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ORIGINAL ARTICLE

The function of DREAM gene mediated by NF-kB signal pathway in the pathogenesis of osteosarcoma

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Summary

Purpose: *To explore the function of DREAM gene mediated by NF-kB signal pathway in the pathogenesis of osteosarcoma.*

Methods: *This study included 13 Sprague Dawley (SD) rats with osteosarcoma (treatment group) and 13 healthy rats (control group). NF-kB, DREAM and P105 mRNAs expression levels were determined using quantitative PCR (qPCR). The expression levels of NF-kB, DREAM and P105 proteins were evaluated using ELISA and western blot. Also, DREAM protein expression in rats was determined by immunofluorescence.*

Results: *NF-kB and DREAM levels in the treatment group were significantly higher than those in the control group ($p<0.05$). However, P105 mRNA expression level in the treatment group was significantly lower than in the control group ($p<0.05$). Results obtained from ELISA and western blot showed that NF-kB and DREAM expression levels in the treatment group were significantly higher than in the control group ($p<0.05$). NF-kB and DREAM levels in the treatment group were 4.3±0.12 μg/l and 6.8±0.21 μg/l, respectively. These levels in the control group were 0.96±0.11 μg/l and 1.25±0.18 μg/l, respectively. P105 expression level in the treatment group was 0.37±0.11 μg/l which was significantly lower than that in the control group (1.63±0.21 μg/l) ($p<0.05$). Immunofluorescence results showed that DREAM expression level was significantly higher in the treatment group ($p<0.05$).*

Conclusion: *NF-kB signal pathway promoted the expression of DREAM gene and also promoted the pathogenesis and worsening of osteosarcoma.*

Key words: *DREAM gene, glial cells, immunofluorescence, NF-kB signal pathway, osteosarcoma, P105 gene*

Introduction

Malignancies caused by habits, lifestyle and environmental factors are increasing [1]. Osteosarcoma is a malignancy of bone with increased morbidity and its clinical manifestations include persistent and progressive ostealgia leading to intractable pain [2], which impact the patient quality of life (QoL). Statistical data [3] show that the morbidity of osteosarcoma increases by 0.68% each year [4]. In 2015, there were 124,000 patients with osteosarcoma in China, which emphasizes the importance of investigating the mechanism of osteosarcoma occurrence and development as well as the need for developing of new diagnostic and treatment methods [5]. Recent studies found that NF-kB has been involved in many signaling pathways [6]. For example, abnormal levels of key modulators such as Toll Like Receptor 4 (TLR4) in NF-kB signaling pathway were detected in breast and colon cancers [7,8]. It has been shown that the expression level increased with deterioration of the patient condition. It was reported that the downstream regulatory element antagonist modulator (DREAM) was an important member of neuronal calcium sensor (NCS) family, and was involved in

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intracellular release of neurotransmitters, the activity of Ca^{2+} channel and gene transcription [9]. It has also been reported that DREAM could bind to the promoter region and inhibit the expression of the target genes [10]. DREAM has been reported to be involved in the transmission of pain, yet the relationship between DREAM and osteosarcoma, and the pathway through which DREAM is involved in this disease is not completely understood [11].

In this study we tried to explore the relationship between NF-kB-based DREAM gene and osteosarcoma in order to provide theoretical and experimental evidence for the diagnosis and treatment of this malignant disease.

Methods

General information

In this study, 13 SD rats with osteosarcoma, aged 4.1±1.2 weeks on average were used as the treatment group. This included 5 male and 8 female animals. Thirteen healthy SD rats (5 males and 8 females) were used for the control group. The average age for the control group was 4.5±1.1 weeks.

Reagents

RAN extract kit (Xinmai Biological Technology Co., Ltd.,China), Quantitative Fluorescence PCR kit (ABI,USA), rabbit anti-human NF-kB, rabbit anti-human DREAM, rabbit anti-human P105 monoclonal antibodies (ACRIS,USA), HRP-labeled mouse anti-rabbit polyclonal antibodies (secondary antibodies, Suzhou, Jinweizhi, China), fluorescence-labeled secondary antibody (Thermo,USA), 10% goat serum (Suzhou, Bosai, China), immunohistochemistry kit (Roche, USA). Other reagents were purchased from Shanghai Guoyao Co., Ltd, China.

Instruments

Quantitative Fluorescence PCR amplifier (ABI,USA), microplate reader (Beijing Liuyi, China), protein microelectrophoresis apparatus (Beijing Liuyi, China), gel imaging system (BIO-RAD, USA), low-temperature and high-speed centrifuge (Hellich, Germany), fluorescence microscope (Olympus, Japan).

