Expression Profiles of the Phosphatase and Tensin Homolog (PTEN), CDH1, and CDH2 Genes, and the Cell Membrane Protein, CD133, in the Ishikawa Human Endometrial Adenocarcinoma Cell Line

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Background: This study aimed to investigate the expression profile of the phosphatase and tensin homolog (PTEN) gene, the cadherin genes, CDH1 and CDH2, and the cell membrane glycoprotein, CD133, in the Ishikawa human endometrial adenocarcinoma cell line.

Material/Methods: The Ishikawa endometrial carcinoma cell groups included cells transfected with the pLVX-puro lentiviral expression vector (the Ishikawa-puro group) and cells transfected with the pLVX-puro-PTEN lentiviral expression vector (the Ishikawa-PTEN group). The mRNA expression of the cadherin genes, CDH1 and CDH2, was detected by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The expression levels of the transmembrane glycoprotein CD133, a cancer stem cell marker, was detected by flow cytometry.

Results: The expression of CDH1 and CDH2 mRNA in the Ishikawa-PTEN cells was lower than in the control cells. CD133 expression was lower in the Ishikawa-PTEN cells compared with the control cells.

Conclusions: This in vitro study showed that in Ishikawa endometrial carcinoma cells, downregulation of PTEN was associated with the expression of the CDH1 and CDH2 genes and upregulated expression of the cell membrane glycoprotein, CD133, which are associated with epithelial-mesenchymal transition (EMT) in malignancy. These findings support the need for further studies to investigate the potential role of PTEN in invasion and metastasis in endometrial carcinoma.

MeSH Keywords: Cadherins • Endometrial Neoplasms • Epithelial-Mesenchymal Transition • PTEN Phosphohydrolase

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Background

Endometrial cancer is one of the most common malignant tumors of the reproductive tract in women, and the molecular mechanisms involved in the etiology and pathogenesis continue to be investigated. The phosphatase and tensin homolog (PTEN) gene was the first tumor suppressor gene with phosphatase activity to be identified [1,2]. The phosphatase activity of PTEN can inhibit signaling pathways involved in oncogenesis, including the phosphatidylinositol-3-kinases (PI3K)/protein-serine-threonine kinase (AKT) pathway and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, resulting in the inhibition of cell growth and increasing cell apoptosis [1–4].

Studies have shown that mutation or deletion of the PTEN gene and the abnormal expression of the PTEN protein are present in endometrial carcinoma, breast carcinoma, glioma, prostate carcinoma, colorectal carcinoma, gastric carcinoma, and other tumors [5–8]. However, the association between expression of the PTEN gene and in endometrial carcinoma remains to be investigated. Currently, there have been few studies on the use of PTEN gene transfection in endometrial carcinoma cells in vitro to investigate the effects on the expression of factors associated with epithelial-mesenchymal transition (EMT), which may indicate the involvement of PTEN in invasion and metastasis in endometrial carcinoma.

Therefore, this study aimed to investigate the expression profile of the PTEN gene, the cadherin genes, CDH1 and CDH2, and the cell membrane glycoprotein, CD133, in the Ishikawa human endometrial adenocarcinoma cell line, including cell transfection with PTEN.

Material and Methods

Materials

The Ishikawa endometrial carcinoma cell line was purchased from Nanjing KeyGen Biotech Co., Ltd. (cat. no. KG314). The cells were cultured in RPMI 1640 medium containing inactivated fetal bovine serum (FBS) and 8,000 U/ml penicillin and 8 mg/ml streptomycin. The lentivirus packaging transfection reagent and the Common Plasmid Small Extraction kit I were purchased from Taihe Biotechnology Co., Ltd. (Beijing, China). UltraSYBR Mixture (High ROX) was purchased from Taihe Biotechnology Co., Ltd. (Beijing, China). The primers were synthesized by Igenbio Inc. (Chicago, IL, USA).

Anti-human CD133-phycoerythrin (PE) was purchased from Guangzhou Jetway Biotechnology Co., Ltd. (Guangzhou, China) (cat. no. 8512133841). All reagents were prepared on the day of use.

Cell lines and cell culture

The Ishikawa endometrial carcinoma cell groups included cells transfected with the pLVX-puro lentiviral expression vector (the Ishikawa-puro group) and cells transfected with pLVX-puro-PTEN lentiviral expression vector (the Ishikawa-PTEN group). The culture medium used was RPMI-1640 complete medium with 10% FBS. The cells were cultured in an incubator with 5% CO₂ at 37°C and 95% relative humidity.

