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MiR-20a lowers chemosensitivity of liver cancer Huh-7 cells *via* regulating NF-κB expression

B.-M. YANG[1], J.-R. ZHAO[2], T.-T. HUO[2], M.-L. ZHANG[3], X.-H. WU[1]

[1]Department of Hepatobiliary Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, China
[2]Department of Neurology, Hebei General Hospital, Shijiazhuang, China
[3]Department of Emergency, The Second Hospital of Hebei Medical University, Shijiazhuang, China

Abstract. – OBJECTIVE: The aim of this study was to explore the regulatory effect of micro ribonucleic acid (miR)-20a on nuclear factor-κB (NF-κB) in liver cancer Huh-7 cells, and to elucidate its influence on the chemosensitivity of Huh-7 cells.

MATERIALS AND METHODS: Huh-7 cells with overexpression of miR-20a or knockout of miR-20a were first constructed. Quantitative polymerase chain reaction (qPCR) was adopted to detect the expression level of miR-20a in each group of cells. The sensitivity of cells to cisplatin and doxorubicin in each group was measured using methyl thiazolyl tetrazolium (MTT) assay, and the 50% inhibitory concentration (IC50) was calculated. Hoechst 33258 staining was performed to detect the apoptosis of cells in each group. Furthermore, the expression levels of apoptosis-associated proteins and the NF-κB signaling pathway-related proteins in each group of cells were determined via Western blotting.

RESULTS: The expression level of miR-20a in blank control group was considerably higher than that in knockout group ($p<0.01$). Meanwhile, cells in overexpression group exhibited a notably higher expression level of miR-20a than blank control group ($p<0.01$). Cells in knockout group had dramatically enhanced sensitivity to doxorubicin and cisplatin ($p<0.01$), with a prominently decreased IC50 value ($p<0.01$). However, cells in overexpression group exhibited remarkably weakened sensitivity ($p<0.01$) and increased IC50 value ($p<0.01$). After treatment with doxorubicin and cisplatin, the apoptosis level of cells rose substantially in knockout group ($p<0.01$), whereas declined significantly in overexpression group ($p<0.01$). Moreover, knockout group exhibited a notably elevated expression level of Caspase-3 ($p<0.01$), and a considerably decreased ratio of B-cell lymphoma 2 (Bcl-2)/Bcl-2 associated X protein (Bax) ($p<0.01$). The expression level of Caspase-3 declined remarkably ($p<0.01$), however, the ratio of Bcl2/Bax increased substantially ($p<0.01$) in overexpression group. The expression level of NF-κB inhibitor beta (NF-κBIB) was markedly up-regulated ($p<0.01$), while the expression levels of Livin and Survivin declined remarkably ($p<0.01$) in knockout group. Furthermore, overexpression group had a considerably decreased expression level of NF-κBIB ($p<0.01$), but notably increased expression levels of Livin and Survivin ($p<0.01$).

CONCLUSIONS: MiR-20a up-regulates the expressions of the downstream proteins Livin and Survivin, decreases the expressions of apoptosis-associated proteins, weakens the sensitivity of cells to chemotherapy drugs and lowers the apoptosis level of cells by activating the NF-κB signaling pathway in liver cancer Huh-7 cells.

Key Words:
Liver cancer, MiR-20a, NF-κB signaling pathway, Chemosensitivity.

Introduction

At present, primary liver cancer is clinically the sixth most common cancer, whose morbidity rate in males is higher than that in females. Live cancer is characterized by high mortality rate, poor prognosis and high recurrence rate. Among all types of liver cancer, hepatocellular carcinoma is the most common one, accounting for more than 80% of total cases[1,2]. Currently, the clinical treatments for liver cancer include surgical resection, chemotherapy, liver transplantation and radiofrequency ablation. Since they have not been definitely diagnosed until the middle-advanced stage, chemotherapy is a conventional treatment method for most patients with liver cancer[3]. The sensitivity of chemotherapeutic drugs is an important factor for the success of chemotherapy. Multiple researches have shown that the current crucial challenges for the treatment of liver can-

Corresponding Author: Xiaohui Wu, MD; e-mail: xhwu@hbmu.edu.cn

cer are to potentiate the sensitivity of liver cancer cells to chemotherapy drugs and weaken the resistance of liver cancer cells to drugs[4].

