Original Paper

Overexpression of Long Non-Coding RNA NNT-AS1 Correlates with Tumor Progression and Poor Prognosis in Osteosarcoma

Hui Ye^{a,b} Jinkuang Lin^b Xuedong Yao^b Yizhong Li^b Xiaobin Lin^b Hai Lu^a

^aDepartment of Orthopedics, Academy of Orthopedics, The Third Affiliated Hospital of Southern Medical University, Guangdong, ^bDepartment of Orthopedics, The Second Affiliated Hospital of Fujian Medical University, Fujian, China

Key Words

Lncrna • NNT-AS1 • Osteosarcoma • Biomarker

Abstract

Background/Aims: Increasing evidence demonstrates that long non-coding RNAs (lncRNAs) play critical regulatory roles in cancers, including osteosarcoma. A previous study showed that Nicotinamide Nucleotide Transhydrogenase-antisense RNA1 (NNT-AS1) was aberrantly expressed in several types of cancer. However, the potential biological roles and regulatory mechanisms of NNT-AS1 in osteosarcoma progression remain unknown. **Methods:** Quantitative RT-PCR was performed to examine the expression of NNT-AS1 in human tissues and cells. The biological functions of NNT-AS1 were determined by CCK-8, colony formation, Flow cytometry and Transwell assays *in vitro*. A mouse xenograft model was performed to investigate the effect of NNT-AS1 on tumor growth *in vivo*. **Results:** In this study, we found the expression of NNT-AS1 was significantly increased in tumor tissues compared to adjacent normal tissues. Furthermore, upregulated NNT-AS1 expression predicted poor prognosis and was an independent and significant risk factor for osteosarcoma patient survival. Further experiments revealed that NNT-AS1 knockdown significantly inhibited cell proliferation by inducing cell cycle arrest and promoting apoptosis in osteosarcoma cells. Moreover, NNT-AS1 silencing suppressed cell migration and invasion *in vitro*. In a tumor xenograft model, knockdown of NNT-AS1 suppressed tumor growth of OS-732 cells *in vivo*. **Conclusions:** Taken together, these findings indicate that NNT-AS1 functions as an oncogene in osteosarcoma and could be a novel diagnostic and therapeutic target for osteosarcoma.

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Hai Lu

Department of Orthopedics, Academy of Orthopedics, Guangdong Province, The Third Affiliated Hospital of Southern Medical University, 183 Zhongshan Avenue West, Guangzhou, 510665, (China); Tel. +86-20-62784240, E-Mail dr_yehui@163.com

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Introduction

Osteosarcoma is the most common primary malignancy of bone and frequently occurs in young adults and adolescents [1, 2]. With the advancement of radical surgery and neoadjuvant chemotherapy, the survival rates of osteosarcoma patients have dramatically improved in recent decades [3-6]. However, more than 20% of osteosarcoma patients die from tumor recurrence or metastasis, which remain major obstacles in bone cancer treatment [3, 7]. Therefore, there is an urgent need to explore the cellular and molecular mechanisms that regulate cancer cell growth and metastasis-related cellular activities.

Long non-coding RNAs (lncRNAs) are a novel class of RNA transcripts, longer than 200 nucleotides in length and with no protein-coding capacity [8, 9]. Increasing evidence indicates that lncRNAs interact with nucleic acids or proteins in diverse biological processes, including cell proliferation, cell differentiation, chromatin remodeling, and drug resistance [9-11]. Previous studies have shown that lncRNAs could act as oncogenes or tumor suppressors during the development and progression of cancers, such as hepatocellular carcinoma, lung cancer, pancreatic cancer, osteosarcoma, and colon cancer [12-17]. For example, lncRNA DGCR5 was decreased in hepatocellular carcinoma tissues and serum, and low DGCR5 expression was associated with HbsAg and vascular invasion. This finding suggests that DGCR5 is an independent negative prognostic factor for hepatocellular carcinoma [12]. HNF1A-antisense 1 (HNF1A-AS1) was highly overexpressed in esophageal adenocarcinoma and promoted tumor growth and metastasis by mediating the expression of cancer-related lncRNA H19 [18]. Recent studies showed that NNT-AS1 was upregulated in colorectal cancer specimens and that knockdown of NNT-AS1 reduced cell proliferation, invasion, induced apoptosis, and inhibited tumor growth and metastasis via inhibiting the epithelial-mesenchymal transition (EMT) and inactivating the MAPK/Erk signaling pathway [19]. Nevertheless, the expression and potential roles of NNT-AS1 during osteosarcoma tumorigenesis and metastasis remain unclear.

