

RESEARCH ARTICLE

BCRP Expression in VX2 Rabbit Liver Tumours and its Effects on Tumour Recurrence, Metastasis and Treatment Tolerability

Cai-Xia Li*, Kai Zhang, Fu-Bo Xie

Abstract

Objective: This study aimed to investigate the effects of BCRP expression on tumor recurrence, metastasis and treatment tolerability. **Methods:** A VX2 rabbit liver tumor model was established. Division was randomly into 4 groups: namely saline control group; A group, given hydration lipiodol; B group, Ad-p53; and C group, Ad-p53+hydration lipiodol. After the intervention, samples were collected to detect the BCRP, MMP-2, VEGF and PCNA. **Results:** The expression of BCRP, MMP-2, PCNA and VEGF in tumors in Group A had no significant difference when compared with the control group, while in B and C group, the values were significantly lower ($P < 0.05$). BCRP positive expression in metastatic lesions significantly increased ($P < 0.05$), and was correlated with MMP-2 ($X^2=6.172, P = 0.0131$). **Conclusions:** BCRP may play an important role in mediating liver cancer multidrug resistance to chemotherapy, and may be correlated with tumor recurrence and metastasis, which leads to weakened treatment effect. Ad-P53 can down-regulate the expression of related genes, playing a role in multidrug resistance reversal and increased sensitivity in liver cancer treatment.

Keywords: VX2 rabbit liver tumor - intervention - BCRP - MMP-2 - VEGF - PCNA

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Introduction

China is a highly-incident-liver-cancer country, with the number of new cases each year accounting for 55% of the whole world, and the incidence increases year by year. 80% clinical cases of liver cancer have lost the chances of operation before the treatment, though palliative care therapy via the hepatic artery chemoembolization intervention has achieved good short-term effects, and patients can survive with existence of tumor, the long-term outcome is not satisfactory (Song et al., 2012; Takayasu et al., 2012). The intrinsic or acquired therapeutic resistance would often lead to the failure of treatment. Among the drug transporter proteins, the overexpression of membrane transport protein ATP binding cassette G2 (ABCG2) has close relation with multi-drug resistance of tumors (Hampras et al., 2010). Many studies have confirmed that BCRP, a new breast cancer resistance protein, belonging to a superfamily of ABC, is not only expressed in normal tissues, such as brain tissue, bile and intestinal mucosa, etc, but also in a large number of tumor tissues. The high expression of BCRP in tumor cells could specifically transport mitoxantrone, methotrexate, camptothecin and many anticancer drugs, forming the important cause of tumor cell resistance (Robey et al., 2009; Ni et al., 2010; Getz et al., 2011). Recent researched suggested that BCRP had protective effects on stem cell differentiation and development, and would have a large number expression

in cancer stem cells (An et al., 2010; Ding et al., 2010; Natarajan et al., 2012), which might provide the theoretical support of BCRP's potential overcoming the tumor resistance.

Researches find that, multidrug resistance (MDR) is the most important cellular defense mechanism for malignant tumor to drug attack (Oh et al., 2010), and an important reason for treatment failure and tumor recurrence and metastasis, which is a problem urgent to be solved in clinic. It is cross-resistance phenomenon of tumor cells to a variety of unrelated drugs with different molecular structures, cellular targets and action mechanisms. The emergence of MDR is a complicated process involving multiple factors (Yusuf et al., 2010). At present, the known involved mechanisms of MDR are as follows: 1) expression of ATP binding cassette (ABC) transporters including BCRP, P-glycoprotein, etc.; 2) expression of lung resistance-related protein gene; 3) increase of glutathione and glutathione S-transferase content; 4) DNA repair enzyme and DNA isomerase (TOPO) content decrease and activity reduction; 5) increase of dihydrofolate reductase content and activity or gene mutation. Most data show that, MDR can be used as an indicator for prognosis. In tumor patients with MDR, the prognosis is generally poor (Maier et al., 2010; Yusuf et al., 2010). For tumor MDR reversal, a variety of effective reversal agents to P-gp, MRP, GST, DNA repair related enzymes (TOPO) and MDR protein have been discovered.