Micro ribonucleic acids (miRNAs) are a kind of important non-coding RNAs. They have been observed involved in the post-transcriptional regulation of over 30% coding genes in the body and multiple processes such as cell proliferation, differentiation, apoptosis and signal transduction[5]. Withers et al[6] have found that numerous miRNAs participate in regulating the resistance of tumor cells to drugs. Meanwhile, they also target the apoptotic mechanism and the PI3K/protein kinase B (Akt) signaling pathway in the body to modulate the sensitivity of tumor cells to chemotherapeutic drugs. MiR-20a, located on chromosome 13, is highly expressed in multiple tumor cells and functions as an oncogene[7]. Active oncogenes in cells can prominently increase the phosphorylation level of inhibitor of κB (IkB), decrease the content of nuclear factor-κB inhibitor beta (NF-kBIB) and help NF-kB enter the nuclei and bind to the promoter region of target genes, thereby regulating cell response[8]. Yang et al[9] have demonstrated that the NF-kB signaling pathway can be activated by miR-20a to weaken the sensitivity of gastric cancer cells to cisplatin. In recent years, the influence of miR-20a on the chemosensitivity of liver cancer cells has not been fully elucidated. Therefore, the aim of this study was to explore the effects of miR-20a on the chemotherapy of liver cancer cells *via* functional assays. Our findings might help to provide theoretical bases for the clinical treatment of liver cancer patients.

Materials and Methods

Materials and Instruments

Human liver cancer Huh-7 cell lines (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China), methyl thiazolyl tetrazolium (MTT), cisplatin and doxorubicin (Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS) and TRIzol (Invitrogen, Carlsbad, CA, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibico, Rockville, MD, USA), SYBR Premix Ex Taq kit and Prime Script RT reagent kit (TaKaRa, Komatsu, Japan), Hoechst 33258 kit and bicinchoninic acid (BCA) protein assay kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), radioimmunoprecipitation assay (RIPA) lysis buffer and protease inhibitor (Wuhan Servicebio Technology Co., Ltd., Wuhan, China), and antibodies (Abcam, Cambridge, MA, USA).

Cell incubator (Thermo Fisher Scientific, Waltham, MA, USA), ultraviolet spectrophotometer (Coulter, Miami, FL, USA), quantitative polymerase chain reaction (qPCR) instrument (Illumina Eco, San Diego, CA, USA), microplate reader (BioTek, Biotek Winooski, VT, USA), electrophoresis apparatus (Corning, Corning, NY, USA), thermostatic water bath (Shanghai Yiheng Technology Co., Ltd., Shanghai, China), fluorescence microscope (Nikon, Tokyo, Japan) and pipette (Eppendorf, Hamburg, Germany). The origin of other reagents was stated in the paper.

Construction and Culture of Liver Cancer Cell Lines with Overexpression or Knockout of MiR-20a

Huh-7 cell lines with overexpression or knockout of miR-20a were constructed by GenePharma Co., Ltd. (Shanghai, China). Cells with empty plasmid were used as normal controls. All cells were assigned into three groups, including: blank control group, overexpression group, and knockout group. After recovery, the cells were cultured in DMEM containing 10% FBS in a 5% CO_2 incubator at 37°C until they grew to the logarithmic phase. When cell density reached about 80%, they were sub-cultured. The third-generation cells were seeded into 96-well plates at the adjusted density of 2×10^4 cells/well. Next, they were continued to be cultured until covered about 80% of the culture flask for experiments.

Determination of MiR-20a Expression Level in Liver Cancer Cells Via qPCR

Cells in blank control group, overexpression group and knockout group were first harvested and cultured. Then, they were inoculated into 6-well plates at the adjusted density of 2×10^5 cells/well. When the cells grew to 80% of the culture flask, total RNAs were extracted using TRIzol reagent. The purity and concentration of extracted total RNAs were measured. Subsequently, they were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using the reverse transcription kit under the following conditions: 37°C for 15 min and 85°C for 5 s. GenBank was searched for the sequences of target genes, and primers for qPCR were designed and synthesized by Invitrogen (Carlsbad, CA, USA). Glyceraldehyde-3-phosphate dehydro-

genase (GAPDH) was used as an internal reference (Table I). The qPCR system was prepared using 10 μL of SYBR® Premix Ex Taq™ II (2×), 1.0 μL of primer mix, 1.0 μL of cDNAs, and DEPC-treated water added until the total volume of 20 μL. Specific conditions were as follows: 95°C for 30 s, 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. Relative expression level of miR-20a in cells was calculated using the $2^{-\Delta\Delta Ct}$ method.

