·Basic Research·

Ezrin and inducible nitric oxide synthase in malignant proliferation of salivary gland pleomorphic adenoma

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[Abstract] Background and Objective: Ezrin and inducible nitric oxide synthase (iNOS) may play an important role in regulating tumor proliferation, invasion, and metastasis. This study was to investigate the expression of Ezrin and iNOS and their correlation to malignant proliferation of salivary gland pleomorphic adenoma (PA). Methods: Expressions of Ezrin, iNOS and nuclear Ki-67 antigen were detected by immunohistochemistry in common type (n=40), active type (n=25) and malignant (n=11) pleomorphic adenoma. Results: Expressions of Ezrin, iNOS in common type, active type and malignant pleomorphic adenoma were gradually increased (P<0.05). There was a significant positive correlation between the expression of Ezrin and iNOS (P<0.05). Ki-67 proliferation index (Ki-67 PI) was increased with the increasing of Ezrin and iNOS expressions (P<0.05). Conclusion: Overexpression of Ezrin and iNOS may promote proliferation and malignant transformation of salivary gland pleomorphic adenoma.

Key words: pleomorphic adenoma, Ezrin, inducible nitric oxide synthase, Ki-67 nuclear antigen, proliferation

Pleomorphic adenoma (PA) or known as mixed tumor is the most common tumor of salivary gland, which takes up 50% of reported epithelial salivary tumors. The tumor cells frequently invade membrane and surrounding salivary tissues. They have a tendency of recurrence after surgery and can undergo malignant transformation. It is considered as a borderline tumor with properties between malignancy and benignity. The Ki-67 nuclear antigen is used to determine the activeness of cell proliferation and is an indicator for growth status of benign and malignant tissues. Proliferating cell nuclear antigen (PCNA) can more directly reflect the proliferative status of cells.[1] Ezrin protein is a member with the most representativeness in the ERM family of membrane-cytoskeleton cross-linker protein (Ezrin/Radixin/Moesin) and it is closely related to proliferation, metastasis, and prognosis of the tumor.[2-4] Inducible nitric oxide synthase (iNOS) and its product, NO, play an important role in proliferation, angiogenesis, and prognosis of the tumor.[5,6] Ezrin protein and iNOS are highly expressed in many types of human tumors.[3] This research used immunohistochemical analysis to measure the expressions of Ezrin protein, iNOS, and Ki-67 nuclear antigen in normal, active, and malignant type of PA, so as to investigate the role of Ezrin protein and iNOS in the proliferation and malignant transformation of PA.

Materials and Methods

Clinical data. Tissue samples of PA,which were surgically removed and embedded in paraffin blocks for storage in the Pathology Department, were obtained from the Otolaryngology Department in the Second Affiliated Hospital of Sun Yet-Sun University from January 1998 to December 2007. There were 40 cases of normal type, 25 cases of active type, and 11 cases of malignant type (which were pathologically diagnosed as "cancer in PA"). All lesions were located in parotid glands, salivary glands, and minor salivary glands of the upper palate. The ages of patients were between 17- and 83-year-old, with a medium age of 42.5-year-old. There were 35 males and 41 females. All cases of normal and active PA were diagnosed for the first time and they did not receive any anti-tumor treatment. All malignant cases of PA were derived from benign tumors.

The pathological diagnoses of benign and malignant PA were based on the new classification for otolaryngological tumors after detailed observation and description. For the standard to determine the type of PA, please refer to Table 1.[7,8] In this research, carcinosarcoma (or known as true malignant PA) and metastatic PA were not included in 11 cases of malignant PA. All samples were confirmed by pathologists in our hospital and conformed to original diagnoses.

Test agents. Rat anti-human monoclonal antibody of Ezrin and Rabbit anti-human monoclonal antibody of iNOS were the products of US NeoMaker Company and purchased from Jingmei Bioengineering Company. The rat anti-human monoclonal antibody of Ki-67 nuclear antigen, SP immunohistochemical test kit, DAB colorization kit, and hematoxylin stain were the products of US Sigma Company and were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd.

