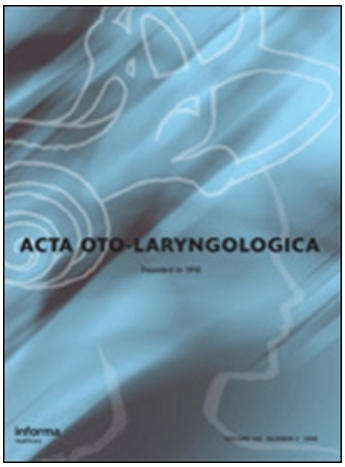

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ORIGINAL ARTICLE

Expression of PTEN and phosphorylated Akt in human cholesteatoma epithelium

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Abstract
Conclusion. We found a reduced PTEN and an increased phosphorylated Akt (p-Akt) expression in cholesteatoma epithelium when compared with retro-auricular (RA) skin. The negative correlation between cholesteatoma PTEN and p-Akt may suggest that cellular survival mechanisms may be involved in cholesteatoma epithelial hyperplasia. *Objectives*. The tumor suppressor PTEN regulates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and modulates cell cycle progression and cell survival. We hypothesized that PTEN might be involved in this pathway mechanism in cholesteatoma. *Materials and methods*. Western blotting and immunohistochemistry were used to examine the expression of PTEN and p-Akt in 16 cases of cholesteatoma and paired cases of RA skin. *Results*. In cholesteatoma, p-Akt expression was significantly higher than in RA skin, whereas PTEN expression was significantly lower in cholesteatoma when compared with skin ($p < 0.05$). Immunohistochemical analysis showed that weak PTEN immunoreactivity was observed in the nuclei of cholesteatoma epithelium, whereas strong PTEN immunoreactivity was detected in the nuclei of skin. Also, strong p-Akt immunoreactivity was observed in the cytoplasm of cholesteatoma epithelium, whereas very weak or no p-Akt immunoreactivity was observed in the RA skin. Furthermore, we found that a significant inverse correlation exists between PTEN and p-Akt expression ($r = -0.796$).

Keywords: *PTEN, Akt, cholesteatoma, middle ear*

Introduction

Cholesteatoma, which is characterized by the presence of keratin-producing squamous epithelium in the middle ear, mastoid, or petrous apex, is still a major problem in the practice of otolaryngology. Epithelial cells of cholesteatoma are characterized by certain tumor-like features such as increased proliferation, atypical differentiation, and chromosomal aberrations [1,2], although cholesteatoma is not a malignancy. The molecular and cellular processes resulting in the clinical hallmarks of cholesteatomas, namely migration, uncoordinated proliferation, altered differentiation, and aggressiveness, have not been fully understood. A recent report by Huisman et al. shows that the keratinocyte of cholesteatoma epithelium may be protected against programmed cell death, and the most plausible mechanism for the survival process of epithelial cells is the phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B (PI3K/Akt/PKB) signaling pathway [3]. Activated Akt by phosphorylation at position Ser 473 has been demonstrated to increase keratinocyte survival [4]. However, the mechanism of Akt activation in cholesteatoma epithelium is still unknown. The phosphatase and tensin homologue deleted on chromosome ten (PTEN) is known as a tumor suppressor gene located on human chromosome 10q23 [5]. PTEN has been shown to play an important role in the pathogenesis of a variety of human cancers [6,7]. As a lipid phosphatase PTEN can dephosphorylate phosphatidylinositol (3,4,5) triphosphate (PIP3) at the 3-prime position of the inositol ring. PIP3 is the phosphorylation product of the interaction of PI3K and phosphatidylinositol (4,5) biphosphate (PIP2) [5]. The conversion from PIP2 to PIP3 by PI3K leads to the activation of Akt and other downstream effectors. Thus, PTEN

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antagonizes signaling through the PI3K pathway, and inhibits Akt activation. Indeed, a recent report shows that loss of PTEN function increases the level of phosphorylated Akt in clinical specimens of endometrial carcinoma [7]. High levels of PTEN are associated with low levels of p-Akt, which leads to the induction of apoptosis [8]; hence, loss of PTEN function leads to increased activity of Akt and subsequently cell survival [6,9]. Therefore, it is reasonable that investigation of PTEN expression may be a key step for understanding cellular survival in cholesteatoma epithelium. We hypothesized that PTEN may be involved in the PI3K/Akt/PKB signaling pathway and an inverse correlation between PTEN and p-Akt level may be related to the cellular hyperplasia mechanism in cholesteatoma epithelium. To determine the role of PTEN in cholesteatoma, the expression of PTEN and p-Akt in middle ear cholesteatoma epithelium was examined and compared to that of normal retro-auricular (RA) skin. We used Western blot analysis to determine the quantitative expression of PTEN and p-Akt protein in middle ear cholesteatoma and normal RA skin.