Influence of MiR-20a on the Chemosensitivity of Liver Cancer Cells

Transfected cells in blank control group, overexpression group and knockout group were first collected. After seeded into 96-well plates at the adjusted density of 2×10^4 cells/well, the cells were cultured with complete medium in a 5% CO_2 incubator at 37°C for 48 h. Subsequently, the cells were added with cisplatin or doxorubicin at different concentrations (250 ng/mL, 500 ng/mL, 750 ng/mL, 1000 ng/mL and 2000 ng/mL) and cultured for another 24 h. Next, the cells were treated with 20 μL of 5 mg/mL MTT solution for another 4 h. After discarding the medium, dimethyl sulfoxide (DMSO) was added moderately. Absorbance of cells in each group at 490 nm was measured using a micro-plate reader. Finally, the proliferation of cells in each group was quantified, and the 50% inhibitory concentration (IC50) of cisplatin and doxorubicin was calculated.

Influence of MiR-20a on Liver Cancer Cell Apoptosis

Transfected cells in blank control group, overexpression group and knockout group were first collected. After cell density adjusted to 2×10^4 cells/well, they were inoculated into 6-well plates and cultured in complete medium in a 5% CO_2 incubator at 37°C. 48 h later, the cells were added with 1000 ng/mL cisplatin or doxorubicin, followed by culture for another 24 h. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min and washed with phosphate-buffered saline (PBS) for 3 times. Afterwards, the cells were incubated with Hoechst 33258 dye at 50 μL/well at room temperature for 10 min in dark. Next, the cells were photographed by a fluorescence microscope, and the nuclei of apoptotic cells showed bright blue. Cell apoptosis was finally calculated in each group.

Influence of MiR-20a on the Relevant Proteins in Liver Cancer Cells

Transfected cells in blank control group, overexpression group and knockout group were first harvested. Then the cells were seeded into 6-well plates at the adjusted density of 2×10^4 cells/well, and cultured with complete medium in a 5% CO_2 at 37°C for 48 h and with 1000 ng/mL cisplatin for another 24 h. With discarding the medium, each group of cells was collected for later use. Subsequently, an appropriate amount of RIPA lysis buffer was added in cells in each group to extract total proteins. The concentration of extracted proteins was determined using the BCA protein assay kit. Next, the loading buffer system at an equal concentration was prepared with the protein diluent in moderate, followed by boiling in thermostatic water bath for 15 min. Protein samples were separated by electrophoresis at the constant voltage of 80 V, and transferred onto polyvinylidene difluoride (PVDF) membranes by the wet transfer method. After sealing with freshly prepared 5% skim milk powder for 1 h, the membranes were incubated with primary antibodies against rabbit Caspase-3, rabbit-B-cell lymphoma 2 (Bcl-2), rabbit Bcl-2 associated X protein (Bax), rabbit NF-kBIB, rabbit Survivin, rabbit Livin and rabbit GAPDH at 4°C overnight. On the next day, the membranes were washed with Tris-Buffered Saline with Tween-20 (TBST) for 5 min ×3 times, and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 2h. Next, the membranes were washed again with TBST for 5 min ×3 times. Immuno-reactive bands were developed using the prepared developing solution in a developer, and relative expression levels of proteins were finally calculated.

Table I. QPCR primers.

	Sequence
MiR-20a	Forward: 5'-TGCGCTAAAGTGCTTATAGTGC-3' Reverse: 5'-CCAGTGCAGGGTCCGAGGTATT-3'
GAPDH	Forward: 5'-AATGCCTCCTGCACCACCAAC-3' Reverse: 5'-AAGGCCATGCCAGTGAGCTTC-3'

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental results were presented as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Based on the test for homogeneity of variance, Bonferroni test was performed for pairwise comparisons of the data conforming to homogeneity of variance, whereas Welch's test for pairwise comparisons of the data meeting heterogeneity of variance. $p<0.05$ was considered statistically significant.

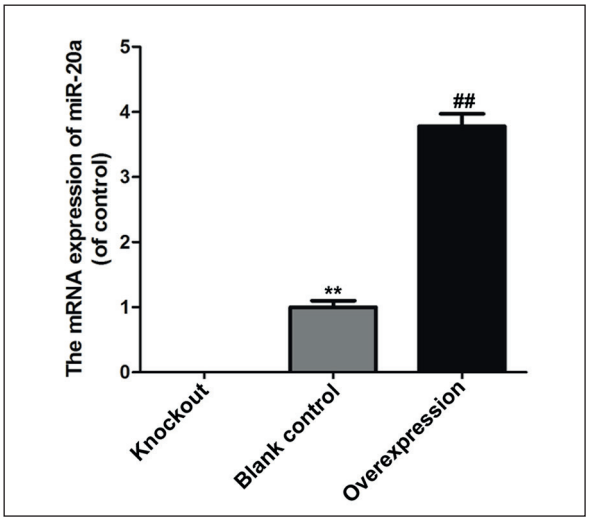

Figure 1. Expression level of miR-20a in each group of cells determined *via* qPCR: Cells in blank control group had a remarkably higher expression level of miR-20a than knockout group. The expression level of miR-20a in overexpression group was markedly higher than blank control group (**$p<0.01$ *vs.* knockout group, $^{\#\#}p<0.01$ *vs.* blank control group).