Experimental procedures. All samples were fixated in 4% paraformaldehyde and embedded in paraffin blocks, before they were sequentially cut into slides with the thickness of 4 m. They were then adhered to pre-treated glass slides. The SP immunohistochemical analysis was performed by following procedures provide in the test kit to determine the expressions of Ezrin protein, iNOS, and Ki-67 nuclear antigen in normal, active, and malignant PA. Citrate buffer was added and these samples were heated in microwave for antigen repair for 17 min. DAB colorization was then performed and these samples were stained by hematoxylin, dehydrated and sealed. PBS was used to replace primary antibodies for negative control. Slides with known positive expressions of Ezrin protein, iNOS, and Ki-67 nuclear antigen were used as positive control.

Deterimation of results. Ezrin protein and iNOS were primarily expressed in cytoplasm of tumor cells and were presented in brownish yellow or brownish granules. In normal cells, they were occasionally expressed, but primarily in the cell membrane. The evaluation criteria were based on the rate of positive tumor cells and the intensity of stain (For each specimen, ten randomly selected viewfields under 400x magnification was observed and at least 1,000 cells were counted). Based on the classification standard by Mathew,[9] these samples were grouped accordingly: negative (-) for non-expression; positive (+) for < 50% of cells with positive expression or light stain; strong positive (++) for ⩾ 50% of cells with positive expression and deep stain. The stain to indicate positive expression of Ki-67 nuclear antigen could be observed in nucleus, while it could also exist in cytoplasm of some tumor cells, where the nucleus was in brown or both the nucleus and cytoplasm were brown. The negative result was identified when only cytoplasm but not the nucleus was stained or when both nucleus and cytoplasm were not stained. Under microscopy with 400x magnification, 100 cells were counted in each viewfield out of all 10 randomly selected fields. Ki-67 PI = total number of positive cells / 1,000 x 100%.

Statistical analysis. SPSS13.0 software was used

Table 1 The classification standard of pleomorphic adenoma

	Growth pattern	Peplos invasion	Tumor constituent	Heteromorphism	Nuclear phase
Common type	One centre	-	Less cells	-	Without karyokinesis
	slow		regular		
Active type	Multicentre	+	More cells	+	With karyokinesis
	fast		irregular		higher karyoplasmic ratio

for statistical analysis. The ^{2}-test was performed to analyze the intergroup variation in the expressions of Ezrin protein and iNOS. The comparison of Ki-67 PI between groups was performed using variance analysis and the LSD-t-test. $P < 0.05$ indicated statistically significant.

Results

Expressions of Ezrin protein and iNOS in benign and malignant PA and their correlations. The positive expressions of Ezrin protein and iNOS were primarily in the cytoplasm of tumor cells and they were in brownish yellow or brown (Fig. 1). In 40 cases of normal PA, there were 23 cases with positive expression of Ezrin protein (57.5%) and 20 cases with positive expression of iNOS (50%). In 25 cases of active PA, there were 18 cases with positive expression of Ezrin protein (72.0%) and 18 cases with positive expression of iNOS (72.0%). In 11 cases of malignant PA, there were 11 cases with positive expression of Ezrin protein (100%) and there were 10 cases with positive expression of iNOS (90.9%). As cell proliferation became active and malignancy of tumor worsened, the rate of positive expression of Ezrin protein and iNOS gradually increased and its intensity amplified. Most malignant PA had strong positive expression of Ezrin protein and iNOS. The expressions of Ezrin protein and iNOS were much higher in malignant PA than in normal and active PA, and were higher in active PA than in normal PA (Table 2). The variations had statistical significance (Ezrin protein: $\chi^2 = 10.6$, $P = 0.005$; iNOS:$\chi^2 = 8.231$, $P = 0.016$). In 76 cases of benign and malignant PA, 44 cases were reported to have positive expressions of Ezrin protein and iNOS (54.0%), while 20 cases had no Ezrin protein and iNOS (26.3%). There were 11 cases with positive expression of Ezrin protein and negative expression of iNOS (14.5%). Analyzed by the χ^2-test, the expressions of Ezrin protein and iNOS in benign and malignant PA were positively correlated ($\chi^2 = 35.62$,

