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ORIGINAL ARTICLE

A potential diagnostic marker for osteosarcoma: hsa_circ_0005721

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Summary

Purpose: *To detect the expression level of hsa_circ_0005721 in osteosarcoma specimen and plasma of osteosarcoma patients, and to analyze the clinical significance of hsa_circ_0005721 as a diagnostic marker for osteosarcoma.*

Methods: *Expression levels of hsa_circ_0005721 in osteosarcoma specimen and osteosarcoma cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between hsa_circ_0005721 expression difference and overall survival in osteosarcoma was analyzed by Kaplan-Meier method. Expression level of hsa_circ_0005721 was stably downregulated in U-2OS and HOS cells by shRNA transfection. Proliferative potential in osteosarcoma cells regulated by hsa_circ_0005721 was assessed by colony formation and 5-Ethynyl-2'- deoxyuridine (EdU) assay. Differentially expressed hsa_circ_0005721 in the plasma of healthy controls, benign bone tumor patients and osteosarcoma patients was determined by qRT-PCR. The diagnostic capacity of hsa_circ_0005721 in osteosarcoma was examined by receiver operating characteristic (ROC) curves.*

Results: *hsa_circ_0005721 was upregulated in osteosarcoma specimen and osteosarcoma cell lines. High level of hsa_circ_0005721 predicted poor prognosis in osteosarcoma patients. In vitro experiments showed that knockdown of hsa_circ_0005721 suppressed proliferative ability in osteosarcoma cells. Compared with that in healthy controls and benign bone tumor patients, plasma level of hsa_circ_0005721 was higher in osteosarcoma patients. ROC curves demonstrated the diagnostic potential of hsa_circ_0005721 in osteosarcoma.*

Conclusions: *hsa_circ_0005721 is upregulated in osteosarcoma samples, which acts as an oncogene responsible for aggravating the progression. hsa_circ_0005721 can be a promising diagnostic marker for osteosarcoma.*

Key words: *osteosarcoma, hsa_circ_0005721, proliferation, diagnostic marker*

Introduction

Osteosarcoma is a highly malignant, osteogenic tumor derived from primary mesenchymal cells. The metastasis and recurrence rates are relatively high. Back to the emergence of comprehensive therapy, more than 90% of osteosarcoma patients die of lung metastasis [1]. Osteosarcoma mainly affects adolescents aged 15-19 years (>50%) and the elderly aged 60-85 years. In particular, osteosarcoma mainly affects men, with male to female ratio 1.4:1 [2]. Osteosarcoma can occur in any bone tissues, including distal femur and proximal tibia (>50%), axial bones (10%), pelvis and proximal humerus [3]. Because of the strong invasiveness and metastasis of osteosarcoma, adolescent patients often have to accept amputation or limb salvage surgery. Postoperative quality of life and physical and psychological trauma to them are overwhelming [4]. It is urgent to clarify the molecular mechanism of osteosarcoma, which is conductive to provide novel ideas for developing targeted therapy.

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CircRNAs are a novel type of endogenous, non-coding RNAs. They are produced by reverse splicing of precursor mRNAs. There are many splicing forms of circRNAs, mainly including exon cyclization and intron cyclization. CircRNAs produced by exon cyclization are the majority type, and they are mainly distributed in the nucleus [5,6]. Currently, circRNAs have become biomarkers owing to the characteristics of high abundance, conservatism, stability and specificity. CircRNAs are not only stably expressed in tumor tissues, but also in body fluids, such as exosomes and blood. Liquid biopsy is more convenient, less invasive and easier acquisition than traditional biopsy. Hence, circRNAs display great potentials as cancer biomarkers [7]. It is reported that circ_0021093 drives hepatocellular carcinoma progression via the miR-766-3p/MTA3 axis, presenting prognostic capacity [8]. Downregulation of hsa_circ_0001649 is a signal for poor prognosis in retinoblastoma [9]. hsa_circ_0001649 is responsible for regulating proliferative and apoptotic capacities in retinoblastoma by activating the AKT/mTOR pathway. CircEXOC6B and circN-4BP2L2 have been identified as novel prognostic biomarkers for epithelial ovarian cancer [10,11].

