

Effect of mutated I κ B α transfection on multidrug resistance in hilar cholangiocarcinoma cell lines

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Supported by China Postdoctoral Science Foundation, No. 2002031291

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Received: 2004-05-31 **Accepted:** 2004-06-28

Abstract

AIM: To explore the expression effect of mutated I κ B α transfection on multidrug resistance gene (MDR-1) in hilar cholangiocarcinoma cells by inhibiting the activity of nuclear transcription factor- κ B (NF- κ B).

METHODS: We used the mutated I κ B α plasmid to transfect QBC939HCVC+ cells and QBC939 cells, and electrophoretic gel mobility shift assay (EMSA) to detect the binding activity of NF- κ B DNA and the effect of the transfecting mutated I κ B α plasmid on multidrug resistance gene (MDR-1) in hilar cholangiocarcinoma cells and its expression protein (P-GP).

RESULTS: Plasmid DNA was digested by restriction enzymes XbaI and Hind III, and its product after electrophoresis showed two bands with a big difference in molecular weight, with a size of 4.9 kb and 1.55 kb respectively, which indicated that the carrier was successfully constructed and digested with enzymes. The radioactivity accumulation of QBC939HCVC+ and QBC939 cells transfected with mutated I κ B α plasmid was significantly lower than that of the control group not transfected with mutated I κ B α plasmid. Double densitometer scanning showed that the relative signal density between the transfection group and non-transfection group was significantly different, which proved that the mutated I κ B α plasmid could inhibit the binding activity of NF- κ B DNA in hilar cholangiocarcinoma cells. Compared to control group not transfected with m I κ B α plasmid, the expression level of MDR-1 mRNA in the QBC939 and QBC939HCVC+ cells transfected with mutated I κ B α plasmid was lower. The expression intensity of P-GP protein in QBC939 and QBC939HCVC+ cells transfected with mutated I κ B α was significantly lower than that of the control group not transfected with mutated I κ B α plasmid.

CONCLUSION: The mutated I κ B α plasmid transfection can markedly reverse the multidrug resistance of hilar cholangiocarcinoma cells. Interruption of NF- κ B activity may become a new target in gene therapy for hilar cholangiocarcinoma.

Key words: Hilar cholangiocarcinoma; I κ B α ; NF- κ B; MDR-1 gene; Transfection

Chen RF, Li ZH, Kong XH, Chen JS. Effect of mutated I κ B α transfection on multidrug resistance in hilar cholangiocarcinoma cell lines. *World J Gastroenterol* 2005; 11(5): 726-728

<http://www.wjgnet.com/1007-9327/11/726.asp>

INTRODUCTION

With the discovery of the anti-apoptosis effects of nuclear transcription factor- κ B (NF- κ B) and the research on the activity inhibition of NF- κ B, especially the breakthrough progress in the study on the phosphorylation and degradation mechanism of the I κ B α of NF- κ B, a new way to diagnose and treat hilar cholangiocarcinoma has been explored by inhibiting the activity of NF- κ B through gene therapy^[1-3]. Mutated I κ B α means that the amino acid residues of I κ B α at the sites of 32 and 36 are substituted by alanines (S_{32,36}→A), which block the phosphorylation and degradation of I κ B α by IKKs, and exclusively bind to the activated NF- κ B and subsequently suppress the inappropriate or excessive activation of NF- κ B^[4-6]. In this paper, we used mutated I κ B α to block the activation pathway of NF- κ B to explore the effect of mutated I κ B α transfection on multidrug resistance (MDR-1) in hilar cholangiocarcinoma cell lines.

MATERIALS AND METHODS

Materials

QBC939 cells were provided by Professor Wang Shuguang of Southwest Hospital, Third Military Medical University. QBC939HCVC+ cells were prepared by us and stored in our laboratory. *E. coli* DH5 α was stored in our laboratory by general methods. Mutated I κ B α (S32, 36→A) plasmid was presented by Professor Baldwin of North Carolina State University. CLONfectin (Clontech), Trizol (promega), access RT-PCR system (Promega), RNAzol (TEST INC), sense and anti-sense primers of MDR-1 and β 2-M, were synthesized by Shanghai Sangon Bioengineering and Technology Service Co., Ltd.

Methods

Transformation, identification and maxi preps of mutated I κ B α plasmid We used mutated I κ B α plasmid to transform the calcified *E. coli* DH5 α , screen the positive clones, amplify and extract and purify the plasmid DNA. The plasmid was stored at -20 °C, for transfection use.

Cell culture and transfection QBC939 cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), and mutated I κ B α plasmid was transfected to QBC939 and QBC939HCVC+ cell lines in log phase using the liposome-mediated method, and compared with the control group.

EMSA test QBC939 and QBC939HCVC+ cell lines transfected with mutated I κ B α plasmid were examined by electrophoretic gel mobility shift assay (EMSA). Extraction of nuclear protein was referred to the method of Ichikawa *et al*^[7]. The method of Coomassie brilliant blue G250 was used to determine the protein concentration of supernatant. EMSA test was performed

according to operation manual of Promega Company provided with the gel shift assay kit.