Results

Expression Level of MiR-20a in Each Group of Cells

Huh-7 cell lines with overexpression or knockout of miR-20a were first constructed. Meanwhile, cells transfected with the empty plasmid were used as normal controls. The expression level of miR-20a in cells was determined *via* qPCR. As shown in Figure 1, the expression level of miR-20 in blank control group was considerably higher than that in knockout group ($p<0.01$). However, cells in overexpression group had a notably higher expression level of miR-20a than those in blank control group ($p<0.01$).

Chemosensitivity of Liver Cancer Cells

The viability rate of cells in each group was measured *via* MTT assay. It was found that under the same concentration, the viability rate of cells in blank control group declined substantially when compared with overexpression group ($p<0.01$). However, cells in knockout group had a considerably lower viability rate than those in blank control group ($p<0.01$) (Figure 2). Next, IC50 values of cisplatin and doxorubicin in each group of cells were calculated. The results demonstrated that, after treatment with doxorubicin or cisplatin, cells in blank control group exhibited a remarkably higher IC50 value than knockout group ($p<0.01$). Meanwhile, the IC50 value in cells in overexpression group was markedly higher than that in blank control group ($p<0.01$) (Figure 3).

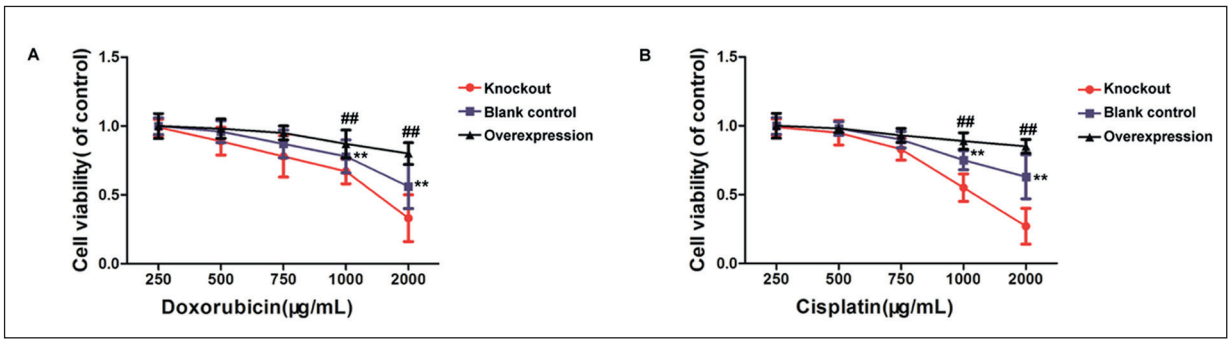

Figure 2. Proliferation of cells in each group. (**A**) Cells treated with doxorubicin, and (**B**) cells treated with cisplatin. The viability rate of cells in knockout group was substantially lower than blank control group and overexpression group (**$p<0.01$ *vs.* knockout group, $^{\#\#}p<0.01$ *vs.* blank control group).

