European Review for Medical and Pharmacological Sciences 2017; 21: 2829-2834

High expression of long non-coding RNA XIST in osteosarcoma is associated with cell proliferation and poor prognosis

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Abstract. – OBJECTIVE: Osteosarcoma is one of the most common primary bone malignancies. Long non-coding RNAs (lncRNAs) have recently emerged as key regulators of osteosarcoma. The aim of present study was to explore the prognostic value of long non-coding RNA XIST (XIST) in osteosarcoma and XIST's relation to the cell proliferation in osteosarcoma *in vitro*.

PATIENTS AND METHODS: The XIST expressions were detected in osteosarcoma tissues and their paired adjacent normal tissues from 145 osteosarcoma patients by using qRT-PCR. The association between XIST expression and clinicopathological factors, as well as survival rates, was analyzed. The possibility of XIST as a prognostic biomarker for osteosarcoma was examined by Cox proportional hazard regression model. MTT assays were conducted to explore the impact of XIST overexpression on the proliferation of osteosarcoma cells.

RESULTS: The results showed that XIST was significantly up-regulated in osteosarcoma tissues and cell lines, and high XIST expression was significantly associated with advanced tumor size (p=0.009), advanced clinical stage (p=0.001) and present distant metastasis (p=0.009). Kaplan-Meier analysis showed that increased XIST expression was associated with poor overall survival of patients. Univariate and multivariate analysis suggested that XIST expression was an independent prognostic factor for the survival of patients with osteosarcoma. Furthermore, we found that knockdown of XIST significantly suppressed the proliferation of osteosarcoma cells *in vitro*.

CONCLUSIONS: XIST was suggested to have a tumor promoter effect, and thus, to be a predictor of outcome in patients with osteosarcoma.

Key Words:
LncRNA, XIST, Osteosarcoma, Proliferation, Prognosis.

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy and derives from primitive bone-forming mesenchymal cells[1,2]. Conventionally, chemotherapy has been used to improve patient survival. However, this therapeutic approach has reached a plateau[3]. Over the last decade, the development in surgical technology and combined therapeutic strategies have significantly improved the overall survival of the patients with OS[4,5]. However, the prognoses were unsatisfactory in patients with undetectable metastases at diagnosis[6]. An early diagnosis is pivotal for patients and for the treatment of this malignant tumor. However, up to date, most clinicopathological factors cannot be sufficient to accurately predict prognosis of patients[7,8]. Thus, identifying biomarkers for precisely predicting prognosis or for future targeted therapy is needed to improve the outcome of this malignant disease. Recently, lncRNAs with length greater than 200 nucleotides have gained prominence[9]. More scholars have revealed that lncRNAs play a potent role in the processes of cell proliferation, differentiation, apoptosis, and cancer metastasis[10,11]. Several studies have shown that aberrantly expressed lncRNAs are associated with tumorigenesis in various cancers. For instance, Zhu et al[12] reported that lncRNA HULC silencing suppressed the potential for the invasion, adhesion and migration of glioma cells by regulating ESM-1 via the PI3K/Akt/mTOR signaling path. Cai et al[13] found that over-expression of lncRNA CCAT2 promoted breast tumor growth by regulating the Wnt signaling pathway. Tuo et al[14] indicated that lncRNA

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Table I. RT-PCR primers for amplification of expression XIST.

Primer	Primer sequence (5'-3')
XIST F	TCAGCCCATCAGTCCAAGATC
XIST R	CCTAGTTCAGGCCTGCT-TTTCAT
GAPDH F	GAAGGTGAAGGTCGGAGTC
GAPDH R	GAAGATGGTGATGGGATTTC

UCA1 promoted breast cancer cell growth through decreasing tumor suppressive miR-143. Recently, some researches revealed that aberrant expressions of lncRNAs were significantly associated with diagnosis and prognosis of tumor patients including OS[15,16], suggesting the potential of lncRNAs as a candidate independent marker for OS. X inactivate-specific transcript (XIST), derived from XIST gene, is a newly identified lncRNA. Previous findings showed that XIST expression was up-regulated in different types of tumor, such as non-small cell lung cancer[17], human nasopharyngeal carcinoma[18] and hepatocellular carcinoma[19]. However, to our best knowledge, the effect of XIST in OS has not been reported. In the present work, we firstly determine the expression levels of XIST in OS, and further explore the prognostic value of XIST in patients with OS.

