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***Basic Study***

**Multidrug resistance reversal of hepatocellular carcinoma by metformin through inhibiting *NF-κB* gene transcription**

Wu W *et al.* MDR in HCC

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**Abstract**

**AIM:** To interfere the activation of nuclear factor-κB (NF-κB) with metformin on effects of reversing multidrug resistance (MDR) for hepatocellular carcinoma (HCC).

**METHODS:** Expressions of P-glycoprotein (P-gp) and NF-κB in human HepG2 or HepG2/ADM cells were analyzed, inhibited by pCMV-NF-κB-siRNA with or without metformin, and quantified by western blotting or fluorescence quantitative PCR. Cell viability was tested by CCK-8 assay. Cell cycle or cell apoptosis was measured by flow cytometry or Annexin-V-PE/7- ADD double staining assay.

**RESULTS:** The P-gp over-expressions in HepG2 and HepG2/ADM cells were closely related to mdr1 mRNA (3.310 ± 0.154) and NF-κB mRNA (2.580 ± 0.040). NF-κB gene transcription was inhibited by specific siRNA with significantly down-regulation of P-gp and enhanced HCC cell chemosensitivity to doxorubicin. After pretreatment with metformin, HepG2/ADM cells were sensitized to doxorubicin and confirmed with P-gp decreasing through NF-κB signal pathway. The synergistic effect of both collaboration were founded in HepG2/ADM cells with proliferation inhibition, cell cycle arrest and inducing cell apoptosis and the same molecular mechanism that inhibited the P-gp expression via NF-κB signaling pathway.

**CONCLUSION:** Metformin via silencing NF-κB signaling could effectively reverse MDR of HCC by down-regulated MDR1/P-gp expression.

**Key words:** Hepatocellular carcinoma; Multidrug resistance; Reversal; Metformin

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**Core tip:** Metformin might target AMP-activated protein kinase mammalian target of rapamycin pathway, suppressed hypoxia‑inducible factor-1α and transcriptionally down-regulated P-gp and MDR-associated protein 1, suggesting that metformin may reverse MDR by targeting the AMPK/mTOR/HIF-1α/P-gp and MRP1 pathways. In the present study, HepG2/ADM cells pretreatment with metformin were sensitized to doxorubicin and confirmed with P-gp decreasing through NF-κB pathway. The synergistic effects were founded in the cells with proliferation inhibition, cell cycle arrest and inducing apoptosis and inhibited the P-gp expression *via* NF-κB pathway and effectively reversed MDR by down-regulated MDR1/P-gp expression.

Wu W, Yang JL, Wang YL Yao M, Wang L, Gu JJ, Cai Y, Shi Y, Yao DF. Reversing multidrug resistance with metformin *via* silencing NF-κB in hepatocellular carcinoma cells. *World J Hepatol* 2016; In press

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the most common cancers and causes of mortality worldwide[1-3]. Due to the lack of specific symptoms, the fast majority of HCCs were diagnosed at late and/or advanced stages[4,5]. Although recent advances in surgical techniques and interventional therapy have improved survival, however, the emergence of multidrug resistance (MDR) to a series of clinical chemotherapeutics with different structures or different target sites with severely blocks the successful management of HCC[6,7]. The well recognized mechanism of classical MDR is the significant over-expression of human MDR1 gene encoding MDR1/P-glycoprotein (P-gp) that acts as an efflux pump in cell surface[8,9]. Intracellular anti-cancer drugs increasingly flow from cells through the efflux pump, thus drug concentrations becomes lower and cancer cells becomes resistant to chemotherapeutic drugs, such as doxorubicin[10,11].

Recently, some studies have found diverse anticancer effects of metformin in the cells of lung, gastric, endometrial, breast, and other types of cancer[12,13]. Metformin exhibits anti-proliferative effects in tumor cells *in vitro* and *in vivo*[14,15]. Metformin might target the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway[16,17], suppressed the hypoxia-inducible factor-1α (HIF-1α)[18,19] and transcriptionally down-regulated P-gp and MDR-associated protein 1 (MRP1). Collectively, suggesting that metformin may reverse MDR by targeting the AMPK/mTOR/HIF-1α/P-gp and MRP1 pathways[20,21]. In additional, the activation of nuclear factor-kappa B (NF-κB) pathway plays an important role in the development of HCC[22-24], but whether it is related to MDR and its molecular mechanisms still remain be explored[25,26]. In this study, the models of human resistant HepG2/adriamycin (HepG2/ADM) cells were used to silence NF-κB gene transcription by specific small interference RNA (siRNA) on MDR1 regulation and combining with metformin down-regulating P-gp expression to observe the effect on reversing MDR of HCC.

