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Transcriptional suppression of breast cancer resistance protein (BCRP) by wild-type p53 through the NF-κB pathway in MCF-7 cells

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ABSTRACT

Breast cancer resistance protein (BCRP) has been shown to confer multidrug resistance, but the mechanisms of its regulation are poorly understood. Here, we investigate the effects of wild-type and mutant p53, and nuclear factor kappa-B (NF-κB) (p50) on BCRP promoter activity in MCF-7 cells. Our results demonstrated that wild-type p53 markedly suppressed BCRP activity and enhanced the chemosensitivity of cells to mitoxantrone, whereas mutant p53 had little inhibitory effect. After inhibition of NF-κB, similar results were obtained. Following knockdown of endogenous p53, BCRP and p50 expressions were increased, and the chemosensitivity of the cells to mitoxantrone was decreased. We conclude that wild-type p53 acts as a negative regulator of *BCRP* gene transcription.

1. Introduction

The development of multidrug resistance (MDR) by tumor cells is a major obstacle to successful chemotherapy for cancer. One pivotal mechanism by which tumor cells can become resistant to cytotoxic drugs used in chemotherapy is the increased expression of certain ATP-binding cassette (ABC) transporters which including P-glycoprotein (P-gp, MDR1), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP) [1]. These proteins are thought to function as energy-dependent efflux pumps of a variety of structurally diverse chemotherapeutic agents, thereby decreasing intracellular drug accumulation. Comparison of BCRP protein sequences with that of P-gp and MRP revealed that, unlike P-gp and MRP, which are arranged in two repeated halves, BCRP is a half-transporter consisting of only one nucleotide binding domain followed by one membrane-spanning domain. This cell line (MCF-7/AdrVp) was selected by continuous exposure to doxorubicin in combination with verapamil to avoid development of resistance due to expression of P-gp. Interestingly, MCF-7/AdrVp cells also did not express the multidrug resistance-associated protein MRP [2]. BCRP was also discovered by investigating cell lines selected for resistance to mitoxantrone – a poor substrate for P-gp and MRP, which is a high-affinity substrate for BCRP [3]. BCRP expression status is a significant determinant of sensitivity of cancer cells to its substrate anticancer agents.

P53 is a critical tumor suppressor in the human cells that mediates growth arrest, senescence and apoptosis in response to several cellular stresses and often referred to as "the guardian of the genome". Mutations of the p53 tumor suppressor gene have been estimated to occur in close to 50% of human tumors and p53 mutation is not a random process [4]. According to the p53 mutation database (www.iarc.fr/p53), the majority of DNA mutations are located in the core domain responsible for DNA binding, such as codons 175, 245, 248, 249, 273, and 282. The hot spot mutations at codons 175 and 248 occur with highest frequency in human cancers. P53 functions primarily as a transcription factor which exerts its downstream functions by activating or repressing a large number of its downstream genes [5,6]. The majority of the promoter studies seem to confirm a downregulation of the MDR1 or MRP promoter by wild-type p53 (wt-p53) and an upregulation or activation by certain mutants of p53 [7,8].

Up to now, whether *p53* gene can regulate *BCRP* gene expression has not been reported. The mechanisms of BCRP overexpression in drug-resistant cells are not clearly understood. In this study, we investigated the role of *p53* gene in the regulation of human *BCRP* gene expression. Disclosing the relationship between

Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; BCRP, Breast cancer resistance protein; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; NF-κB, nuclear factor kappa-B; RNAi, RNA interference; wt-p53, wild-type p53; PBS, phosphate balanced solution; EMSA, electrophoretic mobility shift assay

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p53 gene and *BCRP* gene could give rise to new evidence for an explanation of the mechanisms of drug resistance.

2. Materials and methods

2.1. Materials

The human breast cancer cell line MCF-7 and JAR (a human choriocarcinoma cell line overexpressing endogeneous BCRP) were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The wild type (wt) p53 expression plasmid, pC53-SN3, and the empty vector, the mutants of pC53–175 and pC53–248 were kindly provided by Dr. Liang Cao at the University of Hong Kong of China and their constructions have been described elsewhere [9]. Plasmid pAVU6 + 27 (a gift of Dr. ML He, University of Hong Kong, China) was used to generate a plasmid that expressing hairpin RNAs. The nuclear factor kappa-B (NF-κB) promoter/luciferase reporter plasmid (pNF-κB) was a gift of Dr. Cao L (University of Hong Kong, China) [10]. pBabe-IκBα was constructed by IκBα mutant gene into EcoRI sites of retroviral plasmid pBabe.

2.2. Constrution of BCRP promoter-luciferase plasmids

Genomic DNA was extracted from the human JAR cells using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The promoter region of the human BCRP gene, a 358 bp fragment was amplified by PCR using the following primers: 5′-CCGCTCGAGGAGGTACT GATCAGCCCAATGAG-3′ and 5′-CCCAAGCTTAGCGCTGACACGAACTTCCTAAG-3′. The promoter fragment was cloned into the Hind III and Xbal I sites in the pGL2-Basic (Promega, Madison, WI). All of the constructed DNAs were purified through Qiagen columns (Qiagen) and confirmed by restruction analysis and sequencing.

