Survivin up-regulates the expression of breast cancer resistance protein (BCRP) through attenuating the suppression of p53 on NF-κB expression in MCF-7/5-FU cells

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A B S T R A C T
Both breast cancer resistance protein (BCRP, ABCG2) and apoptosis-related molecules are involved in the development of multidrug resistance (MDR) in cancer cells. However, the association of BCRP with apoptosis-related molecules in the development of MDR is unknown. In this study, we investigated the changes in expression levels of BCRP, Survivin, p53, Bcl-2, Bcl-xL, or Bax in cultured MCF-7 and MCF-7/5-FU cells, and explored whether these changes affected the expressions of BCRP. Our data showed that the protein and mRNA expression levels of BCRP, Survivin and Bcl-2 were significantly higher in MCF-7/5-FU cells than in MCF-7 cells, while p53 expression lower in MCF-7/5-FU cells than in MCF-7 cells. Knockdown of Survivin or Bcl-2 in MCF-7/5-FU cells and overexpression of Survivin in MCF-7 cells revealed that Survivin had significant association with BCRP expression. Luciferase reporter gene assays showed that Survivin up-regulated BCRP expression at transcriptional level and this response was mediated through NF-κB(p50) pathway. However, may be due to the physical interaction between p53 and Survivin, p53 directly affected Survivin-regulated BCRP expressions. Interestingly, we found that Survivin would suppress p53 expression. Furthermore, our data revealed that Survivin itself had no apparent effect on NF-κB(p50) expression when knockdown of p53 in MCF-7 cells; whereas p53 exerted significant inhibitory action on these when knockdown of Survivin. In conclusion, through down regulation of p53 expression, Survivin attenuates the suppressing effect of p53 on NF-κB(p50) expression and then enhances BCRP expression. This may represent a novel strategy for reversal of BCRP drug transporter activity to modulate MDR in cancer cells.

1. Introduction

Breast cancer, the most commonly diagnosed type of cancer, is the second leading cause of cancer-related deaths in women, accounting for 26% of all new cancer cases (Noordhuis et al., 2004). Chemotherapy plays an important role in the management of breast cancer, but the efficacy of this treatment is limited by drug resistance, especially the multiple drug resistance (MDR) (Ross, 2000; Stavrovskaya, 2000; Banerjee and Bertino, 2002).

MDR in tumor treatment is characterized by resistance to a broad spectrum of structurally unrelated cytotoxic drugs, leading to inefficient killing of the cancer cells and chemotherapeutic failure in patients with solid tumors, such as breast cancers (Kuo, 2007; Chuthapisith et al., 2006). Overexpression of membrane ATP-binding cassette (ABC) family, which functions as an energy-dependent efflux pump to extrude antitumor agents from the cytoplasm, thus reducing intracellular drug concentrations to sublethal levels, is one of the mechanisms in development of MDR (Doyle et al., 1998; Natarajan et al., 2012; Schinkel and Jonker, 2003; Doyle and Ross, 2003). There are three human ABC transporters primarily associated with the MDR phenomenon, namely P-glycoprotein (P-gp, MDR1), multidrug resistance-associated...
protein (MRP) and breast cancer resistance protein (BCRP, ABCG2) (Doyle et al., 1998; Natarajan et al., 2012; Schinkel and Jonker, 2003; Doyle and Ross, 2003). Among these ABC transporters, BCRP is a characterized xenobiotic half-transporter protein identified first in breast cancer cell line MCF-7/AdrVp and has a MDR phenotype despite the addition of a P-gp blocking agent (Doyle et al., 1998). Multiple studies have shown that BCRP may confer resistance to mitoxantrone (MX), anthracyclines, methotrexate (MTX), topoisomerase I inhibitors, gefitinib, doxorubicin, and 5-FU, which is particularly relevant to the problem of MDR in breast cancer (Doyle and Ross, 2003; Ji et al., 2010; Yuan et al., 2009). Therefore, BCRP expression status appears to be a significant determinant of sensitivity of cancer cells to its substrate anticancer agents (Robey et al., 2007; Natarajan et al., 2012), and blocking BCRP-mediated active efflux may provide a therapeutic benefit for cancers.

Nevertheless, despite promising preclinical and early clinical data, reversing agents for ABC transporters have failed to show important benefits in terms of response to chemotherapy or the length of survival in oncologic practice (Yu et al., 2013). This indicates that unknown molecules or mechanisms are responsible for the development of MDR. In fact, BCRP gene transcription is regulated by many transcription trans-activating factors in the BCRP promoter (Natarajan et al., 2012). Hence, quite a few molecules susceptible to affect these BCRP gene trans-activating factors may alter BCRP expression and then influence BCRP-mediated MDR. As a result, investigating the precise molecular mechanisms for BCRP gene expression may lead to identification of a novel molecular target to modulate BCRP-mediated MDR.

