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Circular RNA circ-NT5C2 acts as a potential novel biomarker for prognosis of osteosarcoma

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Abstract. – OBJECTIVE: Recent evidence suggests that circular RNAs (circRNAs) play important roles in multiple diseases, including cancer. Circ-NT5C2 was reported to be up-regulated in osteosarcoma. However, the clinical significance of circ-NT5C2 remains largely unclear. The aim of the current study was to investigate the value of circ-NT5C2 for the prognosis of patients with osteosarcoma.

PATIENTS AND METHODS: the expr of circ-NT5C2 in osteosarcoma tissues a responding normal tissues were explore quantitative real-time polymerase chain reac (qRT-PCR) experiments. The association of c NT5C2 expression with clinico gical fa tors or the prognosis of oste patients was also analyzed. Kaplan r sur l analysis was performed to ana the as ation of circ-NT5C2 expression w erall and disease-free sur l (D tients. Univariate and multiv e Cox- ssion analyses were used t ntify risk for poor prognosis.

RESULTS: da wed a sig ficant increase of c -NT5C2 e sion in osteosarcoma tissu mpared with ent normal bone tissues < 0.01). In addition found that the level rc-NT in osteosarcoma was strongly lated clinical stage ($p = 0.006$) and dista sis (p 001). Importantly, patients w gh exp on of circ-NT5C2 had a er O = 0 and DFS ($p = 0.001$) than th with l ession of circ-NT5C2. Finally, regres analyses showed that high ci 5C2 expression might be an independent ameter to predict poor prognosis.

CONCLUSIONS: Our findings indicated that c-NT5C2 is significantly up-regulated in osarcoma tissues. Circ-NT5C2 may represent a marker of prognosis in osteosarcoma.

Key Words:
Circ-NT5C2, Osteosarcoma, Prognosis.

Introduction

Osteosarcoma is the most common primary malignancy mainly affecting childhood and adolescence, accounting for approximately 20% of primary somas in bone[1,2]. It is characterized for m the metaphyseal regions of long b the appendicular skeleton[3]. Despite the multidisciplinary synthetic treatments chemotherapy, radiotherapy, and surgical , the overall survival rate of osteosarcoma has not been substantially improved and approximately 35% of patients will die within 5 years[4,5]. Like other tumors, the progression of osteosarcoma is a multistep process with accumulation of genetic and epigenetic changes[6]. Up to date, in order to improve the prognosis of osteosarcoma patients, multiple research groups focused on the identification of biomarkers with high specificity and sensitivity for early detecting osteosarcoma and predicting the prognosis of this tumor[7,8]. Circular RNA (circRNA) is a novel type of RNA molecule formed by a covalently closed loop that regulates the gene expression at the transcriptional or post-transcriptional level by modulating microRNAs or other molecules[9]. CircRNA were first identified in RNA viruses as early as the 1970s[10]. However, for quite a long time, researchers misconstrue them as 'splicing rubbish'. In recent years, the biological function of circRNAs has attracted more and more attention in the field of biology[11]. Growing evidence[12-14] shows that circRNAs may be involved in the development and progression of various disease, including tumors. Recent advances, including high-throughput sequencing and bioinformatics, indicated that circRNAs are aberrantly expressed

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in various tumors, such as lung cancer[15], laryngeal cancer[16], papillary thyroid carcinoma[17] and colorectal cancer[18]. All these findings make circRNAs a promising candidate for a new biomarker of carcinogenesis. CircRNA circ-NT5C2, also named hsa_circ_0092509, was a newly identified circRNA. Up to date, just one study by Liu et al[19] reported that circ-NT5C2 expression was significantly up-regulated in osteosarcoma patients through high-throughput human circular RNA microarray. Furthermore, they performed *in vivo* and *in vitro* and found that circ-NT5C2 silencing suppressed osteosarcoma cells proliferation and invasion, indicating that circ-NT5C2 may serve as a tumor promoter in osteosarcoma. However, its clinical significance in osteosarcoma is still unclear. Our present study aimed to investigate whether the increased expression of circ-NT5C2 can be used as a prognostic biomarker in osteosarcoma patients.

Patients and Methods

Patients and Tissue Samples

A total of 170 pairs of osteosarcoma and their matched adjacent normal bone re obtained from patients who underwent surgery at the Department of Orthopedics of the Affiliated Hospital of Qingdao University. These patients (111 males, 59 females) were engaged in te work without smoking habits with a of 22.6 years (SD, ± 3.4 years). A tumor were confirmed pathologically f the specimens obtained from surgery. The rs were staged according to the Enn g stag stem and were graded based o World He ganization (WHO) clas ation c eria. Sy treatment was based Chine guideline for osteosarcoma. Co ple i thologi data of the patients f whi speci s were collected wer ilable. The ic nological characteristi a gastric ca patients are summari h Ta This study was approved by the Research Eth ommittee of the Affiliate tal of Qingd iversity, (Qingdao, C). Informed consent were obtained from atients.