This study aimed to uncover clinically significant circRNAs for non-invasive diagnosis of osteosarcoma. A previous study has shown that hsa_circ_0005721 is abnormally expressed in osteosarcoma tissues by the next-generation sequencing technology. In this article, we detected hsa_circ_0005721 levels in cancer specimen and plasma of osteosarcoma patients, and explored its diagnostic potential.

Methods

Subjects and specimen collection

Cancer and adjacent normal tissues were collected from 50 osteosarcoma patients. They did not have chemotherapy, radiotherapy and immune therapy before surgery. All specimens were postoperatively diagnosed as osteosarcoma. Enneking stage of osteosarcoma was assessed by the Union Internationale Contre le Cancer (UICC). In addition, plasma samples from age-matched healthy controls (n=16), benign bone tumor patients (n=16) and osteosarcoma patients (n=20) were collected and stored at -80°C. This study got approval by the Ethics Committee of Yantaishan Hospital and was conducted after signed informed consent form was received from each subject. Experimental protocols conformed to the Helsinki Declaration.

Cell culture

Human osteoblast cell line (hFOB) and osteosarcoma cell lines (143B, U-2OS, HOS and Saos-2) were maintained at American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added in the culture medium. Cells in the logarithmic growth phase were collected for experiments.

Transfection

U-2OS and HOS cells implanted in 24-well plates were transfected with HBLV-GFP-PURO or HBLV-has_circ_0005721-shRNA-GFP-PURO (Multiplicity of Infection (MOI) = 20). Stably transfected cells were screened by puromycin. Transfection efficacy was examined by quantitative real-time polymerase chain reaction (qRT-PCR) at 48 h.

RNA extraction

Total RNA was extracted from cell or tissue lysate using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 250 μL of chloroform were added in 1 mL of TRIzol mixed with cell or tissue lysate. After gentle mixture for 30 s and centrifugation at 4°C, the aqua phase was mixed with the same volume of pre-cold isopropanol. The centrifuged precipitant was washed in 75% ethanol, air dried and diluted in 30 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA concentration and purity were determined using NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA). Qualified RNAs were stored at -80°C.

qRT-PCR

Qualified RNA was reversely transcribed into complementary DNA (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The cDNA was subjected to qRT-PCR using the SYBR Green Master Mix (Applied Biosystems, San Diego, CA, USA) at 95°C for 5 min, and 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30s. Primer sequences were: has_circ_0005721: 5'-AGCAGAGAAAGGGTCGTTGG-3' (forward) and 5'-CTCCTCTCCCAAGCTCAGACT-3' (reverse); PCNA: 5'-CCTGCTGGGATATTAGCTCCA-3' (forward) and 5'-CAGCGGTAGGTGTCGAAGC-3' (reverse); glyceraldheyde 3-phosphate dehydrogenase (GAPDH): 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (forward) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (reverse); U6: 5'-GCTGAGGTGACGGTCTCAAA-3' (forward) and 5'-GCCTCCCAGTTTCATGGACA-3' (reverse).

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were inoculated in 96-well plates. 10 μL of EdU was applied in each well for cell labeling. After three h, cells were incubated with 4% methanol for 20 min, followed by phosphate buffered saline (PBS) washing and incubation with 0.5% Triton X-100 (Sigma, San Francisco, CA, USA) for another 20 min. 1 mL of Hoechst 33342 (Sigma, San Francisco, CA, USA) was applied for nuclei staining in the dark. Finally, cells were washed in PBS and captured.

Colony formation assay

Cells (4×10^2) were inoculated in the 6 cm dish and cultured for 14 days. Visible colonies were fixed in 4% paraformaldehyde for 30 min and stained using 0.5% crystal violet for 30 min. The number of visible colonies was counted.