Current evidences have suggested that most chemotherapeutic agents exert their anticancer activity by inducing apoptosis; therefore, anti-apoptosis may be a major factor limiting the effectiveness of reversing agents for ABC transporters in cancer therapy. For example, overexpression of Bcl-2 or Bcl-xL (Alla et al., 2012; Wersig et al., 2007), down-regulation of Bax (Chandrika et al., 2010), mutations or deletion of p53 (Jones et al., 2012; Metzinger et al., 2006), and overexpression of Survivin (Zheng et al., 2012), have been demonstrated to inhibit apoptosis and be involved in the development of MDR. However, few studies have explored the association of BCRP with apoptosis-related molecules in the development of MDR.

In present study, we, therefore, investigate whether the changes in expression levels of Survivin, Bcl-2, Bcl-xL or Bax affect the expression of BCRP by means of cultured MCF-7 cancer cells and 5-fluorouracil (5-FU)-resistant MCF-7/5-FU cells.

2. Materials and methods

2.1. Reagents

Culture medium RPMI-1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), OPTI-MEM medium, fetal bovine serum and Lipofectamine 2000 were purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). The plasmid pcDNA3-myc was purchased from Invitrogen Inc., and the plasmid pSUPERIOR was purchased from Clontech Co. (Mountain View, CA, USA). Anti-human Survivin antibody, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-p53, anti-NF-κB(p50), anti-β-actin, HRP-conjugated antimouse, anti-rabbit, anti-goat IgG, and Protein A/G plus-agarose were purchased from Santa-Cruz Biotechnology Inc. (Santa Cruz, CA, USA), p53 and NF-κB(p50) siRNA plasmid were purchased from Santa-Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Survivin antagonist YM155 was purchased from Selleck Chemicals LLC (Houston, TX, USA). Mouse monoclonal anti-human BCRP antibody was purchased from Calbiochem Inc. (La Jolla, CA, USA). Chemiluminescent Western Detection kit was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The Plasmid Midi kit was purchased from Qiagen Inc. (Valencia, CA, USA). BCRP promoter luciferase plasmid (pBCRP-luc), wild-type p53 plasmid (p53-SN3), the mutants of p53-175 and p53-248 have been described in our previous study (Wang et al., 2010).

2.2. Cell culture

MCF-7, a human breast adenocarcinoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and its 5-fluorouracil resistant cell line MCF-7/5-FU cells were cloned from parental drug-sensitive MCF-7 cells. Briefly, MCF-7/5-FU cells were developed by step-wise selection as previously described (Zheng et al., 2010). MCF-7 cells were routinely maintained in DMEM medium and MCF-7/5-FU cells were cultured in RPMI 1640. Both media were supplemented with 10% fetal bovine serum. In addition, cells were also cultured in the above media containing Survivin antagonist YM155 (20 nmol/L). The cells were grown in a humidified atmosphere at 37° C in 5% CO2.

2.3. Knockdown of the gene expression by RNA interference

To knockdown gene expression, a short oligonucleotide that targets human Survivin messenger RNA (Survivin siRNA) 5′-TGAGAGCAGCATGACC-3′ and its scrambled siRNA sequence 5′-AAAGTTGTTGTTTGTACT-3′, Bal-2 siRNA sequence 5′-GTACATCATTATAAAGGTGT-3′ and its scrambled siRNA sequence 5′-ACTACGTGTATAGCTGT-3′, p53 siRNA sequence 5′-GACCTCCAGTGAATCTAC-3′ and its scrambled siRNA sequence 5′-TTCCTCCAGGCTGTCAGT-3′, NF-κB(p50) siRNA sequence 5′-GACATGTAATCTACATG-3′ and its scrambled siRNA sequence 5′-TTTCCTCCAGGCTGTCAGT-3′ were used. These siRNAs or scrambled siRNAs were inserted into the pSUPERIOR vector to generate pSUPERIOR-siRNA or pSUPERIOR-siRNA plasmids, and the insertions were confirmed by DNA sequence analysis. Transfections were performed by electroporation using Nucleofector system (Amansa Biosystems, Cologne, Germany) according to the manufacturer’s protocol. The transfected cells were selected using neomycin for 2 weeks, and the expressions of miRNA and protein were determined using quantitative reverse transcription real-time PCR and Western blot analyses. MCF-7/5-FU cells transfected with pSUPERIOR-Survivin-siRNA were named MCF-7/5-FU/siSVV cells. MCF-7/5-FU cells transfected with pSUPERIOR-siRNA were named MCF-7/5-FU/siSVV-con cells. MCF-7/5-FU cells transfected with pSUPERIOR-Bcl-2-siRNA were named MCF-7/5-FU/siBcl2 cells. MCF-7/5-FU cells transfected with pSUPERIOR-siRNA were named MCF-7/5-FU/siBcl2-con cells and so on.

