Original Article
Expression of phosphorylated Akt/mTOR and clinical significance in human ameloblastoma

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Abstract: This study aimed to evaluate the expression of AKT and phosphorylated AKT (p-Akt) in human ameloblastoma (AB). Immunohistochemistry showed human AB was positive for Akt and Akt expression was mainly found in the cytoplasm of epithelial cells. The Akt expression in AB was significantly higher than that in normal oral mucosa (NOM), but still lower than that in oral squamous cell carcinoma (OSCC). NOM was negative for p-Akt, but AB was positive for p-Akt. In some AB tissues, p-Akt expression was found in both cytoplasm and nucleus. Akt expression in AB was significantly different from that in NOM and OSCC. The p-Akt in AB was markedly higher than that in NOM, but lower than that in OSCC. mTOR expressed in cytoplasm in AB, but not in NOM. P-mTOR expressed on cell membrane in NOM, while in cytoplasm and nucleus in AB. Results of western blot assay showed that Akt expression was found in all the AB tissues, and increased in tissues with malignant transformation. In addition, the p-Akt expression also markedly increased in AB, but was still lower than that in OSCC tissues. Compared to NOM, mTOR and p-mTOR expression significantly increased in AB. BandScan 5.0 software was used to detect the optical density of protein bands. Results showed p-Akt, mTOR and p-mTOR expression in AB was markedly different from that in control group.

Keywords: Human ameloblastoma, oral squamous cell carcinoma, Akt

Introduction

Serine/threonine kinase Akt and mammalian target of rapamycin (mTOR) are downstream molecules of phosphoinositide 3-kinase (PI3K) signaling pathway and involve the regulation of multiple signaling pathways such as those related to cell apoptosis, cell cycle and cell proliferation. Akt has been found to play important roles in the occurrence and development of tumors. A variety of studies have confirmed that Akt may be a potential oncogene in humans and over-expression of phosphorylated Akt/PKB (p-Akt/PKB) has been found in some cancers including gastric cancer, ovarian cancer, pancreatic cancer, and breast cancer. Recent studies reveal that p-Akt activation is persistent in some malignancies including endometrial cancer, liver cancer, prostate cancer, colon cancer, colorectal cancer, follicular thyroid cancer and lung cancer, and the abnormal expression of Akt is closely related to the their occurrence, development and sensitivity to radiotherapy and chemotherapy [1]. West et al treated human tracheal epithelial cells with nicotinamide and found that Akt was rapidly activated in these cells which were resistant to apoptosis induced by UV or hydrogen peroxide [2]. Nanawin et al [3] found p-Akt expression increased significantly in human colorectal cancer and p-Akt could facilitate the proliferation of cancer cells and inhibit their apoptosis to promote the occurrence and development of cancer cells. In addition, studies also confirmed that the extent of Akt activation is associated with the clinical progression and pathological processes of cancers such as the extent and depth of invasion, lymph node metastasis and pathological stage. Metastasis is a biomarker of the malignancy of cancers and a major cause of high morbidity and mortality. In ovarian cancer, the increased p-Akt expression is related to the highly invasive capability of cancer cells. In breast cancer and prostate cancer, p-Akt not only promotes the cancer development, but is related to the type of invasion of breast cancer, and cancers at stage III/IV have significantly increased p-Akt expression [4]. In studies on the pancreatic duct adenocarcinoma, elevated p-Akt expression has been found to be an inde-
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Independent risk factor of a poor prognosis. Tsao et al [5] proposed that Akt activation was an early event in the progression of lung cancer and closely related to the metastasis of malignancy. Jiang et al [6] found that the well differentiated esophageal cancer at stage II-IIa and without lymph node metastasis had a significantly lower Akt protein expression when compared with poorly differentiated esophageal cancer at stage IIIb-IV and without lymph node metastasis. Further investigation reveals that Akt is able to promote the invasion and metastasis of cancer cells. Above findings suggest that Akt over-expression may predict a poor prognosis. mTOR, one of the most related proteins with cell proliferation and apoptosis, is a downstream molecule directly regulated by Akt in the P13K/Akt pathway. Recently, mTOR signaling transduction pathway has been found to control many cellular processes which play important roles in the genesis and development of different tumors. These cellular processes include regulations of cell apoptosis, transcription, translation, metabolism, angiogenesis and cell cycle. There are always genetic changes and activations by biochemical limitations of mTOR signaling transduction pathway in the early stage of malignant transformation and the progressive stage of tumors. Meanwhile, the extent of the activation of this pathway is an important indicator to judge the prognosis of patients with tumors [7-10].

