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Original Article

miR-144 reverses chemoresistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Chemoresistance occurrence is a major cause of treatment failure in HCC. Currently, extensive research has revealed diverse mechanisms for chemoresistance, but the molecular mechanisms underlying the role of miRNAs in resistance to 5-FU are not confirmed in HCC cells. By quantitative real-time polymerase chain reaction (qRT-PCR) analysis, we found that miR-144 was significantly decreased in HCC cell lines. It has been further demonstrated that miR-144 were significantly down-regulated in Bel-7402/5-FU cells compared with parental Bel-7402 cells by qRT-PCR and western blot. The expression of Nrf2 was reversely correlated to that of miR-144 in HCC cells. Moreover, Enhancement of 5-FU-induced cytotoxicity and apoptosis are resulted from the transfection with miR-144 mimics in Bel-7402/5-FU cells. Mechanically, miR-144 promoted nuclear factor erythroid-2-related factor-2 (Nrf2) mRNA degradation by directly targeting the Nrf2 3'untranslated region (3'UTR). In addition, ectopic expression of miR-144 in Bel-7402/5-FU cells reduced the levels of Nrf2 and inhibited the transcription of Nrf2-dependent HO-1 gene, thus contributing to 5-FU sensibilization. Conversely, re-expression of Nrf2 partly attenuated the chemosensibilization of miR-144. Our study showed that miR-144 serves as a potential chemoresistance-reversal agent in hepatocellular carcinoma cells, which is at least partly due to the down-regulation of Nrf2-dependent antioxidant pathway.

Keywords: Hepatocellular carcinoma, miR-144, chemoresistance, Nrf2

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors, especially in sub-Saharan Africa, Southeast Asia, and Eastern China [1]. It causes approximately 600,000 deaths every year. Currently, liver transplantation and surgical resection are potential curative treatments for early HCC; however, most asymptomatic HCC patients are diagnosed at advanced stage [2]. The systemic chemotherapy is not an effective treatment for patients with HCC, and since the high occurrence of intrinsic and acquired drug resistance is mainly responsible for the extremely poor prognosis of HCC [3]. The mechanisms of developing resistance in HCC are poorly understood. Therefore, it is important to understand the molecular mechanisms contributing to chemotherapy resistance in order to develop new therapeutic strategies in overcoming acquired resistance in HCC.

MicroRNAs (miRNAs) are small non-coding RNAs that function as negative gene regulators. miRNAs post-transcriptionally repress complementary target gene expression and can contribute to the regulation of diverse cellular processes, including cell proliferation, differentiation, invasion, and apoptosis [4-6]. Currently, accumulating studies indicate that up- or down-regulation of some miRNAs contributes to chemoresistance in several cancers. In ovarian cancer cell-lines, up-regulation of miR-214 and miR-214 has been reported to enhance cisplatin resistance by inhibiting PTEN and ALK7, respectively [7, 8]. However, to date, the role of miRNAs in resistance to 5-fluorouracil (5-FU) in HCC cells is not fully understood.

Table 1. The primers used in the reactions

Gene		Primer
miR-144	F	TACTGCATCAGGAACTGACTGGA
	R	GTGCAGG GTCCGAGGT
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'
Nrf2	F	5'-ACACGGTCCACAGCTCATC-3'
	R	5'-TGCCTCCAAGTATGTCAATA-3'
GAPDH	F	5'-ACCACAGTCCATGCCATCAC-3'
	R	5'-TCCACCACC CTGTTGCTGTA-3'

Emerging evidence shows that miR-144 is down-regulated in many cancers including HCC [9]. In the present study, we hypothesize that down-regulation of miR-144 might contribute to acquisition of resistance to 5-FU in HCC cells. Nrf2 was further identified as a direct target of miR-144 in HCC cells. Ectopic expression of Nrf2 can partially attenuated chemosensibilization effect of miR-144 in HCC cells.

Materials and methods

HCC tissues, cell line and cell culture

23 patients were chose from January 2011 and December 2013, who received resection of HCC at Tangdu Hospital, Fourth Military Medical University. None of these 23 patients received neoadjuvant or adjuvant chemotherapy before operation. Our study was approved by the ethics committee of the Fourth Military Medical University and written informed consents were obtained from all the patients. The HCC Bel-7402 cell line and 5-FU selected drug-resistant Bel-7402/5-FU cells were obtained from Nanjing Keygen Biotech (Nanjing, China). Bel-7402 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin, and 100 mg/mL streptomycin. Bel-7402/5-FU cells were cultured in the above-mentioned medium with the addition of 20 μg/mL 5-FU until at least 2 weeks before the experiment. Exponentially growing cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The chemoresistance of Bel-7402/5-FU cells had been verified in our previous works [10].

In vitro drug sensitivity assay

Cells were plated at 5×10^3 cells/well in 96-well plates. After transfection with RNA oligos for 72 h, cells were cultured in fresh medium with or without different concentration of 5-FU or CDDP (Tasly Pharmaceutical Co, Tianjin, China) for 48 h, and then a conventional tetrazolium-based (MTT) assay was performed. The IC_{50} value of 5-FU/CDDP was calculated using Prism software (GraphPad Software, La Jolla, CA). Three independent experiments were performed.

