Association between expression of nuclear receptor co-activator 5 protein and prognosis in postoperative patients with osteosarcoma

YA WU[1,2*], JIAN WU[3*], QI-RONG DONG[1] and NAI-ZHOU GUO[3]

[1]Department of Orthopedics, Second Affiliated Hospital of Suzhou University, Suzhou, Jiangsu 215004; Departments of [2]Orthopedics and [3]Laboratory Medicine, The First People's Hospital of Yancheng City, Yancheng, Jiangsu 224005, P.R. China

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Abstract. The aim of the present study was to investigate the association between the expression of nuclear receptor co-activator 5 protein (NCOA5) and the prognosis of post-operative patients with osteosarcoma. Human osteosarcoma samples were collected from 145 patients and normal bone tissues were collected from 100 individuals as controls. Immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR) were employed to measure the levels of NCOA5 protein in cases of human osteosarcoma. The results from the RT-PCR analysis demonstrated that the positive rate of NCOA5 mRNA expression in human osteosarcoma was 17.24% (25/145). The positive rate in normal bone tissues was 84.00% (84/100), which was significantly higher compared with that of human osteosarcoma tissues (χ^2=33.166; P<0.001). IHC staining indicated that the positive rate of NCOA5 protein in the osteosarcoma samples was 26.21% (38/145). The positive rate in normal bone tissues was 82.00% (82/100), which was significantly increased compared with that of human osteosarcoma tissues (χ^2=28.166; P<0.001). NCOA5 mRNA and protein expression levels were consistent in human osteosarcoma tissues, and were lower than in control tissues. The expression of NCOA5 was low in human osteosarcoma tissues, while it was high in normal bone tissues. These low NCOA5 expression levels were associated with postoperative survival of human osteosarcoma.

Correspondence to: Dr Qi-Rong Dong, Department of Orthopedics, Second Affiliated Hospital of Suzhou University, 1055 Sanxiang Road, Suzhou, Jiangsu 215004, P.R. China
E-mail: 65141271@qq.com

Dr Nai-Zhou Guo, Department of Laboratory Medicine, The First People's Hospital of Yancheng City, 166 Yulong Road, Yancheng, Jiangsu 224005, P.R. China
E-mail: piaoxue1982717@sina.com

*Contributed equally

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Introduction

Osteosarcoma is the most common primary tumor of bone tissue; it is most common in young people and exhibits a high degree of malignancy (1,2). The treatment of osteosarcoma is neoadjuvant chemotherapy and surgery (3). The five-year survival rate remains at 60-70%, and there has been no significant increase during the past 10 years (4). The primary reason for this is that the mechanism of pathogenesis is not clear. The formation and development of the tumor is a multifactorial, multi-stage and a gradual process; early diagnosis and timely intervention are essential for the prognosis of patients (5). In previous years, the associations between cell proliferation and cell signaling have become an area of interest (6,7).

Previous studies have identified that nuclear receptor co-activator 5 (NCOA5) is a nuclear receptor co-regulator that is widely expressed (8,9). NCOA5 is also a co-regulator for the estrogen receptor and orphan nuclear hormone receptor co-activator BD73 (10). The transcription of NCOA5 is mediated by estrogen receptors to achieve homeostatic regulation of human B cells. NCOA5 gene defects may lead to cancer and diabetes (11,12).

To investigate the association between the expression of NCOA5 and the prognosis of postoperative patients with osteosarcoma, immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR) were employed in the present studyt to measure the levels of NCOA5 protein in patients with human osteosarcoma, using normal bone tissues as the controls. The association between these groups and the mechanism of the formation of osteosarcoma were explored to create novel targets for early diagnosis of this disease.

