

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

W.-B. NIE^{1,2}, L.-M. ZHAO³, R. GUO⁴, M.-X. WANG², F.-G. YE⁵

¹Qingdao University Medical College, Qingdao, Shandong, China

²Department of Orthopedics, Shanxian Central Hospital, Shanxian, Shandong, China

³Department of Orthopedics, Gaomi People's Hospital, Weifang, Shandong, China

⁴Department of Oncology, Shanxian Central Hospital, Shanxian, Shandong, China

⁵Department of Orthopedics, Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

Wen-Bo Nie and Li-Ming Zhao contributed equally to this work

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 expression of circ-NT5C2 in osteosarcoma tissues and corresponding normal tissues were explored by quantitative real-time polymerase chain reaction (qRT-PCR) experiments. The association of circ-NT5C2 expression with clinicopathological factors or the prognosis of osteosarcoma patients was also analyzed. Kaplan-Meier survival analysis was performed to analyze the association of circ-NT5C2 expression with overall survival (OS) and disease-free survival (DFS) of patients. Univariate and multivariate Cox-regression analyses were used to identify risk factors for poor prognosis.

RESULTS: Our data showed a significant increase of circ-NT5C2 expression in osteosarcoma tissues compared with adjacent normal bone tissues ($p < 0.01$). In addition, we found that the level of circ-NT5C2 in osteosarcoma was strongly correlated with clinical stage ($p = 0.006$) and distant metastasis ($p = 0.001$). Importantly, patients with high expression of circ-NT5C2 had a shorter OS ($p = 0.006$) and DFS ($p = 0.001$) than those with low expression of circ-NT5C2. Finally, Cox regression analyses showed that high circ-NT5C2 expression might be an independent prognostic parameter to predict poor prognosis.

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

Key Words:

Circ-NT5C2, Osteosarcoma, Prognosis.

Introduction

Osteosarcoma is the most common primary bone malignancy mainly affecting childhood and adolescence, accounting for approximately 20% of all primary sarcomas in bone^{1,2}. It is characteristically found in the metaphyseal regions of long bones in the appendicular skeleton³. Despite the multidisciplinary synthetic treatments such as chemotherapy, radiotherapy, and surgical resection, 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 changes⁶. 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 molecules⁹. CircRNA were first identified in RNA viruses as early as the 1970s¹⁰. 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 biology¹¹. Growing evidence¹²⁻¹⁴ 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

in various tumors, such as lung cancer¹⁵, laryngeal cancer¹⁶, papillary thyroid carcinoma¹⁷ and colorectal cancer¹⁸. 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 al¹⁹ 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 tissues and their matched adjacent normal bone were

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 teaching work without smoking habits with a mean age of 22.6 years (SD, \pm 3.4 years). All the tumors were confirmed pathologically from the specimens obtained from surgery. The tumors were staged according to the Enneking staging system and were graded based on the World Health Organization (WHO) classification criteria. System treatment was based on Chinese guideline for osteosarcoma. Complete clinicopathological data of the patients from which the specimens were collected were available. The clinicopathological characteristics of the gastric cancer patients are summarized in Table I. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University, (Qingdao, China). Informed consents were obtained from all patients.

RNA Extraction and qPCR Analysis

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

Table I. Circ-NT5C2 expression and clinicopathologic features in osteosarcoma patients.

Variable	Number	circ-NT5C2 expression		p-value
		Low	High	
Age (y)				0.565
< 25	89	40	49	
\geq 25	81	44	37	
Gender				0.215
Male	111	51	60	
Female	59	33	26	
Tumor size (cm)				0.207
< 8	97	52	45	
\geq 8	73	32	41	
Drinking				0.707
Yes	77	37	40	
No	93	47	46	
Alkaline phosphatase				0.546
Normal	91	43	48	
Abnormal	79	41	38	
Anatomic location				0.121
Tibia/femur	91	50	41	
Elsewhere	79	34	45	
Clinical stage				0.006
IIA	89	53	36	
IIB/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 qRT-PCR, three replicates of each sample were amplified in a 20- μ L 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 \pm standard deviation (SD). The differential expression of circ-NT5C2 between osteosarcoma tissues and normal brain tissues was evaluated by independent sample *t*-test. The χ^2 -test was used to assess circ-NT5C2 expression with respect to clinicopathological factors. Survival curves were calculated using the Kaplan-Meier method and were analyzed using the log-rank test. Survival data 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 biological 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 non-tumor 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

