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Research Paper

Small interfering RNA-mediated silencing of G-protein-coupled receptor 137 inhibits growth of osteosarcoma cells

Hao Li, Xiaodong Fu, Yingjian Gao, Xiaomiao Li*, Yi Shen, Weili Wang

Department of orthopaedics, South Campus, Ren Ji Hospital, School of Medicine, Shanghai Jiaotong University, China

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ABSTRACT

Purpose: Osteosarcoma is the most widespread primary carcinoma in bones. Osteosarcoma cells are highly metastatic and frequently develop resistance to chemotherapy making this disease harder to treat. This identifies an urgent need of novel therapeutic strategies for osteosarcoma. G-Protein-coupled receptor 137 (GPR137) is involved in several human cancers and may be a novel therapeutic target.

Methods: The expression of GPR137 was assessed in one osteoblast and three human osteosarcoma cell lines via the quantitative real-time polymerase chain reaction and western blot assays. Stable GPR137 knockdown cell lines were established using an RNA interference lentivirus system. Viability, colony formation, and flow cytometry assays were performed to measure the effects of GPR137 depletion on cell growth. The underlying molecular mechanism was determined using signaling array analysis and western blot assays.

Results: GPR137 expression was higher in the three human osteosarcoma cell lines, Saos-2, U2OS, and SW1353, than in osteoblast hFOB 1.19 cells. Lentivirus-mediated small interfering RNA targeting GPR137 successfully knocked down GPR137 mRNA and protein expression in both Saos-2 and U2OS cells. In the absence of GPR137, cell viability and colony formation ability were seriously impaired. The extent of apoptosis was also increased in both cell lines. Moreover, AMP-activated protein kinase α, proline-rich AKT substrate of 40 kDa, AKT, and extracellular signal-regulated kinase phosphorylation levels were down-regulated in GPR137 knockdown cells.

Conclusions: The results of this study highlight the crucial role of GPR137 in promoting osteosarcoma cell growth *in vitro*. GPR137 could serve as a potential therapeutic target against osteosarcoma.

1. Introduction

Osteosarcoma is the most common histological form of primary bone neoplasms and is often found in children and young adults. Although osteosarcoma is not a common cancer compared to others, more than 800 new osteosarcoma cases are diagnosed annually in the United States. Unfortunately, half of these are in children and teenagers [1]. Chemotherapy has been the primary treatment of high-grade malignant osteosarcoma and there has been substantial progress in improving the survival rate. However, the results continue to be unsatisfactory in the case of metastatic osteosarcomas and accompanying complex pathological conditions because the molecular cause of the disease remains unknown [2]. Current detailed investigations on the pathogenesis, progression, and prognosis of osteosarcoma have identified several molecules that might effectively modulate the initiation and progression of the disease. These include vascular endothelial growth factor (VEGF), mTOR, p53, Rb, APEX1, VEGFA, and c-myc [3]. Particularly, the importance and applicability of targeted therapy in osteosarcoma have been reviewed due to its high tendency to metastasize [4].

G-Protein-coupled receptors (GPRs) are the largest family of membrane-bound receptors and have been identified as key factors in many cancer types [5]. Cancer cells can evade immune detection and proliferate autonomously by hijacking the functions of GPRs [6]. Therefore, GPRs have become novel drug targets.

GPR family members play critical roles in the development and metastasis of osteosarcoma [7]. GPR137 belongs to a particular group of GPRs: orphan G protein-coupled receptors [8,9]. The GPR137 gene was discovered using homology screening and shares identity with a prostate-specific odorant-like orphan GPR that has been used as a diagnostic marker of prostate cancer [10]. Importantly, GPR137 is involved in several human cancers. Zong et al. [11] showed that GPR137 was highly expressed in high grade gliomas, and small interfering (si) RNA-mediated knockdown of GPR137 significantly suppressed their tumorigenic ability. A recent study revealed that GPR137 could modulate colon cancer cell proliferation and cell cycle progression [12].

* Corresponding author.
E-mail address: drlixm@126.com (X. Li).

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However, the role of GPR137 in human osteosarcoma has not been well-studied.

Given the ability to silence any gene through RNA interference (RNAi), siRNA has received mounting attention in targeted therapy. Importantly, the potential of siRNA-mediated gene silencing in the treatment of osteosarcoma has been studied. Zhang et al. [13] demonstrated that siRNA-mediated knockdown of the β-catenin gene reduced the invasion ability of MG63 osteosarcoma cells.

In the current study, we measured the expression of GRP13 in different osteosarcoma cells. With the aim to evaluate the roles of GRP13 played in osteosarcoma formation and development, we knocked it down in upregulated cells lines by using RNAi, determined the cell viability, colony formation and apoptosis change after GRP13 silence and discussed the mechanism.