Statistics

Data were expressed as mean ± standard deviation and processed by SPSS 22.0 (IBM, Armonk, NY, USA). Differences between groups were analyzed by two-tailed t-test. Disease-free survival and overall survival in osteosarcoma patients expressing high or low level of hsa_circ_0005721 were compared by Kaplan-Meier method and log-rank test. Diagnostic potential of hsa_circ_0005721 in osteosarcoma was evaluated by depicting receiver operating characteristic (ROC) curves. P<0.05 was considered as statistically significant.

Results

Upregulation of hsa_circ_0005721 in osteosarcoma specimen

QRT-PCR was conducted to determine hsa_circ_0005721 levels in 50 cases of osteosarcoma tissues and adjacent normal ones. It was shown that hsa_circ_0005721 was upregulated in cancer tissues, which was consistent with the previous report (Figure 1A). Notably, higher level of hsa_circ_0005721 was detected in advanced-stage osteosarcoma tissues than that of early stage ones (Figure 1B). Furthermore, we classified osteosarcoma specimens based on the tumor size (8 cm). The data revealed that hsa_circ_0005721 level was higher in larger osteosarcoma tissues, which was positively related to tumor size in osteosarcoma (Figure 1C). The above results demonstrated the vital function of hsa_circ_0005721 in osteosarcoma. Recruited osteosarcoma patients were divided into two groups based on the cut-off value of hsa_circ_0005721 level in their cancer tissues. Kaplan-Meier curves and log-rank test indicated that overall survival were worse in osteosarcoma patients expressing high level of hsa_circ_0005721 compared with those expressing low level (Figure 1D). It is suggested that hsa_circ_0005721 may be a prognostic indicator for osteosarcoma.

Knockdown of hsa_circ_0005721 suppressed osteosarcoma proliferation

As expected, hsa_circ_0005721 was upregulated in osteosarcoma cell lines (Figure 2A). U-2OS and HOS cells expressed the highest level of hsa_circ_0005721 than the other tested osteosarcoma cell lines, which were used for generating hsa_circ_0005721 knockdown models by transfection of sh-hsa_circ_0005721 (Figure 2B). Knockdown of hsa_circ_0005721 in U-2OS and HOS cells markedly decreased the numbers of cell colonies (Figure 2C) and positive EdU-stained cells (Figure 2D), indicating suppressed proliferative ability. PCNA level was markedly downregulated by knockdown of hsa_circ_0005721, further supporting our findings (Figure 2E).

