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ORIGINAL ARTICLE

hsa_circ_0005721 triggers proliferation, migration and invasion of osteosarcoma by upregulating the linear transcript TEP1

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Summary

Purpose: *This study aimed to illustrate the biological role of hsa_circ_0005721 in the development of osteosarcoma and the molecular mechanism.*

Methods: *hsa_circ_0005721 levels in 30 pairs of osteosarcoma and non-tumor tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Functional experiments were conducted to assess the influence of hsa_circ_0005721 on proliferative, metastatic and apoptotic rates of osteosarcoma cells. The downstream target of hsa_circ_0005721 and their co-regulatory mechanism in malignant development of osteosarcoma were analyzed by dual-luciferase reporter assay and rescue experiments, respectively.*

Results: *hsa_circ_0005721 was upregulated in osteosarcoma tissues and cell lines. Knockdown of hsa_circ_0005721 suppressed proliferative and metastatic rates of U-2OS and Saos-2 cells, and stimulated apoptosis. Serving as a ceRNA, hsa_circ_0005721 upregulated the linear transcript TEP1 by competitively binding miR-16-5p, thus exerting its biological functions in regulating osteosarcoma development.*

Conclusions: *This study for the first time identified the upregulated hsa_circ_0005721 in osteosarcoma, which triggers the malignant development of osteosarcoma by upregulating the linear transcript TEP1.*

Key words: *hsa_circ_0005721, miR-16-5p, TEP1, osteosarcoma*

Introduction

Osteosarcoma is a common mesenchymal primary malignant tumor. Osteosarcoma cells directly differentiate into bones or bone-like tissues [1,2]. Osteosarcoma mainly affects children and adolescents. Because of the high malignant level and rapid progression, osteosarcoma cells can easily metastasize to lungs through circulating blood in the early phase. In the past decade, the 5-year survival of osteosarcoma has sharply increased from 10-20% to 60-70%. However, there are still a large number of osteosarcoma patients lose their lives [3-5]. It is urgent to explore new biomarkers of osteosarcoma for improving the clinical prognosis.

CircRNAs are a novel type of noncoding RNAs that are extensively present in the cytoplasm of eukaryotic cells [6]. Recent evidence has shown that circRNAs are competitive, endogenous RNAs involved in gene expression regulation and cancer development. It is well known that circRNAs differ from traditional linear RNAs in the structure and their high stability [7-9]. Actually, circRNAs are multifunctional in cell behaviors [10,11].

hsa_circ_0005721 is located on chr14: 20871535-20874559, and its associated gene symbol is TEP1. Zhu et al [12] identified through next generation sequencing that hsa_circ_0005721 is

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abnormally upregulated in osteosarcoma tissues [12]. In the present study, we aimed to explore the biological role of hsa_circ_0005721 in regulating the malignant development of osteosarcoma and the possible mechanism.

Methods

Subjects and samples

Thirty pairs of osteosarcoma and non-tumor tissues were collected. Samples were labeled and stored in liquid nitrogen for use. Recruited patients did not have history of anti-cancer treatment or other tumors. This study was approved by the research Ethics Committee of First Affiliated Hospital, Heilongjiang University of Chinese Medicine and complied with the Helsinki Declaration. Informed consent was obtained from subjects and their parents.

Cell culture

Human osteoblast cell line (hFOB) and osteosarcoma cell lines (143B, U-2OS, HOS and Saos-2) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) at 37°C, 5% CO_2.

Cell transfection

Transfection plasmids were synthesized by Ribobio (Guangzhou, China). Cells were seeded in 6-well plates and cultured to 80% density. After 48-h transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), cells were collected for verifying transfection efficacy and functional experiments. Sequences of hsa_circ_0005721 siRNA were: AAGAGCAGAGAAAGGGTCGTT.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Tissue or cell samples were processed by TRIzol (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. After purification, qualified RNAs were reversely transcribed to complementary DNAs (cDNAs) and subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Tokyo, Japan). Relative levels of PCR products were calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6. Primer sequences were as follows. hsa_circ_0005721: Forward: 5'-CCCCGCTCTCCAGGGAT-3' and reverse: 5'-GGGTCTCTGCCCCTTTCTTC-3'; miR-16-5p: Forward: 5'-TAGCAGCACGTAAATATTGGCG-3' and reverse: 5'-TGCGTGTCGTGGAGTC-3'; TEP1: Forward: 5'-CCACCCTCTCTAGTCTAAAGAGC-3' and reverse: 5'-CAGCTTGCGTCATGTGAGATA-3'; GAPDH: Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; U6: Forward: 5'-GCTGAGGTGACGGTCTCAAA-3' and reverse: 5'-GCCTCCCAGTTTCATGGACA-3'.