In the current study, we investigated the role of NNT-AS1 in osteosarcoma. We found that the expression of NNT-AS1 was remarkably upregulated in osteosarcoma tissues and cell lines, and high NNT-AS1 expression was associated with larger tumors, advanced Enneking stage, metastasis and poor prognosis. Furthermore, we investigated the biological functions of NNT-AS1 in osteosarcoma cell proliferation, migration and invasion *in vitro* and tumor growth *in vivo*.

Materials and Methods

Patients and samples

A total of 126 freshly frozen OS samples and paired adjacent normal tissue samples were collected from patients who underwent surgical resection between 2007 and 2010 at the Department of Orthopedics in The Third Affiliated Hospital of Southern Medical University (Guangzhou, China). Written informed consent was obtained from all patients who participated in this study. This study was approved by the ethics committee of The Third Affiliated Hospital of Southern Medical University and conducted in accordance with guidelines provided by the Ethics Committees and Institutional Review Boards. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until use.

Cell lines and cell cultures

Immortalized human osteoblast cells (hFOB1.19) and 6 cell lines (U2OS, Saos2, OS-9901, SOSP-9607, MG-63 and OS-732) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). hFOB1.19 cells were cultured in Dulbecco's minimal essential medium/Ham's F12 (HyClone, Logan, UT, USA). Other human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO₂ humidified incubator.

Immunohistochemistry

The dissected tissues from xenograft tumors were fixed in a 10% formalin solution and processed to obtain paraffin-embedded tissue. After slides were deparaffinized and dehydrated, endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at room temperature. Then, slides were blocked with 10% normal goat serum for 30 min and subsequently incubated with antibody against C-myc (Abcam, Cambridge, MA, USA) overnight at 4°C. After washing with PBS, slides were incubated with biotinylated secondary antibody at room temperature for 1 h. Antigen expression was detected using the DakoCytomation LSAB2 system-HRP (Dako). Slides were counterstained with hematoxylin, dehydrated and mounted. Negative controls were performed by replacing the specific primary antibody with phosphate buffered saline. Evaluation of C-myc staining in tissues was assessed by two independent professional pathologists who were blinded to patient clinical status and outcomes. C-myc expression scores were determined by the area of staining and the percentage of positive cells according to previous work [20].

Reverse transcription and real-time PCR

Total RNA was extracted from clinical samples and cell lines with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative PCR was performed with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on an ABI7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequence-specific forward and reverse primers sequences for NNT-AS1 were 5'-CTGGAATCCCTGCTACTCAGGA-3' and 5'-GCCATGTGATATGCCTGCTC-3' respectively. Forward and reverse primers sequences for GAPDH were 5'-GTCAACGGATTGGTCTGTATT-3'; and the reverse primer was 5'-AGTCTTCTGGGTGGCAGTGAT-3' respectively. GAPDH was used as the endogenous control and the relative expression of NNT-AS1 was determined by the 2^{-ΔΔCt} method.

Cell proliferation and colony formation assay

For the cell proliferation assay, 2×10³ cells in 100 μL medium were seeded into a 96-well plate in quadruplicate. 100 μL of Cell Counting Kit-8 (CCK-8) (Dojindo; Kumamoto, Japan) was added to each well at 24, 48, 72 and 96 h after the cells were seeded. The absorbance was measured at a wavelength of 450 nm.

For cell colony analysis, approximately 800 single cells were seeded into each well of a 6-well plate in quadruplicate. Cells were cultured for 2 weeks in a 37°C incubator. Cells were washed by PBS and fixed by 4% paraformaldehyde, stained with 1% crystal violet for 20 min, and then washed with water. The number of colonies with > 50 cells was counted manually under an inverted microscope.

Flow cytometry analysis

For cell apoptosis assays, cells were harvested and gently resuspended in 100 μL of binding buffer containing 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI), and then incubated for 15 min at room temperature in the dark. The stained cells were analyzed by flow cytometry (BD Biosciences, San Diego, CA, USA).