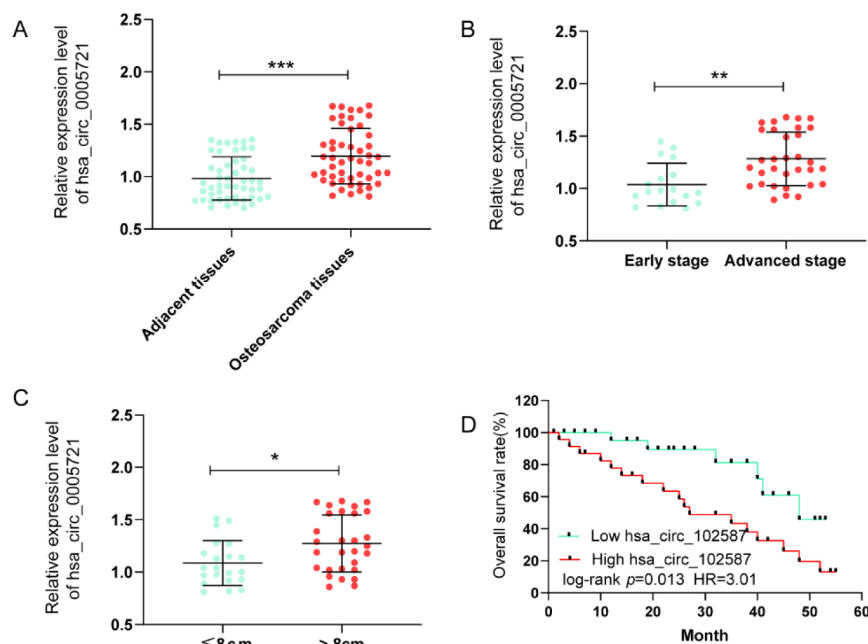

Figure 1. Upregulation of hsa_circ_0005721 in osteosarcoma specimen. **A:** hsa_circ_0005721 levels in osteosarcoma tissues (n=50) and adjacent normal tissues (n=50) detected by qRT-PCR. **B:** hsa_circ_0005721 levels in early stage and advanced osteosarcoma tissues detected by qRT-PCR. **C:** hsa_circ_0005721 levels in osteosarcoma tissues with large and small tumor size detected by qRT-PCR. **D:** Overall survival in osteosarcoma patients expressing high and low level of hsa_circ_0005721.*p<0.05; **p<0.01; ***p<0.001.

Increased plasma level of hsa_circ_0005721 in osteosarcoma patients

Very recently, circRNAs in the circulating peripheral blood have shown diagnostic potentials. Here, we detected plasma level of hsa_circ_0005721 in healthy controls (n=16), benign bone tumor patients (n=16) and osteosarcoma patients (n=20) by qRT-PCR. No significant difference in plasma level of hsa_circ_0005721 was discovered between healthy controls and benign bone tumor patients. However, plasma level of hsa_circ_0005721 was higher in osteosarcoma patients than in those with benign bone tumor patients (Figure 3A). Furthermore, higher abundance of hsa_circ_0005721 was detected in the plasma of osteosarcoma patients with Enneking stage IIB+III than those with I+IIA (Figure 3B). Since we have detected the differential expressions of hsa_circ_0005721 in healthy controls, benign bone tumor patients and osteosarcoma patients, it is speculated that hsa_circ_0005721 may be used for diagnosing osteosarcoma. Subsequently, ROC curves demonstrated that hsa_circ_0005721 level was able to distinguish healthy controls from osteosarcoma patients (AUC=0.7924, p=0.0024, Figure 3C). It also distinguished benign bone tumor patients from osteosarcoma patients (AUC=0.7563, p=0.0090, Figure 3D). Therefore, hsa_circ_0005721 was a promising diagnostic marker for osteosarcoma.

Discussion

At present, neoadjuvant therapy is the major standardized treatment for osteosarcoma, which greatly influences the prognosis of osteosarcoma patients [12]. However, the overall efficacy of neoadjuvant therapy remains 60% owing to the side effects and drug resistance [13]. In addition, chemotherapy efficacy determined by tumor necrosis rate takes a long time and prolongs the therapeutic period for osteosarcoma patients [14,15]. Non-invasive examinations by detecting plasma indicators are convenient and highly repeatable. We aimed to develop specific biomarkers for osteosarcoma, thus improving survival of affected people.

CircRNAs have been well concerned because of their unique structures and functions. Unlike traditional linear RNAs, circRNAs are featured by covalent closed loop structure lacking 5' or 3' end [16]. Previously, circRNAs were considered as mistaken RNAs during the splicing process and underestimated. With the improvement on bioinformatic analyses and high-throughput sequencing, abundant circRNAs have been discovered [17]. It is now considered that circRNAs are diversely functional, with the typical characteristics of tissue and development specificities. Besides, circRNAs exert sponge effects on miRNAs, thereafter blocking target gene translation or regulating the interaction with RNA-binding proteins [18,19]. Biological

