Int J Clin Exp Pathol 2015;8(11):14885-14892
www.ijcep.com /ISSN:1936-2625/IJCEP0010087

Original Article

P15, MDM2, NF-κB, and Bcl-2 expression in primary bone tumor and correlation with tumor formation and metastasis

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Received May 10, 2015; Accepted June 26, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Primary bone tumor is one of the most common malignant tumors in skeletal system. It seriously affected bone movement and development with unclear pathogenesis. In this paper, rabbit VX-2 malignant bone tumor model was applied to explore apoptotic genes P15, MDM2, NF-κB and Bcl-2 correlation with primary bone tumor occurrence and metastasis. 0.3 ml rabbit VX-2 tumor cell suspension (1×10^6/ml) was injected to the marrow cavity of the right tibia condyle to establish the rabbit malignant bone tumor model, while equal amount of the saline was injected to the left tibia as control. Real-time PCR was applied to determine P15, MDM2, NF-κB and Bcl-2 expression level. Immunohistochemistry was performed to detect the abovementioned genes expression in lung, stomach, kidney and bladder. Compared with control, P15 expression level in the inoculation site surrounding tissues decreased obviously following the inoculate time elongation ($P<0.05$), while Bcl-2, MDM2 and NF-κB expression significantly increased ($P<0.05$). Bcl-2 showed significant correlation with MDM2 and NF-κB ($P<0.05$). At the 2, 4, 6 weeks, Bcl-2, MDM2 and NF-κB in lung, Bcl-2 in kidney, and Bcl-2 and MDM2 in bladder positively expressed ($P<0.05$), whereas P15 gene exhibited no significant positive expression in these tissues ($P>0.05$). P15, MDM2, NF-κB, and Bcl-2 genes expression levels can effectively reflect malignant bone tumor growth of rabbit tibia. MDM2, NF-κB and Bcl-2 genes involved in primary bone tumors metastasis directly. It has important clinical significance for early diagnosis and treatment of primary bone tumor.

Keywords: Bone tumor, apoptosis, P15, MDM2, NF-KB, Bcl-2

Introduction

Primary bone tumor is a type of malignant tumor derived from bone tissue mainly presented as osteosarcoma, fibrosarcoma and chondrosarcoma. Of which osteosarcoma accounts for about half of all the body malignant tumors. It mostly appeared in the end of long bone in 10-20 years old teenagers and old man over 60 years old [1]. Primary malignant tumor development has bad influence on skeleton movement. Tumor cell proliferation, growth and migration greatly increase the possibility of distant organ metastasis [2]. P15 gene can affect a variety of tumor cell proliferation and growth through inhibiting cyclin dependent kinase 4/6 to block cell cycle in G1 phase [3]. Murine double minute 2 (MDM2) is a new kind of cell apoptotic suppressor gene belongs to the IAP apoptosis inhibition family. It plays an important role in lung cancer and bladder cancer metastasis. Nuclear factor κB (NF-κB) is a nuclear transcription factor that participated in gastric cancer development by activating multiple inflammatory genes transcription including tumor necrosis factor and interleukin [4]. As a kind of classic proto-oncogene, B-cell lymphoma 2 (Bcl-2) has been found play a crucial regulatory role in a variety of malignant tumor cells apoptosis and distant metastasis, such as lung cancer, liver cancer, gastric cancer, kidney cancer and bladder cancer, etc. [5]. They play an important role in the process of tumor formation and metastasis, which has very important biological significance for malignant tumor early diagnosis and treatment. However, there is still lack of reports about the abovementioned genes correlation with bone tumor metastasis.