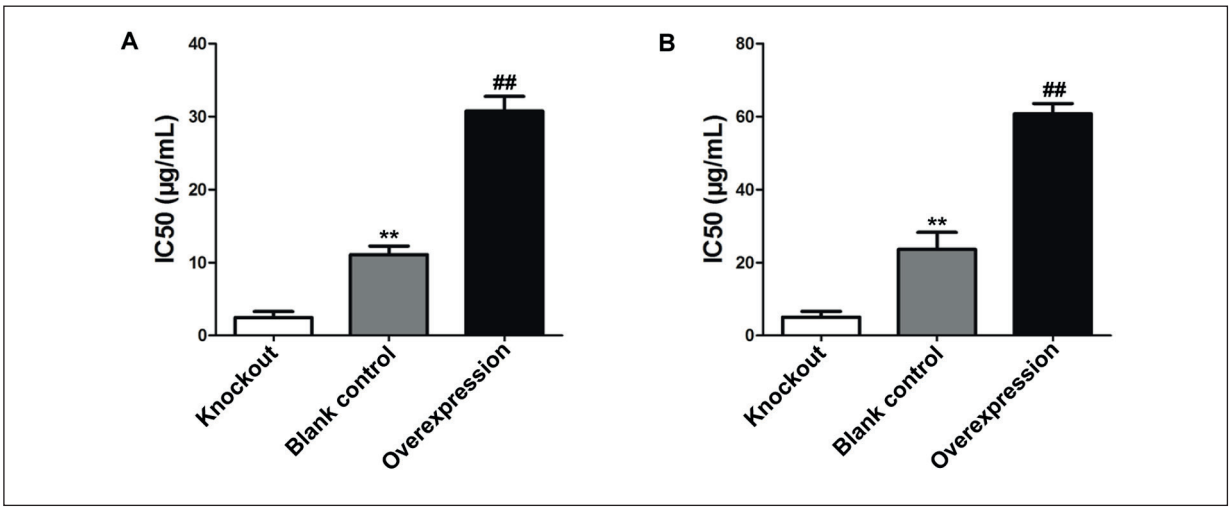

Figure 3. IC50 values of doxorubicin and cisplatin in each group of cells. (**A**) Cells treated with doxorubicin, and (**B**) cells treated with cisplatin. IC50 values of doxorubicin and cisplatin in knockout group were substantially lower than blank control group and overexpression group (**p<0.01 *vs.* knockout group, $^{\#\#}p$<0.01 *vs.* blank control group).

Apoptosis Level of Liver Cancer Cells

Hoechst 33258 staining was used to detect the apoptosis level of cells in each group after doxorubicin treatment. As shown in Figure 4, the apoptosis level of cells in blank control group was significantly lower than that of knockout group (p<0.01). Meanwhile, the apoptosis level of cells in overexpression group was significantly lower than that of blank control group (p<0.01). The apoptosis level of cells in each group after cisplatin treatment was shown in Figure 5. The results indicated that the apoptosis level of cells in blank control group was significantly lower than that of knockout group (p<0.01). Furthermore, the apoptosis level of cells in overexpression group was significantly lower than blank control group (p<0.01).

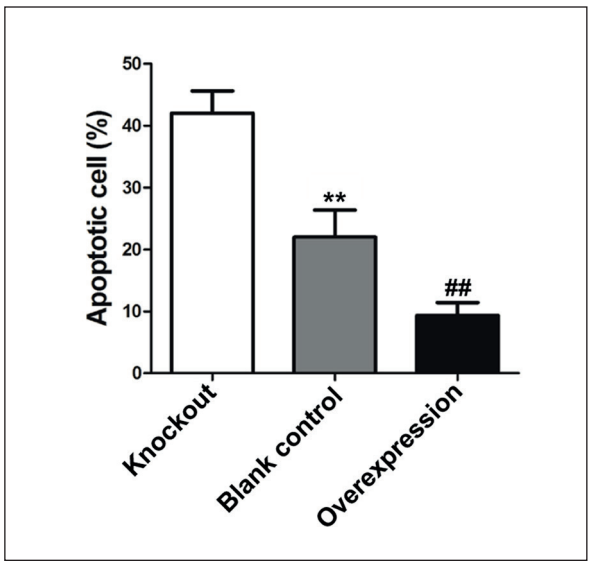

Figure 4. Apoptosis level of cells determined *via* Hoechst 33258 staining after treatment with doxorubicin: The apoptosis level of cells in overexpression group was substantially lower than knockout group and blank control group (**p<0.01 *vs.* knockout group, $^{\#\#}p$<0.01 *vs.* blank control group).

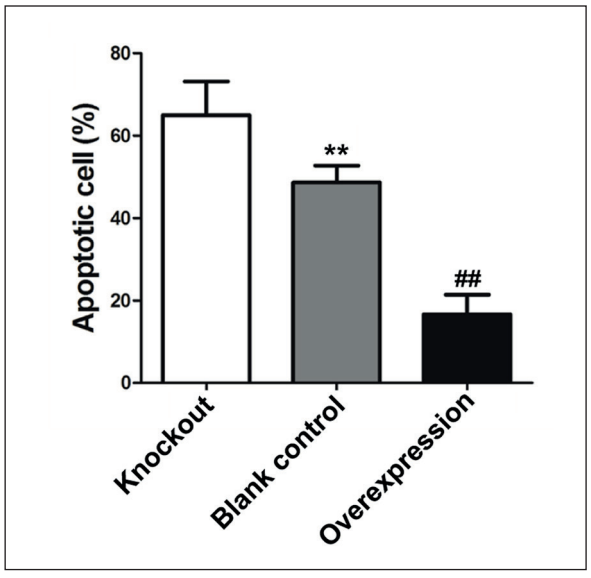

Figure 5. Apoptosis level of cells determined *via* Hoechst 33258 staining after treatment with cisplatin: The apoptosis level of cells in overexpression group was substantially lower than knockout group and blank control group (**p<0.01 *vs.* knockout group, $^{\#\#}p$<0.01 *vs.* blank control group).

