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Original Article

miR-21 is involved in resistance of Huh7 cells to cisplatin by regulating Wnt signaling via PTEN not CK1

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Abstract: It is not well-known how microRNAs (miRNAs) affect cisplatin resistance in hepatocellular carcinoma (HCC). In this study, it was found that miR-21 levels were significantly increased in HCC patients after cisplatin chemotherapy and cisplatin resistant Huh7 (Huh 7 R) cells. miR-21 mimics were used to upregulate miR-21 levels in Huh7 cells while miR-21 inhibitors were used to downregulate miR-21 levels in Huh7-R cells. Relative mRNA levels and protein levels were detected by real-time PCR and Western blotting, respectively. Cell proliferation was tested by CCK-8 assay. The results showed that forced expression of miR-21 decreased cisplatin (10 μg/mL)-induced inhibition of cell proliferation in Huh7 cells. Both mRNA and protein levels of Wnt were elevated remarkably when Huh7 cells were treated with miR-21 mimics. Wnt expression was also significantly increased in Huh7-R cells, compared with Huh7 cells. Expression of PTEN, a negative regulator of Wnt signaling, was decreased by miR-21 mimics and its expression was lower in Huh7-R cells. Wnt signaling was activated by PTEN inhibition. PTEN overexpression impaired miR-21 upregulation-induced cell proliferation and inhibition of Wnt signaling decreased the role of miR-21 in cisplatin resistance. In conclusion, miR-21 is involved in regulating resistance of Huh7 cells to cisplatin by activating Wnt signaling via PTEN.

Keywords: Hepatocellular carcinoma, cisplatin, chemoresistance, miR-21, Wnt, PTEN, CK1

Introduction

Primary liver cancer is the second leading cause of cancer deaths, worldwide, and its major histological type is hepatocellular carcinoma (HCC). Incidence of HCC is highest in Asian countries, specifically Eastern and Southeastern Asian countries [1]. Systematic chemotherapy is important for HCC treatment, especially advanced HCC [2]. However, due to unclear underlying mechanisms causing chemoresistance, progress in HCC chemotherapy has been limited. It is necessary and urgent to reveal the cellular and molecular mechanisms for development of chemoresistance.

Cisplatin has been widely used in HCC chemotherapy to inhibit cancer cell DNA replication. Unfortunately, chemoresistance to cisplatin limits its efficacy to a high degree. Many studies have indicated that microRNAs (miRNAs) play crucial roles in the development or regulation of cisplatin chemoresistance. For example, Keremu et al. demonstrated that cisplatin resistance could be overcome in osteosarcoma through miR-199a-modulated inhibition of HIF-1α [3]. Xiong et al. revealed that iASPP promotes epithelial-mesenchymal transition and confers cisplatin resistance in cervical cancer cells through miR-20a-FBXL5/BTG3 signaling [4]. Lin et al. suggested that miR-218 increases sensitivity to cisplatin in esophageal cancer cells *via* upregulating survivin expression [5]. Shi et al. revealed that downregulation of miR-26b induces cisplatin resistance in nasopharyngeal carcinoma by repressing JAG1 [6].

However, how miRNAs affect cisplatin resistance in HCC is not well-known. Previous studies have indicated that downregulation of miR-199a-5p increases cisplatin resistance by activating autophagy in HCC cells [7] and upregulated miR-130a increases cisplatin resistance by regulating RUNX3 and Wnt signaling in HCC cells [8]. This present study investigated whether miR-21 is involved in the regulation of cis-

Table 1. Sequences of the primers

Genes	Sequences (5'→3')
miR-21-F	GTGCAGGGTCCGAGGT
miR-21-R	GCCGCTAGCTTATCAGACTGAGTGT
PTEN-F	GCAGAAAGACTTGAAGGCGTA
PTEN-R	TTGGCGGTGTCATAATGTCT
Wnt-F	GCAAGCGGAACCTGGAAGTC
Wnt-R	GAAGAGATGGCGTACACGAAGG
CK1-F	CACCCACCAGCACATTCCCTA
CK1-R	GGAGCGGCAGAAGTTGAGGTAT

platin resistance in HCC cells and further explored possible mechanisms.