**MATERIALS AND METHODS**

***Cell culture***

Human hepatoma cell lines (HepG2), HepG2/ADM cell lines and hepatocyte LO2 cell lines were purchased from Aibio Biotech Company (Shanghai, China). The LO2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, KeyGen Biotech Co., Ltd, Nanjing, china) containing 10% fetal bovine serum (FBS, Invitrogen, United States), penicillin (100 U/mL)/streptomycin (100 U/mL), at 37℃ with 5% CO2. HepG2 and HepG2/ADM cells were cultured in RPMI 1640 (KeyGen Biotech Co., Ltd, Nanjing, China) complete medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL)/streptomycin (100 U/mL) at 37℃ in a humidified incubator containing 5% CO2.

***Western blotting***

The cultured cells were washed with phosphate buffered saline (PBS) twice and lysed in phenylmethane sulfonyl fluoride (PMSF, Beyotime, Nantong, China) cell lysis buffer (1:1000), and the protein concentrations were determined by the bicinchoninic acid (BCA, Beyotime, Nantong, China) protein assay kit. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF, Millipore, United States) membranes. After blocking with 5% skim milk Tris-buffered saline with tween (TBST) at room temperature for 3 h, the membranes were incubated with the primary antibody overnight at 4 ℃. The primary antibodies were diluted as follows: p65 and P-p65 (rabbit anti-human, 1: 1000, Cell Signaling, United States), MDR1 (rabbit anti-human, 1: 500, Abcam, United States) and β-actin (mouse anti-human, 1: 2000, internal reference, proteintech, United States). Then the membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (mouse or rabbit anti-human, 1:1000, Univ-bio, Nanjing, china) for 2.5 h at room temperature. At last, the samples were detected by Quantity One software with the electrochemi- luminescence kit (Millipore, United States) using gel imaging acquisition system. All Western blotting were repeated three times.

***Real-time quantitative PCR***

The cultured cells were digested by trypsin. Total RNA was extracted with TRIzol (Invitrogen, United States) reagent according to the protocol of the manufacturer. The quantity of total RNA was determined based on absorbance at 260 nm, and the purity of total RNA was analyzed based on the absorbance ratio at 260 and 280 nm (A260/280). Reverse transcription of total RNA to complementary DNA (cDNA) was performed with RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, CA, United States). PCR was carried by SYBR®Premix Ex TaqTMII kit (TaKaRa, Dalian, China), and GAPDH as internal reference. Sequence[27] as following: NF-κB/p65 (forward: 5’-CT ATCAGTCAGCGCATCCAG-3 and reverse: 5’-GCCAGAGTTTCGGTTCACT C-3’). mdr1 (forward: 5’-CCGGTTTGGAGCCTACTTG-3’ and reverse: 5’-TCCAA TGTGTTCGGCATTAG-3’). GAPDH (foward: 5’-CAAGGTCATCCATGACAAC TTTG-3’ and reverse: 5’-GTCCACCACCCTGTTGCTGTAG-3’). Real-time PCR was performed as follows: Initial denaturation at 94 ℃ for 2 min, and then 95 ℃ for 10 s, 55 ℃ for 30 s, 70 ℃ for 45 s for 40 cycles. The amplification specificity was confirmed by the melting curves. Ct values were calculated based on duplicates and normalized to GAPDH. The relative expression was calculated using the 2−ΔΔCt method. All PCR were repeated three times.

