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Original Article

Relationship between expression of apoptosis-related genes in osteosarcoma and cancer cell invasion processes

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Abstract: Primary bone cancer is the most common malignant tumor in the skeletal system, which seriously affect the function of bone movement and support, the process of inducing apoptosis of cancer cells is a hot issue in the research of malignant tumor in recent years, study on the mechanism of apoptosis of bone cancer cell can provide an important theoretical basis for the prevention and treatment of bone cancer. This paper intends to study the expression of apoptosis related gene consist of NF-kappa B and Bcl-2 in osteosarcoma and their application in the development and metastasis of cancer. Animal model of rabbit with VX-2 bone malignant tumor was constructed by injecting rabbit VX-2 bone tumor cells into tibial bone marrow cavity of rabbit, the pathology characteriatics of animal model with osteocarcinoma were evaluated at different time after transplantation, the relationship between the invasion process of osteocarcinoma cells and the expression change of apoptosis related gene consists of NF-κB, Bcl-2, Apaf-1 in animal transplantation area surrounding tissue was measured by By QRT PCR and immunohistochemistry methods. Through the detection of animal model with osteocacinoma and pathological process, it was found that the tumor cells begin to invade surrounding tissues after transplantation of 3 weeks, and at the same time the expression of NF-κB and Bcl-2 gradually increased in the level of protein and mRNA, but the expression of Apaf-1 decreased. The expression level of NF-κB and Bcl-2 increased and Apaf-1 decreased with the develpoment of osteocarcinoma invasion.

Keywords: Osteocarcinoma, NF-κB, Bcl-2, Apaf-1, animal model

Introduction

Primary osteocarcinoma is a malignant tumor originated from bone tissue, which is common in the growth and development of young people under the age of twenty, the morbidity of primary osteocarcinoma accounted for 6% [1] in the population with malignant tumors under the age of twenty. Primary osteocarcinoma can seriously affect the motor function and the health of patients. At present, surgery and radiochemotherapy is still the main strategy for the treatment of bone cancer [2]. But the five year survival rate of patients after surgical treatment is still less than 20%, the survival rate of patients can be improved by the combination of various drugs and chemotherapy, but there are still differences, tolerance and relapse of drug reaction [3, 4]. The study on the regulation mechanism, especial the mechanism of cell proliferation, apoptosis and invasion has important sense to the research and therapy of osteocarcinoma in the level of cell and molecular.

Apoptosis is a complex process, various genes including oncogenes and tumor suppressor genes are involved in the regulation of apoptosis [5, 6]. P53 is a tumor suppressor gene, which is able to check the integrity of DNA in the interphase, if it is found the damage that the cells will be blocked in interphase [7]. NF-κB is a nuclear transcription factor, which regulates a variety of genes involved in the development of cancer and the expression of TNF in malignant tumor cells, recently, it has been found that NF-κB plays an important role in the drug resistance of malignant tumors [8, 9]. Bcl-2 is a kind

Table 1. Chemical synthesis of nucleic acid sequences for cell transfection

Name	Sequence
β-actin	F: 5'-CGTGGACATCCGCAAAGACCT-3'
	R: 5'-AGCCAGAGCAGTGATCTCCTTC-3'
NF-κB	F: 5'-TCTGTTTCCCCTCATCTTTCC-3'
	R: 5'-TGGGTGCGTCTTAGTGGTAT-3'
Bcl-2	F: 5'-GGGGCTACGAGTGGGATGC-3'
	R: 5'-GCGGTAGCGGCGGGAGAAGT-3'
Apaf-1	F: 5'-CCTCTCATTTGCTGATGTCG-3'
	R: 5'-TCACTGCAGATTTTCACCAGA-3'

of inhibitor of apoptosis gene, which can induce the release of apoptosis factors such as cytochrome C in mitochondria [10]. Apaf-1 is a kind of apoptosis enzyme activation factor, which plays an important role in the mitochondrial apoptosis pathway [11]. This paper intends to study apoptosis related gene consists of NF-κB, Bcl-2 and Apaf-1 expression in osteosarcoma and its role in the occurrence and metastasis of osteosarcoma.

