Expression and significance of Kruppel-like factor 6 gene in osteosarcoma

Kai Chen · Yu Chen · Xiao-dong Zhu · Yu-shu Bai ·
Xian-zhao Wei · Chuan-feng Wang · Zi-qiang Chen
Ming Li

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Abstract

Purpose Osteosarcoma is primary malignant tumour of bone. Kruppel-like factor 6 (KLF6) is a tumor suppressor gene frequently inactivated in a number of human cancers and a ubiquitously expressed zinc-finger transcription factor. The present study aimed to first explore the relationship between the expression level of the KLF6 gene in osteosarcoma and the occurrence of bone tumours.

Methods KLF6 mRNA and protein expression levels in osteosarcoma and normal bone tissue were assayed by real-time quantitative PCR and immunohistochemistry. KLF6 mRNA and protein expression levels in osteosarcoma cells and normal osteoblasts were detected by semi-quantitative reverse transcription PCR and Western blotting, respectively.

Results Both the expression of KLF6 mRNA and protein in osteosarcoma cells and tissues were significantly lower than that in normal cells and tumour-adjacent tissues.

Conclusions KLF6 is a putative tumor suppressor gene involved in osteosarcoma which can be used as a new therapeutic target and an important marker for early diagnosis and postoperative monitoring.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone and often occurs in young people (ten–20 years old) or the elderly population (50–60 years old) [1]. The degree of malignancy and metastasis rate of osteosarcoma are very high.

Distant metastasis, especially lung metastasis, is the main reason for the treatment failure and death of most patients with osteosarcoma [2]. Clinical diagnosis of osteosarcoma is difficult because of covert morbidity features. Once diagnosed, bone tumours have migrated and thus cause death. The major therapeutic strategy in clinic is methotrexate, cisplatin, doxorubicin, ifosfamide and other drug-based chemotherapy, surgery and postoperative chemotherapy [3, 4], which have been demonstrated to improve the average five-year survival of osteosarcoma patients by 60 %. However, development of drug resistance is a key element in the failure of chemotherapy treatment [5, 6]. Moreover, to some extent, the surgery and chemotherapy will cause damage to the human body and produce possible toxic side effects in most organs, thereby affecting the quality of life of patients [7]. It is undeniable that the average five-year survival of osteosarcoma after surgery is maintained at a lower level of 30 %. Therefore, there is an urgent need to explore new technologies to improve the survival rate of osteosarcoma. As the rapid development of molecular genetics and molecular biology, genetic diagnosis and treatment have recently become a promising approach for cancer therapy [8–10]. The use of genetic diagnostic and therapeutic technologies play an active prevention and treatment role for early diagnosis of osteosarcoma and lung metastases [11]. Hence, it is necessary to search for candidate tumour-related genes and further clarify the molecular mechanism of osteosarcoma tumorigenesis.

Kruppel-like factor 6 (KLF6) is one of the family members of the Kruppel-like transcription factors [12]. Kruppel-like family proteins play important roles in regulating cell growth, proliferation, differentiation and angiogenesis [13]. KLF6 is a ubiquitously expressed transcription factor with zinc lipid structure in a variety of cells. Recent studies show that KLF6 is a ubiquitous tumor suppressor gene [14, 15]. Somatic cell mutations or allelic loss down-regulates KLF6 gene expression. KLF6 expression is closely associated with the

Kai Chen and Yu Chen contributed equally to this work.

K. Chen · Y. Chen · X.-d. Zhu · Y.-s. Bai · X.-z. Wei · C.-f. Wang ·
Z.-q. Chen · M. Li (✉)

Department of Orthopaedics, Changhai Hospital,
Second Military Medical University,
Shanghai 200433, China
e-mail: mingliming@hotmail.com

occurrence of liver cancer, prostate cancer, colorectal cancer, malignant glioma, oesophageal cancer and lung cancer [15–21]. In the present study, quantitative PCR and immunohistochemistry were used to detect the KLF6 mRNA and protein expression in 32 cases of osteosarcoma tissue collected from clinical samples as well as in osteosarcoma cell lines MG63 and Saos2. The relationship between the changes of KLF6 gene expression and the occurrence of osteosarcoma was investigated. This work may provide an important reference value for early diagnosis and gene therapy of osteosarcoma.

