Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Histone methyltransferase SETD2 regulates osteosarcoma cell growth and chemosensitivity by suppressing Wnt/β-catenin signaling

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ARTICLE INFO

Article history:
Received 22 May 2018
Accepted 26 May 2018
Available online xxx

Keywords:
SETD2
Osteosarcoma
Chemosensitivity
Cancer stem cell
Wnt/β-catenin signaling

ABSTRACT

SETD2 is a histone methyltransferase that catalyzes the trimethylation of lysine 36 on histone 3. SETD2 is frequently found to be mutated or deleted in a variety of human tumors, whereas the role of SETD2 in oncogenesis of osteosarcoma has never been defined. Here in our study, we uncovered that SETD2 regulates tumor growth and chemosensitivity of osteosarcoma. Overexpression of SETD2 significantly inhibited osteosarcoma cell growth *in vitro* and *in vivo*. Moreover, SETD2 significantly enhanced cisplatin-induced apoptosis in osteosarcoma cells and inhibited cancer stem cell properties in OS cells. SETD2 regulates Wnt/β-catenin signaling and its downstream gene c-myc, CD133 and cyclin D1. We further revealed that SETD2 upregulates H3K36me3 modification in GSK3B loci and promotes its transcription, which lead to β-catenin degradation. Together, our study delineates SETD2 function in osteosarcoma as an important regulator of Wnt/β-catenin signaling, and suggests SETD2 as a novel target in diagnosis and combined chemotherapy of osteosarcoma.

1. Introduction

Osteosarcoma (OS) is the most common primary bone malignant tumor in children and adolescents [1,2]. Current treatments for osteosarcoma usually include surgery dissection combined with systemic chemotherapy, and introduction of adjuvant chemotherapies has improved the long-term survival rate impressively [3,4]. Cisplatin is a first-line chemotherapy drug commonly used for clinical treatment of osteosarcoma [4]. When administered intravenously in OS patients, cisplatin demonstrated a response rate of approximately 30%, which means a significant proportion of OS patients are intrinsically resistant to cisplatin [5]. However, the underlying mechanisms of cisplatin resistance in these OS patients are still unclear.

The SETD2 protein is a histone methyltransferase that specifically catalyzes the trimethylation of lysine 36 on histone 3(H3K36me3) [6]. SETD2 is implicated in DNA repair, chromosome segregation and RNA splicing process [7–9]. And SETD2 mutation or deletion are common in human tumors, including clear cell renal cell carcinoma (about 20%), and in approximately 5%–10% bladder cancer, lung cancer, melanoma, and endometrial cancer [10,11]. Research reported that SETD2 modulates alternative splicing to inhibit intestinal tumorigenesis in mice model [12]. Moreover, low expression of SETD2 was correlated with poor prognosis in gastric cancer [13]. Nevertheless, its role in tumorigenesis and target signaling pathways regulated remains largely undetermined.

Here in our study, low expression of SETD2 was observed in OS tissue, and we revealed that SETD2 suppresses OS cell growth in *vitro and in vivo*. SETD2 overexpression enhances the cisplatin sensitivity in OS cells by regulating Wnt/β-catenin signaling and cancer stem cell characteristics. Thus, our results highlight SETD2 may serve as a target for the treatment of human osteosarcoma.

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https://doi.org/10.1016/j.bbrc.2018.05.176
0006-291X/

2. Materials and methods

2.1. Human specimen analysis

OS specimens and paired paracarcinoma normal tissue were obtained from patients who underwent surgical treatment from Changzheng Hospital. The study was approved by by Research Ethics Committee of Changzheng Hospital.

2.2. RNA extraction and RT-qPCR

Total RNA was extracted by RNAiso Plus reagent (Takara), and 1.5 μg of total RNA from cultured cells was reverse transcribed using the PrimeScriptP RT Reagent Kit (Takara) according to manufacturer's instructions. All quantitative real-time reverse transcription polymerase chain reactions (RT-qPCRs) were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). The amplified transcript level of each specific gene was normalized to that of Actin. The primers used were listed in Table S1.

2.3. Western Blot

Total protein of cell lines was extracted with RIPA lysis buffer (Beyotime; P0013B). Protein concentration was then quantified using BCA assay. 30 μg of protein lysate was separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore), immunoblotted with primary antibodies and horseradish peroxidase–conjugated secondary antibodies in PBST (phosphate buffered solution with 0.5% Tween-20) with 5% albumin bovine. The following antibodies were used in this study: anti-Actin (Abways; ab00035), anti-SETD2 (Santa Cruz Biotechnology; sc-99451), anti-phospho AKT (Cell Signaling Technology; 4058), anti-Notch1 (Abways; CY2762), anti-β-catenin (Abways; CY3523).

