Experimental Study of Doxorubicin Interventional Chemotherapy in the Treatment of Rabbit VX2 Renal Transplantation Carcinoma

Zhao Shuyuan[*1], Yu Hui[1] and Du Nan[2]

[1]Peripheral intervention department of zhumadian central hospital, henan province, China

[2]Traditional Chinese medine hospital of zhumadian of Henan province, China

Abstract

Objective to study the effect of doxorubicin interventional chemotherapy on rabbit VX_2 renal transplantation carcinoma and its mechanism. Methods Thirty healthy New Zealand white rabbits were chosen to establish VX_2 renal transplantation carcinoma models. The experimental rabbits were randomly divided into three groups with 10 rabbits in each group. The rabbits in the control group (negative control), doxorubicin group and cisplatin group were treated with saline, 5mg/kg doxorubicin and 2mg/kg cisplatin respectively. The tumor volume was monitored with B-mode ultrasonography. The rabbits were anesthetized and killed after two weeks of interventional chemotherapy. The changes of Bcl-2 and Bax at the levels of mRNA and protein were analyzed with real-time PCR and immunehistochemistry. Results The efficacy of interventional chemotherapy was evaluated with tumor volume changes monitored by B-mode ultrasonography. The tumor volume of control group and doxorubicin group was 1.29±0.60cm3 and 0.47±0.12 cm3 respectively. Further fluorescence quantitative PCR detection results showed that doxorubicin could reduce the Bcl-2 expression and increase the Bax expression (P<0.05).The result of immunohistochemistry was consistent with that of fluorescence quantitative PCR. Conclusions The effect of doxorubicin interventional chemotherapy on renal transplantation carcinoma is obvious and the mechanism may be related to the down-regulation of Bcl-2 expression and up-regulation of Bax expression thus inducing the apoptosis of tumor cells.

***Corresponding Author**: Zhao Shuyuan, Peripheral intervention Department of zhumadian central hospital, henan province, china; E-mail: zhaoshyhn@163.com

DOXorubicin (DOX) is a kind of antitumor drug commonly used in clinical which belongs to the cell cycle non-specific drugs and has the characteristics of broad antitumor spectrum and having a certain effect for a variety of tumors [1-4].Interventional chemotherapy is a treatment method achieved by application of equipments, technologies and methods of radiodiagnostics. That is to say, puncture and intubation are performed with cannula and guide wire to achieve the purpose of local administration. With intensive study of interventional technique, it has been widely used in clinical with evident effects. Although it has been relatively clear that doxorubicin induces apoptosis by affecting DNA synthesis, its molecular mechanisms and expression and regulation of signaling molecules such as relevant oncogenes and cancer suppressor genes involved in the process of inducing apoptosis still need to be studied intensively[1, 2].In our study, we will treat rabbit

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VX_2 renal transplantation tumor models with doxorubicin interventional chemotherapy to investigate the mechanisms of doxorubicin promoting cancer cells apoptosis by changing the expressions of Bcl-2 and Bax [3].

Materials and Methods

Materials

Laboratory Animals

Thirty New Zealand white rabbits, weighing 2.5-3.0 kg and male, were purchased from Slac Laboratory Animal LLC. All rabbits were fed with standard fodder, freely took food and drank water during the experiment. The laboratory was well ventilated and nature lighting day and night was achieved. The temperature was maintained at 18~25℃

VX_2 Tumor Cell Lines

VX_2 squamous cell carcinoma lines were from ATCC and preserved in our laboratory.

Pharmacological Agents

Doxorubicin and Bax/Bcl-2 polyclonal antibody immunohistochemistry kit were purchased from Santa Cruz Biotechnology. Napental was purchased from Sigma. Tissue RNA extraction kit (RNeasy Plus Mini Kit) was purchased from QIAGEN. Reverse transcription kit (iScript cDNA Synthesis Kit) and real-time PCR fluorescence quantification kit (Sso Advanced SYBR Green Super mix) were purchased from Bio-Rad.