RNA oligos and transfection

miR-144 mimics/inhibitors/negative control (NC) were purchased from Shanghai GenePharma Company (GenePharma, Shanghai, China). The sequences of RNA oligos are described as before [11]. Cells in the exponential growth phase were seeded in 6-well plates (5×10^5 cells/well). After 24 h, Bel-7402/5-FU cells were transfected with 100 nM of the miR-144 mimics alone or miR-144 mimics combined with inhibitors or NC, while 100 nM of the miR-144 inhibitor or 100 nM miRNA inhibitor control was transferred into Bel-7402 cells, using lipofectamine 2000 (Invitrogen, Long Island, NY, USA) according to the manufacturer's protocol. Cells were collected for further analysis after 48 h transfection.

Transfection with Nrf2 expression vector for rescue experiments and analysis of cell apoptosis

Cells were seeded at 1.5×10^5 cells/well into 6-well plates. The following day, Bel-7402/5-FU cells were transfected with 100 nM miR-144 mimics alone or 100 nM miR-144 mimics combined 10 ng pcDNA3.1-flag-Nrf2 (GenePharma, Shanghai, China) by Lipofectamine 2000.

For apoptosis analysis, cells were harvested after incubated with the indicated concentrations of 5-FU/CDDP treatment for 48 h, washed in ice cold PBS, and fixed in ice-cold ethanol. Cells were incubated with annexin V/propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room-temperature, and the cells were analyzed using FACS (BD, Franklin Lakes, NJ, USA).

RNA isolation and real-time quantitative PCR

Total RNA was extracted from Bel-7402 and Bel-7402/5-FU cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) or mirVana kit (Ambion, Austin, TX, USA) according to the man-

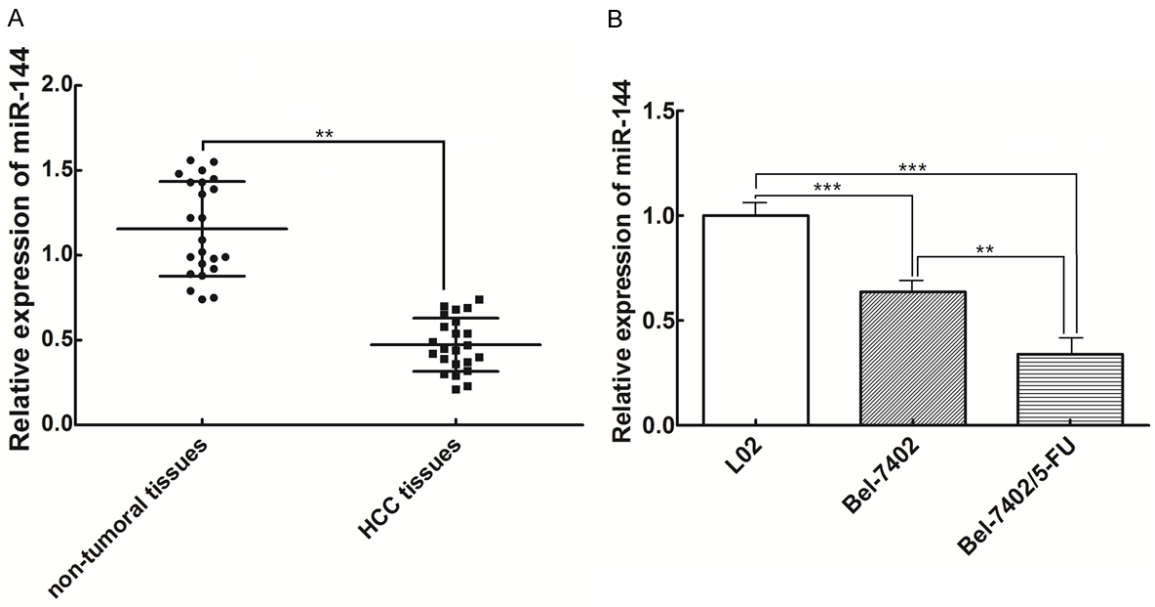


Figure 1. Expression of miR-144 in HCC. miR-144 was decreased in HCC. The expression of miR-144 was measured by qRT-PCR in 23 paired HCC and non-tumor tissues (A) and two HCC cell lines and L02 cells (B). *P<0.05, **P<0.01, ***P<0.001, compared with control.

ufacturer's instructions, followed by reverse transcription. Real-time quantitative PCR (qPCR) detection of genes was performed using SYBR Green Master Mix (ABI, Foster City, CA, USA) by LightCycler System (Roche, Pleasanton, CA, USA). Detection of miR-144 in cell lines was performed using TaqMan microRNA assays. The amplification of miR-144 and Nrf2 were normalized to U6 and GAPDH, respectively, using $2^{-\Delta\Delta Ct}$ method. The primers used in the reactions are listed in **Table 1**. All reactions were run in triplicate and all experiments were run in triplicate.

