miR-340 Reverses Cisplatin Resistance of Hepatocellular Carcinoma Cell Lines by Targeting Nrf2-dependent Antioxidant Pathway

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Abstract

Many chemotherapeutic agents have been successfully used to treat hepatocellular carcinoma (HCC); however, the development of chemoresistance in liver cancer cells usually results in a relapse and worsening of prognosis. It has been demonstrated that DNA methylation and histone modification play crucial roles in chemotherapy resistance. Currently, extensive research has shown that there is another potential mechanism of gene expression control, which is mediated through the function of short noncoding RNAs, especially for microRNAs (miRNAs), but little is known about their roles in cancer cell drug resistance. In present study, by taking advantage of miRNA effects on the resistance of human hepatocellular carcinoma cells line to cisplatin, it has been demonstrated that miR-340 were significantly downregulated whereas Nrf2 was upregulated in HepG2/CDDP (cisplatin) cells, compared with parental HepG2 cells. Bioinformatics analysis and luciferase assays of Nrf2-3'-untranslated region-based reporter construct indicated that Nrf2 was the direct target gene of miR-340, miR-340 mimics suppressing Nrf2-dependent antioxidant pathway and enhancing the sensitivity of HepG2/CDDP cells to cisplatin. Interestingly, transfection with miR-340 mimics combined with miR-340 inhibitors reactivated the Nrf2 related pathway and restored the resistance of HepG2/CDDP cells to CDDP. Collectively, the results first suggested that lower expression of miR-340 is involved in the development of CDDP resistance in hepatocellular carcinoma cell line, at least partly due to regulating Nrf2-dependent antioxidant pathway.

Keywords: Hepatocellular carcinoma cell lines - miR-340 - Nrf2 - cisplatin - chemoresistance

Introduction

Recent research indicated great progress has been achieved in the molecular characterization of human hepatocellular carcinoma (HCC). Despite the wide use of multiple therapies including chemotherapy toward molecular pathways involved in neoplastic transformation and progression, HCC still confers a poor prognosis when diagnosed at mature stages, mainly due to acquired resistance of HCC to traditionally therapeutic regimens (Hsieh et al., 2011). Comprehensive research show that there are several potential mechanisms of cancer-specific drug resistance, such as genetic hypothesis (random drug-induced mutation) and epigenetic hypothesis (the drug-induced nonmutational alterations of gene function). In addition, recent findings have shown that cancer stem cells and epithelial–mesenchymal transition-type cells also mediate drug resistance (Lavi et al., 2014). However, the underlying mechanisms of acquired resistance to chemotherapeutic agents are still poorly understood. Therefore, it should be addressed that the identification of other avenues is urgently needed.

MicroRNAs, a group of short non-coding RNAs, have been recognized as gene expression modulators by interacting with the 3'-untranslated region (UTR) of mRNAs for translational inhibition or mRNA decay (Gaur et al., 2007). Emerging evidences have shown that microRNAs are implicated in kinds of essential tumor cellular processes, such as cell proliferation, invasion, and apoptosis (Shi et al., 2008). Recent research indicated that knock-down or restored expression of specific miRNAs by miRNA inhibitors or mimics could regulate the acquired drug resistance in cancer cells (Giovannetti et al., 2012, Hu et al., 2014). For instance, it was indicated that the enhanced sensitivity of breast cancer patients to anthracycline-based chemotherapy may associate with the deletion of chromosome 11q, a region containing miR-125b gene (Wang et al., 2013). However, the molecular
mechanisms of miRNAs in the acquisition of drug resistance by cancer cells still remain elusive.

In the present study, HepG2 cell lines resistant to cisplatin were established and miRNA microarray and a quantitative real-time PCR (Q-PCR) were used to detect the differential expressed microRNAs in HepG2/CDDP and HepG2 cells. It has been found that the expression level of miR-340 was significantly down-regulated in HepG2/CDDP cells. Conversely, the expression level of Nrf2 and its downstream genes were significantly greater in HepG2/CDDP cells compared with their parental counterparts. Moreover, bioinformatics analysis and the luciferase assays verified that miR-340 modulates the expression of the Nrf2 gene, an important factor in drug resistance, and this interaction may impair the acquired resistance of cancer cells to chemotherapeutic agents. Prospectively, the current findings suggest that miR-340 restoration could be a potential therapeutic approach to overcome the drugresistance in human hepatocellular carcinoma cancer cells.