For cell cycle analysis, cells were harvested and fixed with 70% cold ethanol at 4°C overnight. Then, cells were incubated with PI for 10 min, and the DNA content was determined by flow cytometry.

Cell migration and invasion assays

Cell migration assays were performed using 24-well Transwell plates (8.0-μm pore membranes; Costar). Approximately 5 × 10⁴ OS cells transduced with NC or shRNA were plated in the upper chamber in a serum-free medium. The lower chambers were filled with 600 μL of medium containing 10% FBS as a chemoattractant. After a 36 h incubation, the bottom of the chamber insert was stained with 1% crystal violet for 20 min. For the cell invasion assay, the procedure was similar to the cell migration assay, except that Transwell membranes were pre-coated with Matrigel (BD Biosciences). The cells that had invaded through the membrane to the lower surface were photographed and counted under an inverted microscope in 6 random fields with a 200x magnification.

Protein extraction and western blot analysis

Cellular protein was lysed in RIPA Lysis Buffer (Cwbiotech, Beijing, China) supplemented with complete protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Equal amounts of protein were separated by

10% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad). After the membranes were blocked with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies for AKT (Cell Signal Technology, Danvers, MA, USA), Phospho-Akt (Ser473) (Cell Signal Technology), MMP-2 (Cell Signal Technology), MMP-9 (Cell Signal Technology), BCL-2 (Abcam), C-myc (Abcam), CyclinD1 (Abcam) and GAPDH (Cwbiotech) at 4°C overnight.

Xenograft Model

All mouse experiments were performed with the approval of the Animal Studies Ethics Committee of The Third Affiliated Hospital of Southern Medical University according to the Guide for the Care and Use of Laboratory Animals. OS-732 cells (1×10^7) transduced with NC or XIST-shRNA were subcutaneously injected into the right flank of 4-week-old BALB/c nude mice (Shanghai Slaccas Laboratory Animal Company). Tumor length and width were measured every week after injection using calipers. At 5 weeks after injection, the mice were sacrificed and the tumor tissues were collected for further IHC analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean $\pm$ standard deviation (SD) from at least three separate experiments. Differences between groups were analyzed using Student's t test and the χ^2 test. Survival curves were plotted using the Kaplan-Meier method and the log-rank test. $P < 0.05$ was considered statistically significant.

Results

NNT-AS1 expression is increased in osteosarcoma tissues and cell lines

To assess the expression of NNT-AS1 in osteosarcoma tissues, the expression of NNT-AS1 was detected in 126 paired osteosarcoma cancer samples and their corresponding adjacent non-tumor tissues by qRT-PCR. As shown in Fig. 1A and 1B, the expression level of NNT-AS1 was significantly overexpressed in the primary tumor tissue of patients, while there was no difference in NNT-AS1 expression between benign tissues and healthy controls (Fig. 1B). Meanwhile, the expression of NNT-AS1 was remarkably upregulated in with higher Enneking stage (IIB-III) osteosarcoma tissues compared with lower Enneking stage (IA-IIA) osteosarcoma tissues (Fig. 1C). In addition, we explored the expression of NNT-AS1 in 6 osteosarcoma cells (U2OS, Saos2, OS-9901, SOSP-9607, MG-63 and OS-732) and osteoblast cells (hFOB1.19). Our results indicated that NNT-AS1 expression was

Fig. 1. NNT-AS1 expression is increased in OS tissues. (A) Relative expression of NNT-AS1 in osteosarcoma tissues compared with their adjacent healthy tissues. (B) The expression of NNT-AS1 was significantly upregulated in osteosarcoma samples compared to the adjacent non-malignant tissues. (C) The upregulation of HOTAIR was significantly associated with advanced Enneking stage. (D) qRT-PCR was performed to detect the expression of NNT-AS1 in an osteoblastic cell line (hFOB1.19) and the osteosarcoma cell lines (U2OS, Saos2, OS-9901, SOSP-9607, MG-63 and OS-732). ** $p < 0.01$.

increased in all 6 osteosarcoma cell lines compared with hFOB1.19 (Fig. 1D). These results showed that NNT-AS1 may function as an oncogene in osteosarcoma.