Patients and methods

Patient samples. Samples of human osteosarcoma were collected between February 2012 and June 2017 from 145 patients who received surgical resection at The First People's Hospital of Yancheng City (Yancheng, China), and had been diagnosed by pathological confirmation. Detailed clinical data were available for all the patients, including sex, age, differentiation, invasion depth, lymph node metastasis and Union for International Cancer Control stage tumor node metastasis (TNM) stage (13), and none had received preoperative chemotherapy or radiotherapy. Human osteosarcoma patients

included 75 males and 70 females, (age range between 15-63 years; average age, 40.9±11.6 years). Normal bone tissue specimens were collected by surgical resection from 100 individuals to serve as a control group. These included 55 males and 45 females, (age range 16-68 years; average age, 34.4±10.3 years). No statistically significant differences were detected in age or sex between the two groups. The protocol used in the present study was in accordance with the Medical Ethics and Human Clinical Trial Committee of The First People's Hospital of Yancheng City, (Identification No. HMU Ethics 20120003). Written informed consent was obtained by all participants in the present study.

Immunohistochemical staining. The EnVision and DAB chromogenic reagent kit (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for immunostaining to detect the distribution of NCOA5 (Antibody Diagnostic; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Immunohistochemical procedures were performed in strict accordance with the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). The EnVision and 3,3'-diaminobenzidine chromogenic reagent kits (Antibody Diagnostic; Applied Biosystems) were used for immunohistochemical staining. All slide staining was performed under the same conditions: The tissue was sliced to 4-μm thickness, dehydrated with 21% sucrose, de-waxed and subjected to antigen retrieval in 0.01 mol/l citric acid pH 6.0 at 95˚C for 10 min. Normal goat serum (10%; Toyobo Life Science, Osaka, Japan) was placed on the slide and incubated for 10 min at room temperature. Slides were then incubated with the corresponding specific primary antibodies (mouse anti-osteonection/NCOA5; cat. no. ab70831; Abcam, Cambridge, UK; 1:1,000) for 1.5 h at room temperature. Tissue sections were then washed with PBS 3 times for 3 min. Subsequently, tissue sections were incubated with secondary antibody horseradish peroxidase-conjugated mouse anti-osteonection/NCOA5 IgG (1:1,000; cat. no. ab125508; Abcam) for 30 min at room temperature. Proteins were detected using 3,3'-Diaminobenzidine (5 min at room temperature), while the nucleus was stained with 10% hematoxylin at 37˚C for 7 min. Tissue samples were then dehydrated in 75, 85, 95 and 100% gradient ethanol series, cleared by 1% xylene and sealed with rubber cement. Each batch of staining contained a positive control sourced from patients with confirmed osteosarcoma [with the known positive section reagent (cat. no. ab92431; Abcam; 1:1,000)] and a negative control, in which the corresponding specific antibody was replaced by PBS.

Samples with nuclei with yellow or tan reactant particles were considered positive. A total of four independent experiments were conducted, and four fields of view were assessed by optical microscopy at x200 magnification. Analysis of the staining scores was semi-quantitative: Final staining scores were calculated by multiplying the positive cell percentage score and the staining intensity score. The positive cell staining percentage score criteria were as follows: 0-15%, 0; >15-30%, 1; >30-45%, 2; and >45%, 3. These staining scores were then classified as follows: >2, negative (-); 2-4, weakly positive (+); 4-6, positive (++); and ≥6, strongly positive (+++). For the convenience of statistical analysis, samples within the (-) group were defined as the negative expression group (-), and samples within the (+), (++) and (+++) groups were defined as the positive expression group (+).

Detection of NCOA5 mRNA expression by RT-PCR. Total RNA was isolated from tissues using TRIzol® reagent (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). A total of 2 μg RNA was reverse transcribed to complementary DNA using the QuantiNova Reverse Transcription kit (50) according to the manufacturer's protocol (cat. no. 205411; Qiagen GmbH, Hilden, Germany). Samples were then amplified by semi-quantitative PCR with β-actin as a reference. The sequences of the primers (Sangon Biotech., Co., Ltd., Shanghai, China) are listed in Table I. Thermocycler conditions were as follows: Pre-denaturation at 94˚C for 4 min, followed by 30 cycles of 94˚C for 10 sec, 55˚C for 30 sec and 72˚C for 60 sec.

Amplification of NCOA5 by PCR was examined using 1% agarose gel electrophoresis. The absorbance value of the sample gene and the reference (β-actin) were read, and the results were expressed as the ratio (sample value/reference value). If the ratio of osteosarcoma to reference absorbance values was positive, NCOA5 mRNA expression was considered to be positive; if it was negative, NCOA5 mRNA expression of the sample was categorized as negative.