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Gene therapy is the key to solve the problem of MDR (Maier et al., 2010), but at present it is mainly focused on P-gp gene and some cancer-related genes (e.g., P53). Compared to other related protein, the effective specific reversal agent for BCRP has not been found until now.

VX2 rabbit liver tumor is a hypervascular liver cancer model established in big animals, and is suitable for experimental research of transcatheter arterial chemoembolization (TACE). It is a good animal model of liver cancer, based on which a series of experimental studies, including imaging, therapeutics and pharmacokinetics of antitumor drugs, can be performed. Relevant basic and clinical researches on expression of MDR-associated factors have been reported (Robey et al., 2007; Abaan et al., 2009; Schwavedissen et al., 2010; Wittgen et al., 2011), while there is still no study about the impact of VX2-interventional treatment on MDR gene in rabbit liver cancer. Previously, our team has studied the establishment of common VX2 rabbit liver tumor model, and assessed the changes in tumor foci using imaging methods including DSA and MR. In addition, we have conducted the interventional therapy using P53 in treatment of VX2 rabbit liver tumor.

In this study, the intervention with different regimens was used to treat VX2 rabbit liver tumor. The expressions of BCRP and other factors reflecting tumor invasion and metastasis (e.g., MMP-2, VEGF and PCNA) in tumor tissue were observed. The correlations of BCRP expression with tumor recurrence and metastasis and treatment tolerability were investigated. In addition, the regulatory effect of Ad-P53 intervention on BCRP expression was observed, and the significance of gene therapy for MDR reversal and increase of tumor focus sensitivity to treatment were studied. The objective is to provide a basis for gene therapy and further improving the clinical therapeutic effect of MDR reversal agent, a new chemotherapy sensitizer.

Materials and Methods

China is a highly-incident-liver-cancer country, with the number of new cases each year accounting for 55% of the whole world, and the incidence increases year by year. 80% clinical cases of liver cancer have lost the chances of operation before the treatment, though palliative care therapy via the hepatic artery chemoembolization intervention has achieved good short-term effects, and patients can survive with existence of tumor, the long-term outcome is not satisfactory (Song et al., 2012; Takayasu et al., 2012). The intrinsic or acquired therapeutic resistance would often lead to the failure of treatment. Among the drug transporter proteins, the overexpression of membrane transport protein ATP binding cassette G2 (ABCG2) has close relation with multi-drug resistance of tumors (Hampras et al., 2010). Many studies have confirmed that BCRP, a new breast cancer resistance protein, belonging to a superfamily of ABC, is not only expressed in normal tissues, such as brain tissue, bile and intestinal mucosa, etc, but also in a large number of tumor tissues. The high expression of BCRP in tumor cells could specifically transport mitoxantrone, methotrexate, camptothecin and

many anticancer drugs, forming the important cause of tumor cell resistance (Robey et al., 2009; Ni et al., 2010; Getz et al., 2011). Recent researches suggested that BCRP had protective effects on stem cell differentiation and development, and would have a large number expression in cancer stem cells (An et al., 2010; Ding et al., 2010; Natarajan et al., 2012), which might provide the theoretical support of BCRP's potential overcoming the tumor resistance.

Researches find that, multidrug resistance (MDR) is the most important cellular defense mechanism for malignant tumor to drug attack (Oh et al., 2010), and an important reason for treatment failure and tumor recurrence and metastasis, which is a problem urgent to be solved in clinic. It is cross-resistance phenomenon of tumor cells to a variety of unrelated drugs with different molecular structures, cellular targets and action mechanisms. The emergence of MDR is a complicated process involving multiple factors (Yusuf et al., 2010). At present, the known involved mechanisms of MDR are as follows: 1) expression of ATP binding cassette (ABC) transporters including BCRP, P-glycoprotein, etc.; 2) expression of lung resistance-related protein gene; 3) increase of glutathione and glutathione S-transferase content; 4) DNA repair enzyme and DNA isomerase (TOPO) content decrease and activity reduction; 5) increase of dihydrofolate reductase content and activity or gene mutation. Most data show that, MDR can be used as an indicator for prognosis. In tumor patients with MDR, the prognosis is generally poor (Maier et al., 2010; Yusuf et al., 2010). For tumor MDR reversal, a variety of effective reversal agents to P-gp, MRP, GST, DNA repair related enzymes (TOPO) and MDR protein have been discovered. Gene therapy is the key to solve the problem of MDR (Maier et al., 2010), but at present it is mainly focused on P-gp gene and some cancer-related genes (e.g., P53). Compared to other related protein, the effective specific reversal agent for BCRP has not been found until now.