Materials and methods

Subject selection

Acquired cholesteatoma tissues diagnosed clinically and confirmed pathologically after surgery were used in this study. All cholesteatomas were extended to the mastoid, with varying degrees of attic bone destruction with keratin retention. In total, 16 samples of cholesteatoma were obtained from patients, ranging in age from 45 to 60 years (mean age 52), who had undergone middle ear surgery for cholesteatomatous otitis media between November 2006 and May 2007 at the Department of Otolaryngology, Kyung Hee University Hospital, Seoul, Korea. Normal RA skin as a control was obtained during ear surgery. The Institutional Review Boards (IFC/IRB) of the Kyung Hee University East-West Neo Medical Center (Seoul, Korea) approved the protocol of this study (IRB approval no. KHNMC IRB2008-007). The harvested tissues were kept in Eppendorf tubes and frozen at -70°C before use.

Western blotting

Tissues from cholesteatoma and normal RA skin were homogenized in a lysis buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM $Na_2P_2O_7$, 10 mM NaF, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM vanadate, and 1 mM PMSF. All chemicals used in Western blotting were purchased from Sigma (St Louis, MO, USA). Tissue homogenates were incubated for 20 min at 4°C and centrifuged at 25 000 *g* for 30 min at 4°C. The protein level of the supernatant was determined using the BCA assay (Pierce, Rockford, IL, USA). Protein sample (30 μg) was separated by SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) by electrophoresis. The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with polyclonal antibodies against p-Akt (Ser473) and Akt (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), monoclonal antibodies against PTEN (1:500 dilution; BD Biosciences, San Jose, CA, USA), and β-actin (1:5000 dilution; Sigma). The primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were visualized by chemiluminescence using Supersignal (Pierce). The relative density of each band on Western blots was measured and analyzed by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA, USA). Background in films was subtracted from the optical density measurements. The gels shown in figures are representative of results from three separate experiments.

Immunohistochemistry

Cholesteatoma and normal RA skin were fixed in 4% formaldehyde, dehydrated in graded ethanols (70%, 95%, and 100%), and finally embedded in paraffin. Paraffin-embedded tissues were cut into 10 μm sections using a paraffin block cutter (Sahndon, UK) and mounted on adhesive slides (poly-L-lysin, Sigma no. P8920). For immunohistochemistry, sections were de-paraffinized followed by rehydration and treated in an autoclave at 121°C for 10 min in a 10 mmol/L citric acid buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation in 0.5% H_2O_2 in methanol for 20 min. Sections were then processed for immunohistochemistry using antibodies against PTEN (1:50 dilution, diagnostic BioSystems, Pleasanton, CA, USA) and p-Akt (1:50 dilution, Cell Signaling Technology) for 2 h at room temperature. The ABC method was used to detect cells labeled specifically using a Vectastain kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine (DAB) was used as a substrate for peroxidase. Images were collected using an Olympus microscope and SPOT™ (Diagnostic Instrument Inc.). In all immunohistochemistical controls, reaction to the substrate was absent if the primary antibody was

omitted or if the primary antibody was replaced by a non-immune control antibody. Some sections were counterstained with hematoxylin to visualize the nuclei of the epithelium.

Data analysis

The data obtained in this study showed normal distribution when checked by the Shapiro-Wilk test. Data are expressed as mean $\pm$ SD. To compare the means of paired variables, we used the paired samples t test. The level of significance was set at $p < 0.05$. To determine the association between p-Akt and PTEN, the Pearson's correlation test based on the results of Western blot analysis was used to calculate correlations. Correlation was considered significant at the 0.05 level. The SPSS10 software package (SPSS, Chicago, IL, USA) was used for the calculations.

