

Basic Study

Chemosensitization of HepG2 cells by suppression of NF- κ B/p65 gene transcription with specific-siRNA

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Abstract

AIM: To investigate small interfering RNA (siRNA)-mediated inhibition of nuclear factor-kappa B (NF- κ B) activation and multidrug-resistant (MDR) phenotype formation in human HepG2 cells.

METHODS: Total RNA was extracted from human HepG2 or LO2 cells. NF- κ B/p65 mRNA was amplified by nested reverse transcription polymerase chain reaction and confirmed by sequencing. NF- κ B/p65 was analyzed by immunohistochemistry. Specific-siRNA was transfected to HepG2 cells to knock down NF- κ B/p65 expression. The effects on cell proliferation, survival, and apoptosis were assessed, and the level of NF- κ B/p65 or P-glycoprotein (P-gp) was quantitatively analyzed by enzyme-linked immunosorbent assay.

RESULTS: HepG2 cells express NF- κ B/p65 and express relatively less phosphorylated p65 (P-p65) and little P-gp. After treatment of HepG2 cells with different doses of doxorubicin, the expression of NF- κ B/p65, P-p65, and especially P-gp were dose-dependently upregulated. After HepG2 cells were transfected with NF- κ B/p65 siRNA (100 nmol/L), the expression of NF- κ B/p65, P-p65, and P-gp were downregulated

significantly and dose-dependently. The viability of HepG2 cells was decreased to 23% in the combination NF- κ B/p65 siRNA (100 nmol/L) and doxorubicin (0.5 μ mol/L) group and 47% in the doxorubicin (0.5 μ mol/L) group ($t = 7.043$, $P < 0.001$).

CONCLUSION: Knockdown of NF- κ B/p65 with siRNA is an effective strategy for inhibiting HepG2 cell growth by downregulating P-gp expression associated chemosensitization and apoptosis induction.

Key words: Hepatocellular carcinoma; Nuclear factor- κ B; Multidrug-resistant; Chemosensitization; Small interference RNA; P-glycoprotein

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Core tip: Hepatic nuclear factor-kappa B (NF- κ B) signaling pathway could be a potential target for designing highly effective therapeutic agents for the chemoprevention of hepatocellular carcinoma (HCC). Specific siRNAs used in combination with doxorubicin could enhance doxorubicin cytotoxicity in human HepG2 cells by downregulating NF- κ B and P-glycoprotein expression. Stable NF- κ B inhibition and chemosensitization could significantly inhibit tumor cell proliferation. Thus, the modulation of NF- κ B might represent an advance in HCC therapy efficacy and is worthy of further research and investigation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and causes of mortality in China^[1,2]. A great deal of progress in understanding the mechanisms of hepatocarcinogenesis has been achieved in recent years^[3-5]. Many genes, such as protooncogenes, tumor suppressor genes, and growth factor genes, have been suggested to play an important role in this process^[6]. Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is involved in HCC development and progression. Tumorigenic protein (HBx or core protein of HCV) activates a variety of signaling pathways, including nuclear- transcription factor kappa B (NF- κ B) and tumor necrosis factor (TNF)- α ^[7,8]. Abnormal activation of NF- κ B has been shown to modulate the transcription and expression of many genes in hepatocarcinogenesis^[9,10]. NF- κ B is formed by five subunits, p65, RelB, C-Rel, p50, and p52. The p50/p65 dimers play the common biological

role and can be activated by chemotherapy drugs, cytokines, and viruses induced into nucleus^[11-13].

HCC is one of the most resistant tumors to systemic chemotherapy, and doxorubicin is one of the chemotherapy drugs used in HCC^[14,15]. Both NF- κ B and P-glycoprotein (P-gp) have been described as important mediators of chemotherapy-induced cell death^[16-18]. Because the first exon of multidrug resistance (MDR) gene promoter contains the NF- κ B binding sequence, it may be one of the downstream target genes of NF- κ B^[19,20]. A variety of chemotherapeutic drugs can induce formation of the MDR phenotype in malignant cells, thereby restricting the efficacy of these drugs. P-gp is encoded by the MDR gene and is a membrane bound ATP-dependent flow pump. It can pump drugs out of the cell with the energy provided by ATP, and this is the main mechanism underlying the MDR phenomenon^[21,22]. However, the role of NF- κ B signaling pathway in chemotherapy-induced MDR remains to be elucidated. In this study, we used small interference RNA (siRNA)-mediated inhibition of NF- κ B expression in combination with chemotherapy drugs to explore the role of NF- κ B in human HepG2 cell growth.