Protein isolation and 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 10% SDS-PAGE, subsequently transferred onto a PVDF membrane (Millipore, MA, USA). Western blotting was performed as described previously in our studies [10]. The primary antibodies against Nrf2, HO-1 and β-actin were purchased from Santa Cruz, CA. Protein levels were expressed quantitatively by using Quantity One software (Bio-Rad Life Science, Shanghai, China), with protein expression normalized to β-actin.

Vector construction and dual-luciferase reporter assay

Wild type (WT) or Mutant (Mut) 3'-UTR of Nrf2 were cloned into the pGL3-Basic vector (Promega, Madison, WI, USA) respectively, and then constructed pGL3/Nrf2-WT and pGL3/Nrf2-Mut recombinant vector after sequencing. HEK-293 cells cultured in 24-well plate were co-transfected with 100 nM of miR-144 mimic or mimic-NC and pGL3/Nrf2-WT or pGL3/Nrf2-Mut using Lipofectamine 2000 reagent. The luciferase activity was measured after 48 h transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well.

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.

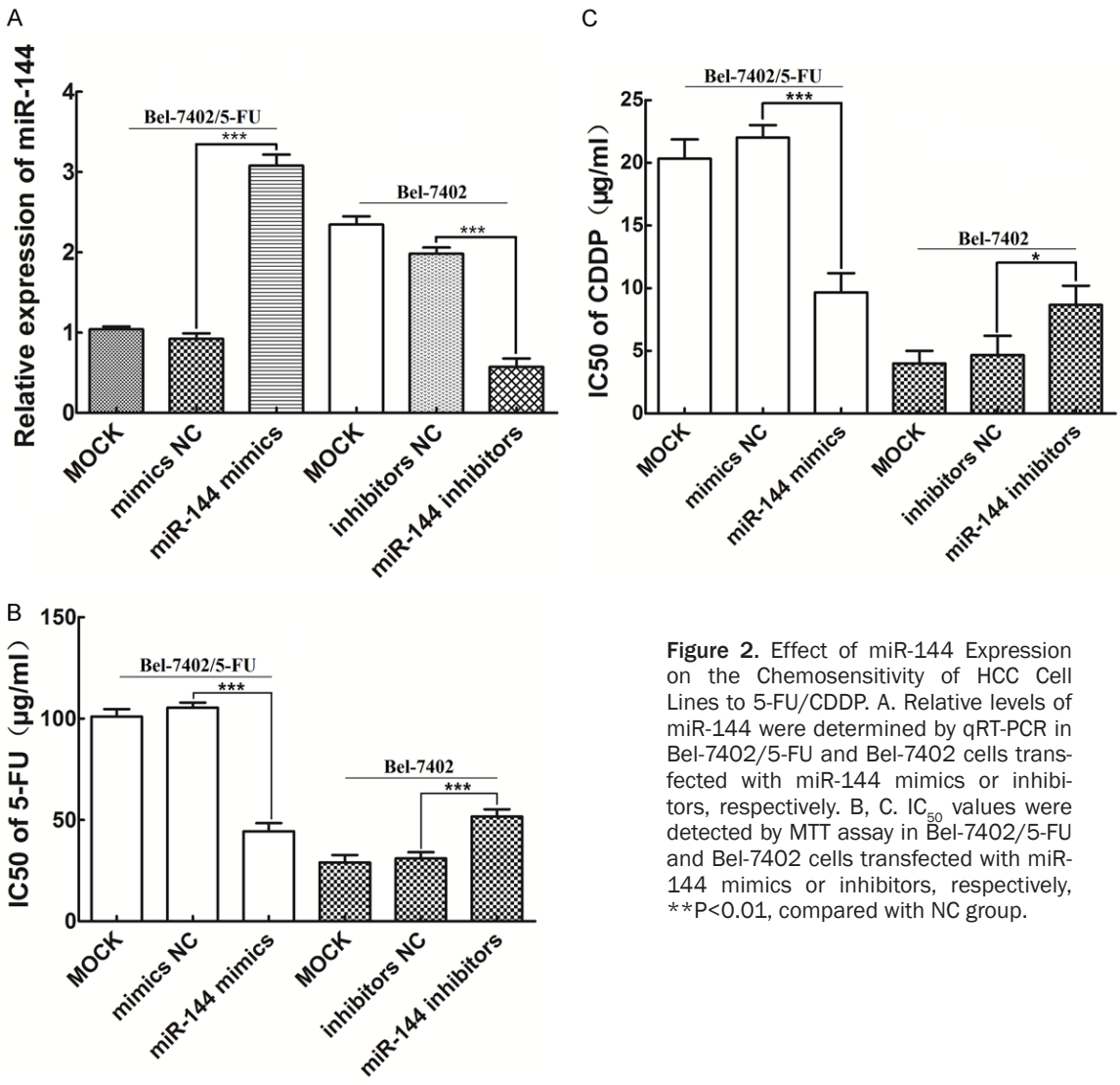


Figure 2. Effect of miR-144 Expression on the Chemosensitivity of HCC Cell Lines to 5-FU/CDDP. A. Relative levels of miR-144 were determined by qRT-PCR in Bel-7402/5-FU and Bel-7402 cells transfected with miR-144 mimics or inhibitors, respectively. B, C. IC_{50} values were detected by MTT assay in Bel-7402/5-FU and Bel-7402 cells transfected with miR-144 mimics or inhibitors, respectively, **P<0.01, compared with NC group.