Materials and methods

Samples and cell lines

Blood samples from 19 HCC patients, before or after cisplatin-based combination chemotherapy, between April 2010 and December 2011, were collected. Median age of the patients was 61, ranging from 48 to 75. Protocols used for patients were approved by the Protection of Human Subjects Committee of our hospital and informed consent was obtained from all patients.

Huh7 human hepatoma cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO_2 incubator. Cisplatin-resistant Huh7-R subline was established by continuous exposure to increasing concentrations of cisplatin for more than 12 months [9].

Solution preparation

Cisplatin was dissolved into DMSO, while DKK-1 human (Dickkopf WNT signaling pathway inhibitor 1) was dissolved into PBS. All were then diluted to required concentrations by the cultured medium. All of the reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from blood samples and Huh7 or Huh7-R cells by TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). Reverse transcription (RT) PCR was performed using PrimeScript RT reagent kit (Takara, Dalian, China) and primers (**Table 1**). Real-time PCR was performed, according to protocol of the PrimeScript RT reagent kit, on a real-time PCR system (ViiA 7, ABI, Thermo Fisher, Hudson, NH, USA). ΔCt values were normalized to reference gene levels.

RNA interference

mirVana®miRNA inhibitor for hsa-miR-21-5p (MH10206) and mirVana®miRNA mimic for hsa-miR-21-5p (MC10206) were obtained from Thermo Fisher, as well as their controls. RNA interference was performed according to instructions for Lipofectamine®2000 (Invitrogen, Thermo Fisher).

For PTEN knockdown experiments, cells were transfected with PTEN siRNA (GS5728, Qiagen) [10]. pcDNA-PTEN was constructed to overexpress PTEN by introducing a BamHI-EcoRI fragment containing a PTEN precursor into the same sites in pcDNA3.1.

Cell proliferation assay

Cell proliferation was detected using Cell Counting Kit-8 (Sigma-Aldrich). Huh7 or Huh7-R cells were plated in 96-well plates (4×10^4 cells per well), in triplicate, and cultured in the medium. Cells were treated with corresponding reagents for a period of time and number of cells per well was measured by the absorbance (450 nm) of reduced WST-8.

Western blotting analysis

Protein was extracted from cells using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich). Western blotting analysis was performed, as previously reported, to detect Wnt (ab91226, 1:1000) [11], PTEN (ab31392, 1:1000) [12], and GAPDH. All antibodies were purchased from Abcam (Cambridge, UK).

Statistical analysis

Data are from at least three separate experiments and are shown as mean ± SD. Differences were analyzed using Student's *t*-test (two groups) or one-way analysis of variance (three or more groups), followed by Tukey's post hoc test. α was set as 0.05.

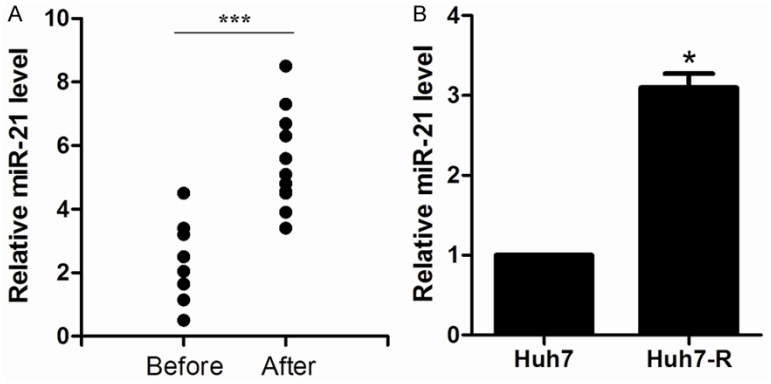

Figure 1. miR-21 levels were significantly increased after cisplatin combination therapy and in cisplatin-resistant Huh7 (Huh7-R) cells. A. miR-21 levels were detected in HCC patient blood samples before and after cisplatin combination therapy (n=19). Total RNA was extracted and miR-21 levels were analyzed in each sample by real-time PCR. U6 was used as a reference for miRNAs. Relative expression was calculated with respect to a tissue. The results were shown as Log ($2^{-\Delta\Delta Ct}$). ***P<0.001. B. Huh7-R cell line was established by continuous exposure to increasing concentrations of cisplatin more than 12 months and miR-21 levels were analyzed by real-time PCR. *P<0.05, *vs* Huh7.

