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Circular RNA circNASP modulates the malignant behaviors in osteosarcoma via miR-1253/FOXF1 pathway

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ABSTRACT

Increasing evidences demonstrate that circular RNAs (circRNAs) serve as essential regulators in various human diseases, including cancer. However, the study on circRNA function in osteosarcoma (OS) is just emerging. In the present study, we screened out a novel circRNA termed circNASP which was significantly upregulated in OS tissues compared to adjacent normal tissues. We found that circNASP knockdown dramatically inhibited the proliferation, cell cycle progression and invasion of OS cells. Moreover, we showed that circNASP expression was positively correlated with tumor size and metastasis in OS patients. In terms of mechanism, we found that circNASP acts as a sponge of miR-1253 targeting FOXF1 in OS cells. By inhibiting miR-1253 availability, circNASP promoted FOXF1 expression. Rescue assays indicated that inhibition of miR-1253 could significantly reverse the effects of circNASP knockdown on OS cell proliferation and invasion while restoration of FOXF1 rescued the proliferation and invasion of OS cells transfected with miR-1253 mimics. Taken together, our findings demonstrated that circNASP contributes to malignant behaviors of OS cells by miR-1253/FOXF1 pathway, which suggested circNASP/miR-1253/FOXF1axis might be a potential therapeutic target.

1. Introduction

Osteosarcoma (OS) is the most prevalent primary bona tumor among adolescents and adults [1]. OS is characterized with high metastatic potential and rapid progression [2]. Thus OS contributes to a large amount of cancer-associated deaths worldwide every year [3]. Although some advances achieved on therapeutic strategies such as radical surgery and neoadjuvant chemotherapy, the outcomes of OS clinical prognosis still remain poor [4]. The 5-year survival is 60%–70% in OS patients even without tumor metastasis [5]. Hence, there is an urgent requirement to investigate the molecular mechanism of OS occurrence and progression, which will benefit for the development of novel effective therapeutic targets.

Circular RNA (circRNA) belongs to a class of endogenously expressed noncoding RNA (ncRNA), which is characterized with a covalently closed loop structure [6]. Most circRNA is conserved and expressed in a tissue- or cell type-specific manner [7]. As the development of sequencing technology, more and more circRNAs are identified in various tissues. Increasing evidences indicate that circRNA exerts very important functions in a diversity of physiological and pathological processes, such as cell survival, proliferation and invasion [8,9]. It has been observed that circRNAs are often aberrant expressed and involved in cancer progression [10]. For example, hsa_circ_0000977 is overexpressed and promotes pancreatic ductal adenocarcinoma progression [11]. Circular RNA circ-ITCH is involved in bladder cancer progression via sponging miR-17/miR-224 [12]. In OS, several circRNAs were identified as oncogenes or tumor suppressors, such as hsa_circ_0001564 [13] and hsa_circ_0009910 [14]. Nevertheless, the functions of most circRNAs in OS remain largely unknown.

In the present study, we screened out a highly expressed circRNA termed circNASP (ID: hsa_circ_0092340) in OS tissues by bioinformatics method. circNASP is derived from NASP. Through functional experiments, we found that circNASP knockdown significantly inhibited OS cell proliferation, arrested cell cycle progression and reduced the invasion. Mechanistically, we showed that circNASP serves as a sponge of miR-1253 that targets FOXF1.

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Via inhibiting miR-1253 availability, circNASP enhances FOXF1 expression in OS cells. Through a series of rescue assays, we demonstrated that circNASP promotes OS cell proliferation and invasion by regulating miR-1253/FOXF1 pathway. In summary, our findings identified the essential role of circNASP/miR-1253/FOXF1 regulatory loop in OS progression.