***Cell viability assay***

Cell viability was evaluated by cck-8 kit (Dojindo, Japan). The experiment groups were divided into blank, negative control and experimental group. Briefly, Logarithmic growth phase cells were digested by trypsin, the cells suspension liquid seeded in100 μL with 96-well plates. Toxicity tests were performance with different concentrations of ADM adding to 96-well plates in experimental group. The micro-plates were pre-cultured at 37℃ in a humidified incubator containing 5% CO2, and exchange liquid in a fixed time. Then 10 μL/well CCK-8 solution were added at 37 ℃ for 4 h. The absorbance (A) was measured by a microplate reader at the wave length of 450 nm. Cell survival rate = Aexp/Acon×100%. Values of IC50 were evaluated by the Graphpad prism5 software. Each individual experiment was performed at least three times.

***Metformin treatment***

HepG2/ADM cells were divided into three groups: blank, control and experiment. The experimental group were treated with 1 μmol/L metformin for 24 h, and continued to be cultured for 48 h with 1.5 μmol/L doxorubicin. The control groups were only treated with doxorubicin. The blank groups did not do any treatment.

***Analysis of cell apoptosis***

HepG2/ADM cells were treated with drugs for 48 h, and continued to be cultured for 24 h with another culture solution. Cells were harvested by tyrisin without EDTA and washed with cold PBS twice. Cell cycle or cell apoptosis (*n* = 3) was measured by flow cytometry or Annexin-V-PE/7-ADD double staining assay (BD, United States).

***Plasmid construction and cell transfection***

NF-κB-siRNAs were designed according to the previously reported sequences[28] and synthesized by the Biomics Company (Nantong, China) according to Rel A sequence obtained from Gene ID 5970. Forward, 5′-TGCTGTTCATCTCCTGAAAGGAGGC CGTTTTGGCCACTGACTGACGGCCTCCTCAGGAGATGAA-3′ and reverse, 5′-C CTGTTCATCTCCTGAGGAGGCCGTCAGTCAGTGGCCAAAACGGCCTCCTTT CAGGAGATGAAC-3′. Negative-siRNA: F, 5′-TGCTGAAATGTACTGCGCGTGG AGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′ and R, 5′-CC TGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGC GCAGTACATTTC-3′. Each siRNA was inserted to a pcDNA™ 6.2-GW/EmGFPmiR vector (Invitrogen, United States). The experiment groups were divided into blank control, negative siRNA control and NF-κB/p65 siRNA transfection. HepG2/ADM cells were planted into microwell plates in the density of 70%. The plasmids were transfected into cells for incubating for 24 h according to the manufacturer’s instructions. The medium in another day was removed and replaced with the fresh one, and the transfection efficiency was observed with a fluorescence microscope. These experiments were performed in triplicate.

***Statistical analysis***

Data was expressed as the mean ± SD. Statistical analyses were done using the SPSS21.0 software package. Differences between groups were assessed using analysis of variance or t-test. *P* ≤ 0.05 was regarded as statistically significant.

**RESULTS**

***Expression of P-gp, mdr1, and NF-kB among different liver cell lines***

The levels of P-gp, mdr1, and NF-kB expressions among different liver cell lines are shown in Figure 1. The proliferation of HepG2 and HepG2/ADM cells were decreased along with the increased of the concentration of doxorubicin, and the ability of proliferation was higher in HepG2/ADM cells than that in HepG2 cells. At 24, 48 and 72 h, the IC50 values of HepG2 cells to doxorubicin were 0.489, 0.221 and 0.224 μmol/L, respectively, and the IC50 values of HepG2/ADM cells to doxorubicin were 4.166, 1.522 and 1.380 μmol/L, respectively. The resistance index (RI, μmol/L) of HepG2/ADM cells was 8.519 at 24 h, 6.874 at 48 h and 6.166 at 72 h, respectively. There was almost no P-gp expression in LO2 cells. Different degree expression of P-gp protein in HepG2 and HepG2/ADM cells, but the P-gp expressions in HepG2/ADM cells were significantly higher than that in HepG2 cells (Figure 1A and B). The p-p65 expressions were significantly increased, meanwhile the expression of p65 were significantly decreased in HepG2/ADM cells (Figure 1C and D). The level of mdr1 mRNA and NF-κB mRNA were 3.310 ± 0.154 and 2.580 ± 0.040, respectively in HepG2/ADM cells, and were 0.084 ± 0.038 and 0.607 ± 0.032, respectively in HepG2 cells, the former was significantly higher than the latter (*P* < 0.01). Relative transcript levels (2-∆∆ct)were 9.381 ± 0.750 and 3.927 ± 0.069, respectively (Figure 1E).