MATERIALS AND METHODS

Cell line and cultures

Human hepatoma cell (HepG2) and normal liver cell (LO2) lines were purchased from the Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China) and cultured at 37 °C with 5% CO₂ in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (FCS). The cells were digested by parynzyme. Recombinant human TNF- α was purchased from the Prepro Tech, Inc. (Rocky Hill, NJ, United States). Doxorubicin and all chemicals not otherwise specified below were purchased from Sigma (St Louis, MI, United States).

Synthesis of NF- κ B complementary DNA

To the cells (5×10^5), 1.0 ml of Trizol reagent (Promega, Madison, WI, United States) was added. Total RNA was isolated according to standard procedures and the protocols outlined by the manufacturer. RNA purity was estimated from the ratio of absorbance (A) readings at 260 and 280 nm, with an A_{260/280} ratio between 1.8 and 2.0 indicating sufficient purity. The RNA samples were kept frozen at -85 °C until required. For synthesis of NF- κ B complementary DNA (cDNA), 2 μ g of total RNA was denatured in the presence of random hexamers (100 pMol/L, Promega) and reverse-transcriptase (Gibco, Carlsbad, CA, United States) at 23 °C for 10 min, 42 °C for 60 min, 95 °C for 10 min, on ice for 5 min, and then stored at -20 °C for polymerase chain reaction (PCR) amplification.

PCR amplification of the NF- κ B gene

A set of primers, NF- κ B-P1 (sense), 5'-AGCACA

GATACCACCAAGAC-3' (nt398-417) and NF- κ B-P2 (antisense), 5'-TGGTCCCGTGAAATACACCT-3' (nt523-542) were designed according to NF- κ B/p65 sequences obtained from Genbank (NM-021975) and synthesized in the Shanghai Institute of Cell Biology, Chinese Academy Sciences, China, and the size of amplified fragment was 145 bp. The PCR amplification consisted of initial denaturation at 94 °C for 5 min, followed by 94 °C for 25 s, 55 °C for 30 s, and 72 °C for 90 s for 30 cycles. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genome was used as a control. A set of primer sequences was GAPDH-1 (sense), 5'-AGAAGGCTGGGGCTCATTG-3' and GAPDH-2 (antisense), 5'-AGGGGCCATCCACAGTCTTC-3', and the size of designed fragment was 258 bp. The amplified products were separated by electrophoresis on 2% agarose gels with ethidium bromide staining. The fragment sizes were evaluated using DNA markers (Promega) as molecular weight standards, and Molecular Imager Gel DocTM System (Biorad, Hercules, CA, United States).

Synthesis NF- κ B/p65 siRNA and cell transfections

The human NF- κ B/p65 siRNA was purchased from the Biomix Biotechnologies Co. (Nantong, China). The sequences of siRNA were 5'-GAUGAGAUUCUCCUACUGUdTdT-3' for p65 and 5'-UUCUCCGAACGUGU CACGUTTdTdT-3' for negative control. Cells at 50% confluence were transfected with 100 nmol/L NF- κ B/p65 siRNA using LipofectamineTM 2000 Transfection Reagent (Invitrogen), according to the manufacturer's specifications. The cells were seeded the day before transfection, using RPMI-1640 with 10% FCS without antibiotics. After 2 d, the cells were subjected to Western blotting analysis or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay in the presence or absence of doxorubicin.

MTT assays

The effect of doxorubicin on the viability of HepG2 cells was studied using the MTT Cell Proliferation and Cytotoxicity Assay Kit purchased from the Nanjing KeyGen Biotech CO., China. MTT assay was performed in 96-well flat-bottomed plates (Nunc, Rochester, NY, United States). Approximately 5×10^4 cells were seeded in 100 μ L of drug free media and incubated for 24 h before drug treatment or siRNA transfection. Different concentrations of doxorubicin were added for 24 h. Then, 10 μ L of MTT solution was added and incubated for 4 h, and, subsequently, 100 μ L formazan solution was added and incubated for 4 h. The amount of soluble formazan produced, by cellular reduction of MTT, was measured at 570 nm. Approximate IC₅₀ values were determined from a dose response curve. Data were derived from at least three independent experiments ($n = 3$). The effects of doxorubicin with or without NF- κ B/p65 siRNA on the viability of HepG2 cells were studied using MTT

assay. Twenty four hours after seeding, the cells were transfected with NF- κ B/p65 siRNA or negative control siRNA. After 24 h, doxorubicin was added for an additional 24 h. The MTT assays were then performed, as described above.

Western blotting

For Western blotting, the cytoplasmic proteins were purified from cells cultured in 6-wells plates and lysed with a hypotonic buffer (20 mmol/L Tris-buffer, pH 8.0, 150 mmol/L NaCl, 100 mmol/L NaF, 10% of glycerol, 1% of Nonidet P-40, 1 mmol/L PMSF, 40 μ g/mL leupeptin, and 20 μ g/mL arotinin) for 30 min at 4 °C. After centrifuged, equal amounts of protein (25 μ g/lane) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline (TBS), containing 2% glycine and 3% non-fat dried milk overnight at 4 °C, and then incubated with specific primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, United States) to NF- κ B/p65, phosphorylated p65, P-gp, and β -actin for 2 h at 37 °C. Membranes were then incubated with horseradish peroxidase-labeled secondary antibody for 1.5 h at 37 °C. The reaction was developed using a chemiluminescence detection system.