I Extracti and qPCR Analysis

RNA s extracted from tissues with TRIzol (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. ed RNA (100 ng) was reverse-transcribed

Table I. Circ-NT5C2 expression and cl thologic fe s in osteosarcoma patients.

Variable	Nu	circ-NT5C2 expression		*p*-value
		Low	High	
Age (y)				0.565
< 25	89	40	49	
≥ 25	44	37		
Gender				0.215
Male	111	51	60	
Female	59	33	26	
Tumor si m)				0.207
< 8	97	52	45	
>	73	32	41	
D g				0.707
Ye	77	37	40	
No	93	47	46	
line p ta				0.546
mal	91	43	48	
ormal	79	41	38	
location				0.121
	91	50	41	
Elsewhere	79	34	45	
linical stage				0.006
A	89	53	36	
III	71	31	50	
Distant metastasis				0.001
Absence	104	62	42	
Presence	66	22	44	

to the first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). For qRTPCR, three replicates of each sample were amplified in a 20-μL reaction mixture containing SYBR Green reaction mix (Promega, Madison, WI, USA) and 0.5 mM of primer, and analyzed using a Roche Light-Cycler (Sigma-Aldrich, St. Louis, MO, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. The primers were utilized as described in Table II. Relative levels of gene expression were quantified by the standard $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). The differential expression of circ-NT5C2 between osteosarcoma tissues and normal brain tissues was evaluated by independent sample *t*-test. The x^2-test was used to assess circ-NT5C2 expression with respect to clinicopathological factors. Survival curves were calculated using the Kaplan-Meier method and [illegible] analyzed using the log-rank test. Survival [illegible] were evaluated using univariate and multivariate Cox proportional hazards models. All tests were two tailed and results with $p < 0.05$ were considered statistically significant.

Results

Expression of circ-NT5C2 Is Up-Regulated in Osteosarcoma

To explore the potential effect of circ-NT5C2, we compared the expression level of circ-NT5C2 between tumor and corresponding adjacent normal bone tissues in 170 osteosarcoma patients. As shown in Figure 1, our data showed that the expression level of circ-NT5C2 in osteosarcoma tissues was significantly higher than that in adjacent normal tissues ($p < 0.01$).

Table II. Primer sequences used for PCR.

Real-time PCR primer	Sequence (5′-3′)
Circ-NT5C2-F	AGTCCTAAGTTTTCCACTTCA
Circ-NT5C2-R	AGGTGCCAGTAGCATTTTAGAC
GAPDH-F	CGACCACTTTGTCAAGCTCA
GAPDH-R	AGGGGTCTACATGGCAACTG

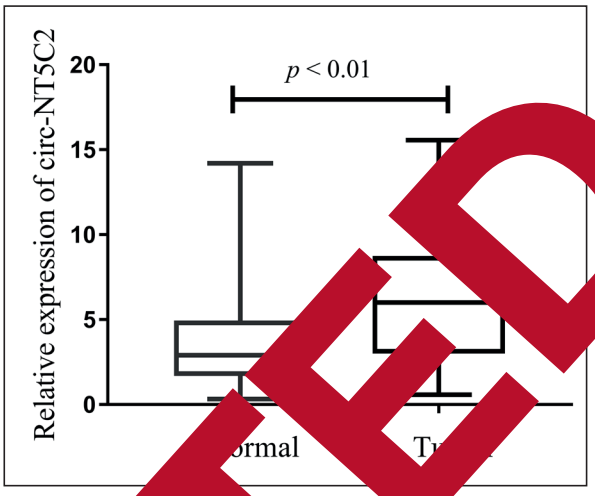

Figure 1. [illegible] circ-NT5C2 [illegible] upregulated in osteosarcoma. Comparison of circ-NT5C2 levels in osteosarcoma tissues and normal control, including 170 paired cancer tissues and their adjacent non-cancerous hepatic tissues. The average circ-NT5C2 expression was normalized using GAPDH expression.