Patients and Methods

Patients and Tissue Samples

All experiments involving patients were approved by the Institutional Review Boards of Linzi District People's Hospital. Written informed consent was obtained from all subjects. 145 patients who were admitted to the Department of Orthopedics in Linzi District People's Hospital were included in the study. None of the patients had received chemotherapy or radiation therapy before the surgery. The specimens were immediately frozen in liquid nitrogen and stored at -80°C until use. The clinical stage of these OS patients was classified according to the sixth edition of the tumor-node-metastases classification of the Union for International Cancer Control. The clinicopathological information of the patients was summarized in Table II.

Cell Culture

The human OS cell lines HOS, SAOS-2, SOSP-9607, MG63, and U2-OS and hFOB 1.19 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Haiding, Beijing, China) with 10% FCS and 1% penicillin/

Table II. Clinicopathological characteristics of 145 osteosarcoma patients.

Clinicopathological features	Number of cases	XIST expression High	Low	*p*
Age				NS
<30 years	59	25	33	
≥30 years	86	49	37	
Gender				NS
Male	78	41	37	
Female	67	34	33	
Tumor size				0.009
>8 cm	53	35	18	
≤8 cm	92	40	52	
Anatomic location				NS
Tibia/femur	92	48	44	
Elsewhere	53	27	26	
Serum level of lactate dehydrogenase				NS
Elevated	86	46	40	
Normal	59	29	30	
Serum level of alkaline phosphatase				NS
Elevated	79	42	37	
Normal	66	33	33	
Clinical stage				0.001
I/II	72	27	45	
III/IV	73	48	25	
Distant metastasis				0.009
Absent	101	45	56	
Present	44	30	14	

Table III. Univariate and multivariate analysis of overall survival in 145 patients with osteosarcoma.

Variables	Univariate log-rank test (p)	Cox multivariable analysis (p)	Relative risk
XIST expression (high *vs*. low)	0.004	0.011	5.762
Clinical stage (I/II *vs*. III/IV)	0.001	0.007	7.235
Distant metastasis (absent *vs*. present)	0.011	0.008	4.368
Tumor size (cm) (>8 *vs*. ≤8)	0.013	-	-

streptomycin (Gibco, Haiding, Beijing, China). The cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Small RNA Interference

Small interfering RNA that targeted XIST RNA (si-XIST) and scrambled negative control (si-NC) was generously provided by Life Technologies. Human OS SAOS-2, MG63s and hFOB 1.19 cell line were transfected with si-XIST or si-NC, using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, GmbH, Hilden, Germany) and cDNA synthesized using SuperScript® VILO™ cDNA Synthesis Kit (ThermoFisher Scientific, Pudong, Shanghai, China) according to the manufacture's protocol. Amplification and detection were carried out using the SYBR Premix Ex Taq (TaKaRa, Dalian, China) on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR analysis was undertaken using gene-specific primers, and the sequences were shown in Table I. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal controls. The relative level of mRNA was calculated as $2^{-\Delta\Delta Ct}$.

MTT Mapping Cell Growth Curve

Cell proliferation was determined by MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Briefly, following transfection, the cells were seeded in 96-well plates (2 x 104 cells per well) and incubated at 37°C. At different time points (24, 48 or 72 h), 20 μL of MTT (5 mg/mL, Life Technologies, Carlsbad, CA, USA) was added. After incubation at 37°C for 4 h, 150 μL DMSO was added. Viable cell numbers were estimated by measurement of the optical density (OD) at 450 nm.

Statistical Analysis

All data are presented as mean values ±SD and analyzed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was estimated by Student's *t*-test and X^2-test. Survival curves were plotted using the Kaplan-Meier method, and differences between survival curves were compared by the log-rank test. A Cox proportional hazards modeling of the factors potentially related to survival was performed to identify those useful factors. $p<0.05$ was considered statistically significant.

