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Original Research

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, controling 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 effictiveness.

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 1. Primers information.

Gene name	Genetic code
Pten	forward: 5′-TGGATTCGACTTAGACTTGACCT-3′
	reverse: 5′-GGTGGGTTATGGTCTTCAAAAGG-3′
AKT	forward: 5′-GGACAACCGCCATCCAGACT-3′
	reverse: 5′-GCCAGGGACACCTCCATCTC-3′
Raptor	forward: 5′-CTAATTATTCGGTAACTGACTTGA-3′
	reverse: 5′-ACAGTTCAGCCATCACTTGGA-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

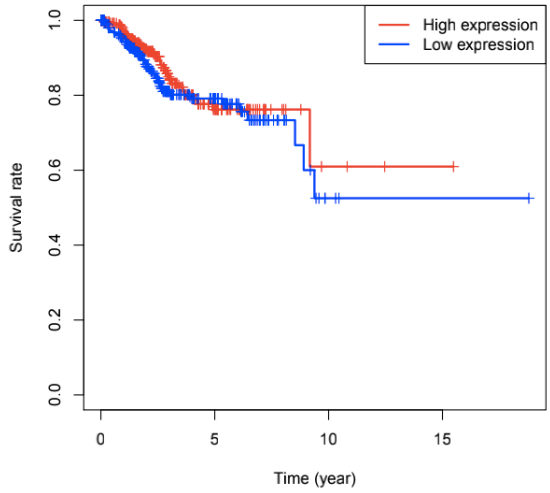

Fig. 1. The survival analysis of *Pten* in EC based on the TCGA database.

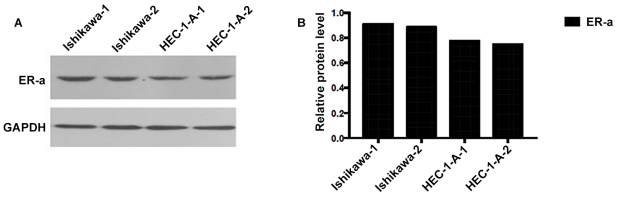

Fig. 2. The expressions of ER-alpha in Ishikawa and HEC-1-A. (A) Western blot results showed Ishikawa and HEC-1-A cell lines were positive for ER-alpha, and GAPDH was an internal control. (B) The quantitative results of ER-a protein in Ishikawa and HEC-1-A cells.

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-

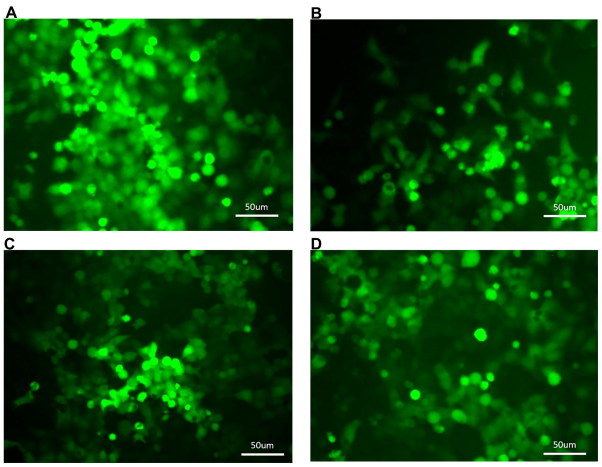

Fig. 3. Fluorescence expressions in Ishikawa and HEC-1-A cell lines after transfection. (A) *Pten* enhanced transfection group in Ishikawa cells (Ishikawa+LV-Pten). (B) The empty plasmid transfection group in Ishikawa cells (Ishikawa+NC). (C) The *Pten* interference fragment transfection group in HEC-1-A cells (HEC-1-A+LV-Pten-RNAi). (D) The empty plasmid transfection group in HEC-1-A cells (HEC-1-A+NC).