Overexpression of NNT-AS1 is associated with malignant status and overall survival in OS patients

To evaluate the relationship between NNT-AS1 expression and clinicopathological parameters, we divided the 126 patients into high and low NNT-AS1 expression groups based on the median value of NNT-AS1 expression. As shown in Table 1, the NNT-AS1 expression level was significantly higher in larger tumors (diameter >5 cm, $P=0.0333$), more advanced Enneking stage tumors ($P=0.0041$), and metastasis ($P=0.0233$). Nevertheless, no significant relationship was observed between NNT-AS1 expression and other clinical parameters (Table 1). Moreover, Kaplan-Meier analysis showed that the overall survival rate (Fig. 2A) and disease-free survival rate (Fig. 2B) of the high NNT-AS1 expression group was significantly worse than that of the low NNT-AS1 expression group. These results suggest that NNT-AS1 is involved in osteosarcoma aggressiveness and progression.

Knockdown of NNT-AS1 repressed osteosarcoma cell proliferation by affecting cell apoptosis and cell cycle

Since NNT-AS1 expression was associated with tumor size, we explored the biological functions of NNT-AS1 by short hairpin RNA (shRNA)-mediated NNT-AS1 downregulation in osteosarcoma cell lines. Two NNT-AS1-targeted shRNAs (shRNA#1 and shRNA#2) could downregulate the expression of NNT-AS1 in both MG-63 and OS-732 cells by qRT-PCR (Fig. 3A). CCK-8 assays indicated that depletion of NNT-AS1 significantly suppressed MG-63 and OS-732 cell proliferation compared with a control group (NC) (Fig. 3B). Data from a colony formation assay also revealed that inhibition of NNT-AS1 caused a substantial reduction in cell survival (Fig. 3C). To investigate potential mechanisms underlying the growth-inhibitory effects of NNT-AS1 knockdown, we assessed apoptosis and cell cycle in MG-63 and OS-732 cells. Compared to the si-NC transfected cells, NNT-AS1 knockdown resulted in a significant increase in MG-63 and OS-732 cell apoptosis (Fig. 3D). Cell cycle analysis suggested that depletion of NNT-AS1 decreased the number of MG-63 and OS-732 cells in S phase compared to cells transfected with si-NC (Fig. 3E). These results indicate that NNT-AS1 is involved in osteosarcoma cell proliferation partially by promoting cell cycle progression and inhibiting cell apoptosis.

Table 1. Correlation between NNT-AS1 expression and clinicopathological parameters of osteosarcoma

Characters	NNT-AS1 expression level		P-value
	Low (N=63)	High (N=63)	
Gender			0.4525
Female	24	19	
Male	39	44	
Age, y			0.5894
≤20	34	38	
>20	29	25	
Tumor size (cm)			0.0333
<5	40	27	
≥5	23	36	
Enneking stage			0.0041
IA-IIA	42	25	
IIB-III	21	38	
pathologic fracture			0.8346
No	49	47	
Yes	14	16	
Location			0.7226
Femur	28	26	
Tibia	20	18	
Other	15	19	
Metastasis			0.0233
No	48	36	
Yes	15	27	

*Fisher's exact tests, and χ^2 tests for all the other analysis.

Fig. 2. High NNT-AS1 expression is associated with worse survival in osteosarcoma. (A) The overall survival (OS) of osteosarcoma patients with high NNT-AS1 expression was less than that of patients with low NNT-AS1 expression. (B) Kaplan-Meier analysis shows the disease-free survival curves of patients with high NNT-AS1 expression was shorter than that of patients with low NNT-AS1 expression.

Fig. 3. NNT-AS1 knockdown inhibits cell proliferation in vitro. (A) The expression of NNT-AS1 in MG-63 and OS-732 cells transduced with NC or NNT-AS1 shRNA was measured using qRT-PCR. (B) The CCK-8 assay and (C) colony formation assay were used to evaluate the proliferation of the MG-63 and OS-732 cells after being transduced with NC or NNT-AS1 shRNA. Flow cytometry was performed to assess cell apoptosis (D) and cell cycle progression (E) of MG-63 and OS-732 cells after NNT-AS1 knockdown.

