Original Article

Cisplatin chemotherapy-induced miRNA-210 signaling inhibits hepatocellular carcinoma cell growth

Huan Yu[1], Guangyue Shi[2]

[1]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Renal Cancer and Melanoma, Peking University Cancer Hospital & Institute, Beijing 100142, China; [2]Department of Medical Oncology, the Affiliated Tumor Hospital, Harbin Medical University, Harbin 150001, China

Contributions: (I) Conception and design: G Shi; (II) Administrative support: None; (III) Provision of study materials or patients: H Yu; (IV) Collection and assembly of data: H Yu; (V) Data analysis and interpretation: H Yu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Guangyue Shi. Department of Medical Oncology, the Affiliated Tumor Hospital, Harbin Medical University, Harbin 150001, China. Email: sgy2012@126.com.

Background: Chemotherapy has improved the survival of hepatocellular carcinoma (HCC) patients, but the underlying mechanisms are still not fully understood. MicroRNAs (miRNAs) are critical regulators in carcinogenesis and involved in response to cancer therapy. However, the correlation between miRNAs and chemotherapy is not well-established, and the detailed mechanisms and responsive targets remain unclear. Here, we investigated the function and mechanism of miR-210 in HCC chemotherapy with cisplatin.

Methods: This study involved samples from patients after HCC surgery, including tumor and non-tumor liver tissues. Total RNA was extracted from the fresh tissue samples and the levels of miR-210 were assessed by qRT-PCR analysis. Cisplatin treatment was performed in HepG2 and PLC cell lines, and ephrin A3 (EFNA3) levels were determined by Western blotting.

Results: We observed that miR-210 expression was up-regulated in HCC tissues and correlated with HCC progression. Notably, HCC patients underwent recurrences after chemotherapy showed high levels of miR-210 expression in tumors, indicating that miR-210 might be involved in regulating the chemotherapeutic efficacy. We also demonstrated that cisplatin treatment decreased the expression of miR-210 and increased the expression of miR-210 target EFNA3 in HCC cells. Moreover, miR-210 overexpression prevented the effects of cisplatin and rescued HCC cell growth, and miR-210 inhibition contributed to improved chemosensitivity of cisplatin in HCC cells.

Conclusions: Our findings defined a novel mechanism underlying the efficacious effects of cisplatin chemotherapy in HCC, and miR-210-induced EFNA3 signaling might be a potential target of cisplatin in HCC treatment.

Keywords: MicroRNA-210 (miRNA-210); ephrin A3 (EFNA3); cisplatin; hepatocellular carcinoma (HCC); chemotherapy; recurrence

Submitted Aug 27, 2018. Accepted for publication Mar 08, 2019.
doi: 10.21037/tcr.2019.03.26
View this article at: http://dx.doi.org/10.21037/tcr.2019.03.26

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent type of cancer affecting the global population, with mortality rate ranking third in the world (1,2). Although successful local therapies, such as surgery or transcatheter arterial chemoembolization, have been adopted after primary diagnosis through several effective biomarkers like AFP, patients with HCC still develop a high rate of recurrence due to local invasion and intrahepatic metastasis (3).

Conventional therapies using cytotoxic agents showed low response rate (4). Hepatic arterial infusion chemotherapy (HAIC) is an option for patients with HCC, which can maximize drug concentrations in the liver while keeping systemic exposure low (5).

MicroRNAs (miRNAs) are small, 20- to 24-nucleotide and non-coding RNAs found in diverse organisms and have a broad impact on gene expression through post-transcription suppression or translational repression (6,7). Previous studies indicated that deregulation of miRNAs was common in HCC tumorigenesis (8-11). For instance, miR-199a/b-3p is consistently decreased in HCC, and its decrement significantly correlates with poor survival of HCC patients (12). Another study showed that microRNA-125a-5p was significantly downregulated in 80% HCC tissues and accompanied with upregulation of several oncogenes, such as sirtuin-7, matrix metalloproteinase-11, and c-Raf (13). Recent reports also indicated specific miRNAs as prognostic factors for chemotherapy in several solid cancers (14,15). These findings suggest that miRNAs not only act as oncomiRs or tumor suppressive miRNAs but function as chemotherapeutic targets. miR-210 is involved in diverse biological and pathological processes (16,17). For instance, miR-210 modulates endothelial cell response to hypoxia by targeting ephrin A3 (EFNA3), and miR-210 can inhibit oxygen-glucose deprivation through suppression of the apoptosis of PC12 cells (18,19). The level of serum miR-210 is correlated with hepatitis B virus infection in the liver (20,21). However, the functions of miR-210 in HCC and chemotherapy remain unknown.

In this study, we showed that miR-210 expression was upregulated in HCC tissues and correlated with recurrence of HCC patients who received chemotherapy. We also demonstrated that miR-210-induced EFNA3 signaling was a therapeutic target of cisplatin in HCC cells.