Results

XIST Expression was Increased in OS Tissues and Cell Lines

To determine the expression pattern of XIST in human OS, we analyzed the expression of XIST in 145 paired OS and adjacent noncancerous tissues by qRT-PCR. We found that XIST expression was significantly increased in OS tissues compared with that in corresponding noncancerous bone tissues ($p<0.01$, Figure 1A). Then, we also measured XIST levels in OS cell lines (HOS, SAOS-2, SOSP-9607, MG63, and U2-OS) and hFOB 1.19 cell line. The results of PCR showed that the expression of XIST was significantly higher in all five-cell lines, compared to those in hFOB 1.19 cell line (Figure 1B).

Association Between Clinicopathological Characteristics and XIST Expression in OS Patients

To further explore the role of XIST in determining the clinical significance of glioma, we analyzed the relationships of the XIST with various clinical features of OS. As shown in Table II, the X^2 test indicated that XIST expression was associated with tumor size ($p=0.009$), clinical stage ($p=0.001$), and distant metastasis ($p=0.009$). However, no statistically significant associations of XIST expression with age, gender, anatomic lo-

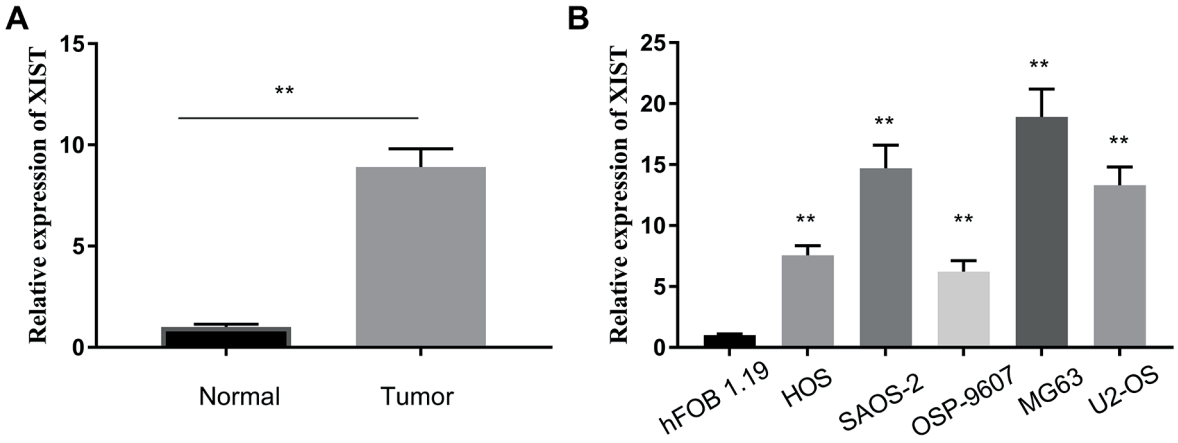

Figure 1. XIST expression is frequently up-regulated in osteosarcoma tissues and osteosarcoma cell lines. ***(A)*** The expression level of XIST in osteosarcoma tissues and matched adjacent non-cancerous tissues were determined by RT-PCR. ***(B)*** The levels of XIST in five osteosarcoma cell lines and normal osteoblast cell line hFOB1.19 were detected by qRT-PCR. $^{*}p<0.05$. $^{**}p<0.01$.

cation, serum level of lactate dehydrogenase were found (all $p>0.05$).

High-Expression Level of XIST Predicted Poor Prognosis in OS Patients

Given that up-regulation of XIST was significantly associated with clinical stage and distant metastasis, we hypothesized that XIST might affect the prognosis of OS patients. We assessed the correlation between the levels of l XIST expression and overall survival through Kaplan-Meier analysis and log-rank test. As shown in Figure 2, the data showed that increased XIST expression was associated with poor overall survival of patients with OS ($p<0.001$). Then, we further explore whether XIST expression could be an independent prognostic factor for overall survival. Table III showed the results of Cox´s proportional hazards model. XIST expression was an independent prognostic factor for the survival of patients with OS.

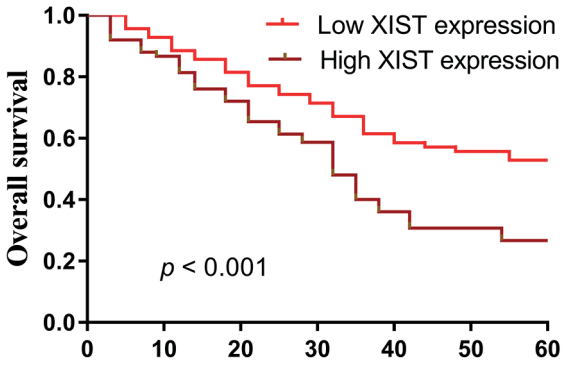

Figure 2. Kaplan-Meier overall survival curves of patients with osteosarcoma according to relative expression of XIST.