Table 2 Expressions of Ezrin, iNOS and nuclear Ki-67 antigen in 76 cases of benign and malignant pleomorphic adenoma

Tissue	Cases	Ezrin		iNOS		Ki-67 PI
		+	−	+	−	($\bar{x}$±s)
Common type	40	23	17	20	20	23.775±5.886
Active type	25	18	7	18	7	56.560±9.566
Malignant	11	11	0	10	1	95.727±4.982

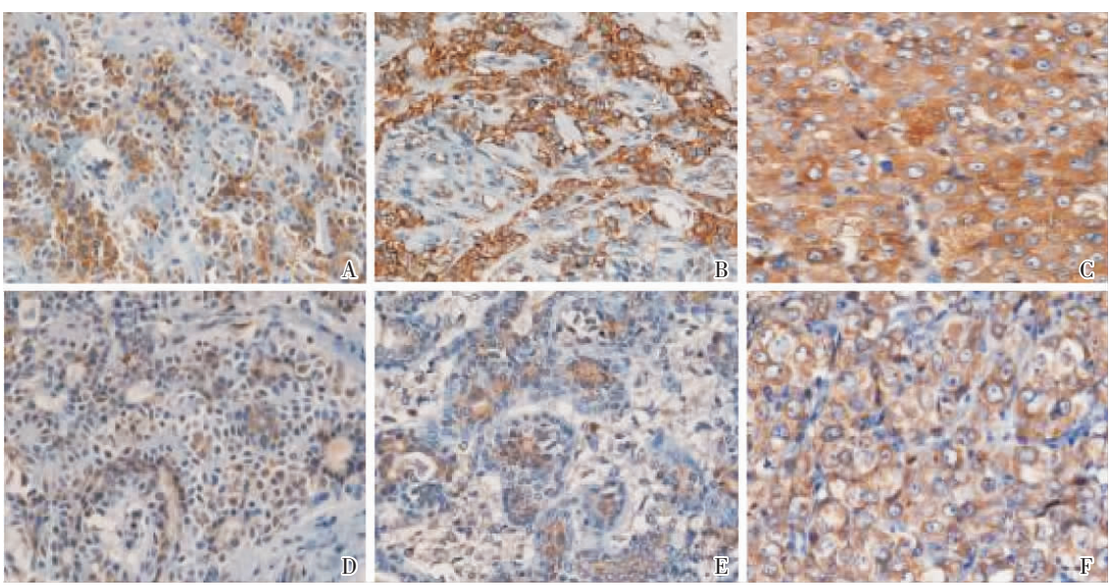

Figure 1 Expressions of Ezrin and iNOS in the cytoplasm of common type, active type and malignant pleomorphic adenoma cells (DAB×400)

A-C: Expression of Ezrin was gradually increased in common type, active type and malignant pleomorphic adenoma. D-F: Expression of iNOS was gradually increased in common type, active type and malignant pleomorphic adenoma.

r= 0.685, P< 0.001).

Expression of Ki-67 nuclear antigen in benign and malignant PA. Positive expression of Ki-67 nuclear antigen primarily showed brownish yellow or brown color in nucleus (Fig. 2). Ki-67 PI was gradually increased in the order of normal, active, and malignant type of PA (Table 2). The variations in Ki-67 PI among the three groups had statistical significance (F-value = 477.65, P < 0.001).

Expressions of Ezrin protein and iNOS in benign and malignant PA and their relationships to Ki-67 PI. In 76 cases of benign and malignant PA, the Ki-67 PIs in the cases with negative and positive expression of Ezrin protein were 26.625 13.256 and 52.923 26.856, respectively, and the variation between the two had statistical significance (t = 5.713, P < 0.001). The Ki-67 PIs in the cases with negative and positive expression of iNOS were 28.286 16.182 and 54.292 27.338, respectively, and the variation between the two had statistical significance (t= 5.209, P< 0.001). It suggested that in benign and malignant PA, patients with high expressions of Ezrin protein and iNOS also had a high level of Ki-67 PI.