Influences of MiR-20a on the Expression Levels of Apoptosis-Associated Proteins In Liver Cancer Cells

The expression levels of apoptosis-associated proteins in each group of cells were determined using Western blotting. The results indicated that the expression level of Caspase-3 was substantially lower in blank control group than knockout group ($p<0.01$), whereas the ratio of Bcl-2/Bax was prominently higher ($p<0.01$). However, overexpression group exhibited a remarkably lower expression level of Caspase-3 ($p<0.01$) and a higher ratio of Bcl-2/Bax ($p<0.01$) than blank control group (Figure 6).

Influence of MiR-20a on NF-kB Signaling Pathway

Western blotting was performed to detect the expression levels of the NF-kB signaling pathway-related proteins. It was discovered that cells in blank control group exhibited a markedly lower expression level of NF-kBIB ($p<0.01$) and higher expression levels of Livin and Survivin ($p<0.01$) than those in knockout group. Compared with blank control group, the expression level of NF-kBIB declined prominently in overexpression group ($p<0.01$), while the expression levels of Livin and Survivin rose substantially ($p<0.01$) (Figure 7).

Discussion

Low sensitivity to chemotherapeutic drugs is the leading cause of treatment failure and high recurrence rate of liver cancer patients. Massive studies have confirmed that epigenetic changes play a vital role in the chemosensitivity of tu-