Methods

Establishment of an Animal Model

The conservation of VX_2 cells was taken out from the liquid nitrogen, recovered and subcultured. They were made into cell suspension (the concentration of viable cells was about 10^7/ml) [5, 6]. 2ml of precooled cell suspension was taken out and injected into the subcutaneous parts of bilateral hind legs of the experimental rabbits. After 15 days, the tissues at the edge

of tumors were scissored with ophthalmic scissors and mixed and suspended with PBS. The rabbits were anesthetized with napental at a dosage of 30mg/kg and fixed in a prone position. The VX_2 tumor mass suspension was injected into the lower pole of right kidney under the guidance of B-mode ultrasonography to establish VX_2 renal transplantation carcinoma model. The rabbits were fed normally.

Interventional Chemotherapy

The model animals were randomly divided into three groups and there were 10 rabbits in each group. When the tumor grew to about 1.5cm under the monitoring of B-mode ultrasonography, interventional chemotherapy was performed. Three groups were treated two times per week for two weeks with 5 mg/kg doxorubicin, 2mg/kg cisplatin and saline respectively via renal artery perfusion. Follow-up B-mode ultrasonography examination was performed after two weeks of interventional treatment. To measure the maximum diameter (l) and minimum diameter (h) in the largest section of tumor and calculate the tumor volume according to the formula: $V= l\, h^2/2$ [7]. After the treatment, the animals were killed with air embolism and the tumor samples were collected.

Bioinformatic Analysis

The signaling pathways Bcl-2 and Bax involved in and interaction proteins were preliminarily analyzed using protein interaction online analysis tool (STRING9.05) and protein signaling pathway database (KEGG PATHWAY Database) (http://www.genome.jp). STRING database (http://string-db.org) integrates the experimental data, corresponding results of PubMed abstract and bioinformatics prediction results which is a system searching for known or predicted interactions between proteins.

Experimental Method of Fluorescent Quantitative PCR

The total RNA of tumor tissue was extracted according to the method of Trizol in the instruction of total RNA extraction Kit. According to the process of reverse transcription Kit, RT extracting total RNA of tissue and RT-cDNA and real-time PCR

testing were performed. To inquiry the mRNA sequences of Bcl-2 and Bax in NCBI database and design the real-time PCR primers. All primers were synthesized by Invitrogen Corporation. The specific sequences were shown in the Table1.

Table 1: Primer information of Real-time PCR

Primer	Sequence
GAPDH upstream primer	ACCACAGTCCATGCCATCAG
GAPDH downstream primer	CCACCACCCTGTTGCTGTAG
Bcl-2 upstream primer	GGATTGTGGCCTTCTTTGAG
Bcl-2 downstream primer	CCAAACTGAGCAGAGTCTTC
Bax upstream primer	TCCACCAAGAAGCTGAGCGAG
Bax downstream primer	GTCCAGCCCATGATGGTTCT

Table 2: PCR reaction system and amplification temperature cycle parameters

Components	Volume per Reaction
Sso Advanced SYBR Green Super mix	5μl
Forward primer (10μM)	0.3μl (300nM)
Reverse primer (10μM)	0.3μl (300nM)
cDNA template	100ng
Nuclease-free water	Up to 10μl

Immunohistochemistry

Tissue processing and slice

To take the tumor tissue, cut it into small pieces the thickness of which was no more than 5mm and make a mark on them. They were fixed in 10% neutral formalin for more than 24h, rinsed with tap water, on the next day dehydrated in gradient alcohol, embedded in paraffin and sliced (thickness of 3 to 5µm).

Immunohistochemical Staining

The paraffin slices were conventionally dewaxed to water and the endogenous peroxidase was obturated with hydrogen peroxide ($3\%H_2O_2$). Immunohistochemical staining was performed with Bcl-2 and Bax polyclonal antibody immunohistochemical staining kit. The positive cells were brown. Immunohistochemical data was analyzed using Image-ProPlus software. First of all, to select the area with color of dye in the picture (AOI, Area Of Interesting), measure the Integrated Option Density (IOD) of the area, then select and measure effectively statistical area and calculate the IOD mean value in the area (IOD/area, Density mean).The density mean was used as semi quantitative parameter of the expression level of Bcl-2 and Bax genes [8].