Table 1. Primers used for PCR

Gene	Sequence	Amplification length
GAPDH	5'-TGG GGA TGA AGG TCG GAGTG-3'	216 bp
	5'-CAG CCT TGA CGG TGC CAT GGA AT-3'	
p15	5'-TGGCTCTGACCACTCTGC-3'	285 bp
	5'-AGCGAATTCGGGTGGGAAATTGGGGTAAGAA-3'	
Bcl-2	5'-GGG GCT ACG AGT GGG ATG C-3'	320 bp
	5'-GCG GTA GCG GCG GGA GAA GT-3'	
MDM2	5'-GCCUGGCUCUGUGUGUAAUdTdT-3'	195 bp
	5'-AUUACACACAGAGCCAGGCdTdT-3'	
NF-κB	5'-TCTGTT TCC CCT CAT CTTTCC-3'	185 bp
	5'-TGGGTG CGT CTT AGT GGT AT-3'	

In this study, we applied real-time PCR to determine P15, MDM2, NF-κB and Bcl-2 expression level in rabbit malignant bone tumor model, and detect their expression and morphology changes in lung, stomach, kidney and bladder by immunohistochemistry to explore the role of apoptotic gene P15, MDM2, NF-κB and Bcl-2 on primary bone tumors formation and metastasis.

Materials and methods

Experimental animals

Twenty New Zealand rabbits at 3 months old and weighted 2.0-3.0 kg were purchased from Harbin medical University laboratory animal center and raised in standard captivity.

Modeling

Under aseptic condition, VX2 tumor cells suspension was injected to the rabbit hind leg muscle. The tumor diameter reached 5 cm after two to three weeks [6]. The tumor tissue was extracted in sterile under anesthesia. The tumor was cut into 1 mm^3 pieces and filtered in Hanks fluid through cell strainer. After centrifuged at 1000 r/min for 5 min, cell suspension was collected. MTT was applied to calculate the tumor cell survival rate. Rabbit VX2 tumor cell line was purchased from Harbin medical University laboratory animal center.

The rabbit was anesthetized by injecting 3% sodium pentobarbital (1.5 ml/kg body weight) through ear vein under aseptic condition. Right tibia was exposed after the rabbit was fixed. Tibial metaphysis was punctured by 18 # needle through proximal tibial articular surface for about 2.0 cm in depth. The wound was sealed by bone wax after injecting 0.25 ml tumor cells suspension. The left tibia received the same operation and was injected 0.3 ml saline as control.

Bone tumor pathological feature

The rabbit was anesthetized by injecting 0.5% sodium pentobarbital (1.5 ml/kg body weight) at 1, 2, 4, 6 weeks after inoculation. Pathology characteristics of the right tibia bone marrow cavity surrounded bone and muscle was checked by DF-312A-2500 mA X-ray machine, while the tibia on the left side was set as control. Cortical bone, periosteum, cartilage, and muscle tissue pathological destruction was recorded. Pathological characteristics such as bone density decreases, periosteal edema, periosteal new bone and skeletal muscle sarcoma were observed to determine bone tumor proliferation and growth.

Real time-PCR

The muscle tissue at 1 cm under the knee was separated after euthanizing the rabbit at 0, 1, 2, 4, 6 weeks after seeding. Total RNA was extracted from the tumor tissue. The cDNA was synthesized using the RNA. Each real-time RT-PCR reaction (in 20 μL) contained 2.5×SYBR Green Real-time PCR Master Mix (TIANGEN), 0.5 μM primers and 0.5 μL of template cDNA. The cycling conditions for real-time RT-PCR reaction consisted of an initial, single cycle of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 70°C. Gene expression levels were quantified relative to the expression of GAPDH using an optimized comparative Ct (ΔΔCt) value method [7]. All real time PCR reagents were bought from Shanghai Novland Co., LTD. The primers for p15, Bcl-2, MDM2, NF-kB and GADPH were listed in **Table 1**.