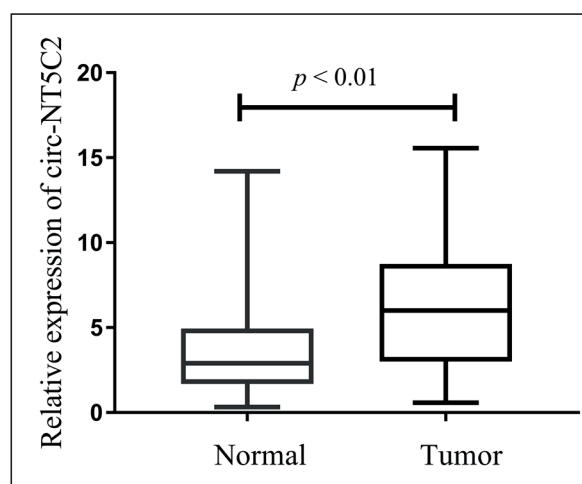


Figure 1. Expression of circ-NT5C2 is upregulated in osteosarcoma. Comparison of circ-NT5C2 levels in osteosarcoma tissues and normal control, including 170 paired cancerous tissues and their adjacent non-cancerous hepatic tissues. The average circ-NT5C2 expression was normalized using GAPDH expression.

Associations 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 chemotherapy²⁰. 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 disease²¹. Currently, several researchers^{22,23} fo-

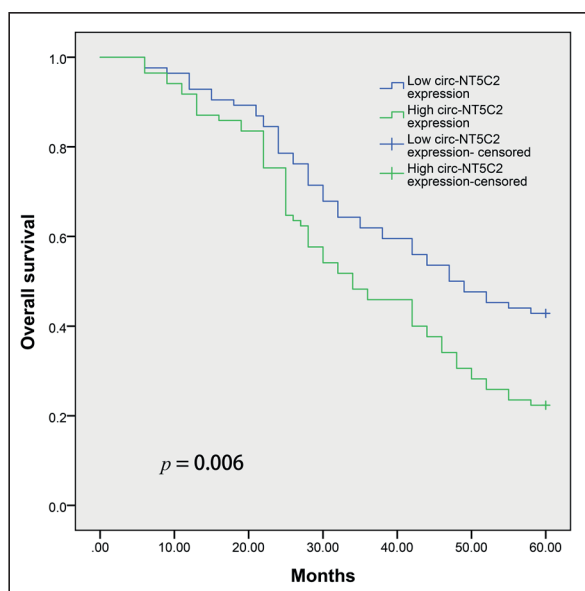


Figure 2. Kaplan-Meier survival curves for cervical cancer patients according to the expression of circ-NT5C2. OS rate of osteosarcoma patients with high circ-NT5C2 was significantly poorer compared to those patients with low circ-NT5C2 ($p = 0.006$).

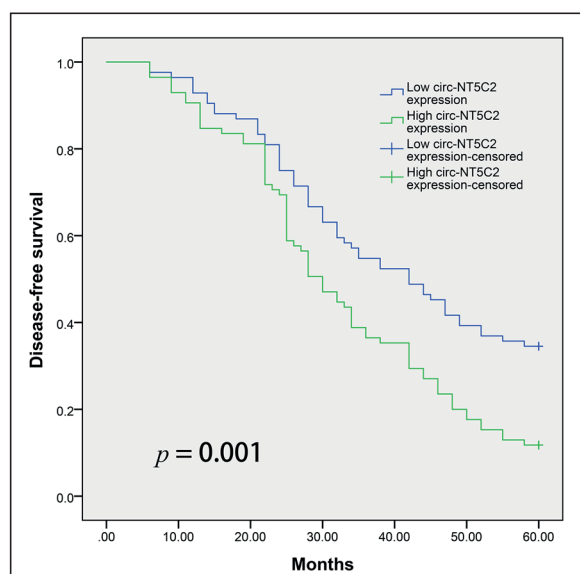


Figure 3. Kaplan-Meier survival curves for cervical cancer patients according to the expression of circ-NT5C2. DFS rate of osteosarcoma patients with high circ-NT5C2 was significantly poorer compared to those patients with low circ-NT5C2 ($p = 0.001$).

cused 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.

