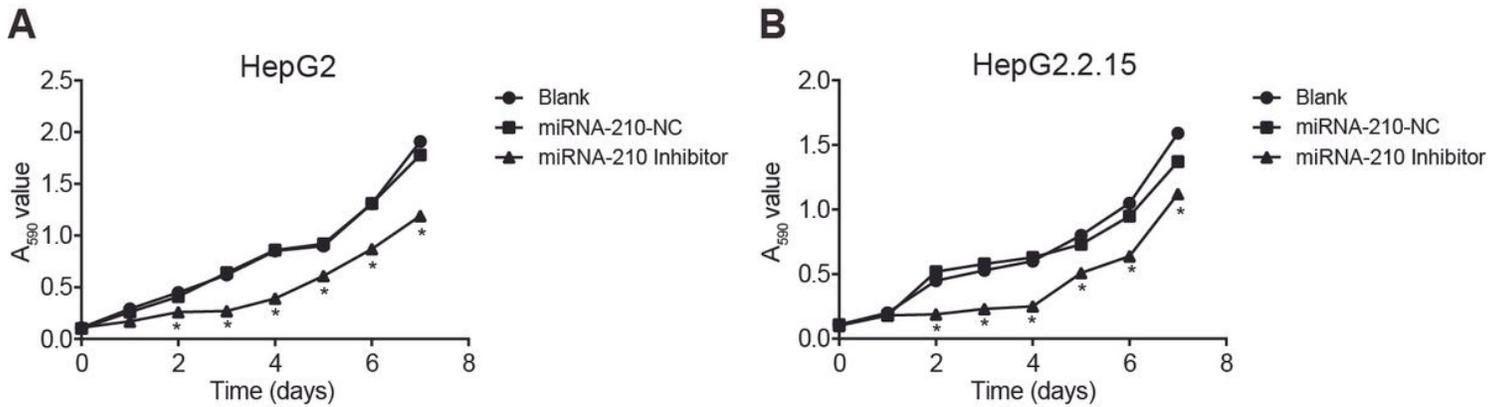
Figures

Figure 1

miRNA-210 was overexpressed in chronic hepatitis B cirrhosis and hepatitis B virus infected liver cancer. (A) miRNA-210 expression was markedly increased in liver tissues of Cirrhosis group and Liver cancer group when compared with that in Control group. * P < 0.05 when compared with Control group. # P < 0.05 when compared with Cirrhosis group. (B) miRNA-210 expression was much elevated in HepG2 and HepG2.2.15 cells when compared with that in HL-7702 cells. * P < 0.05 when compared with HL-7702 cells. # P < 0.05 when compared with HepG2 cells.

Figure 2

miRNA-210 inhibitor transfection successfully inhibited miRNA-210 expression in HepG2 and HepG2.2.15 cells. miRNA-210 expression was successfully inhibited in HepG2 (A) and HepG2.2.15 (B) cells by miRNA-210 inhibitor transfection. ** $P < 0.01$ when compared with Blank group and miRNA-210-NC group.
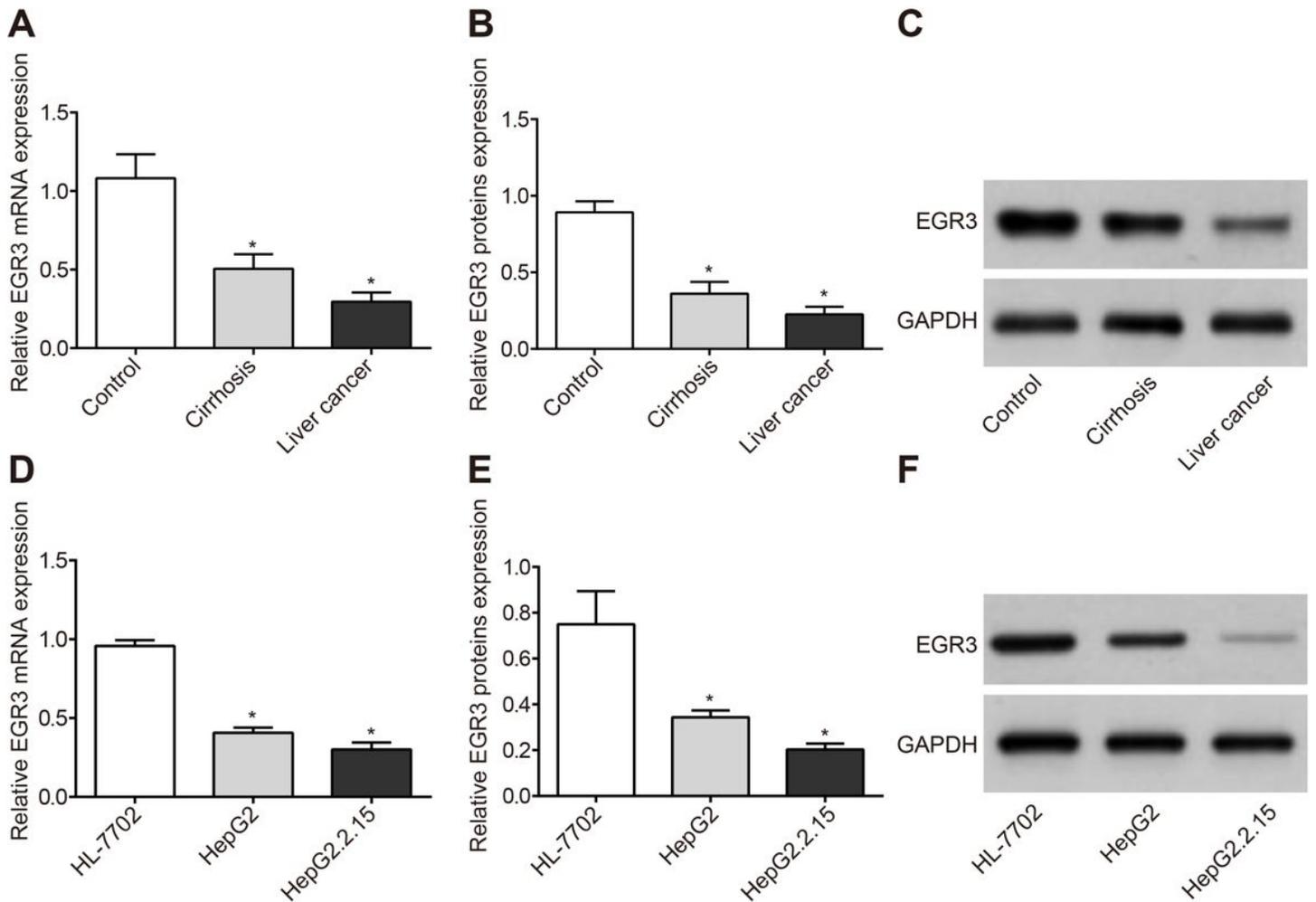