Statistical Analysis

The experimental data was processed with SPSS statistical software package. The results are expressed as the mean ± standard deviation (x±SD). The data was examined with variance analysis and t test. Findings with $P < 0.05$ were considered to be statistically different. （*P<0.05; ** P<0.01).

Results

Establishment of Rabbit VX_2 Renal Transplantation Carcinoma Models

The tumor growth was monitored by B-mode ultrasonography. It indicated the success of modeling that the echo of the lower lobe of right kidney was uneven, the dark halo around it was visible and occupying lesion with punctate high-level echo and rich blood flow was seen in the kidney and its envolope. When the tumor grew to about 1.5cm under the monitoring of B-mode ultrasonography, interventional chemotherapy was started.

Efficacy Judgment of Interventional Chemotherapy

The efficacy of interventional chemotherapy was judged preliminarily with the volume of tumor. After two weeks of

interventional chemotherapy, the rabbits were killed. The nodular lesion with complete capsule and rich blood flow in the lower lobe of right kidney was shown. The tumors were removed and the maximum and minimum diameters in the largest section of them were calculated. The volume of tumor was calculated according to the formula: $V = l\,h^2 / 2$. The volumes of tumors after different interventional chemotherapy were shown in table 3.

Table 3: Tumor volume after different interventional chemotherapy ($\bar{x} \pm SD$)

Measurement Treat	l(cm)	h(cm)	V(cm^3)
Control	1.64±0.11	1.26±0.28	1.29±0.60
DOX	1.23±0.25	0.87±0.21	0.47±0.12
Cisplatin	1.27±0.32	0.92±0.25	0.54±0.38

Bioinformatic Analysis

The interaction proteins of Bcl-2 and Bax were searched with STRING9.05.Through bioinformatic analysis, it can be seen that the interaction proteins of Bcl-2 were mainly BAX,BBC3,BECN1,BAD and BID.KEGG PATHWAY Database showed that Bcl-2 and Bax commonly participated in multiple signaling pathways associated with apoptosis and DNA synthesis. Bioinformatic analysis provided important information for the study on Bcl-2 and Bax participating in signaling pathways and playing their biological effects.
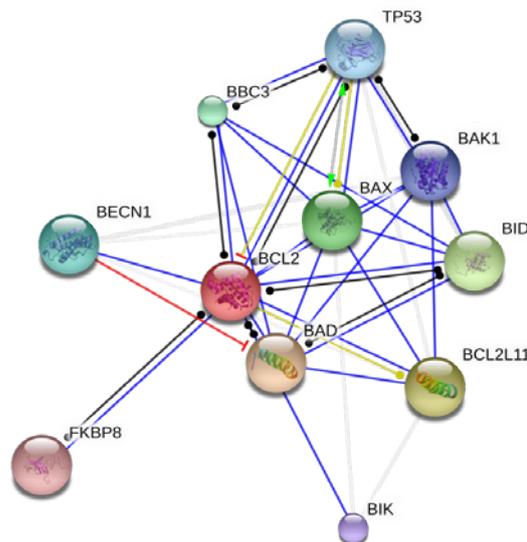
Figure: 1

Figure 1: Bcl-2 interaction proteins analyzed with STRING software

Expression Changes of Bcl-2 and Bax in Tumor tissue at mRNA Level Detected with Fluorescent Quantitative PCR