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 al²⁶ 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 al²⁷ 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 al²⁸ 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	p-value
Univariate analysis						
Age	0.834	0.514-1.556	0.326	0.722	0.571-1.732	0.211
Gender	0.819	0.489-1.316	0.519	0.775	0.614-1.679	0.336
Tumor size	1.234	0.644-1.944	0.143	1.442	0.548-1.734	0.119
Drinking	1.548	0.919-2.216	0.332	1.328	0.782-2.022	0.134
Alkaline phosphatase	0.939	0.546-1.667	0.177	1.232	0.778-2.012	0.113
Anatomic location	1.371	0.824-2.219	0.343	1.421	0.893-2.531	0.226
Clinical stage	3.556	1.456-5.328	0.005	2.835	1.228-4.132	0.015
Distant metastasis	3.894	1.652-6.643	0.001	3.216	1.267-5.437	0.006
Circ-NT5C2 expression	3.136	1.424-5.137	0.005	2.457	1.234-4.438	0.009
Multivariate analysis						
Clinical stage	3.231	1.134-4.784	0.008	2.679	1.044-4.138	0.019
Distant metastasis	3.427	1.347-5.452	0.003	2.895	1.139-5.022	0.013
Circ-NT5C2 expression	2.884	1.215-4.569	0.008	2.133	1.037-4.037	0.011

cells by acting as a sponge of miR-1253 targeting FOXF1. Liu et al¹⁹ found that circ-NT5C2 expression was significantly up-regulated in osteosarcoma tissues and could be used as a potential biomarker for distinguishing osteosarcoma tissues from normal bone tissues. In addition, they perform the loss-of-function assay and found knockdown of circ-NT5C2 suppressed osteosarcoma cells proliferation and invasion by targeting miR-448, suggesting that circ-NT5C2 serve as a tumor promoter in osteosarcoma progression. However, the prognostic value of circ-NT5C2 in osteosarcoma has not yet been reported. In this study, circ-NT5C2 was found to be significantly overexpressed in osteosarcoma tissues compared with normal bone tissues, revealing that it acts as an oncogene in the development of osteosarcoma. Then, the results of a clinical assay indicated that a high level of circ-NT5C2 expression was correlated with clinical stage and distant metastasis suggesting that circ-NT5C2 might be involved in the carcinogenesis and metastasis of osteosarcoma. Moreover, we firstly reported that patients with lower levels of circ-NT5C2 expression had better survival than those with higher levels of circ-NT5C2 expression. Furthermore, multivariate Cox analysis showed that circ-NT5C2 could serve as an independent prognostic biomarker, indicating that high circ-NT5C2 level was a promising biomarker for prognosis of osteosarcoma patients. However, exact mechanisms underlying circ-NT5C2 in osteosarcoma progression warrant further investigation.

Conclusions

We, for the first time, showed that circ-NT5C2 was frequently upregulated in osteosarcoma, and it could be a useful poor prognostic biomarker and a potential therapeutic target for patients with osteosarcoma. Moreover, large-scale studies are warranted 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.

References

- 1) OTTAVIANI G, ROBERT RS, HUH WW, PALLA S, JAFFE N. Sociooccupational and physical outcomes more than 20 years after the diagnosis of osteosarcoma in children and adolescents: limb salvage versus amputation. *Cancer* 2013; 119: 3727-3736.
- 2) OTTAVIANI G, JAFFE N. The epidemiology of osteosarcoma. *Cancer Treat Res* 2009; 152: 3-13.
- 3) MOORE DD, LUU HH. Osteosarcoma. *Cancer Treat Res* 2014; 162: 65-92.
- 4) ANDO K, HEYMANN M-F, STRESING V, RÉDINI KMF, HEYMANN D. Current therapeutic strategies and novel approaches in osteosarcoma. *Cancers* 2013; 5: 591-616.
- 5) BROADHEAD ML, CLARK JC, MYERS DE, DASS CR, CHOONG PF. The molecular pathogenesis of osteosarcoma: a review. *Sarcoma* 2011; 2011: 959248.

