



IMMUNOHISTOCHEMICAL LOCALIZATION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN PLEOMORPHIC ADENOMA OF SALIVARY GLAND: CORRELATION WITH CYCLOOXYGENASE-2 AND TUMOR PROLIFERATIVE ACTIVITY

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ABSTRACT

Objectives: to investigate immunohistochemically localization pattern and distribution of inducible Nitric Oxide Synthase (iNOS) in pleomorphic adenoma (PA) of salivary glands and to find a correlation of its expression with Cyclooxygenase-2 (COX-2) and tumor proliferative activity.

Material and Methods: 18 cases of PA were subjected to immunohistochemical staining for iNOS, COX-2 and proliferative cell nuclear antigen (PCNA) using streptavidin-peroxidase technique. 10 samples of normal salivary glands tissues (NSG) were used as a control. COX-2, iNOS and PCNA expression were statistically compared.

Results: in PA, expression of iNOS was increased as that of COX-2 when compared with the control. The expression intensity and pattern of distribution of studied proteins were different within the tumor tissue. The strong expression was localized mainly at the tumor periphery while the center of tumor showed weak or even negative expression. The PCNA-LI mean percentage was higher in the periphery of the tumors. The low expression scores of iNOS were detected in 4 cases, moderate in 5 cases and high in 7 cases, whereas the expression scores of COX-2 were observed in 5 cases (low), in 9 cases (moderate) and 5 cases (high). The expression scores of iNOS and COX-2 were significantly correlated ($P \leq 0.05$). Significant correlation of expression scores of COX-2 and iNOS with PCNA-LI ($P \leq 0.01$) were found.

Conclusion: this study revealed that COX-2 and iNOS expression was increased significantly in PA. Their pattern of expression was more in the periphery of the lesion than the center. The labelling index of PCNA was higher in the periphery of the lesion indicating that the tumorous and proliferative activity of PA is more at tumor tissues near the margins suggesting that the aggressiveness of PA is at the tumor peripheries.

KEY WORDS: Pleomorphic adenoma, iNOS, COX-2, PCNA, Immunohistochemistry

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INTRODUCTION

Several molecules including COX-2 and iNOS have been identified to have essential roles in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis^(1, 2). In response to pro-inflammatory agents, iNOS continuously produces sustainable concentrations of nitric oxide (NO)⁽¹⁾ which consider as one of the major mediator of chronic inflammation⁽¹⁾. It was reported that NO has a role in tumorigenesis and may modulate several cellular processes such as cell proliferation, survival and migration. In addition, it can modulate angiogenesis, drug resistance, DNA modifications and inactivation of DNA repair enzymes^(1, 3). COX is an enzyme that have cyclooxygenase and peroxidase activities and it constitute two isoforms of enzyme⁽²⁾. COX-1 which expressed constitutively in most tissues producing prostaglandins for homeostatic functions^(2,4,5). Whereas, COX-2 is activated by various stimuli at the sites of inflammation such as growth factors, inflammatory cytokines and oncogenes⁽²⁾.

Upregulation of iNOS and COX-2 have been found in various human tumors⁽⁶⁻¹²⁾. iNOS was upregulated in hepatic carcinoma, thyroid carcinoma, squamous cell carcinoma and ameloblastoma^(3,6,7,9,11,13) while upregulation of COX-2 was reported in colonic carcinoma⁽¹⁴⁾, gastric carcinoma⁽¹⁶⁾, prostate carcinoma⁽¹⁷⁾, high grade gliomas⁽¹⁸⁾, malignant melanoma⁽¹⁹⁾ and in thyroid carcinomas⁽²⁰⁾.

Upregulation of COX-2 in salivary gland tumors has been demonstrated in the previous reports⁽²¹⁻²²⁾. In our previous studies on salivary gland tumors, we verified that expression of COX-2 was significantly upregulated in mucoepidermoid carcinomas and Adenoid cystic carcinoma when compared with PA⁽²³⁾. We also reported that upregulated expression of both iNOS and COX-2 might have a role in malignancy in mucoepidermoid carcinomas of salivary gland⁽²⁴⁾.

PCNA, a proliferation marker previously used in several neoplasms correlated to clinical outcomes⁽²⁵⁾. Also, PCNA expression level have been increased in biologically aggressive neoplasms including salivary gland tumors^(26,27).

Although expression of COX-2, iNOS and PCNA have been studied in benign and malignant salivary gland tumors. The present study focused on their immunolocalization and distribution pattern within the PA tissues and the correlation between them.

MATERIAL AND METHODS

Eighteen cases of PA of salivary glands diagnosed based on the clinical and histopathological examination were retrieved from archives of Oral Pathology Department, Faculty of Dentistry, Tanta University. All samples were excised from intraoral minor salivary gland tumors with normal salivary tissue (NSG). The patients' recorded data were retrieved from the archives. Samples of NSG tissues adjacent to the tumor of 10 cases were used as control.