Knockdown of NNT-AS1 suppressed osteosarcoma cell migration and invasion

To further assess the effect of NNT-AS1 on the migration and invasion of osteosarcoma cells, Transwell migration assays were performed. Transwell migration assays indicated that NNT-AS1 silencing markedly repressed MG-63 (Fig. 4A) and OS-732 (Fig. 4B) migration and invasion compared to si-NC. Furthermore, based on the above effects of NNT-AS1 on osteosarcoma cells, we detected the expression of AKT, p-AKT, BCL-2, C-myc, CyclinD1, MMP-2 and caspase-9 by western blot. As shown in Fig. 4C, the protein levels of p-AKT, BCL-2, C-myc, CyclinD1, MMP-2 and MMP-9 were significantly decreased in NNT-AS1 knockdown MG-63 and OS-732 cells (Fig. 4C). These results indicate that NNT-AS1 plays a role in enhancing the invasion ability of osteosarcoma cells.

Stable knockdown of NNT-AS1 inhibited tumor growth in vivo

To further explore the effects of NNT-AS1 on the growth of osteosarcoma xenografts in nude mice, OS-732 cells stably transfected with shRNA#1 were inoculated into male nude mice. Five weeks later, *in vivo* study showed that xenograft tumors formed by the NNT-AS1-silenced cells had smaller volumes and weighed less than those formed by NC-treated cells

Fig. 4. NNT-AS1 silencing inhibits osteosarcoma cell migration and invasion. (A) Transwell migration and invasion assays were performed to evaluate the invasive ability of MG-63 cells transduced with NC or NNT-AS1 shRNA. (B) Transwell migration and invasion assays were performed to evaluate the invasive ability of OS-732 cells transduced with NC or NNT-AS1 shRNA. (C) Western blot analysis of total AKT, p-AKT, BCL-2, C-myc, CyclinD1, MMP-2 and MMP-9 proteins in osteosarcoma cells with or without NNT-AS1 knockdown. ** $p < 0.01$.

Fig. 5. Inhibition of the growth of OS-732 xenografts by NNT-AS1 knockdown in vivo. (A) Representative xenograft tumors formed by OS-732 cells transduced with NC or NNT-AS1 shRNA. (B) Tumor growth was significantly slower in mice injected with NNT-AS1 knockdown cells. (C) Tumor weights as shown in A. (D) The tumor/body weight ratio. (E) The body weight of BALB/c (nu/nu) mice. (F) The expression of NNT-AS1 was measured in xenografts tumors. (G) Immunohistochemical staining with a Ki67 antibody was performed on paraffin-embedded sections of xenografts tumors from OS-732 cells with or without NNT-AS1 knockdown. ** $p < 0.01$.

(Fig. 5A-D). However, there was no statistically significant difference in body weight between the NC group and NNT-AS1-silenced group (Fig. 5E). qRT-PCR revealed that NNT-AS1 was significantly decreased in tumors from the NNT-AS1-silenced group (Fig. 5F). In addition, the tissues resected from the xenograft tumors showed that the tumors developed from NNT-AS1-silenced cells displayed lower C-myc staining than those of the NC group (Fig. 5G). Taken together, these results are consistent with the *in vitro* findings, which confirmed the proto-oncogenic role of NNT-AS1 in osteosarcoma tumorigenesis.

Discussion

Deregulation of lncRNAs has been shown to contribute to initiation and progression in various human cancer types, including hepatocellular carcinoma, lung cancer, pancreatic cancer, osteosarcoma and colon cancer [13, 15, 16, 20]. Even though more and more lncRNAs have been reported to play crucial roles in osteosarcoma [15, 17, 21], the roles of lncRNAs in osteosarcoma are remain poorly defined. The identification of osteosarcoma-associated lncRNAs was needed to better understand the initiation and progression of osteosarcoma.

In this study, we found that NNT-AS1 was obviously upregulated in osteosarcoma tissues and cell lines, compared to the adjacent non-tumor tissues and immortalized human osteoblast cells. Statistical analysis revealed that high NNT-AS1 expression was associated with tumor size, advanced Enneking stage, metastasis and poor prognosis. Thus, we explored the biological function of NNT-AS1 in osteosarcoma by silencing NNT-AS1. Our *in vitro* shRNA-mediated NNT-AS1 silencing resulted in inhibition of cell proliferation and motility.