RNA extraction

Samples were thawed and 500 µl RNA Plus were added. Samples were then centrifuged at 1000 g for 10 min at 4°C and the supernatant was discarded. Chloroform (200 µl) was added and mixed well, and the mixture was placed on ice for 15 min. Samples were then centrifuged at 1000 g for 15 min at 4°C. The supernatant was transferred into a RNase-free EP tube, and equal volume of isopropanol was added. After 10 min on ice, samples were centrifuged at 1000 g for 5 min at 4°C and the supernatant was discarded. Ethanol (750 µl,75%) was added and mixed well. Samples were then centrifuged at 1000 g for 10 min at 4°C and the supernatant was discarded. Residual ethanol was removed and appropriate volume of RNase-free water was added. The extracted RNA was weighted and used for reverse transcription.

qPCR

qPCR was performed according to the manufacturer's instructions (TAKARA, Tokyo, Japan). Primers were synthesized by Shanghai Sangon Biological Technology Co., Ltd., China. The sequences of these primers are presented in Table 1.

Table 1. The primers for qPCR

Primers	*Sequences*
NF-kB-F	CTAGCTAGCTACGGCATCGATCG
NF-kB-R	CGTAGGAGTCGATCGATATAGCTACG
Dream-F	CGTAGGCTAGCATGATCGATAGCC
Dream-R	CTAGGAGATCGATCGATCGATCGAC
P105-F	CGTAGCTAGCTACGAGGCATCGATC
P105-R	CGTAGCTACGATCGATAGATCAGTC
GAPDH-F	GTCGATGGCTAGTCGTAGCATCGAT
GAPDH-R	TGCTAGCTGGCATGCCCGATCGATC

ELISA

ELISA was performed as described in the instructions (TAKARA). The protein standard was diluted (1:50) with the Assay Buffer in the ELISA kit, and the standard curve was plotted. The sample was diluted with PBS (pH=7.2, 1:100) and 100 µl of sample were added into each well of 96-well plates. The sample was incubated with 50 µl assay buffer at room temperature for 2 hrs, and 3,3', 5,5' tetramethylbenzidine (TMB) substrate was then added for visualization. The absorbance was measured at a wavelength of 495 nm. The NF-kB, DREAM and P105 levels were calculated based on the standard curve [12].

Western blotting

This was carried out according to the manufacturer's instructions for protein extraction (AXYGEN, Tewksbury, USA). Samples (0.5 mg) were collected from in both groups and 20 µl of the sample were used for SDS-PAGE. Proteins were then transferred onto PVDF membrane, which was blocked for 2 hrs. The membrane was incubated with primary antibody (1:1000) at room temperature followed by 2-h incubation with secondary antibody at room temperature. The membrane was washed 5 times (10 min each) [13] and finally electrochemical luminescence (ECL) was used for visualization [9].

Immunohistochemistry procedures

Samples obtained from both groups were fixed with 10% formaldehyde and embedded with paraffin. Parafin sections (4 µm in thickness) were fixed on the slides and heated at 70°C for 1 hr. Sections were dewaxed with xylol and dehydrated with graded absolute ethanol; residual ethanol was removed by ultrapure water. The sections were washed with PBS (pH 7.2) for 5 min×5 times. Finally, the sections were placed in an autoclave (121°C, 2 min), and after cooling, the sections were placed in phosphate buffer saline (PBS) at room temperature for 30 min. The sections were incubated in 0.3% Triton X-100 buffer for 30 min and washed with 0.01 M PBS for 5 min (2-3 times). The sections were then blocked in 10% goat serum for 2 hrs and incubated with primary antibody (1:1000) at room temperature for 2 hrs and then washed with PBS for 10 min (3-5 times). Then, the sections were incubated with secondary antibody (1:700) at room temperature for 20 min and washed with PBS for 10 min (3-5 times). Finally, the sections were placed on polylysine-coated slides, mounted with glycerol:PBS (7:3) and observed under a microscope.

Statistics

SPSS version 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Categorical data were compared by chi-square test with $\alpha = 0.05$, $p<0.05$ indicating significant difference and $\alpha=0.01$, $p<0.05$ indicating very significant difference.

Results

NF-kB mRNA expression level

Total RNA was extracted and RNA was examined with electrophoresis. As shown in Figure 1A, the total RNA was not significantly degraded, indicating that the extracted RNA could be used in qPCR (Figure 2). NF-kB mRNA expression level in the treatment group was 48.2-fold higher than that in the control group (Figure 1) ($p<0.05$).