Immunohistochemistry

The cells were fixed with 10% formaldehyde, and then the streptavidin-peroxidase (S-P) method with empirical procedure directions was performed. Phosphate buffered saline (PBS) was used to substitute for the primary antibody and served as a negative control. The positive material of NF- κ B/p65 was a brown-yellow fine particle layer localized in the nucleus or cytoplasm. NF- κ B/p65 staining was evaluated semi-quantitatively according to the percentage of positive cells.

Enzyme-linked immunosorbent assay

The nuclear protein was extracted after cell transfection, according to the instructions for the nuclear and cytoplasmic protein extraction kit, and quantified spectro-photometrically using the BCA assay kit (Beyotime, Haimen, China). The level of NF- κ B/p65 was detected according to the human NF- κ B/p65 enzyme-linked immunosorbent assay kit (Cusabio Biotech, Wuhan, China), with 30 μ L of complete combining buffer, 10 μ L of nuclear protein extraction agent, and 20 μ L of complete lysis buffer (CLB). The positive control consisted of 2.5 μ g of provided nuclear extract diluted in 20 μ L of CLB per well; the blank well contained only 20 μ L of CLB. Twenty microliters of the appropriate standard diluted in the CLB was added to each well. Solutions were incubated with mild agitation for 1 h at room temperature. Each well was washed three times with 200 μ L of washing buffer, and then 100 μ L of diluted NF- κ B antibody was added. The plate

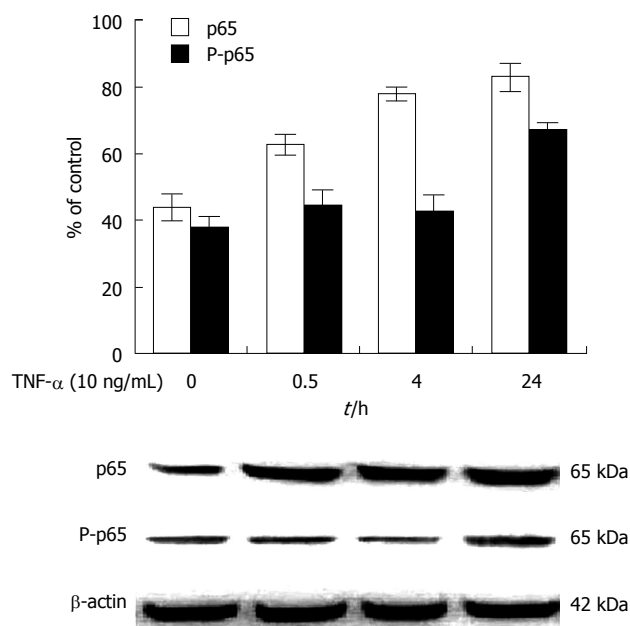


Figure 1 Comparative analysis of nuclear factor- κ B/p65 and P-p65 expression in human HepG2 cells induced with tumor necrosis factor- α . A: The ratios of nuclear factor- κ B (NF- κ B) p65 and P-p65 expression to β -actin in HepG2 cells with 10 ng/mL of tumor necrosis factor (TNF)- α at time points, data are means of independent triplicate experiments; B: The expression of NF- κ B/p65 and P-p65 by Western blot analysis, NF- κ B/p65 and P-p65 were 65 kDa, and β -actin, the control, was 42 kDa.

was covered and incubated for 1 h with mild agitation, washed four times, and 100 μ L of Developing Solution was added. After 10 min incubation in the dark, 100 μ L of stop solution was added, and within 5 min, the A450 was measured with a spectrophotometer and reference wavelength at 655 nm. NF- κ B level was calculated according to a standard curve.

Statistical analysis

Data was expressed as the mean \pm standard deviation (SD). Statistical analyses were done using the SPSS 10.0 software package (Chicago, IL, United States). Differences between groups were assessed using Fisher's exact test or the χ^2 test. $P \leq 0.05$ was regarded as statistically significant.