Recent study has shown that NNT-AS1 was significantly increased in colorectal cancer tissues and could be used as a biomarker of poor prognosis [19]. Consistent with this previous study, our results revealed that NNT-AS1 was significantly upregulated in osteosarcoma tissues and was positively associated with poor prognosis. These data indicate that NNT-AS1 might act as an oncogene in the development of osteosarcoma. Uncontrolled cell proliferation and metastasis are the major cause of cancer. The shRNA-mediated NNT-AS1 silencing reduced CRC cell proliferation and colony formation *in vitro*. Knockdown of NNT-AS1 mediated CRC cell migration and invasion, which possibly attenuated EMT and the MAPK/Erk signaling pathway [19]. In our present study, NNT-AS1 silencing suppressed cell proliferation, migration and invasion *in vitro*. In addition, flow cytometry analysis showed that knockdown of NNT-AS1 contributed to a significant increase in cell apoptosis. To further elucidate the regulatory mechanism of NNT-AS1's involvement in cell proliferation, migration and invasion, proteins involved in proliferation, migration and invasion were detected by western blotting. Our results indicated that NNT-AS1 silencing remarkably decreased the expression of phosphorylated levels of AKT, C-myc, BCL-2, MMP-2 and MMP-9. lncRNAs are highly upregulated in liver cancer (HULC) and promote cell proliferation and invasion via the PI3K/Akt/mTOR signaling pathway in glioma [22]. lncRNA AK023948 was found to be a positive regulator of AKT contributing to breast tumor progression [23]. Other studies have reported that lncRNAs mediate tumor progression via CyclinD1, MMP-2 and MMP-9, which are important in cancer development [24, 25]. NNT-AS1 silencing impaired tumor growth *in vivo*, also confirming that NNT-AS1 acts as a proto-oncogene in osteosarcoma.

Conclusion

In summary, our study provides the first evidence that the dysfunction of NNT-AS1 expression is associated with the progression of osteosarcoma. We show that inhibition of NNT-AS1 suppresses cell proliferation and tumor growth *in vitro* and *in vivo*. Knockdown of NNT-AS1 suppresses migration and invasion abilities *in vitro*. Other possible biological roles of NNT-AS1 will be explored in future studies.

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Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