Association between circ-NT5C2 Expression and the Clinicopathological Characteristics of the Osteosarcoma Patients

To explore whether circ-NT5C2 was associated with the development and progression of osteosarcoma, we divided osteosarcoma patients into two groups (High and Low) based on the median value of circ-NT5C2 expression levels. The relationship between clinicopathologic characteristics and circ-NT5C2 expression levels in individuals with osteosarcoma are summarized in Table I. We found that the level of circ-NT5C2 in osteosarcoma was strongly correlated with clinical stage ($p = 0.006$) and distant metastasis ($p = 0.001$), suggesting that circ-NT5C2 may play a positive regulator in clinical progression of osteosarcoma patients. However, no significant difference in circ-NT5C2 expression was observed with age, gender, tumor size, drinking, alkaline phosphatase and anatomic location ($p > 0.05$).

Correlation Between circ-NT5C2 Level and Prognosis in Osteosarcoma Patients

Furthermore, to investigate the prognostic value of circ-NT5C2 expression in patients with osteosarcoma, the detailed clinical information of patients with malignant osteosarcoma was reviewed. The prognostic performance of circ-NT5C2 was evaluated using Kaplan-Meier anal-

ysis. As shown in Figure 2 and 3, we found that the patients with a high circ-NT5C2 expression had shorter OS (p = 0.006) and DFS (p = 0.001) times than those with a low circ-NT5C2 expression. To determine the possibility of circ-NT5C2 as an independent risk factor for poor prognosis, we further performed univariate and multivariate Cox regression analysis. We observed that clinical stage, distant metastasis, and circ-NT5C2 expression were potential predictors for DFS and OS (Table III). Moreover, multivariate Cox regression analysis suggested that circ-NT5C2 was an independent prognostic indicator for DFS (HR = 2.884, 95% CI 1.215-4.569, p = 0.008) and OS (HR = 2.133, 95% CI 1.037-4.037, p = 0.011) in patients with osteosarcoma.

Discussion

Osteosarcoma is a high-grade malignant bone tumor. The primary treatment is a combination of surgery and neoadjuvant chemotherapy[20]. However, these treatment methods often have significant side effects and inadequately treat the disease. So finding new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategies and outcomes of this disease[21]. Currently, several researchers[22,23] focused on the study of ncRNAs, such as miRNAs, long noncoding RNA and circRNAs. Importantly, miRNAs and long noncoding RNAs have been well studied in cancers. However, only a few studies explored the expression and biological function of circRNAs in cancers. In this study, our attention focused on circ-NT5C2.

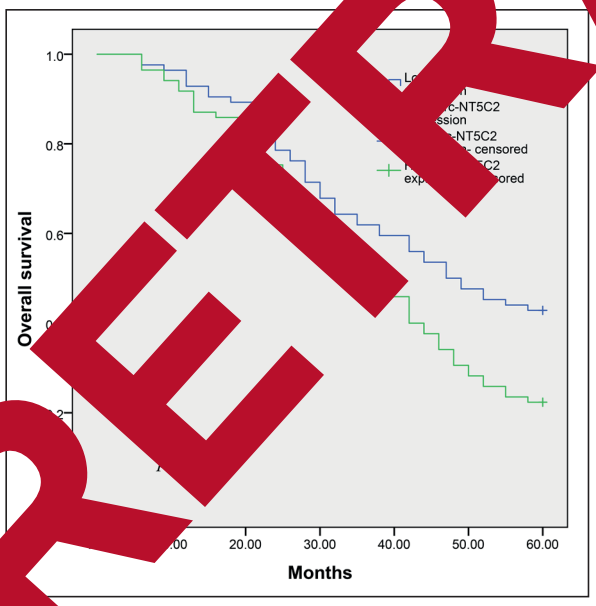

Figure 2. Kaplan-Meier survival curves for cervical cancer patients according to the expression of circ-NT5C2. OS rate of osteosarcoma patients with high circ-NT5C2 was significantly poorer compared to those patients with low circ-NT5C2 (p = 0.006).

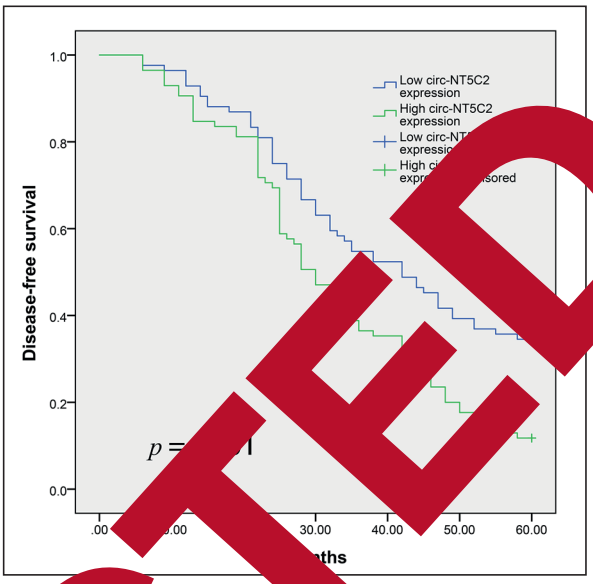

Figure 3. Kaplan-Meier survival curves for cervical cancer patients according to the expression of circ-NT5C2. DFS rate of osteosarcoma patients with high circ-NT5C2 was significantly poorer compared to those patients with low circ-NT5C2 (p = 0.0