Methods

Tissue samples and cell lines

This study involved samples from patients after HCC surgery, including tumor and non-tumor liver tissues. The patient characteristics can be seen in *Table S1*. RNA of non-cancer liver samples from at least 3 donors constituted the total RNA pool, and the detailed information for each donor was obtained from Ambion Inc. This study was approved by the ethical board of the Affiliated Tumor Hospital of Harbin Medical University.

Huh7, Bel402, and SMMC7721 cells were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences. HepG2, Hep3b, and PLC cells were obtained from the American Type Culture Collection. All the cells were cultured in DMEM (Hyclone) supplied with 10% fetal bovine serum (Hyclone) at 37 ℃ in 5% CO_2.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the fresh tissue samples and cultured cells with TRIzol (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis was performed to assess the levels of miR-210 by TaqMan probes (Invitrogen, Carlsbad, CA, USA) according to the manufacturer. Endogenous U6 snRNA was used as a control. Relative expression of miR-210 was calculated by the equation $2^{-\Delta\Delta Ct}$ ($\Delta CT = CT_{miR\text{-}210} - CT_{U6}$). The value of the relative expression ratio <1.0 was considered as low expression in the tumor relative to the non-tumorous control, whereas others were considered as high expression. Primers for qRT-PCR are shown in *Table S2*.

Constructs, reagents

The 3'-UTR of the human *EFNA3* mRNA was cloned in pRL-TK (Promega). Mutation of the targeted sequence was created by using a QuickChange Site-Directed Mutagenesis kit (Stratagene). Sequences of primers are shown in *Table S2*. miR-210 and control miRNA mimic/inhibitor were purchased from Dharmcon Inc. Cisplatin was obtained from Hospira Australia Pty Ltd.

Luciferase assay

HepG2 and PLC/PRF/5 cells were seeded in 24-well plates (1×10^5 cells per well). When the cells grew to a density of 70% confluence, they were transfected with pRL-TK luciferase reporter plasmid (50 ng per well), pGL3-control firefly luciferase plasmid (10 ng per well). Effectene (QIAGEN) was used to carry out the transfections. Passive lysis buffer (Promega) was used to prepare the cell lysates 48 h after transfection, and the Dual-Luciferase Reporter Assay Kit (Promega) was used to measure luciferase activities.

Cell proliferation and apoptosis assay.

CCK-8 (DOJINDO) assay was performed to measure the

cellular proliferation rate of hepatocarcinoma cells. HepG2 cells were subjected to apoptosis assay. Forty-eight hours after transfection with miR-210 mimic/scramble, the cells were subjected to apoptosis analysis using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer by the FACS Calibur Flow Cytometer (Becton Dickinson).

Western blot

HepG2 and PLC cells were lysed using M-PER (Pierce). Supernatants were collected by centrifugation. Proteins were resolved on Tris-HCl gradient gel (Bio-Rad) and transferred to nitrocellulose membrane. After room temperature blocking (5% BSA) for 1 hour, the membranes were incubated overnight at 4 ℃ with antibodies for EFNA3 (Cell Signaling Technology), and GAPDH (Abcam) according to the manufacturer. After incubated with HRP-conjugated secondary antibodies from Santa Cruz (CA, USA) for 2 hours, the membranes were visualized using the ECL-Plus kit (Amersham Biosciences).

Statistical analysis

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test (two-tailed) was performed to compare 2 groups unless otherwise indicated (χ^2 test), and one-way analysis of variance was performed to analyze 3 groups' data. Correlations between miRNA expression and clinicopathological features were analyzed, non-parametric tests such as the Mann-Whitney U-test and the Kruskal-Wallis test were performed for differences between 2 groups and between 3 or more groups respectively. Each experiment was repeated at least 3 times. P values <0.05 were considered statistically significant.

Results

miR-210 is a prognostic factor of HCC chemotherapy

To elucidate the role of miR-210 in HCC chemotherapy, we first determined the correlation between miR-210 expression and HCC progression using the entire TCGA HCC cohort (372 patients). Although no significant difference in the expression of miR-210 was observed between the HCC tissues and the matched normal controls (*Figure 1A*), we noticed that miR-210 upregulation was significantly associated with a more advanced tumor phenotype (P<0.001) (*Figure 1B,C*) and a more extensive vascular invasion (P=0.011) (*Figure 1D*). Kaplan-Meier survival analysis demonstrated that lower miR-210 levels in patients were correlated with longer overall survival (OS) and disease-free survival (DFS) (*Figure 1E,F*). We also performed qRT-PCR analysis to determine the expression of miR-210 in a total of 45 pairs of fresh HCC tissue samples and adjacent non-cancer tissues. The results showed that miR-210 expression was significantly upregulated in HCC tissues compared with the matched adjacent non-cancer liver tissues (here denoted as normal control) (*Figure 1G*). The correlation between miR-210 expression and the TNM stage of HCC patients was further explored, we found that miR-210 upregulation was associated with advanced tumor stage (P<0.05) (*Figure 1H*). Notably, we explored whether better chemotherapy clinical potency was associated with lower miR-210 levels. Follow-up study was further performed within 45 cases of HCC patients underwent chemotherapy treatment after resection, and we divided these patients into 2 groups according to recurrence rate 3 years after resection. We found that HCC patients underwent recurrence had a higher miRNA-210 expression in the tumors (*Figure 1I*). These results indicate that miR-210 is involved in HCC development and may serve as a prognostic biomarker for HCC patients receiving chemotherapy.