2.4. Overexpression of gene in MCF-7 cells

Overexpression of p53 in MCF-7 cells has been described in our previous study (Wang et al., 2010). To overexpress Survivin in MCF-7 cells, the full-length coding region of Survivin was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primers (GenBank: NM_001168; forward primer 5′-GAATTCAGTCGGGACACCTTG-3′; reverse primer 5′-CTTCTCCAGGACCAGAGTCCG-3′) with internal EcoRI/Xhol sites (underlined). After being cloned by vector pGEM-T (Promega, Madison, WI, USA), the Survivin cDNA fragment was inserted into the EcoRI/Xhol site of the pcDNA3-myc expression vector. The full-length sequence was determined by sequencing. The pcDNA3-myc or pcDNA3-myc-survivin plasmids were transfected into MCF-7 cells using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. 1.0 mg/ml of genetin sulfate (Invitrogen) was added. Geneticin-resistant clones were obtained.
Table 1
The primer sequence for real-time PCR.

<table>
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<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Access number</th>
<th>Annealing temperature</th>
<th>Product</th>
</tr>
</thead>
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<td>BCRP</td>
<td>Forward: 5'-TGGCTTACACCTCAAGCAGCAGC-3'</td>
<td>NM_004827</td>
<td>57 °C</td>
<td>67 bp</td>
</tr>
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<td></td>
<td>Reverse: 5'-TGCTCGCCATTGCAATGTTG-3'</td>
<td></td>
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<td></td>
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<tr>
<td>Survivin</td>
<td>Forward: 5'-AAAGAAGGCGGCGGCTTCTGCA-3'</td>
<td>NM_001168</td>
<td>60 °C</td>
<td>185 bp</td>
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<tr>
<td>Bcl-2</td>
<td>Forward: 5'-ATGGTCTGAGGGCCCTGCAACC-3'</td>
<td>NM_000633</td>
<td>58 °C</td>
<td>196 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGAGGACATCTTAGAGAGACCC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bax</td>
<td>Forward: 5'-CAAAGCTTGTGCTGAGAGGC-3'</td>
<td>NM_138763</td>
<td>58 °C</td>
<td>188 bp</td>
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<td>NM_138578</td>
<td>58 °C</td>
<td>100 bp</td>
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<tr>
<td>Bcl-xl</td>
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<td>AB082923</td>
<td>57 °C</td>
<td>194 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ATGGGCAATGAGGGGTC-3'</td>
<td>NM_001101</td>
<td>57 °C</td>
<td>76 bp</td>
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</table>

by limiting dilution method. The selected cells were harvested to determine the expression of Survivin using reverse transcription real-time PCR and Western blot analyses. MCF-7 cells transfected with pcDNA3-myc-survivin were named MCF-7/SV cells. MCF-7 cells transfected with pcDNA3-myc were named MCF-7/SV-con cells.

2.5. Western blot analysis

Cells treated under various conditions were lysed with a lysis buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 100 mM NaF, 2.0 mM EDTA, 1% NP-40, 1.0 μg/ml leupeptin, 1.0 μg/ml antipain, and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). The protein content in the cell lysates was determined using a BCA protein-assay kit. The extracts (40 μg of protein) were fractionated on polyacrylamide–SDS gels and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked with a solution containing 3% skim milk and incubated overnight at 4 °C with each of the following antibodies: anti-BCRP, anti-Bcl-2, anti-Bcl-xl, anti-Survivin, anti-Bax, anti-p53 and anti-NF-κB/p50. Subsequently, the membranes were incubated with horseradish peroxidase-coupled anti-rabbit or mouse IgG antibodies (Amersham Biosciences) for 1 h at room temperature. The reactive proteins were visualized using ECL plus (Amersham Biosciences) according to the manufacturer’s instructions. As an internal standard, anti-β-actin mouse mono-clonal antibody was used as the primary antibody to detect β-actin protein.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA was isolated using TRIZOL (Invitrogen). One microgram of purified total RNA was used for the real-time PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). cDNA was subjected to quantitative real-time PCR by using SYBR Premix Ex Taq (Takara Biomedical, Siga, Japan) and the ABI Prism 7000 detection system (Applied Biosystems, Foster, CA) in a 96-well plate according to the manufacturer’s instructions. Primers were designed and validated by Invitrogen Biotechnology Co. Ltd. The sequences of primers used in reverse transcription PCR were listed in Table 1. As an internal control for each sample, the β-actin gene was used for standardization. Cycle threshold (Ct) values were established, and the relative difference in expression against β-actin expression was determined according to the 2–ΔΔCt method of analysis and compared with the expression in control cells. All assays were done in triplicate.