Statistical methods. SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The χ^2 test was used to compare the distribution of NCOA5 between osteosarcoma and normal bone tissues. The Kaplan-Meier survival analysis with log-rank test was performed to analyze the association between the expression levels of the proteins in cancer tissue or other clinicopathological characteristics and the survival rate of patients. Associations between the expression of NCOA5 and overall survival of postoperative patients with osteosarcoma were assessed using Cox regression analysis. $P<0.05$ was considered to indicate a statistically significantly difference.

Results

Cell nucleus staining distribution of NCOA5 in human osteosarcoma and normal bone tissues. The positive rate of NCOA5 staining in human osteosarcoma tissues was 26.21% (38/145), which was significantly lower compared with the positive rate of NCOA5 in normal bone tissues, which was 82.00% (82/100; $P<0.05$; Fig. 1).

NCOA5 mRNA expression in human osteosarcoma and normal bone tissues. The results from RT-PCR indicate that mRNA NCOA5 was expressed in osteosarcoma and normal bone tissues ($P<0.05$; Table I). The positive rate of NCOA5 mRNA expression in osteosarcoma was 17.24% (25/145), which was significantly lower compared with that in normal bone tissue (84.00%, 84/100; $\chi^2=33.166$; $P<0.001$; Fig. 2). Compared with the normal bone tissue, the rate of NCOA5 expression was significantly lower in pulmonary metastatic osteosarcoma tissues.

Table I. Primer sequences for reverse transcription-polymerase chain reaction analysis.

Primers	Primer sequences (5'-3')	Product size (bp)
NCOA5		
Forward	CTGCTGGCAGACAACAGGTA	344
Reverse	CTGTTTGCTGCTGTGGAAAA	344
B-actin		
Forward	TGACGTGGACATCCGCAAAG	231
Reverse	CTGGAAGGTGGACAGCGAGG	231

NCOA5, nuclear receptorco-activator 5.

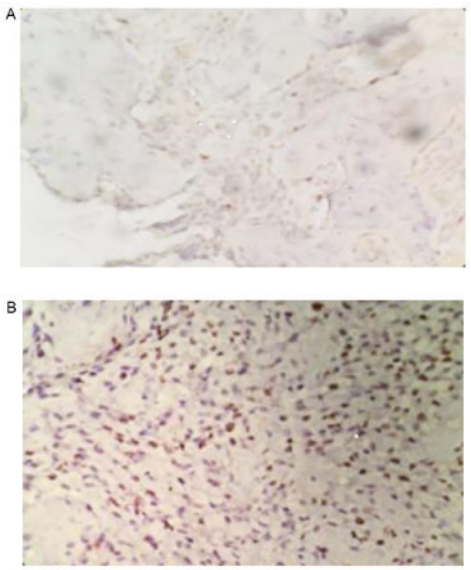

Figure 1. EnVision immunohistochemistry of NCOA5 staining in human osteosarcoma and normal bone tissues at x200 magnification. (A) Weak positive staining result for NCOA5 in human osteosarcoma. (B) Strong positive staining result for NCOA5 in normal bone tissues. NCOA5, nuclear receptor co-activator 5.

Association between the expression of NCOA5 mRNA and the clinicopathological characteristics of osteosarcoma. The expression levels of NCOA5 mRNA and protein in osteosarcoma were consistent and were lower than those in the normal controls. NCOA5 exhibited a low level of expression in osteosarcoma, and was not associated with sex, age or tumor size. However, it was associated with recurrence and metastasis (P<0.01; Table II).

Association between the expression of NCOA5 and overall survival of postoperative patients with osteosarcoma. The overall survival time of NCOA5-positive patients was (60.92±3.45) months after a median follow-up of 61.5 months (61.5±77.3 months). The overall survival of NCOA5-negative patients was (25.68±5.65) months. Kaplan-Meier survival analysis demonstrated that there was a significant difference between these data (χ^2=0.990; P=0.031; Fig. 3).