Materials and methods

Selection of research objects

Twenty New Zealand white rabbits aged about 3 months were selected as the study subjects, experimental animals were purchased from Shandong University Academy of sciences, the weight of all the experimental rabbit at the range of 2.5-3.0 kg, the average weight was 2.68±0.26 kg, all rabbits eat and drink freely, before accepting this experiment, 2 weeks of adaptive feeding.

Rabbits were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second Hospital of Shandong University.

Establishment of osteocarcinoma model

VX2 cell line was purchased from cell bank of Chinese Academy of Sciences, DMEM culture medium (Hyclon) carrying penicillin and streptomycin (sigma) double antibody was used, and 10% fetal bovine serum (Hyclon) was added. After recovery culture and trypsin (Abcam) digestion, the VX2 cells were resuspended and counted for injection to animals.

All rabbits were anesthetized according to the dose of 1.5 mL/kg injected with a dose of 0.5%. Then the abdomen is fixed on the operating table, the hind limbs were shaved to disfect the skin, operating knife blade was used to cut 1 cm of the wound in the upper tibia skin, and the piercing and puncture was made with syringe needle, VX2 0.2 mL cell suspension was injected into the left hind limb of the bone marrow cavity, and the right hind limb was injected with the same volume of saline as the control. Bone wax was used to seal pinhole, and then the wound was cleaned and the skin was stitched.

Evaluation of pathological features of osteocarcinoma model

After surgery 1, 3, and 5 weeks, all the rabbits were anesthetized with an injection of 1.5 mL/kg dose of 0.5%, the pathology features of tumor in left tibia marrow cavity and tibia surrounding muscle tissue were detected by HRQ-2000AE veterinary ultrasound, the change of bone density, periosteum edema, rhabdomyosarcoma occurrence, formation of new bone were recorded in order to evaluate the proliferation and growth of osteocarcinoma. In the 1, 3, 5 weeks after the completion of the operation, the experimental rabbits were treated with euthanasia. Operation tissues were divided into two parts, one part of the tissues were carried out 10% neutral formalin fixation and embedding for immunohistochemical study, another part of the tissues were immediately used to extract RNA for qRT-PCR detection.

QRT-PCR detection

QRT-PCR primer was designed according to the mRNA sequnence of NF-κB, Bcl-2, Apaf-1 gene (Genebank accession number: NM_003998, NM_000633, NM_013229), the amplified primers were shown in **Table 1**, mRNA relative expression level of NF-κB, Bcl-2 and Apaf-1 were detected with β-actin as the internal reference. Tissue RNA Extraction Kit (QIAGEN) was used to extract tissue samples RNA, qRT-PCR kit (TianGen) was used for qRT-PCR reaction. The analysis software V2.02 to analyze, and the U6 gene sequences were regarded as internal reference, the results were expressed with 2-ΔΔCt [11].

Immunohistochemistry (IHC)

The protein expression of NF-κB, Bcl-2 and Apaf-1 was detected in protein level by IHC. The