Results

miR-144 was down-regulated in HCC cells

We examined the expression of miR-144 in a panel of 23 matched-pair of HCC and non-tumor tissues by qRT-PCR. miR-144 was significantly downregulated in HCC tissues compared with that of the non-cancerous liver tissues (**Figure 1A**). The expression of miR-144 was significantly decreased in Bel-7402 and Bel-7402/5-FU cells, as compared with normal human liver cell line L02, and the expression of miR-144 was remarkably reduced in Bel-7402/5-FU cells compared with that in Bel-7402 cells (**Figure 1B**).

miR-144 regulated chemosensitivity of HCC

Here, we further investigate the potential role of miR-144 in modulating the chemosensitivity of HCC. MTT assay suggested that Bel-7402/5-FU cells transfected with miR-144 mimics showed lower IC_{50} value of 5-FU/CDDP than those transfected with a no-target control (**Figure 2A-C**). When Bel-7402 cells were transfected with miR-144 inhibitors, the IC_{50} of 5-FU/CDDP was significantly increased as compared with that in the inhibitor NC group (**Figure 2A-C**). These results indicated that miR-144 might modulate the chemosensitivity of HCC.

Nrf2/HO-1 pathway was activated in chemoresistant Bel-7402/5-FU cells

Western blotting has been performed to determine whether activation of Nrf2/HO-1 is involved in acquired resistance to 5-FU in both Bel-7402 and Bel-7402/5-FU cells. Results indicated that the protein levels of Nrf2 and

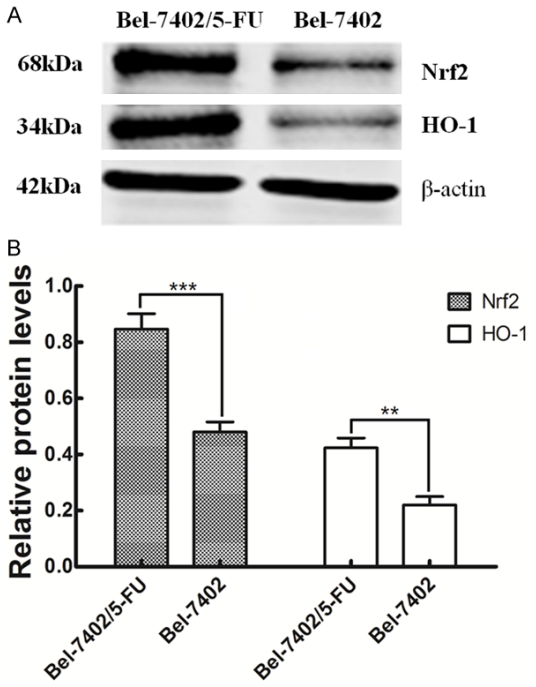


Figure 3. Nrf2/HO-1 pathway is activated in chemoresistant Bel-7402/5-FU cells. A. The protein levels of Nrf2 and its downstream gene was detected by western blot in Bel-7402/5-FU and Bel-7402 cells. B. Columns, mean of three independent experiments; Data were shown as mean ± SD, **P<0.01, ***P<0.001, compared with parental.

HO-1 were significantly elevated in Bel-7402/5-FU cells compared with Bel-7402 cells (**Figure 3**). These results indicated that Nrf2-dependent defensive system was fully activated in Bel-7402/5-FU cells to acquired chemo-resistance. In conclusion, these results indicated that activation of Nrf2-dependent defensive system was associated with acquired chemoresistance in Bel-7402/5-FU cells.

Bioinformatics prediction of miR-144 target and luciferase detection assay

Computational analysis revealed that Nrf2 was the target gene of the miR-144 by using miRNA databases (TargetScan, miRanda, and MiRDB). A putative miR-144 binding site was found located within the 3'UTR of Nrf2 (**Figure 4A**). To further assess whether Nrf2 is a direct target of miR-144, the luciferase reporter vector with the Nrf2 3'-UTR including the putative target site for miR-144 downstream of the luciferase gene (pGL3-Nrf2) and a mutant version (pGL3-Nrf2-mut) were constructed. The reporter vectors were co-transfected with miR-144 mimics or mimic-NC into Bel-7402/5-FU cells, and it showed that miR-144, but not its mutant form, specifically reduced Nrf2 mRNA levels in a dose-dependent manner (**Figure 4B**). Moreover, Bel-7402 cells with co-transfected with pGL3-Nrf2 vector and miR-144 inhibitor exhibited a more than 1.3-fold increase in luciferase activity compared to control group (P<0.0001). Conversely, Bel-7402/5-FU cells co-transfected with pGL3-Nrf2 and miR-144 mimics displayed a more than 1.9-fold reduction in luciferase activity compared to control group (P<0.0001). Meanwhile, it has not shown any statistically significant change in luciferase activity following the co-transfection of pGL3-Nrf2-mutation vector and miR-144 mimics or miR-144 inhibitor (**Figure 4C**). Collectively, these results indicated a direct interaction between miR-144 and Nrf2 mRNA in Bel-7402 and Bel-7402/5-FU cells lines.