VX2 rabbit liver tumor is a hypervascular liver cancer model established in big animals, and is suitable for experimental research of transcatheter arterial chemoembolization (TACE). It is a good animal model of liver cancer, based on which a series of experimental studies, including imaging, therapeutics and pharmacokinetics of antitumor drugs, can be performed. Relevant basic and clinical researches on expression of MDR-associated factors have been reported (Robey et al., 2007; Abaan et al., 2009; Schwavedissen et al., 2010; Wittgen et al., 2011), while there is still no study about the impact of VX2-interventional treatment on MDR gene in rabbit liver cancer. Previously, our team has studied the establishment of common VX2 rabbit liver tumor model, and assessed the changes in tumor foci using imaging methods including DSA and MR. In addition, we have conducted the interventional therapy using P53 in treatment of VX2 rabbit liver tumor.

In this study, the intervention with different regimens was used to treat VX2 rabbit liver tumor. The expressions of BCRP and other factors reflecting tumor invasion and metastasis (e.g., MMP-2, VEGF and PCNA) in tumor tissue were observed. The correlations of BCRP

Table 1. BCRP Expression in Each Treatment Group

Group	+	++	+++	Positive rate (%)	Overexpression rate (%)
A	1	2		60	40
B	1	1		40*	20*
C	0	1		20*	20*
Control	1	2		60	40

No significant difference between A group and the control group ($P > 0.05$); significant differences existed between B, C group when compared with the control group ($P < 0.05$); * $P < 0.05$

Table 2. MMP-2, PCNA and VEGF Expression in Each Group

Group	N	MMP-2	PCNA	VEGF
		+	+	+
A	5	5	4	4
B	5	2*	2*	3*
C	5	1*	1*	2*
Control	5	5	5	4

* $P < 0.05$

expression with tumor recurrence and metastasis and treatment tolerability were investigated. In addition, the regulatory effect of Ad-P53 intervention on BCRP expression was observed, and the significance of gene therapy for MDR reversal and increase of tumor focus sensitivity to treatment were studied. The objective is to provide a basis for gene therapy and further improving the clinical therapeutic effect of MDR reversal agent, a new chemotherapy sensitizer.

Results

General observation

A large coagulation necrosis could be seen in the liver necrosis area, with clear edge, and a gray strip at the edge, namely the inflammatory infiltration zone. Necrosis area was hard, pale and dull, totally different from the pre-treatment fish-shaped bright color. 5 cases of control group were found newborn liver metastases, A, B and C group found 4, 2 and 1 cases newborn lesions, respectively. The newborn lesions located in or near the edge of necrosis, nodular or zonal distribution, with bright color and fish-shaped hard texture.

DSA performance

DSA showed that the majority of the tumor was supplied by the left hepatic artery, only a small were supplied by the right hepatic artery. In the arterial phase, tumor local artery increased, thickened, tortuous and gathered into a group, tumor local vascular reticulum increased and disordered, and visible blood pool could be seen partially. In the parenchymal phase, ring staining exhibited from the edge of the tumor, and gradually turned nodular staining. Tumor angiogenesis sometimes might be pushed, destructed or formed into a ball-shape.