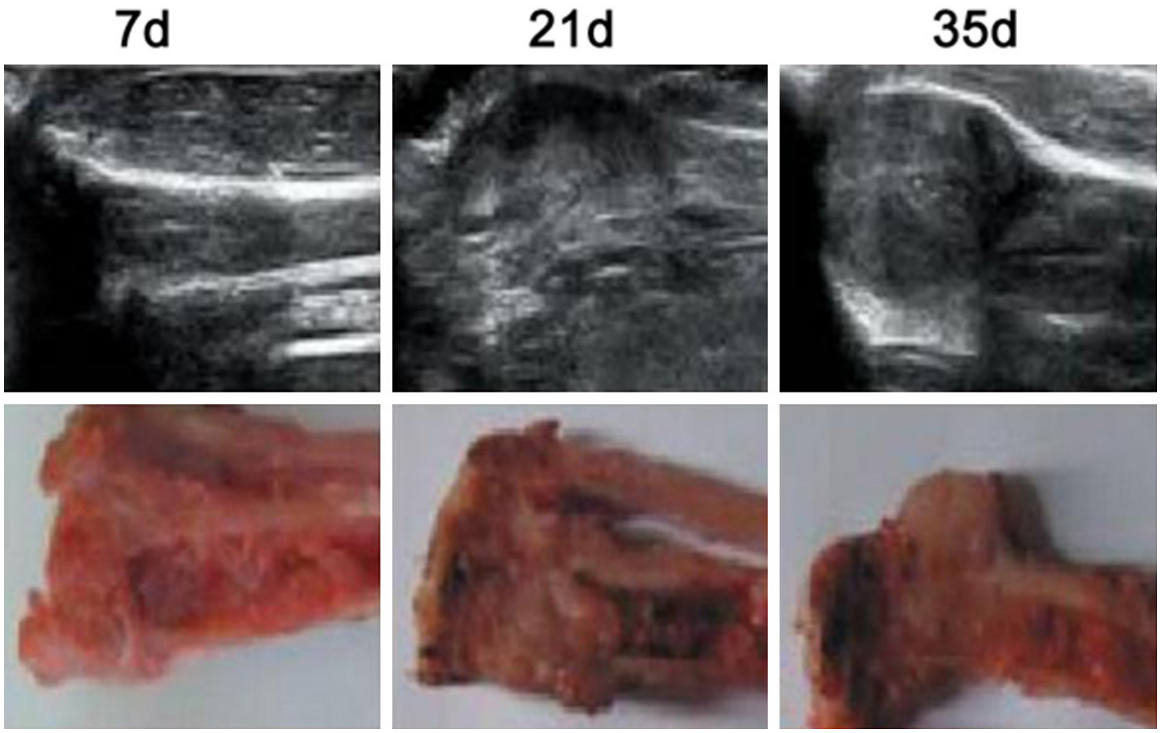

Figure 1. Pathological characteristics at different stage after bone cancer modeling.

main steps are as follows: the tissue samples obtained from osteocarcimona model of rabbit were fixed by routine paraffin embedded, after routinely dewaxing and hydration, non-specific antibody binding site was closed, monoclonal antibody of mice anti-NF-κB, Bcl-2 and Apaf-1 (ProteinTech) were added respectively to carry out immune reaction, washing, and then biotin labeled second antibody was added to carry out immune reaction, horseradish peroxidase labeled streptavidin was added to carry out affinity reaction, after washing and coloration, and the coloration was ended, hematoxylin was added to redye, the sections were observed under the CX3 microscope (OLYMPUS). At high magnification, according to the degree of staining of the cytoplasm the scores were evaluated: there is no staining: 0 score; there is superficial staining: 1 score; there is moderate staining: 2 scores; there is deeper staining: 3 scores; the scores of single cancer cell is higher or equal to 2 scores are regarded as positive, the positive ratio is the ratio of positive cells number dividing total cells number.

Statistical analysis

JMP10.0 software (SAS) analysis was used for statistical analysis, the results were expressed with average ± standard deviation, ANOVA one-way method was used to analyze the comparison between groups, $P<0.05$ indicated that it had statistical significance.

Results

Experimental model

The osteocarcinoma model of rabbit was induced by application of VX2 tumor cell xenograft, the pathological characteristics of osteocarcinoma model of animal was analyzed by two-dimensional ultrasound detection at different time points, results are shown in **Figure 1**. It was found 1 week after operation two dimensional ultrasound examination of the tibia of rabbits was performed, it was found that cortical bone of the tibia was continuous, bone marrow cavity showed low echo, there was slight abnormal echo. After 3 weeks of inoculation, the two dimensional ultrasound examination was found that there was uneven echo mass in the tibial metaphysis, contrast enhanced ultrasound showed diffuse growth of the tumor, observation the opening tibia found internal tumors grew well, but did not infiltrate into the surrounding tissue. But 5 weeks after inoculation, two dimensional ultrasound examination showed that the tumor in the bone marrow cavity was aggressive and broke through the soft

Table 2. Expression of NF-κB, Bcl-2 and Apaf-1 of tissues at different times by qRT-PCR