Knockdown XIST Inhibited OS Cell Proliferation in vitro

To check whether XIST influenced OS cells proliferation, XIST inhibitor was transfected into SAOS-2 and MG-63 cells. The transfection and down-expression efficiency of XIST were detected by qRT-PCR (Figure 3 A,B). The effect of XIST on proliferation of human OS cell lines was evaluated by MTT assay. The results demonstrated that inhibiting XIST significantly reduced SAOS-2 and MG-63 cell growth (Figure 3 C, D). These results suggest that XIST expression in OS cells was important for proliferation.

Discussion

OS is the most frequent primary bone tumor. Despite total surgical resection of the primary lesion, approximately 80% of patients go on to develop metastases[20]. Identification of prognostic markers is very important in the development of improved therapeutic strategies. Recently, lncRNAs have become research hotspots. Growing lncRANs have been identified as independent markers for predicting the clinical outcome of OS patients, such as lncRNA TUG1[21], lncRNA MALAT1[22] and lncRNA HOTTIP[23]. In the present study, we evaluate the expression of XIST in term of osteosarcoma. Previous researches have suggested that XIST served as oncogene in progression of tumor.

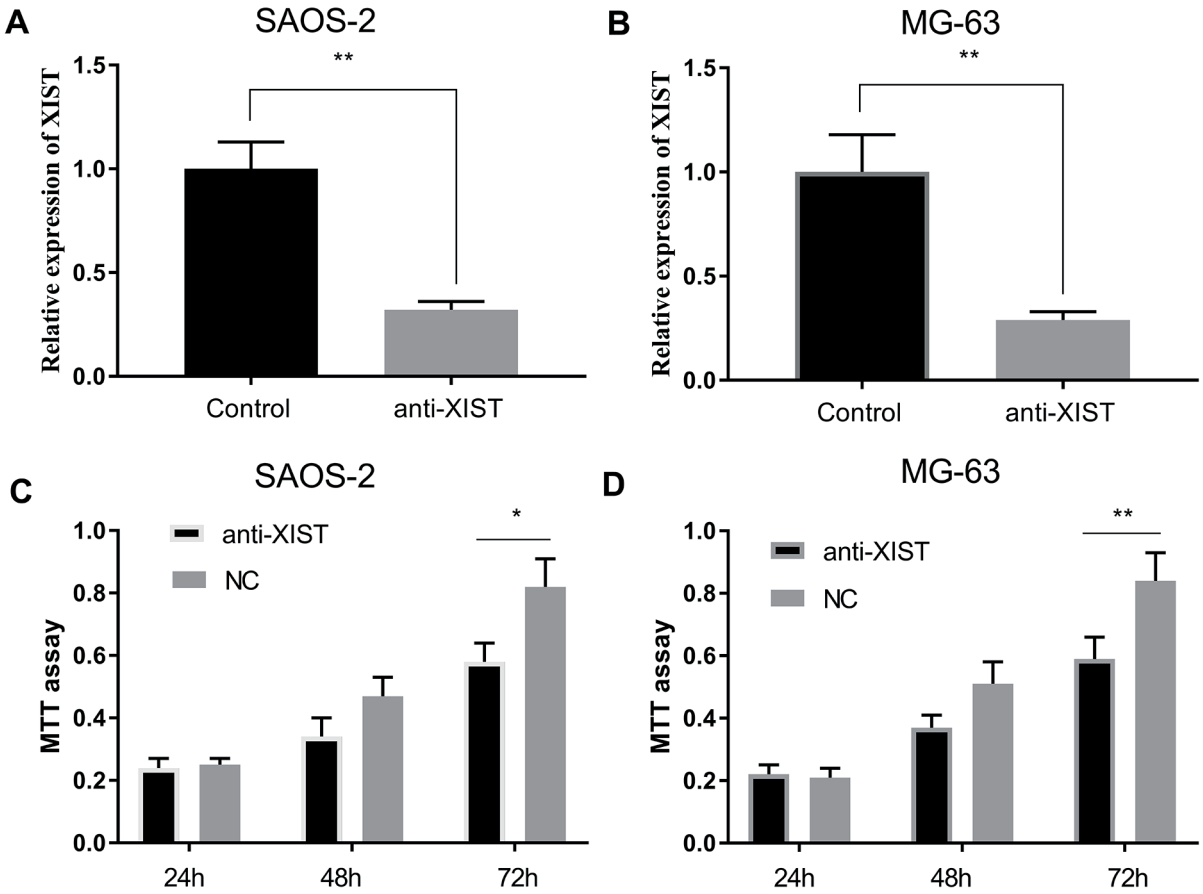

Figure 3. Overexpression of XIST inhibited osteosarcoma cells growth *in vitro*. ***(A, B)*** qRT-PCR analysis of XIST in SAOS-2 and MG63 cells transfected with XIST-inhibitor or negative control oligonucleotides. ***(C, D)*** Cell viability was measured by the MTT assay 24-72 h post-transfection after transfecting of XIST-inhibitor or negative control oligonucleotides. $^{*}p<0.05$. $^{**}p<0.01$.