RESULTS

Expression of NF- κ B/p65 and P-p65 in HepG2 cells with TNF- α

The comparative analysis of NF- κ B/p65 and P-p65 expression in human HepG2 cells induced with TNF- α are shown in Figure 1. The ratio of NF- κ B/p65 and the relative expression of P-p65 to β -actin were increased in HepG2 cells treated with a time course of 10 ng/mL of TNF- α (Figure 1A), as shown with Western blotting (Figure 1B). The expression of NF- κ B/p65 or P-p65 was slight in HepG2 cells. Incubation of HepG2 cells with TNF- α (10 ng/mL) significantly increased the expression of NF- κ B/p65 at 0.5, 4, and 24 h relative to that at 0 h ($P < 0.01$). The expression of P-p65 was significantly

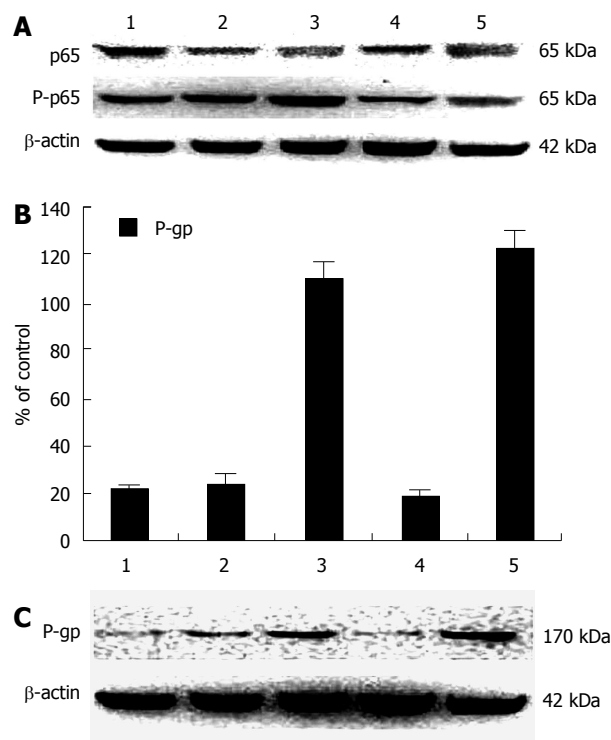


Figure 2 Expressions of nuclear factor- κ B/p65, P-p65, and P-gp induced by different doses of doxorubicin in HepG2 cells. Lane 1, control without doxorubicin; Lane 2, 0.5 μ mol/L of doxorubicin for 4 h; Lane 3, 0.5 μ mol/L of doxorubicin for 24 h; Lane 4, 1.0 μ mol/L of doxorubicin for 4 h; and Lane 5, 1.0 μ mol/L of doxorubicin for 24 h. A: The expressions of NF- κ B/p65 and P-p65 in HepG2 cells with different doses of doxorubicin were analyzed by western blotting. NF- κ B/p65 and P-p65 shows a 65 kDa band, and β -actin shows a 42 kDa band as control; B: The relative levels of P-glycoprotein (P-gp) expression to β -actin in HepG2 cells with different doses of doxorubicin at different times, data are means of independent triplicate experiments; C: The analysis of P-gp expression in HepG2 cells with different doses of doxorubicin by western blotting. P-gp, 170 kDa; β -actin, 42 kDa as control protein. The action time of HepG2 cells with different doses of doxorubicin.

higher ($P < 0.01$) at 24 h than at 0, 0.5, and 4 h^[23].

Expression of p65, P-p65 and P-gp in HepG2 cells with doxorubicin

The expression of NF- κ B/p65, P-p65, and P-gp induced by different doses of doxorubicin in HepG2 cells are shown in Figure 2. The expression of NF- κ B/p65 and P-p65 with different doses of doxorubicin was analyzed using Western blotting (Figure 2A). There were no significant changes between NF- κ B/p65 or P-p65 with various doses or incubation times of doxorubicin. The ratio of P-gp expression to β -actin in human hepG2 cells with different doses of doxorubicin at different times (Figure 2B) was analyzed by Western blotting (Figure 2C). P-gp expression in cells without doxorubicin was minimal, with no significant change found in the cells with doxorubicin at 4 h. P-p65 expression at 24 h was 6- or 7-fold higher than that at 4 h.

Specific siRNA downregulated NF- κ B/p65, P-p65, and P-gp expression

The downregulation of NF- κ B/p65, P-p65, and P-gp expression in HepG2 cells after siRNA transfection