miR-144 regulated chemosensitivity by inhibiting Nrf2/HO-1 pathway

Since Nrf2 was directly targeted by miR-144, we hypothesized that miR-144 might increases sensitivity of drug resistance Bel-7402/5-FU cells to 5-fluorouracil via down-regulation of Nrf2 protein. In order to validate this hypothesis, Bel-7402/5-FU cells were transfected with either miR-144 mimics or miR-144 mimics combined with miR-144 inhibitors. The mRNA and protein levels of Nrf2 and HO-1 were assessed 48 after transfection. The mRNA and protein levels of Nrf2 and HO-1 were significantly decreased in miR-144 mimics transfected cells compared with that of negative control. Interestingly, transfection with miR-144 mimics combined with miR-144 inhibitors reactivated the Nrf2 related pathway (**Figure 5A-C**).

To further confirm these results, Bel-7402/5-FU cells were co-transfected with miR-144 mimics and pcDNA3.1-flag-Nrf2 or miR-144 mimics alone. It has been shown that miR-144 mimics restored the sensitivity of Bel-7402/5-FU cells to 5-FU, and a marked increase in apoptosis cells with Annexin V staining was detected by flow cytometry in miR-144 mimics transfected cells compared with negative controls. Meanwhile, 5-FU-induced apoptosis was significantly suppressed in Bel-7402/5-FU cells co-transfected with pcDNA3.1-flag-Nrf2 and miR-144 mimics, resulted in partly restoration of the resistance to 5-FU in Bel-7402/5-FU