***Effects of Metformin on HepG2/ADM cells***

The effect of metformin be related to concentration and time on the proliferation of HepG2/ADM cells are shown in Table 1. There were no significant different that effected in HepG2/ADM cells when the concentration of metformin were less than 3 mmol/L, but has different degrees inhibited on the proliferation of HepG2/ADM cells when metformin concentrations were between 3-10 mmol/L (*P* < 0.05). The HepG2/ADM cells were divided into experimental group and control group. After pretreatment with metformin the experimental group cells were joined into different concentration doxorubicin. The effect of adriamycin combined with metformin on the proliferation of HepG2/ADM cells are shown in Table 2. After treatment with metformin HepG2/ADM cells were more sensitive to adriamycin.

***Metformin promoting HepG2/ADM cell apoptosis***

The levels of HepG2/ADM cell apoptosis among the experimental (treated with metformin plus adriamycin), the control (only treated with adriamycin) and the blank (without adriamycin or metformin) group are shown in Figure 2. After the cells were pretreated with 1mM metformin for 24 h, then joined adriamycin, MDR1 in HepG2/ADM cells down-regulated, the cell cycle of G0/G1 blocked and promoted apoptosis when joined metformin. Significant difference of the apoptosis rates were found among the different groups (F = 3726.97, *P* < 0.001), with significantly higher in the experimental groups (22.17 ± 0.37%) more than those in the control groups (14.86 ± 0.21%) or the blank groups (4.17 ± 0.13%).

***Metformin reversing MDR via NF-κB pathway***

Metformin reversing the MDR of HCC via the NF-κB pathway are shown in Figure 3. The levels of P-gp expression in the HepG2/ADM cells were decreased with the dose of increasing metformin, and the phosphorylated p65 expression in the nucleus was also decreased. Metformin could down-regulate the P-gp expression by inhibiting NF-kB activation in a dose or time dependent manner.

***Synergistic effect of metformin plus NF-κB****-****siRNA***

The synergistic effects of reversing MDR by metformin combined with siRNA are shown in Figure 4. The HepG2/ADM cells were divided into three groups: untreated, metformin and the metformin combined with NF-κB-siRNA. In the third groups NF-κB-siRNA were transfected into HepG2/ADM cells for 24 h, and then were treated with 1 mmol/L metformin for 48 h. The levels of P-gp expression were 0.91 ± 0.24, 0.63 ± 0.13 and 0.22 ± 0.02 (*F* = 14.47, *P* = 0.005) in untreated, metformin and the metformin combined with NF-κB-siRNA groups, respectively. The expression of P-gp were significant reduced in treated with metformin plus NF-κB-siRNA groups than that in only treated with metformin groups (*t* = 5.39, *P* = 0.006).

**DISCUSSION**

Recent advances of HCC in surgical techniques and interventional therapy have improved survival of the patients[6,7,29]. However, the emergence of MDR to a series of clinical chemotherapeutics with different structures or target sites with severely blocks the successful management of HCC and still is a difficult problem to be solved in clinical practice[30,31]. MDR with a complex phenomenon in HCC could result from several biochemical mechanisms including decreased drug influx, increased drug efflux, altered cell cycle checkpoints, altered drug targets, increased drug metabolism and/or resistance to drug-induced apoptosis. Therefore, it is very important to find safe and effective reversal MDR agents for HCC[32]. In the present study, the HepG2/ADM cells with higher NF-κB expression were used to reverse MDR of HCC by metformin with silencing *NF-κB* gene transcription.

Anti-cancer drug efflux is one of the most common mechanisms in HCC MDR that encountered and mediated by ATP-binding cassette transporters[33,34], such as P-gp encoded by MDR1 gene that located in downstream of NF-κB signal pathway. The P-gp expression regulated by MDR1 is the most important and common cause of MDR, and weakened the apoptosis of cancer cells induced by chemotherapeutic drugs. Both of P-gp expression and NF-κB activation are linked positive closely with HCC progression[35]. Usually NF-κB takes part in gene transcription by means of homodimers or heterodimers, such as p50/p65, p65/p65, p65/Rel, *etc.* In the quiescent cells, they are predominantly cytoplasmic, associating with members of inhibitory IκB family and forming NF-κB/IκB complexes without activity. Both levels ofP-gp and NF-kB at protein or transcriptional level were significantly higher (Figure 1), with p65 expression decreasing in HepG2/ADM cells, indicated that abnormal P-gp and NF-kB expression could associate with the MDR information of HCC[20].