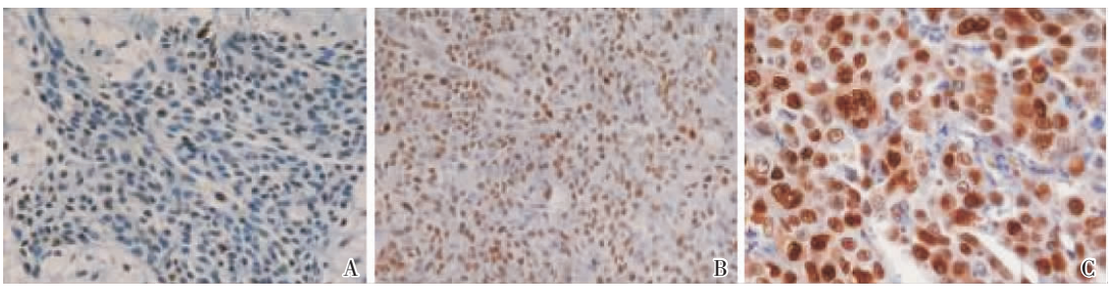

Figure 2 Expression of Ki-67 in common type, active type and malignant pleomorphic adenoma (DAB ×400)

Discussion

Active proliferation of tumor cells is an important aspect for kinetic study of tumor growth. The measurement of proliferative activities in cells can determine the malignancy and biological properties of the tumor. Currently, there are many methods to measure the activities of tumor proliferation, and the measurement of Ki-67 nuclear antigen is one of them. Ki-67 is a non-histone nuclear antigen. The gene is located on 10q5 of human chromosome. Except in G_0 phase, it is expressed in all phases of cell cycle and it reaches the peak during M phase. It participates in mitosis. Its expression level is closely related to activeness of cell proliferation, and thus, its measurement can reflect the status of tumor proliferation. It is regarded as a reliable indicator for assessing malignant proliferation of tumor cells.[1] Katori et al.[10] found that Ki-67 was more strongly expressed in PA and adenocarcinoma than in sialadenitis, and they believed the high expression of Ki-67 could be an important reference index for early carcinogenesis of PA. In this research, by measuring the expressions of Ki-67 in normal, active, and malignant PA, we found that Ki-67 PI was gradually increased in the order from normal, active, to malignant PA, along with the increase in expressive activities of Ki-67 nuclear antigen. This result suggests that there is a variation in proliferation between normal PA and active PA. Additionally, during the malignant transformation of PA, this proliferative property became stronger in tumor cells, in which it finally attained the state of infinite growth. This suggests that Ki-67 is closely related to malignant transformation of PA. The expression level of Ki-67 was increased as the proliferative activities increased. Ki-67 nuclear antigen could be used as a reliable marker for evaluating malignant proliferation of PA.

Ezrin protein is the mostly investigated cytoskeleton linking protein in the family of ERM proteins. Its encoding gene is villin2 and is located between 6q25.2-q26 on the chromosome and is composed of 585 amino acids. It has three domains: the α-helix region in the middle, the amino group terminal in direction of membrane, and the hydroxyl terminal extending to cytoplasm.[11] Ezrin protein, by mediating linkage between membrane and cytoskeleton, participates in the signal transduction systems for tyrosine kinase receptor, Fas, and Rho. It plays important roles in physiopathological processes, such as mitosis, cell proliferation, adhesion, and cell migration.[2,12-14] In recent years,