Results

Quantitative expression of PTEN and p-Akt in cholesteatoma

To investigate the expression of PTEN and p-Akt in cholesteatoma, Western blot analysis was performed by using the specific antibody against p-Akt, Akt, and PTEN. The stromal levels of PTEN and p-Akt are too low to influence Western blotting (see the section on immunohistochemistry, below). We first examined the expression of PTEN in middle ear of cholesteatoma and compared it with that of normal RA skin. As visualized in a representative Western blot of samples from 4 patients (Figure 1A), the level of PTEN expression was markedly reduced in all cholesteatoma from 16 patients when compared with that of normal skin. The level of Akt expression was not changed. Quantitative analysis of Western blots showed that the level of PTEN expression was significantly lower in cholesteatoma than that in control skin (Figure 1B). By contrast, the level of p-Akt expression was markedly increased in cholesteatoma epithelium when compared with that of normal skin (Figure 1C). In addition, relative PTEN and p-Akt levels in representative Western blots were 0.684, 0.476, 0.383, and 0.636 in cholesteatoma and 2.094, 7.750, 1.886, and 1.625 in normal RA skin, respectively. The relative protein level was normalized to the Western blot intensity of β-actin.

Immunohistochemistry of PTEN and p-Akt expression in cholesteatoma epithelium

Immunohistochemistry was performed by using the specific antibody against p-Akt and PTEN. There was no signal in the negative control tissues. Immunohistochemical study revealed a decrease in PTEN expression in cholesteatoma as compared with that in normal RA skin (Figure 2A). Normal RA skin tissue showed a strong PTEN immunoreactivity in the nuclei of epithelium. There was a difference in expression profile: basal and suprabasal PTEN expression in control epithelium and weak, basal expression in cholesteatoma epithelium. By contrast, cholesteatoma showed a weak PTEN immunoreactivity in the nuclei of cholesteatoma

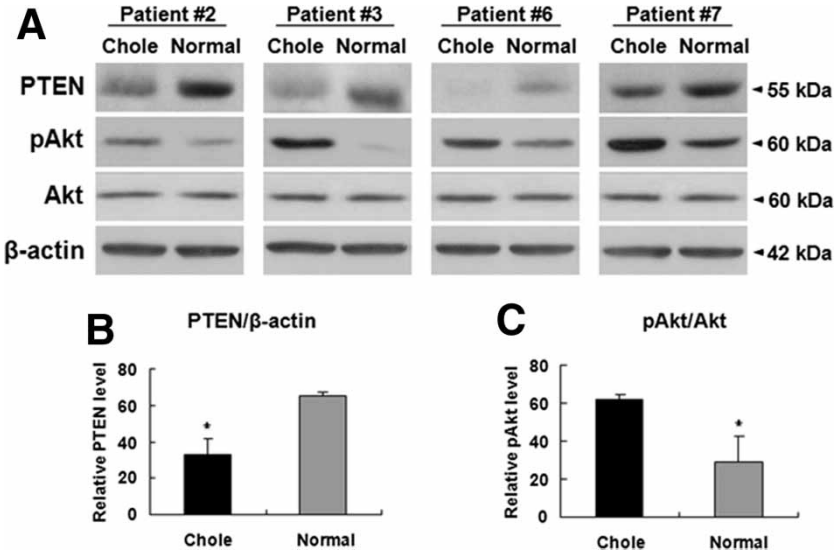

Figure 1. Western blot analysis of PTEN and p-Akt in cholesteatoma and normal retro-auricular (RA) skin. (A) Representative Western blots of PTEN and p-Akt in cholesteatoma and normal RA skin from four patients. The level of Akt expression was not changed. (B, C) Quantitative analysis of Western blots of PTEN and p-Akt from 16 patients. Note that PTEN expression was significantly decreased in cholesteatoma whereas p-Akt level was significantly higher in cholesteatoma as compared with control skin. β-Actin was used as an internal control. Data are presented as mean $\pm$ SD of three independent experiments. $^{*}p < 0.05$.