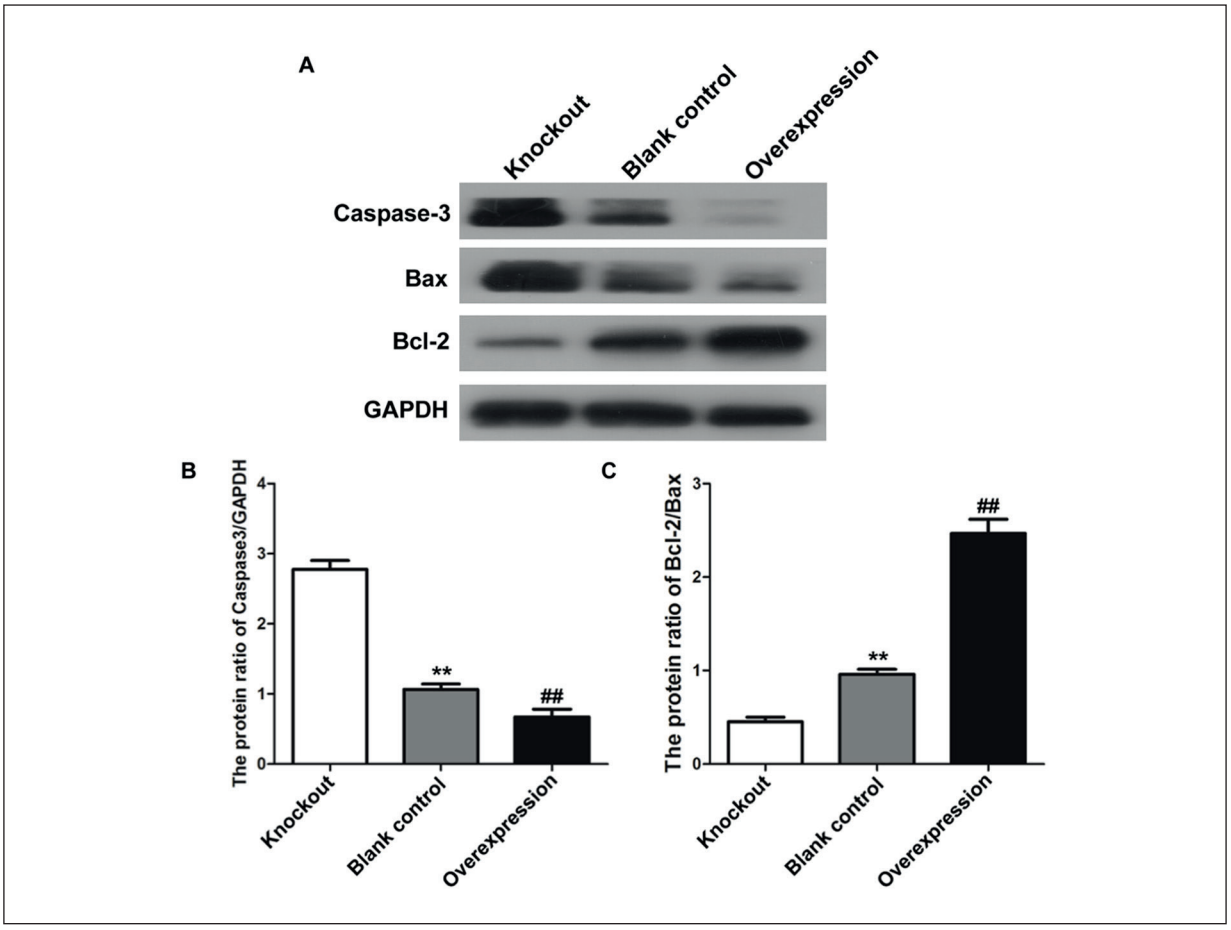

Figure 6. Expression levels of apoptosis-associated proteins measured *via* Western blotting. (**A**) Protein bands (**B**) statistics of Caspase-3 protein expression and (**C**) statistics of ratio of Bcl-2/Bax. The expression level of Caspase-3 in knockout group was dramatically higher than blank control group and overexpression group. However, the ratio of Bcl-2/Bax declined considerably when compared with blank control group and overexpression group (**$p<0.01$ *vs.* knockout group, $^{\#\#}p<0.01$ *vs.* blank control group).

