Interaction of Pten gene and AKT/mTOR pathway in endometrial adenocarcinoma proliferation

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Abstract

Objective: The Pten/AKT/mTOR pathway is one of the most critical pathways in tumor proliferation. The present research aimed to analyze the interaction between Pten gene and AKT/mTOR pathway in endometrial cancer cell lines. Methods: TCGA analysis was used to study the relationship between Pten expression and survival of endometrial cancer patients. Human endometrial cancer cell lines with low Pten expression (Ishikawa) and with high Pten expression (HEC-1-A) were selected. Plasmid transfection was used to regulate the Pten expression in the cell lines. QRT-PCR and Western Blot were adopted to detect Pten/AKT/mTOR expressions in tumor cells. Western blot of Ki-67 and CCK-8 were adopted to detect the activity of cells proliferation. A p < 0.05 was considered to be statistically significant. Results: The TCGA analysis showed the Pten expression was associated with survival of endometrial cancer patients significantly. Plasmid transfections elevated Pten expression in Ishikawa and decreased Pten expression in HEC-1-A cells. After the plasmid transfection, with overexpression of Pten in Ishikawa cell line, the Western Blot and QRT-PCR revealed the AKT/mTOR pathway is restrained, leading to decreased cell proliferation; with Pten decreased in HEC-1-A cells, the AKT/mTOR pathway is activated, leading to increased cell proliferation. Conclusions: A decreased expression of Pten gene in Ishikawa and HEC-1-A cell lines could activate AKT/mTOR pathway and promote tumor cells proliferation.

Keywords: Pten/AKT/mTOR pathway; endometrial cancer; tumor proliferation

1. Introduction

Endometrial cancer (EC) is one of the major gynecologic cancers, and 65,620 new cases were diagnosed in 2020 in the United States [1]. Based on histopathology, EC can be classified into estrogen-dependent EC (type I), accounting for 80–90% and not estrogen-dependent (type II), accounting for 10–20% [2,3].

At the present, surgery is the main treatment method for this disease [4]. But surgical treatment may not be possible for patients who cannot tolerate surgery, patients who desire fertility preservation, or patients with advanced-stage disease [5]. Chemotherapy and hormonal therapy have limitations [5]. Therefore, studying the mechanism of tumorigenesis may provide bio-therapeutic target for patients with EC.

The Pten gene was first discovered in 1997, located at 10q23.3, with 200kb in length, containing 9 exons and 8 introns [6]. The Pten gene plays an important role in maintaining the normal physiological activities of cells by regulating multiple pathways, controlling the proliferation and migration of tumor cells, and apoptosis. Mutations of the Pten gene can be found in many types of cancers [6].

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin PI3K/AKT/mTOR pathway is one of the downstream pathways of Pten, associated with tumor cell proliferation [7]. Abnormal expression of the Pten/AKT/mTOR signaling pathway has been found in liver, gastrointestinal, breast, lung, leukemia and other malignant tumors, which changes metabolism and proliferation of tumor cells [8–12], and is associated with poor prognosis [9,13]. This research aimed to analyze the interaction of the Pten/AKT/mTOR pathway in EC cell lines.

2. Materials and methods

2.1 The Cancer Genome Atlas (TCGA) analysis

The TCGA database (https://portal.gdc.cancer.gov) is the most authoritative cancer gene database, including gene expression, miRNA data, copy number variation, methylation, and single nucleotide polymorphisms (SNP). We downloaded the original DNA expression data of EC and analyzed it.

2.2 Cell culture

The HEC-1-A and Ishikawa cell lines were purchased from the National Collection of Authenticated Cell Cultures, Shanghai, China. The Pten gene is highly expressed in HEC-1-A, while its expression in Ishikawa is low. Both cell lines are positive for estrogen receptor-alpha (ER-a). The HEC-1-A and Ishikawa cells were stored in liq-
uid nitrogen and maintained in dulbecco’s modified eagle medium (DMEM) before using.

2.3 Reagents and transfection

In each cell line, three groups of blank control (BC), negative control (NC), overexpression (OE) or knockdown (KD) were established. BC group was without lentivirus transfection; NC group was transfected by naked lentivirus; OE group was transfected by lentivirus with segment enhancing \( Pten \) expression; and KD group was transfected by lentivirus with segment silencing \( Pten \) expression. Human \( Pten \) (NM 000314.8) cDNA was cloned into pLVX-IRES-Zsgreen vector. Lipo2000 (Thermo Fisher, America, catalog 11668027, Carlsbad, California, USA) were used to transfect cells, according to manufacturer’s protocol. Fluorescence-based microscopy was used to observe the transfection effectiveness.