**Materials and Methods**

**HCC cell line and cell culture**

The human hepatocellular carcinoma HepG2 cell line and HepG2/CDDP were cultured using Dulbecco’s modified Eagle’s medium (HyClone) containing 10% newborn bovine serum (Gibco BRL, Grand Island, NY) and 100U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. The drugresistant phenotype, HepG2/CDDP was successfully established by stepwise selection at 5 μg/ml after prolonged (>6 months) exposure of HepG2 cells to gradually increasing concentrations of CDDP. The IC₅₀ (inhibitory concentration to produce 50% cell death) values were 22.4 and 8.2 μg/ml for HepG2/CDDP and parental HepG2 cells, respectively. The CDDP-resistant cells were cultured in drug free medium for one week prior to experimentation. Exponentially growing cells were used in all experiments.

**In Vitro drug sensitivity assay**

The proliferation of the cells was calculated by the CCK-8 (Doxjindo, Kumamoto, Japan) assay according to the manufacturer’s instructions. Cells were plated at 5x10⁴ cells/well in 96-multiwell plates. After transfection with RNA oligos for 72 h, optical density were measured to an apoptosis assay. Briefly, 1x10⁴ washed cells were incubated with annexin V/propidium iodide for 15 min at room temperature followed by flow cytometry using two-color fluorescence-activated cell sorting analysis (BD Biosciences).

**miRNA microarrays and data analysis**

Total RNA was extracted from HepG2 and HepG2/CDDP cells using TRizol Reagent (Invitrogen) according to the manufacturer’s instructions and size fractionated (<200 nucleotides) by a mirVana kit (Ambion, Austin, USA) and then labeled with Cy3 and Cy5 fluorescent dye. Dye switching was used to eliminate the dye bias. Pairs of labeled samples were applied for hybridization to dual-channel microarrays on Microfluidics chip. Raw data were normalized and adjusted using the GenePix Pro 4.0 software. The Student’s t-test analysis was employed for HepG2 and HepG2/CDDP samples, and miRNA with p values<0.05 were chosen for cluster analysis using a hierarchical method and average linkage and Euclidean distance metric.

**RNA oligos and transfection**

Cells in the exponential growth phase were seeded in 6-well plates (5x10⁴ cells/well). After 24h, HepG2/CDDP cells were transfected with 100nM of the miR-340 mimics alone or miR-340 mimics combined with inhibitors or negative control, while 100nM of the miR-340 inhibitor or 100nM miRNA inhibitor control was transferred into HepG2 cells, using lipofectamine 2000 (Invitrogen, Long Island, NY, USA) according to the manufacturer’s protocol. All the RNA oligos were purchased from Shanghai GenePharma Company (Shanghai, China). The sequences are miR340 mimic, F: 5’-UUUAAGCAAGUGACUAGUUGU-3’, R: 5’-UCAGCUCAUGCGUCGUUUAUAAUAAU-3’, miR-340 inhibitor : 5’-AAAGCUAGCUCAUGCGUCGUUUAUAAU-3’, negative control for miRNA mimic, F: 5’-UCAGCUCAUGCGUCGUUUAUAAUAAU-3’, R:5’-ACUGACGACGACGCUAGATT-3’. Cells were collected for further analysis after 48h transfection.

**Transient transfection with Nrf2 plasmid for rescue experiments and Celluar apoptosis assay**

Cells were seeded at 1.5x10⁴ cells/well into 6-well plates. The following day, HepG2/CDDP cells were transfected with 100 nM miR-340 mimics alone or 100 nM miR-340 mimics combined 10 ng expression vectors for Nrf2-FLAG (Shanghai Genechem Co.,Ltd.). Lipofectamine 2000 (Invitrogen) was used in the transient transfection experiments. After transfection, cells were incubated with the indicated concentrations of CDDP treatment for 48 h and then were subjected to an apoptosis assay. Briefly, 1x10⁴ washed cells were incubated with annexin V/propidium iodide for 15 min at room temperature followed by flow cytometry using two-color fluorescence-activated cell sorting analysis (BD Biosciences).