Immunohistochemistry

Lung, stomach, kidney, and bladder were extracted and maintained in 10% formalin after euthanizing the rabbit at 0, 1, 2, 4, 6 weeks

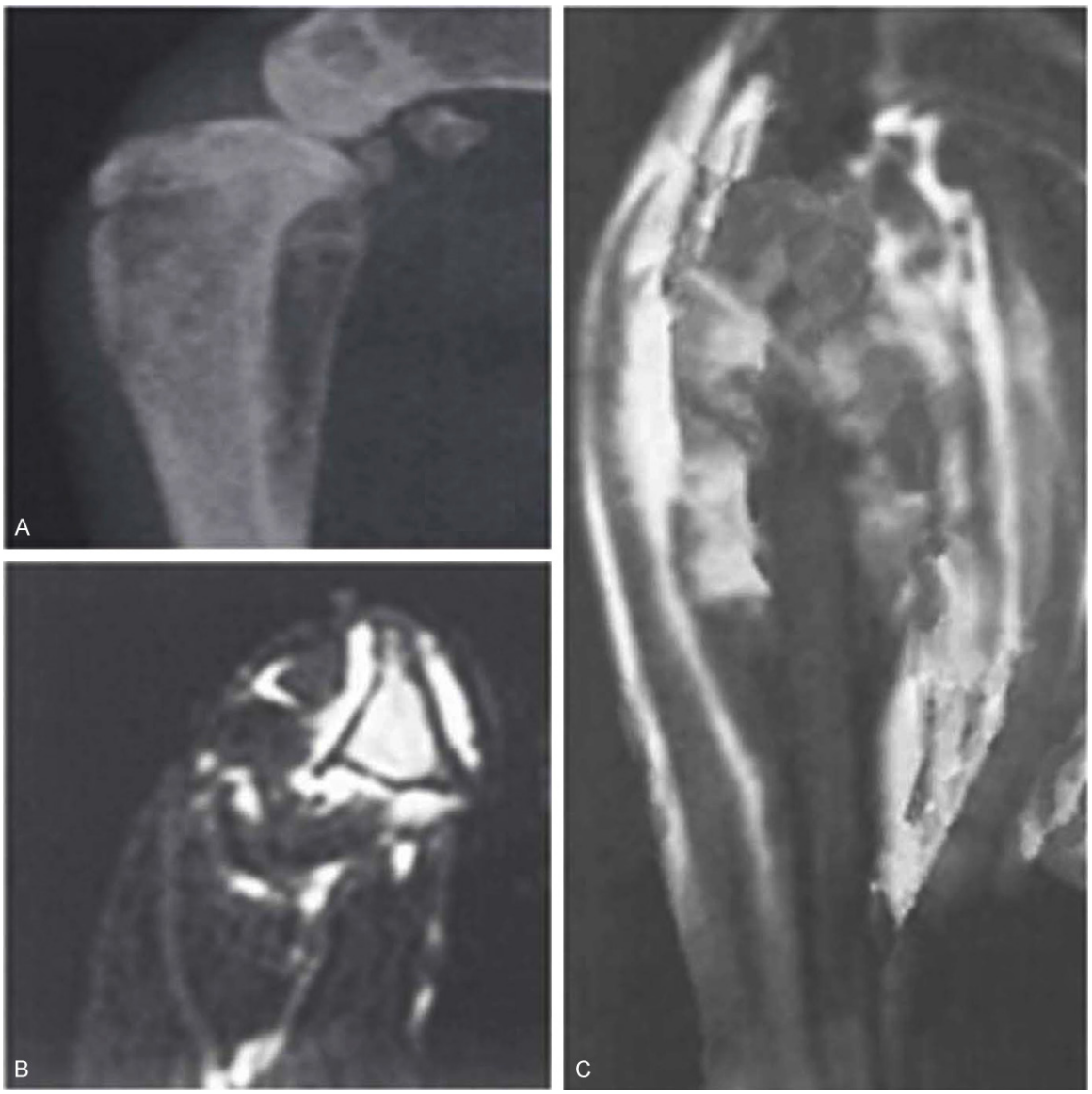

Figure 1. X-ray detection of tibia surrounding tissue pathology characteristics. A. First week, normal tibia structure; B. Second week, bone cortex destruction. C. Fourth and sixth week, tibial periosteal thickening and skeletal muscle sarcomatoid structure.

after seeding. Pathological tissue section was prepared for SP immunohistochemical staining. Positive cells appeared brown granules. Improved Shimzu method was applied as 10 visions cells were counted and proliferation index was calculated by the number of positive staining cells percentage [8]. Expression rate <50% was considered negative, while 50% or higher was positive. All steps were in strict accordance with the immunohistochemical kit manual. Morphological changes were observed and apoptosis related gene P15, MDM2, NF-κB, and Bcl-2 expressions were measured to speculate their relevance with bone tumor metastasis. All immunohistochemical kits were provided by Bioleaf co., LTD. SP immunohistochemical reagent were provided by Beijing Xin Xing Tang biological technology co., LTD. DAB solution were provided by Beijing Hapten and Protein Biomedical Institute.