2.7. Luciferase reporter gene assay

MCF-7 cells were plated in the wells of a 24-well plate at a density of 5 × 10^4 cells per well and incubated overnight to subconfluent. One microgram of luciferase reporter plasmid containing fragments of the Survivin plasmid DNA (pcDNA3-myc-Survivin), or the control-luciferase plasmid (pcDNA3-myc-con), pBCRP-luc plasmid, in combination with 1 μg of pRL-TK renilla plasmid (Promega), were transfected into MCF-7 cells using the Lipofectamine 2000 reagent (Invitrogen) according to a protocol provided by the manufacturer. Luciferase and renilla signals were measured at 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instructions. Three independent experiments were performed and the data are presented as mean ± SD.

2.8. Immunoprecipitation (IP) assay

Protein preparations were similar to those for Western blotting. 1.0 mg of total protein was incubated with 20 μl of control IgG or anti-BCRP, anti-Survivin or anti-p53 antibody overnight at 4 °C with gentle rocking after adjusting the volume to 1.0 ml with ice-cold RIPA buffer. Then 20 μl protein A/G-plus-agarose was added to the complex with incubation at 4 °C for 2 h on the rocker platform. The immune complex was collected by centrifugation at 1000 × g for 30 s at 4 °C and washed three times with PBS, and then resuspended with sample loading buffer and boiled for 10 min and resolved by SDS-PAGE and electrotransferred onto PVDF membrane as previously mentioned. The membrane was incubated and immunoblotted with anti-BCRP, anti-Survivin or anti-p53 antibody. Protein–antibody complex was detected by chemiluminescence.

2.9. Statistical analysis

All experiments were repeated at least three times. SPSS 13.0 was used for the statistical analyses. Data are presented as the mean ± SD. Comparisons were made using a one-way ANOVA or paired t test. p < 0.05 was considered statistically significant.

3. Results

3.1. Expression levels of BCRP, Survivin, Bcl-2 and p53 in MCF-7/5-FU cells

Western blot analysis (Fig. 1A) illustrated that BCRP protein could be detected in both the MCF-7 and MCF-7/5-FU cells, but BCRP protein was obviously overexpressed in anticancer drug-resistant
MCF-7/5-FU cells, suggesting that overexpression of BCRP is an important molecular mechanism involved in the development of MDR in MCF-7/5-FU cells.

However, some anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bax were also associated with the development of MDR. In an attempt to investigate whether the expression levels of these proteins in MCF-7/5-FU cells were different from their parental sensitive cells, we compared several well-documented resistance-related molecules, namely, Survivin, Bcl-2, Bcl-xL, Bax and p53 expression between MCF-7/5-FU cells and MCF-7 cells.

**Fig. 1.** The expression of BCRP, Survivin, Bcl-2, Bcl-xL, Bax and p53 in MCF-7/5-FU and MCF-7 cells. (A) Western blotting analysis: samples (40 μg) of the total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and incubated with primary antibodies against BCRP, Bax, Bcl-2, Bcl-xL, Survivin, p53 and β-actin, and then with a horseradish peroxidase-conjugate as the secondary antibody. (B) qRT-PCR analysis of the mRNA expressions of BCRP, Survivin, Bcl-2, Bax, Bcl-xL and p53 in MCF-7/5-FU and MCF-7 cells. Total RNA was extracted, and the various mRNA levels were determined by real-time polymerase chain reaction. The result was representative of 5 independent experiments. An asterisk indicates a significant difference when compared with the control (**p < 0.01).
and MCF-7 cells. As shown in Fig. 1A, Survivin and Bcl-2 were much higher in MCF-7/5-FU cells than those in MCF-7 cells, whereas p53 was lower in MCF-7/5-FU cells than in MCF-7 cells. Bcl-xL and Bax had no significant difference between MCF-7/5-FU and MCF-7 cells.