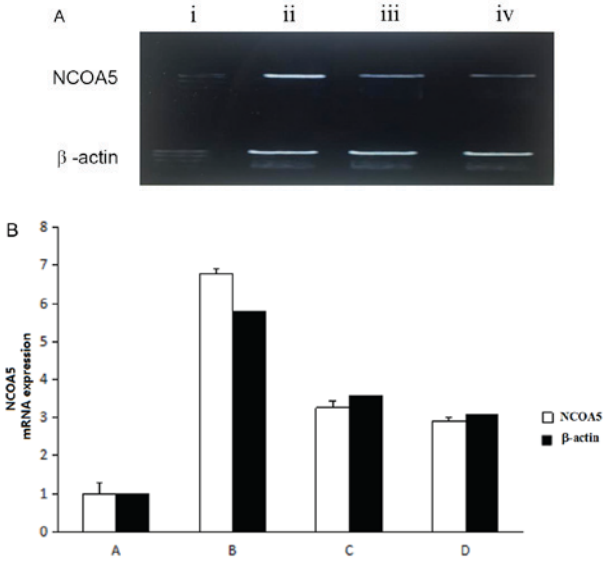

Figure 2. NCOA5 mRNA expression in osteosarcoma and normal bone tissues. (A) Western blot analysis and (B) quantification of mRNA expression of the (i) negative control group, (ii) normal bone tissue group, (iii) human osteosarcoma without pulmonary metastasis group and (iv) human osteosarcoma with pulmonary metastasis group. NCOA5, nuclear receptor co-activator 5.

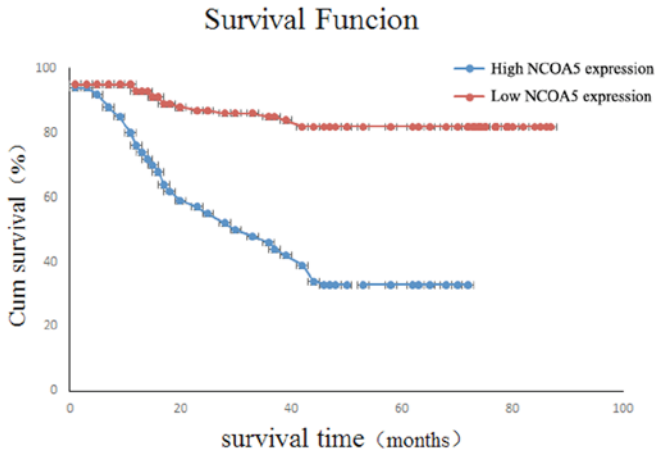

Figure 3. NCOA5 expression and survival analysis of patients with osteosarcoma. NCOA5, nuclear receptor co-activator 5.

In addition, adjuvant therapy, sex, age, gross morphology, tumor invasion depth and lymph node metastasis were not identified as independent factors affecting the overall survival of postoperative patients (Table III).

Discussion

Previous studies have indicated that the occurrence and development of tumors is a complicated process (14); disorders in the regulation of cell growth and proliferation may affect the occurrence and development of tumors (15). Concurrently, the abnormal expression of tumor-associated genes, and the abnormal activation of cell signal transduction and cell proliferation cycle are also involved in numerous aspects of tumor development (16,17). Cell growth and proliferation in the human body are affected and controlled by a number of

Table II. Association between NCOA5 mRNA and protein expression with clinicopathological features in osteosarcoma.

Characteristics	n	NCOA5 mRNA positive rate, n (%)	χ^2	P-value	NCOA5 protein positive rate, n (%)	χ^2	P-value
Sex			0.190	0.663		0.008	0.927
Male	75	15 (20.0)			12 (16.0)		
Female	70	11 (15.7)			11 (15.7)		
Age (years)			0.121	0.728		0.005	0.945
<40	73	13 (17.8)			12 (16.4)		
≥40	72	10 (13.9)			13 (18.1)		
Tumor diameter (cm)			0.351	0.553		0.072	0.789
≥10	63	11 (17.5)			10 (15.9)		
<10	82	13 (15.9)			15 (18.3)		
Pulmonary metastasis			7.601	0.006		7.601	0.006
Yes	65	26 (40.0)			26 (40.0)		
No	80	5 (6.3)			5 (6.3)		

The χ^2 test was used to compare the distribution of NCOA5 with clinicopathological features in osteosarcoma. P<0.05 was considered to indicate a statistically significant difference. NCOA5, nuclear receptor co-activator 5.