- Collins M, Wilhelm M, Conyers R, Herschtal A, Whelan J, Bielack S, Kager L, Kuhne T, Sydes M, Gelderblom H, Ferrari S, Picci P, Smeland S, Eriksson M, Petrilli AS, Bleyer A, Thomas DM: Benefits and adverse events in younger versus older patients receiving neoadjuvant chemotherapy for osteosarcoma: Findings from a meta-analysis. *J Clin Oncol* 2013;31:2303-2312.
- Bielack S, Carrle D, Casali PG: Osteosarcoma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2009;20 Suppl 4:137-139.
- Hasei J, Sasaki T, Tazawa H, Osaki S, Yamakawa Y, Kunisada T, Yoshida A, Hashimoto Y, Onishi T, Uno F, Kagawa S, Urata Y, Ozaki T, Fujiwara T: Dual programmed cell death pathways induced by p53 transactivation overcome resistance to oncolytic adenovirus in human osteosarcoma cells. *Mol Cancer Ther* 2013;12:314-325.
- Mirabello L, Troisi RJ, Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the Surveillance, Epidemiology, and End Results Program. *Cancer* 2009;115:1531-1543.
- Carrle D, Bielack S: Osteosarcoma lung metastases detection and principles of multimodal therapy. *Cancer Treat Res* 2009;152:165-184.
- Zhou W, Hao M, Du X, Chen K, Wang G, Yang J: Advances in targeted therapy for osteosarcoma. *Discov Med* 2014;17:301-307.
- Sakamoto A, Iwamoto Y: Current status and perspectives regarding the treatment of osteo-sarcoma: Chemotherapy. *Rev Recent Clin Trials* 2008;3:228-231.
- Mattick JS: The genetic signatures of noncoding RNAs. *PLoS Genet* 2009;5:e1000459.
- Ulitsky I, Bartel DP: LincRNAs: Genomics, evolution, and mechanisms. *Cell* 2013;154:26-46.
- Kung JT, Colognori D, Lee JT: Long noncoding RNAs: Past, present, and future. *Genetics* 2013;193:651-669.
- Mercer TR, Dinger ME, Mattick JS: Long non-coding RNAs: Insights into functions. *Nat Rev Genet* 2009;10:155-159.
- Huang R, Wang X, Zhang W, Zhangyuan G, Jin K, Yu W, Xie Y, Xu X, Wang H, Sun B: Down-Regulation of lncRNA DGCR5 correlates with poor prognosis in hepatocellular carcinoma. *Cell Physiol Biochem* 2016;40:707-715.
- Chen Y, Li C, Pan Y, Han S, Feng B, Gao Y, Chen J, Zhang K, Wang R, Chen L: The emerging role and promise of long noncoding RNAs in lung cancer treatment. *Cell Physiol Biochem* 2016;38:2194-2206.
- Zhou M, Diao Z, Yue X, Chen Y, Zhao H, Cheng L, Sun J: Construction and analysis of dysregulated lncRNA-associated ceRNA network identified novel lncRNA biomarkers for early diagnosis of human pancreatic cancer. *Oncotarget* 2016;7:56383-56394.
- Zhao H, Hou W, Tao J, Zhao Y, Wan G, Ma C, Xu H: Upregulation of lncRNA HNF1A-AS1 promotes cell proliferation and metastasis in osteosarcoma through activation of the Wnt/beta-catenin signaling pathway. *Am J Transl Res* 2016;8:3503-3512.
- Li S, Hua Y, Jin J, Wang H, Du M, Zhu L, Chu H, Zhang Z, Wang M: Association of genetic variants in lncRNA H19 with risk of colorectal cancer in a Chinese population. *Oncotarget* 2016;7:25470-25477.
- Chen R, Wang G, Zheng Y, Hua Y, Cai Z: Long non-coding RNAs in osteosarcoma. *Oncotarget* 2017
- Yang X, Song JH, Cheng Y, Wu W, Bhagat T, Yu Y, Abraham JM, Ibrahim S, Ravich W, Roland BC, Khashab M, Singh VK, Shin EJ, Yang X, Verma AK, Meltzer SJ, Mori Y: Long non-coding RNA HNF1A-AS1 regulates proliferation and migration in oesophageal adenocarcinoma cells. *Gut* 2014;63:881-890.
- Wang Q, Yang L, Hu X, Jiang Y, Hu Y, Liu Z, Liu J, Wen T, Ma Y, An G, Feng G: Upregulated NNT-AS1, a long noncoding RNA, contributes to proliferation and migration of colorectal cancer cells *in vitro* and *in vivo*. *Oncotarget* 2017;8:3441-3453.
- Wang F, Yang H, Deng Z, Su Y, Fang Q, Yin Z: HOX antisense lincRNA HOXA-AS2 promotes tumorigenesis of hepatocellular carcinoma. *Cell Physiol Biochem* 2016;40:287-296.

- 21 Xie CH, Cao YM, Huang Y, Shi QW, Guo JH, Fan ZW, Li JG, Chen BW, Wu BY: Long non-coding RNA TUG1 contributes to tumorigenesis of human osteosarcoma by sponging miR-9-5p and regulating POU2F1 expression. *Tumour Biol* 2016;37:15031-15041.
- 22 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.
- 23 Koirala P, Huang J, Ho TT, Wu F, Ding X, Mo YY: LncRNA AK023948 is a positive regulator of AKT. *Nat Commun* 2017;8:14422.
- 24 Lu X, Zhou C, Li R, Deng Y, Zhao L, Zhai W: Long noncoding RNA AFAP1-AS1 promoted tumor growth and invasion in cholangiocarcinoma. *Cell Physiol Biochem* 2017;42:222-230.
- 25 Lv QL, Hu L, Chen SH, Sun B, Fu ML, Qin CZ, Qu Q, Wang GH, He CJ, Zhou HH: A long noncoding RNA ZEB1-AS1 promotes tumorigenesis and predicts poor prognosis in glioma. *Int J Mol Sci* 2016;17