Statistical analysis

All statistical analyses were performed using SPSS18.0 software (Chicago, IL). Numerical data were presented as means and standard

Table 2. P15, NF-κB, MDM2 and Bcl-2 gene expression and correlation analysis in bone tumor tissue

Index	Group	Detection time/w				
		0	1	2	4	6
P15/%	Control	0.02	0.04	0.08	0.12	0.10
	Experimental group	0.05	0.13	0.85※	1.13※	1.32※
NF-κB/%	Control	0.07	0.09	0.11	0.10	0.12
	Experimental group	0.03	0.22#	0.76※,#	1.14※,#	1.34※,#
MDM2/%	Control	0.05	0.04	0.08	0.13	0.12
	Experimental group	0.02	0.36#	0.93#,※	1.23#,※	1.42#,※
Bcl-2/%	Control	0.02	0.06	0.09	0.14	0.11
	Experimental group	0.04	0.24#	0.83#,※	1.22#,※	1.35#,※

※P<0.05, compared with control; #P<0.05, compared with experimental groups at different time points.

Table 3. Bcl-2 expression in different organs of rabbit bonetumor model

Detection time/w	N	Expression rate/%			
		Lung	Stomach	Kidney	Bladder
0	5	20±1.93	23±1.07	18±1.95	20±2.01
1	5	24±2.23	25±1.13	20±2.05	22±2.13
2	5	56±2.11※	29±1.12	51±2.16※	56±1.92※
4	5	58±2.14※	32±1.08	65±2.13※	63±1.84※
6	4	86±2.22※	38±1.06	72±2.24※	78±2.43※

※P<0.05, positive expression compared with control.

deviation (± SD). Differences between multiple groups were analyzed by one-way ANOVA. *P*<0.05 was considered as significant difference. Spearman correlation and chi-square tests were used to evaluate the relationship between immunohistochemical detected gene and cancer distant metastasis.

Results

Tibia bone tumor pathology characteristics detected by X-ray

No significant tissue pathological change was observed within the first week after inoculation. In the second week, X-ray detection found that local bone destruction began to appear. Tibial periosteal thickening and skeletal muscle sarcomatoid structure were clearly observed at the 4th and 6th week, and skeletal muscle fibrosis became worse (**Figure 1**).

P15, NF-κB, MDM2 and Bcl-2 gene expression in bone tumor tissue at different times

P15, NF-κB, MDM2 and Bcl-2 relative expression levels showed no obvious differences in control at different time points (P>0.05). Following the inoculation time prolonged, P15 gene expression level declined obviously in the experimental group, while NF-κB, MDM2 and Bcl-2 levels elevated. They showed significant differences compared with control at the 2nd, 4th, and 6th week (P<0.05). Spearman correlation revealed that P15 presented no significant correlation with the rest three genes (r=0.22, P=0.22), while NF-κB, MDM2 and Bcl-2 were markedly positively correlated with each other between the expression level of (r=0.85, P=0.03; r=0.72, P=0.04; r=0.91, P=0.02) (**Table 2**).

Immunohistochemical detection of Bcl-2 expression in different organs

At one week after inoculation, Bcl-2 expression was negative in viscera tissues. It began to express in the lung, kidney, and bladder from the second week and showed an elevation trend. Its expression increased obviously in the abovementioned organs at the 2nd, 4th, and 6th week compared with control (P<0.05). However, Bcl-2 expression was negative in stomach and showed no significant difference (P>0.05) (**Table 3** and **Figure 2**).

