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Knockdown of HuR represses osteosarcoma cells migration, invasion and stemness through inhibition of YAP activation and increases susceptibility to chemotherapeutic agents

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ABSTRACT

This study aims to explore the roles and related mechanisms of HuR in osteosarcoma (OS) cells migration, invasion, stemness and chemotherapeutical sensitivity. Here, we found that HuR exhibited higher level in OS tissues compared with the adjacent normal tissues. Knockdown of HuR with lentivirus infection suppressed OS cells migration and invasion, and thus the epithelial-mesenchymal transition (EMT) process. Additionally, HuR knockdown inhibited OS cells stemness. Mechanistically, YAP was identified as a direct target of HuR in OS cells, and HuR knockdown decreased its expression. Moreover, YAP transcriptional activity was attenuated by HuR knockdown, and RNA immunization co-precipitation (RIP) assay showed that HuR directly bound with YAP. Importantly, YAP overexpression rescued the inhibition of HuR knockdown on OS cells migration, invasion and stemness. Furthermore, HuR knockdown enhanced adriamycin sensitivity in OS cells, this effect was attenuated by YAP overexpression too. Importantly, HuR and YAP expression was positively correlated in OS tissues. Therefore, HuR acts as a tumor promoter by enhancing YAP expression in OS cells.

1. Introduction

Osteosarcoma (OS) is a common bone primary malignancy with strong invasiveness and bad prognosis [1]. Since the early diagnosis rate is low, the treatment method is relatively single, and the slow development of basal and clinical studies, the prognosis of patients with OS was not improved obviously, the 5-year survival rate was less than 30% [2]. Thus, it is necessary to elucidate the underlying mechanisms contributing to OS development, which could facilitate the development of OS diagnosis or treatment.

HuR, also known as ELAVL1, is an RNA binding protein (RBP) which could modulate various genes mRNA stability and expression via directly binding to them, like p53, TNF, and VEGF [3]. HuR expression has been identified to be increased in various tumors, such as breast cancer [4], lung cancer [5] and pancreatic cancer [6]. Additionally, myocardial knockdown of HuR attenuates post-MI inflammatory response and left ventricular dysfunction [7], and HuR could regulate nuclear import of protein [8]. However, HuR roles and related mechanisms in OS progression, especially in OS cell invasion and stemness, have never been investigated.

Hippo signaling pathway was firstly identified in drosophila by Huang et al, which could regulate cell proliferation, migration and apoptosis [9]. As the key executor, YAP could modulate various downstream effectors expression which could promote tumor progression when Hippo pathway is inactivated [10]. Recent studies have shown that YAP transcription activity is positively correlated with tumor migration, EMT and stemness [11]. Thus, YAP has been identified as a potential target for treating cancer and verteporfin, an inhibitor of YAP attenuated the acquired resistance to BRAF inhibitor in melanoma stem cells. [12], and verteporfin could induce cell apoptosis in melanoma stem cells [13]. Recent studies have indicated that Hippo/YAP signaling pathway is involved in OS chemoresistance [14], and YAP could be regarded as a potential prognostic value in conventional OS [15]. However, the detailed mechanisms contributing to the modulation on YAP transcription activity in OS progression are still unclear.

Here, we detected HuR expression level in OS and adjacent normal tissues, and found that HuR expression was significantly increased in OS tissues compared with that in adjacent normal tissues. Lentivirus infection was performed to construct OS cells with HuR stable knockdown. We found that HuR knockdown could block OS cells migration, invasion, EMT and stemness. Moreover, we showed that HuR could directly bind with YAP, and increase YAP mRNA stability, expression and transcriptional activity. Furthermore, the inhibition of HuR knockdown on OS cells progression could be rescued by YAP

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overexpression. Notably, HuR and YAP expression exhibited a positive correlation in OS tissues. Therefore, our results suggest that HuR could enhance OS cells migration, invasion and stemness via regulating YAP activity.

2. Material and methods

2.1. OS clinical samples

A total of 41 OS and normal adjacent tissue specimens were obtained from the TongRen Hospital from October 2014 to June 2017. All patients were not received chemotherapy. The Ethics Committees of TongRen Hospital approved this study, and written informed consent from all patients was obtained before operation. Additionally, paired mRNA profiling data were downloaded from the TCGA data portal (GSE42352, http://cancergenome.nih.gov). The dataset from the Tumor Osteosarcoma public (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi), which includes 127 OS samples, was obtained as a validation set. The R2 platform was used to analyze the microarray (http://r2.amc.nl).