In the experiment, expression changes of Bcl-2 and Bax at mRNA level after interventional chemotherapy were studied using the method of relatively quantitative real-time PCR with GAPDH as a reference. Figure 2 showed that both doxorubicin and cisplatin could reduce the expression of Bcl-2 and raise the expression of Bax ($P<0.05$).The relative expression of Bcl-2 in the doxorubicin group was lower than that in the cisplatin group.
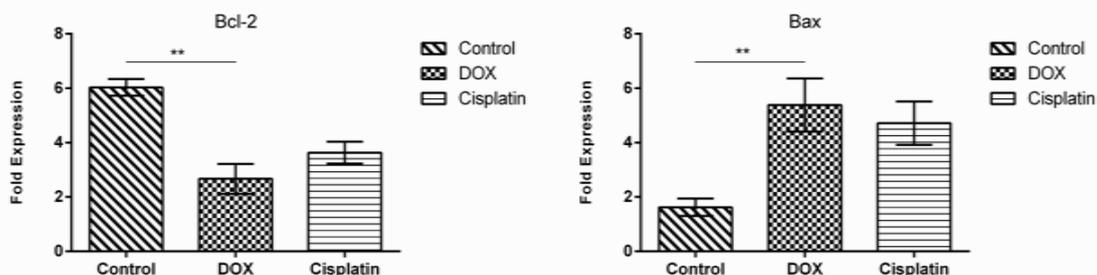

Figure 2: Related gene expression detected with real-time PCR

Immunohistochemistry Results

The expressions of Bcl-2 and Bax genes in the tumor tissues at tissue level were detected with immunohistochemistry after the interventional chemotherapy. The positive cells were brown. The immunohistochemical data analysis was performed with Image-ProPlus (IPP) software. The density mean in the selected area was used as semi quantitative parameter of the expression of Bcl-2 and Bax genes. The Bcl-2 protein was 0.77 ± 0.25 in the control group and 0.32 ± 0.11 in the doxorubicin group. The Bax protein was 0.35 ± 0.09 in the control group and 0.53 ± 0.12 in the doxorubicin group.
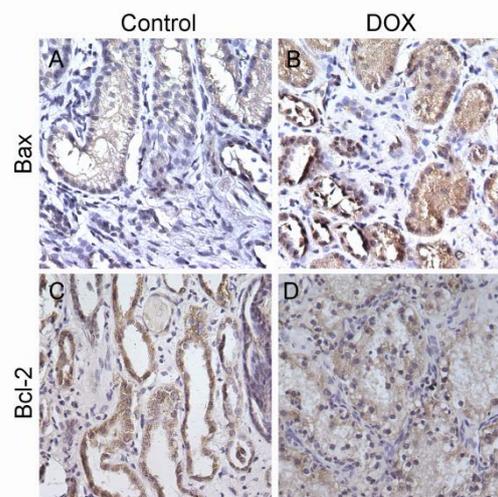
Figure: 3

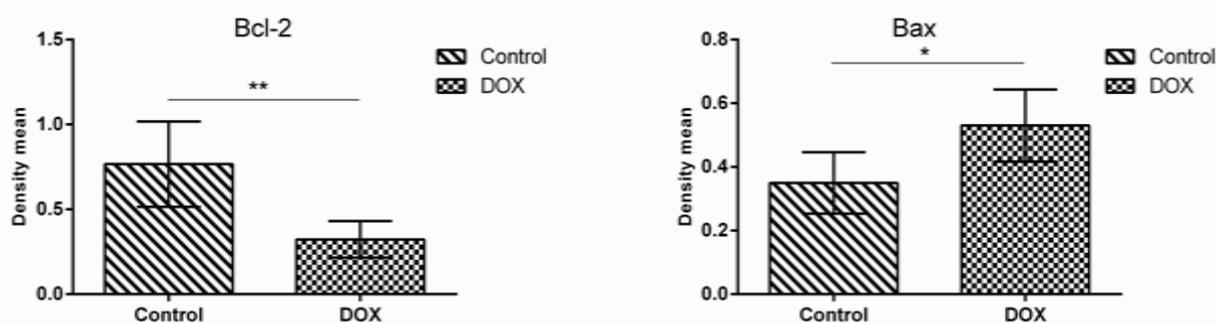**Figure 3:** Expression of Bcl-2 and Bax detected with immunohistochemistry

Figure 4: Semi-quantitative results of expression level of Bcl-2 and Bax genes

Discussion

As a broad spectrum antitumor drug, doxorubicin is already very mature in clinical. It promotes the apoptosis of tumor cells through inhibiting the synthesis of RNA and DNA (especially RNA). However, small molecular drugs usually have multiple target effects. In the meanwhile of treating diseases, there are many side effects inevitably which hinders the application of the drugs. Therefore, it is crucial for clinical application of a drug and research and development of new drugs to know the biological targets of a drug and drug-mediated signaling pathways as clearly as possible [9, 10].