However, the expression of Akt in AB is not clearly reported, so the present study aimed to evaluate the expression of AKT and phosphorylated AKT (p-Akt) in AB tissue.

Materials and methods

Sample collection

AB tissues (n = 80) and OSCC tissues (n = 5) were collected from the Department of Stomatologic Pathology of China Medical University from 2007 to 2010. NOM tissues (n = 5) was obtained from the patients receiving surgical removal of wisdom teeth and tooth germ tissues were from the abortus in the No202 Hospital of PLA. Informed consent was obtained from subjects concerned for the use of samples. Tissues were rapidly collected and placed in liquid nitrogen for further use. Classification was done according to the WHO criteria (2005). The primary AB was found in 16 patients, recurrence AB in 12 (9 with recurrence after curettage, and 3 after surgical resection; time interval to recurrence: 4 months to 14 years), and malignant transformation of AB in 2 patients. There were 16 males and 14 females with a median age of 44 years (range: 16-72 years). AB was mainly found in patients aged 31-49 years (36.7%; 11/30). In addition, AB was found in the mandibular bone (80.0%, 24/30; 9 in left mandibular bone, 9 in right mandibular bone and 6 in mandibular symphy- sis), maxillary bone (10.0%, 3/30; 2 in left maxillary bone and 1 in right maxillary bone), gingiva (n = 1) and parapharyngeal space of left palate (n = 1) and left cheek (n = 1). Solid / polycystic AB accounted for 83.3% (25/30), unincystic AB for 3.3% (1/30), desmoplastic AB for 3.3% (1/30), and peripheral AB for 10% (3/30). Moreover, of 25 patients with solid AB, follicular type was found in 14 patients, plexiform type in 5, acanthoma type in 3, basal cell type in 2 and keratotic type in 1.

Detection of Akt, p-Akt, mTOR and p-mTOR expression in human AB by immunohistochemistry

Tissues were embedded in paraffin and cut into sections which were deparaffinized (100% ethanol I and II [10 min for each], 95% ethanol [5 min], 75% ethanol [5 min]). After washing in 0.01 M PBS thrice, sections underwent antigen
retrieval in citrate buffer (50 ml; 0.1 M citrate [9 ml], 0.1 M sodium citrate [41 ml]; pH = 6.0) for 10 min. When the sections were cooled to room temperature, sections were treated with 0.3% H$_2$O$_2$ at room temperature for 10 min. After washing in PBS thrice, sections were blocked in 10% BSA-PBST (containing 0.2% Tween 20) at room temperature for 1 h. The blocking solution was removed, and then sections were incubated with goat Akt (1:100), p-Akt (1:100), mTOR (1:100) or p-mTOR (1:400) antibodies in 1% BSA-PBST at 40°C overnight. Following washing in PBS thrice (10 min for each), sections were incubated with biotin conjugated rabbit anti-goat IgG at room temperature for 2 h. After washing in PBS thrice, sections were treated with Streptavidin-HRP at room temperature for 1 h. After washing in PBS, visualization was done with DAB. Counteracting was done with hematoxylin and mounting with neutral gum. Sections were observed under a light microscope and representative photographs were captured.

Detection of protein expression of Akt, p-Akt, mTOR and p-mTOR by Western blot assay

Extraction of proteins: tissues were mixed with 5 volumes of RIPA buffer (1×PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor) followed by homogenization. After centrifugation at 10000 g for 10 min at 4°C, and supernatant was collected and stored at -70°C.

Protein quantification: 3 ml of Coomassie Brilliant Blue G250 solution (0.4 g of Coomassie Brilliant Blue G250, 100 ml of 95% ethanol, 200 ml of 85% H$_3$PO$_4$, deionized water [final: 2000 ml]), 5 μl of sample and 95 μl of deionized water were mixed. Control group: 3 ml of Coomassie Brilliant Blue G250 solution, 5 μl of RIPA buffer, and 95 μl of deionized water were mixed. The optical density was measured at 595 nm with a visible spectrophotometer, and standard curve was delineated. The protein concentration was determined according to the standard curve.