2.3. Generation and characterization of p53-overexpression cell lines MCF-7/wtp53

The MCF-7 cells were plated in the wells of a 6-well plates at a density of 1.0×10^5 cells per well and incubated overnight to $90 \sim 95\%$ confluent and transfected with 5.0 μg of wt-p53 expression plasmid DNA or the empty vector DNA for pCMV-Neo-Bam by using Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, G418 (800 μg/ml) was added to the medium and was supplemented in the media throughout the cloning process. After growing the cells in the presence of G418 for 3 weeks, a few colonies formed in plates transfected with p53 plasmids, compared with about 100 times more colonies with empty vector transfection. Individual colonies were isolated with a cloning ring from the transfection plates and expanded as established cell lines (MCF-7/wtp53, and MCF-7/pCMV). Other cell lines established similar to the above method.

2.4. Transient transfection and luciferase reporter gene assays

MCF-7 cells were plated in the wells of a 24-well plates at a density of 1.0×10^5 cells per well and incubated overnight to $90 \sim 95\%$ confluent. Using Lipofectamine 2000 kit (Invitrogen) as instructed by the manufacturer, plasmid DNA (2.0 μg) and DNA for cotransfection were transfected into the cells. Luciferase plasmids were cotransfected with 1.0 μg β-galactosidase or Renilla control vector (Promega) to monitor transfection efficiency. For luciferase assays, cells were washed three times with 2.0 ml of ice-cold phosphate balanced solution (PBS) and were lysed in 150 μl of lysis buffer. Samples were assayed in a Lumi-Scint Luminometer (Bioscan, Washington, USA) using 20 μl of cell lysate and 100 μl of Luciferase assay reagent (Promega). Luciferase activity was measured a 10 s delay and 30 s integration time and was normalized to β-galactosidase or Renilla luciferase activity to determine transfection efficiency.

2.5. Transfection of RNA interference plasmid

The short-hairpin-RNA-encoding complementary single-stranded oligonucleotides corresponding to p53 were designed according to the literatures [11]. These oligonucleotides 5′-tcgacGACTCCAGTGGTAATCTACttcaagagaGTAGATTACCACTGGAGTCtttttt-3′ and 5′-ctagaaaaaGACTCCAGTGGTAATCTACtctcttgaaGTAGATTACCACTGGAGTCG-3′; 5′-tcgacAGCTTCATAAGGCGCATGCttcaagagaGCATGCGCCTTATGAAGCTttttt-3′ and 5′-ctagaaaaaAGCTTCATAAGGCGCATGCtctcttgaaGCATGCGCCTTATGAAGCTG-3′, were annealed and cloned into pAVU6+27 vector to generate p53 (pAVU6 + 27/sip53) and control construction (pAVU6+27/sicontrol), respectively. Then, MCF-7 cells were transfected with the above constructions using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

2.6. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) assay

Total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen) and quantified by UV absorbance spectroscopy. BCRP, NF-κB (p50), β-actin mRNA transcripts were detected using RT-PCR assays. A 206 bp BCRP cDNA fragment was amplified with the primers 5′-CACCTTATTGGCCTCAGGAA-3′ (sense) and 5′-CCTGCTTGGAAGGCTCTATG-3′ (antisense); a 176 bp NF-κB (p50) cDNA fragment with the primers: 5′-CTGGAAGCACGAATGACAGA-3′ (sense) and 5′-TGAGGTCCATCTCCTTGGTC-3′ (antisense); a 194 bp p53 cDNA fragment with the primers: 5′-CCAGCCAAAGAAGAAACCAC-3′ (sense) and 5′-TATGGCGGGAGGTAGACTGA-3′ (antisense). As an internal control, amplification of β-actin mRNA (309 bp) was carried out with the primers 5′-ACCGTGGAGAAGAGCTACGA-3′ (sense) and 5′-GTACTTGCGCTCAGAAGGAG-3′ (antisense).

2.7. Western blot and electrophoretic mobility shift assays (EMSA)

Western blotting and EMSA analyses were carried out as previously described [12,13]. Biotin-labeled double-strand NF-κB oligonucleotides (5′-CCCGACCTGGGGAAACCCGGGCGCTGGGG-3′) containing the NF-κB (p50) binding site in the BCRP promoter and mutant NF-κB oligonucleotides (5′-CCCGACCTTTTTTAATTTGGGGCGCTGGGG-3′) were obtained from Zhaorui Biotech Co., Ltd. (Shanghai, China) and used as probes (underlining indicates NF-κB binding site).

2.8. Chromatin immunoprecipitation assay (ChIP)

Immunoprecipitation experiments with an anti- NF-κB (p50) antibody (Santa Cruz Biotech., CA) to examine protein-DNA interactions were done using the chromatin immunoprecipitation assay kit (Upstate, Charlottesville, VA) following manufacturer's instructions. To analyze the target regions (NF-κB-binding site at −27/−18 regions in the BCRP promoter), the DNA samples were amplified by PCR, forward; 5′-CGGGAGTGTTTGGCTTGTC-3′; reverse: 5′-CAGAGCTGAACGCAGTGGC-3′ that generated a 233-bp PCR product.

2.9. Cytotoxicity assay

Cytotoxicity of mitoxantrone on cells was assessed by MTT as described previously [14]. Cells were counted and then cultured in 96-wells plate, and treated with those anticancer drugs of