2.4 RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated by TRIzol (Invitrogen™, 15596018, Carlsbad, California, USA). RNA was transcribed to cDNA using PrimeScript™ RT reagent Kit (TaKaRa, RR047A, Ōtsu, Shiga, Japan), then cDNA was used as the template to perform qRT-PCR, using SYBR Green (Bio-Rad, 1725151, Hercules, California, USA) for molecular probes. Glyceraldehyde phosphate dehydrogenase (GAPDH) was detected as a control to normalize the threshold value. The primers information was followed (Table 1).

<table>
<thead>
<tr>
<th>Gene name</th>
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| \( Pten \) | forward: 5'-TGGATTCGACTTAGACTTGACCT-3'  
reverse: 5'-GGTGGGTTATGGTCTTCAAAAGG-3' |
| \( AKT \) | forward: 5'-GGACAACCGCCATCCAGAC-3'  
reverse: 5'-GCCAGGGACACCTCCATCTC-3' |
| \( Raptor \) | forward: 5'-CTAATTATTCGGTAACTGACTTGA-3'  
reverse: 5'-ACAGTTCAACCACTACCTTGGA-3' |
| \( Rictor \) | forward: 5'-GCTAGGTGCATTGACATACAACA-3'  
reverse: 5'-AGTGCTAGTTCACAGATAATGGC-3' |
| \( GAPDH \) | forward: 5'-TCGGAGTCAACGGATTTGGT-3'  
reverse: 5'-TTCCCGTTCTCAGCCTTGAC-3' |

2.5 Protein extraction and western blotting tris buffered saline (TBS)

The total protein was extracted from cell lines in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific™, 89901, Carlsbad, California, USA). Samples were separated in 10% sodium dodecyl sulfate-polyacrylamide (SDS) polyacrylamide gels and transferred to nitrocellulose membranes. The protein was measured by enhanced chemiluminescence (ECL) blotting analysis system. The primary antibodies included anti-\( Pten \) (Abcam, ab267787, Cambridge, UK), anti-PI3K (P85) (Abcam, ab182651, Cambridge, UK), anti-p-AKT (S473) (Abcam, ab81283, Cambridge, UK), anti-p-AKT (T308) (Abcam, ab38449, Cambridge, UK), anti-Raptor (Abcam, ab40768, Cambridge, UK), anti-Rictor (Abcam, ab70374, Cambridge, UK), anti-ER-a (Abcam, ab32063, Cambridge, UK), anti-Ki-67 (Abcam, ab16667, Cambridge, UK) and anti-GAPDH (Abcam, ab8245, Cambridge, UK).

2.6 Cell proliferation

Cell viability was measured by Ki-67 (Abcam, ab16667) and Cell Counting Kit-8 (CCK-8) (Abcam, ab228554).

2.7 Statistics

All experiments were repeated 3 times independently. Data were presented as mean ± standard deviation, and all statistical analyses were performed by SPSS (Version 22.0, IBM, USA). Statistical significance was analyzed by Stu-
Fig. 3. Fluorescence expressions in Ishikawa and HEC-1-A cell lines after transfection. (A) Pten enhanced transfection group in Ishikawa cells (Ishikawa+LV-Pten). (B) The empty plasmid transfection group in Ishikawa cells (Ishikawa+NC). (C) The Pten interference fragment transfection group in HEC-1-A cells (HEC-1-A+LV-Pten-RNAi). (D) The empty plasmid transfection group in HEC-1-A cells (HEC-1-A+NC).

Fig. 4. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in Ishikawa cells. (A) Pten mRNA increased by 77% ($p < 0.05$). (B) AKT mRNA decreased by 56% ($p < 0.05$). (C) Raptor mRNA decreased by 45% ($p < 0.05$). (D) Rictor mRNA decreased by 33% ($p < 0.05$).

Fig. 5. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in HEC-1-A cells. (A) The Pten mRNA decreased by 48% ($p < 0.05$). (B) The AKT mRNA increased by 79% ($p < 0.05$). (C) The Raptor mRNA increased by 69% ($p < 0.05$). (D) The Rictor mRNA increased by 59% ($p < 0.05$).

Fig. 6. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in Ishikawa cells. (A) The western blot of the Pten/AKT/mTOR signaling pathway in Ishikawa cells. (B) Pten protein increased by about 37% ($p < 0.05$). (C) p-AKT (T308) decreased by 27% ($p < 0.05$). (D) p-AKT (S473) decreased by 38% ($p < 0.05$). (E) p-Raptor decreased by 25% ($p < 0.05$). (F) p-Rictor decreased by 7% ($p > 0.05$).

3. Results

3.1 TCGA analysis

Data on 512 cases were available for analysis, including a normal group (n = 35) and a tumor group (n = 477). We did a survival analysis based on the high or low expression of the Pten gene, which proved that Pten expression was statistically significantly associated with survival ($p = 0.037$) (Fig. 1).