CCK-8 assay

Cells were seeded in 96-well plates with 4×10^3 cells suspended in 200 μL of medium per well. They were induced with 10 μL of CCK-8 solution per well at the indicated time points cell counting kit-8 (CCK-8). After cell culture in the dark for 2 h, absorbance at 450 nm was detected for plotting cell viability curves.

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

Cells were seeded in 12-well plates (5×10^4 cells/well), labeled by 50 μM EdU (RiboBio, Guangzhou, China) for 2 h, and dyed using AdoLo and 4',6-diamidino-2-phenylindole (DAPI) in the dark. EdU-labeled cells were captured for calculating EdU-stained cell ratio.

Flow cytometry

Cells were stained with Annexin V-FITC (fluorescein isothiocyanate) in the dark, and subjected to flow cytometry for assessing the apoptotic rate (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell assay

5×10^4 cells suspended in serum-free medium were seeded in a Transwell chamber pre-coated with 200 mg / mL Matrigel (diluted in serum-free medium at a ratio of 1:6, coated on the bottom and dried overnight). Medium containing 10% FBS was added to the bottom as an inducer. After 24-h cell culture, invasive cells to the bottom were fixed, and dyed in 0.2% crystal violet. They were captured in 5 random fields per well for cell counting. Migration assay was similarly conducted using a Transwell chamber in the absence of Matrigel (Corning, Corning, NY, USA).

Dual-luciferase reporter assay

Luciferase vectors were constructed based on the predicted binding site in miR-16-5p 3'UTR. They were co-transfected into cells with miR-16-5p mimics or NC for 48 h. Luciferase activity (Promega, Madison, WI, USA) was measured for three times and the average value was calculated.

Statistics

SPSS 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (La Jolla, CA, USA) were used for statistical analyses. Data were expressed as mean ± standard deviation. Differences between groups were compared by the t-test. Correlation between expression levels of two genes in osteosarcoma tissues was assessed by Pearson correlation test. $P<0.05$ was considered as statistically significant.

Results

Upregulation of hsa_circ_0005721 in osteosarcoma

In comparison to non-tumor tissues, hsa_circ_0005721 was upregulated in osteosarcoma tissues (Figure 1A). Similarly, hsa_circ_0005721 was highly expressed in osteosarcoma cell lines (Figure 1B). The stability of hsa_circ_0005721 in osteosarcoma cells was detected and showed that

the half-life of hsa_circ_0005721 in Actinomycin D-induced U-2OS and Saos-2 cells exceeded 24 h, which was shorter than 12 h of its linear transcript TEP1 (Figure 1C). In addition, RNase R induction did not affect hsa_circ_0005721 level, but remarkably decreased TEP1 level (Figure 1D).

Knockdown of hsa_circ_0005721 suppressed proliferative, migratory and invasive abilities of osteosarcoma cells, but stimulated cell apoptosis

We constructed si-hsa_circ_0005721 and examined its intervention efficacy in U-2OS and Saos-2 cells by qRT-PCR (Figure 2A). As CCK-8 curves