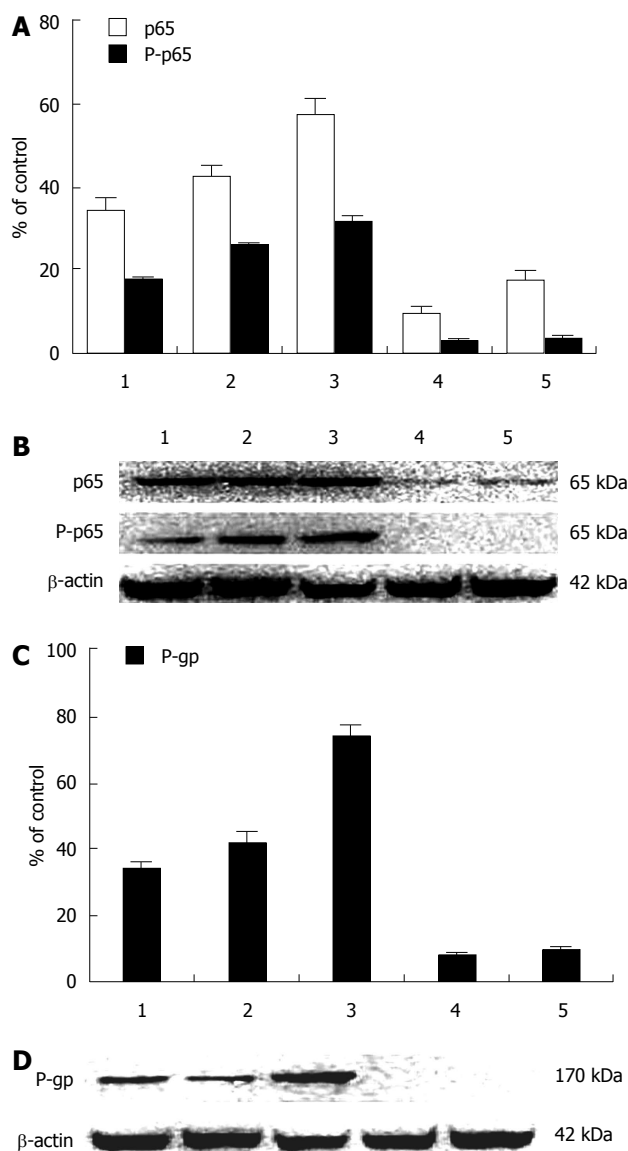


Figure 3 Downregulation of nuclear factor- κ B/p65, P-p65, and P-gp expression in HepG2 cells after siRNA transfection. 1, control; 2, negative-siRNA for 48 h; 3, 0.5 μ mol/L of doxorubicin for 48 h; 4, nuclear factor- κ B (NF- κ B)/p65 siRNA for 48 h; 5, 100 nmol/L of NF- κ B/p65 siRNA for 24 h, then added 0.5 μ mol/L of doxorubicin for another 24 h. A: The ratios of NF- κ B/p65 and P-p65 expression in HepG2 cells after NF- κ B/p65 siRNA transfection, data are means of independent triplicate experiments; B: The analysis of NF- κ B/p65 and P-p65 expression in HepG2 cells after NF- κ B/p65 siRNA transfection by western blotting; C: The relative levels of P-gp expression in HepG2 cells after NF- κ B/p65 siRNA transfection, all values are means of independent triplicate experiments; D: The analysis of P-gp expression in HepG2 cells after NF- κ B/p65 siRNA transfection by western blotting. The molecular weight NF- κ B/p65, P-p65, and P-gp were 65 kDa, 65 kDa, and 170 kDa, respectively; and β -actin, molecular weight 42 kDa, was the control protein.

is shown in Figure 3. In HepG2 cells transfected with siRNA, the ratios of NF- κ B/p65, P-p65, or P-gp expression to β -actin (Figure 3A and C) were decreased significantly, and this was confirmed by Western blotting (Figure 3B and D). The expression of NF- κ B/p65, P-p65, and P-gp was inhibited significantly ($P < 0.001$) in compared with the control or doxorubicin group.