Gene	Group	QRT-PCR detection time (week)			
		0	1	3	5
NF-κB	Control group	0.07	0.09	0.11	0.12
	Experimental group	0.05	0.13#	0.36*,#	0.78*,#
Bcl-2	Control group	0.02	0.03	0.06	0.07
	Experimental group	0.04	0.25*,#	0.56*,#	0.89*,#
Apaf-1	Control group	0.51	0.49	0.47	0.48
	Experimental group	0.51	0.46	0.21*,#	0.06*,#

*, Compared with control group, P<0.05; #: In the same group, the same index was statistically significant at different time points, P<0.05.

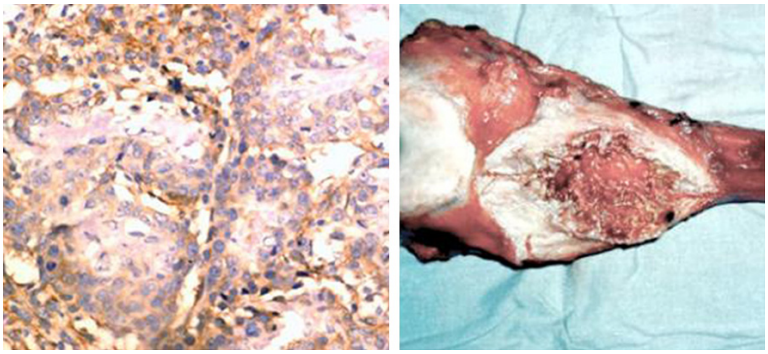

Figure 2. CD99 immumohistochemical staining in osteosarcoma.

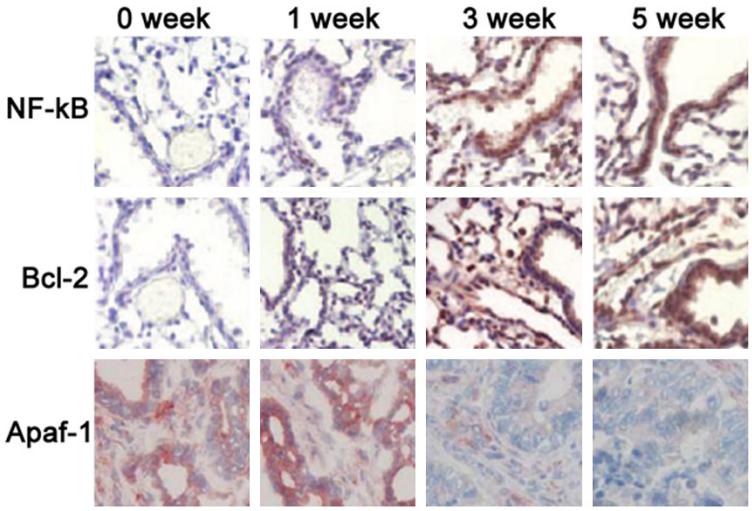

Figure 3. Immumohistochemical examination for expression of apoptosis related proteins.

tissue surrounding the bone, after ultrasound contrast, no contrast agent perfusion was found in the center of the tumor, bone tissues were severely damaged and pathological fracture occurred, necrosis occurred in the center of the tumor, but the boundary of the cell was active to invade the surrounding soft tissue to inform an ill defined mass. The experimental results showed it has been successfully constructed VX2 tumor cell Xenograft and induced osteocarcinoma model of rabbit.

QRT-PCR detection

The mRNA expression of NF-κB, Bcl-2 and Apaf-1 in surrounding tissue of tibia in different period after inoculation was analyzed by qRT-PCR, the results was shown as **Table 2**. It was found that after 1 week inoculation, the expression of NF-κB and Bcl-2 in surrounding tissue of tibia was higher than that in control group (P<0.05), and the difference between Apaf-1 and control group was not significant, after 3 weeks of inoculation, compared with control group, the expression of NF-κB and Bcl-2 in surrounding tissue obviously increased (P<0.05), and the expression of Apaf-1 obviously decreased (P<0.05). By comparing the expression of three genes at different time points, we found that the expression level of NF-κB and Bcl-2 gradually increased with the longer time after vaccination (P<0.05) In contrast, the expression level of Apaf-1 decreased gradually (P<0.05).