Results

Cisplatin induces upregulation of miR-21 in vivo and in vitro

This study detected expression of miRNAs in HCC patient blood samples, finding that miR-21 levels were significantly increased in HCC patients after cisplatin chemotherapy (**Figure 1A**). A cisplatin-resistant hepatoma cell line (Huh7-R) was then established by continuous exposure of Huh7 cells to cisplatin, also finding that miR-21 levels were significantly higher in Huh7-R cells than in Huh7 cells (**Figure 1B**). These findings indicate that miR-21 might be involved in cisplatin resistance in hepatoma cells.

Forced expression of miR-21 decreases cisplatin-induced inhibition of cell proliferation

To study the role of miR-21 in cisplatin-induced inhibition of cell proliferation, Huh7 cells were treated with cisplatin plus miR-21 mimics while Huh7-R cells were treated with cisplatin plus miR-21 inhibitors. Cell proliferation was detected by CCK-8 assay. miR-21 expression levels were markedly increased after miR-21 mimics treatment (**Figure 2A**). Cisplatin (10 μg/mL) treatment significantly inhibited Huh7 cell proliferation, compared with PBS control, and forced expression of miR-21 decreased cisplatin-induced inhibition of cell proliferation in Huh7 cells (**Figure 2B**). Cisplatin treatment modestly inhibited Huh7-R cell proliferation, whereas miR-21 inhibitors markedly increased cisplatin-induced inhibition of Huh7-R cells (**Figure 2C**). These data suggest that upregulated miR-21 resists cisplatin-induced inhibition of hepatoma cell proliferation.

miR-21 activates Wnt signaling and inhibits PTEN

Wnt signaling, activated in HCC cells, is closely related to cell proliferation [8, 13]. **Figure 3A** shows that miR-21 mimics markedly increased Wnt mRNA levels in Huh7 cells (**Figure 3A** left) and Wnt mRNA levels in Huh7-R cells were significantly higher than in Huh7 cells (**Figure 3A** right). Protein expression of Wnt was consistent with its RNA expression (**Figure 3B**).

PTEN [14] and CK1 [15] are negative regulators of Wnt signaling. This study investigated whether they are targets of miR-21. As shown in **Figure 3C** and **3D**, expression of PTEN was decreased by miR-21 mimics in Huh7 cells and was also lower in Huh7-R cells, compared with Huh7 cells. However, miR-21 could not affect CK1 expression in Huh7 cells (**Figure 3E** left). Expression of CK1 in Huh7-R cells and Huh7 cells was similar (**Figure 3E** right). These data indicate that miR-21 activates Wnt signaling and inhibits PTEN.

Cisplatin-induced upregulation of miR-21 enhances cisplatin resistance by activating Wnt signaling and inhibiting PTEN

In investigating how miR-21 enhances cisplatin resistance in hepatoma cells, Huh7 cells were transiently transfected with Wnt signaling reporter TOPFlash, or the negative control FOPFlash, along with PTEN-siRNA or siRNA control. In cells transfected with siRNA control, Wnt3a treatment was unable to markedly increase TOPFlash activity. However, in cells transfected with PTEN-siRNA, Wnt3a caused a