miR-210 inhibition contributes to improved chemosensitivity of cisplatin in HCC cells

As mentioned above, HCC patients with low levels of miR-210 in their tumors represented a high response rate to cisplatin chemotherapy. Therefore, we further performed experiments to validate this finding in HCC cells (*Figure 2*). We knocked down the expression of miR-210 in HepG2 cells by transfection with a specific inhibitor of miR-210 (Anti-210) (*Figure 2B*). And then these cells were treated with cisplatin after the IC50 value was determined (34.9 mg/L). The following cell growth analysis revealed that miR-210 inhibition led to decreased cell proliferation in HepG2 cells (*Figure 2C*). We further performed apoptosis assay and observed that miR-210 inhibition promoted cisplatin-induced apoptosis in HepG2 cells (*Figure 2D,E*). Together, these data indicate that miR-210 suppression contributes to the improved efficacy of cisplatin in HCC cells.

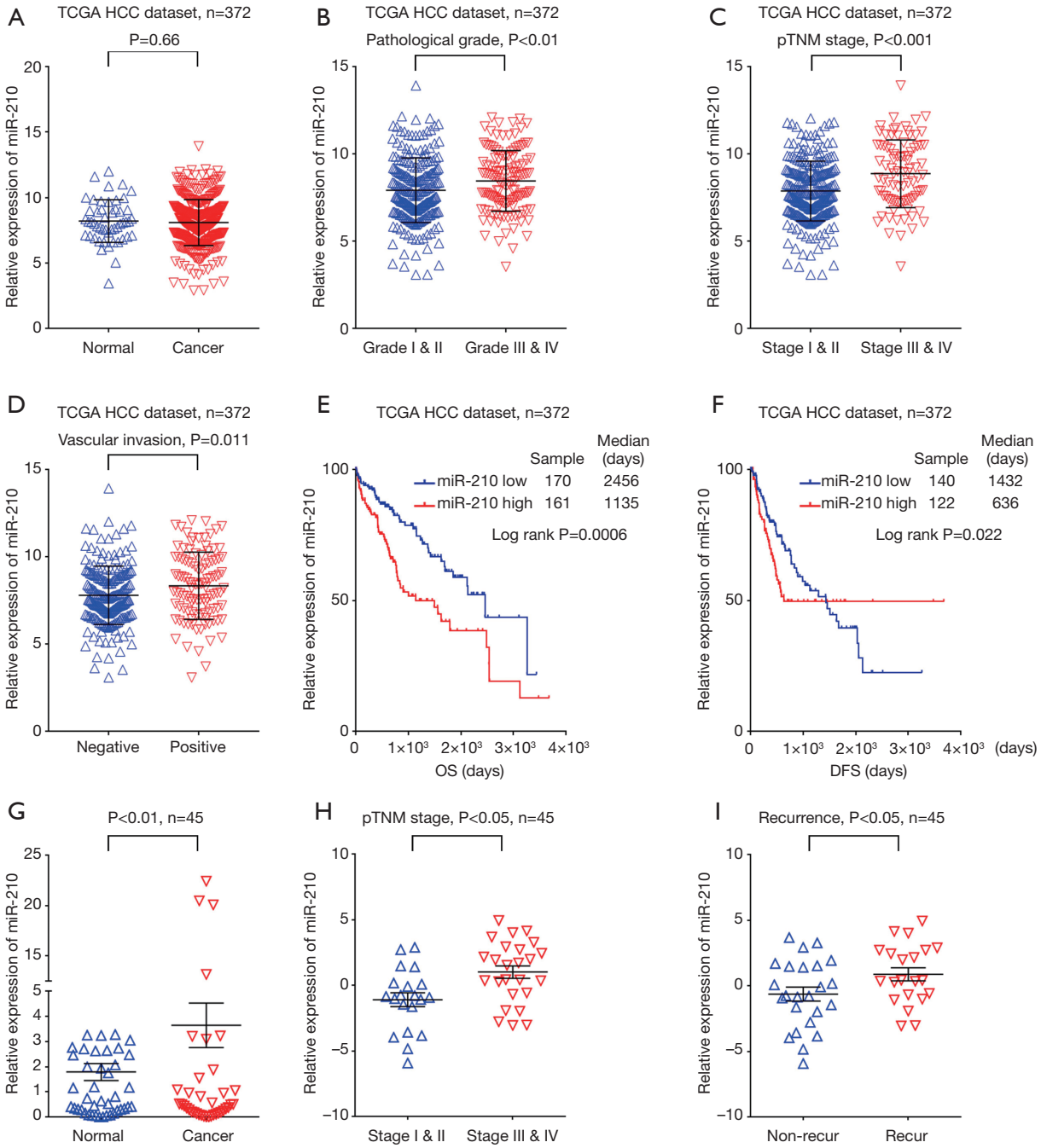

Figure 1 miR-210 is a prognostic factor for HCC chemotherapy. (A) The expression of miR-210 between the HCC tissues and the matched normal controls in 372 TCGA HCC cohort; (B,C) HCCs with high levels of miR-210 represented a more aggressive tumor phenotype; (D) HCCs with high levels of miR-210 represented a more extensive vascular invasion; (E,F) HCC patients with high levels of miR-210 represented shorter OS and DFS; (G) miR-210 expression was upregulated in our 45 HCC tissue samples compared with the matched normal liver tissues examined by using qRT-PCR analyses; (H) miR-210 upregulation was associated with a more aggressive tumor phenotype in our 45 HCC samples; (I) HCC patients with high levels of miR-210 tended to recur after chemotherapy. OS, overall survival; DFS, disease-free survival; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