SDS-PAGE: 10% separating gel and 4% stacking gel were prepared for SDS-PAGE.

Preparation of proteins and electrophoresis: 30 μg of proteins, 1× Reducing sample buffer (5× Reducing Sample Buffer: 0.312 M Tris-HCl [pH 6.8]), 10% SDS, 25% β-mercaptoethanol, 0.25% bromophenol blue, 50% glycerin and additional 1×PBS were mixed (final volume: 10-20 μL). Then, the mixture was centrifuged and boiled for 5 min followed by centrifugation at 10000 rpm for 5 min. Protein samples were loaded, followed by electrophoresis at 80 V.

Protein transferring: The gel was taken out and useless gel was removed. The remaining gel was placed in the transfer buffer (3.03 g of Tris, 14.4 g of glycin, 850 ml of water and 150 ml of methanol), followed by balancing for 30 min. PVDF membrane and filter with similar size to the gel were prepared and then immersed in the above buffer for 5 min. Then, the filter, PVDF and gel were placed on the instrument avoiding the bubble between them, and protein transferring was done at 25 V for 0 min.

Blocking: PVDF membrane was washed in 1×PBS for 2-3 min and then incubated with blocking buffer (5% non-fat milk and 0.1% Tween20 in 1×PBS) over night. The membrane was treated with above Coomassie bright blue R250 overnight, and the staining solution was removed by washing. The protein bands were observed.

Hybridization: The blocking solution was removed, and the membrane was washed with washing solution (0.1% Tween20, 1×PBS) for 5 min. After incubation with UBC9 antibody in hybridization solution (1% BSA, 0.1% Tween20, 1×PBS) (1: 1000) at room temperature for 2 h, the membrane was washed thrice (10 min for each). After incubation with secondary antibody (1:5000) at room temperature for 1 h, the membrane was washed in washing buffer thrice (10 min for each).

Visualization with BCIP/NBT: Protein bands were analyzed with an automatic gel image analysis system and the optical density was determined.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. Data are expressed as mean ± standard deviation. Quantitative data were compared with t test between groups and one way analysis of variance among groups. A value of $P < 0.05$ was considered statistically significant.
**Results**

**Expression and distribution of Akt and p-Akt in AB**

Immunohistochemistry showed Akt expression was at a low level in NOM, but p-Akt expression was not observed in NOM (Figure 1A). In AB, Akt expression was strong positive and p-Akt expression increased significantly. Akt expression was mainly found in the cytoplasm of epithelial cells of cancer tissues (Figure 1B). When compared with OSCC tissues, the expression of Akt and p-Akt reduced significantly (Figure 1C). In some tissues, p-Akt expression was observed in both cytoplasm and nucleus. Statistical analysis showed Akt expression in AB was markedly different from that in NOM and OSCC, and p-Akt expression in AB was significantly higher than that in NOM, but markedly lower than that in OSCC. These findings suggest that AB is a benign tumor (Table 1).

**Expression and distribution of mTOR and p-mTOR in AB**

mTOR did not expressed in NOM, but abnormally expressed in AB (Figure 2A, 2B). p-mTOR in NOM was dyed to be claybank which located on the cell membrane and in the nucleus. However, p-mTOR expressed abnormally in cytoplasm and nucleus in AB, and the staining intensity also increased. The expression on the cell membrane decreased, while abnormal expression in nucleus was always companied with abnormal expression in cytoplasm (Figure 2C-E).

**Expression of Akt and p-Akt in different tissues by Western blot assay**

Western blot assay was employed to detect the expression of Akt and p-Akt in 30 fresh AB tissues, and 5 NOM tissues and 5 OSCC tissues served as controls. Results showed Akt expression was observed in all the tissues, but increased in tissues with malignant transformation. The p-Akt expression increased signifi-