The mRNA expressions of BCRP, Survivin, Bcl-2, Bcl-xL, Bax and p53 are shown in Fig. 1B. BCRP, Survivin and Bcl-2 mRNA were significantly higher in MCF-7/5-FU cells than in MCF-7 cells, p53 mRNA was lower in MCF-7/5-FU cells than in MCF-7 cells (all \( p < 0.01 \)). Bcl-xL and Bax mRNA expressions had no significant difference between MCF-7/5-FU and MCF-7 cells.

These results indicated that BCRP, Bcl-2, p53 and Survivin might co-participate in the 5-FU induced multidrug resistance of MCF-7 cells.

3.3. Survivin regulates BCRP expression at the transcriptional level

To further test the effect of Survivin on the BCRP gene expression, we performed luciferase reporter gene assays. The outcomes are shown in Fig. 3. When MCF-7 cells were co-transfected with different doses of pcDNA3-myc-Survivin expression plasmid and BCRP promoter luciferase (pBCRP-luc) reporter construct, the transcriptional activity of the BCRP promoter was gradually upregulated by Survivin in a dose-dependent manner (Fig. 3 lanes 2–4 versus lane 1) (\( p < 0.05 \) or \( p < 0.01 \)). These outcomes confirmed that Survivin could regulate BCRP expression and indicated the Survivin-regulated BCRP expression was done at the transcriptional level.

3.2. Survivin but not Bcl-2 has significant association with BCRP expression

Our previous study had shown that p53 suppressed BCRP transcription through NF-κB(p50) pathway. In this study, we explored whether the expression change of Bcl-2 or Survivin had association with BCRP expression.

First, we knocked down the expression of Bcl-2 or Survivin in MCF-7/5-FU cells by using Bcl-2 or Survivin siRNA. As shown in Fig. 2A and B, MCF-7/5-FU cells showed significantly decreased mRNA and protein levels of BCRP and Survivin following transfection with the Survivin siRNA (all \( p < 0.01 \)). However, the mRNA and protein expression of BCRP remained unchanged in MCF-7/5-FU cells transfected with the Bcl-2 siRNA, except that Bcl-2 distinctly decreased. Meanwhile, in the mock siRNA transfected group, neither BCRP nor Survivin or Bcl-2 mRNA and protein expression levels were changed.

Further, we introduced the Survivin gene expression vector, pcDNA3-myc-Survivin, into MCF-7 cells to investigate the effect of the overexpression of Survivin on the expression of BCRP. After transfection with pcDNA3-myc-Survivin, the mRNA and protein expression of BCRP and Survivin significantly increased in MCF-7 cells (all \( p < 0.01 \)). While the BCRP or Survivin mRNA and protein expressions in MCF-7 cells were not altered in the empty plasmid pcDNA3-myc transfected group (Fig. 2C and D).

These results indicated that Survivin but not Bcl-2 had significant association with BCRP expression.

Fig. 4. Survivin-regulated BCRP gene transcription through NF-κB pathway. MCF-7 cells were transfected with pcDNA3-myc-Survivin expression plasmid (pcDNA-Survivin) in the presence or absence of Survivin antagonist YM155, or co-transfected with NF-κB(p50) siRNA plasmid or its corresponding scrambled siRNA. After being cultured, the expression of Survivin, NF-κB(p50) and BCRP were determined using Western blot analysis and quantitative reverse transcription real-time PCR (qRT-PCR) analysis. (A) Western blot analysis for Survivin, NF-κB(p50) and BCRP protein expression in MCF-7 cells (control), or cells transfected with pcDNA-Survivin in the presence or absence of Survivin antagonist YM155 or co-transfected with pcDNA-Survivin and NF-κB(p50) siRNA plasmid or its corresponding scrambled siRNA; (B) qRT-PCR analysis for Survivin, NF-κB(p50) and BCRP mRNA expression in MCF-7 cells (control), or cells transfected with pcDNA-Survivin in the presence or absence of Survivin antagonist YM155 or co-transfected with pcDNA-Survivin and NF-κB(p50) siRNA plasmid or its corresponding scrambled siRNA. Values were expressed with mean ± SD (\( n = 3 \)). Statistical significance compared with MCF-7 cells: *\( p < 0.05 \), **\( p < 0.01 \), compared with MCF-7 cells; *\( p < 0.01 \) compared with cells transfected with pcDNA-Survivin in the absence of Survivin antagonist YM155.
3.4. Survivin up-regulates BCRP gene transcription through NF-κB(p50) pathway

The results above demonstrated that Survivin BCRP expression was done at the transcriptional level. However, Survivin is not a classic transcription factor therefore it is unlikely to bind to the BRCR promoter to induce transcription. To gain further insight into the mechanisms, we transfected MCF-7 cells with pcDNA3-myc-Survivin expression plasmid in the presence or absence of Survivin antagonist YM155, or co-transfected MCF-7 cells with pcDNA3-myc-Survivin expression plasmid and NF-κB(p50) siRNA to test whether Survivin up-regulated BCRP gene transcription would be mediated through NF-κB(p50) pathway (Fig. 4).