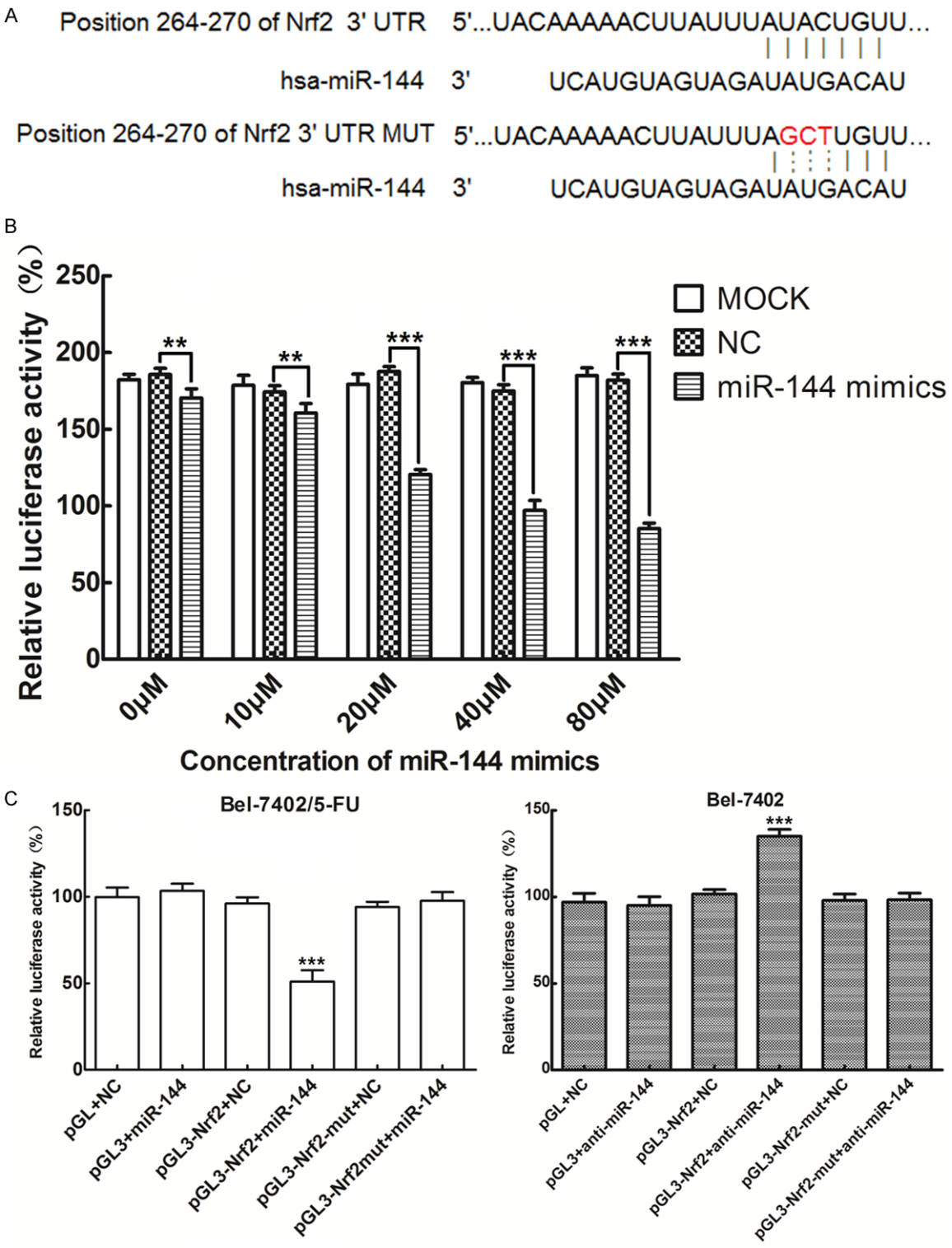


Figure 4. miR-144 Directly Targets Nrf2. A. Schematic of the two predicted seed regions in the 3'UTR of Nrf2 and mutated 3'UTR. B. Dose-dependent suppression of Nrf2 expression in the luciferase report assay after in Bel-7402/5-FU cells transfected with miR-144 mimics or a negativecontrol (NC). C. Luciferase assay in Bel-7402/5-FU and Bel-7402 