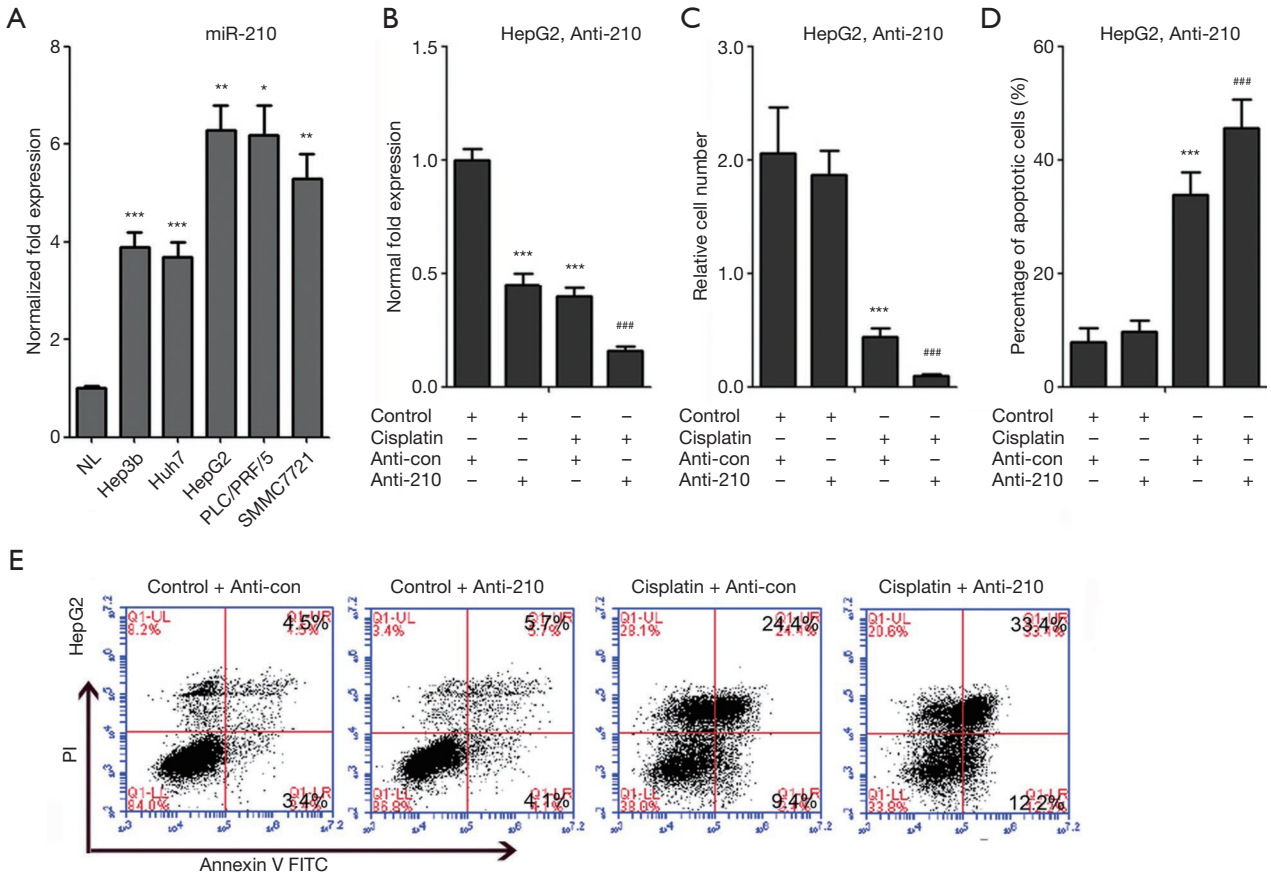

Figure 2 miR-210 expression is downregulated in HCC cells with cisplatin treatment. (A) qRT-PCR analysis was conducted to quantify the miR-210 level in Hep3b, Huh7, HepG2, PLC/PRF/5, and SMMC7721 cells. Data were normalized to the level of NL. *P<0.05, **P<0.01, ***P<0.001 *vs.* NL. (B) qRT-PCR analysis was conducted to determine the expression of miR-210 in HepG2 cells treated with control (PBS) or cisplatin in combination with Anti-con or Anti-210. ***P<0.001 *vs.* Control + Anti-con, ###P<0.001 *vs.* cisplatin + Anti-con. (C) CCK-8 assay was used to determine the cell growth of HepG2 cells in 24 h intervals up to 96 h and the values of 48 h were shown. ***P<0.001 *vs.* Control + Anti-con, ###P<0.001 *vs.* cisplatin + Anti-con. (D,E) Apoptosis assay was employed in HepG2 cells, which showed that miR-210 inhibition led to increased apoptotic cells upon cisplatin treatment. Percentage of apoptotic cells was shown in the panel D. ***P<0.001 *vs.* Control + Anti-con, ###P<0.001 *vs.* cisplatin + Anti-con. NL, normal liver tissue; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR; EFNA3, Ephrin A3.

EFNA3 is a direct target of miR-210 in HCC cells

The best way to elucidate the function of miR-210 is to identify its direct targets. EFNA3 was validated as a miR-210 effector in peripheral nerve sheath tumor cells (22). In this study, we further confirmed this finding in HCC cells. We performed dual-luciferase reporter assay and observed that endogenous