Compared to the controls, MCF-7 cells transfected with pcDNA3-myc-Survivin expression plasmid had significant increase of NF-κB(p50) protein and mRNA levels (p < 0.01). Correspondingly, the mRNA and protein levels of BCRP were also up-regulated by Survivin. However, both the expression levels of NF-κB(p50) and BCRP up-regulated by Survivin were attenuated when Survivin antagonist YM155 was added into the culture media (p < 0.01). Knockdown of NF-κB by using NF-κB(p50) siRNA had the similar effects with YM155. Moreover, the up-regulatory effects of Survivin on BCRP expression were almost completely blocked when MCF-7 cells were co-transfected with pcDNA3-myc-Survivin expression plasmid and NF-κB(p50) siRNA.

These results suggested that Survivin up-regulated BCRP gene transcription was mediated through NF-κB(p50) pathway.

3.5. Survivin-induced BCRP expressions are directly associated with the expression levels of p53

Since both Survivin and p53 regulated BCRP transcription through NF-κB(p50) pathway, we hypothesized that p53 might be directly associated with the Survivin-regulated BCRP expressions. To test this hypothesis, we first determined whether p53 would physically interact with Survivin using immunoprecipitation assays. Fig. 5A shows that the protein immunoprecipitated from the cell lysates of MCF-7/5-FU cell using anti-BCRP antibody was found no formation of complex with anti-Survivin. The same, the protein immunoprecipitated using anti-Survivin antibody was found no formation of complex with anti-BCRP (Fig. 5B). However, the protein immunoprecipitated using anti-Survivin antibody was found to form complex with anti-p53 (Fig. 5B). Also, the protein immunoprecipitated using the anti-p53 antibody formed complex with anti-Survivin (Fig. 5C). Because neither BCRP nor p53 or Survivin was contained in the control IgG immunoprecipitation complex, the immunoprecipitation of p53 with Survivin was specific. These findings showed that Survivin directly interacted with or bound to p53.

Further, we examined whether the expressions of p53 would affect Survivin to activate BCRP promoter in MCF-7 cells using luciferase reporter gene assays. As shown in Fig. 3, when different doses of wild-type p53 (pcP53-SN3) or mutant p53 (pcP53-175 and pcP53-248) expression plasmids, pcDNA3-myc-Survivin expression plasmid and BCRP promoter luciferase (pBCRP-luc) reporter construct were co-transfected into MCF-7 cells, wild-type p53 limited, in a dose-dependent manner, the activities of the BCRP promoter (Fig. 3 lanes 5–7 versus lane 4) (p < 0.05 or p < 0.01), which suggested that the expression levels of p53 would directly affect Survivin-induced BCRP expressions.

3.6. Survivin induces BCRP expression through attenuating the suppression of p53 on NF-κB(p50) expression

Since the expression levels of p53 would directly affect Survivin-induced BCRP expressions, we first investigated whether...
Survivin overexpression would repress p53 expression in MCF-7 cells. Fig. 6 shows the expressions of p53 detected in MCF-7 cells by transfection with the plasmids which coded the Survivin gene or Survivin siRNA. MCF-7 cells transfected with the pcDNA3-myc-Survivin expression plasmid (MCF-7/SVV cells) showed significantly decreased protein and mRNA levels of p53 compared to the control cells transfected with the empty plasmid pcDNA3-myc (MCF-7/SVV-con cells) (p < 0.05); whereas, the cells transfected with Survivin siRNA (MCF-7/siSVV cells) exhibited distinctly increased protein and mRNA levels of p53 compared to the control cells transfected with the mock siRNA (MCF-7/siSVV-con cells) (p < 0.05).