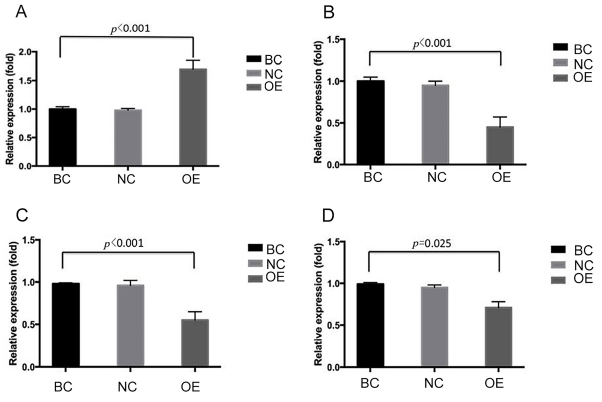

Fig. 4. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in Ishikawa cells. (A) *Pten* mRNA increased by 77% ($p < 0.05$). (B) AKT mRNA decreased by 56% ($p < 0.05$). (C) Raptor mRNA decreased by 45% ($p < 0.05$). (D) Rictor mRNA decreased by 33% ($p < 0.05$).

dent's *t*-test, $p < 0.05$ was considered to be statistically significant.

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).

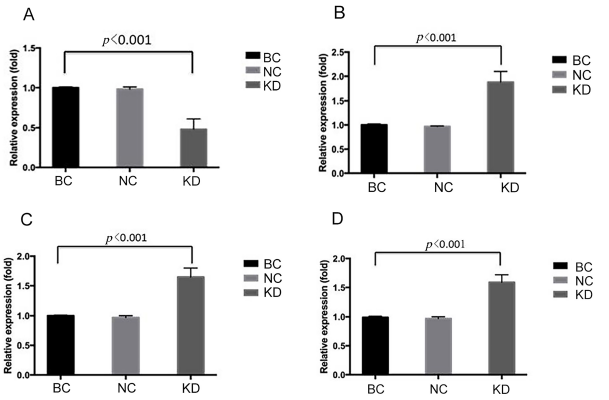

Fig. 5. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in HEC-1-A cells. (A) The *Pten* mRNA decreased by 48% ($p < 0.05$). (B) The AKT mRNA increased by 79% ($p < 0.05$). (C) The Raptor mRNA increased by 69% ($p < 0.05$). (D) The Rictor mRNA increased by 59% ($p < 0.05$).

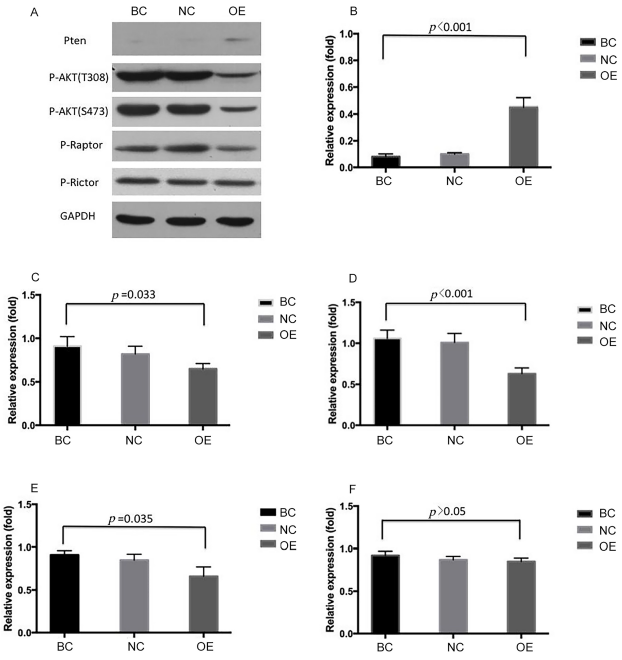

Fig. 6. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in Ishikawa cells. (A) The western blot of the Pten/AKT/mTOR signaling pathway in Ishikawa cells. (B) *Pten* protein increased by about 37% ($p < 0.05$). (C) p-AKT (T308) decreased by 27% ($p < 0.05$). (D) p-AKT (S473) decreased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

3.2 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).