miR-210 inhibited the luciferase activity of wild-type *EFNA3* 3'-UTR when compared with mutant *EFNA3* 3'-UTR in HepG2 and PLC/PRF/5 cells (*Figure 3A*). Because miR-210 expression was significantly upregulation in advanced HCC tissues and cell lines, we next knocked down the expression of miR-210 in HCC cells to investigate its effect on the expression of EFNA3. HepG2 and PLC/PRF/5 cells were transfected with a miR-210 inhibitor, and qRT-PCR analysis revealed that miR-210 expression was decreased upon Anti-210 treatment (*Figure 3B*). Remarkedly, our western blot analysis indicated EFNA3 protein level was upregulated by miR-210 inhibition in HepG2 and PLC/PRF/5 cells (*Figure 3C*). Altogether, these data indicate EFNA3 is the target of miR-210 in HCC cells.

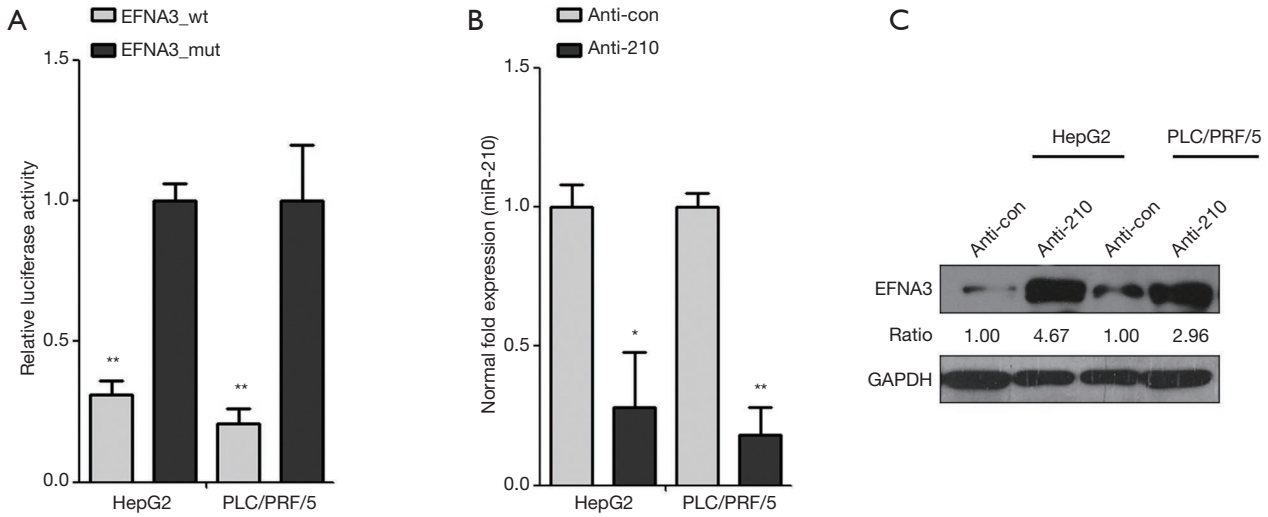

Figure 3 EFNA3 is a direct target of miR-210 in HCC cells. (A) Luciferase gene reporter assay revealed that endogenous miR-210 inhibited the luciferase activity of wild-type EFNA3 3'-UTR reporter whereas mutant the matched seed sequence abolished the interaction in HepG2 and PLC/PRF/5 cells. **P<0.01 *vs.* EFNA3-wt. (B) Anti-210 treatment led to decreased miR-210 levels in HepG2 and PLC/PRF/5 cells shown by qRT-PCR analysis. *P<0.05 *vs.* Anti-con, **P<0.01 *vs.* EFNA3-wt. (C) Western blot was performed to determine the protein levels of EFNA3 in HepG2 and PLC/PRF/5 cells with miR-210 inhibition. GAPDH serves as loading control. EFNA3, ephrin A3; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