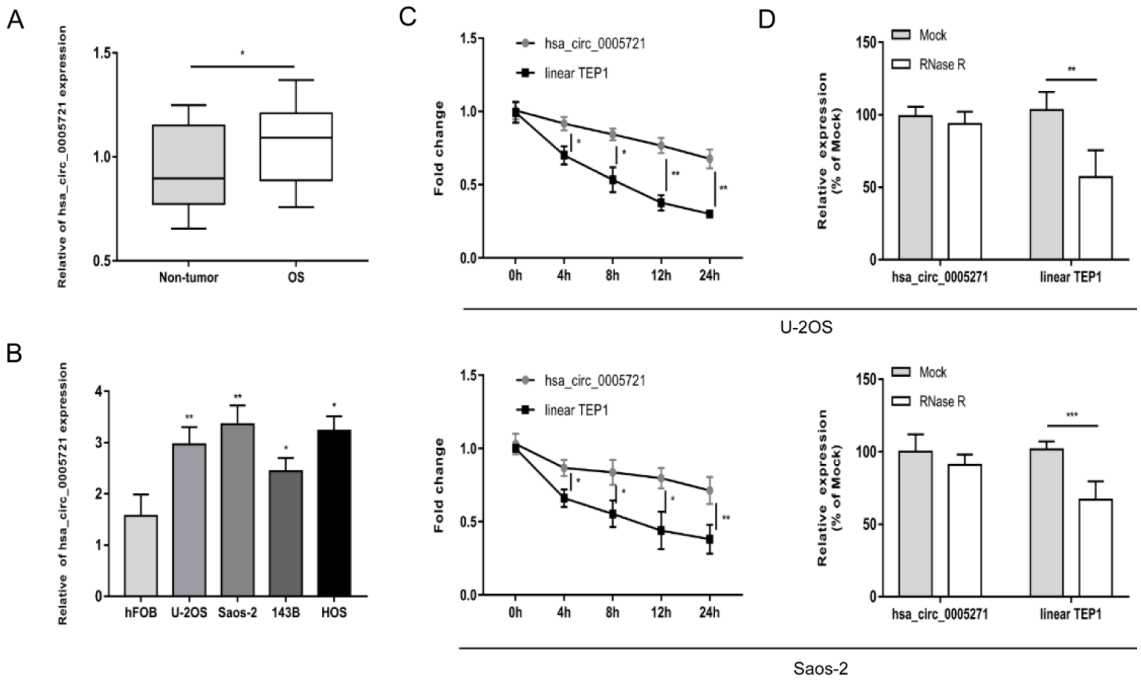

Figure 1. Upregulation of hsa_circ_0005721 in osteosarcoma. **A:** hsa_circ_0005721 levels in osteosarcoma and non-tumor tissues. **B:** hsa_circ_0005721 levels in osteosarcoma cell lines. **C:** Half-life of hsa_circ_0005721 and TEP1 in Actinomycin D-induced U-2OS and Saos-2 cells. **D:** Stability of hsa_circ_0005721 and TEP1 in RNase R-induced U-2OS and Saos-2 cells. *p<0.05; **p<0.01; ***p<0.001.