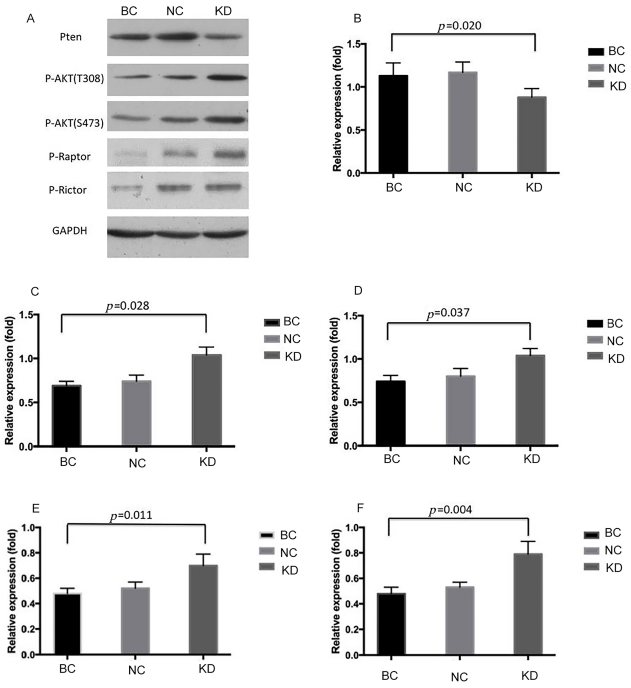

Fig. 7. The mRNA quantitative analysis of Pten/AKT/mTOR signaling pathway in HEC-1-A cells. (A) The western blot of Pten/AKT/mTOR signaling pathway in HEC-1-A cells. (B) *Pten* protein decreased by about 23% ($p < 0.05$). (C) p-AKT (T308) increased by 34% ($p < 0.05$). (D) p-AKT(S473) increased by 29% ($p < 0.05$). (E) p-Raptor increased by 32% ($p < 0.05$). (F) p-Rictor increased by 40% ($p < 0.05$).

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).

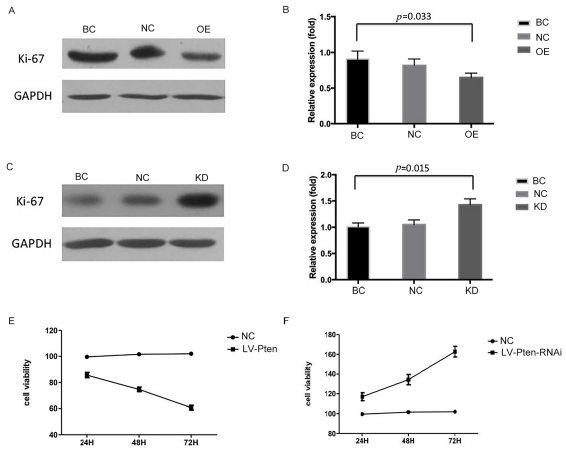

Fig. 8. Proliferation abilities of Ishikawa and HEC-1-A cells after transfection. (A) Western blot of Ki-67 in Ishikawa cells. (B) With *Pten* overexpression in Ishikawa cells, Ki-67 decreased by 29% ($p < 0.05$). (C) Western blot of Ki-67 in HEC-1-A. (D) With *Pten* inhibition in HEC-1-A cells, Ki-67 increased by 70% ($p < 0.05$). (E) The cell viability declined in Ishikawa cells with *Pten* overexpression. (F) The cell viability improved in HEC-1-A cells with *Pten* knockdown. LV-Pten: *Pten* enhanced fragment transfection group. LV-Pten-RNAi, *Pten* interference fragment transfection group.

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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