**Real-time quantitative PCR (qRT-PCR) Analysis for miRNA**

Expression of mature miRNAs were assayed using SuperTaq Polymerase and EzoOmines™ One-Step qPCR Kit (Biomics Biotechnologies Co., Ltd, Nantong, China) according to the manufacturer’s instructions. Reactions contained EzOmines™ miRNA qPCR Detection Primer Set specific for human miR-125b, miR-141, miR-200b, miR-34a, miR-21, miR-7, miR-199-3a-p, miR27b, let-7, miR-340, miR-103a, miR-205, miR-429, miR-345, miR-28, miR-206, and miR-106a. U6 gene was used for detecting the gene amplification and normalizing the each sample. The mRNA levels of Nrf2 pathway related
genes were detected by SYBR RT-PCR kits detection system (Toyobo Japan) with the following cycles: 95°C for 1 min; 95°C for 15 s; 58°C for 20 s and 72°C for 20 s (40 cycles); and 72°C for 5 min. Primer sequences were Nrf2, F: 5'-TGAGGTGTTCGCTCAGTT-3'; R: 5'-CTTCTGTCAGTTGCTCTGG-3', NQO1: F: 5'-GGTTCAGGCGATGTTCTACGG-3'; R: 5'-GCTGGAGGAGGAGATTGAGCG-3', HO-1: F: 5'-CTGGAGGAGGAGATTGAGCG-3'; R: 5'-ATGGCTGGTGTGTAGGGGAT-3', β-actin, F: 5'-CCACACCTTCTACATAGACG-3'; R: 5'-GGTCTCACAACATGATCTGGG-3'. The data were collected and calculated using the comparative Ct method and normalized to β-actin.

**Western blot analysis**

Cells were seeded in 6-well plates (5×10^5 cells/well). After the transfection of RNA oligos for 72 h, cells were harvested and homogenized with lysis buffer. Total protein was subjected to 12% SDS-PAGE, subsequently transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Western blotting was performed as described previously. The primary antibodies against Nrf2, NQO1, HO-1 and β-actin were purchased from Santa Cruz, CA. Protein levels were quantified using Quantity One software (Bio-Rad Life Science, Shanghai, China) to obtain the ratio of the optical density of the target protein to that of β-actin.

**Vector construction and dual-luciferase reporter assay**

3′-untranslated region (UTR) of Nrf2 which was predicted to interact with miR-340 or a mutant sequence with the putative target sites were inserted into the KpnI and SacI sites of pGL3 promoter vector (Invitrogen). They were named as pGL3-Nrf2-wild-type and pGL3-Nrf2-mut.

For the luciferase assay, cells seeded at 1.5×10^5 per well in 24-well plates were co-transfected with 50 ng luciferase reporter plasmids containing wild-type or mutated 3′UTR of Nrf2 and 100 nM miR-340 mimics or inhibitor by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol as described, and the pRL-TK vector (Promega) was used as an internal control. The relative luciferase activity was measured after 48 h transfection using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Independent experiments were performed in triplicate.

**Statistical analysis**

Each sample was performed in triplicate. All values were presented as mean ± SD. Statistical significance was analyzed by Student’s t test or one-way ANOVA followed by the Student–Newman–Keuls comparison method using SPSS11.0 software (Chicago, IL). p values less than 0.05 were considered significant. GraphPad Prism 4.0 was used for data analysis.