many researchers found that Ezrin gene is closely related to many biological properties of tumors such as growth, proliferation, infiltration, metastasis, and prognosis.[2–4,15,16] Speaking of salivary tumor, Wang et. al. [17] studied adenoid cystic carcinoma of salivary gland and found that the high expression level of Ezrin protein might have to do with biological properties, such as incidence, progression, and metastasis of the tumor. In this research, the rates of positive Ezrin protein in normal, active, and malignant PA were gradually increasing, as well as their intensities. This was in accordance to research results on other human tumors. As the cell proliferation became active and the malignancy of tumor worsened the expression of Ezrin protein also increased. This result suggests that the elevation in the expression of Ezrin protein is positively correlated to the activeness in cell proliferation of PA. The effect of Ezrin protein in the incidence, progression, and malignant proliferation of PA still requires further investigation. Variations in the intensity of positive expression and rate of positive expression of Ezrin protein in normal, active, and malignant PA suggest that some reasons may cause abnormal expression of Ezrin to lead to the incidence and malignant transformation of PA.

NO and its primary synthase iNOS play many roles in the incidence, progression, and metastasis of tumors at the molecular level, such as inhibition on apoptosis and promotion on angiogenesis. Many experiments have proven that the expression of iNOS in tumor of neck and head is significantly increased to promote proliferation, infiltration, and metastasis of tumor. Chen et al.,[5,18] from a series of studies on lingual carcinoma, found that iNOS has important roles in growth, differentiation, lymphatic metastasis, and angiogenesis of the tumor; in the *in vitro* environment, they further discovered that silencing of iNOS gene exerted an inhibitory effect on the proliferating activities of lingual squamous cell carcinoma. Brennan et al.[19] discovered that in PA, the expression of iNOS was much higher than that in normal salivary tissues. Zhang et al.[20] in the study on adenoid cystic carcinoma of salivary gland showed that high expression of iNOS could promote angiogenesis of tumors and affect its prognosis. This study, based on histological morphology, classified PA and found that there was variation in expression of iNOS in normal, active, and malignant PA. As the activeness of tumor proliferation of PA increased, the expression rate of iNOS also increased, as well as its intensity and malignancy. The abnormally high expression of iNOS could achieve certain degrees of effect in the incidence and malignant transformation of PA.

In PA tissues, patients with high expressions of Ezrin protein and iNOS would also have an elevated level of Ki–67 PI, and the two factors had a synergic effect. It is suggested that Ezrin and iNOS are closely related to mitosis and proliferation of PA, as Ki–67 PI is increased along with elevation of Ezrin and iNOS. This further proves that the two have promotional effects on proliferation of PA, even though its underlying mechanism still requires further investigation. Glynne et al.[21] found that the binding of iNOS and ERM–binding phosphoprotein–50 (EBP50) in the epithelial cells controls the synthesis of NO and affects its biological effect.

PA is a borderline tumor and is a group of heterogeneous tumors from the biological aspect. Thus, it is hard to determine its prognosis in clinical practice. This research results showed the high expressions of Ezrin and iNOS, as well as their imbalance, could be related to proliferation and malignant transformation of PA. It, at the protein expression level, supports the classification standard in the pathological diagnosis of the disease, and to some degrees, indicates the potential for malignant transformation in the active type of PA. It also provides new evidence for further research to unravel the malignant proliferative mechanism of PA. Ezrin and iNOS could become one of the prognostic indices for PA in clinical practice. In addition, the synergic expression of Ezrin protein and iNOS could promote abnormal proliferation and malignant transformation by regulating synthesis of NO. The synergic effect of the two in the incidence and progression of PA is possible, but its underlying mechanism requires further study.

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References

[1] Schluter C, Duchrow M, Wohjemberg C, et al. The cell proliferation-associated antigen of Ki-67:a very large, ubiquitious nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins [J]. J Cell Biol, 1993,123(3): 513-522.

[2] Fais S, Demilito A, Lozupone F. The role of FAS to ezrin association in FAS-mediated apoptosis [J]. Apoptosis, 2005, 10(5):941-947.

[3] Hunter KW. Ezrin, a key component in tumor metastasis [J]. Trends Mol Med, 2004,10(5):201-204.

[4] Weng WH, Ahlen J, Astrom K, et al. Prognostic impact of immunohistochemical expression of ezrin in highly malignant soft tissue sarcomas [J]. Clin Cancer Res, 2005,11(17):6198-6204.