Materials and methods

Materials and reagents

From April 2008 to December 2010, 32 archived paraffin blocks of osteosarcoma biopsy specimens were obtained from patients ($n=32$) at our hospital, including 20 males and 12 females whose ages were from 12 to 48 years old (the average age was 19). All patients did not receive radiotherapy and chemotherapy before surgery. They were diagnosed with osteosarcoma by postoperative pathological examination. Five cases of normal bone tissue were collected as a control. Human osteosarcoma cell lines MG63, Saos2, and normal control bone cell line hFOB1.19 were obtained from our laboratory. KLF6-antibody was purchased from Santa Cruz (CA, USA). Immunohistochemistry substance P (SP) kit and diaminobenzidine (DAB) colour reagent were purchased from the New Biotechnology Development Co., Ltd. in Fuzhou, China.

Immunohistochemistry for detecting KLF6 protein expression in tissues

The paraffin sections (5 μm) were roasted overnight at 70 °C, dewaxed and then immersed in the distilled water following routine methods. For antigen retrieval, specimens were incubated with 10 mM citrate buffer (pH 6.0) by incubating at 92–98 °C for 15 min. Avidin-biotin-peroxidase complex immunohistochemical staining was used after slow rewarming. Positive hepatocellular carcinoma section was regarded as a positive control and PBS (0.01 mol/L, pH 7.4) instead of primary antibody as a negative control. Immunohistochemistry analysis was determined as Fromowitz et al. described [22] with some modification. The results were divided into four levels (–, +, ++, +++) according to the scores of positive signal strength and number of positive cells.

Quantitative real-time PCR detection of KLF6 mRNA expression in osteosarcoma

Total RNA were extracted from osteosarcoma and normal bone tissues using Trizol reagent, and 1 μg total RNA was

used in a reverse transcription reaction for synthesis of cDNA. TaqMan fluorescent probes (Ambion's) were used to detect KLF6 expression in each tissue. The β -actin mRNA expression was considered as an internal control. The reaction system was 20 μL : 1 μL TaqMan microRNAs assay (20 $\times$), 10 μL Taqman 2 $\times$ Universal PCR master Mix, 7.67 μL Nuclease-free water, and 1.33 μL cDNA. The PCR reaction conditions were 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min, followed by 42 repeated step-cycles. Each sample contained KLF6 and β -actin gene primers adding the corresponding TaqMan probe for quantitative PCR reaction. Blank control was also used. Each reaction had three-repeated wells. A standard curve was constructed using the logarithmic concentration of the serial dilutions as X axis and the corresponding threshold cycle (Ct) as Y axis.

Detection of KLF6 mRNA expression in cell lines by semi-quantitative RT-PCR

RT-PCR was performed to detect KLF6 mRNA expression in osteosarcoma cell lines MG63 and Saos2 as well as in the negative control bone cells hFOB1.19. Total RNA was extracted from cells at logarithmic phase using Trizol reagent. The AMV reverse kit was used to synthesize cDNA. The sequences of the primers were as follows: KLF6 sense strand 5'-TGACGAGGAGAGAGAGCTT GAAA-3'; and antisense strand 5'-CAACATCCAGGGTC GACAGAAT-3'; β -actin sense strand 5'-CCCAGCACA ATGAAGATCAAGATCA-3'; and β -actin antisense strand 5'-ATCTGCTGGAAGGTGGACAGCGA-3'. The reaction system was 25 μL , including 2 μL cDNA, 2 μL dNTP, 1 μL primer(each) and 0.5 μL Taq polymerase (0.5 U/L). The reaction parameters were denaturation at 95 °C for 5 min, 94 °C 30 s, 53 °C 30 s, and 72 °C 45 s, followed by 32 repeated step-cycles, and then 72 °C for 10 min. PCR products (5 μL) were separated by 2 % agarose gel electrophoresis and analysed by using the gel imaging analysis system and camera.