2.4. CCK-8 assay

Cell viability was assessed using Cell Counting Kit-8 (Beyotime; C0038) according to the manufacturer's protocol. 2000 cells were seeded into one well of a 96-well plate, add 10ul CCK-8 solution per well and incubating for 2 h, A450 was read at indicated time points.

2.5. Colony formation assay

1000 cells were seeded in 6-well plates. Cells were stained with Crystal Violet Staining Solution (Beyotime; C0121) after ten days. And colonies contained ≥20 cells were counted.

2.6. Flow cytometry

Cells were seeded in a six-well plate, and after 48 h post treatment, the cells were harvested for Flow Cytometry analysis. Apoptotic cells were analyzed with 7AAD (BD Biosciences; 559925) and Annexin-V-PE (BD Biosciences; 556421), cells were stained for 30 min before performing flow cytometry. Data was analyzed with FlowJo 7.6 (Treestar Inc).

2.7. In vivo tumorigenesis assay

Lentiviral-transduced cells (2×10^6 143 B cells) in 0.2 mL of medium were subcutaneously injected into the right armpits of the nude mice. Tumor size was evaluated every 7 days. Tumor volume was calculated by formula V = 1/2 (a*b*b), where *a* denotes the major tumor axis and *b* the minor tumor axis. The mice were sacrificed 3 weeks after injection, and the tumor weight was quantified. All animal experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

2.8. Cell lines, plasmids, reagents, and cell transduction

Human OS cell lines 143 B and HOS were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle medium and containing 10% fetal calf serum. Transfection of cell lines was performed by using FuGENE® HD Transfection Reagent (Promega) according to the manufacturer's instructions. To knock down human SETD2 gene, gene-specific short hairpin RNA was cloned to pLVX-shRNA1 plasmid (Clontech). SETD2-KD1: GCCCTTCGCAGTGTTTGTCT, SETD2-KD2: GCTCAGAGTTAACGTTTGA, and a scramble sequence was negative control. Then, lentivirus was packaged using psPAX2 and pMD2G, the 3-plasmid system. To obtain the stable cell lines, lentivirus supernatant was added to 143 B and HOS cells, followed by screening with 2 mg/mL puromycin for 2 weeks.

2.9. Sphere formation assay

Cells were seeded with a density of 2000 cells/well in six-well ultra-low attachment plates (Corning; 3261) in DMEM/F12 (Gibco) culture medium supplemented with 10 ng/ml human EGF (PeproTech; AF-100-15) and 10 ng/ml human bFGF (PeproTech; 100-18 B). After culture for 10 days, 143 B cells efficiently formed spheres. Then spheres larger than 100uM were quantitated under microscopy. The experiments were performed in triplicate.

2.10. ChIP-qPCR analysis

1×10^6 control and SETD2-OE 143 B cells were crosslinked and lysed. UCD-300 (Bioruptor) was used to shear crosslinked DNA to ~200–1000 base pairs in length. ChIP was performed according to manufacturer's instructions of Chromatin Immunoprecipitation Kit (17–371; Millipore), followed by qPCR for quantification of ChIP-enriched DNA. The antibodies used for ChIP were anti-H3K36me3 (Active Motif; 61021) and normal mouse IgG (Active Motif; 12–371 B). Primer sequences are listed in Table S2.

2.11. Statistical analysis

Data in the figures are presented as mean ± SD. Analysis were performed with statistical software SPSS 22.0 and statistical significance was determined by 2-tailed Student's *t*-test or 1-way ANOVA. For all statistical tests, a P value of <0.05 was considered to be statistically significant.

3. Results

3.1. SETD2 expression is downregulated in human OS

To explore the role of SETD2 in OS, we first assessed SETD2 expression in OS patients. Immunohistochemistry analyses revealed a significantly lower SETD2 expression in osteosarcoma tissue compared with paired normal biopsies (Fig. 1A and B). RT-qPCR results also showed that SETD2 expression was significantly lower in OS tissues compared with in their normal counterparts (Fig. 1C). These data suggested SETD2 as a potential biomarker for OS and imply a potential role of SETD2 in OS tumorigenesis.

Please cite this article in press as: C. Jiang, et al., Histone methyltransferase SETD2 regulates osteosarcoma cell growth and chemosensitivity by suppressing Wnt/β-catenin signaling, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.176