Metformin is a safe, low-cost drug, and therefore remains one of the most commonly prescribed drugs worldwide[16,36]. The anticancer effects of metformin indicate the possibility that certain diabetes-associated types of cancer[37,38] may be circumvented and have anti-proliferative potential against cancer cells or reversal MDR, *in vitro* and *in vivo*[39,40]. However, the precise molecular mechanisms whereby metformin works in cancer prevention remain multi-factorial and ill-defined. Metformin affected HepG2/ADM cell proliferation in a dose or time dependent minner (Table 1). There were no significant different in HepG2/ADM cells with less than 3 mmol/L, and the cells treated with metformin between 3-10 mmol/L were more sensitive to adriamycin with promoting cell apoptosis (Figure 2) and significantly higher inhibition in the experimental groups more than those in the control groups or the blank groups (Table 2), suggesting that metformin could increase the sensitivity of HepG2/ADM cells to anti-cancer drug.

There are few studies on the MDR effect in HCC by metformin. A developing siRNA strategy is a powerful technique to inhibit specific gene expression, which has highlighted the potential use of siRNA molecules to study gene function or explore new HCC therapeutic agents[41,42]. The expression of NF-κB gene transcription was inhibited by specific siRNA with significantly down-regulating P-gp and enhancing the chemosensitivity of HCC cells to doxorubicin and confirmed with the mechanism of decreasing P-gp *via* the NF-κB signal pathway. The synergistic effects of both collaboration were founded in HepG2/ADM cells with cell proliferation inhibition, cell cycle arrest, and inducing cell apoptosis, the data confirmed that the metformin could enhance the HepG2/ADM cells sensitivity to adriamycin and reverse MDR via the NF-κB signaling passway (Figure 4).

In conclusion, the MDR formation still is one of major causes of HCC chemotherapy failure with a wide variety of chemical structures and mechanisms[43,44]. Although specific NF-κB siRNA is powerful small molecule reagents designed to silence expressions of NF-κB and MDR1/P-gp related to MDR information to increase tumor cell sensitivity to anti-cancer drugs, however, how to apply metformin plus interfering NF-κB activation for effective reversing MDR of HCC still need to explore with more works in future.

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**COMMENTS**

***Background***

Hepatocellular carcinoma (HCC) multidrug resistance (MDR) to a series of clinical chemotherapeutics with different structures or different target sites with severely blocks the successful management of HCC. The mechanism of classical MDR is the significant over-expression of MDR1/P-glycoprotein (P-gp) that acts as an efflux pump in cell surface. Intracellular anti-cancer drugs increasingly flow from cells through the efflux pump, thus drug concentrations becomes lower and cancer cells becomes resistant to chemotherapeutic drugs, such as doxorubicin.

***Research frontiers***

Metformin could target AMP-activated protein kinase mammalian target of rapamycin pathway, suppressed hypoxia‑inducible factor-1α and transcriptionally down- regulated P-gp and MDR-associated protein 1, suggesting that metformin may reverse MDR by targeting the AMPK/mTOR/HIF-1α/P-gp and MRP1 pathways. However, metformin plus NF-κB pathway that might effectively reverse MDR of HCC remains to be explored.

***Innovations and breakthroughs***

Recently, there are few studies that the application of metformin on MDR effects of HCC. In this study, the present data suggested that the abnormal expressions of MDR1/P-gp and NF-κB activation during HCC development was related to MDR information, which might be down-regulated through inhibiting activation of NF-κB signaling pathway by specific siRNA. The collaboration of metformin with interfering *NF-κB* gene transcription could effectively reverse the MDR of HCC.

