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Research paper

RING domain of zinc finger protein like 1 is essential for cell proliferation in endometrial cancer cell line RL95-2

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ABSTRACT

Endometrial cancer (EC) is the fourth most common cancer in women and exhibits increasing incidence and mortality. Some reports showed that the 5-year survival rate of EC was closely associated with the diagnosed stage. It is urgent to screen for sensitive and specific targets to improve early detection and EC therapy. In our study, we found that zinc finger protein like 1 (ZFPL1) was highly expressed in EC tissues and the EC cell line RL95-2, as detected via RT-qPCR and western blot analysis. Immunocytochemistry results showed that ZFPL1 was localized in the Golgi complex dependent on the C-terminal transmembrane domain. The MTT and EdU stains were employed to examine the effect of ZFPL1 on cell proliferation. We found that the silencing of ZFPL1 blocked cell proliferation and the expression of p-Akt308 and p-Akt473 but improved the protein level of PTEN. The overexpression of ZFPL1 and ZFPL1ΔTMD (deletion of the transmembrane domain) promoted cell proliferation and induced the expression of p-Akt308 and p-Akt473. However, the overexpression of ZFPL1ΔRING (deletion of the RING domain) caused loss of the function of ZFPL1 in cell proliferation and the PI3K/Akt pathway. In summary, ZFPL1 induced RL95-2 cell proliferation and was involved in PI3K/Akt pathway, suggesting the oncogenic role of ZFPL1 during EC development. Additionally, the RING domain was essential for the function of ZFPL1. These findings provided a new biomarker for EC diagnosis and therapy.

1. Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies worldwide (Hussein and Soslow, 2018). Of significance, the incidence and mortality rates for EC have been increasing in developed and developing countries (Makker et al., 2017). The risk factors for EC are complex, including family history, unopposed estrogen therapy, obesity and hyperinsulinemia (Nead et al., 2015). A previous study has showed that approximately 70% of EC patients who were diagnosed at the early stages were associated with an overall 5-year survival rate of 95%. However, 30% of EC patients who were diagnosed at an advanced stage were associated with a 5-year survival rate of 69% (Troisi et al., 2018). Consequently, early diagnosis is a major issue to appropriately manage EC and decrease mortality associated with EC (Martinez-Garcia et al., 2018). Therefore, understanding the mechanism of EC and developing specific biomarkers is essential to favor early EC detection and the improvement of outcomes among women with advanced EC (Talhouk et al., 2017).

Zinc finger proteins are one of the most abundant groups of proteins and are involved in the regulation of several cellular processes (Fedotova et al., 2017). The most important and abundant zinc-finger domain proteins consist of C2H2, really interesting new gene (RING), plant homeodomain, Lin-II, IsI-1 and Mec-3 (Cassandri et al., 2017). The RING family is the largest type of E3 ubiquitin ligases, and it plays an essential role in the regulation of several biological events (Dove and Klevit, 2017). Recent findings have highlighted the essential roles of RING family in cancer progression (Chasapis and Spyroulias, 2009). For example, the expression of RING finger protein 113A is closely associated with poor prognosis of esophageal squamous cell carcinoma patients (Wang et al., 2018). Ring finger protein 6 plays a pivotal oncogenic role in colorectal tumorigenesis via activating the Wnt/β-catenin pathway (Liu et al., 2018). However, few RING finger proteins are

Abbreviation: EC, endometrial cancer; ZFPL1, zinc finger protein like 1; RING, really interesting new gene; EdU, 5-ethynyl-2′-deoxyuridine; MTT, diphenyl tetrazolium bromide

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explored during the progression of EC.

Zinc finger protein like 1 (ZFPL1), which is isolated from the 11q13 region, encodes a putative protein of 310 amino acids (Hoppener et al., 1998). A previous study has revealed that ZFPL1 is a conserved integral membrane protein and interacts with the cis-Golgi matrix protein GM130 to maintain the structure and functional integrity of the cis-Golgi (Chiu et al., 2008). A recent report has shown that the knockdown of ZFPL1 induces cell death via autophagy rather than apoptosis, suggesting the important role of ZFPL1 in the human gastric carcinoma cell lines NCI-N87 and BGC-823 (Xie et al., 2017). However, the exact role of ZFPL1 during tumor development remains elusive. In our study, we employed EC as a research model to investigate the function of ZFPL1 during cancer progression. During the experiments, we found that ZFPL1 was highly expressed in EC tissues and the cell line RL95-2 and was localized in the Golgi complex. MTT assay and EdU staining showed that the silencing of ZFPL1 blocked RL95-2 cell proliferation. We also found that the knockdown of ZFPL1 suppressed the phosphorylation of Akt308 and Akt473; however, the overexpression of ZFPL1 exerted the opposite effect on cell proliferation and the expression of the above genes. These data revealed that ZFPL1 induced cell proliferation and involved in the activation of PI3K/Akt pathway in the EC cell line RL95-2, indicating that ZFPL1 served as an oncogene during EC development. These findings provided a new biomarker for EC diagnosis and therapy.

2. Materials and methods

2.1. Reagents

Human endometrial cancer cell line RL95-2 and endometrium epithelial cells EEC (KeyGen Biotech. Inc., Nanjing, China); negative control (NC) siRNA, ZFPL1-homo-212 siRNA, ZFPL1-homo-293 siRNA and ZFPL1-homo-374 siRNA (Sangon Biotech, Shanghai, China); Lipofectamine 2000 and 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen, USA); Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) (Gibco, USA); 2 × One-step SYBR Real-time RT-PCR Kit (BioTeke Corporation, Beijing, China); TRIzol buffer, 4′6-diamidino-2-phenylindole (DAPI), Radio-immunoprecipitation assay (RIPA) buffer and diphenyl tetrazolium bromide (MTT) (Sigma, USA); Rabbit anti-Akt (D120056), anti-p-Akt308 (D153568), anti-p-Akt473 (D155022), anti-PTEN (D261095) antibodies and HRP-conjugated Goat anti-rabbit IgG (D110058) (BBI Life Science, Shanghai, China); Rabbit anti-ZFPL1 antibody (ab26057, abcam, USA); Enhanced chemiluminescence (ECL) kit (Thermo Scientific, San Jose, CA, USA); Golgi-Tracker Red (Beyotime, Shanghai, China).