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**Table 1. Expression of Akt and p-Akt in different tissues (IOD, x ± SD)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Akt</th>
<th>p-Akt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABs</td>
<td>7264.27 ± 135.96*</td>
<td>4193.52 ± 95.77*</td>
</tr>
<tr>
<td>OSCC</td>
<td>9933.18 ± 101.34*</td>
<td>5489.99 ± 128.67*</td>
</tr>
<tr>
<td>NOM</td>
<td>1281.3 ± 90.52</td>
<td>237.83 ± 40.69</td>
</tr>
</tbody>
</table>

Footnotes: *P < 0.05 vs. NOM.
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Significantly in AB tissues, but was still lower than that in OSCC tissues (Figure 3). These findings were consistent with those from immunohistochemistry. BandScan 5 software was used to determine the optical density of protein bands, and results showed p-Akt expression in AB was significantly different from that in NOM and OSCC tissues (Figure 4).

Expression of mTOR and p-mTOR in different tissues by Western blot assay

p-mTOR expression was observed in all the tissues (Figure 5A). Expression of p-mTOR in AB (1.06 ± 0.53) was significant higher than that in NOM (0.22 ± 0.05) (P = 0.022). mTOR expression was also observed in all the tissues (Figure 5B). Expression of mTOR in AB (1.27 ± 0.57) was also significant higher than that in NOM (0.12 ± 0.05) (P = 0.029).

Discussion

Abnormal activation of signal transduction is an important step in the occurrence and development of cancers. PI3K/Akt signaling pathway is aberrantly activated in a majority of cancer types, and plays crucial roles in the proliferation, survival, cell movement, resistance to apoptosis, angiogenesis, metastasis and resistance to chemotherapy and radiotherapy. Akt is also known as protein kinase B (PKB) and persistent activation of Akt is closely related to the occurrence and development of cancers. Akt is a core component of PI3K/Akt signaling pathway and has a molecular weight of about 60 kDa. Akt is a serine/threonine protein kinase encoded by pro-oncogene c-akt. Following activation by PI3K, Akt may activate or inhibit downstream target proteins (such as Bad, caspase-9, NF-kB, Forkhead, mTOR, Par-4, P21) via phosphorylation, induce cell proliferation via multiple cytokines and promote cell survival via some pathways. The activation of PI3K/Akt signaling pathway is regulated by a variety of factors. The conformational change in dimmers and the direct binding between Ras and p110 may cause PI3K activation [11], which leads to the production of phosphatidylinositol 3, 4, 5-triphosphate (PIP3). PIP3 binds to the PH domain of intracellular Akt, resulting in the translocation of Akt from cytoplasm to cell membrane and Akt acquiring catalytic activity. Phosphoinositide-dependent kinase (PDK) is also involved in the Akt activation. PDK1 phosphorylates the Thr308 of Akt protein, but has no effect on its Ser473. However, PDK2 (such as integrin-linked kinase [ILK]) protein kinase may phosphorylate Ser473 of Akt protein, resulting in Akt activation [12]. Of note, only the complete phosphorylation of Akt at

Figure 2. A. Negative expression of mTOR in NOM (Immunohistochemistry; SP method). B. Positive expression of mTOR in AB (Immunohistochemistry; SP method). C. Positive expression of p-mTOR on cell membrane and in nucleus in NOM (Immunohistochemistry; SP method). D. E. Abnormal expression of p-mTOR in cytoplasm and nucleus in AB (Immunohistochemistry; SP method).
Ser473 and Thr308 can Akt exhibit its functions. The activated Akt may activate or inhibit its downstream proteins (such as Bad, Caspase9, NF-κB, Forkhead and mTOR) via phosphorylation to regulate the proliferation, apoptosis and migration of cells.

P13K/Akt/mTOR signaling pathway is closely related to cell growth and proliferation [13]. P13K/Akt and LKB1/AMPK signaling pathways function through TSC. Akt phosphorylates TSC2 to prevent TSC complex affecting the activities of Rheb and thus to activate mTOR. mTOR and its related factors play important roles in the genesis and development of tumors [12, 14]. Activated mTOR could lead to quick proliferation of tumor cells, increased secretion of tumor proteins, fast cell cycles and short G1 phase, which facilitate the quick development of tumor [15]. Activation of mTOR signaling transduction pathway could inhibit cell apoptosis induced by multiple irritations, promote progression of cell cycles, accelerate cell survivals an proliferations, be involved in angiogenesis, paly an important role in genesis of tumors, and in the meanwhile be involved in the invasion and metastasis of tumors. The invasion and metastasis of tumors is the main factor which affect the prognosis, while degradation of extracellular matrix is an essential procedure of tumor invasion and metastasis.