***Applications***

The abnormal expression of MDR1/P-gp in HCC was related to MDR information, which could be down-regulated through inhibiting activation of NF-κB signaling pathway by specific siRNA and increasing sensitivity of HCC cells to chemotherapy drugs. Interfering NF-κB activation with metformin have showed more effective to reverse MDR of HCC. However, how to apply metformin plus interfering NF-κB activation for effective reversing MDR of HCC still need to explore with more works in future.

***Terminology***

Metformin is a safe, low-cost drug. The anticancer effects of metformin indicate the possibility that certain diabetes-associated types of cancer may be circumvented. Indeed, many retrospective meta-analyses have shown that metformin possesses anti-cancer activities and decreases the incidence of primary cancer development in those taking metformin routinely, and a multitude of clinical cancer trials are actively assessing its benefits in non-diabetic population who have already developed cancer. However, the precise molecular mechanisms whereby metformin works in cancer prevention remain multi-factorial and ill-defined.

***Peer-review***

Authors have done excellent work in this present study. They have explored the interfering *NF-κB* gene transcription by specific siRNA on MDR1 gene regulation and combining with metformin. The application of interfering *NF-κB* activation with metformin showed more effective to reverse MDR of HCC.

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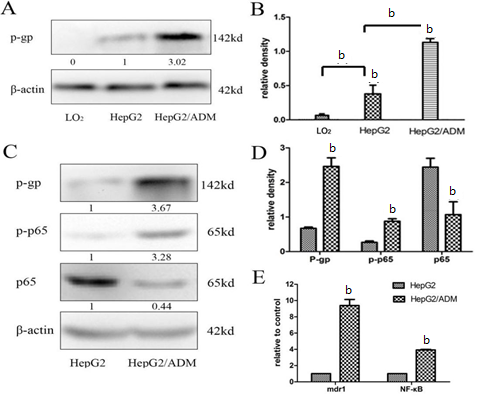
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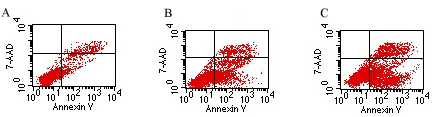
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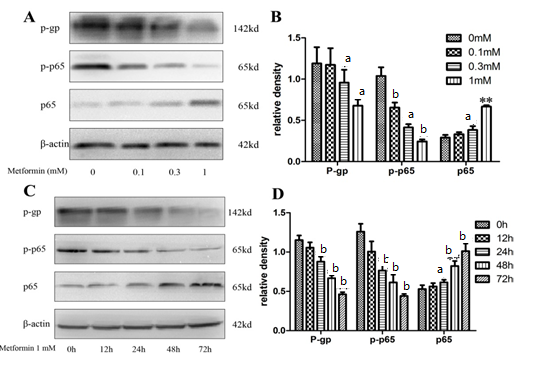
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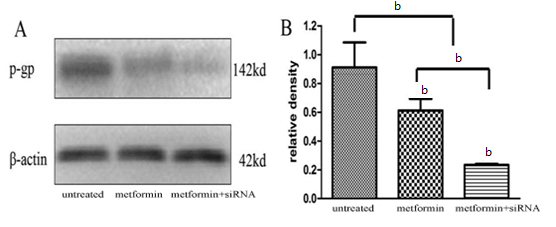
**Figure 1 the level of P-gp, mdr1 and NF-kB Expression among different cell lines**. A, C: The levels of P-gp and NF-kB expressions in HepG2 or HepG2/ADM cells were determined by Western blotting, the number indicated the ratio from HepG2/ADM cells to HepG2 cells (*n* = 3, Mean ± SD); B, D: The gray intensity images of Figure 1-A and Figure 1-C using Image J software; E: The levels of mdr1 and NF-κB mRNA expression were determined by qRT-PCR. b*P* < 0.01 (*n* = 3, mean ± SD), compared with hepG2 or LO2 cell lines.



**Figure 2 Metformin enhanced apoptosis rate of HepG2/ADM cells inducing by Adriamycin.** The cell early apoptosis were measured by Annexin-V-PE/7-ADD double staining assay in triplicate. A: The blank group (without treated with adriamycin or metformin). B: The control group (only treated with adriamycin); and C: The experiment group (treated with metformin plus adriamycin).