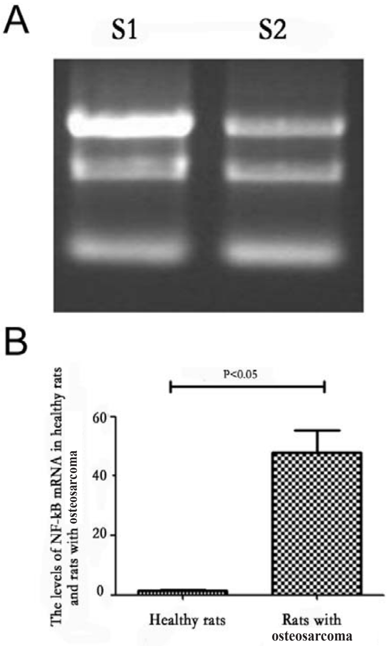

Figure 1. NF-kB mRNA expression level in healthy rats and rats with osteosarcoma determined by electrophoresis and qPCR. **A:** The electrophoresis of total RNA, S1 and S2 represents the total RNA extracted from different rats; **B:** qPCR results.

DREAM mRNA expression level

DREAM mRNA in the treatment group was 65.3-fold higher than that in the control group (Figure 2) ($p<0.05$).

P105 mRNA expression level

P105 mRNA expression level in the treatment group was 40.4-fold higher than that in the control group (Figure 3) ($p<0.05$).

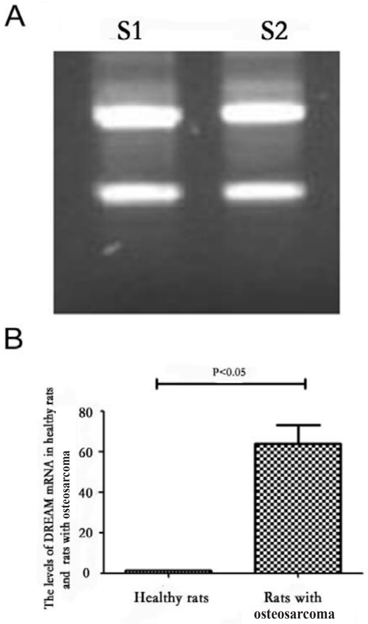

Figure 2. DREAM mRNA expression level in healthy rats and rats with osteosarcoma determined by electrophoresis and qPCR. **A:** The electrophoresis of total RNA, S1 and S2 represents the total RNA extracted from different rats; **B:** qPCR results.

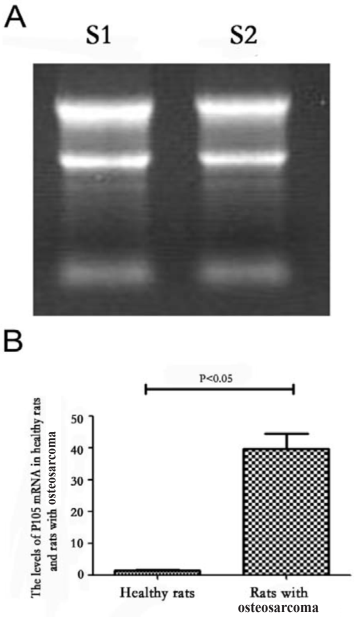

Figure 3. P105 mRNA expression level in healthy rats and rats with osteosarcoma determined by electrophoresis and qPCR. **A:** The electrophoresis of total RNA, S1 and S2 represents the total RNA extracted from different rats; **B**: qPCR results.

NF-kB, DREAM and P105 proteins expression levels

Results obtained from ELISA revealed that the expression levels of NF-kB (4.3±0.12 µg/l) and DREAM (6.8±0.21 µg/l) in the treatment group were significantly higher than those in the control group (0.96±0.11 µg/l, 1.25±0.18 µg/l, respectively) ($p<0.05$) (Figure 4). P105 protein expression level the treatment group (0.37±0.11 µg/l) was significantly lower than that in the control group (1.63±0.21 µg/l) ($p<0.05$) (Figure 4).

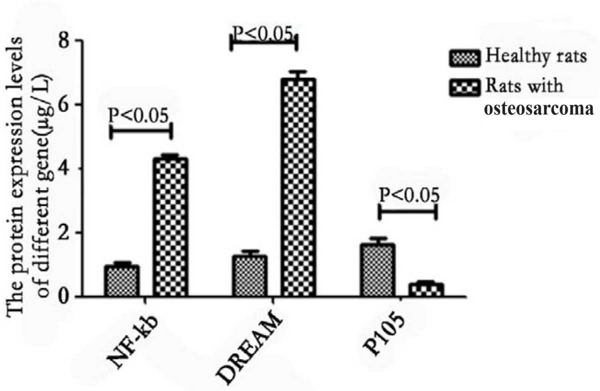

Figure 4. NF-kB, DREAM and P105 expression levels in healthy rats and rats with osteosarcoma (ELISA).