[5] Chen WL, Yang L, Zeng SG, et al. Effect of using RNA interference to alter iNOS gene expression on the proliferation of tongue squamous cell carcinoma cell line Tca8113 [J]. Br J Oral Maxillofac Surg, 2008,46(6):435-438.

[6] Gallo O, Masini E, Morbidelli L, et al. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer [J]. J Natl Cancer Inst, 1998,90(8):587-596.

[7] Li J. Advancement of medical imaging diagnosis in maxillofacial tumors [J]. Zhonghua Kou Qiang Yi Xue Za Zhi, 2006, 41 (8): 474-477. [in Chinese]

[8] Chen Y, Tian KU, Geng, N, et al. Significance of matrix metalloproteinase and its tissue inhibitors in the biological behaviors of pleomorphic adenoma[J]. Zhonghua Kou Qiang Yi

[9] Xue Za Zhi, 2005, 40(1): 58-61. [in Chinese]
Mathew J, Hines JE, Obafunwa JO, et al. CD44 is expressed in hepatocellular carcinomas showing vascular invasion [J]. J Pathol, 1996,179(1):74-79.

[10] Katori H, Nozawa A, Tsukuda M. Increased expression of cyclooxygenase-2 and Ki-67 are associated with malignant transformation of pleomorphic adenoma [J]. Auris Nasus Larynx, 2007,34(1):79-84.

[11] Gary R, BretscherA. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site [J]. Mol Biol Cell, 1995,6(8):1061-1075.

[12] Hiscox S, Jiang WG. Ezrin regulates cell-cell and cell-matrix adhesion, a possible role with E-cadherin/b-catenin [J]. J Cell Sci, 1999,112(18):3081-3090.

[13] Krieg J, Hunter T. Identification of the two major epidermal growth factor-induced tyrosine phosphorylation sites in the microvillar core protein ezrin [J]. J Biol Chem, 1992,267 (27):19258-19265.

[14] Takahashi K, Sasaki T, Mammoto A, et al. Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/ moesin initiates the activation of the Rho small G protein [J]. J Biol Chem, 1997,272(37):23371-23375.

[15] Moilanen J, Lassus H, Leminen A, et al. Ezrin immunoreactivity in relation to survival in serous ovarian carcinoma patients [J]. Gynecol Oncol, 2003,90(2):273-281.

[16] Khanna C, Wan X, Bose S, et al. The membrane cytoskeleton linker ezrin is necessary for osteosarcoma metastasis [J]. Nat Med, 2004,10(2):182-186.

[17] Wang YY, Chen WL, Yang ZH, et al. Ezrin expression in adenoid cystic carcinoma of salivary gland and its influence on metastasis, proliferation and invasion of the tumor[J]. Zhonghua Kou Qiang Yi Xue Za Zhi, 2009, 44 (4): 203-207. [in Chinese]

[18] Chen WL, Li HG, Pan ZB, et al. Expression of nitric oxide synthase in squamous cell carcinoma of tongue and its correlation to prognosis [J]. Zhonghua Kou Qiang Yi Xue Za Zhi, 1998, 33(5): 152-154. [in Chinese]

[19] Brennan PA, Umar T, Buckley J, et al. Expression of nitric oxide synthase in pleomorphic adenomas of the parotid [J]. Br J Oral Maxillofac Surg, 2000,38(4):338-342.

[20] Zhang J, Peng B, Chen X. Expressions of nuclear factor kappaB, inducible nitric oxide synthase, and vascular endothelial growth factor in adenoid cystic carcinoma of salivary glands: correlations with the angiogenesis and clinical outcome [J]. Clin Cancer Res, 2005,11(20):7334-7343.

[21] Glynne PA, Darling KE, Picot J, et al. Epithelial inducible nitric-oxide synthase is an apical EBP50-binding protein that directs vectorial nitric oxide output [J]. J Biol Chem, 2002, 277(36):33132-33138.