CircRNAs play crucial roles during cancer initiation and progression. Up to date, several circRNAs has been reported to be dysregulated and serve as tumor suppressor or tumor promoters in several tumors, including osteosarcoma[24,25]. For instance, Zhang et al[26] found that circRNA UBAP2 was significantly up-regulated and associated with poor prognosis in osteosarcoma patients. *In vitro* and *in vivo* experiments showed that overexpression of circRNA UBAP2 promotes osteosarcoma growth by binding miR-143. Zhu et al[27] revealed that circular RNA PVT1 had a promotive role in osteosarcoma and was able to contribute to doxorubicin and cisplatin resistance of osteosarcoma cells by regulating ABCB1. The clinical assay showed that circular RNA PVT1 expression could be a promising candidate for early detection and prognosis in osteosarcoma patients. Huang et al[28] indicated that circNASP was highly expressed in osteosarcoma and its knockdown dramatically inhibited the proliferation, cell cycle and invasion of OS

Table III. Univariate and multivariate Cox regression analyses for DFS and OS in patients with osteosarcoma.

Variables	DFS			OS		
	HR	95% CI	*p*-value	HR	95% CI	
Univariate analysis						
Age	0.834	0.514-1.556	0.326	0.722	0.571-1.73	0.211
Gender	0.819	0.489-1.316	0.519	0.775	0.614-1.67	0.336
Tumor size	1.234	0.644-1.944	0.143	1.442	0.548-1.734	119
Drinking	1.548	0.919-2.216	0.332	1.328	0.7 -022	
Alkaline phosphatase	0.939	0.546-1.667	0.177	1.232	0 -2.012	
Anatomic location	1.371	0.824-2.219	0.343	1.421	3-2.53	0.2
Clinical stage	3.556	1.456-5.328	0.005	2.835	228-4.1	0.015
Distant metastasis	3.894	1.652-6.643	0.001	3.216	67-	006
Circ-NT5C2 expression	3.136	1.424-5.137	0.005	2	38	09
Multivariate analysis						
Clinical stage	3.231	1.134-4.784	0.008	79	1.04	0.019
Distant metastasis	3.427	1.347-5.452	0.003		1.139-5.	0.013
Circ-NT5C2 expression	2.884	1.215-4.569	0.008	2.	1.037-4.03	0.011

cells by acting as a sponge of miR-1253 targeting FOXF1. Liu et al[19] found that circ-NT5C2 expression was significantly up-regulated in osteosarcoma tissues and could be used as a potential biomarker for distinguishing osteosar a tissues from normal bone tissues. In a they perform the loss-of-function assay d found knockdown of circ-NT5C2 suppre osteosarcoma cells proliferation and invasion targeting miR-448, suggesting c-NT5C serve as a tumor promote arcoma progression. However, th gnost alue of circ-NT5C2 in osteosar has reported. In this stud irc as fou o be significantly ov pressed steosarcoma tissues compare normal b sues, revealing that it oncogene e devel-opment of ost sarco hen, the results of a clinical a indicated t high level of circ-NT5C2 pression was cor d with clinical stag distar etastasis suggesting that circ-NT mig involved in the carcinogenesis and i of os rcoma. Moreover, we y re d th tients with lower levels -NT ession had better survival tha hose w igher levels of circ-NT5C2 ex Furthermore, multivariate Cox anal-si hat circ-NT5C2 could serve as an dependent prognostic biomarker, indicating high circ-NT5C2 level was a promising bio-m for prognosis of osteosarcoma patients. However, exact mechanisms underlying circ-NT5C2 in osteosarcoma progression warrant further investigation.

Conclusions

for the fi me, showed that circ-NT5C2 was egulated in osteosarcoma, and it coul seful poor prognostic biomarker nd a potential therapeutic target for patients with rcoma. Moreover, large-scale studies are d to investigate the prognostic value of the circ-NT5C2 level for patients with osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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