The 293T cell line, derived from human embryonic kidney 293 cells, is a highly transfectable cell line. Frozen 293T cells were removed from storage in liquid nitrogen. After thawing over a 37°C water bath, the 293T cells were inoculated into a 25 cm² culture flask, and DMEM containing 10% calf serum was added and cultured at 5% CO₂ at a temperature of 37°C and 95% relative humidity in an incubator. When the cells grew into a monolayer and reached cell confluence of >90%, the medium was removed, and 500 μl of 0.25% trypsin was added. The cells were digested at room temperature and observed under a microscope. When the cells became shrunken and rounded, DMEM was added immediately, and the medium was repeatedly pipetted to form a cell suspension. Cells were counted using a hemocytometer, and the cell density was adjusted using DMEM containing 10% calf serum. Cells were inoculated into a 10 cm cell culture dish, at approximately 5×10⁴ cells per dish in 10 ml of complete medium. The cells were cultured in an incubator at 95% relative humidity in 5% CO₂ at 37°C. After 24 h, when the cell density reached 80%, they were cultured in a dish measuring 150 mm.

Lentivirus packaging

On the day of cell lentivirus packaging, after 24 h of separation from the dish, 293T cells were re-plated with 20 ml of DMEM and 10% FBS with 4 mM glutamine (Gln), and incubated for 2 h in 5% CO₂ at 37°C, and 95% relative humidity. A total of 27 μg GSTM5 lentivirus plasmids and two helper plasmids were added to 1 ml of saline, then gently mixed. After standing for 5 min, three times the amount of plasmid was added to the transfection reagent in polyethyleneimine, which was incubated at room temperature for 20 min then slowly added into the 150 mm culture dishes and shaken gently. This time point was identified as the beginning of transfection. After 6 h of transfection, the cells were rinsed twice with a moderate amount of PBS, and the medium was replaced with DMEM and 10% FBS with 4 mM Gln and 1% penicillin/streptomycin.
**Table 1.** The primers for GAPDH, CDH1, and CDH2.

| Primer   | Forward (5’→3’)                  | Reverse (5’→3’)                  | Length |
|----------|----------------------------------|----------------------------------|--------|
| GAPDH    | TGCACCAACAACTGCTTAGC             | GGCATGGAGCTGTGGCATGAG             | 87 bp  |
| CDH1     | CACCCAGGGCTTGGGATTTTG            | TGGGGCTTTCTACATACCC              | 140 bp |
| CDH2     | ATCCGTTATCCTTGTGCTG             | GTCCTGCTTTCTTCTTCC              | 153 bp |

CDH – cadherin; bp – base pair.

**Virus collection**

The supernatant was collected at 48 h and 72 h after transfection, centrifuged at 1,000 rpm for 5 min, and the supernatant was filtered with a 0.45 μm micropore filter. The solution was centrifuged at 50,000×g at 4°C for 2 h in an ultrahigh-speed refrigerated centrifuge. The supernatant was discarded, the viral precipitate was fully dissolved with 1 ml PBS, and then filtered with a 0.22 μm micropore filter and dispensed into a 100 μl/tube, and the concentrated aliquots were stored in a −80°C freezer.

**Ishikawa cell passage and plating**

RPMI 1640 complete medium, trypsin-EDTA, and PBS were pre-warmed to room temperature. The culture medium from the dish was removed, and the cells were washed twice with PBS to remove the residual serum. After the PBS was removed, an appropriate amount of trypsin-EDTA was added, then gently oscillated to ensure even coverage of the surface of the culture vessel. After digestion for about 1 min, the cells appeared rounded and the gaps between them were seen to enlarge when viewed under the microscope. The digestion was terminated by the addition of 2 ml RPMI 1640 complete medium. After pipetting the liquid from the culture and gently blowing the surface of the culture dish repeatedly, the cells were removed from the culture dish, transferred into a centrifuge tube, and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and 5 ml of complete DMEM was added. The culture medium was repeatedly pipetted to produce a cell suspension, and a small sample was counted. According to the results of the cell counting, 0.5×10^5 cells/well were inoculated into a six-well plate, and three wells were inoculated.