For example, Chen et al[24] found that XIST was significantly up-regulated in gastric cancer tissues and cell lines. *In vitro* overexpression of XIST promoted cell proliferation, migration and invasion by affecting the expression of miR-101. Fang et al[17] reported that XIST is over-expressed in non-small cell lung cancer, and its increased level is associated with shorter survival. Functionally, knockdown of XIST promoted NSCLC cells proliferation, migration and invasion by repressing KLF2 expression. Kobayashi et al[25] showed that high expression of XIST was significantly associated with poor overall survival rates of cervical cancer. These results highlight the effect of XIST in development of tumors. However, the effect of XIST in OS remained largely unknown. In the present study, we firstly determined XIST expression by qRT-PCR. Our findings showed that the expression levels of XIST in OS tissues were significantly higher than those in corresponding noncancerous tissues. In addition, the increased XIST expression correlated well with tumor size, clinical stage and distant metastasis. Moreover, to explore the effect of XIST in prognosis of patients of OS, we performed the Kaplan-Meier method and the data showed that the patients with higher XIST levels exhibited poorer prognosis. More importantly, we proved that XIST could be an independent prognostic factor in patients with OS by Cox proportional hazards model. These results indicated XIST served as a tumor promoter. To explore whether XIST could affect the behavior of OS cells, OS cells were transfected with XIST inhibitor control. The data of PCR confirmed the down-expression of XIST in OS cells. Then, we performed the MTT and found that knockdown of XIST significantly suppressed cell proliferation of OS cells. There experiments validated our findings that XIST functioned as a tumor promoter in OS.