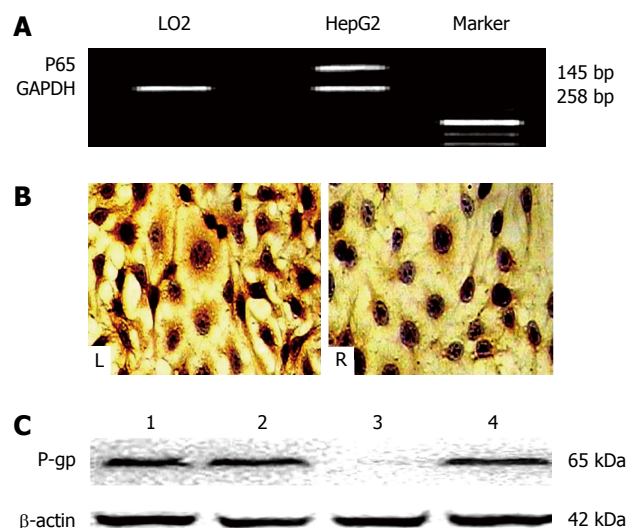


Figure 4 Expression of nuclear factor- κ B or nuclear factor- κ B/p65 mRNA in HepG2 cells before and after siRNA transfection. A: The expression of nuclear factor (NF)- κ B/p65 mRNA was higher in HepG2 cells than LO2 cells before siRNA transfection. The fragments (145 bp) of NF- κ B/p65 mRNA were amplified by reverse transcription polymerase chain reaction, separated on 2% agarose gel, and stained with ethidium bromide; B: Immunohistochemical staining with anti-NF- κ B/p65 (streptavidin-peroxidase, original magnification $\times 40$); NF- κ B/p65 positive material was a brown-yellow fine particle layer and localized in the cytoplasm and nucleus of HepG2 cells (L); the expression of NF- κ B/p65 positive material in the cytoplasm and nucleus of HepG2 cells with siRNA transfection (R); C: Western blotting of NF- κ B/p65 in cytoplasm and nucleus of HepG2 cells with siRNA transfection. Lanes 1, cytoplasm of HepG2 cells; Lanes 2, nucleus of HepG2 cells; Lanes 3, cytoplasm of HepG2 cells with 100 nmol/L of siRNA transfection; and Lanes 4, nucleus of HepG2 cells with 100 nmol/L of siRNA transfection. p65, 65 kDa; β -actin, 42 kDa, as the control protein.

Expression alteration of NF- κ B in HepG2 cells with siRNA

The expression of NF- κ B/p65 and NF- κ B/p65 mRNA in HepG2 cells before and after siRNA transfection is shown in Figure 4. The expression of NF- κ B/p65 mRNA was significantly higher in HepG2 cells than LO2 cells ($P < 0.001$, Figure 4A) before siRNA transfection. The relative ratio of NF- κ B/p65 mRNA to GAPDH was 1.13 ± 0.03 in HepG2 cells and 0.29 ± 0.07 in LO2 cells. NF- κ B/p65 positive material was a brown-yellow fine particle layer and localized in cytoplasm and nucleus of HepG2 cells; and this expression pattern was different in the cytoplasm and nucleus of HepG2 cells transfected with siRNA [$P < 0.001$, Figure 4B(L) vs Figure 4B(R)]. The incidence of NF- κ B/p65 expression decreased significantly in the cytoplasm of HepG2 cells with siRNA transfection but not in nucleus of HepG2 cells (Figure 4C), as confirmed by Western blotting.

HepG2 cells with siRNA sensitized to doxorubicin

The effects of human HepG2 cell viability with doxorubicin are shown in Figure 5A. The viability of HepG2 cells was analyzed after the cells were incubated with different doses of doxorubicin for 24 h. Drug cytotoxicity in HepG2 cells was dose-dependent, and the IC_{50} was between 0.5-1.0 μ mol/L during 24 h. The viability of HepG2 cells with combination siRNA

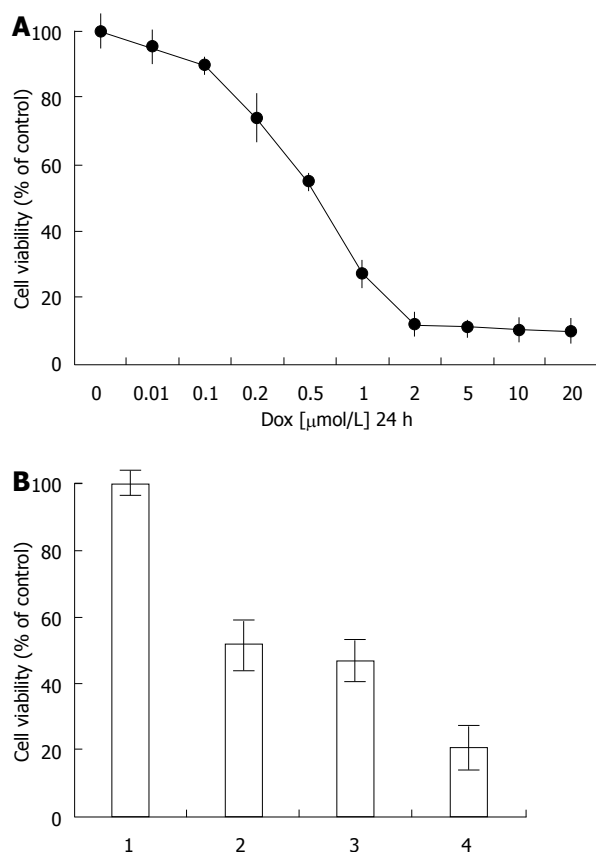


Figure 5 The viability of human HepG2 cells with doxorubicin after nuclear factor- κ B/p65 siRNA transfection. A: The effects of doxorubicin on HepG2 cells by MTT assay during a 24 h period. All values are means of independent triplicate experiments. Dox, doxorubicin; B: Nuclear factor- κ B (NF- κ B)/ siRNA inhibition of NF- κ B/p65 sensitized the HepG2 cells to doxorubicin, 1, control; 2, 0.5 μ mol/L of doxorubicin for 48 h; 3, negative siRNA for 24 h, then added 0.5 μ mol/L of doxorubicin for another 24 h; 4, 100 nmol/L of NF- κ B/p65 siRNA for 24 h, then added 0.5 μ mol/L of doxorubicin for another 24 h. Data were derived from three independent experiments ($n = 3$).