Analysis of KLF6 protein expression in cell lines by Western blotting

Cell lysate (200 μL) was separately added into MG63, Saos2, and hFOB1.19 cells, and 1×10^7 cells were collected by digestion with 0.25 % trypsin (0.02 % EDTA). After centrifuging at 3000 g for 5 min at 4 °C, 50 μL cell lysate was added to the cell pellet and the tissue cell was suspended with oscillator on ice for 30 min. After centrifuging at 12000 g for 15 min at 4 °C, the supernatant was collected. Protein concentration was quantitated by using bicinchoninic acid method. The sample buffer was added to the protein and boiled for 10 min. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12 % gel. The proteins were then transferred onto polyvinylidene fluoride membrane and

Table 1 Pathological examination of the 32 cases of osteosarcoma biopsy specimens

Information on clinical pathology	Number of cases	KLF6 protein-positive cases	
		Number of cases	Percent (%)
Gender			
Male	20	16	80.0
Female	12	19	75.0
Age (years)			
≥18	18	14	77.8
<18	14	11	78.6
Position			
Femur	15	12	80.0
Tibia	12	9	75.0
Other	5	4	80.0
Dahlin type			
Osteoblastoma type	9	6	66.7
Chondroblastoma type	7	5	71.4
Fibroblast type	7	6	85.7
Others	9	7	77.8

blocked with Tris buffer saline solution containing 5 % nonfat dry milk at 4 °C for 2 h and incubated with the KLF6 antibody overnight at 4 °C. The membrane was washed by Tris-buffered saline/Triton X-100 buffer and then horseradish peroxidase-labeled IgG was added and incubated at room temperature for 90 min. The imaging was performed by chemiluminescent detection system.

Results

KLF6 protein expression is detected in tissues by immunohistochemistry

In 32 osteosarcoma cases, the ratio of male-to-female was 1.67:1, and the average age was 19 years old. The osteosarcoma

mainly developed in the tibia and femur (27 cases, 84.4 %), and the major type included the osteoblast cell, the chondroblastic-type and fibroblast-type (23 cases, 71.9 %; Table 1).

Immunohistochemical staining revealed that KLF6 protein expressed both in nucleus and cytoplasm. KLF6 expression was 100 % in five cases of normal bone tissue sections. Expression of KLF6 protein was 25/32 (78.1 %) in 32 cases of osteosarcoma. The level of KLF6 protein in 25 KLF6-positive expressed cases of osteosarcoma samples was significantly lower than normal tissue (Fig. 1).

KLF6 expression in osteosarcoma tissues are detected by real time PCR detection

In order to confirm KLF6 expression, the real time PCR was performed and results showed that KLF6 mRNA levels in 32 cases of osteosarcoma were lower than normal bone tissue. The starting concentration of KLF6 cDNA in the matched normal bone (0.396 ± 0.081) was about seven times greater than that in osteosarcoma (0.058 ± 0.024), which were calculated through β -actin-standardization of KLF6 cDNA. The standardized values of the KLF6 starting copy number were compared using Student's t-tests by SPSS 12.0 software. The results indicated that the difference of the KLF6 gene expression level was significant ($t=3.683$, $P<0.05$) between osteosarcoma and normal bone tissue. Thus, the results suggested that KLF6 expression was significantly reduced in primary osteosarcoma.

KLF6 mRNA expression levels in osteosarcoma cells

Semi-quantitative RT-PCR results showed that KLF6 mRNA expressed both in osteosarcoma cell lines MG63 and Saos2, as well as in the control bone cells hFOB1.19; their expression levels were 0.41 ± 0.03 , 0.39 ± 0.04 and 2.50 ± 0.11 , respectively. Statistical analysis showed that KLF6 mRNA expression levels in MG63 and Saos2 cells were significantly lower than the normal control cells hFOB1.19 ($P<0.05$) (Fig. 2). There was no significant difference of KLF6 mRNA expression between two osteosarcoma cell lines ($P>0.1$).