Cisplatin exhibits clinical efficacy through miR-210-induced EFNA3 signaling in HCC cells

We next investigated whether cisplatin held efficacious effects on HCC treatment through miR-210 induced signaling. Firstly, the expression of miR-210 was determined in several HCC cell lines, including Hep3b, Huh7, HepG2, PLC/PRF/5, and SMMC7721. The results revealed that miR-210 expression was increased in these cells compared with primary human hepatocytes (NL) (*Figure 2A*). Next, the above cells were treated with cisplatin, and qRT-PCR analysis showed that miR-210 expression was downregulated by cisplatin in a dose-dependent manner (*Figure 4A*). The IC50 values in HepG2 and PLC/PRF/5 cells were estimated, and then we treated these two cells with cisplatin (HepG2, 34.9 mg/L; PLC, 21.1 mg/L). qRT-PCR analysis showed that miR-210 expression was significantly downregulated in HepG2 and PLC/PRF/5 cells upon cisplatin treatment (*Figure 4B,C*). Because cisplatin treatment led to a decrease of miR-210 levels and EFNA3 is a direct functional target of miR-210 in HCC cells as stated above, EFNA3 protein levels might be increased in HCC cells with cisplatin treatment. Western blot analysis confirmed this hypothesis (*Figure 4D*). Altogether, these findings indicate that miR-210-mediated EFNA3 pathway is a potential chemotherapeutic target of cisplatin in HCC cells.

miR-210 overexpression prevents the suppression of cisplatin treatment on HCC cell growth

Given the relationship between miR-210 and cisplatin efficacy, we further performed a rescue assay to elucidate the role of miR-210 in HCC chemotherapy with cisplatin. To this end, miR-210 mimic was transfected into HepG2 cells following cisplatin treatment to re-modify the levels of miR-210 and its target. qRT-PCR analysis showed that cisplatin treatment led to a decrease in miR-210 expression and this downregulation was partially rescued by miR-210 overexpression in HepG2 cells (*Figure 5A*). In addition, the cell proliferation assay demonstrated that miR-210 overexpression repressed cisplatin-induced proliferation arrest of HepG2 cells (*Figure 5B*). We also performed apoptosis analysis in HepG2 cells and observed that miR-210 overexpression repressed apoptosis induced by cisplatin (*Figure 5C,D*). We also observed that EFNA3 protein was increased after cisplatin treatment and decreased with miR-210 overexpression (*Figure 5E*). In summary, these findings suggest that cisplatin holds clinical benefits for HCC therapy at least partly through regulating miR-210-induced EFNA3 signaling.