**Ishikawa cell transfection**

When the Ishikawa cells were fully adherent and had grown to 50% confluence, the transfection was performed. Before transfection, 10 μg/ml of polybrene was incubated with the cells for 1 h and was then replaced with 1,000 μl of fresh complete DMEM in each well. A volume of 100 μl of the pLVX-puro-PTEN lentiviral expression vector (the Ishikawa-puro group) was added into one well, and another well was supplemented with 100 μl of the pLVX-puro lentiviral expression vector (the Ishikawa-puro group). A further well of cells was used as a blank control group. The cells were incubated at 95% relative humidity with 5% CO2 at 37°C. After transfection for 6 h, the culture medium was aspirated and replaced with RPMI 1640 complete medium, and the cells were returned to the incubator at 35% CO2 and 37°C with 95% relative humidity. After 48 h, the cells were subcultured and collected for further study.

**Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) for expression levels of CDH1 and CDH2 mRNA**

The extraction of total RNA from Ishikawa cells was performed with TRIzol® reagent. The first cDNA chain was synthesized from 10 μl RNA with the HiFiScript kit. The PCR primers were designed using Primer Premier 5.0 software (Table 1), with GAPDH as an internal reference. The PCR reaction conditions were as follows: incubation at 42°C for 5 min, 85°C for 15 min, and a further 40 cycles at 98°C for 10 min, 98°C for 15 sec, and 60°C for 34 sec with the collection of the fluorescence signals. At the end of the cycles, the melting curve was obtained at between 60°C and 98°C.

**CD133 expression by flow cytometry**

In the Ishikawa-puro group, a small fraction of cells was collected into an Eppendorf tube without the CD133 antibody, as a blank control group (Control). In the study group, 5 μl of anti-CD133 antibody was added to samples from the Ishikawa-PTEN group and the Ishikawa-puro group. The cells were incubated in the dark for 30 min, centrifuged at 1,000 rpm for 5 min, and washed twice with PBS. A volume of 500 μl PBS was added, and the solution was resuspended. The rate of positive detection of the CD133 cell membrane marker was detected by flow cytometry.

**Statistical analysis**

Data were analyzed using SPSS version 20.0 software (IBM, Chicago, IL, USA). Data were expressed as the mean±standard deviation (SD) and underwent analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant difference.
Results

Expression levels of CDH1 and CDH2 mRNA in Ishikawa cells

In different samples, in order to reflect the differential expression in the gene to be tested, factors such as the experimental error and sample size should be included. Therefore, samples were normalized to the cycle quantification value (Cq value) of the reference gene. The difference in gene expression was expressed by the relative quantification (RQ) value derived from the Cq value. The results showed that the expression of CDH1 mRNA in Ishikawa-PTEN cells was 0.62 times greater than in the Ishikawa-puro cells. The expression of CDH2 mRNA in Ishikawa-PTEN cells was 2.07 times greater than that of Ishikawa-puro cells (Figure 1). The difference between the two groups was statistically significant (P<0.05).

Table 2. Expression rates of CD133 by flow cytometry.

| Group          | R2 (%) (gated on P01.R1) | R3 (%) (gated on P01.R1) |
|----------------|--------------------------|--------------------------|
| Control        | 0.95                     | 0.63                     |
| Ishikawa-puro  | 74.26                    | 61.60                    |
| Ishikawa-PTEN  | 70.06                    | 56.00                    |

PTEN – phosphatase and tensin homolog. Ishikawa endometrial carcinoma cells transfected with the pLVX-puro lentiviral expression vector were the Ishikawa-puro group and cells transfected with pLVX-puro-PTEN lentiviral expression vector were the Ishikawa-PTEN group.

The positive expression rate of CD133 was compared between the Ishikawa-puro group and the Ishikawa-PTEN group. The Ishikawa-PTEN group showed a decreasing trend compared with the Ishikawa-puro group (Table 2). The results of the

Figure 2. Expression of CD133 by flow cytometry in the control cells. CD133 cell membrane expression in the control group by flow cytometry. Each red dot shows a CD133-positive cell.
flow cytometry analysis of the blank control group (without the CD133 antibody) is shown in Figure 2, the Ishikawa-puro group is shown in Figure 3, and the Ishikawa-PTEN group is shown in Figure 4.