2. Material and methods

2.1. Reagents and plasmids

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum were purchased from Hyclone (Logan, UT, USA). Lipofectamine 2000 and TRIzol Reagent were obtained from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). The antibodies used were as follows: rabbit anti-GPR137 (Proteintech Group, Chicago, IL, USA), rabbit anti-phospho-AMP-activated protein kinase (AMPK)α (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-proline-rich AKT substrate of 40 kDa (PRAS40) (Cell Signaling Technology), rabbit anti-phospho-extracellular signal-regulated kinase (ERK)1/2 (Cell Signaling Technology), rabbit anti-AKT (Proteintech Group), rabbit anti-phospho-AKT (Cell Signaling Technology), rabbit anti-ERK (Proteintech Group), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (Proteintech Group), and goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA). Lentiviral vector pFH-L and the lentiviral packaging auxiliary carriers pVSVG-I and pCMV△R8.92 were purchased from Duruobio (Shanghai, China). SYBR Green Master Mix Kits and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human embryonic kidney cell line 293 T, osteoblast hFOB 1.19 cells, and three human osteosarcoma cell lines (Saos-2, U2OS, and SW1353) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in DMEM supplemented with 10% heat inactivated foetal bovine serum and maintained in a humidified atmosphere of 5% CO_2.

2.3. Lentivirus vectors construction and infection

siRNA targeting GPR137 (NM_001170726.1) and non-silencing control siRNA were transformed into stem-loop-stem oligos and cloned into the pFH-L vector. The sequences used were: GPR137 siRNA (siGPR137): 5′-GACGCTTATGAACCTCTACTT-3′, and control siRNA (siCon): 5′-TTCTCCGAACGTGTCACGT-3′. The generated vectors were confirmed by DNA sequencing followed by transfection of the silencing and non-silencing siRNA-containing lentiviruses into 293 T cells. For the transfection, 293 T cells were cultured in 10 cm dishes at a density of 1×10^7 using standard protocols. Briefly, the medium was replaced 2 h prior to transfection with serum-free DMEM. The plasmids, including siRNA, packaging vector pHelper 1.0, and VSVG expression plasmid pHelper 2.0, were then added to 200 μL of Opti-MEM and 15 μL of Lipofectamine 2000. Following 48 h of transfection, the lentiviral particles were harvested by ultracentrifugation (80,000 g) at 4 °C for 30 min. Then, osteosarcoma cells (5×10^4 cells per well) were infected with the prepared lentiviruses (siGPR137 or siCon) in 6-well plates. Successful infections were confirmed after 96 h of infection by observing green fluorescent protein-expressing cells under a fluorescence microscope.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

GPR137 mRNA levels were measured by qRT-PCR analysis using the 2× SYBR Green Master Mix Kit with the DNA Engine Opticon™ System (MJ Research, Waltham, MA, USA). The PCR reaction mixture added to each vial contained 10 μL of 2× SYBR Green Master Mix, 0.8 μL of forward and reverse primers (2.5 μM), and 5 μL of cDNA (2 ng). The PCR experiment was carried out using the following protocol in a BioRad Connect RT-PCR platform: initial denaturation at 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing extension at 60 °C for 20 s. Actin was used as the housekeeping mRNA reference. The primers (5′ to 3′) used for GPR137 expression analysis were: GPR137: forward (ACCTGGGGAACAAAGGCTAC) and reverse (TAGGACCGAGAGGCAAAGAC); actin: forward (GTGGACATCC GCAAAGAC) and reverse (AAAGGGTGTAACGCAACTA). Relative gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot analysis

After infection with lentivirus for five days, cells were washed, collected, and lysed with 2× sodium dodecyl sulphate (SDS) sample buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS, and 10% glycine on ice for 30 min. Then, the supernatant was collected by centrifuging (12,000 × *g* for 15 min). The protein content was measured by the Lowry method and 20 μL of each sample containing 140 μg protein were electrophoresed on a 10% SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with specific primary antibodies overnight at 4 °C, followed by secondary antibody treatment for another 2 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) by exposing to X-ray film. Bands were quantified using an ImageQuant densitometry scanner (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. 3-(4,5-Dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT) assay

Cells were cultured in 96-well plates at 2×10^3 cells/well and transfected with siRNA containing lentivirus for 1–5 days. At the end of each incubation period, 20 μL of MTT solution (5 mg/mL) were added to each well and incubated at 37 °C for 4 h. The formazan crystals were dissolved in 200 μL of acidic isopropanol (10% SDS, 5% isopropanol. and 0.01 M HCl). Finally, the optical density was measured using a microplate reader at 595 nm.

2.7. Colony formation assay

To further investigate the effect of GPR137 silencing on the proliferation of osteosarcoma cells, the colony formation assay was performed. Briefly, transfected cells (500 cells/well) were seeded into six-well plates and incubated for two weeks, replacing the medium every three days. After the incubation period, the cells were washed, fixed with 4% paraformaldehyde, and stained with freshly prepared diluted crystal violet dye for 20 min. Finally, the colonies were visualized and counted under a light/fluorescence microscope.

2.8. Cell apoptosis analysis

The influence of GPR137 depletion on cell apoptosis was analysed by flow cytometry. Lentivirus-transduced and control cells were seeded on 6 cm culture dishes at a density of 2×10^5 cells/dish and incubated