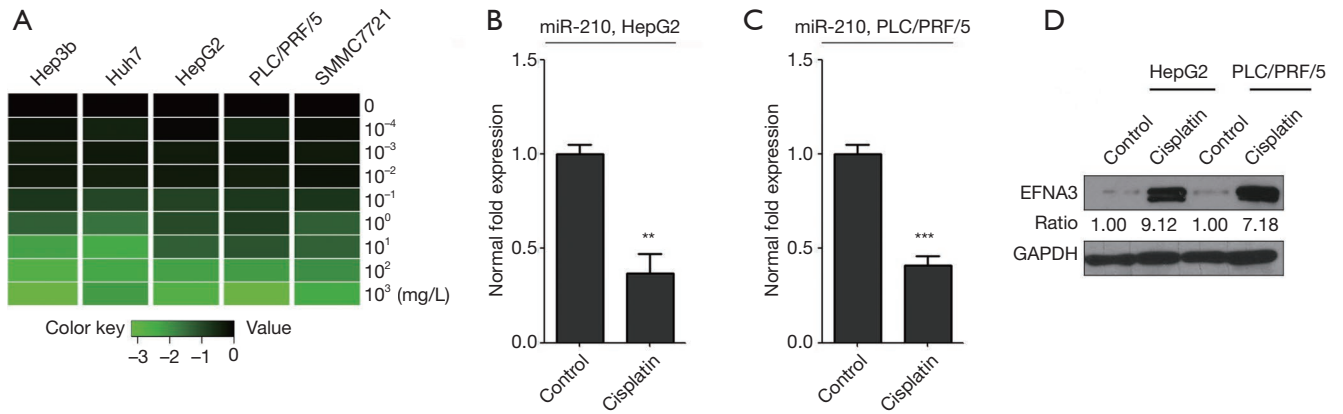

Figure 4 miR-210 inhibition leads to improved efficacy of cisplatin in HCC cells. (A) qRT-PCR analysis was conducted to determine the expression of miR-210 in several HCC cells with cisplatin on different concentration (0–10^3 mg/L). (B,C) qRT-PCR analysis of miR-210 expression in HepG2 (B) and PLC/PRF/5 (C) cells treated with Control (PBS) and cisplatin. Each assay was run in triplicate. **P<0.01, ***P<0.001 *vs.* Control. (D) Immunoblots was conducted to determine the protein levels of EFNA3 in HepG2 and PLC/PRF/5 cells treated with cisplatin. GAPDH serves as loading control. HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

Discussion

In the present work, we identified miR-210 as a miRNA involved in chemosensitivity of HCC. miR-210 was overexpressed in human advanced HCC and miR210 overexpression predicts higher disease stage, poor overall and disease-free survival as well as high incidence of recurrence after chemotherapy. We found that miR-210 could be inhibited by the drug cisplatin, and miR-210-mediated the effects of cisplatin by targeting EFNA3.

HCC is a kind of life-threatening solid cancer and chemotherapy is a critical therapeutic strategy for this disease. Cisplatin is a clinical drug for treatment of human HCC (23). However, the mechanisms underlying the cisplatin-mediated inhibition of HCC and the chemoresistance are not fully understood.

Increasing evidence clearly indicated that deregulation of miRNAs was involved in tumorigenesis and progression. In this pathological process, specific miRNAs function as oncomiRs or tumor suppressive miRNAs via their direct targets. Recent studies further demonstrated that miRNA expression signature served as prognostic biomarkers for cancer patients (24-26). However, the role of specific miRNAs, such as miR-210, in the chemotherapy or chemoresistance was not defined. Our data showed that miR-210 was a potential biomarker to predict the prognosis of HCC patients underwent chemotherapy according to the finding that HCC patients with high miR-210 levels in their tumors tended to recur.

We further observed that the expression of miR-210 was inhibited in HCC cells with cisplatin treatment, suggesting miR-210-induced signaling might be a potential target of cisplatin in HCC. We further confirmed this data by determining a direct bona fide effector of miR-210 in cisplatin-treated HCC cells, and we noticed that cisplatin-induced increased EFNA3 protein levels via miR-210. A rescue assay was conducted to clearly demonstrate the role of miR-210 in cisplatin exhibiting its suppressive impact on HCC cell growth, which revealed that miR-210 overexpression led to a significant alteration of cisplatin-induced cell growth arrest. These findings implicated that the miR-210-EFNA3 signaling is critically involved in the anti-tumor effect of cisplatin in HCC cells. Further *in vivo* growth or PDX experiments will be important to provide a full profile of miR-210-EFNA3 signaling in cisplatin efficiency in human HCC.

Conclusions

Our data provide the evidence that miR-210 is an effective biomarker for predicting the efficacy of HCC patients receiving chemotherapy. Targeting miR-210-induced signaling might be a novel strategy to improve the prognosis of HCC patients treated with cisplatin.

Acknowledgments

Funding: None.

