Distinct roles of parafibromin in the extracellular environment, cytoplasm and nucleus of osteosarcoma cells

Jia-Jun Zhu, Yan Cui, Kai Cui, Xi Li, Zhi-Yu Zhang

Department of Orthopaedics, The 4th Affiliated Hospital of China Medical University, Shenyang, P. R. China

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Abstract: Osteosarcoma (OS) is the most common primary malignant bone tumor. Parafibromin-inactivating mutations have been reported in various malignancies. In this study, the effects and relevant mechanisms of ectopic parafibromin expression were identified in the extracellular environment, cytoplasm and nucleus of OS cells. Our results indicate that parafibromin located in the nucleus can induce apoptosis and G_1 phase arrest in OS cells. Parafibromin was found to suppress the MEK/ERK and PI3K/AKT signaling pathways, leading to activation of caspase 3 and caspase 9. Overall, these studies demonstrate the anti-tumor activity of parafibromin in the OS cell line, and provide insight into relevant mechanisms that may lead to novel treatments for OS.

Keywords: Parafibromin, osteosarcoma cell, proliferation, apoptosis, cell cycle

Introduction

Osteosarcoma, the most common primary malignant bone tumor, accounts for 5% of childhood cancers and represents the fifth most frequent tumor in young adults [1]. Better knowledge of oncogenic processes in osteosarcoma could lead to the development of new therapeutic approaches [2].

Parafibromin is encoded by the hyperparathyroidism 2 (HRPT2) gene located on chromosome 1q31, whose germline mutation leads to hereditary hyperparathyroidism-jaw tumor (HPT-JT) syndrome [3]. Parafibromin-inactivating mutations have been reported in various malignancies, such as parathyroid cancer [4], major renal cell tumor [5], lung cancer [6]. Previous studies have demonstrated that parafibromin overexpression could inhibit colony formation and proliferation, and induce cell cycle arrest in oral squamous cell carcinoma cell [7], colon carcinoma cell [8], and cervical cancer cell [9].

In our study, the effects and relevant mechanisms of ectopic parafibromin expression was examined in the extracellular environment, cytoplasm and nucleus of osteosarcoma cells.

Methods

Cell culture

The human osteosarcoma cell lines, MG-63 and HOS, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C with 5% CO_2.

Transfection

Parafibromin expression vector was constructed by PCR amplification of human CDC73 natural ORF mammalian expression plasmid (HG11656-UT, Sino Biological Inc, Beijing, China) utilizing the primers (Sangon Biotech, Shanghai, China): 5'-TTCAAGTCTATGGGGCGGAGG-3' (forward) and 5'-TACCGTCGACATGATTCTCCGAG-3' (reverse), and cloning into the mammalian expression vector pEGFP-C2 (Clontech Laboratories, Inc., Shanghai, China). Cells were transfected with pEGFP-C2-parafibromin plasmid at 70% conflu-
ence 20 to 24 h after seeding on dishes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. As the methods of Hahn et al. [10], site directed mutagenesis was carried out on pEGFP-C2-parafibromin to convert basic amino acids within each signal to neutral amino acids using the QuickChange II site directed mutagenesis kit (Stratagene, CA, USA).

**RT-PCR**

Cellular RNA was isolated using TRIzol reagent (Invitrogen). cDNA was then synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. PCR amplification of cellular cDNA was performed in 15 μl mixtures. RT-PCR conditions were: one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 72°C for 45 sec, 58°C for 45 sec, and final extension at 72°C for 10 min. Finally, products were resolved by 1% agarose gel electrophoresis, and visualized by ethidium bromide staining and a UV imaging system (UVP, Upland, CA, USA).

**Fluorescence**

Parafibromin expression was indicated by using EGFP. Cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China). Photographic images were taken using an Olympus CX71 fluorescence microscope (Olympus, Tokyo, Japan).

**Colony formation assay**

Cells (300 cells/well) were seeded in twenty-four-well plates. After 24 h, cells were treated with various concentrations of recombinant parafibromin protein (CSB-MP750783HU, CUSABIO® Biotech Inc., Wuhan, China) (0, 100, 200, 300, 400, or 500 ng/ml). Three days later, colonies were stained with 0.05% crystal violet (Beyotime) and counted in 4 to 5 random fields for each of the duplicate samples by using a microscope at 100 × magnification.