Detection of expression of MDR-1 gene in transfected cells with RT-PCR The single-step method of guanidine thiocyanate was used to extract the total RNA, which was then quantitated by an UV spectrophotometer and identified by agarose gel electrophoresis. Reverse transcription-PCR reaction was performed in a sensitive single-tube two-enzyme system, (Promega Company). The reaction volume of 50 μ L contained 20 μ L special RNAase free water, 10 μ L AMV/Inf 5, 1 μ L dNTP mixture (2.5 mmol/L), 10 μ L sense/anti-sense primer mixture, 2 μ L MgSO₄ (25 mmol/L), 1 μ L AMV reverse transcriptase (5 u/ μ L), 1 mL Tfi DNA polymerase (5u/ μ L), 3 μ g RNA sample, MDR-1 sense primer (5'-GTACCCATCATTGCAQATAGC), anti-sense primer (5'-CAAACCTTCTGCTCCTGAGTC-3'). PCR reaction conditions were at 94 $^{\circ}$ C for 40 s, at 56 $^{\circ}$ C for 45 s; at 72 $^{\circ}$ C for 1min for 30 cycles. PCR products were identified by 2% agarose gel electrophoresis, and stained with ethidium bromide (EB). Electrophoresis bands of DNA were analyzed with a gel scanner for determining the photon intensity. The ratio of MDR-1 to β -2-M was regarded as the reference data of expression levels to determine the relative quantity of MDR-1 to PCR products.

Immunohistochemistry determination of the expression of P-GP protein in transfected cells The general immunohistochemistry adopted labeled-streptavidin biotin system (LSAB), and the positive and negative control groups were set up. When over 5% cytoplasm or cell membrane showed brown-yellow in color, the staining was regarded as the positive P-GP.

RESULTS

Identification of mutated I κ B α plasmid

Plasmid DNA was digested by restriction enzymes XbaI and *Hand*III, and its product after electrophoresis showed two bands with a big difference in molecular weight (Figure 1), with a size of 4.9 kb and 1.55 kb respectively, which indicated the carrier was successfully constructed and digested with enzymes.

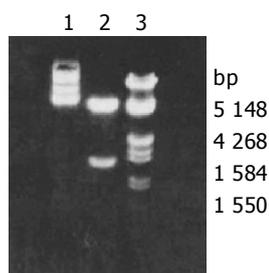


Figure 1 Macrorestriction map of mutated I κ B α Plasmid. Lane 1: Plasmid DNA; lane 2: Plasmid DNA cut by XbaI and *Hand*III; lane 3: DNA marker.

Inhibition of mutated I κ B α plasmid transfection on NF- κ B DNA binding activity

The radioactivity accumulation of QBC₉₃₉HCVC+ and QBC939 cells transfected with mutated I κ B α plasmid was significantly lower than that of the control group not transfected with mutated I κ B α plasmid. Double densimeter scanning showed that the relative signal density between the transfection group and non-transfection group was significantly different, which proved that the mutated I κ B α plasmid had the function of inhibiting the binding activity of NF- κ B DNA in hilar cholangiocarcinoma cells.

In order to examine the specificity of EMSA technology, this experiment set up a negative control and a competitive reaction control. The reaction solution of the negative control without addition of the labeled probe showed no band after electrophoresis (Band 3 in Figure 2). The reaction solution of

the competitive reaction control after adding the 30 \times non-labeled probe showed no avidity band of NF- κ B after electrophoresis (Band 1 in Figure 1). The experiment indicated that the non-labeled probes bound to NF- κ B competitively, causing the labeled probes to decrease remarkably or almost completely. These two experiments proved that the activity detection of NF- κ B in this research had a specificity.

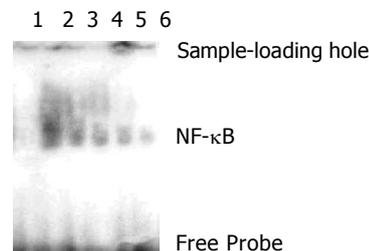


Figure 2 EMSA of QBC₉₃₉ and QBC₉₃₉HCVC+ cells transfected with mutated I κ B α plasmid. Lane 1: Control group; lane 2: QBC₉₃₉HCVC+ cells without transfected with mutated I κ B α ; lane 3: QBC939 cells not transfected with mutated I κ B α ; lanes 4 and 5: QBC₉₃₉HCVC+ cells transfected with mutated I κ B α ; lane 6: QBC939 cells transfected with mutated I κ B α .

Effect of mutated I κ B α plasmid transfection on the expression of MDR-1 and P-GP in hilar cholangiocarcinoma cells

Compared with control group, the expression level of MDR-1 mRNA in QBC939 and QBC939HCVC+ cells transfected with mutated I κ B α plasmid was lower (Figure 3). The expression intensity of P-GP protein in QBC939 and QBC939HCVC+ cells transfected with mutated I κ B α was significantly lower than that in the control group not transfected with mutated I κ B α plasmid (Figures 4A, B).