2. Materials and methods

2.1. Patient samples

A total of 39 freshly frozen OS samples and paired adjacent normal tissue samples were collected from patients who underwent surgical resection at The Fifth Affiliated Hospital of Wenzhou Medical University. The clinical parameters of OS patients are listed in Table 1. Written informed consent was obtained from all patients who participated in this study. This study was approved by the ethics committee of The Fifth Affiliated Hospital of Wenzhou 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.

2.2. Cell culture and transfection

The human osteosarcoma cell lines (143B and MG63) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin/streptomycin solution. All the cell lines were cultured at 37 °C in a humidified incubator containing 5% CO_2.

For knockdown, specific siRNAs were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences were provided by GenePharma and shown as follows: si-circNASP, 5′-GATTATGGAATCTCTGTCTCT-3'. Cell transfection and co-transfection were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Cell proliferation assay

MTT assay was used to evaluate cell proliferation. Transfected cells were seeded into 96-well plates (2000 cells per well) and cultured for 1, 2, 3, and 4 days. MTT (5 mg/ml) was added to each well for 4 h at 37 °C. Then, the medium was removed, and the reaction was stopped by 200 μl DMSO. Absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA).

Table 1
Clinical parameters of OS patients enrolled in this study.

Pathological characteristics	circNASP expression		*p* value
	low	high	
All cases	20	19	
Age			0.731
<25	13	14	
≥25	7	5	
Gender			0.748
Male	11	12	
Female	9	7	
Enneking stage			0.025
I + IIA	13	5	
IIB/III	7	14	
Size (cm)			0.026
<5	14	6	
≥5	6	13	
Lung metastasis			0.009
Yes	7	15	
No	13	4	

2.4. Transwell invasion assay

Twenty-four-well plates containing transwell chambers with 8-μm pore size polycarbonate membrane inserts (Corning Incorporated, Corning, NY, USA) were used for cell invasion assay. For invasion assay, transwell membranes were pre-coated with Matrigel (BD Biosciences), 5×10^4 cells in serum-free RPMI DMEM medium were seeded in each well. After 24 h of incubation, cotton swabs were used to remove the cells inside the upper chamber, while the cells on the other side of the membrane were fixed and stained with 0.5% crystal violet solution. Five random fields were counted for each well.

2.5. RNA isolation and qRT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen), followed by removal of DNA with the TurboDNase Kit (Ambion). Quantification of extracted RNA was performed using NanoDrop. cDNA synthesis was performed using PrimeScriptRT reagent KIT (Takara) using 1000 ng of total RNA. QRT-PCR was performed using the SYBR Select Master Mix (Applied Biosystems) on an ABI 7900 system (Applied Biosystem). The level of GAPDH was used as a control. The Ct value was calculated based on the ΔΔCt-method. Fold change of gene expression was expressed as $2^{-\Delta\Delta Ct}$ method.

2.6. Target prediction

Circular RNA Interactome (https://circinteractome.nia.nih.gov) was used to predict the potential miRNAs targeting circNASP. The potential targets of miR-1253 were predicted using the TargetScan (http://www.targetscan.org), microRNA (http://www.microrna.org/) and miRDB (http://www.mirdb.org). The common genes of these algorithms were selected for further analysis.

2.7. Cell cycle analysis

After transfection, cells were washed in PBS and fixed in 70% cold ethanol for 2 h and the cell cycle analysis was performed by Cell Cycle Analysis Kit (Lianke, China) according to the manufacturer's instructions. Flow cytometry (FACScan, BD Biosciences, USA) was performed to analyze cell cycle.

2.8. Luciferase reporter assay

OS cells were seeded into a 24-well plate. Cells were co-transfected with wild-type or mutated circNASP or FOXF 3′-UTR reporter plasmids, and with miR-1253 mimics or negative controls. Luciferase assays were conducted 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

2.9. Statistical analysis

All experiments were conducted at least three times. Data were displayed as mean ± SD. Analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL, USA). Student's t-test was used for differences between two groups and one-way ANOVA for multiple comparisons. A p-value < 0.05 was considered statistically significant.

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