**Pharmacological agents**

IGF-1 is a PI3K/mTOR agonist described previously [11], and was purchased from R&D Systems. Cells transfected with pEGFP-C2-parafibromin plasmid were incubated with IGF-1 (100 ng/ml) for 24 hours.

**Apoptosis**

According to the manufacturer’s instructions (KeyGEN, Nanjing, China), cells were resuspended in 200 μl binding buffer containing Annexin V-FITC (0.5 μg/ml), and incubated at room temperature in the dark. After 20 min, 400 μl binding buffer was added and samples were immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson Medical Devices, Shanghai, China).

**Cell cycle**

Cells were harvested, and fixed with 70% ethanol at 4°C. After centrifugation (1500 × g for 5 min), supernatants were discarded, cellular DNA was stained with 10 μM propidium iodide (KeyGEN), and samples were analyzed by a FACSCalibur flow cytometer (Becton Dickinson).

**Western blot**

Protein extracts were resolved on SDS-PAGE, followed by electrotransfer to nitrocellulose membranes (Bio-Rad, Philadelphia, PA, USA). Following a blocking step in 5% milk in TBST, membranes were incubated with primary and secondary antibodies. Cell signaling-related proteins were probed using the following antibodies: parafibromin, Akt, phospho-Akt, PI3K, phospho-PI3K, MEK, phospho-MEK, ERK, and phospho-ERK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin (Santa Cruz) was used as an internal control. The reaction was followed by probing with peroxidase-coupled secondary antibodies, including anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG antibodies at dilutions ranging from 1:1000 to 1:2000 (ZS-BIO, Beijing, China), and binding results were visualized by enhanced chemiluminescence (Santa Cruz).

**Statistical analysis**

Differences between groups within experiments were examined by ANOVA test. Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS, Inc.) and significance was established at p < 0.05.
Results

Parafibromin-expressing OS cells

We investigated the consequence of exogenously expressed parafibromin expression in OS cells. MG-63 and HOS cells were transfected with pEGFP-C2-parafibromin (wild-type) or pEGFP-C2-parafibromin mutagenesis (mutagenesis), and parafibromin expression was measured by using RT-PCR and western blot. The results of RT-PCR and western blot analysis confirmed exogenous expression of parafibromin expression in OS cells after transfection (Figure 1). Fluorescence analysis showed wild-type parafibromin was located in the nucleus of OS cells and mutational parafibromin was located in cytoplasm of OS cells (Figure 1).

The distinct effects of parafibromin in extracellular environment, cytoplasm and nucleus of OS cells

Recombinant parafibromin and mutational parafibromin showed no anti-tumor effect on OS cells by using colony formation assay (Figure 2A and 2B, p > 0.05). However, wild-type parafibromin could inhibit proliferation of both MG-63 and HOS cells (Figure 2B, p < 0.05). Annexin V-FITC and PI double staining was used to detect apoptotic cells. The apoptotic ratio was 18-21 times higher in cells expressing wild-type parafibromin protein than that of recombinant parafibromin or mutational parafibromin treated cells (Figure 2C and 2D, p < 0.05). Cell cycle was examined by using PI staining. Wild-type parafibromin protein could increase the cell number in G1 phase, while no changes were found in recombinant parafibromin or mutational parafibromin treated cells (Figure 2E). IGF-1 is a PI3K/mTOR agonist commonly used to active the P13K/AKT signaling pathway [11]. In this study, we found that IGF-1 could reverse the anti-tumor effect of wild-type parafibromin in OS cells (Figure 2).