3.2 The expression of ER-alpha protein in cell lines

The western blot confirmed that Ishikawa and HEC-1-A were positive for ER-a (Fig. 2). After transfection of 48 hours, stable expressions of fluorescent signal were shown in each group under fluorescent microscope, indicating a successful transfection (Fig. 3).
3.3 The mRNA and protein expressions of Pten/AKT/mTOR pathway in cell lines after transfection

In Ishikawa cells, the mRNA level of Pten in OE group increased significantly compared to the BC group; while the mRNA of AKT, Raptor, and Rictor decreased significantly ($p < 0.05$) (Fig. 4). In HEC-1-A cells, Pten mRNA in KD group decreased ($p < 0.05$), while the AKT, Raptor, and Rictor decreased significantly ($p < 0.05$) (Fig. 5).

The proteins of Pten/AKT/mTOR signaling pathway in Ishikawa (Fig. 6) and HEC-1-A cells (Fig. 7) were confirmed similar to the mRNA levels shown above.

3.4 The effect of the Pten/AKT/mTOR pathway on proliferation

After 48 hours of transfection, the expression of Ki-67 protein was detected by western blotting (Fig. 8A–D). In Ishikawa cells, compared with the BC group, the Ki-67 in OE group was significantly lower ($p = 0.03$) (Fig. 8A,B). In HEC-1-A cells the Ki-67 in KD group was significantly higher ($p = 0.02$), compared with the NC group (Fig. 8C,D).

In CCK-8, with transfection progress, the cell viability of Ishikawa cells with Pten overexpression gradually decreased; on the contrary, the cell viability of HEC-1-A cells with Pten knockdown gradually increased (Fig. 8E,F).

4. Discussion

Previous studies in lung, prostate, colon, and bladder cancer show that with tumor progression, the expression of the Pten gene tends to be lower [14–19]. A slight reduction in Pten expression in animal models could have a major impact on cancer susceptibility [20]. Mice with Pten ± heterozygous deletion (Pten ±) could develop endometrial atypical hyperplasia, and ab 20% even progressed to high differentiated cancer [21].

AKT/mTOR is a classic pathway for tumor cell proliferation [22–25]; and AKT regulates tumor-associated cell processes including cell growth, cell cycle progression, survival, migration, epithelial-mesenchymal transition and angiogenesis [26]. Loss of Pten induces abnormal activation of the AKT/mTOR pathway and promotes tumor cell growth, and proliferation [27]. Primary Pten mutation with activation of the AKT pathway is rare in EC, but the activation of the AKT pathway caused by other reasons often occurs in EC making the disease progress rapidly [26,28]. After knocking out Pten gene in mice, the AKT pathway was abnormally activated [29]. A similar effect can be obtained if Pten inhibitors were used [28,30]

In our study, after 48 hours of transfection, QRT-PCR suggested that when Pten mRNA was highly expressed, the AKT, Rictor, and Raptor of Pten downstream were decreased, and vice versa ($p < 0.05$). This is roughly consistent with the regulation of Pten/AKT/mTOR in other tumors.
In the Western blot, comparing with the BC group, p-AKT (T308), p-AKT (S473) and p-Raptor proteins in Ishikawa and HEC-1-A cell lines were regulated by the expression of Pten \((p < 0.05)\), which was consistent with the results of qRT-PCR [31,32].

After the plasmid transfection, with overexpression of Pten in the Ishikawa cell line, cell proliferation decreased, while a decline in Pten expression in the HEC-1-A cell line resulted in increased cell proliferation. The above results suggest that the expression level of Pten in cell lines of EC is inversely related to the activity of tumor proliferation, which is consistent with the results of other studies, including renal cell carcinoma, glioblastoma and bladder cancers [33–36].

5. Conclusions and limitations

In Ishikawa and HEC-1-A cell lines, the decreased expression of the Pten gene could activate AKT/mTOR pathway and promoting the proliferation activity of tumor cells, which is worthy of further investigation as a potential immunotherapy target. The HEC-1-A and Ishikawa cell lines were used for transfection experiments; as their Pten expression levels were opposite (HEC-1-A with Pten highly expressed, Ishikawa with low Pten expression), the experimental results are reliable. However, we did not perform animal experiments, which should be considered for future research.

Abbreviations

EC, Endometrial cancer; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; ER-a, estrogen receptor-a; qPCR, quantitative real-time polymerase chain reaction; BC, blank control; NC, negative control; OE, overexpression; KD, knockdown; CCK-8, Cell Counting Kit-8.

Author contributions

DT—Project development, Performed the research, Data Collection and management, Statistical analysis, Manuscript writing. MRX—Obtaining funding, Critical revision of the manuscript, Supervision. XZ—Project development, Performed the research, Data Collection and management, Statistical analysis, Critical revision of the manuscript, Supervision, Obtaining funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

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