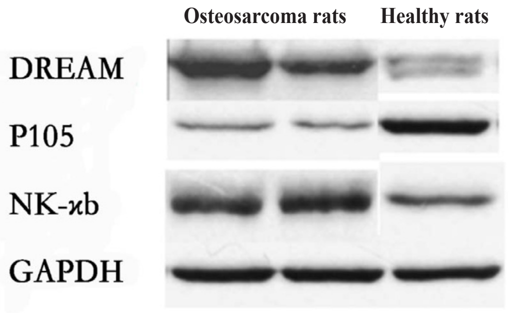

Figure 5. NF-kB, DREAM and P105 expression levels in healthy rats and rats with osteosarcoma (Western blot). NF-kB and DREAM were significantly higher and P105 was significantly lower in rats with osteosarcoma compared with healthy rats.

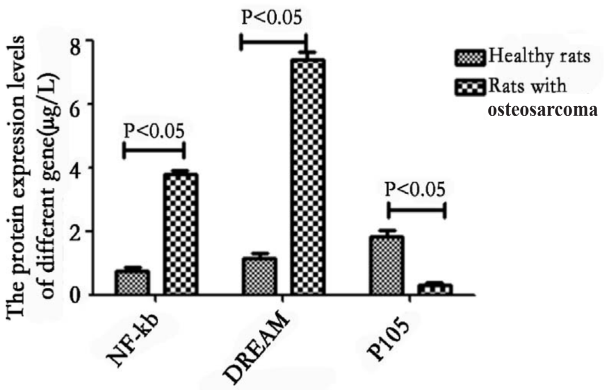

Figure 6. Semi-quantitative measurement of the Western blot results. Expression of NF-kB and DREAM were significantly higher and P105 was significantly lower in rats with osteosarcoma compared with healthy rats.

Results obtained from western blot revealed that the expression levels of NF-kB and DREAM in the treatment group were significantly higher than those in the control group ($p<0.05$).

Compared with the control group, P105 protein expressin level was significantly lower than that in the treatment group ($p<0.05$; Figure 5). These results were consistent with the semi-quantitative measurement of the proteins in the gel imaging system (Figure 6).

Immunofluorescence

The intensity of fluorescence in the tissue lesions in the treatment group was significantly higher than that in the control group (Figure 7). This suggested that the DREAM protein expression level in osteosarcoma tissue was significantly higher than that in the bone tissue in healthy rats.

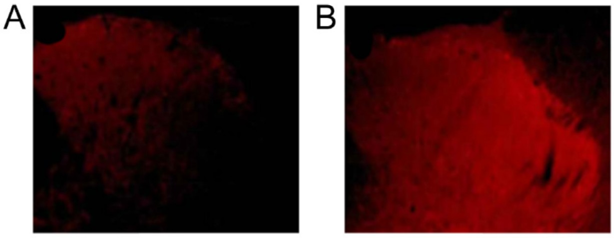

Figure 7. DREAM expression levels in healthy rats and rats with osteosarcoma (immunofluorescence). Red indicates positive expression of DREAM. **A:** healthy rats. **B:** rats with osteosarcoma.

Discussion

Previous evidence [13] showed that anaesthetics injection could significantly inhibit nerve ending, affect the release of neurotransmitters in nerve cells, and delay nerve conduction [14]. Thus inhibition of nerve cells could indirectly inhibit the femoral pain caused by osteosarcoma. In this study, we detected significantly higher levels of NF-kB and DREAM mRNAs expression in rats with osteosarcoma suggesting that NF-kB and DREAM were significantly related to osteosarcoma. However, the expression level of P105 (a protein that inhibited NF-kB) was significantly lower in rats with osteosarcoma. NF-kB and DREAM protein expression levels were significantly higher in rats with osteosarcoma, however on the contrary, the expression level of P105 was significantly lower in rats with osteosarcoma, which was consistent with the result of qPCR.

We concluded that DREAM gene could mediate the pathogenesis and lead to deterioration of osteosarcoma through NF-kB signaling pathway.

This study did not investigate the interaction between DREAM gene and osteosarcoma cells [15], and how DREAM gene promoted the generation and proliferation of osteosarcoma cells [16]. A previous study [17] showed that DREAM gene could regulate the cell cycle [18] and accelerate cell proliferation through promoting the production of tubulins [19,20].

Conflict of interests

The authors declare no confict of interests.

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