2.2. Clinical samples

A total of 30 endometrial carcinomas tissues were collected from EC patients who had undergone surgical resection at the Department of Gynecology of Qilu Hospital of Shandong University. None of the patients had been administered chemotherapy or radiotherapy. Thirty adjacent non-tumors were collected as the normal control. The disease stage of the endometrial specimens was classified according to the FIGO 2009 criteria (Pecorelli, 2009) as follows: 15 cases with FIGO staging I–II and 15 cases with FIGO staging III–IV. The tumor specimens were independently confirmed by two pathologists. The research protocol was approved by the Qilu Hospital of Shandong University Ethics Committee.

2.3. Cell culture and transfection

RL95-2 cells were cultured with DMEM containing 10% FBS under 5% CO_2 in an incubator at 37 °C. At a density of 70–90%, the RL95-2 cells were transfected with siRNA-targeted ZFPL1-homo-212, ZFPL1-homo-293 or ZFPL1-homo-374 using Lipofectamine 2000 according to the manufacturer's instructions. NC siRNA was used as the negative control. After 48 h, the cells were collected for further investigation. The sequences of siRNA were as follows: ZFPL1-homo-212 sense: 5′-gcuucgaacaucgggucaatt-3′, antisense: 5′-uugacccgauguucgaagctt-3′; ZFPL1-homo-293 sense: 5′-ggcuccaagauagcgacuatt-3′, antisense: 5′-uagucgcuaucuuggagcctt-3′; ZFPL1-homo-374 sense: 5′-gcuaugaucucuuucacugtt-3′, antisense: 5′-cagucaaagagaucauagctt-3′; NC siRNA sense: 5′-uucuccgaacgugucacgutt-3′, antisense: 5′-acgugacacguucggagaatt-3′.

2.4. Real-time qPCR (RT-qPCR)

Total RNA was isolated from tissues or RL95-2 cells using TRIzol reagent. The quantity and quality was measured using the Nano Drop-2000 ultramicrospectrophotometer (Thermo, San Jose, CA, USA). RT-qPCR was performed using the 2 × One-step SYBR Real-time RT-PCR kit in a real-time PCR system (CFX Connect™, Bio-Rad, USA) according to the manufacturer's protocol. The relative expression level of ZFPL1 was quantified using the internal control GAPDH via the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The parameters for RT-qPCR were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s, and a final extension at 72 °C for 5 min. The primers for RT-qPCR were as follows: ZFPL1 forward primer: 5′-acgacccgccttgtctgctat-3′, reverse primer: 5′-gctctgggctcaccacctcatc-3′. GAPDH forward primer: 5′-gccaaaagggtcatcatctc-3′, reverse primer: 5′-gtagaggcagggatgatgttc-3′. The experiments were conducted in triplicate with independent experimental samples.

2.5. Vector Construction and immunocytochemistry

Appropriate DNA sequences encoding ZFPL1 and different truncated mutants were inserted into pcDNA3.1-GFP plasmid or pcDNA3.1 plasmid: ZFPL1ΔRING, in which the RING domain (aa 53–100) was deleted; ZFPL1ΔTMD, in which the transmembrane domain (aa 265–287) was deleted. The inserts were verified through DNA sequencing. RL95-2 cells were seeded into 6-well plates. At a density of 70–90%, the cells were pre-incubated with DMEM for 1 h. Five micrograms of plasmid DNA (pcDNA3.1-GFP, pcDNA3.1-ZFPL1-GFP, pcDNA3.1-ZFPL1ΔRING-GFP or pcDNA3.1-ZFPL1ΔTMD-GFP) with 5 μg of Lipofectamine 2000 was suspended in 250 μL of DMEM. After 5 min, the mixture was added into the medium for cell incubation. After 48 h, the cells were cultured with Golgi-Tracker Red solution for 30 min at 37 °C. After being washed with PBS, the signals were captured using a laser confocal microscope (Zeiss, Germany) from five non-overlapped fields. ZFPL1 was marked with green, and the Golgi complex was visualized as red.

2.6. MTT assay and EdU detection

Cell proliferation was measured using the MTT assay and EdU stain kit. The cells were seeded into 6-well plates and were transfected with plasmid DNA or siRNA. After 48 h, the transfected cells were seeded into 96-well plates for the MTT assay. After 0, 24, 48 and 72 h, the cells were incubated with 10 μL of MTT (5 mg/mL) for another 4 h at 37 °C, followed by removal of the culture medium and addition of 150 μL dimethyl sulfoxide. The optical density (OD) value at the wavelength of 560 nm was determined using a microplate reader (Thermo, USA).

RL95-2 cells were seeded into 24-well plate and were transfected with plasmid DNA or siRNA. After 48 h, the cells were exposed to 50 μM of EdU for 4 h at 37 °C and were fixed with freshly prepared 4% paraformaldehyde solution for 20 min at room temperature. After being washed with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS at 37 °C for 30 min. After PBS washes, the cells were reacted with 100 μL of 1 × Apollo reaction cocktail for 30 min, were stained with DAPI (1 μg/mL) for 30 min and were visualized under a fluorescence microscope (Nikon, Japan).