To further clarify the effect of p53 on Survivin-induced BCRP expression, we investigated the expression levels of NF-κB(p50) and BCRP when MCF-7 cells were co-transfected with pcDNA3-myc-Survivin expression plasmid or empty plasmid pcDNA3-myc and p53 siRNA plasmid, or co-transfected with wild-type p53 (pC53-SN3) or its corresponding scramble plasmid and Survivin siRNA plasmid, respectively. As shown in Fig. 7A and B, cells co-transfected with Survivin expression plasmid and p53 siRNA plasmid had significantly higher expression levels of Survivin protein and mRNA compared to cells co-transfected with Survivin empty plasmid and p53 siRNA plasmid (all p < 0.01); however, the expression levels of NF-κB(p50) and BCRP had no apparent difference between these two groups (all p > 0.05). These results suggested that Survivin had no direct effect on NF-κB(p50) expression/function. In cells co-transfected with wild-type p53 expression plasmid and Survivin siRNA plasmid, the expression levels of p53 protein and mRNA are significantly higher than those in cells co-transfected with scramble p53 plasmid and Survivin siRNA plasmid, whereas both the expression levels of NF-κB(p50) and BCRP are significantly lower than those in cells co-transfected with scramble p53 plasmid and Survivin siRNA plasmid (all p < 0.01) (Fig. 7C and D).

These data demonstrated that Survivin-induced BCRP expression was mediated through down-regulation of p53 expression and then abrogating p53-suppressed NF-κB(p50) expression.

4. Discussion

In the present study, we demonstrated that the expression of BCRP were up-regulated by the overexpression of Survivin in the anticancer drug-resistant cell line MCF-7/5-FU compared with its parental cell line MCF-7, and the change in up-regulated BCRP expression elicited by the overexpression of Survivin might be mediated through down regulation of p53 expression and then attenuation of the suppressing effect of p53 on NF-κB(p50) expression. This suggested that Survivin might be involved in the regulation of the BCRP-mediated MDR.

BCRP has emerged as an important MDR factor because it confers cross-resistance to several structurally unrelated classes of cancer chemotherapeutic agents. However, reversal of ABC drug transporter has failed to provide clinical benefit (Yu et al., 2013), indicating that there may be other mechanisms or unknown molecules responsible for MDR. Indeed, due to the fact that most chemotherapeutic agents exert their anticancer activity by inducing apoptosis, effort has been made to understand the actions of anti-apoptotic/apoptotic pathways in the development of MDR (Alla et al., 2012; Wesarg et al., 2007; Chandrika et al., 2010; Jones et al., 2012; Metzinger et al., 2006). Furthermore, the evidences
that various molecular mechanisms of MDR co-express in malignant cells have been discovered by several studies (Huang et al., 1997; Chauhan et al., 2012). Nevertheless, these, almost no study has explored the association of BCRP with apoptosis-related molecules in MDR cancer cells.

In the present study, our results showed that the MDR cancer cells MCF-7/5-FU expressed higher levels of BCRP. Survivin and Bcl-2 compared with its parental cell line MCF-7; meanwhile, lower levels of p53 were also detected in MCF-7/5-FU cells. As a result, the development of MDR in MCF-7/5-FU cells might be associated with several molecules or mechanisms. This phenomenon is similar with the studies by Huang et al. (1997) and Chauhan et al. (2012).

Interestingly, when we selectively knocked down the expression of Survivin, MCF-7/5-FU cells showed significantly decreased the mRNA and protein levels of BCRP; whereas the mRNA and protein expression of BCRP remained unchanged in these cells knocked down for Bcl-2. On the contrary, the mRNA and protein expressions of BCRP were found to be increased in MCF-7 cells transfected with Survivin expression plasmid. These findings indicated that Survivin but not Bcl-2 might be involved in the regulation of the BCRP expression.

Survivin, a unique member of the inhibitor of apoptosis protein (IAP) family, is highly expressed in cancers but undetectable in most normal adult tissue and is known to negatively regulate apoptosis by directly or indirectly binding and inhibiting the caspase within the mitochondria (Altieri, 2003; O’Connor et al., 2000; Song et al., 2003). Besides apoptosis inhibition, Survivin controls cell proliferation and promotes angiogenesis (Ryan et al., 2006). As a result, Survivin has been argued to function as a multifunctional protein (Ryan et al., 2006). Due to this multifunction, it is not surprising that the increased Survivin expression in cancers is closely associated with tumorigenesis, poor prognosis, and drug resistance. In fact, studies have shown that patients who have tumors with high Survivin mRNA expression reveal shorter survival and increased rate of recurrence (Ikeguchi and Kaibara, 2002; Swana et al., 1999).

Moreover, Survivin is involved in the resistance to chemotherapy and may serve as a radio- and chemo-resistance factor (Zhang et al., 2005; Tran et al., 2002; Rodel et al., 2003; Altieri, 2006). Thus, Survivin potentially plays a key role in resistance to anticancer drugs. Remarkably, our present data further indicated that the role of Survivin in resistance to anticancer drugs might partially be due to Survivin-enhanced BCRP expression.