transfection and doxorubicin is shown in Figure 5B. Viability in cells treated with doxorubicin (0.5 μ mol/L) for 24 h was reduced to 51% compared with the control group. In cells transfected with negative siRNA for 1 d and then treated with doxorubicin for 1 d, the cell viability was reduced to 47% compared with control. There was no significant difference between the doxorubicin only group and the combination negative-siRNA and doxorubicin group. In cells transfected with siRNA for 1 d and then treated with doxorubicin for 1 d, the cell viability was reduced to 23%. The cell viability with siRNA and doxorubicin was significantly lower than that in the doxorubicin only group ($P < 0.001$). In HepG2 cells transfected with siRNA and treated with doxorubicin, the expression of NF- κ B/p65, P-p65, and P-gp was decreased relative to doxorubicin treatment alone.

DISCUSSION

HCC is one of the most common cancers and one of the most resistant tumors to systemic chemotherapy^[24,25].

Chronic infections with HBV and HCV are etiologically linked to hepatitis, liver cirrhosis, and HCC. Both viruses may induce activation of NF- κ B in hepatocytes, which plays a crucial role in the regulation of cell growth and apoptosis^[26-28]. NF- κ B signaling complexes in both viruses in HCC tumor and non-tumor tissues may disclose possible common mechanisms in hepatocarcinogenesis^[29,30]. NF- κ B and P-gp are important mediators of chemotherapy-induced cell death. In the present study, we evaluated siRNA-mediated inhibition of NF- κ B expression and application of chemotherapy to explore the anti-cancer effect on HepG2 cells.

Doxorubicin is a topoisomerase II inhibitor and can destruct the synthesis of DNA, induce NF- κ B activation, and inhibit cell apoptosis^[31,32]. The viability of HepG2 cells was analyzed in cells incubated with different doses of doxorubicin for 1 d. However, extracellular stimulation, including antineoplastic agents, mitogens, hormones, cytokines, and growth factors, can upregulate the expression of the MDR gene. We showed TNF- α can induce NF- κ B expression, as shown by the time-dependent increase of NF- κ B/p65 in HepG2 cells incubated with TNF- α (10 ng/mL) (Figure 1). In addition, P-p65, the active form of NF- κ B/p65 that is phosphorylated at Ser⁵³⁶ of the C-terminal transactivation domain during the phosphorylation and degradation of I κ B, was increased with 24 h of TNF- α treatment^[13]. Our results indicated that HCC is a tumor with high NF- κ B expression.

In the process of liver formation or liver regeneration, activity of MDR1 is abnormal. The human MDR gene family includes two members, MDR1 and MDR2, and abnormally regulated MDR1 is closely linked to the MDR phenotype^[15,17]. NF- κ B was moderately expressed in resting HepG2 cells. In cells treated with doxorubicin, NF- κ B/p65 and P-p65 expression was significantly and dose-dependently increased (Figure 2A). However, the P-gp expression in HepG2 cells was related to drug dose and treatment duration. Interestingly, the expression of P-p65 at 24 h was 6-fold to 7-fold higher than that at 4 h (Figure 2B and C). Doxorubicin induced P-gp expression in HepG2 cells, whereas the control HepG2 cells expressed very low P-gp. The first exon of MDR gene promoter contains the NF- κ B binding sequence, and a variety of chemotherapeutic drugs can induce formation of MDR phenotype in malignant cells that restrict the efficacy of drugs. These data suggest that activated P-gp is involved in the formation of the MDR phenotype.

NF- κ B activation in tumor cells was resistant to doxorubicin-based chemotherapy^[16]. The NF- κ B/p65 pathway acts as a bridge in the process of doxorubicin-mediated upregulation of P-gp expression. The expression of NF- κ B/p65, P-p65, and P-gp was significantly downregulated in HepG2 cells transfected with siRNA (Figure 3) compared with cells in the control or doxorubicin alone groups. The mRNA

expression of NF- κ B/p65 and NF- κ B/p65 in HepG2 cells before and after siRNA transfection was also significantly different (Figure 4). The NF- κ B/p65 mRNA expression or the ratio of NF- κ B/p65 mRNA to GAPDH was significantly higher in HepG2 cells than that in LO2 cells before siRNA transfection. After HepG2 cells were transfected with siRNA, positive NF- κ B/p65 expression decreased significantly in cytoplasm but not in nucleus, suggesting that inhibiting NF- κ B activity can enhance the cytotoxicity of drugs in liver cancer cells.