**Discussion**

Malignant tumors, including endometrial carcinoma, are characterized by the properties of local invasion and metastasis, which are associated with poor prognosis. Epithelial-mesenchymal transition is recognized as an important requirement for the local invasion of malignant cells [9,10]. EMT was first described in the development of mammalian embryos and involves the transformation of epithelial cells into mesenchymal cells, which increase the chance of invasion and is associated with tumor stem cell characteristics [11–14]. Downregulation of epithelial markers is followed by upregulation of the expression of mesenchymal markers, which causes epithelial cells to lose their cell polarity and intercellular junctions. In EMT, there is loss in epithelial cell adhesion and an increase in the expression of extracellular matrix metalloproteinases (MMPs), which
facilitates cancer cell invasion [15]. There are many markers involved in the process of EMT, including epithelial markers, such as CDH1 (E-cadherin) and cytokeratin, and interstitial markers, including CDH2 (N-cadherin), vimentin and α-smooth muscle actin [16,17].

Cadherins are calcium-dependent transmembrane glycoproteins that mediate cell junctions in the epithelial tissue to maintain tissue polarity and integrity [18,19]. Cadherins include E-cadherin, P-cadherin, and N-cadherin, and changes in the expression of E-cadherin and N-cadherin are considered as key steps in the mechanism of tumor EMT [20,21]. CD133 is a member of the prominin family and is a transmembrane glycoprotein with a molecular weight of 120 kD. CD133 is composed of 865 amino acids and is expressed in mesenchymal stem cells, endothelial progenitor cells, and other cells [22,23]. CD133 expression has been identified in several types of tumor stem cells, which have the biological characteristics of a malignant phenotype and invasive ability [24–26]. Therefore, CD133 is currently recognized as a cell membrane marker of tumor stem cells. Dahching et al. [27] isolated and detected CD133-positive cells from Ishikawa human endometrial adenocarcinoma cells, and showed that these cells had a stronger clonogenic ability, chemotherapy tolerance, and tumorigenicity in vitro.

In the present study, quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and flow cytometry were used to study CDH1, CDH2, and CD133 in Ishikawa-PTEN cells. The results showed that the expression of CDH1 mRNA in Ishikawa cells transfected with the pLVX-puro-PTEN lentiviral expression vector (Ishikawa-PTEN cells) was lower than that in Ishikawa- cells transfected with the pLVX-puro lentiviral expression vector (Ishikawa-puro cells). The expression of CDH2 mRNA in Ishikawa-PTEN cells was higher than that in Ishikawa-puro cells. These results indicated that, in Ishikawa cells studied in vitro, the PTEN gene regulated the expression profile that was characteristic for EMT. The knockdown of PTEN increased the expression of CDH1, with low expression of CDH2. The positive expression rate of CD133 in Ishikawa-PTEN cells was lower than that in Ishikawa-puro cells. Given the biological characteristics of tumor stem cell expression of CD133, these results suggest that the PTEN gene may have a role in reducing the malignant phenotype and invasive ability Ishikawa human endometrial adenocarcinoma cells studied in vitro. These preliminary findings require further functional in vitro studies, including cell migration studies, and in vivo studies.

**Conclusions**

The aims of this study were to investigate the expression profile of the phosphatase and tensin homolog (PTEN) gene, the cadherin genes, CDH1 and CDH2, and the cell membrane glycoprotein, CD133, in the Ishikawa human endometrial adenocarcinoma cell line. Ishikawa cells were transfected with PTEN, and the expression levels of CDH1 and CDH2 mRNA and the CD133 cell membrane glycoprotein were evaluated and compared between the cell groups. Downregulation of PTEN was associated with the expression of the CDH1 and CDH2 genes and upregulated the expression of CD133, which are associated with epithelial-mesenchymal transition (EMT) in malignancy. These findings support the need for further studies to investigate the potential role of PTEN in invasion and metastasis in endometrial carcinoma.

**Conflict of interest**

None.