Wild-type parafibromin suppresses the PI3K/AKT and MEK/ERK signaling pathways in OS cells

Western blot was performed to identify the mechanism of apoptosis induced by wild-type parafibromin. While total levels of P13K, AKT, MEK, and ERK showed no changes, the levels of phospho-P13K, phospho-AKT, phospho-MEK, and phospho-ERK were observed to be significantly lower in wild-type parafibromin expressing cells (Figure 3). Following the inhibition of the MEK/ERK and PI3K/AKT pathways, activation of caspase 3 and caspase 9 was observed in wild-type parafibromin expressing cells (Figure 3). To test the hypothesis that wild-type parafibromin can induce apoptosis in OS cells by suppressing the P13K/AKT and MEK/ERK signaling pathways, IGF-1 was used to treat OS cells as a control. IGF-1 could up-regulated the levels of phospho-P13K, phospho-AKT, phospho-MEK, and phospho-ERK in parafibromin transfected OS cells (Figure 3). In combination, these results suggest that wild-type
Figure 2. Anti-tumor roles of parafibromin in vitro. A. Cell proliferation of OS cells treated with different concentrations of recombinant parafibromin was detected by using colony formation assay. B. Cell proliferation of OS cells treated with recombinant parafibromin, wild-type parafibromin and mutational parafibromin. C, D. Apoptotic ratio of cells were determined from Annexin-V/PI double-staining assays. The histogram indicates statistically significant results (p < 0.05). E. Cell cycle changes were determined by staining with PI. The red arrow indicates G1 phase arrest. Untreated: OS cells without treatment; Recombinant: OS cells treated with recombinant parafibromin; Wild-type: OS cells were transfected with pEGFP-C2-parafibromin plasmid; Mutagenesis: site directed mutagenesis was carried out on pEGFP-C2-parafibromin. IGF-1 was used as a positive control.
Parafibromin and osteosarcoma

Discussion

Downregulated expression of parafibromin protein promoted the pathogenesis, proliferation, differentiation, and mobility of many cancers [12-14]. Lin et al. [15] reported that overexpression of wild-type parafibromin induced apoptosis in transfected HeLa cells. In our study, we also found that parafibromin could induce apoptosis and G1 arrest in OS cells. These data indicated that parafibromin is a suppressor in cancer cells.

The main finding of this study is that distinct anti-tumor roles of parafibromin in the extracellular environment, cytoplasm, and nucleus of OS cells. We confirmed that recombinant parafibromin showed no effects on OS cells. It means that parafibromin in extracellular environment could not play anti-tumor roles in OS cells. Lack of receptor of parafibromin in OS cell surface may be the main reason for losing its function. Hahn et al. [10] reported that parafibromin has three distinct nucleolar localization signals (NoLS) at amino acid residues 76-92 (NoLS 1), 192-194 (NoLS 2), and 393-409 (NoLS 3) in addition to its known nuclear localization (NLS) signal at 125-139. In this study, we constructed mutational parafibromin which is located in the cytoplasm of OS cells by using site-directed mutagenesis. Interestingly, we found that mutational parafibromin showed no anti-tumor roles in OS cells. Parafibromin could play its anti-tumor roles only in the nucleus of OS cells. Furthermore, we confirmed the mechanisms of parafibromin in OS cells. Inhibition of MEK/ERK and PI3K/AKT pathways could induce genes that play major roles in cell cycle, apoptosis, and angiogenesis [16]. In our study, we found that parafibromin could induce dephosphorylation of MEK, ERK, PI3K and AKT. To test the hypothesis that parafibromin can induce apoptosis in OS cells by suppressing the PI3K/AKT and MEK/ERK signaling pathways, IGF-1 was used to treat OS cells as a control. IGF-1 is an agonist commonly used to activate the PI3K/mTOR signaling pathway [11]. In this study, IGF-1 was found to induce the proliferation of OS cells, and to inhibit apoptosis in OS cells. More importantly, the mechanism of parafibromin in the OS cells was determined using IGF-1 as a positive control.

Collectively, parafibromin could play its anti-tumor roles only in the nucleus of OS cells, and
these involved suppression of the MEK/ERK and PI3K/AKT signaling pathways. Moreover, apoptosis was induced by activation of caspase 3 and caspase 9 following the inhibition of the MEK/ERK and PI3K/AKT pathways. These results provide valuable insight into potential novel treatments for OS.

Disclosure of conflict of interest

None.

Address correspondence to: Zhi-Yu Zhang, Department of Orthopaedics, The 4th Affiliated Hospital of China Medical University, No. 4, Chongshan East Road, Huanggu District, Shenyang 110032, P. R. China. E-mail: zhangzy_4th@sina.com

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