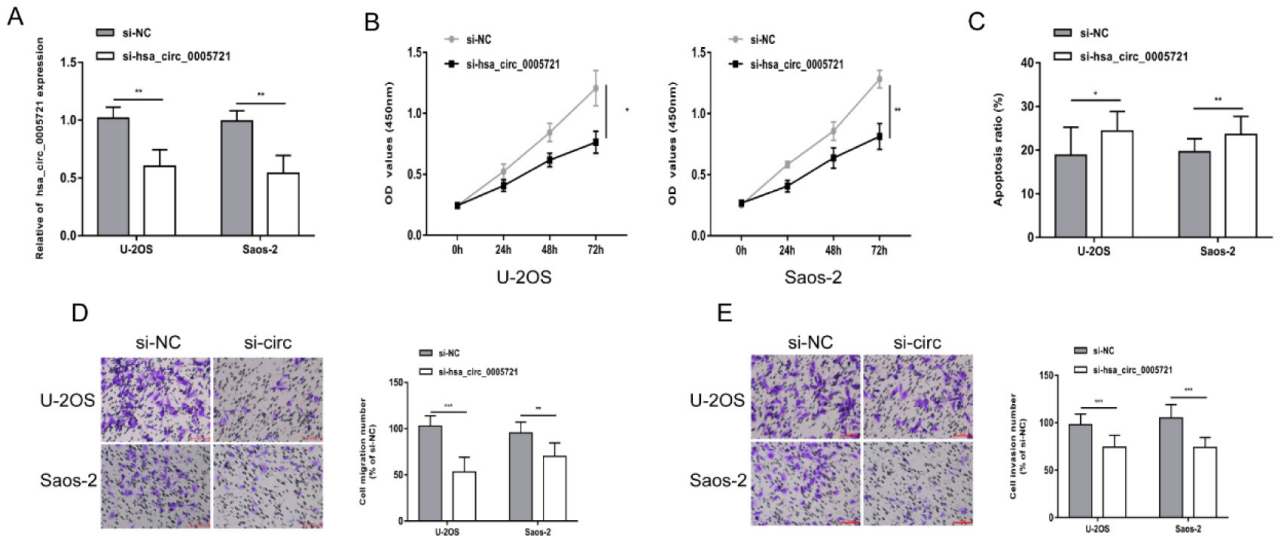

Figure 2. Knockdown of hsa_circ_0005721 suppressed proliferative, migratory and invasive abilities of osteosarcoma cells, but stimulated cell apoptosis. **A:** Intervention efficacy of si-hsa_circ_0005721 in U-2OS and Saos-2 cells. **B:** Viability in U-2OS and Saos-2 cells intervened by hsa_circ_0005721. **C:** Apoptotic rate in U-2OS and Saos-2 cells intervened by hsa_circ_0005721. **D:** Migration in U-2OS and Saos-2 cells intervened by hsa_circ_0005721 (200×). **E:** Invasion in U-2OS and Saos-2 cells intervened by hsa_circ_0005721 (200×). *p<0.05; **p<0.01; ***p<0.001.

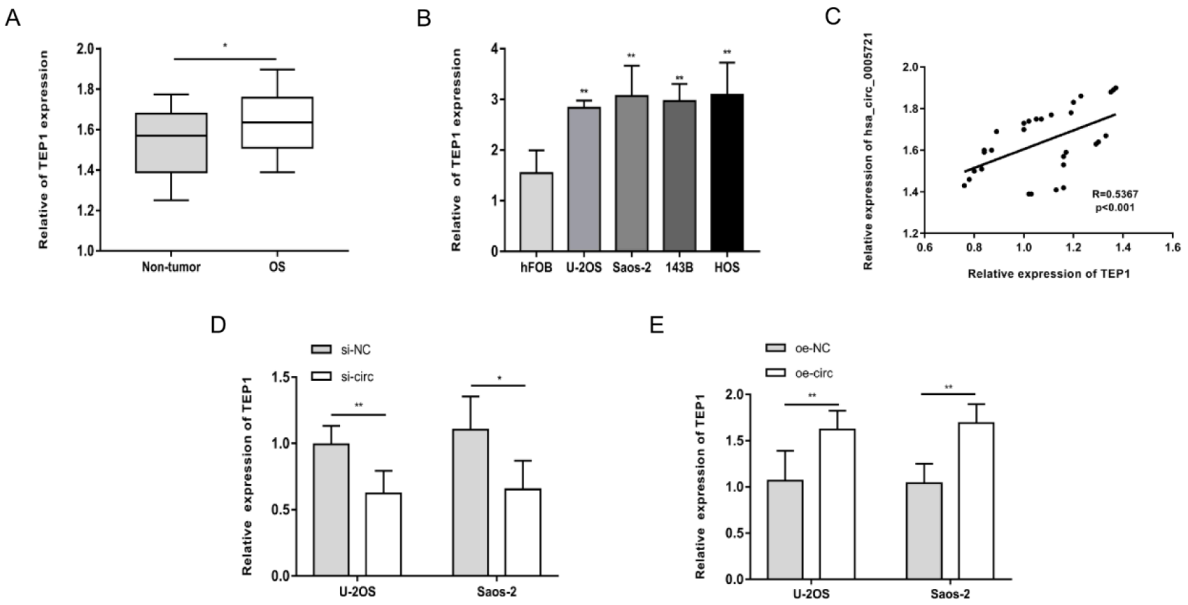

Figure 3. hsa_circ_0005721 upregulated TEP1. **A:** TEP1 levels in osteosarcoma and non-tumor tissues. **B:** TEP1 levels in osteosarcoma cell lines. **C:** A positive correlation between TEP1 and hsa_circ_0005721. **D:** TEP1 level in U-2OS and Saos-2 cells intervened by hsa_circ_0005721. **E:** TEP1 level in U-2OS and Saos-2 cells overexpressing hsa_circ_0005721. *p<0.05; **p<0.01.

