Cellular Signalling 45 (2018) 43–53

Contents lists available at ScienceDirect

Cellular Signalling

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

Overexpression of the 14-3-3γ protein in uterine leiomyoma cells results in growth retardation and increased apoptosis

Qi Shen[a], Xiaoli Hu[a], Lulu Zhou[a], Shuangwei Zou[a], Lu-Zhe Sun[b,*], Xueqiong Zhu[a,*]

[a] Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China
[b] Department of Cell Systems & Anatomy, University of Texas Health Science Center at San Antonio, TX, USA

ARTICLE INFO

Keywords:
Uterine leiomyoma
14-3-3γ
Proliferation
Apoptosis
Bioinformatics
Signaling pathway

ABSTRACT

Protein 14-3-3γ was significantly reduced in human uterine leiomyoma compared to the adjacent normal myometrium tissue. To investigate the possible link between the reduced 14-3-3γ expression and uterine leiomyoma growth, we have overexpressed 14-3-3γ protein in uterine leiomyomal cells and its effects on cell proliferation and apoptosis were analyzed. Over-expression of 14-3-3γ was achieved by transducing into two types of uterine leiomyoma cells (primary culture cells and immortal stem cells) with a 14-3-3γ expressing adenovirus vector. Differentially expressed proteins were screened by the proteomics tool (TMT-LCTMS), followed by PANTHER database analysis to single out specifically modified signaling pathway proteins, which were confirmed by Phospho-MAPK Antibody Array and Western blots analysis. The results showed that increase in 14-3-3γ expression in both two types of human uterine leiomyoma cells inhibited cell proliferation and induced apoptosis. Proteomic screening has found 42 proteins, among 5846, that were significantly affected. PANTHER database and GeneMANIA analysis of the differentially expressed proteins have found that proteins involved in apoptosis signaling and cytoskeletal/adhesion were among the ones affected the most. Further analysis of the key signaling pathways have found that over-expression of 14-3-3γ resulted in reductions in the phosphorylations of multiple signaling molecules, including AKT, pan, ERK1/2, GSK-3 α/β, MEK1/2, Foxo1 and Vimentin. In conclusion, the loss of 14-3-3γ may have causal effects on the growth of uterine leiomyoma, which may function through modifying multiple signaling pathways, including AKT-Foxo and/or MEK1/2-ERK1/2.

1. Introduction

Uterine leiomyomas are among the most common tumors in women, affecting 20% to 50% of reproductive women, which can cause menorrhagia, abnormal uterine bleeding, pelvic pain, infertility and miscarriage [1]. Except invasive surgical procedure, there is no simple, safe and effective way to treat the disease currently. Uterine leiomyomas are generally considered as a hormonal dependent tumor, closely related to estrogen and progesterone, but the mechanism of tumorigenesis is still unclear [2]. In our previous study, we have found that 14-3-3γ exhibited a marked down-regulation in leiomyoma tissues compared with the adjacent normal myometrium, via proteomic and reverse transcription polymerase chain reaction (RT-PCR) techniques [3]. In this study, we have tested the hypothesis that the loss of 14-3-3γ protein may play a causal role in the origin or growth of leiomyoma.

14-3-3γ is a member of 14-3-3 proteins family (7 isoforms: β, γ, ε, η, σ, τ/θ and ξ), a highly conserved phosphoserine/threonine-binding proteins group that regulates diverse cellular processes, including cell cycle progression, transcriptional regulation, apoptosis, and cell proliferation [4,5]. 14-3-3 proteins have been found to play roles in human tumorigenesis, with different subtypes expressed in different cancer tissues and possible different regulatory mechanisms involved [6]. At present, 14-3-3γ has been reported in breast cancer, lung cancer and glioma [7–9]. However, the role of 14-3-3γ in gynecological tumors has not been reported.

Mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signal transduction pathways are capable of regulating the proliferation and apoptosis of uterine leiomyoma cells, which are considered to be the main signal pathways that are responsible for the development of uterine leiomyomas [10,11]. In

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; MAPK, mitogen-activated protein kinases; PI3K/AKT, and phosphatidylinositol 3-kinase/protein kinase B; ERK1/2, extracellular regulated protein kinases 1/2; TMT, tandem mass tag; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; CCK-8, cell counting kit-8; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; LSD, least significance difference

* Corresponding authors at: No. 109 Xueyuan Xi Road, Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027, China.

E-mail addresses: sunl@uthscsa.edu (L.-Z. Sun), zjwzzxq@163.com (X. Zhu).

https://doi.org/10.1016/j.cellsig.2018.01.025
Received 17 December 2017; Received in revised form 22 January 2018; Accepted 25 January 2018

uterine leiomyoma cells, for example, estrogen is capable of increasing cell proliferation via protein kinase C, which further activates the extracellular regulated protein kinases 1/2 (ERK1/2) signaling pathway. Interestingly, in normal myometrium cells, estrogen is also capable of activating protein kinase C, but instead of stimulating MAPK/ERK1/2 signaling, the kinase inactivates MAPK/ERK1/2 pathway, which resulted in low level of cell proliferation activities. The difference in the signaling transductions of the two cells/systems is still unclear. Progesterone induces PI3K/AKT pathway to activate the downstream genes (GSK3, TSC2, Foxo, and others), which can inhibit the leiomyoma cells apoptosis and also promote cell proliferation [11]. 14-3-3γ is a binding partner of both downstream members of MAPK (such as JNK, p38 and ERK1/2) and PI3K/AKT (such as Foxo, BAD and BAX), playing an important role in cell proliferation and apoptosis via preventing the members from dephosphorylation, which further controls the proportion of cytoplasmic and nuclear protein distributions [12]. In this study, we tested a hypothesis that loss of 14-3-3γ by uterine leiomyoma cells plays a causal role in the development of leiomyoma by modifying MAPK and/or PI3K/AKT signaling pathways.

Tandem mass tag (TMT) is a new proteomics technology that can quantify the absolute amount of proteins by specifically labeling the peptides and proteins [13,14]. Compared to the traditional bidirectional gel electrophoresis proteomics, TMT has clear advantages in detection range, reproducibility, high flux, accuracy and high resolution [15]. Since its invention, TMT has been used widely in cancer researches, such as breast-, liver-, and colorectal-cancers and others [16–18].

In this study, TMT was used to identify differently expressed proteins in cells after 14-3-3γ up-regulated, phospho-MAPK antibody array and Western blots were used for further conformation. Overall, our study tried to explore the role of 14-3-3γ in leiomyoma cell proliferation and apoptosis, and related signal pathways, which will help us better learn the biological effects of 14-3-3γ, and also provide experimental basis for clinical treatment on uterine leiomyoma patients.

2. Materials and methods

2.1. Primary tissue collection and stem cell

Uterine leiomyoma tissues were obtained from 40 patients with ages ranged 43 ± 6 years, between October 2013 and July 2015. The tissues were collected during the procedures of uterine myomectomy, subtotal or total hysterectomy. Patients with following characters were excluded: administrations with medicines or hormones within 3 months prior surgery, or with other complications, such as infections, chronic diseases (such as diabetes and hypertension), uterine malignancy and/or adenomyosis (on the basis of tissue pathology). The study protocol was approved by the Research Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University and met the standards of the Declaration of Helsinki. Written consent was obtained from every patient before the procedures.

Human uterine leiomyoma stem cells were from Dr. Ayman Al-Hendy Lab, we have immortalized them with hTERT lentivirus.