cells. Wild-type (pGL3-Nrf2-3'UTR) and mutagenized (Nrf2-3'UTR-mut) reporter vectors were cotransfected with mimics or inhibitors of miR-144 or negative controls. Columns, mean of three independent experiments; Data were shown as mean ± SD, **P<0.01, ***P<0.001, compared with mut group or NC group.

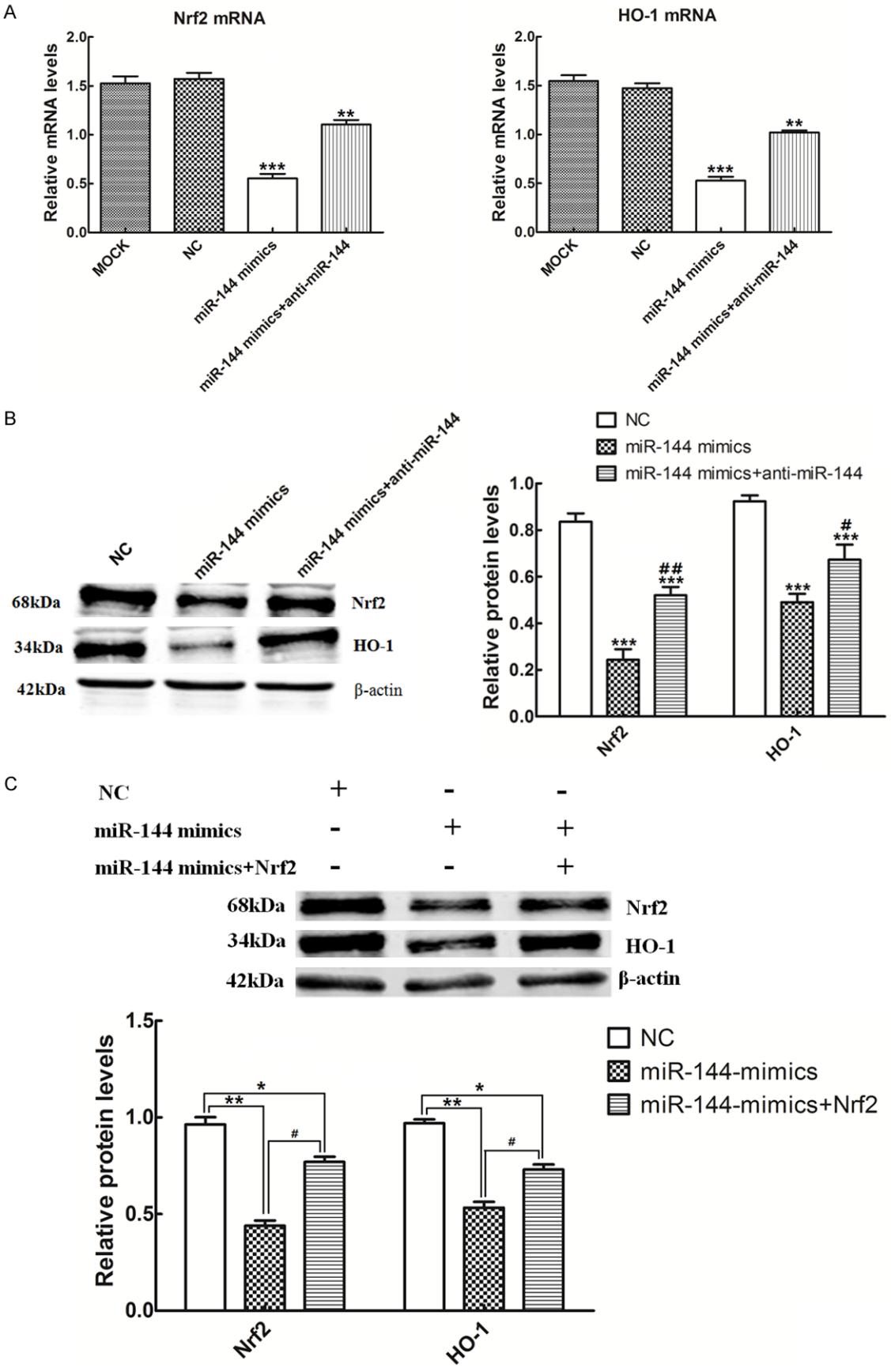
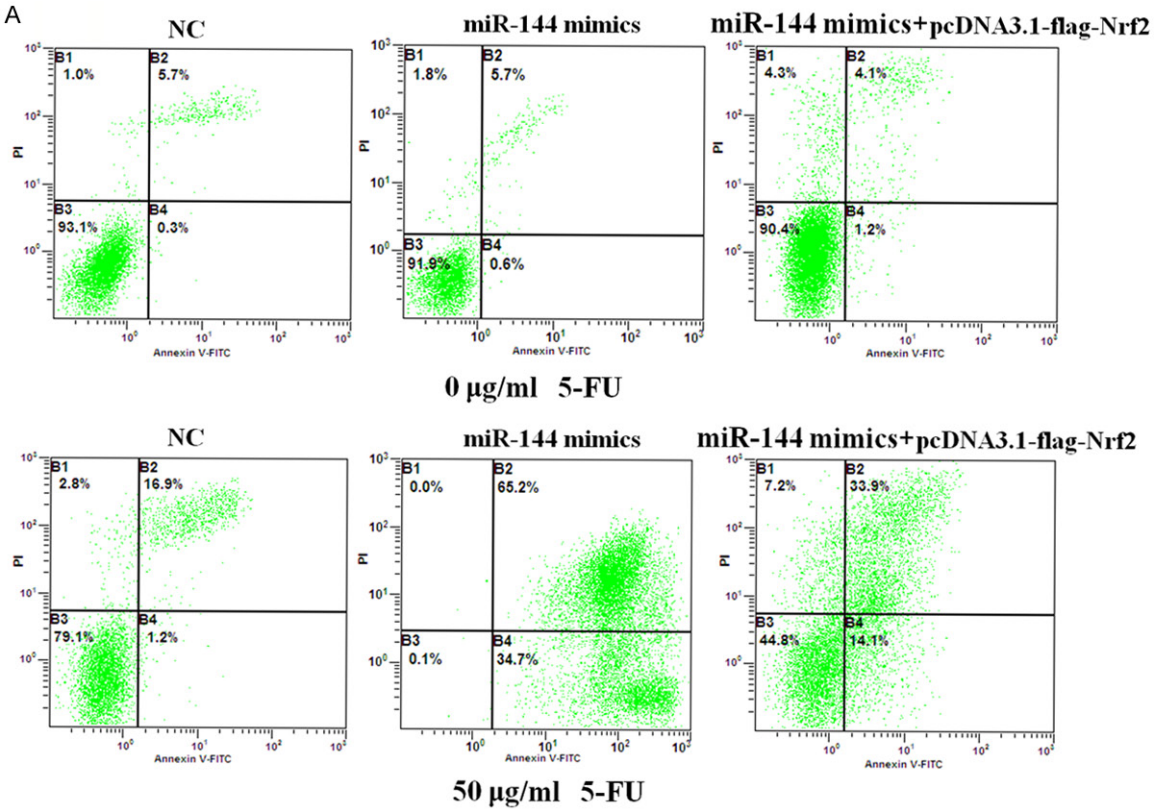