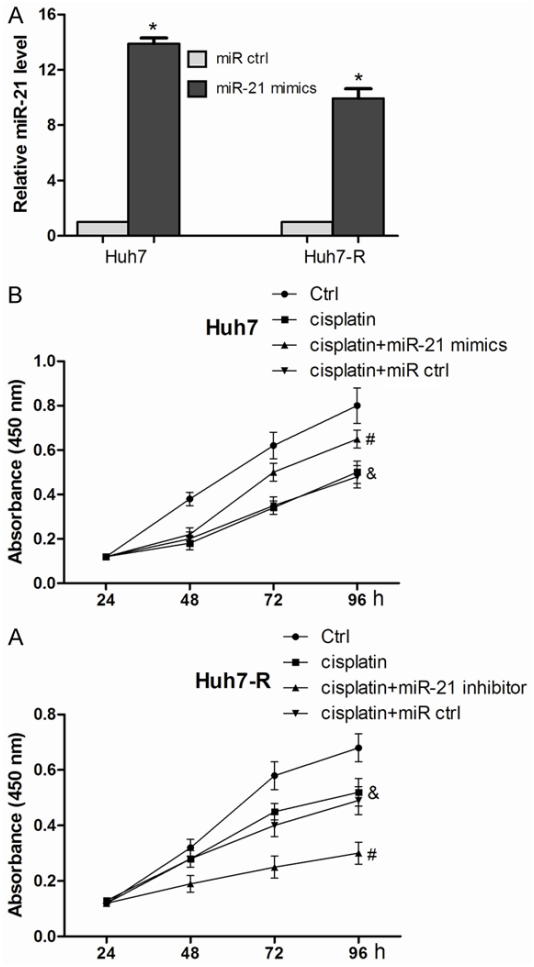

Figure 2. Forced expression of miR-21 decreased cisplatin-induced inhibition of cell proliferation. A. Huh7 or Huh7-R cells were treated with miR-21 mimics and miR-21 expression levels were detected by real-time PCR. B. Huh7 cells were treated with cisplatin (10 μg/mL) or cisplatin plus miR-21 mimics and, at the indicated time points, cell proliferation was detected using CCK-8 assay, according to manufacturer protocol. &P<0.05, cisplatin vs ctrl; #P<0.05, cisplatin+miR-21 mimics vs cisplatin. C. Huh7-R cells were treated with cisplatin or cisplatin plus miR-21 inhibitor, and at the indicated time points, cell proliferation was detected using CCK-8 assay. &P<0.05, cisplatin *vs* Ctrl; #P<0.05, cisplatin+miR-21inhibitor *vs* cisplatin.

significant increase in TOPFlash activity, indicating that Wnt signaling was activated by PTEN inhibition (**Figure 4A**). Furthermore, PTEN overexpression impaired miR-21 upregulation-induced cell proliferation and inhibition of Wnt signaling decreased the role of miR-21 in cisplatin resistance (**Figure 4B**). These data confirm that upregulation of miR-21 enhances cisplatin resistance by regulating Wnt signaling and PTEN in HCC cells.

Discussion

HCC is a fatal malignancy with increasing incidence all over the world. Systematic chemotherapy plays a crucial role in HCC treatment. Cisplatin has been commonly used in HCC chemotherapy. However, resistance of cancer cells to cisplatin frequently leads to tumor recurrence and metastasis [16]. The underlying mechanisms of cisplatin chemoresistance need to be revealed with the goal of improving efficacy and patient prognosis.

Many studies have been published concerning the role of miRNAs in development of chemoresistance in various malignancies. The authors of this present study, also, have made many efforts in this field, revealing how miR-199a-5p [7] and miR-130a [8] exert regulation in Huh7 cell resistance to cisplatin. It was found, previously, that miR-130a levels were significantly higher in HCC patients after cisplatin combination treatment and cisplatin resistant Huh7 cells. Forced expression of miR-130a enhanced cisplatin resistance in Huh7 cells, while downregulation of miR-130a overcame cisplatin resistance in Huh7-R cells. In this study, it was revealed that miR-21 levels increase in cisplatin-treated HCC patients and Huh7-R cells and that miR-21 can activate Wnt signaling to regulate cell proliferation in Huh7 cells. Forced expression of miR-21 decreases cisplatin-induced inhibition of cell proliferation. The question remains whether miR-21 exerts its regulation in Huh7 cell resistance to cisplatin in the same way as miR-130a does.