Figure 3

Down-regulation of miRNA-210 inhibited HepG2 and HepG2.2.15 cells proliferation. From day 2-7, the A590 value of HepG2 (A) and HepG2.2.15 cells (B) in miRNA-210 Inhibitor group was much lower than that in Blank group and miRNA-210-NC group. * $P < 0.05$ when compared with Blank group and miRNA-210-NC group.
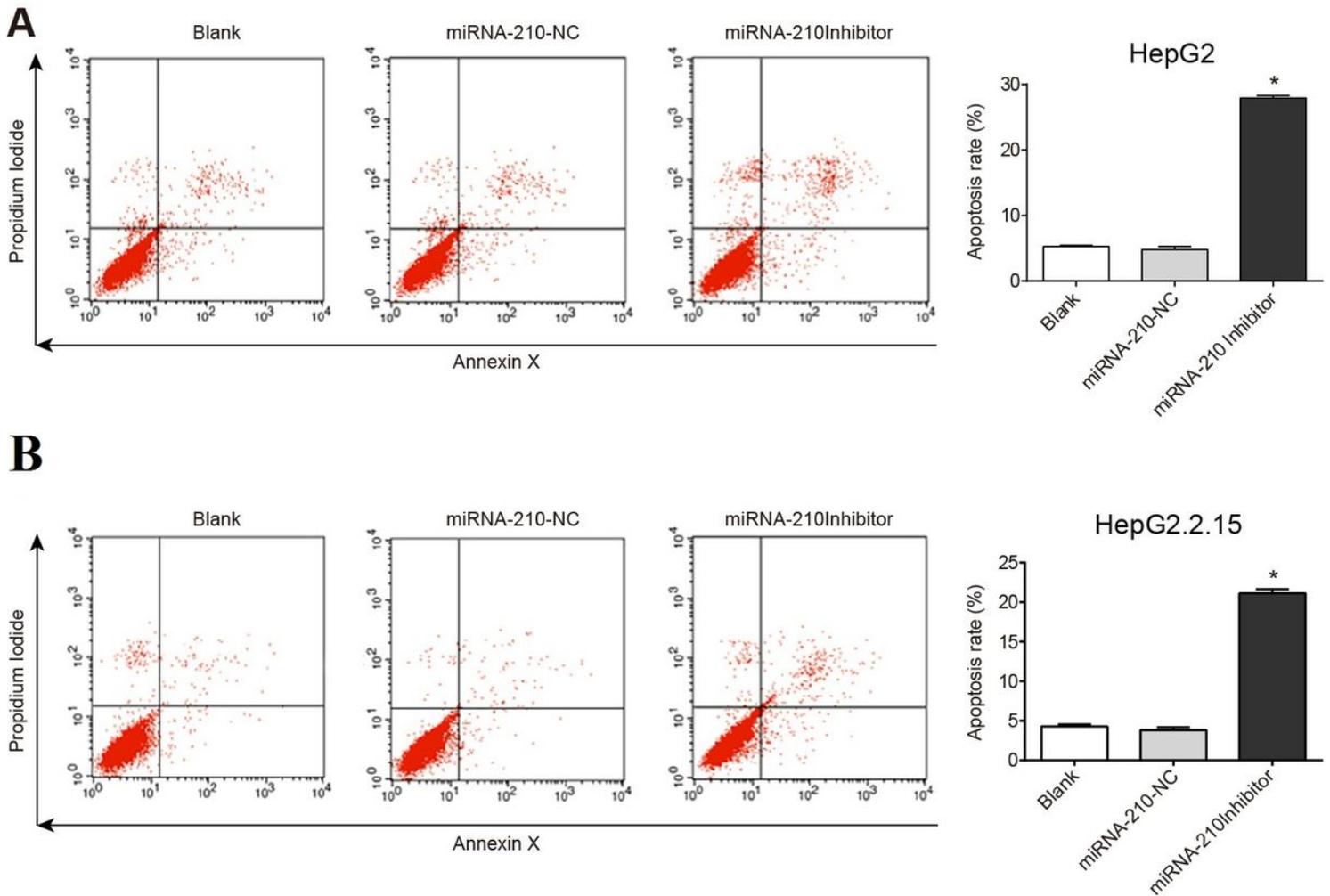
Figure 4