Fig. 1 Expression of KLF6 protein in osteosarcoma and normal bone tissues. KLF6 protein expression was detected by immunohistochemistry (a). Osteosarcoma tissue showed strong cytoplasmic

immunostaining of KLF6 (DAB, $\times 200$). b Normal bone tissues showed negative immunostaining of mirror image complementary antibody

Fig. 2 KLF6 gene expression levels in different cell lines. Semi-quantitative RT-PCR analysis revealed that the expression of KLF6 mRNA in osteosarcoma MG63 and Saos2 were significantly lower than that in the normal cell hFOB1.19

KLF6 protein expression levels in osteosarcoma cells

Western blot analysis showed that KLF6 protein expressed in MG63, Saos2 and hFOB1.19 and its molecular weight was approximately 46 kD; KLF6 protein levels were 0.335 ± 0.025 , 0.382 ± 0.043 and 1.480 ± 0.130 , respectively. As shown in Fig. 3, KLF6 protein expression in osteosarcoma cells MG63 and Saos2 were significantly reduced compared with normal cells hFOB1.19, which was further confirmed by statistical analysis ($P < 0.05$). Identically, there was no significant difference of KLF6 protein expression between two osteosarcoma cell lines ($P > 0.1$).

Discussion

KLF6 gene is located on human chromosome 10 short arm (10p15). The full length of KLF6 is about 7 kb which consists of four exons and its encoded protein contains 283 amino acids, including the transcriptional activity of the N-terminal functional domain (201 amino acids) and C-terminal zinc finger domain (82 amino acids) [14, 15]. The carboxyl terminus of KLF6 shares a common Cys2/His2 zinc finger structure as other members of its family [13]. KLF6 was originally cloned from the rat and human hepatic stellate mesenchymal cells and placental tissue, and it is a ubiquitously expressed nuclear transcription factor involved in growth and development, cell proliferation, differentiation and angiogenesis [13].

Loss of heterozygosity (LOH) and somatic mutations of KLF6 gene were found through the study of prostate cancer

by Narta et al. in 2011, and the KLF6 was first proposed as a new candidate tumor suppressor gene [19]. In addition, KLF6 expression was found significantly decreased in lung cancer [23]. KLF6 gene promoter methylation led to a transcriptional silencing in esophageal cancer cell lines [20]. Recent studies report that LOH and point mutations of KLF6 gene occur in prostate cancer, colorectal cancer, malignant glioma and liver tumours [20]. KLF6 gene alternatively splices in prostate cancer, ovarian cancer and chemotherapy-resistant lung cancer cells [24]. Interestingly, KLF6 may play a tumor suppressor role in tumors via various cell signaling pathways, such as p53-independent upregulation of p21 signaling pathway [16], the interference of cyclinD1 and CDK4 interaction [25] and triggering apoptosis. However, there are no reports about the functional role of KLF6 gene in osteosarcoma and its underlying molecular mechanism.

In the present study, KLF6 gene expression was first detected in 32 cases of clinical osteosarcoma samples. Immunohistochemical analysis revealed that KLF6 protein expression levels in osteosarcoma samples were significantly lower than that in normal bone tissues. This was further confirmed by quantitative PCR detecting the KLF6 mRNA expression. RT-PCR and Western blotting were also performed to detect the KLF6 expression at the cellular level. As shown in Figs. 2 and 3, KLF6 gene expression was significantly reduced in human osteosarcoma cells MG63, Saos2 compared with normal osteoblasts hFOB1.19. These findings suggest that KLF6 gene expression is closely related to the occurrence of osteosarcoma. Lower KLF6 gene expression in bone tissue may indicate a relatively high possibility of the occurrence of osteosarcoma.

Fig. 3 Expression of KLF6 protein in different cell lines. Western blotting showed that the expression of KLF6 protein in osteosarcoma MG63 and Saos2 were lower than that in the normal cell hFOB1.19

However, the study on relationship between KLF6 gene and osteosarcoma is still in the initial stage. The mechanisms that low expression of KLF6 gene can induce the occurrence of tumor remain unclear. Further studies are needed to be conducted on the interaction between KLF6 protein and signaling pathway-related proteins, to find the underlying targets, confirm the role of KLF6 in tumorigenesis and ultimately have a better understanding of the mechanism of bone tumors.

In summary, down-regulation of KLF6 mRNA or protein expression in osteosarcoma cells and tissues could be used as a new early diagnostic marker and therapeutic target for osteosarcoma. This may provide a theoretical basis for post-operative monitoring and has an important clinical significance for poor prognosis of osteosarcoma.

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