Immunohistochemical detection of MDM2 expression in different organs

MDM2 expression was negative at the first week after inoculation. It presented positive expression in the lung and bladder at the 2nd, 4th, and 6th week and showed an escalating trend. MDM2 expression elevated significantly compared with control (P<0.05), while its expression was negative in stomach and kidney with no significant difference (P>0.05) (**Table 4**).

Immunohistochemical detection of P15 expression in different organs

P15 was highly expressed both in the experimental groups and control with no significant

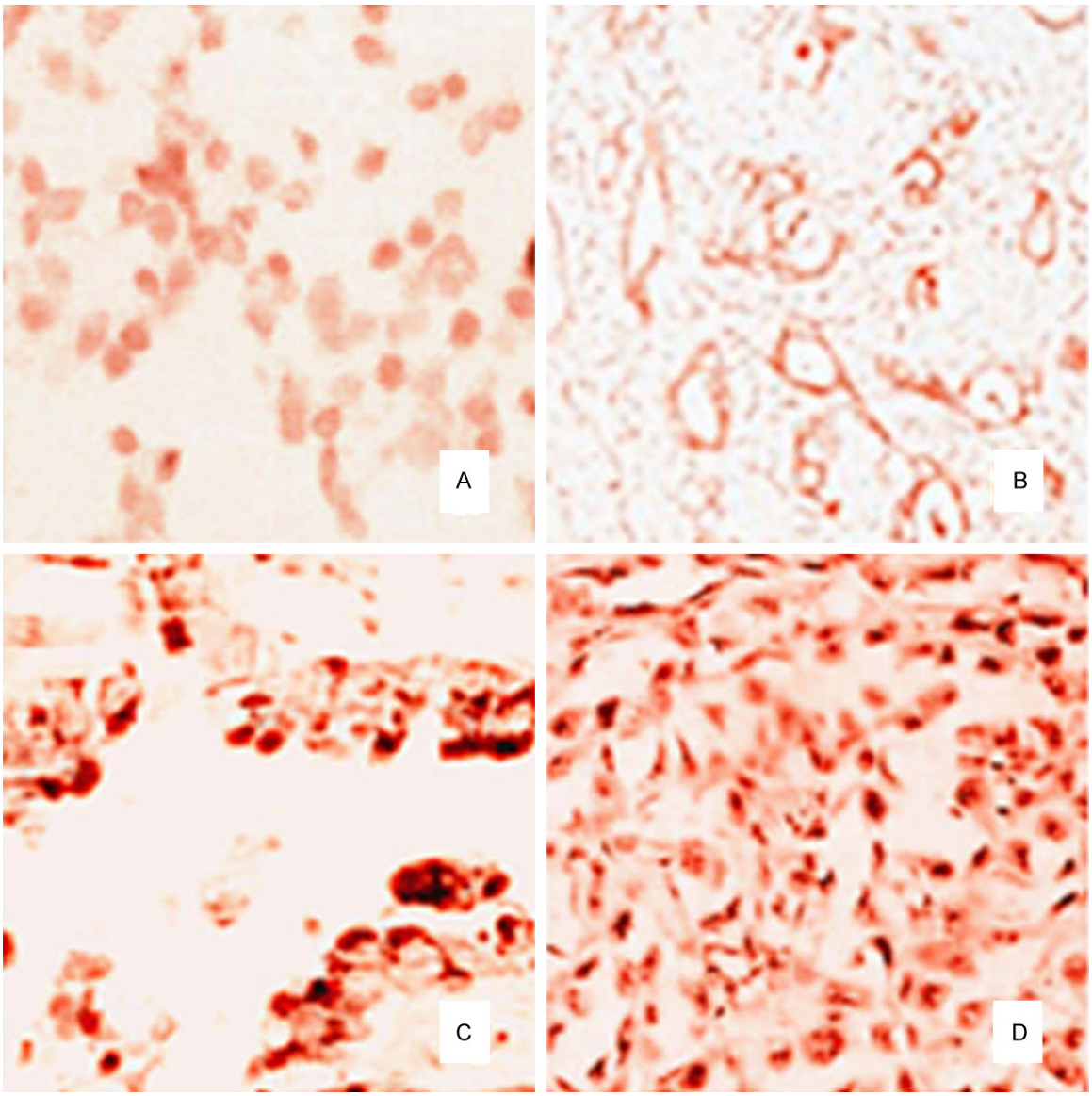

Figure 2. Immunohistochemical detection of Bcl-2 expression in different organs. A. Bcl-2 highly expressed in lung tissue (×200). B. Bcl-2 negatively expressed in stomach tissue (×200). C. Bcl-2 highly expressed in kidney tissue (×200). D. Bcl-2 highly expressed in bladder tissue (×200).

difference (P>0.05), suggesting that P15 was not correlated with tumor metastasis (**Table 5**).

Immunohistochemical detection of NF-κB expression in different organs

NF-κB expressed negatively in all organs at the first week after inoculation. It exhibited positive expression in an escalating trend in the lung from the second week and. NF-κB expression elevated markedly compared with control (P< 0.05), while its expression was negative in stomach, bladder and kidney with no significant difference (P>0.05) (**Table 6**).

Discussion