BCRP expression

After the interventional therapy treatment of hydration lipiodol (0.3-0.5ml) in A group, BCRP expression had no statistically significant difference compared with the

Table 3. Tumors Growth Situation Before and after the Intervention in Each Group

Group	Preoperative tumor average diameter (mm)	Postoperative tumor average diameter (mm)	Tumor diameter growth rate (%)
A	10.05±2.02	13.93±2.00*	33.26±9.00
B	9.97±1.96	13.13±2.04*	31.71±6.60
C	9.93±2.09	13.34±1.81*	34.40±8.25
Control	9.97±2.12	17.79±2.67	80.90±16.21

The tumor diameter showed no significant difference among A, B and C group ($P > 0.05$); while there was significant difference when A, B and C group compared with the control group ($P < 0.05$); * $P < 0.05$

Table 4. BCRP Expression in the Metastasis

Tumor metastasis	N	+	++	+++	Positive rate (%)	Over-expression rate (%)
Yes	12	3*	5*		66.7*	41.7
No	8	0	1		12.5	12.5

* $P < 0.05$

Table 5. Associativity Comparison of MMP-2 and BCRP in the Metastases

	MMP-2	BCRP	X ²	P
+	11	4		
-	2	7	6.172	0.0131

control group, $P > 0.05$. After interventional therapy treatment, namely B group Ad-p53 (1×1011VP/10 ml) and C group Ad-p53 (1×1011VP/10 ml)+hydration lipiodol (0.3-0.5ml), BCRP expression was significantly lower in the tumor when compared with the control group ($P < 0.05$) (Table 1).

MMP-2, PCNA and VEGF expression

After the interventional therapy treatment of hydration lipiodol (0.3-0.5ml) in A group, MMP-2, PCNA and VEGF expressions had no statistically significant difference compared with the control group, $P > 0.05$. After interventional therapy treatment, namely B group Ad-p53 (1×1011VP/10ml) and C group Ad-p53 (1×1011VP/10ml)+hydration lipiodol (0.3-0.5ml), MMP-2, PCNA and VEGF expressions were significantly lower in the tumor when compared with the control group ($P < 0.05$) (Table 2).

Tumors growth

1 week after the VX2 tumor cells plantation and the intervention, tumor foci of A, B and C group increased with different degrees, with no significant difference among groups ($P > 0.05$), while the tumor growth rates of A, B and C decreased significantly when compared with the control group, and the difference was statistically significant ($P < 0.05$). The growth situation of tumors before and after the intervention was shown in Table 3.

BCRP expression in the metastasis

The positive expression rate of BCRP in recurrence and metastasis was significantly higher than those without metastasis ($P < 0.05$), with the positive expression rate as 66.7% and over-expression rate as 41.7% (Table 4).

Associativity comparison

BCRP had associativity with MMP-2, which was related with the high expression of tumor invasion and metastasis ($X^2 = 6.172$, $P = 0.0131$, Table 5).

Discussion

In recent years, interventional treatment of hepatocellular carcinoma has obtained obvious effect, while the tumor recurrence, metastasis and multiple resistances to treatment is its bottleneck limiting the long-term intervention effects. Studies have shown that tumor stem cells, contributing to the tumor recurrence and tolerance to the treatment, might be associated with high expression levels of ABC transporter proteins in stem cells (Bomken et al., 2010). BCRP is a semi-transporter protein with molecular mass 72KD, belonging to superfamily of ABC transporter. BCRP was firstly found in multidrug resistant breast cancer cells, existing as an ATP enzyme-dependent semi binding transporter. The ABC transporter has the function of protecting the body from exogenous substances damage. BCRP would affect the oncology behavior primarily in three aspects: 1) endogenous BCRP would affect absorption, distribution, metabolism and elimination of tumor drug; 2) BCRP expression in tumor cells could discharge tumor drugs, directly causing drug-resistance; 3) BCRP expression in tumor cells could be a signal of metabolic activation, causing the activation of the signaling pathways such as tumor multidrug resistance, self-renewal, invasion, poor prognosis and others. Therefore, BCRP plays an important role in tumor cells resistance, proliferation, metastasis, gene mutation and confrontation of programmed cell death, etc. After the treatment, tumor recurred, at the same time, BCRP gene amplified and overexpressed, suggesting that BCRP might play a role in mediating acquired resistance induced by chemotherapy, the overexpression of BCRP was significantly correlated with the tumor recurrence and treatment tolerability (Bortolomai et al., 2010; Oliveira et al., 2010; Sengupta et al., 2010). The cancer tolerance towards the treatment could be endogenous or acquired.