Detection apoptosis related protein by IHC

The apoptosis related proteins of NF-κB, Bcl-2 and Apaf-1 were analyzed by IHC in protein level, results were shown as **Figure 2**. Three proteins were found in the cytoplasm, by comparing the expression of the three proteins at different time points in the experimental group, we found that the grades of the cytoplasmic staining in the sections of

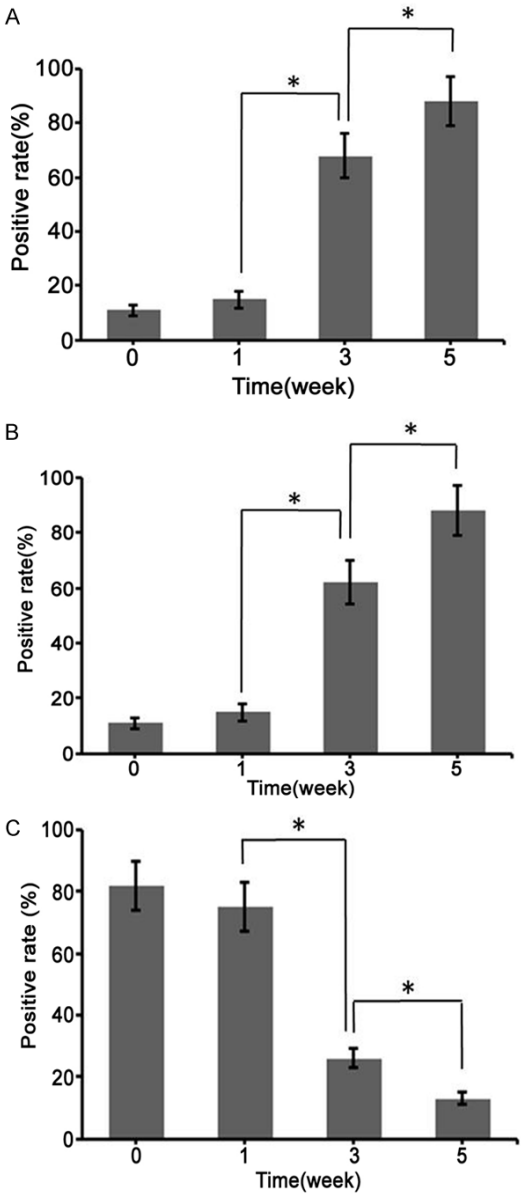

Figure 4. Immunohistochemical analysis of the positive expression of NF-κB, Bcl-2 and Apaf-1. A. Positive rate of NF-κB, verified by immumohistochemical examination. B. Positive rate of Bcl-2, verified by immumohistochemical examination. C. Positive rate of Apaf-1, verified by immumohistochemical examination. *Comparison among groups, P<0.05.

the NF-κB and Bcl-2 after the IHC staining was gradually increased, it showed that the higher the level of NF-κB and Bcl-2 in protein level and it was consistent with the detection results of RT-PCR. Immunohistochemistry analysis of the expression of Apaf-1 in protein levels found that with the longer time after inoculation, the staining grades of Apaf-1 protein was gradually decreased, which showed that the expression level of Apaf-1 in the protein level gradually decreased.

By analyzing the results of the immunohistochemical results, the calculated results are shown in **Figure 3**. The positive rate of NF-nuclear factor kappa Bcl-2 and B expression increased gradually with the increasing of time after inoculation, and the positive rate of Apaf-1 expression decreased gradually, which resultisinagreementwiththeaboveresults.Immunohistochemical analysis **Figure 4** showed the longer time after surgery, the increased positive expression of NF-κB and Bcl-2, while decreased expression of Apaf-1, which was consistent with the above results.