**References:**

1. Li J, Yen C, Liao D et al: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 1997; 275: 1943–47
2. Carnero A, Blanco-Aparicio C, Renner O et al: The PTEN/PI3K/AKT signalling pathway in cancer; therapeutic implications. Curr Cancer Drug Targets, 2008; 8(3): 187–98
3. Lim HJ, Crowe P, Yang JI: Current clinical regulation of PI3K/PTEN/Akt/mTOR signalling in treatment of human cancer. J Cancer Res Clin Oncol, 2015; 141: 671–89
4. Ebbesen SH, Scatrihl M, Biaiuchu CU et al: PTEN loss promotes MAPK pathway dependency in HER2/neu breast carcinomas. Proc Natl Acad Sci USA, 2016; 113: 3030–35
5. Francesca M, Irene T, Flavia S et al: Pilot investigation of the mutation profile of PIK3CA/PTEN genes (PI3K pathway) in grade 3 endometrial cancer. Oncol Rep, 2018; 41: 1560–74
6. Kalyshloz L, Kafshdiz L, Tavibi AD et al: Role of exon 7 PTEN gene in endometrial carcinoma. Asian Pac J Cancer Prev, 2015; 16: 4521–24
7. Chowdhury S, Ongchin M, Wan G et al: Restoration of PTEN activity decreases metastases in an orthotopic model of colon cancer. J Surg Res, 2013; 184: 755–60
8. Klymkowsky MW, Savagner P: Epithelial-mesenchymal transition: A cancer researcher’s conceptual friend and foe. Am J Pathol, 2009; 174: 1588–93
9. Vu T, Datta PK: Regulation of EMT in colorectal cancer: A culprit in metastasis. Cancers (Basel), 2017; 9(12): pii: E171
10. Cha YH, Yook JI, Kim HS et al: Catabolic metabolism during cancer EMT. Arch Pharm Res, 2015; 38: 315–20
11. Li Y, Wang W, Wang W et al: Correlation of TWIST2 up-regulation and epithelial-mesenchymal transition in cervical carcinoma. Gynecol Oncol, 2012; 124: 112–18
12. Dhamija S, Diederichs S: From junk to master regulators of invasion: LncRNA functions in migration, EMT and metastasis. Int J Cancer, 2016; 139: 269–80
13. Rhim AD, Mirek ET, Aiello NM et al: EMT and dissemination precede pancreatic tumor formation. Cell, 2012; 148: 349–61
14. Qian CN, Mei Y, Zhang J: Cancer metastasis: issues and challenges. Cancer, 2017; 36: 108–11
15. Takahashi E, Inoue T, Fujimoto T et al: Epithelial mesenchymal transition-like phenomenon in trabecular meshwork cells. Exp Eye Res. 2014; 118: 72–79
16. Li C, Ma H, Wang Y et al: Excess PLAC8 promotes an unconventional ERK2-dependent EMT in colon cancer. J Clin Invest, 2014; 124: 2172–87

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17. Zuo Li, Zhang J, Liu LZ et al: Cadherin 6 is activated by Epstein-Barr virus LMP1 to mediate EMT and metastasis as an interplay node of multiple pathways in nasopharyngeal carcinoma. Oncogenesis, 2017; 6: 402
18. Nezami MA, Hager S, Garner J: EMT and anti-EMT strategies in cancer. J Cancer Ther, 2015; 11: 1013–19
19. Ding XM: MicroRNAs: Regulators of cancer metastasis and epithelial-mesenchymal transition (EMT). Cancer, 2014; 33: 140–47
20. Komai K, Niwa Y, Sasazawa Y et al: 150 Pirin downregulates E-cadherin gene expression and contributes to EMT. Eur J Cancer, 2014; 50: 51
21. Jin L, Chen J, Li L et al: CRH suppressed TGFβ1-induced epithelial-mesenchymal transition via induction of E-cadherin in breast cancer cells. Cell Signal, 2014; 26: 757–65
22. Abdelbary EH, Rashed HE, Ismail EI et al: Prognostic value of cancer stem cell markers CD133, ALDH1 and nuclear β-catenin in colon cancer. Oncol Transl Med, 2014; 13: 379–85
23. Elbasateeny SS, Salem AA, Abdelsalam WA et al: Immunohistochemical expression of cancer stem cell related markers CD44 and CD133 in endometrial cancer. Pathol Res Pract, 2016; 212(1): 10–16
24. Miller TJ, McCoy MJ, Hemmings C et al: The prognostic value of cancer stem-like cell markers SOX2 and CD133 in stage III colon cancer is modified by expression of the immune-related markers FoxP3, PD-L1 and CD3. Pathology, 2017; 49: 721–30
25. Kim TM, Ko YH, Ha SI et al: Impact of in vitro driven expression signatures of CD133 stem cell marker and tumor stroma on clinical outcomes in gastric cancers. BMC Cancer, 2019; 19: 119
26. Shang C, Lang B, Meng L: Blocking NOTCH pathway can enhance the effect of EGFR inhibitor through targeting CD133+ endometrial cancer cells. Cancer Biol Ther, 2018; 19: 113–19
27. Dahching D, Hwanwun L, Yu-Hsun C et al: Expression of CD133 in endometrial cancer cells and its implications. J Cancer, 2017; 8: 2142–53