Primary bone tumor is a common malignant tumor in skeletal system characterized with strong proliferation and metastasis abilities [9] that seriously influence human bone movement and normal life. In recent years, more and more researches focused on the mechanism of malignancy tumor molecular markers, but there is still lack of investigation primary bone tumor formation and distant metastasis. Establishing a good animal bone tumor model and the reasonable experiment design to explore tumor molecular marker effect in malignant bone

Table 4. MDM2 expression in different organs of rabbit bonetumor model

Detection time/w	N	Expression rate/%			
		Lung	Stomach	Kidney	Bladder
0	5	16±0.21	17±0.17	20±1.02	19±0.16
1	5	18±0.23	19±0.14	22±1.12	29±0.18
2	5	54±0.21※	23±0.12	25±1.05	52±1.12※
4	5	63±0.19※	29±0.09	31±1.26	59±1.21※
6	4	85±0.22※	32±0.16	34±1.14	82±1.32※

※P<0.05, positive expression compared with control.

Table 5. P15 expression in different organs of rabbit bone tumormodel

Detection time/w	N	Expression rate/%			
		Lung	Stomach	Kidney	Bladder
0	5	85±0.23	89±0.14	86±1.05	89±0.58
1	5	80±0.33	83±0.18	82±1.12	85±0.16
2	5	74±0.21	76±0.12	72±1.26	77±0.62
4	5	70±0.19	65±0.09	69±1.09	72±0.24
6	4	64±0.22	62±0.16	53±1.32	56±0.82

Table 6. NF-κB expression in different organs of rabbit bone tumor model

Detection time/w	N	Expression rate/%			
		Lung	Stomach	Kidney	Bladder
0	5	12±0.53	11±0.12	17±0.82	13±0.78
1	5	15±0.57	16±0.32	26±0.72	25±0.56
2	5	54±0.41※	26±0.25	30±1.15	58±0.85
4	5	67±0.59※	30±0.19	35±1.16	68±0.64
6	4	75±0.62※	32±0.16	65±0.84	79±0.71

※P<0.05, positive expression compared with control.

tumor is of great significance to better assess bone tumor progression for timely diagnosis and treatment.