2.2. Primary human cell and stem cell culture

Fresh tissues were washed with cold phosphate-buffered saline (PBS), then cut into small pieces (1 mm^3) and digested with 0.2% (v/v) collagenase II (Invitrogen, Carlsbad, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) for 4 h in a 37 °C with shaking. The dissociated cells were centrifuged at 400 ×*g* for 5 min. The resultant cell deposit was suspended with complete culture medium (DMEM, 10% fetal bovine serum, 100 IU/ml of penicillin G and 100 μg/ml streptomycin) and centrifuged at 400 × g for 5 min. The resultant cells were cultured at a density of 2×10^5 cells/ml under 5% CO_2 at 37 °C in the complete culture medium. Stem cells were cultured with the same complete culture medium above, under 2.5% O_2 and 5% CO_2 at 37 °C. Culture medium was changed every other day. Cells were split as 1:2 every 4 to 6 days. Cells from third passages to the fifth were used for the experiments.

2.3. Staining of α-smooth muscle actin by immunocytochemistry

Uterine leiomyoma cells were identified by the expression of α-smooth muscle actin. Cells were fixed with 4% paraformaldehyde, washed and then permeabilized in PBS containing 0.2% Triton X-100 for 15 min. Cells were then incubated in a serum-free blocking solution for 15 min at room temperature, incubated with mouse monoclonal anti-α-smooth muscle actin antibody (1:100 dilution; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) overnight at 4 °C. After extensive washing with PBS, cells were incubated with biotinylated goat anti-mouse IgG as secondary antibody. After incubation the bound antibodies were visualized using 3,3′-diaminobenzidine. Finally, nuclei were stained with hematoxylin. Cells incubated with PBS, instead of primary antibody, was included as negative control.

2.4. Construction and characterization of recombinant plasmid vectors

Human 14-3-3γ (Genebank: NM_012479.3) gene was synthesized by GenScript company (Shanghai, China). The vector plasmid (pHBAd-MCMV-RFP, Hanbio, Shanghai, China) was digested by the *Bam*HI and *Eco*RI restrictive enzymes, and purified using agarose gel electrophoresis. Then 14-3-3γ gene was subcloned into the pHBAd-MCMV-RFP. The positive recombinant clone, named pHBAd-MCMV-RFP-14-3-3γ, was then transfected and amplified into DH5α by hot shock method and screened by Amp + resistance. The positive clones were finally identified and characterized by PCR and sequencing.

2.5. Virus preparation, purification, and titer determination

The pHBAd-MCMV-RFP-14-3-3γ and the adenovirus helper plasmid pHBAd-BHG (Hanbio, Shanghai, China) were co-transformed into HEK293 cells with lipofectamine 2000 Kit according to the manufacturer's instructions. Six hours after transformation, the medium was replaced with new DMEM, and cytopathic effects were observed every 2 days. The viral particles in the HEK293 cells were prepared by lysis of the cells with three consecutive freeze–thawing cycles in ice-ethanol, then by passing through 0.4 μM syringe filter to remove inadvertent materials. The viral titer (pHBAd-MCMV-RFP-14-3-3γ) was detected by the TCID50 standard method. Finally, the recombinant adenovirus was stored at −80 °C until use. pHBAd-MCMV-RFP-14-3-3γ virus was used to over-express 14-3-3γ proteins, while pHBAd-MCMV-RFP virus was used as a negative control.

2.6. Cell counting kit-8 (CCK-8) assay

To detect the cell proliferation, 1×10^4 cells/well were seeded into 96-well plates and incubated at 37 °C with 5% CO_2. After 24 h of incubation, the cells were treated with 14-3-3γ over-expression virus or control virus. By end of treatments, 10 μl of CCK-8 (Dojindo, Kumamoto, Japan) was added to each well and incubated for an additional 2 h. The reaction product was quantified by spectrophotometry at 450 nm wavelength, and the percentage of viability or number of cells were calculated by formula: (treated cells absorbent / non treated cells absorbent) × 100.

2.7. Flow cytometric assay of apoptosis with APC/7-AAD staining

Cell apoptosis was assayed by flow cytometry after APC/7-AAD staining. APC was used to detect early apoptosis in cellular membrane, 7-AAD was used to detect late apoptosis in cell nucleus. The assay was performed according to the manufacturer's guidelines. Briefly, after