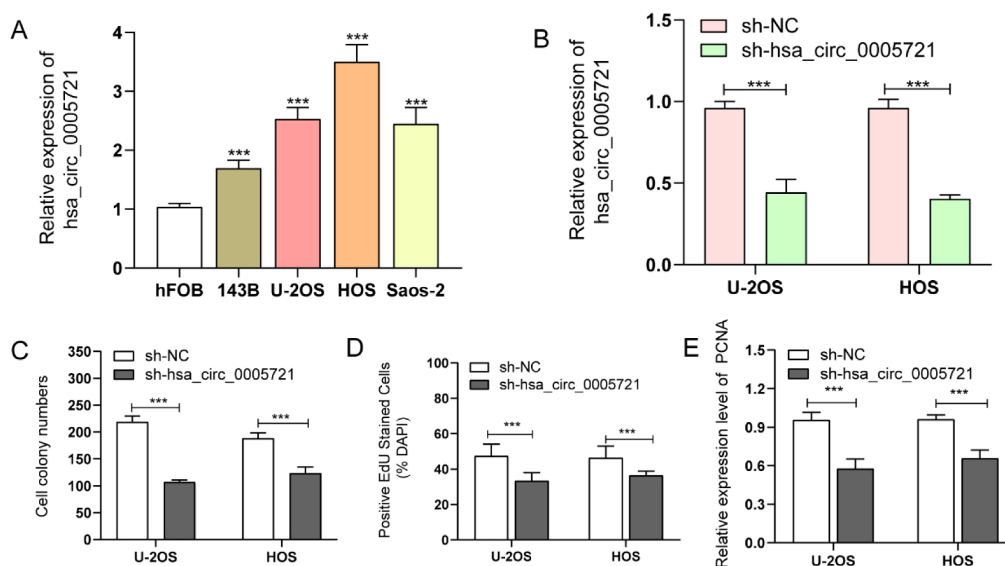

Figure 2. Knockdown of hsa_circ_0005721 suppressed osteosarcoma proliferation. **A:** hsa_circ_0005721 levels in osteosarcoma cell lines detected by qRT-PCR. **B:** Transfection efficacy of sh-hsa_circ_0005721 in U-2OS and HOS cells. **C:** Number of cell colonies in U-2OS and HOS cells transfected with sh-NC or sh-hsa_circ_0005721 detected by colony formation assay. **D:** Positive EdU-stained cells in U-2OS and HOS cells transfected with sh-NC or sh-hsa_circ_0005721 detected by EdU assay. E: Relative level of PCNA in U-2OS and HOS cells transfected with sh-NC or sh-hsa_circ_0005721 detected by qRT-PCR; ***p<0.001.