Discussion

The balance between cell Pro- apoptotic and anti-apoptotic effects is of great significance for normal cell function, when this balance is broken, it may cause cancer. The pathway of apoptosis mainly includes pathway of apoptosis induced by extracellular signal and mitochondrial apoptotic pathway, these two pathways are activated by different mechanisms which can regulate cell apoptosis and other cell behaviors [12, 13]. This article intends to study the expression among the three apoptosis related gene of NF-κB, Bcl-2 and Apaf-1 in osteocarcinoma and the relationship with invasion of osteocarcinoma cells. Osteocarcinoma model was induced by construction VX2 tumor cell xenograft, we analyzed the pathological features of rabbit model of tibial bone cancer transplantation position at different time points, and then the expression of three apoptosis related gene of NF-κB, Bcl-2 and Apaf-1 was detected in mRNA and protein levels respectively by RT-PCR and IHC methods. We found that the promotion of time following transplantation, the invasion effect of osteocarcinoma on surrounding tissues near the transplant area was more and more serious, at the same time, the detection results of three apoptosis related genes of NF-κB, Bcl-2 and Apaf-1 showed that the expression of NF-κB and Bcl-2 increased gradually near transplant area, but Apaf-1 expression decreased gradually. Thus, we speculated that the expression of three genes of NF-κB, Bcl- and Apaf-1 may be associated with invasion process of osteocarcinoma.

NF-κB as a nuclear factor can regulate the expression of a varous of apoptosis suppressor gene to control osteocarcinoma and other malignant tumor's occurrence and development and therapeutic tolerance [14]. Studies showed that NF-κB can regulate the expression of proteins such as XIAP, cIAP1 and Bcl-xL, which regulate cell apoptosis through the mitochondrial apoptotic pathway [15-17]. At the same time, a large number of studies have found that NF-κB can regulate the expression levels of MMP, uPA and IL-8 proteins, and these proteins play an important role in the invasion and metastasis of tumor cells [18]. Our study found that with the process of osteocarcinoma invasion to the surrounding tissues, the expression level of NF-κB in peripheral tissues was gradually increased, which was consistent with others.

The anti apoptotic ability of tumor cells is very important for the survival of metastatic cells and the formation of a new metastasis. BCl-2 plays a very important role in the process of cell apoptosis, which can inhibit the apoptosis of cells [19]. The study showed that Bcl-2 could inhibit the apoptosis of cells by interacting with a variety of proteins, including Caspase-3, Bcl-2 can inhibit the activation of Caspase-3 by binding to Caspase-3, which inhibits cell apoptosis [19]. Apaf-1 is an important protein in the mitochondrial apoptosis pathway, it is able to raise caspase-9 through the formation of apoptotic bodies, and through the role of cytochrome C to further activate Caspase-3, and ultimately induce cell apoptosis [20]. Many studies have confirmed that the expression level of Apaf-1 decreased significantly during the invasion and metastasis of malignant tumors [21]. The results of this study found that in the process of invasion to the surrounding tissues with osteocarcinoma, the expression levels of apoptosis inhibiting genes including NF-κB and Bcl-2 in peripheral tissues increased rapidly, while the pro-apoptotic gene Apaf-1 decreased rapidly, which was consistent with previous research findings.

Malignant osteocarcinoma has strong ability of proliferation and invasion, which is serious harm to human body movement function and life health. In recent years, more and more studies have focused on the occurrence and development of related biomarkers of malignant tumors. Through detection the expression level changes of biomarkers related with the occurrence and development of malignant tumor can provide more information renlated with course of disease for patients to develop a more active and effective treatment strategy. This paper discussed the expression changes of three apoptosis related genes during the course of osteocarcinoma invasion, which can provide more ideas for the future diagnosis of osteocarcinoma patients, at the same time, it also provided some theoretical data for the study of drug target of malignant tumor.

Conclusion

Osteocarcinoma model was constructed successfully by VX2 tumor cell xenograft, through the detection r animal model of osteocarcinoma and pathological process found that three apoptosis related genes of NF-κB and Bcl-2 increased in mRNA and protein levels, but Apaf-1 decreased.

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Disclosure of conflict of interest

None.

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