**Figure 3 Metformin down-regulated P-gp expression *via* NF-kB pathway.** A: The HepG2/ADM cells were treated with different doses of metformin for 24 h, the levels of P-gp and p-p65 expression analyzed by the Western blotting were decreased in a dose dependent manner, meanwhile the cytoplasma p65 increased in a dose dependent manner; B: The gray intensity images of Figure 3-A using Image J software. a*P* < 0.05, b*P* < 0.01 *vs* the blank groups (*n* = 3, Mean ± SD); C: The HepG2/ADM cells were treated with 1mM metformin during different times, the levels of P-gp and p-p65 expression analyzed by the Western blotting were decreased in a time dependent manner, meanwhile the cytoplasma p65 increased in a time dependent manner; D: The gray intensity images of Figure 3C using Image J software. a*P* < 0.05, b*P* < 0.01 *vs* the blank groups (*n* = 3, Mean ± SD).



**Figure 4 Alteration of P-gp expression after cells treated with metformin plus NF-κB**-**siRNA.** A: The alterations of P-gp expression after the cells treated with the different methods. Significant decreasing of the P-gp expression was found in the HepG2/ADM cells treated with the metformin plus NF-κB-siRNA analyzed by Western blotting; B: The gray intensity images of Figure 4A using Image J software. b*P* < 0.01 *vs* the blank groups (*n* = 3, Mean ± SD).

**Table 1 Absorbance value (*n* = 3, mean ± SD) of HepG2/ADM cells treated with different concentrations of metformin**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Time** | **0 (Blank)** | **0.1 mmol/L** | **0.3 mmol/L** | **1 mmol/L** | **3 mmol/L** | **10 mmol/L** |
| 24 h | 1.242 ± 0.03 | 1.233 ± 0.04 | 1.221 ± 0.02 | 1.195 ± 0.00 | 1.189 ± 0.02 | 1.101 ± 0.02a |
| 48 h | 1.744 ± 0.01 | 1.734 ± 0.02 | 1.718 ± 0.04 | 1.703 ± 0.03 | 1.583 ± 0.03a | 1.483 ± 0.01a |
| 72 h | 1.692 ± 0.04 | 1.677 ± 0.01 | 1.650 ± 0.06 | 1.583 ± 0.06 | 1.420 ± 0.06a | 1.300 ± 0.04a |

a*P* < 0.05 *vs* the blank groups.

**Table 2 Effect of adriamycin combined with metformin on the proliferation of HepG2/ADM cells (*n* = 3, mean ± SD)**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Adriacin**  **(μmol/L)** | **24 h** | | **48 h** | | **72 h** | |
| **control** | **metformin** | **control** | **metformin** | **control** | **metformin** |
| 0.0 | 1.434 ± 0.03 | 1.327 ± 0.04a | 1.477±0.08 | 1.357 ± 0.01 | 1.695 ± 0.08 | 1.507 ± 0.05a |
| 0.01 | 1.280 ± 0.06 | 1.160 ± 0.01a | 1.489±0.03 | 1.314 ± 0.03a | 1.505 ± 0.01 | 1.378 ± 0.07a |
| 0.1 | 1.194 ± 0.10 | 1.111 ± 0.09 | 1.418±0.01 | 1.213 ± 0.02a | 1.453±0.02 | 1.249 ± 0.04a |
| 1.0 | 0.847 ± 0.02 | 0.662 ± 0.02a | 0.661±0.01 | 0.661 ± 0.06 | 0.753±0.04 | 0.508 ± 0.04a |
| 5.0 | 0.628 ± 0.08 | 0.458 ± 0.02a | 0.358±0.02 | 0.208 ± 0.03a | 0.347±0.03 | 0.194 ± 0.03a |
| 10.0 | 0.531 ± 0.00 | 0.399 ± 0.01a | 0.162±0.01 | 0.062 ± 0.01a | 0.122±0.01 | 0.049 ± 0.01a |
| 20.0 | 0.284 ± 0.01 | 0.162 ± 0.01a | 0.143±0.01 | 0.051 ± 0.00a | 0.084±0.01 | 0.027 ± 0.00a |

a*P* < 0.05 *vs* the control groups. The proliferation of HepG2/ADM cells calculated with SPSS21.0 were presented as mean ± SD from CCK-8 essay in triplicate.