As expected in this study, the positive expression rate and of overexpression rate BCRP in VX2 rabbit liver tumor cells are as high as 60% and 40%, respectively. This suggests that, BCRP may play a role in mediating the inefficacy of chemotherapy in clinical treatment of liver cancer. The positive expression rate of BCRP in recurrent and metastatic tumor lesion is significantly different with non-metastatic tumor lesion ($P < 0.05$), representing an overexpression. This is consistent with the results of Mullins et al's study (2011). In addition, Mullins et al. (2011) believe that, BCRP can be used as a prognostic indicator, and in tumor patients with MDR, the prognosis is generally poor.

This study also finds that, BCRP has obvious correlation with high expression of MMP-2, which is related with the tumor invasion and metastasis ($X^2=6.172$, $P = 0.0131$). It is speculated that, there is a certain relationship between BCRP expression and tumor invasion, recurrence, metastasis and treatment resistance. Tumor invasion and metastasis is one of key

factors for recurrence and metastasis of hepatocellular carcinoma, and the degradation of extracellular matrix and basement membrane is the key step of tumor invasion and metastasis. McGowan and Duffy (2008) (Lutgendorf et al., 2008) believe that, the expression of MMPs is a reflection induced by the host. The tumor cells can exchange information with host stroma cells by a variety of soluble mediators or membrane-bound molecules. So the host stroma cells produce MMPs and regulate them, facilitating tumor invasion and metastasis.

Matrix metalloproteinase-2 (MMP-2) can hydrolyze not only intercellular matrix components, but also the type IV collagen, main component of the basement membrane, and it's an important indicator of malignant extent and prognosis of tumor cells (Lutgendorf et al., 2008; Lindner et al., 2012). When the tumor exhibits recurrence and metastasis, BCRP gene amplified and overexpressed, and this high expression would affect the activation of various signaling pathways of invasion and poor prognosis, pumping possible differentiation-inducing materials out of cells, thereby preventing cell differentiation, increasing the tumor degree of malignancy, and weakening the treatment of multiple tolerance and treatment effect. P53 gene is a tumor suppressor gene, not only closely associated with tumorigenesis, but also involved in cell growth, differentiation and death regulation. Studies showed that the therapeutic effects of P53 gene on tumor mainly derive from the P53 protein involvement in the anticancer biological functions of regulating cell cycle regulation, DNA repair, cell differentiation and cell apoptosis (Ayed et al., 2010; Lim et al., 2010). This study suggested that Ad-P53 intervention therapy could reduce the BCRP overexpression, so gene therapy might play certain role on reversing multidrug resistance, increasing liver lesions sensitivity to the treatment. Lipiodol hydration and/or Ad-P53 interventional treatment showed growth control effects on rabbit liver VX2 tumor foci. While during the application of adenovirus-mediated wild-type P53 interventional treatment on liver cancer, the expressions of MMP-2, PCNA and VEGF, genes or factors closely related to tumor regeneration and metastasis, and BCRP, breast cancer resistance protein associated with tumor drug resistance, significantly reduced, which might be related with the fact that wild-type p53 could reduce gene or factor, and thus inhibit tumor invasion and metastasis, reverse multidrug resistance, increase tumor sensitivity to treatment. The simple application of lipiodol had no down-regulate effects on related genes.

Of course, the mechanism of MDR of tumor is very complex. It is the result of multi-gene and multi-link actions. Investigating MDR from gene level is expected to provide a theoretical basis for gene therapy, further improving the clinical therapeutic effect of MDR reversal agent, and identifying possible molecular targets for treatment of hepatocellular carcinoma.

In conclusion, BCRP may play an important role in mediating liver cancer multidrug resistance to chemotherapy, and may be corrected with tumor recurrence and metastasis, which leads to treatment tolerability or weakened treatment effect. Ad-P53 can down-regulate the expression of relative genes, playing

a role in multidrug resistance reversal and increased sensitivity in liver cancer treatment.

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