Interventional therapy derives from interventional radiology. Interventional chemotherapy is a treatment method achieved by application of equipments, technologies and methods of radiodiagnostics. That is to say, puncture and intubation are performed with cannula and guide wire to achieve the purpose of local administration which may maintain high medicine concentration in the lesions and give full play to medicine efficacy. Interventional therapy has the characteristics of little injury, accurate positioning, few side effects and complications and quick and definite in effect [11, 12]. Therefore, in recent years, interventional chemotherapy has been widely used for the treatment of tumors.

In our experiment, we investigated the therapeutic effect of doxorubicin interventional chemotherapy on rabbit VX_2 renal transplantation carcinoma model and analyzed the mechanism of doxorubicin interventional chemotherapy hindering the growth of tumor and inducing the apoptosis of tumor cells with tumor volume as a reference. It can be seen from the experimental results that doxorubicin interventional chemotherapy can significantly reduce tumor volume. Compared with the traditional mode of administration, interventional chemotherapy increases the concentration of the drug in the lesion and reduces the side effects of the drug on normal tissue cells due to centralized drug delivery in the lesion. The side effect of a drug is also the emphasis of our research. In the further research, we will mainly study the injury of different drugs to the organs in the traditional mode of administration and interventional chemotherapy.

As a regulator of apoptosis, Bcl-2 is an important factor for maintaining normal cell cycle, which can inhibit apoptosis, including lymphoid hematopoietic cells and nerve cells. Studies have shown that Bcl-2 regulates the cell death through preventing release of mitochondrial cytochrome C, inhibiting Caspase activity and controlling mitochondrial membrane permeability [13]. Bax is also a member of the Bcl-2 family which can bind with Bcl-2 protein and adenovirus homolog protein E18 19K to accelerate the programmed cell death. Therefore, synergistic effect of Bcl-2 and Bax is the important regulator to control the normal apoptosis. In order to further study the protein signaling pathways doxorubicin participating in, we have firstly analyzed the experimentally confirmed signaling pathways by means of bioinformatics. It can be found from KEGG PATHWAY database that Bcl-2 and Bax coordinatedly participate in numerous signaling pathways. Among them, the relatively important signaling pathways include colorectal cancer pathway, Amyotrophic Lateral Sclerosis (ALS) pathway, viral carcinogenesis pathway, apoptosis pathway and neurotrophin signaling pathway. A homologous or heterologous dimer can be formed between the members of Bcl-2 protein family to promote or inhibit the

apoptosis. Meanwhile, there are also synergistic effects between the proteins.Bcl-2 inhibits apoptosis and Bax promotes apoptosis. Studies have proved the expression proportion of Bcl-2 and Bax plays a crucial role in determining whether apoptosis occurs or not [14-16].

In this study, we selected cisplatin as a positive control. Cisplatin is also a antitumor drug commonly used in clinical. Studies have proved that cisplatin can increase the expression of Bax and reduce the expression of Bcl-2 at the level of transcription and translation [17, 18].This conclusion has also been repeated in our study. After doxorubicin interventional chemotherapy of rabbit VX_2 renal transplantation carcinoma model, we detected the expression of Bcl-2 and Bax genes at the mRNA level. We can see that the doxorubicin treatment can significantly reduce Bcl-2 and increase Bax (P<0.01) and the change trend was stronger than that of cisplatin treatment group. Further immunohistochemical studies also got the same results. Cytotoxicity is a double-edged sword which means the inevitable production of toxic side effect when it is used for killing tumor cells. There is also participation of Bcl-2 and Bax in the doxorubicin-induced cardiomyocyte apoptosis of cardiotoxicity [19].Therefore, in-depth study of interventional chemotherapy and clearly understanding the signaling pathways drugs involved in are the focus of our future research.

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