2.2. Cell culture and plasmid acquisition

HEK293T cells and Human OS cell lines SaOS2, 143B, U2OS and MG63 were purchased from the Chinese Academy of Sciences Cell Bank, and were cultured in Dulbecco's Minimum Essential Medium (DMEM) medium (HEK293T, MG63 and U2OS) and 1640 medium (SaOS2 and 143B) which contained 10% fetal bovine serum (FBS, Life Technologies) with 5% CO_2 at 37 °C. 8xGTIIC-luciferase (Plasmid # 34615), a YAP-responsive synthetic promoter driving luciferase expression plasmid, and pGAMA-YAP (Plasmid #74942), a lentiviral overexpression vector for YAP were purchased from addgene.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells with different treatment by TRIzol™ Reagent (Cat # 15596026, ThermoFisher Scientific) following the manufacturer's recommendation, and then was reverse transcribed into cDNA (complementary DNA) using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Cat # K1641, ThermoFisher Scientific) according to the standard procedure. mRNA expression level was examined with SYBR Green Real-Time PCR Master Mixes (Cat # 4385617, ThermoFisher Scientific) on an ABI Prism 7500 Detection System (Applied Biosystems, Inc). GAPDH was used as an internal reference. The qRT-PCR primers were indicated in Supplementary Table S1. $2^{-\triangle\triangle ct}$ method was performed to analyze the relative gene expression levels.

2.4. Western blot

Cells with different treatment were washed with cold-PBS following by adding cell lysis buffer with protease inhibitor. Protein concentration was determined by BCA protein concentration assay kit (Cat#P0009, Beyotime, China). Equal amount of protein with loading buffer was separated with SDA-PAGE, and transferred onto PVDF membranes. Then the membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1.5 h at room temperature, and incubated with corresponding primary antibodies against HuR (ab136542, Abcam), OCT4 (ab181557, Abcam), Nanog (ab109250, Abcam), Caspase-3 (ab18347, Abcam), cleaved-caspase3 (ab32042, Abcam), PARP (ab32138, Abcam), cleved-PARP (ab32064, Abcam), E-cadherin (ab1416, Abcam) and Vimentin (ab8978, Abcam) at 4 °C overnight, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Enhanced chemiluminescence (ECL) kit (Vazyme Biotech Co.,Ltd, Nanjing, China) was used to develop image in Tanon 5200 machine (Tanon, Shanghai, China).

2.5. RNA immunization co-precipitation (RIP) assay

The detailed procedure was mentioned in the previous study [16]. SaOS2 and SaOS2$^{HuR-shRNA}$ were lysed with 100 U/ml RNase inhibitor (Sigma) and 25 mM Tris-HCl buffer (pH 7.5), and incubated with protein-A Sepharose beads pre-coated with 3 μg anti-HuR antibody (Abcam) or control rabbit IgG for 1.5 h at 4 °C. The RNA-protein complexes were pulled-down by protein A/G agarose beads and RNA was extracted with Trizol, followed by detecting YAP level with qRT-PCR.

2.6. Lentivirus plasmid and stable infection cell lines construction

Lentiviral shRNA against human HuR (Lenti-siHuR) and a scramble non-targeting shRNA were purchased from Santa Cruz Biotechnology (sc35619), which were inserted into pLKO.1 (Plasmid # 8453). The lentivirus was packaged in HEK293T cells by co-transfection with pCMV-dR8.2 (Plasmid # 8455) and pMD2.G (Plasmid # 12,259) constructs which were purchased from Addgene. The transfection efficiency is more than 95% for reproducible experiments. After 72 h transfection, supernatants were collected and centrifuged to remove cell debris. SaOS2 cells were infected with the virus with 2 μg/ml Polybrene. After two rounds infection, the infected cells were selected with puromycin (Sigma, 2 μg/ml) for 2 weeks. The infection efficiency was verified by qRT-PCR and western blot assays. SaOS2 cells with HuR stable knockdown was referred as SaOS2$^{HuR-shRNA}$.

2.7. Immunofluorescent assay

Cells were cultured on glass bottom plates, washed with PBS, and then fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100 for 15 min and blocked with 5% BSA in PBS for 1.5 h at room temperature. Then cells were incubated with anti-YAP antibody overnight at 4 °C, and washed with PBS and incubated with FITC-conjugated secondary antibody (Cat # F-2765, ThermoFisher Scientific) for 1 h at room temperature, and washed with PBS for three times again and observed under the confocal microscopy.

2.8. Transfection

Lipofectamine^{TM2000} (Invitrogen) was used for transfection. The detailed procedure was referred to the manufacturer's instruction.

2.9. Luciferase reporter assay

Luciferase reporter assay was performed to detect YAP transcriptional activity in cells with different treatment. Briefly, 8xGTIIC-luciferase plasmid was co-transfected into SaOS2 or SaOS2 $^{HuR-shRNA}$ with β-gal (Ambion, USA) plasmid using Lipofectamine^{TM2000}. After 72 h, the luciferase activity was examined, transfection efficiency was determined using a Luciferase Reporter Assay Kit (cat. no. K801-200, BioVision, Inc., Milpitas, CA, USA). β-gal activity was determined using a β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (cat. no. E2000, Promega Corporation) according to the manufacturer's protocols. β-gal activity was used as a normalization control for luciferase activity.

2.10. Transwell migration and invasion assay

The detailed procedure was referred to the previous study [17].

2.11. Cell sphere formation assay

Cells with different treatment were cultured in ultra-low attachment 24-well plates (Corning, Union City, CA) at 500 cells/well with