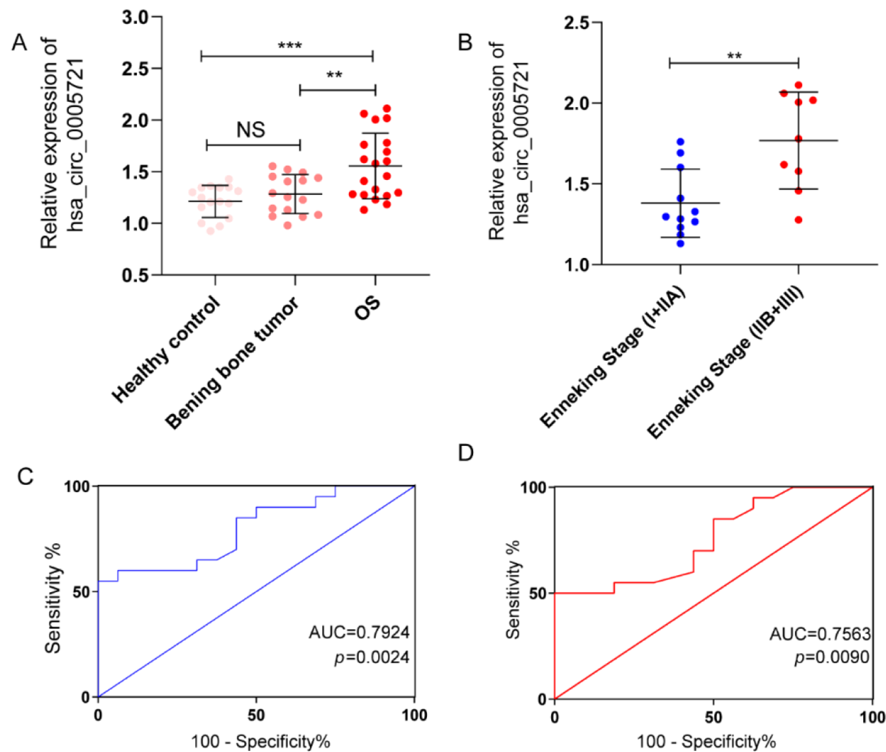

Figure 3. Increased plasma level of hsa_circ_0005721 in osteosarcoma patients. **A:** Plasma level of hsa_circ_0005721 in healthy controls, benign bone tumor patients and osteosarcoma patients detected by qRT-PCR. **B:** Plasma level of hsa_circ_0005721 in Enneking stage I+IIA and IIB+III osteosarcoma detected by qRT-PCR. **C:** ROC curves depicted based on the sensitivity and specificity of hsa_circ_0005721 in distinguishing healthy controls from osteosarcoma patients. **D:** ROC curves depicted based on the sensitivity and specificity of hsa_circ_0005721 in distinguishing benign bone tumor patients from osteosarcoma patients. NS=not significant difference; $^{**}p<0.01$; $^{***}p<0.001$.

functions of circRNAs in regulating osteosarcoma progression have been identified. It is reported that overexpression of circPVT1 induces drug resistance to Adriamycin and cisplatin to osteosarcoma via regulating ABCB1 [20]. Circ_001621 drives proliferative and migratory potentials in osteosarcoma cells by sponge effect on miR-587/VEGF axis [21]. Through activating the AKT / GSK-3β signaling, hsa_circ_0007534 mediates growth and apoptosis of osteosarcoma cells [22].

Latest studies have shown that plasma circRNAs retain high stability in the *in vitro* acid alkali environment and repeated freezing and thawing [23]. Meanwhile, circRNAs are differentially expressed in the plasma of tumor patients and healthy people, displaying specific expression patterns [24]. CircRNA levels are closely linked to tumor progression. As a result, we believe that circRNAs can be potential biomarkers for diagnosis of early-stage tumors [25].

Our findings pointed out that hsa_circ_0005721 was upregulated in osteosarcoma samples. Highly expressed hsa_circ_0005721 was unfavorable to the prognosis in osteosarcoma patients. Subsequently, by generating hsa_circ_0005721 knockdown model in U-2OS and HOS cells, we found that hsa_circ_0005721 was able to promote the proliferative ability in osteosarcoma. Interestingly, plasma level of hsa_circ_0005721 was much higher in osteosarcoma patients than that in healthy controls and benign bone tumor patients. The potential diagnostic value of hsa_circ_0005721 in osteosarcoma was further confirmed by depicting ROC curves. We believe that hsa_circ_0005721 may serve as a potential biomarker for osteosarcoma. Nevertheless, several limitations in this study should be noteworthy. First of all, we only discussed diagnostic significance of hsa_circ_0005721 in osteosarcoma. Other potential circRNAs in osteosarcoma profiling require to be analyzed. Secondly, besides proliferative ability, other cell functions of osteosarcoma regulated by hsa_circ_0005721 should be determined. Thirdly, the exact molecular mechanism of hsa_circ_0005721 on regulating osteosarcoma proliferation needs to be clarified. Last but not least, the *in vitro* findings on the biological functions of hsa_circ_0005721 in osteosarcoma should be validated in *in vivo* models.

Conclusions

hsa_circ_0005721 is upregulated in osteosarcoma samples, which acts as an oncogene for aggravating the progression. hsa_circ_0005721 can be a promising diagnostic marker for osteosarcoma.

Conflict of interests

The authors declare no conflict of interests.

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