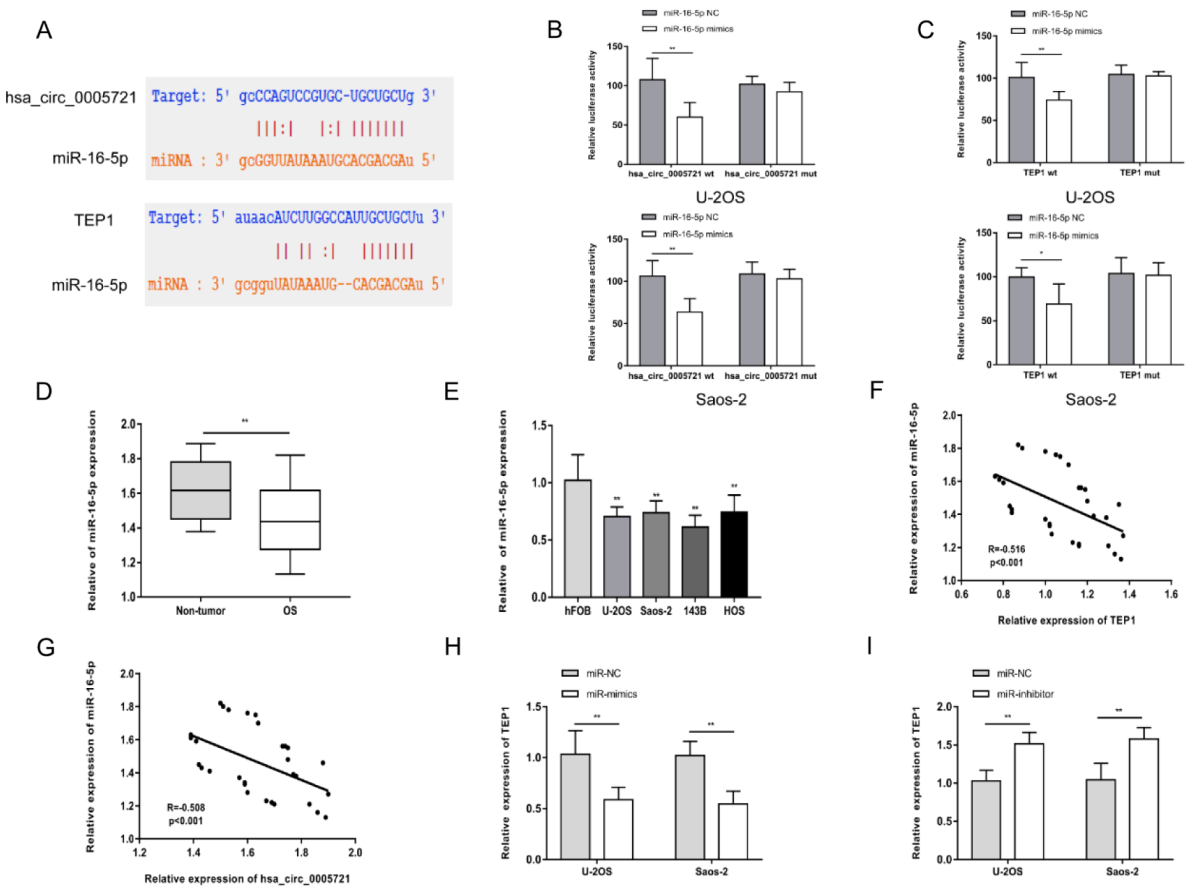

Figure 4. hsa_circ_0005721 upregulated TEP1 by competitively binding miR-16-5p. **A:** Binding sites in miR-16-5p. **B:** Direct binding between miR-16-5p and hsa_circ_0005721. **C:** Direct binding between miR-16-5p and TEP1. **D:** MiR-16-5p levels in osteosarcoma and non-tumor tissues. **E:** MiR-16-5p levels in osteosarcoma cell lines. **F:** A negative correlation between miR-16-5p and TEP1. **G:** A negative correlation between miR-16-5p and hsa_circ_0005721. **H:** TEP1 level in U-2OS and Saos-2 cells overexpressing hsa_circ_0005721. **I:** TEP1 level in U-2OS and Saos-2 cells intervened by hsa_circ_0005721. *p<0.05; **p<0.01.