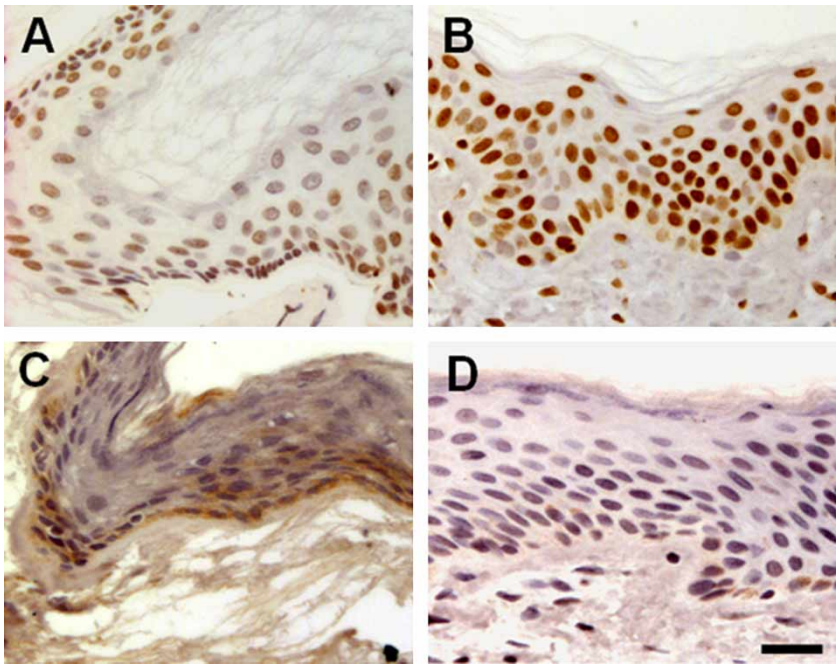

Figure 2. Immunohistochemistry of PTEN and p-Akt in cholesteatoma epithelium and normal retro-auricular (RA) skin obtained from patient no. 2. (A) PTEN in cholesteatoma epithelium; (B) PTEN in normal RA skin; (C) p-Akt in cholesteatoma epithelium; (D) p-Akt in normal RA skin. Note that strong PTEN immunoreactivity is observed in nuclei of the epithelium from normal RA skin, whereas there is weak expression in the nuclei of cholesteatoma epithelium. Also, strong p-Akt immnoreactivity is observed in the cytoplasm of epithelium, especially in the basal and suprabasal layers of cholesteatoma epithelium. Bar = 300 μm.

epithelium. In cholesteatoma tissue, strong p-Akt immunoreactivity was observed in the cytoplasm of epithelium in the basal and suprabasal layers. However, only very weak cytoplasmic p-Akt immunoreactivity was observed in the basal cells of the epithelium in the normal RA skin. Of the pairs examined, all the cholesteatoma epithelium samples showed strong p-Akt immunoreactivity when compared with control tissues. These immunohistochemical observations were consistent with our Western blotting results.

Analysis of the association between p-Akt and PTEN

To find the association between p-Akt and PTEN, we examined the correlation between PTEN and p-Akt expression. The calculations are based on the results of Western blot analysis. Using Pearson's correlation test, we found that significant inverse correlation exists between PTEN and p-Akt expression in cholesteatoma (Figure 3A, $r = -0.796$). However, there was no correlation between PTEN and p-Akt expression in RA skin (Figure 3B, $r = 0.190$).

Discussion

To elucidate the role of PTEN in the pathogenesis of human cholesteatoma, we examined the expression of PTEN and a putative downstream target molecule, p-Akt,0 in the cholesteatoma and compared them with normal RA skin tissues. In this first report of PTEN expression in cholesteatoma, we found that the level of PTEN expression was significantly down-regulated in cholesteatoma when compared with that in normal RA skin. By immunohistochemical study, we also found that normal RA skin tissues showed strong PTEN immunoreactivity in the nuclei of epithelium, whereas cholesteatoma epithelium showed weak immunoreactivity. These results suggest that down-regulation of PTEN expression, which is highly expressed and activated in normal skin, may be involved in the pathogenesis of human

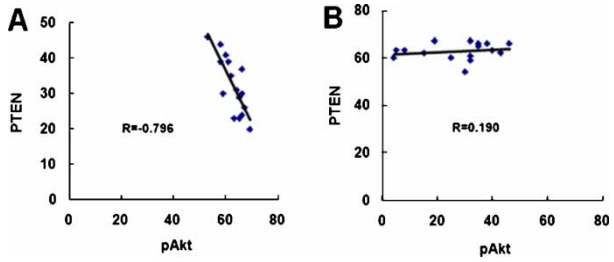

Figure 3. Analysis of correlation between PTEN and p-Akt. The calculations are based on the results of Western blot analysis. (A) The graph shows the correlation between PTEN and p-Akt in cholesteatoma. By Pearson's correlation test, the data show significant inverse correlation between PTEN and p-Akt expression ($r = -0.796$). (B) The graph shows no significant correlation between PTEN and p-Akt in retro-auricular (RA) skin ($r = 0.190$).