Down-regulation of miRNA-210 promoted HepG2 and HepG2.2.15 cells apoptosis. Low miRNA-210 expression promoted HepG2 (A) and HepG2.2.15 (B) cells apoptosis obviously. * P < 0.05 when compared with Blank group and miRNA-210-NC group.
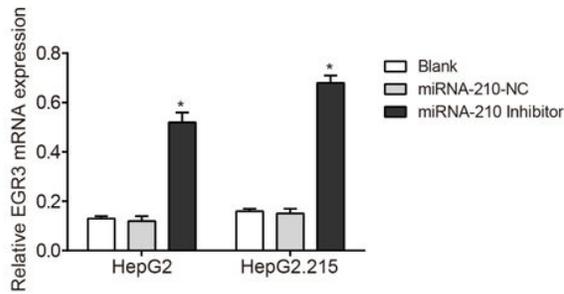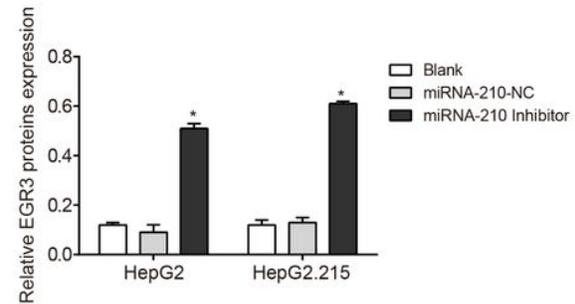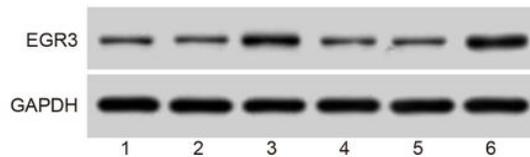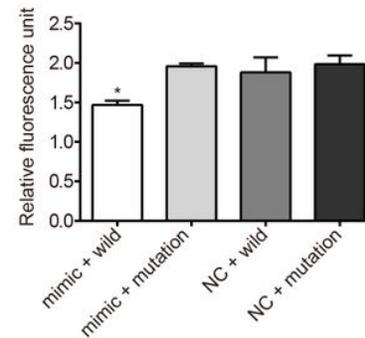
Figure 5

EGR3 was decreased in chronic hepatitis B cirrhosis and hepatitis B virus infected liver cancer. EGR3 mRNA (A) and protein (B and C) expression was significantly decreased in liver tissues of Cirrhosis group and Liver cancer group when compared with Control group. * P < 0.05 when compared with Control group. # P < 0.05 when compared with Cirrhosis group. EGR3 mRNA (D) and protein (E and F) expression was much lower in HepG2 and HepG2.2.15 cells than that in HL-7702 cells. * P < 0.05 when compared with HL-7702 cells. # P < 0.05 when compared with HepG2 cells.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	PCT
Position 180-186 of EGR3 3' UTR 5' ...ACGCCGGCCAGCGGU---CAGGGGCC... hsa-miR-210-5p 3' GUCACACGCCACCGUCCCCGA		7mer-m8	-0.09	60	-0.09	0.031	N/A

Figure 6

EGR3 was directly inhibited by miRNA-210. (A) Prediction of target sites for EGR3 and miRNA-210 binding by Target Scan. (B and C) Inhibition of miRNA-210 promoted EGR3 mRNA and protein expression. * P < 0.05 when compared with Blank group and miRNA-210-NC group. (D) Western blot band of EGR3 protein in each group of cells. The number of 1, 2, and 3 represented HepG2 cells of Blank group, miRNA-210-NC group and miRNA-210 Inhibitor group respectively. The number of 4, 5 and 6 represented HepG2.2.15 cells of Blank group, miRNA-210-NC group and miRNA-210 Inhibitor group, respectively. (E) The targeting relationship between miRNA-210 and EGR3 was detected by dual luciferase reporter gene activity assay. * P < 0.05 when compared with mimic + mutation group, NC + wild group and NC + mutation group.