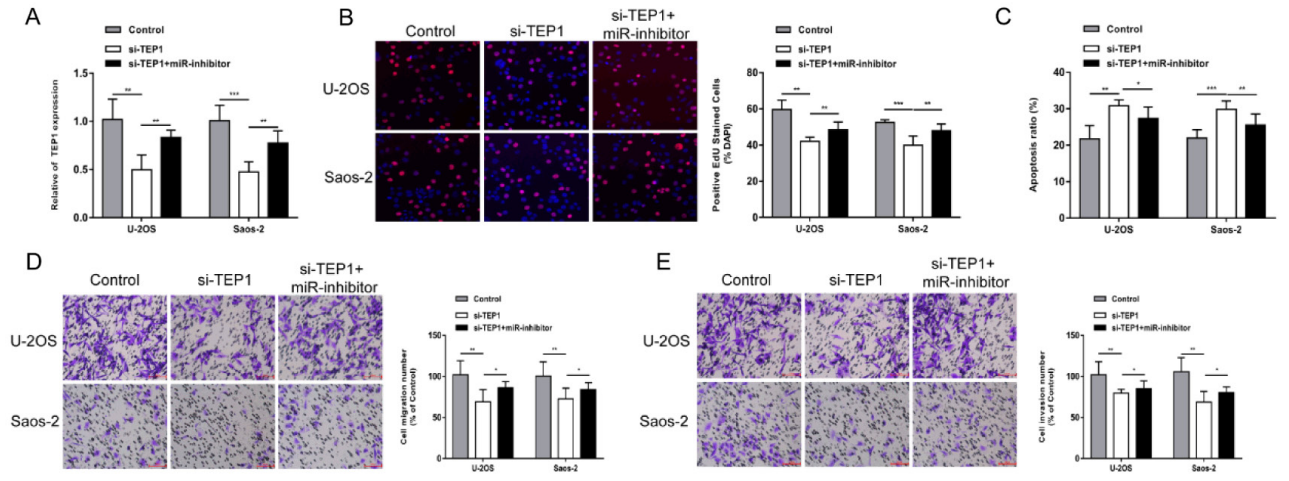

Figure 5. MiR-16-5p was responsible for the role of TEP1 in osteosarcoma development. **A:** TEP1 levels in U-2OS and Saos-2 cells intervened by TEP1 and miR-16-5p. **B:** EdU-positive rate in U-2OS and Saos-2 cells intervened by TEP1 and miR-16-5p. **C:** Apoptotic rate in U-2OS and Saos-2 cells intervened by TEP1 and miR-16-5p. **D:** Migration in U-2OS and Saos-2 cells intervened by TEP1 and miR-16-5p (200×). **E:** Invasion in U-2OS and Saos-2 cells intervened by TEP1 and miR-16-5p (200×). *p<0.05; **p<0.01; ***p<0.001.

revealed, knockdown of hsa_circ_0005721 reduced viability in osteosarcoma cells (Figure 2B). On the contrary, apoptotic rate increased by transfection of si-hsa_circ_0005721 (Figure 2C). Transwell assay uncovered that knockdown of hsa_circ_0005721 not only reduced migratory rate, but also invasive rate of osteosarcoma cells (Figure 2D, 2E).

hsa_circ_0005721 upregulated TEP1

Recent studies indicated that circRNAs display their functions by upregulating corresponding linear transcripts. TEP1, the linear transcript of hsa_circ_0005721, was upregulated in osteosarcoma tissues and cell lines (Figure 3A, 3B). It was positively correlated to hsa_circ_0005721 level in osteosarcoma tissues (Figure 3C). As expected, TEP1 was downregulated by transfection of si-hsa_circ_0005721 in U-2OS and Saos-2 cells, which was upregulated in osteosarcoma cells overexpressing hsa_circ_0005721 (Figure 3D, 3E).