A
Nrf2 mRNA
HO-1 mRNA
Relative mRNA levels
MOCK
NC
miR-144 mimics
miR-144 mimics+anti-miR-144
B
NC
miR-144 mimics
miR-144 mimics+anti-miR-144
68kDa
34kDa
42kDa
Nrf2
HO-1
β-actin
Relative protein levels
C
miR-144 mimics+Nrf2
miR-144-mimics
miR-144-mimics+Nrf2

Figure 5. miR-144 inhibits Nrf2/HO-1 pathway. The mRNA (A) and protein (B) levels of Nrf2 and its downstream gene in the Bel-7402/5-FU cells transfected with miR-144 mimics or a combination of miR-144 mimics and anti-miR-144. Columns, mean of three independent experiments; Data were shown as mean ± SD, ***P<0.001, compared with NC group. #p<0.01 and ##P<0.001 compared with miR-340 mimics group. (C) Bel-7402/5-FU cells were co-transfected with 100 nM miR-144 mimics alone or in combination with pcDNA3.1-flag-Nrf2 or mimics-control, Nrf2 and HO-1 were detected by western blotting. β-actin was used as a loading control.



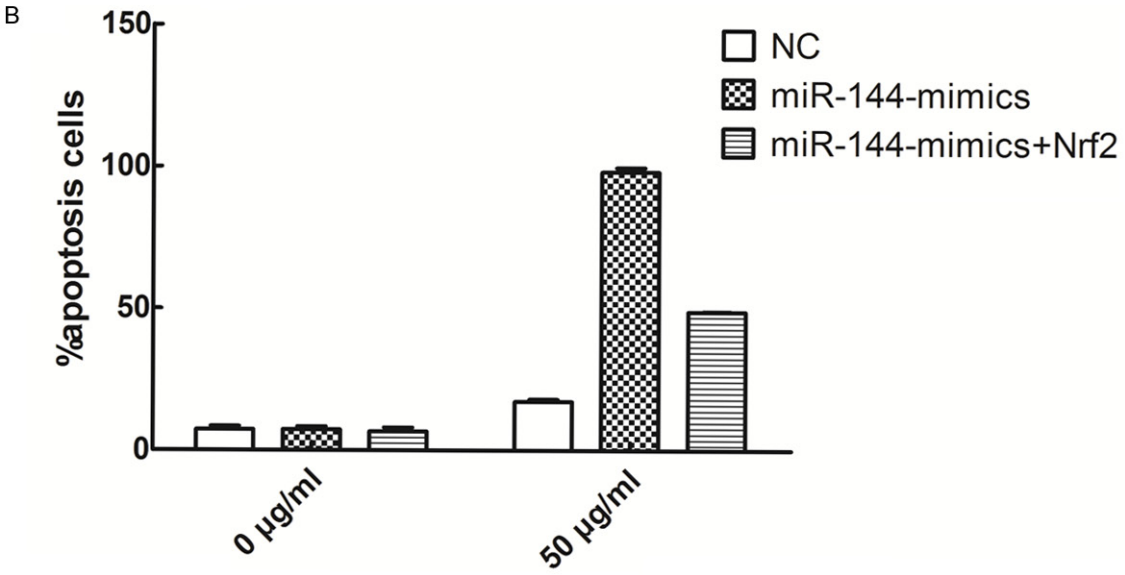


Figure 6. miR-144 regulated chemosensitivity through Nrf2/HO-1 pathway. A, B. The transfected Bel-7402/5-FU were seeded into a 96-well plate at the density of 5×10^3 cells per well and then treated with indicated concentration of 5-FU for 48 h. Then the cells were subjected to apoptosis analysis by annexin-V/PI staining and flow cytometry. Bar graph indicates the relative percentages of apoptotic cells from three independent experiments (right). *P<0.05, **P<0.01 compared with NC group. #p<0.05 compared with miR-340 mimics group.

cells (**Figure 6A**, **6B**). These results suggested that down-regulation of miR-144 was involved in the development of 5-FU resistance by blocking 5-FU-induced apoptosis.

Collectively, these findings suggested that miR-144 might restored the chemosensitivity of hepatocellular carcinoma cell lines to 5-FU at least in part by repressing Nrf2-dependent pathway.

Discussion

Recently, deregulation of miR-497, miR-23a, miR-145, miR-96, miR-203, miR-22 miR-197, miR-21, let-7b and let-7c have been reported to be associated with acquisition of resistance to 5-FU in colorectal cancer or renal cell carcinoma [11-19]. Shi L et al. reported that miR-141 plays a key role in the resistance of HCCs to 5-FU by reactivating the Nrf2-dependent antioxidant pathway [20]. Emerging data demonstrate that miRNAs play an important role in many cancers by conferring chemoresistance. And a revision of the differentially expressed miRNAs may overcome chemoresistance.

Previously, accumulating data have revealed a paradoxical oncogenic and tumor suppressive role of miR-144 in carcinogenesis and cancer progression [21-23]. The role of miR-144 in carcinogenesis and cancer progression seems to be complicated and highly species-specific. Cao et al. found that miR-144 was significantly decreased in HCC tissues and cell lines and forced over-expression of miR-144 remarkably suppressed proliferation and metastasis of HCC cells [24]. Regarding miR-144 acts as a potential tumor suppressor miRNA in HCC, we became interested in the potential role of miR-144 in 5-FU resistance of HCC.

In the present study, we detected the expression of miR-144 in 23 HCC samples and HCC cell lines. We found that miR-144 was most remarkably reduced in Bel-7402/5-FU cells (**Figure 1**). This is the first time for systematically characterized the role of miR-144 in 5-FU resistant of HCC. MTT assay and flow cytometry revealed that transfection of Bel-7402/5-FU cells with miR-144 mimics contributed to the elevated sensitivity of drug-resistant cells to 5-FU/CDDP (**Figures 2B**, **6B**, **6C**). In addition, numerous findings have demonstrated that chemoresistance in a variety of tumors is associated with constitutive activation of Nrf2-mediated antioxidant and detoxification processes [25]. Nrf2 was identified as a direct target of miR-144 containing a conserved binding site within the Nrf2 mRNA 3'UTR by using TargetScan (**Figure 4A**). In this study, it has shown that Nrf2 was up-regulated while miR-144 was down-regulated in Bel-7402/5-FU cells compared with Bel-7402, as obtained from western blotting and qRT-PCR (**Figure 3**). In addition, application of luciferase reporter assay further validated the negative regulation of Nrf2 expression by miR-144 (**Figure 4B**, **4C**). Mechanically, the ectopic expression of miR-144 inhibited the Nrf2 and Nrf2-dependent HO-1 expression, thereby resulting in the enhanced sensitivity of Bel-7402/5-FU cells to 5-FU (**Figures 5**, **6A**). Our work expanded the regulatory role of miR-144 in Nrf2/HO-1 expression and chemoresistance in HCC.

In conclusion, our study presented a novel mechanism that miR-144 could potentially reverse the acquisition of resistance to 5-FU in Bel-7402/5-FU cells at least in part by suppressing Nrf2 expression. This result suggested that miR-144 might be an effective reversal agent on multidrug resistance of HCC in the future.

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Disclosure of conflict of interest

None.

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