**Results**

**MiRNA expression profiles in HepG2 and HepG2/CDDP hepatocellular carcinoma cells**

MiRNA microarrays were used to analyze differentially expressed miRNAs in the HepG2 and its drug-resistant HepG2/CDDP variant cell lines. The cluster analysis suggested that the acquired resistance of HepG2/CDDP cells were mediated by differentially expressed levels of miRNAs. It has been found that 17 miRNAs (nine upregulated and eight downregulated) exhibit significant changes in HepG2/CDDP cells by more than 2-fold or less than 0.5-fold compared to their parental cells (Table1). To confirm the data obtained by microarray analysis, qRT-PCR was independently performed to analyze differentially expressed levels of miR-125b, miR-141, miR-200b, miR-34a, miR-21, miR-7, miR-199-3a-p, miR-27b, let-7, miR-340, miR-103a, miR-205, miR-429, miR-345, miR-28, miR-206, miR-106a detected to be Differentially Expressed in HepG2/CDDP Compared with HepG2 Cells by Microarray were Verified by qPCR. The validated results of the 17 miRNAs suggested that the microarray data were consistent with the qPCR results. It is noteworthy that miR-340 is most abundantly downregulated in HepG2/CDDP compared with HepG2 cells. Columns, mean of three independent experiments; Data were shown as mean±SEM; **p<0.01 vs parental.
their parental cell lines among 17 miRNAs.

**MiR-340regulated chemo-sensitivity of hepatocellular carcinoma cell lines**

To investigate the potential role of miR-340 in CDDP resistance of HCC, CCK-8 assay suggested that HepG2/CDDP cells transfected with miR-340 mimics exhibited greatly reduced the IC_{50} value of CDDP compared with the miRNA mimic negative control (NC) transfected cells (Figure 2A-B), while miR-340 inhibitors were transfected into parental HepG2 cells, the IC_{50} value of CDDP was significantly increased in contrast with its NC or blank control (Figure 2A-B). These results indicated that miR-340 might modulate CDDP resistance of hepatocellular carcinoma cell lines.

**Nrf2 was the target gene of miR-340**

Nrf2 pathway is activated in resistant Hepatocellular Carcinoma cell lines: To determine whether activation of Nrf2 signaling pathway is involved in the acquired resistance, westernblotting has been performed in order to measure the endogenous levels of Nrf2 and its downstream targets including NQO1 and HO-1 in both HepG2 and HepG2/CDDP cells. The result indicated that there were markedely elevated protein levels of Nrf2 and Nrf2-regulated antioxidant genes NQO1 and HO-1 in HepG2/CDDP cells compared to their parental counterpart (Figure 3). These results indicate that Nrf2-dependent defensive system is fully activated in HepG2/CDDP cells to acquire chemo-resistance.

Bioinformatic prediction of miR-340 target and luciferase activity assay: Computational analysis revealed that Nrf2 was the target gene of the miR-340 using miRNA databases (TargetScan, Pictar, and MicroRNA). One conservely putative miR-340 binding site was found within the 3’UTR of Nrf2 (Figure 4A). To validate whether Nrf2 is indeed functionally targeted by miR-340, it has been established a luciferase reporter system containing the putative Nrf2-3’-UTR target site for miR-
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Figure 5. The miRNA (A) and protein(B) levels of Nrf2 and its downstream gene in the HepG2/CDDP cells transfected with miR-340 mimics or a combination of miR-340 mimics and inhibitor. Columns, mean of three independent experiments; Data were shown as mean±SEM, *p<0.05, **p<0.01 vs NC group. †p<0.05 vs miR-340 mimics group.

To further confirm these results, HepG2/CDDP cells were co-transfected with 100 nM miR-340 mimics plus Nrf2-FLAG or miR-340 mimics alone, it has been shown that miR-340 mimics downregulated the expression of Nrf2 and elevated the CDDP sensitivity, and a marked increase in apoptosis cells with annexin V staining was detected by flow cytometry in miR-340 mimics transfected cells compared with negative controls. Meanwhile, co-transfecting Nrf2 FLAG group suppressed the CDDP-induced apoptosis in HepG2/CDDP cells and partly restored the CDDP resistance (Figure 6A-C). These results suggested that lower expression of miR-340 was involved in the development of CDDP resistance by blocking CDDP-induced apoptosis.

Collectively, these findings suggested that miR-340 might modulate CDDP resistance of hepatocellular carcinoma cell lines at least in part by repressing Nrf2-dependent pathway.