It is speculated that doxorubicin-induced upregulation of MDR expression is mediated by activation of the NF- κ B signaling pathway^[33,34]. The drug cytotoxicity on HepG2 cells was dose-dependent, with an IC₅₀ between 0.5–1.0 μ mol/L (Figure 5A). In order to confirm this conjecture, p65-specific siRNA was designed and transfected in HepG2 cells. The cell viability of HepG2 cells with negative-siRNA treated with doxorubicin was reduced to 47% (Figure 5B), whereas viability of cells with NF- κ B/p65-siRNA treated with doxorubicin was reduced to 23%, suggesting that siRNA-mediated inhibition of NF- κ B/p65 downregulated P-gp expression, decreased formation of MDR phenotype, and sensitized tumor cells to drug^[3].

NF- κ B signaling pathway could be a potential target for the design of highly effective therapeutic agents for the chemoprevention of HCC^[35,36]. Specific siRNA combined with doxorubicin can enhance doxorubicin cytotoxicity to HepG2 cells by downregulating NF- κ B and P-gp expression. Furthermore, stable NF- κ B inhibition and chemosensitization can significantly inhibit tumor cell proliferation. Thus, the modulation of NF- κ B may represent an advance in HCC therapy efficacy and is worthy of further research and investigation.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most resistant tumors to systemic chemotherapy, and doxorubicin is one of the chemotherapy drugs used in the treatment of HCC. Nuclear factor-kappa B (NF- κ B) has been described as an important mediator of chemotherapy-induced cell death. Because the first exon of the multi-drug resistance (MDR) gene promoter contains the NF- κ B binding sequence, it may be one of the downstream target genes of NF- κ B. A variety of chemotherapeutic drugs can induce formation of MDR phenotype in malignant cells, thereby restricting the efficacy of drugs. However, the role of NF- κ B signaling pathway in chemotherapy-induced MDR remains to be elucidated.

Research frontiers

Recently, Gu *et al* investigated the inhibitory effects of intervention of the tumor necrosis factor (TNF)- α /NF- κ B signaling pathway on HCC cell proliferation. HepG2 cells were cultured *in vitro* and treated with anti-TNF α mAb to down-regulate its expression or transfected with NF- κ Bp65 siRNA to inhibit its activation, and suggesting that the proliferation of hepatoma cells might be significantly inhibited by intervening in NF- κ B signaling pathway activation,

which promotes cell apoptosis and blocks cell cycling.

Innovations and breakthroughs

Hepatic NF- κ B signaling pathway could be a potential target for designing highly effective therapeutic agents and chemoprevention of HCC. Specific siRNA combined with doxorubicin could enhance the doxorubicin cytotoxicity to human HepG2 cells by downregulating NF- κ B and P-glycoprotein (P-gp) expression. Stable NF- κ B inhibition and chemo-sensitization could significantly inhibit tumor cell proliferation. Thus, the modulation of NF- κ B might represent an improvement in HCC therapy efficacy, and it is worthy of further research and investigation.

Applications

Tumorigenic protein (HBx or core protein of HCV) activates a variety of signaling pathways, including NF- κ B activation and TNF- α . Abnormal activation of NF- κ B modulates the transcription and expression of many genes in hepatocarcinogenesis. Understanding the role of NF- κ B, a master regulator of inflammation and cell death, in the development of hepatocellular injury, liver fibrosis, and HCC, with a particular focus on the role of NF- κ B in different cellular compartments of the liver is important. The application of NF- κ B/p65 siRNA is an effective strategy for inhibiting HepG2 cell growth by down-regulating P-gp expression towards chemosensitization and apoptosis induction.

Terminology

NF- κ B is formed by five subunits, p65, Rel B, C-Rel, p50, and p52. The p50/p65 dimers play the common biological role, and it can be activated by chemotherapy drugs, cytokines, and viruses induced into nucleus. NF- κ B acts as a central link between hepatic injury, fibrosis, and HCC, and it may represent a target for the prevention or treatment of liver fibrosis and HCC. NF- κ B acts as a two-edged sword, and its inhibition may not only exert beneficial effects but also negatively impact hepatocyte viability. Finding appropriate targets or identifying drugs that either exert only a moderate effect on its activity or that can be specifically delivered to nonparenchymal cells will be essential to avoid the potential increase in liver injury associated with NF- κ B blockade in hepatocytes.

Peer-review

Authors have done excellent work in this study. They have investigated small interference RNA (siRNA)-mediated inhibition of NF- κ B activation in human HepG2 cells exposed to an anti-cancer drug. The application of NF- κ B/p65 siRNA is an effective strategy for inhibiting HepG2 cell growth by down-regulating P-gp expression towards chemosensitization and apoptosis induction

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