Table III. Analysis of survival factors in patients with osteosarcoma.

	Univariate analysis				Multivariate analysis			
Clinicopathological features	B	SE	P-value	95%CI	B	SE	P-value	95%CI
Age	0.658	0.604	0.269		0.612	0.598	0.325	
Sex	0.060	0.602	0.940		0.068	0.615	0.915	
Tumor size	1.756	0.765	0.042	1.02-3.08	1.211	0.544	0.031	1.06-3.13
Tumor location	0.052	0.512	0.819		0.063	0.610	0.875	
Pathological type	0.598	0.512	0.294		0.626	0.651	0.312	
Lymph node metastasis	1.796	0.621	<0.001	1.42-4.26	1.826	0.631	<0.001	1.55-4.52
Low NCOA5 expression	3.003	1.071	0.003	1.41-6.08	3.012	1.076	0.003	1.48-6.23

The Kaplan-Meier survival analysis with log-rank test was used to perform univariate analysis examining the association between the expression levels of the proteins in cancer tissue or other clinicopathological characteristics and the survival rate of patients. P<0.05 was considered to indicate a statistically significant difference. CI, confidence interval; NCOA5, nuclear receptor co-activator 5; SE, standard error; B, regression coefficient.

factors (18,19). In particular, cell signaling proteins, growth factors and their receptors, apoptotic proteins and transcription factors, and the changes to these factors are closely associated with the occurrence and development of tumors (20,21).

Previous studies have also revealed that the NCOA5 protein may be associated with interleukin (IL)-6, tumor necrosis factor (TNF)-α and nuclear factor (NF)-κB (22-24). Prevention of the overexpression of IL-6 may limit the occurrence and development of certain types of cancer. TNF-α was significantly increased in NCOA5 gene-deficient animal tumors. However, this gene defect is not reversible *in vivo*, and the specific mechanism requires additional study (25).

Previous studies have also identified that the decrease in NCOA5 expression in esophageal squamous cell carcinoma (ESCC) tissue is associated with the differentiation status of the tumor and the TNM stage, while is not associated with age, sex, weight loss, tumor location or lymph node metastasis (26,27). In addition, the expression of NCOA5 in normal tissues was higher compared with that in tumor tissues. As the level of tumor differentiation and TNM stage are important indicators of the malignant degree of tumors, the results of the present study suggest that the decreased expression of NCOA5 may be involved in the promotion of tumor progression (28-30).

One limitation of the current study is the relatively small sample size. Nevertheless, to the best of our knowledge, the present study is among the largest studies addressing NCOA5 protein expression in osteosarcoma. The results of the present study indicated that the expression of NCOA5 in osteosarcoma was significantly lower compared with that in normal bone tissue. In addition, the expression levels of NCOA5 protein

in benign bone tumor tissues were significantly higher than in osteosarcoma tissues, which may indicate an association between the occurrence and development of tumors and low NCOA5 expression.

The present study demonstrated that the expression of NCOA5 protein in human specimens is closely associated with the occurrence of osteosarcoma. The expression of NCOA5 in osteosarcoma tissues was significantly lower compared with that in normal bone tissue. This indicates that NCOA5 may be a tumor-suppressor gene in humans.

The results of the present study suggested that the expression of NCOA5 is consistent in different types of osteosarcoma tissues, such as those with or without pulmonary metastasis. The expression of NCOA5 in osteosarcoma was significantly lower compared to that in normal bone tissue. The low expression of NCOA5 may be a cause of osteosarcoma, and therefore, it may be important to detect the expression of NCOA5 in osteosarcoma for the diagnosis of this disease.

In the present study, it was also identified that the expression of NCOA5 protein in patients with osteosarcoma was associated with survival prognosis, and the clinical features of the tumor were significantly associated with the survival rate, differentiation and staging.

In conclusion, the results of the present study indicate the potential role of NCOA5 in the progression of osteosarcoma, highlighting its low expression as an independent prognostic factor. Furthermore, additional studies examining NCOA5 may also assist in developing novel therapeutic strategies for osteosarcoma.

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