The migration and adhesion of cancer cells are crucial for the invasion and metastasis of cancers. (1) Increasing movement of cancer cells: AKT/mTOR/p70s6k pathway (mTOR; Rapamycin-associated protein) (p70s6k, Ribosomal protein). The activated p70s6k may promote the remodeling of filaments of actin to promote cell movement [16]. Kim et al constructed thyroid hormone receptor β gene mutation mouse model (TRβPV mouse), thyroid cancer was induced and distal metastasis of thyroid cancer was also present. Moreover, Akt over-expression was observed at primary cancer and metastatic cancer. Blocker of PI3 K/AKT pathway (LY294002) significantly reduced Akt phosphorylation or decreased the expression of Akt downstream proteins, leading to compromised movement of cancer cells [17]. (2) Regulating Akt1: To inhibit Akt1 activity may reduce the migration of cancer cells. There is evidence showing that Akt1 deficient mouse embryonic
fibroblasts showed compromised migration, but Akt1 over-expression restored the cell migration [18], and small molecular Akt1 inhibitor or silencing of Akt1 by RNA interference were found to inhibit the Aur-A over-expression induced migration of LSCCHep2 cells. (3) Regulating bone morphogenetic protein (BMP) expression: Selectively inhibiting PI3K/Akt pathway may inhibit the NF-κB induced promoter activation of BMP-2. In addition, to inhibit NF-κB activity may down-regulate the transcription of Smad1/5/8, a downstream target of BMP-2 signaling pathway, in prostate cancer, and simultaneously inhibit BMP-2 induced phosphorylation of Smad1/5/8. PI3K/Akt-NF-κB signal axis may regulate the transcription and activation of BMP-2-Smad signaling pathway, resulting in bone metastasis of prostate cancer [19]. (4) Reducing intercellular adhesion: epithelial mesenchymal transition (EMT) makes epithelial cells acquire fibroblast-like properties, which reduces the intercellular adhesion and increases cell movement. Akt may affect the morphological characteristics of epithelial cells, cell movement and invasiveness. Activated Akt may promote EMT to down-regulate E-cadherin and β-catenin expression and up-regulate mesenchymal vimentin expression, which increases the movement of cancer cells on the cellulose membrane which was confirmed by Grille et al [20]. Human squamous cell carcinoma cell line (SCC15 cells) transfected with myr-2AKT (activated AKT) had no the morphological features of squamous epithelial cells, but had the characteristics of fibroblasts. Moreover, myr2A-KT trasnfected SCC15 cells had elevated migration and movement [21]. (5) Regulating tumor protein D52 (TPD52) expression: To inhibit TPD52 may reduce the migration of cancer cells. There is evidence showing that over-expression of TPD52 could increase the migration of prostate cancer cells via αvβ3 mediated PKB/Akt activation [22]. (6) Regulating excelluar matrix (ECM): To degrade ECM is effective to promote the invasion and metastasis of cancer cells and play an important role in the cancer progression. Akt may up-regulate the expression of some matrix metalloproteinases (such as MMP-2) in a PI3K/AKT/mTOR dependent manner. Zhang et al found highly invasive Lewis lung cancer cells (H259 cells) had high MMP expression, especially MMP-2 was activated, and Akt may inhibit the expression of MT1-MMP in these cancer cells via PI3K/Akt pathway, resulting in compromised invasiveness of these cells [21]. (7) Affecting angiogenesis in cancers: Cancer cells may express vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in an epidermal growth factor/PI3K/AKT/rapamycin signaling pathway dependent manner, to promote angiogenesis and growth of cancer cells, resulting in the metastasis of cancers [13].

Conclusion

Our results show Akt expression is observed in all the samples, but Akt expression increases significantly in malignant tissues. The expression of p-Akt expression also elevates in AB, which was lower than that in OSCC. In AB, the expression of Akt and p-Akt increases, suggesting the PI3K/AKT signaling pathway is activated in AB. Thus, the activation of components in this pathway may influence the occurrence of AB via above mechanisms.
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Disclosure of conflict of interest

None.

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