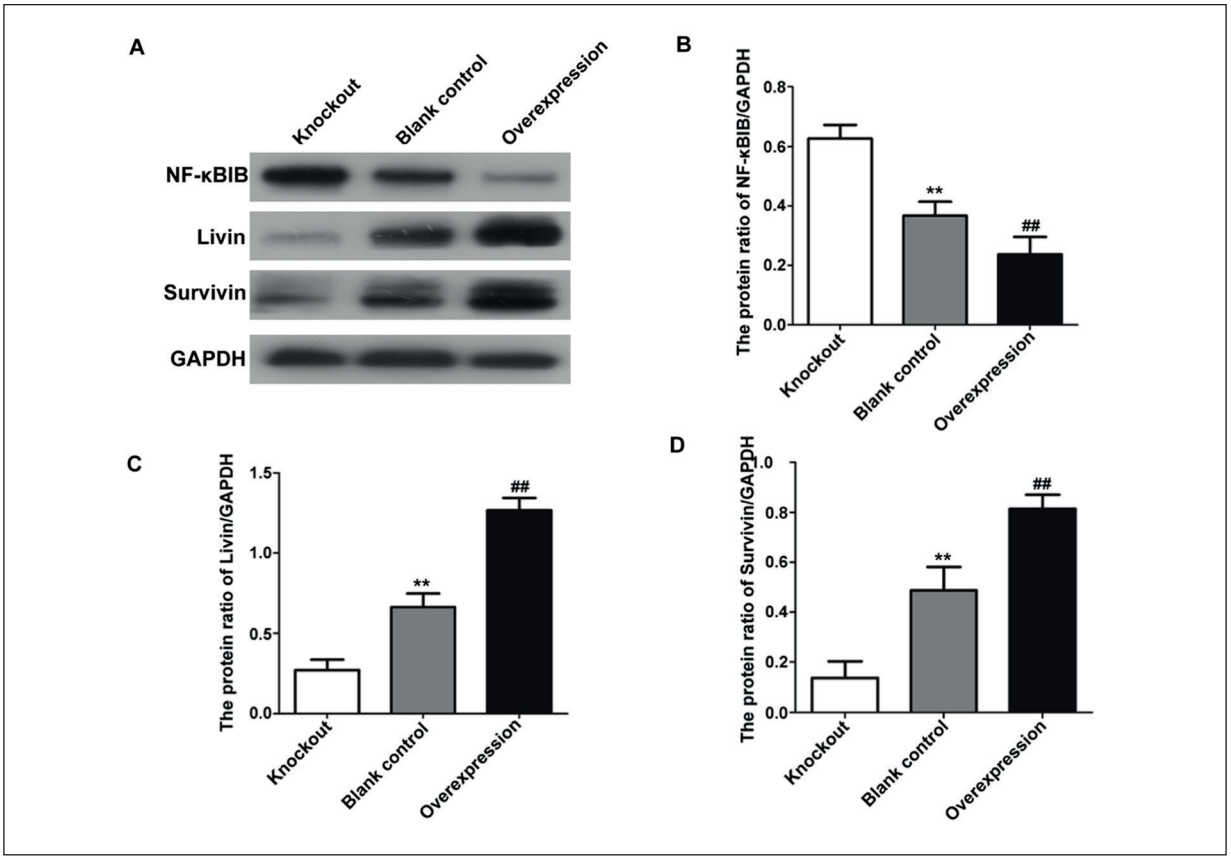

Figure 7. Expression levels of the NF-κB signaling pathway-associated proteins detected *via* Western blotting: (**A**) Protein bands, (**B**) statistics of NF-kBIB protein expression, (**C**) statistics of Livin protein expression and (**D**) statistics of Survivin protein expression. The expression level of NF-kBIB in knockout group was dramatically higher than blank control group and overexpression group. The protein expression levels of Livin and Survivin in knockout group were remarkably lower than blank control group and overexpression group (**$p<0.01$ *vs.* knockout group, $^{\#\#}p<0.01$ *vs.* blank control group).

mors[10,11]. MiR-20a, a membrane in the miR-17-92 family, is highly expressed in multiple tumor cells. Xin et al[12] have discovered that miR-20a is highly expressed in gastric cancer tissues. Meanwhile, it is an important gene affecting the sensitivity of gastric cancer cells to cisplatin. According to Marquez et al[13], the expression level of miR-20a rises notably in resistant liver cancer patients. In the present study, Huh-7 cell lines with overexpression or knockout of miR-20a were first constructed *in vitro*. Subsequently, the cells were treated with different concentrations of doxorubicin or cisplatin. Based on the results, the sensitivity of cells to doxorubicin and cisplatin was enhanced, and the IC50 value was remarkably lowered in knockout group. However, cells in overexpression group exhibited prominently weakened sensitivity to doxorubicin and cisplatin, with a notably increased IC50 value. The above results indicate that miR-20a is closely associated with the sensitivity of liver cancer cells to chemotherapeutic drugs. Additionally, experimental results revealed that after knockout of miR-20a, doxorubicin and cisplatin were more prone to inducing the apoptosis of Huh-7 cells. The opposite conditions were observed in cells in overexpression group.

Large amounts of researches have proven that the NF-kB signaling pathway is involved in cellular processes, such as inflammatory response, immune response, metabolism and cytotoxicity. It is also closely related to the physiological pathways including cell apoptosis[14,15]. Chemotherapeutic agents can restrain the phosphorylation of IkB in cells to accelerate the access of intracellular NF-kB to cell nuclei, and increase the expression of p65 in the nuclei, resulting in tumor cell apoptosis. The NF-kB signaling pathway can be considerably inhibited by increased expression level of NF-kBIB, the inhibitory protein of

NF-kB[16]. Jiang et al[17] have found that activating NF-kB in tumor cells can up-regulate the protein expressions of Bcl-2 and Akt, and down-regulate the protein expression of Caspase-3. This may eventually lead to a markedly lower apoptosis level of cells, as well as drug resistance in cells. According to the findings in the present study, overexpression of miR-20a in Huh-7 cells activated the intracellular NF-kB signaling pathway, thereby down-regulating the expression level of NF-kBIB, up-regulating the expression levels of downstream target proteins Livin and Survivin, decreasing the expression level of Caspase-3, and increasing the expression level of Bcl-2. Livin and Survivin are considered as important members in the anti-apoptosis protein family. Current studies have verified that they play a pivotal role in inhibiting cell apoptosis[18]. Teng et al[19] have manifested that repressing NF-kB in gastric cancer cells can notably increase the expressions of Livin and Survivin, thereby reversing the drug resistance of gastric cancer cells. Besides, Liu et al[20] have demonstrated that miR-20a has the binding site of NF-kB in the promoter region, which can modulate the activation of the NF-kB signaling pathway. It can be inferred from these results that miR-20a regulates the chemosensitivity of liver cancer Huh-7 cells through affecting the activation of the NF-kB signaling pathway.

Conclusions

Summarily, this study corroborates *in vitro* that miR-20a activates the NF-kB signaling pathway in Huh-7 cells to up-regulate the expressions of downstream proteins Livin and Survivin, decrease the expressions of apoptosis-associated proteins, weaken the sensitivity of cells to chemotherapy drugs, and lower the apoptosis level of cells. The novelty of this study was that all our findings may provide theoretical bases for the clinical treatment of patients with liver cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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