hsa_circ_0005721 upregulated TEP1 by competitively binding miR-16-5p

We thereafter explored how hsa_circ_0005721 upregulated TEP1. As predicted in Starbase, miR-16-5p 3'UTR contained binding sequences that paired to sequences of both hsa_circ_0005721 and TEP1 (Figure 4A). Dual-luciferase reporter assay verified that miR-16-5p could bind hsa_circ_0005721 and TEP1 (Figure 4B, 4C). Downregulated miR-16-5p was detected in osteosarcoma tissues and cell lines (Figure 4D, 4E). Moreover, its level was negatively correlated to hsa_circ_0005721 and TEP1 levels (Figure 4F, 4G). TEP1 level in osteosarcoma cells was negatively regulated by miR-16-5p (Figure 4H, 4I).

MiR-16-5p was responsible for the role of TEP1 in osteosarcoma development

In U-2OS and Saos-2 cells transfected with si-TEP1, downregulated TEP1 was further elevated by co-knockdown of miR-16-5p (Figure 5A). Proliferative, migratory and invasive rates of osteosarcoma cells were reduced by knockdown of TEP1, which were partially abolished by silenced miR-16-5p. Conversely, the apoptotic rate of osteosarcoma cells increased by transfection of si-TEP1, and it was reduced after co-transfection of miR-16-5p inhibitor (Figure 5B-5E).

Discussion

CircRNAs are resistant to exonucleases because of the unique closed loop structure [13]. They can be classified into exonic circRNAs, circular intronic RNAs, retained-intron circRNAs, intronic circRNAs and intergenic circRNAs [14]. Owing to the tissue- and sequence-specificity, as well as abundant expressions in the body fluids, circRNAs are considered as promising biomarkers. Previous studies have demonstrated the vital function of circRNAs in osteosarcoma development [8]. It is reported that circTUBGCP3 stimulates proliferation, migration and survival of osteosarcoma cells by binding miR-30b [15]. Highly expressed hsa_circ_0136666 initiates osteosarcoma by activating the miR-593-3p / ZEB2 axis and predicts a poor prognosis [16]. The pathogenesis of osteosarcoma

may be related to circ_ARF3, which upregulates CDK6 by competitively binding miR-1299 [17].

hsa_circ_0005721 is discovered to be upregulated in osteosarcoma. However, its biological functions in affecting osteosarcoma development are unclear. Our results showed that hsa_circ_0005721 was remarkably upregulated in osteosarcoma tissues and cells. *In vitro* knockdown of hsa_circ_0005721 attenuated proliferative, migratory and invasive abilities of osteosarcoma, but stimulated cell apoptosis.

TEP1 level was enhanced by hsa_circ_0005721, which was highly expressed in osteosarcoma samples. Previously, the role of TEP1 has emerged in prostate cancer, bladder cancer and breast cancer [18-20]. Here, we speculated that hsa_circ_0005721 upregulated TEP1 by competitively binding a target miRNA (miR). MiR-16-5p was identified to bind both hsa_circ_0005721 and TEP1 in the 3'UTR. It is a cancer-associated miRNA involved in several types of human cancers [21-24]. In osteosarcoma cells intervened by TEP1, the declined proliferative, migratory and invasive rates could be reversed by knockdown of miR-16-5p. Increased apoptotic rate in osteosarcoma cells transfected with si-TEP1 was further reduced by silence of miR-16-5p. It is suggested that the carcinogenic role of hsa_circ_0005721 in mediating proliferative, metastatic and apoptotic abilities of osteosarcoma cells was achieved by upregulating TEP1 through competitively binding miR-16-5p. Our results provide a new idea for clinical management of osteosarcoma, although the experimental data should be further validated in an animal model.

Conclusions

This study for the first time identified the upregulated hsa_circ_0005721 in osteosarcoma, which triggers the malignant development of osteosarcoma by upregulating the linear transcript TEP1.

Conflict of interests

The authors declare no conflict of interests.

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