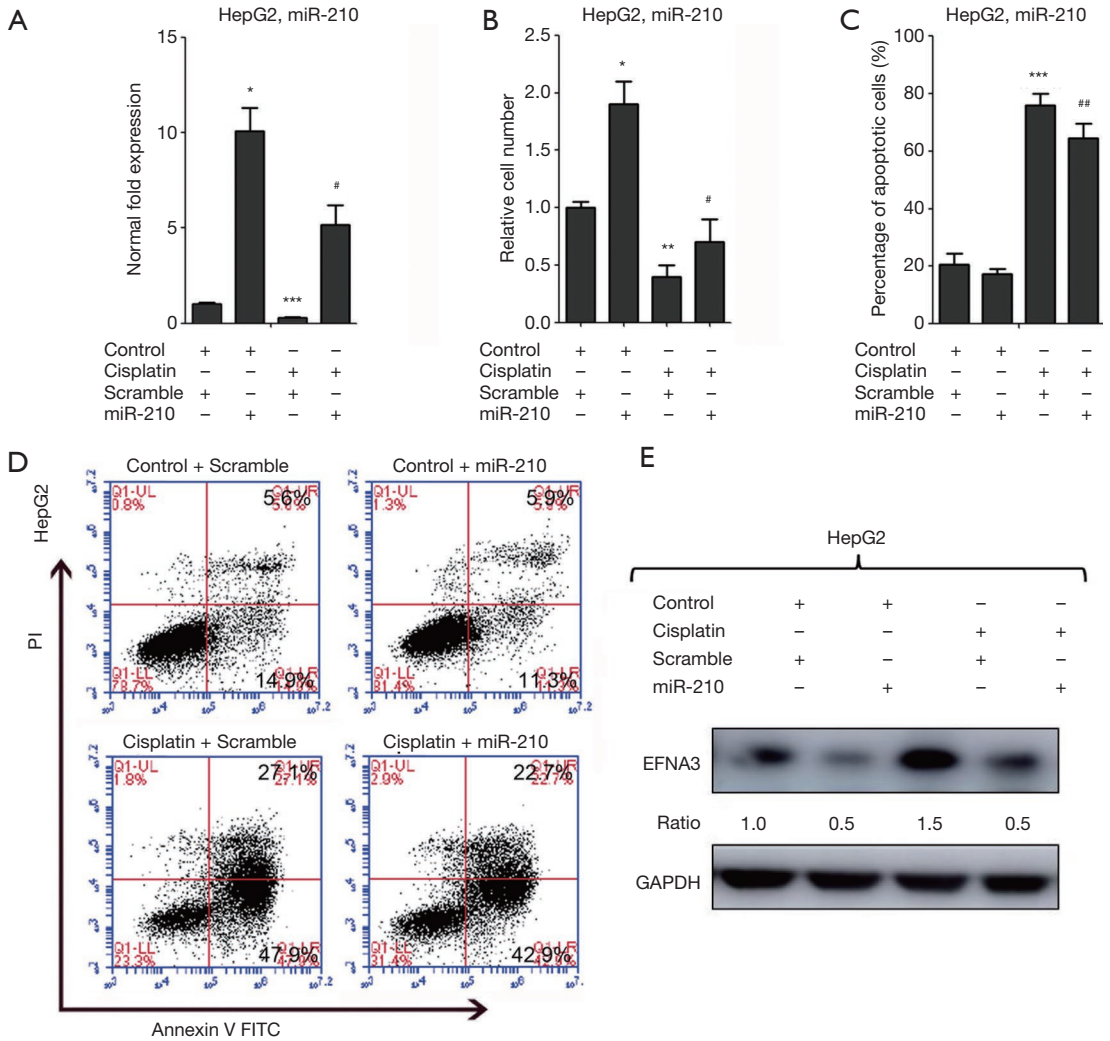

Figure 5 miR-210 overexpression leads to dampened cisplatin inhibitory impact. (A) The expression of miR-210 was evaluated in HepG2 cells treated with cisplatin with or without miR-210 overexpression by using qRT-PCR analysis. *P<0.05, ***P<0.001 *vs.* Control + Scarmble. #P<0.05 *vs.* Cisplatin + Scarmble. (B) Cell growth was determined in HepG2 cells with a different combination of cisplatin and miR-210 mimic by using CCK-8 assay. *P<0.05, **P<0.01 *vs.* Control + Scarmble. #P<0.05 *vs.* Cisplatin + Scarmble. (C,D) Apoptosis assay was employed in the rescue experiments conducted in HepG2 cells. Percentage of apoptotic cells was shown in the panel C. ***, P<0.001 *vs.* Control + Scarmble. ##P<0.01 *vs.* Cisplatin + Scarmble. (E) Western blot analysis was performed to determine the expression of EFNA3 in HepG2 cells conducted rescue assay. GAPDH serves as loading control. EFNA3, ephrin A3; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.03.26). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was previously approved by the ethical board of the Affiliated Tumor Hospital of Harbin Medical University (EAEC 2015-22) and written informed consent was obtained from all patients.

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Cite this article as: Yu H, Shi G. Cisplatin chemotherapy-induced miRNA-210 signaling inhibits hepatocellular carcinoma cell growth. Transl Cancer Res 2019;8(2):626-634. doi: 10.21037/tcr.2019.03.26

Supplementary

Table S1 Characteristics of TCGA-HCC patients

Characteristics	n [%]
Age, years	
<60	160 [45]
≥60	195 [55]
Gender	
Male	243 [68]
Female	113 [32]
TNM stage	
Stage I & II	247 [74]
Stage III & IV	87 [26]
Tumor grade	
Grade 1–2	221 [63]
Grade 3–4	131 [37]
Vascular invasion	
None	196 [65]
Micro	91 [30]
Macro	15 [5]

HCC, hepatocellular carcinoma.

Table S2 Sequences of miR-210, EFNA3 3'-UTR primers

Target ID	Sequence
U6 snRNA RT	5'-AAAATATGGAACGCTTCACGAATTTG-3'
U6 snRNA	
Forward	5'-CTCGCTTCGGCAGCACATATACT-3'
Reverse	5'-ACGCTTCACGAATTTGCGTGTC-3'
miR-210-3p RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCCG-3'
miR-210-3p miRNA	
Forward	5'-CTACAACTGTGCGTGTGACAG-3'
Reverse	5'-GTGCAGGGTCCGAGGT-3'
EFNA3 3'-UTR WT	
Forward	5'-TCTAGACCCGGGCGGCCTTGTG-3'
Reverse	5'-GCGGCCGCGGAGGATAGGTGGG-3'

EFNA3, ephrin A3.