cholesteatoma. Western blot and immunohistochemical analyses also revealed that p-Akt was highly expressed in cholesteatoma as compared with levels in normal RA skin. Furthermore, we found that a significant inverse correlation existed between PTEN and p-Akt. These observations support a possibility that Akt activation accompanied by the loss of PTEN might be an important step in development and/or progression of cholesteatoma. Also, we examined the relationships between PTEN/p-Akt and clinical data such as the degree of hearing loss, bone destruction, and invasion. However, we could not find any relationships between PTEN/p-Akt and any clinical data (data not shown). It has generally been accepted that PTEN down-regulates the PI3K-Akt pathway and PTEN inversely correlates with p-Akt [7]. However, it should be noted that such an inverse correlation is not present in all tumors [10]. Our immunohistochemical results suggest that the basal and suprabasal distribution of PTEN expression in normal skin may be implicated with the role of PTEN as a negative regulator of the PI3K/Akt pathway. On the other hand, the loss of PTEN expression in basal cells of cholesteatoma could explain the high proliferative rate of keratinocytes in cholesteatoma epithelium.

Activation of PI3K-Akt pathway is important for the induction of cell proliferation in the basal layer and terminal differentiation in the upper layers of the normal skin [11]. The Akt signalling pathway is initiated after ligand binding with different growth factor receptors similar to the Ras/mitogen-activated protein kinase (MAPK) pathway, and provides survival signals protecting cells from apoptosis [12]. Once activated, p-Akt phosphorylates and deactivates numerous molecules such as Bad, a Bcl-2 family member promoting cell death, and caspase-9, an initiator of caspase-activated DNAse, resulting in suppression of apoptosis and promoting cell survival [12]. In addition, p-Akt controls cycline-dependent kinase inhibitor, p21, and a p53 upstream regulator, MDM2, affecting the RB and p53 pathways [13,14]. Recently, overexpression of p-Akt has been detected in cholesteatoma epithelium when compared with control skin by immunohistochemical study [3]. These findings are in agreement with our results that p-Akt is highly expressed in cholesteatoma epithelium as compared with normal epithelium. However, the activation mechanisms of Akt in cholesteatoma still have not been fully elucidated.

The expression and activation of PTEN are regulated by kinase phosphorylation, membrane recruitment, or oxidation [15]. Thus, the subcellular localization of PTEN is critical to the regulation of its diverse biological properties. During the G0/G1 phase, the presence of PTEN in the nucleus contributes to the maintenance of cell cycle arrest, partially through the activation of AMPK [16]. When the PI3K/Akt signaling cascade is activated during G1-S transition, PTEN is exported to the cytoplasm. Then cytoplasmic PTEN dephosphorylates the cytoplasmic PIP3 to prevent the constitutive activation of Akt signalling pathways [17]. This equilibrium between PTEN and p-Akt is often disrupted in tumor cells. Thus, PTEN is preferentially expressed in the cytoplasm in a variety of tumors in which the p-Akt cascade is frequently activated. Interestingly, in the nucleus PTEN displays Akt-independent growth-suppressing activities [17]. Our immunohistochemical data show that weak nucleic and perinuclear expression of PTEN was observed in cholesteatoma, suggesting that the loss of PTEN may play a role in hyperplasia of cholesteatoma epithelium.

With regard to down-regulation of PTEN, the loss of PTEN expression seems to be more frequent [18], although mutation of PTEN is uncommon in many human tumor types. Recent results suggest that PTEN may be inactivated by several mechanisms other than mutations and/or deletions in a tissue-specific manner, such as promoter methylation [19]. The precise mechanisms for down-regulation of PTEN in cholesteatoma are to be elucidated in future studies. Nevertheless, our data suggest the potential role of PTEN inactivation in cholesteatoma epithelium via multiple mechanisms, ranging from genetic alteration to epigenetic silencing.

Conclusion

We found that a decreased PTEN expression and an increased p-Akt expression were observed in cholesteatoma epithelium as compared with those in normal RA skin. These observations suggest that the activation of Akt and down-regulation of PTEN may be involved in the cellular hyperplasia in patients with cholesteatoma.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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