A large number of molecular biological studies found that many apoptotic genes play a critical role in malignant tumor cells proliferation and metastasis [10-13]. Once activated, transcripted and amplificated, they can act on cell proliferation and differentiation in different periods, thus control tumor cells abnormal growth and metastasis. Studies showed that P15 gene abnormally expressed in a variety of malignant tumors tissues, such as renal cancer, bladder cancer, tongue squamous cell carcinoma, nasopharyngeal carcinoma, and multiple myeloma, etc. [14]. P15 gene positive expression is negatively correlated with the deterioration degree of ovarian cancer and nasopharyngeal tumor [15]. P15 expression is not related to lymphocyte metastasis and T stage, indicating that P15 gene deletion only participates in the early stage of nasopharyngeal carcinoma (NPC) but not disease progression [16, 17]. P15 gene is a tumor suppressor gene that can block G1-S phase and G2-M phase by inhibiting cyclin-dependent kinases (CDK) 4 and CDK6 activity. In addition, P15 gene methylation and abnormal transcription can lead to its tumor suppressor function deletion, thus enhancing tumor cells growth transfection and metastasis ability. Bcl-2 gene product is an apoptosis inhibitor which overexpression can promote tumor cells proliferation. Real time PCR found Bcl-2 gene abnormal expression in lymphoma patients [18]. After DNA damage, Bcl-2 can inhibit P53 mediated cell apoptosis by enhancing cells resistance to DNA damage factors [19, 20]. MDM2 gene amplification is closely related to tumor cells growth and metastasis [21, 22]. Currently, it was found that MDM2 overexpressed in many tumor tissues, such as bladder cancer, prostate cancer, osteosarcoma, and gliomas [23]. MDM2 can combine with P53 to form compounds, while P53 can induce MDM2 expression. MDM2 can also suppress P53 transcription directly and accelerate it degraded by protease, so as to promote tumor cells proliferation [24]. Transcription factors NF-κB is a dimer composed of P50 and P52 that can promote tumor cell DNA transcription and proliferation [25].

In this paper, we established rabbit tibia malignant bone tumor model, and detected the apoptotic gene MDM2, Bcl-2, P15 and NF-κB expression in the tissue surrounding the tibia bone tumor. We further measured gene expression in multiple organs to determine their correlation with bone tumor formation and metastasis. The results showed that no within no significant pathological changes in the tissues surrounding the tibia in the first week after inoculation; X-ray examination presented bone destruction from the second week after inoculation; tibial periosteal thickening and skeletal muscle sarcomatoid structure can be observed at the 4th week, and skeletal muscle fibrosis became worse. Real time-PCR detection revealed that P15 gene expression significantly

reduced, while MDM2, Bcl-2 and NF-κB overexpressed obviously in the tibia compared with control ($P<0.05$), indicating that these four genes were involved in bone tumor development and regulated tumor cell proliferation. It may be caused by P15 gene methylation, which reduced its expression rate and made it lose the function of inhibiting tumor cell proliferation and differentiation. MDM2 and Bcl-2 overexpressed significantly and showed obvious positive correlation ($P<0.05$), suggesting MDM2 and Bcl-2 may act on cell cycle and apoptosis indirectly through regulating P53 at the same time. NF-κB up-regulation may lead to it transfers from the cytoplasm into the nucleus, promoting tumor cell proliferation and transcription. Skeletal muscle sarcomatoid structure began to appear in the lung, kidney, and bladder tissue from the second week. Among them, MDM2, Bcl-2, and NF-κB expression level elevated obviously in the lung ($P<0.05$); Bcl-2 up-regulated in the kidney ($P<0.05$); MDM2 and Bcl-2 expression increased in the bladder ($P<0.05$); while no obvious gene expression changes in stomach ($P>0.05$). It suggested that P15 gene only participated in the formation process of bone tumor, but not in bone tumor distant metastasis. MDM2, Bcl-2 and NF-κB involved in both bone tumor formation and metastasis to lung. Bcl-2 gene overexpression is associated with bone tumors metastasis to kidney and bladder, while MDM2 gene is participated in bladder metastasis. More tests about their roles and mechanism in bone tumor formation and metastasis were needed.

To sum up, MDM2, Bcl-2, P15 and NF-κB all participate in primary bone tumor formation and development. Of them, P15 gene only related to bone tumor proliferation but not distant metastasis. MDM2, Bcl-2 and NF-κB were related to lung metastasis, Bcl-2 was associated with renal metastasis, and MDM2 and Bcl-2 were involved in bladder metastasis. It provides reference bases for bone tumor early diagnosis and treatment after metastasis.

Disclosure of conflict of interest

None.

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