Conclusions

We suggest that XIST expression was associated with poor prognosis of patients with OS and functioned as a novel prognostic biomarker for patients with this disease.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res 2009; 152: 3-13.
2) Mirabello L, Troisi RJ, Savage SA. International osteosarcoma incidence patterns in children and adolescents, middle ages and elderly persons. Int J Cancer 2009; 125: 229-234.
3) Ferrari S, Serra M. An update on chemotherapy for osteosarcoma. Expert Opin Pharmacother 2015; 16: 2727-2736.
4) Anderson ME. Update on survival in osteosarcoma. Orthop Clin North Am 2016; 47: 283-292.
5) Wang W, Wang ZC, Shen H, Xie JJ, Lu H. Dose-intensive versus dose-control chemotherapy for high-grade osteosarcoma: a meta-analysis. Eur Rev Med Pharmacol Sci 2014; 18: 1383-1390.
6) Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004; 9: 422-441.
7) Anninga JK, Picci P, Fiocco M, Kroon HM, Vanel D, Alberghini M, Gelderblom H, Hogendoorn PC. Osteosarcoma of the hands and feet: a distinct clinico-pathological subgroup. Virchows Arch 2013; 462: 109-120.
8) Rankin KS, Starkey M, Lunec J, Gerrand CH, Murphy S, Biswas S. Of dogs and men: comparative biology as a tool for the discovery of novel biomarkers and drug development targets in osteosarcoma. Pediatr Blood Cancer 2012; 58: 327-333.
9) Flynn RA, Chang HY. Long noncoding RNAs in cell-fate programming and reprogramming. Cell Stem Cell 2014; 14: 752-761.
10) Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12: 861-874.
11) Li J, Wang YM, Song YL. Knockdown of long noncoding RNA AB073614 inhibits glioma cell proliferation and migration via affecting epithelial-mesenchymal transition. Eur Rev Med Pharmacol Sci 2016; 20: 3997-4002.
12) Zhu Y, Zhang X, Qi L, Cai Y, Yang P, Xuan G, Jiang Y. HULC long noncoding RNA silencing suppresses angiogenesis by regulating ESM-1 via the PI3K/Akt/mTOR signaling pathway in human gliomas. Oncotarget 2016; 7: 14429-14440.
13) Cai Y, He J, Zhang D. Long noncoding RNA CCAT2 promotes breast tumor growth by regulating the Wnt signaling pathway. Onco Targets Ther 2015; 8: 2657-2664.
14) Tuo YL, Li XM, Luo J. Long noncoding RNA UCA1 modulates breast cancer cell growth and apoptosis through decreasing tumor suppressive miR-143. Eur Rev Med Pharmacol Sci 2015; 19: 3403-3411.
15) Yang Z, Zhang Y, Zhang X, Zhang M, Liu H, Zhang S, Qi B, Sun X. Serum microRNA-221 functions as a potential diagnostic and prognostic marker for patients with osteosarcoma. Biomed Pharmacother 2015; 75: 153-158.
16) Tang J, Zhao H, Cai H1, Wu H. Diagnostic and prognostic potentials of microRNA-27a in osteosarcoma. Biomed Pharmacother 2015; 71: 222-226.
17) Fang J, Sun CC, Gong C. Long noncoding RNA XIST acts as an oncogene in non-small cell lung cancer by epigenetically repressing KLF2 expression. Biochem Biophys Res Commun 2016; 478: 811-817.
18) Song P, Ye LF, Zhang C, Peng T, Zhou XH. Long non-coding RNA XIST exerts oncogenic functions in human nasopharyngeal carcinoma by targeting miR-34a-5p. Gene 2016; 592: 8-14.
19) Zhuang LK, Yang YT, Ma X, Han B, Wang ZS, Zhao QY, Wu LQ, Qu ZQ. MicroRNA-92b promotes hepatocellular carcinoma progression by targeting Smad7 and is mediated by long non-coding RNA XIST. Cell Death Dis 2016; 7: e2203.
20) Osborne TS, Khanna C. A review of the association between osteosarcoma metastasis and protein translation. J Comp Pathol 2012; 146: 132-142.
21) Ma B, Li M, Zhang L, Huang M, Lei JB, Fu GH, Liu CX, Lai QW, Chen QQ, Wang YL. Upregulation of long non-coding RNA TUG1 correlates with poor prognosis and disease status in osteosarcoma. Tumour Biol 2016; 37: 4445-4455.
22) Gao KT, Lian D. Long non-coding RNA MALAT1 is an independent prognostic factor of osteosarcoma. Eur Rev Med Pharmacol Sci 2016; 20: 3561-3565.
23) Li F, Cao L, Hang D, Wang F, Wang Q. Long non-coding RNA HOTTIP is up-regulated and associated with poor prognosis in patients with osteosarcoma. Int J Clin Exp Pathol 2015; 8: 11414-11420.
24) Chen DL, Ju HQ, Lu YX, Chen LZ, Zeng ZL, Zhang DS, Luo HY, Wang F, Qiu MZ, Wang DS, Xu DZ, Zhou ZW, Pelicano H, Huang P, Xie D, Wang FH, Li YH, Xu RH. Long non-coding RNA XIST regulates gastric cancer progression by acting as a molecular sponge of miR-101 to modulate EZH2 expression. J Exp Clin Cancer Res 2016; 35: 142.
25) Kobayashi R, Miyagawa R, Yamashita H, Morikawa T, Okuma K, Fukayama M, Ohtomo K, Nakagawa K. Increased expression of long non-coding RNA XIST predicts favorable prognosis of cervical squamous cell carcinoma subsequent to definitive chemoradiation therapy. Oncol Lett 2016; 12: 3066-3074.