- 6) OSBORNE TS, KHANNA C. A review of the association between osteosarcoma metastasis and protein translation. *J Comp Pathol* 2012; 146: 132-142.
- 7) ROBL B, PAULI C, BOTTER SM, BODE-LESNIEWSKA B, FUCHS B. Prognostic value of tumor suppressors in osteosarcoma before and after neoadjuvant chemotherapy. *BMC Cancer* 2015; 15: 379.
- 8) KREBS MG, METCALF RL, CARTER L, BRADY G, BLACKHALL FH, DIVE C. Molecular analysis of circulating tumour cells-biology and biomarkers. *Nat Rev Clin Oncol* 2014; 11: 129-144.
- 9) HENTZE MW, PREISS T. Circular RNAs: splicing's enigma variations. *EMBO J* 2013; 32: 923-925.
- 10) SANGER HL, KLOTZ G, RIESNER D, GROSS HJ, KLEIN-SCHMIDT AK. Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc Natl Acad Sci U S A* 1976; 73: 3852-3856.
- 11) QU S, YANG X, LI X, WANG J, GAO Y, SHANG R, SUN W, DOU K, LI H. Circular RNA: a new star of noncoding RNAs. *Cancer Lett* 2015; 365: 141-148.
- 12) BONIZZATO A, GAFFO E, TE KRONNIE G, BORTOLUZZI S. CircRNAs in hematopoiesis and hematological malignancies. *Blood Cancer J* 2016; 6: e483.
- 13) CHEN Y, LI C, TAN C, LIU X. Circular RNAs: a new frontier in the study of human diseases. *J Med Genet* 2016; 53: 359-365.
- 14) KRISTENSEN LS, HANSEN TB, VENØ MT, KJEMS J. Circular RNAs in cancer: opportunities and challenges in the field. *Oncogene* 2018; 37: 555-565.
- 15) ZHAO J, LI L, WANG Q, HAN H, ZHAN Q, XU M. CircRNA expression profile in early-stage lung adenocarcinoma patients. *Cell Physiol Biochem* 2017; 44: 2138-2146.
- 16) PENG N, SHI L, ZHANG Q, HU Y, WANG N, YE H. Microarray profiling of circular RNAs in human papillary thyroid carcinoma. *PLoS One* 2017; 12: e0170287.
- 17) XUAN L, QU L, ZHOU H, WANG P, YU H, WU T, WANG X, LI Q, TIAN L, LIU M, SUN Y. Circular RNA: a novel biomarker for progressive laryngeal cancer. *Am J Transl Res* 2016; 8: 932-939.
- 18) ZHANG R, XU J, ZHAO J, WANG X. Silencing of hsa_circ_0007534 suppresses proliferation and induces apoptosis in colorectal cancer cells. *Eur Rev Med Pharmacol Sci* 2018; 22: 118-126.
- 19) LIU X, ZHONG Y, LI J, SHAN A. Circular RNA circ-NT5C2 acts as an oncogene in osteosarcoma proliferation and metastasis through targeting miR-448. *Oncotarget* 2017; 8: 114829-114838.
- 20) FERRARI S, SERRA M. An update on chemotherapy for osteosarcoma. *Expert Opin Pharmacother* 2015; 16: 2727-2736.
- 21) BISHOP MW, JANEWAY KA, GORLICK R. Future directions in the treatment of osteosarcoma. *Curr Opin Pediatr* 2016; 28: 26-33.
- 22) LI DS, ANIWAER JL, SHEYHIDING I, ZHANG Z, ZHANG LW. Identification of key long non-coding RNAs as competing endogenous RNAs for miRNA-mRNA in lung adenocarcinoma. *Eur Rev Med Pharmacol Sci* 2016; 20: 2285-2295.
- 23) MENG S, ZHOU H, FENG Z, XU Z, TANG Y, LI P, WU M. CircRNA: functions and properties of a novel potential biomarker for cancer. *Mol Cancer* 2017; 16: 94.
- 24) ZHU Q, LU G, LUO Z, GUI F, WU J, ZHANG D, NI Y. CircRNA circ_0067934 promotes tumor growth and metastasis in hepatocellular carcinoma through regulation of miR-1324/FZD5/Wnt/ β -catenin axis. *Biochem Biophys Res Commun* 2018; 497: 626-632.
- 25) DENG N, LI L, GAO J, ZHOU J, WANG Y, WANG C, LIU Y. Hsa_circ_0009910 promotes carcinogenesis by promoting the expression of miR-449a target IL6R in osteosarcoma. *Biochem Biophys Res Commun* 2018; 495: 189-196.
- 26) ZHANG H, WANG G, DING C, LIU P, WANG R, DING W, TONG D, WU D, LI C, WEI Q, ZHANG X, LI D, LIU P, CUI H, TANG H, JI F. Increased circular RNA UBAP2 acts as a sponge of miR-143 to promote osteosarcoma progression. *Oncotarget* 2017; 8: 61687-61697.
- 27) ZHU K, MA X, ZHANG C. Overexpressed circPVT1, a potential new circular RNA biomarker, contributes to doxorubicin and cisplatin resistance of osteosarcoma cells by regulating ABCB1. *Int J Biol Sci* 2018; 14: 321-330.
- 28) HUANG L, CHEN M, PAN J, YU W. Circular RNA circ-NASP modulates the malignant behaviors in osteosarcoma via miR-1253/FOXF1 pathway. *Biochem Biophys Res Commun* 2018; 500: 511-517.