Discussion

CDDP has been clinically used for the treatment of different tumors including Hepatocellular Carcinoma (Yamaguchi et al., 2013). However, like many other chemotherapeutic agents, the clinical effectiveness of CDDP is low due to the emergence of CDDP resistance to cancers, both acquired and intrinsic, according to previous research. Although several factors have been reported, which might contribute to CDDP resistance, the elucidation of their biological mechanisms remains largely unknown, including whether it has been involved in the transition of chemotherapy-sensitive cancer cells to chemotherapy-resistant cancer cells (Yang et al., 2009).

Previously, well-established role of miRNAs have been identified as having an oncogenic or tumor suppressor-like function mediating cell proliferation, apoptosis, metabolic pathways, and signal transduction (Chen et al., 2014).
Recently, dysregulated miRNAs including miR-27b, miR-127, miR-34a, miR-200c, miR-21, miR-214, miR-125, and miR-206 have been reported to be associated with acquisition of resistance to various chemotherapeutic agents such as to CDDP (Robertson and Yigit, 2014). In the present study, differential miRNA expression profiles were obtained by microarray analysis in HepG2 and HepG2/CDDP cell lines. It has been discovered that miR-340 was the most significantly downregulated in HepG2/CDDP cell lines compared to their parental counterparts. Thus, this study, in the first time, validated the data for miRNA microarray by qRT-PCR and then focused on miR-340 (Figure 1). CCK-8 assay revealed that transfection of the HepG2/CDDP cells with miR-340 mimics resulted in the elevated sensitivity of resistant cells to CDDP (Figure 2B, 6B-C). Previous findings combined with current results demonstrate that miRNAs do play a critical role in cancer drug-resistance and that correction of differentially expressed miRNA may have significant applications in the development of targeted therapeutics for overcoming cancer cell resistance.

In addition, numerous findings have revealed that constitutive activation of Nrf2-dependent pathway is associated with chemoresistance in a variety of solid tumors (Bao et al., 2014). Normally, nuclear factor erythroid-2-related factor 2 (Nrf2), a cytoprotective transcription factor, plays a pivotal role in kinds of cellular defensive resistance to oxidative and electrophile insults (Niture et al., 2010). However, possible epigenetic regulation of Nrf2 expression is in need of further elucidation. It has previously reported that miR-144 targets the 3'-UTR of Nrf2 mRNA and regulate expression of Nrf2 in blood cells, which is associated with sickle cell disease (Sangokoya et al., 2010). However, it remains largely unclear whether Nrf2 regulated miRNAs are involved in the acquisition of chemo-resistance to cancer cells. In the present study, it has shown that protein Nrf2 was upregulated while the miR-340 were downregulated in HepG2/CDDP cells compared with HepG2 cells according to the datas of westernbloting and qRT-PCR (Figure 1.3). Thus, the result has been validated the potential role of miR-340 as regulators of the Nrf2 expression. Initially, Nrf2 was identified as a direct target of miR-340 which contains a conserved site complementary within the Nrf2 mRNA 3'UTR by using TargetScan 5.1 prediction algorithm (Figure 4A). The ability of miR-340 to regulate posttranscriptional level of Nrf2 expression was further validated by luciferase reporter assay experiments. (Figure 4B-C). molecularly, it has also found that miR-340 mimics directly target the Nrf2 gene, and inhibited Nrf2 expression and consequently NQO-1 and HO-1 expression. These effects are eliminated in the miR-340 mimics combined with inhibitors group (Figure 5, 6A). According to previous findings, Nrf2 expression is mainy regulated by Nrf2 interaction with Kelch-like ECH-associated protein 1 (Keap1), which leads to degradation of Nrf2 by the ubiquitin-proteasome pathway (He and Ma, 2010), further studies to assess whether MiR-340 inhibits Nrf2 expression through a Keap1-dependent manner were warranted.

In conclusion, the current study has presented a novel mechanism that miR-340 could potentially enhance the sensitivity of HepG2/CDDP cell lines, at least in part, by suppressing Nrf2 expression (Figure 6). The current study might provide new insights into cancer chemotherapy. Moreover, it should also be considered that the application of combined methods that involve impairing Nrf2-dependent pathways to promote therapeutic outcomes. However, our data derived from cell lines in vitro may not be applied to accurate surrogates for clinical tumors.

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