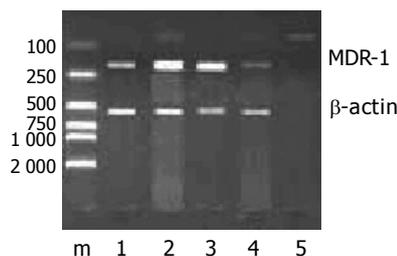


Figure 3 Expression of MDR-1 mRNA in the QBC939 cells transfected with mutated I κ B α . M: DNA marker. Lane 1: The QBC₉₃₉HCVC+ cells transfected with mutated I κ B α ; lane 2: The QBC₉₃₉HCVC+ cells not transfected with mutated I κ B α ; lane 3: The QBC₉₃₉ cells not transfected with mutated I κ B α ; lane 4: The QBC₉₃₉ cells transfected with mutated I κ B α .

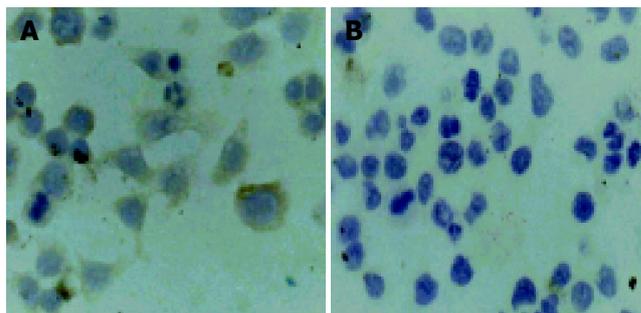


Figure 4 Expression intensity of P-GP protein in QBC939 and QBC939HCVC+ cells transfected with mutated I κ B α . A: Expression of P-GP in the non-transfection Group, LSAB \times 400; B: Expression of P-GP after transfection of mutated I κ B α , LSAB \times 400.

DISCUSSION

I κ B α with a molecular weight of 37KD can be divided into three parts. N-terminal domain can be phosphorylated under signal stimulation and is the basic construction to regulate and control the activity of NF- κ B. In the core of N-terminal domain, there is a repeat sequence comprised of 5 anchored proteins, which are the binding sites of NF- κ B. The C-terminal domain is composed of Pro-Glu-Ser-Thr sequence, which involves in the basic conversion of proteins^[8,9].

In 1995 and 1996, the studies on the mechanism of I κ B α phosphorylation and degeneration made a breakthrough progress, showing that Ser32 and Ser36 play a vital role in the I κ B α phosphorylation and the activation of NF- κ B. Ser32 and Ser36 after mutation are not phosphorylated and degenerated, so that they combine firmly with NF- κ B to stop NF- κ B from entering into the cell nuclei for nuclear transcription^[10]. Therefore, some researchers constructed I κ B α with site mutations at Ser32 and Ser36, which could be used as the super inhibitors. Compared with the I κ B α of wild type, the Ser32 and Ser36 the I κ B α of mutated type are replaced by alanines, the mutated I κ B α is out of the control of I κ B α , continually producing mutated I κ B α , and combines with NF- κ B to form a trimer, reducing the activation level of NF- κ B, which provides a perfect tool for studying the regulation and control of NF- κ B.

The expression of multidrug resistance (MDR) gene in tumor cells is the most crucial obstacle for successful chemical therapy. Once cancer cells express the multidrug resistance gene, drugs with different structures used for chemical therapy would produce drug resistance. MDR-1 is a kind of transmembrane protein P-GP (P-glycoprotein) with a molecular weight of 170kd^[11]. P-GP spreads in tissues. It has been reported that 60% tissues of hilar cholangiocarcinoma could express MDR-1. There are NF- κ B binding sites in the promoter sequence of MDR-1 gene, MDR-1 is under the regulation and control of NF- κ B, which could activate the expression of MDR gene. Therefore, it could be supposed that inhibition of NF- κ B activity may increase the accumulation of poisonous drugs between cells to improve the therapeutic effect.

We have proved that the super activation state of NF- κ B exists in tissues and cells of hilar cholangiocarcinoma. There is evidence that the function of numerous chemical therapeutic drugs for tumor cells is achieved by promoting apoptosis. However, the drugs for chemical therapy can at the same time activate NF- κ B in tumor cells, and activated NF- κ B can motivate

the expression of various anti-apoptosis genes, and reduce the effect of chemical therapeutic drugs. Consequently, people presume that inhibition of NF- κ B can act as an enhancer of chemical therapeutic drugs. Mutated I κ B α plasmid, a inhibitor of NF- κ B, can markedly reduce the expression of MDR-1. The expression of MDR-1 after transfected with mutated I κ B α plasmid is significantly decreased.

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