Generally, gene expression can be regulated at many different levels, but the regulation of BCRP expression is frequently initiated at the level of transcription (Natarajan et al., 2012). In the present study, our luciferase reporter gene assays showed that Survivin was also able to up-regulate the transcriptional activity of BCRP promoter in MCF-7 cells. Nevertheless, Survivin is not a classic transcription factor; it is unlikely to bind to the BCRP promoter to induce BCRP transcription. Therefore, we presume that Survivin-regulated BCRP gene transcription may be mediated through other transcription factors or pathways.

There are various trans-activating factors. Of which, NF-κB mediates resistance against cell death by inducing gene expression and has been found to play important roles in regulation of BCRP gene transcription (Natarajan et al., 2012; Krishnamurthy et al., 2004; Ee et al., 2004; Agarwal et al., 2010; Pradhan et al., 2010; Singh et al., 2011); and 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” (Vousden and Lu, 2002; Fridman and Lowe, 2003). Our most recent study found that wild-type p53 repressed the transcription of BCRP gene through the NF-κB/p50 pathway in MCF-7 cells (Wang et al., 2010). While the present study revealed that Survivin expression was significant higher but p53 expression was lower in MCF-7/5-FU cells than those in MCF-7 cells. Hence, to gain insight into the mechanisms by which Survivin induces the expression of BCRP, we tested whether Survivin-induced BCRP expression would be mediated through NF-κB/p50 pathway and the potential effect of p53 on this pathway. Our data showed that overexpression of Survivin in MCF-7 cells resulted in significant increase of NF-κB/p50 expression levels as well as BCRP mRNA and protein levels, whereas Survivin-induced BCRP expression were almost completely blocked when NF-κB/p50 were knocked down by using NF-κB/p50 siRNA, confirming that Survivin up-regulated BCRP gene transcription was mediated through NF-κB/p50 pathway. By this pathway, NF-κB subunit p50 directly binds to the BCRP promoter to activate BCRP expression (Wang et al., 2010). However, may be due to the physical interaction between p53 and Survivin, our data illustrated that p53 would affect Survivin to induce BCRP promoter activation. In fact, other investigators have shown that p53 inhibits Survivin transcription (Mirza et al., 2002; Hoffman et al., 2002). Interestingly, as reported by Wang et al. (2004), who found that Survivin repressed p53 family at the mRNA and protein levels, our data revealed that Survivin overexpression would down-regulate p53 mRNA and protein expressions in MCF-7 cells. Furthermore, the present study demonstrated that Survivin itself had no direct effect on NF-κB/p50 expression/function when p53 was knocked down in MCF-7 cells; whereas p53 still exerted significant inhibitory action on NF-κB/p50 expression even when Survivin was knocked down. Consequently, Survivin-induced BCRP transcription is likely due to the repression of p53 expression or function by Survivin. Taken together, our data suggest that p53 plays a key role in the Survivin-regulated BCRP expression and, through down-regulation of p53 expression, Survivin attenuates the inhibitory effect of p53 on NF-κB/p50 and then enhances the expression of BCRP. Of course, since p53 may also inhibit Survivin transcription (Mirza et al., 2002; Hoffman et al., 2002), the long-term outcome of modulating Survivin or p53 on BCRP expression and/or drug resistant phenotype would depend on a balance between the expression/function of Survivin and p53. In most cancers, overexpression of Survivin and loss of wild-type p53 expression/function occur (Jones et al., 2012; Metzinger et al., 2006; Zheng et al., 2012), therefore BCRP overexpression and resistance to multiple drugs including mitoxantrone (MX), anthracyclines, methotrexate (MTX), topoisomerase I inhibitors, gefitinib, doxorubicin, and 5-FU have been observed in many cancers (Chen et al., 2011; Doyle and Ross, 2003; Ji et al., 2010; Yuan et al., 2009). Thus, suppress Survivin expression may present a new approach to overcome BCRP-mediated MDR in cancer.

5. Conclusions

The present study has provided evidence that Survivin enhances BCRP expression in MCF-7/5-FU cells, at least in part, though suppressing p53 expression and then attenuating the inhibitory effect of p53 on NF-κB/p50, which suggests that, as an anti-apoptotic protein, Survivin is involved in the BCRP-mediated MDR. This may represent a novel strategy for reversal of BCRP drug transporter activity to modulate chemoresistance in cancer cells.

Conflict of interest statement

None declared.

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