This present study further found that miR-21 targets PETN, not CK1, in Huh7 cells. Ma et al. reported that Wnt/β-catenin signaling was hyperactivated in metastatic breast cancer cells expressing miR-301a and mediated miR-301a-induced invasion and metastasis [17]. miR-301a activates and maintains its activation of Wnt/β-catenin signaling by directly targeting PTEN [17]. PTEN is usually thought of as a tumor suppressor [18]. In this study, it was found that forced expression of miR-21 activates Wnt signaling, while PTEN expression was decreased. Meng et al. demonstrated that miR-21 can modulate gene expression directly at the PTEN 3'-UTR in HCC cell lines (SK-HEP-1, SUN-182, HepG2, PLC/PRF-5) [14]. CK1 is known as a negative regulator of Wnt signaling, as it is a kinase for degrading β-catenin

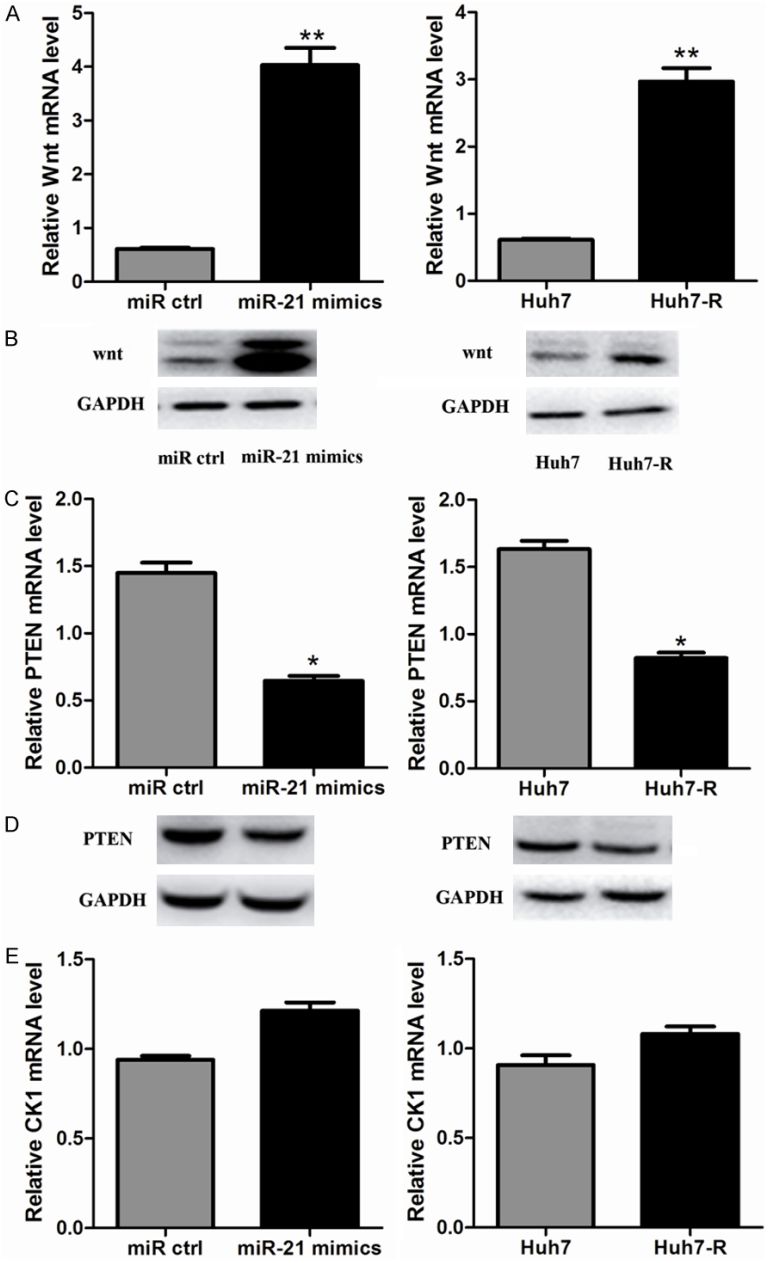

Figure 3. miR-21 activated Wnt signaling and inhibited PTEN. A. Wnt mRNA levels were detected by real-time PCR in Huh7 cells treated with miR-21 mimics or miR ctrl (left), as well as Huh7 cells and Huh7-R cells (right). B. Protein levels of Wnt were detected by Western blotting in Huh7 cells treated with miR-21 mimics or miR ctrl (left), as well as Huh7 cells and Huh7-R cells (right). C. PTEN mRNA levels were detected by real-time PCR in Huh7 cells treated with miR-21 mimics or miR ctrl (left), as well as Huh7 cells and Huh7-R cells (right). D. Protein levels of PTEN were detected by Western blotting in Huh7 cells treated with miR-21 mimics or miR ctrl (left), as well as Huh7 cells and Huh7-R cells (right). E. CK1 mRNA levels were detected by real-time PCR in Huh7 cells treated with miR-21 mimics or miR ctrl (left), as well as Huh7 cells and Huh7-R cells (right). *P<0.05, **P<0.01.

[15]. However, this present study found that forced expression of miR-21 did not affect CK1 expression in Huh7 cells. Thus, it is believed that miR-21 is involved in resistance of Huh7 cells to cisplatin by activating Wnt signaling via PTEN, although the detailed mechanisms require further study.

Many studies have reported that Wnt signaling plays an important role in chemoresistance and that miRNAs are involved in this biological process *via* regulating Wnt signaling pathways [19-23]. It has been previously reported that miR-130a can regulate cisplatin resistance in Huh7-R cells *via* Wnt signaling. In this study, it was also found that miR-21 has a similar function as miR-130a. If these two miRNAs have synergistic effects on Wnt signaling pathways, then how do they coordinate with each other? This question will be investigated further.

In conclusion, miR-21 is involved in regulating Huh7 cell resistance to cisplatin by activating Wnt signaling via PTEN.

Acknowledgements

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Disclosure of conflict of interest

None.

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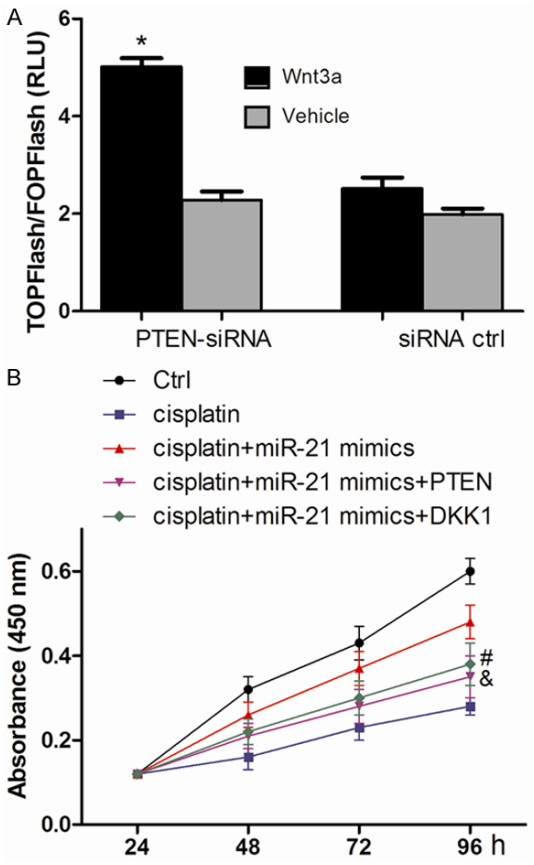

Figure 4. Cisplatin-induced upregulation of miR-21 increased drug resistance by activating Wnt signaling via inhibiting PTEN. A. Luciferase activity of TOPFlash/FOPFlash in Huh7 cells treated with PTEN-siRNA or siRNA control. *P<0.05. B. Huh7 cells were treated with miR-21 or miR-21 plus PTEN or DKK1, and cell proliferation was tested by CCK-8 assay at indicated time points. &P<0.05, cisplatin+miR-21mimics+PTEN vs cisplatin+miR-21 mimics; #P<0.05, cisplatin+miR-21 mimics+DKK1 vs cisplatin+miR-21 mimics.

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