The paraffin blocks of selected cases of PA were cut into sections of 5 μ m thickness subjected for hematoxylin and eosin (HE) staining. All cases were examined to confirm the diagnosis of PA and the presence of NSG tissues associated.

Immunohistochemistry

Immunohistochemical technique used in this study was a peroxidase labeled streptavidin-biotin complex. Serial sections of 5 μ m thickness were obtained from the paraffin blocks of PA cases subjected for immunohistochemical staining as described elsewhere^(23,24). The primary antibodies used were a rabbit polyclonal antibody for iNOS (Thermo Fisher Scientific, Fremont, USA), mouse monoclonal antibody for COX-2 (160112; Cayman, USA) and mouse monoclonal antibody for PCNA

(clone PC10, DakoCytomation, Copenhagen, Den). The primary antibodies were diluted in phosphate-buffered saline (PBS) according to the manufacturer’s instructions. The sections associated with the kits were used as positive control. Normal mouse or rabbit IgGs were used replacing the primary antibodies as negative control.

Analysis of the staining

The positive expression for iNOS and COX-2 was appeared as brown cytoplasmic staining, seen by a light microscope. This cytoplasmic staining intensity and distribution were evaluated in all cases by a reported scoring system with little modification ⁽¹¹⁾. Five random fields of sections at power x 400 were examined under light microscope. The staining intensities of iNOS and COX-2 was graded into 4 scores according to the color of the staining from 0-3 scores as follows 0; negative score (no staining), 1; weakly positive (light yellow), 2; moderately positive (yellowish brown) and 3; strongly positive (dark brown). The proportion of positive staining cells (percentage) was scored as follows, negative (0), <10% positive cells (1), 10% - 50% positive cells (2) and >50% positive cells (3). The total score was calculated as the result of intensity score multiplied with the score of percentage of positive cells for each specimen. The overall results of 1, 2 considered low scores of expression (+). Score 3 and 4 considered moderate scores of expression (++) and score 6 and 9 considered high score of expression (+++) whereas score (-) was negative for iNOS and COX-2.

Analysis of PCNA staining

The percentage of PCNA immunopositive cells (brown stained nuclei) at the center and periphery of the tumor to total number of cells at 400x magnification was calculated in five microscopic fields captured with a camera and the mean was determined automatically using MacBiophotonics ImageJ v.1.44e, analysis software.

Statistics

The data were statistically analyzed using a computer software program SPSS ver. 15.0. The two groups were compared using Student’s t-test and Fisher’s exact test to get statistically significant differences with *p* value less than 0.05. In order to analyze the correlation between markers, a nonparametric Spearman rank-order test was used.

RESULTS

Patients and Clinical Data

The detailed clinical data for 18 studied cases of PA are showed in table1. It revealed that the mean age was 35 years ranging from 10 to 65 years. 8 cases in male and 10 cases in female. The mean size of the tumor was 1.94 cm ranged between 0.5 and 4 cm. The palate was the common site (13 cases) 72.2 %, and the maxilla (5 cases) 27.8 %. No pain complaint recorded in any case.

TABLE (1) Clinical data of studied cases of PA

Site		Sex		Mean size (cm)	Mean Age (year)	
Palate	Maxilla	M	F		M	F
13 (72.2 %)	5 (27.8 %)	8	10	1.94	48	25

iNOS Expression

No staining was observed in negative control. Positive expression of iNOS was observed as a brown staining in the ductal cells of NSG (Fig1A). In PA, iNOS was expressed in the cytoplasm of tumor cells including ductal cells and myoepithelial cells with various staining intensities ranged from mild to strong expression (Fig1B-D). Myoepithelial cells of myxomatous tissue also showed positive expression for iNOS. In the periphery of the lesion, the expression score was moderate to high

in comparison to the center of the lesion, which appeared weak staining or even negative. iNOS expression score are shown in table 2 and chart1.

COX-2 Expression

In the negative controls, no staining was seen. In NSG, the expression of Cox-2 was a cytoplasmic brownish precipitate observed mainly in the duct cells (Fig 2A). In PA, moderate to strong expression of COX-2 was seen in tumor cells of ducts but it was moderate in myoepithelial cells (Fig 2 B-D). The expression distribution pattern was also more at the periphery of PA than in the center of the lesion. COX-2 expression scores are shown in table 2 and chart1.

TABLE (2) Expression scores of iNOS and COX-2 in PA and NSG tissues

Expression Score	iNOS		COX-2	
	PA	NSG	PA	NSG
Negative (-)	2	3	0	2
Low (+)	4	4	5	6
Medium (++)	7	3	9	1
High (+++)	5	0	4	0
Total	18	10	18	10

Correlation of iNOS and COX-2 Expression

The correlation between the expression of both molecules was significantly positive *P- value* =0.024 *P*<0.05 (table 3, Graph 1).

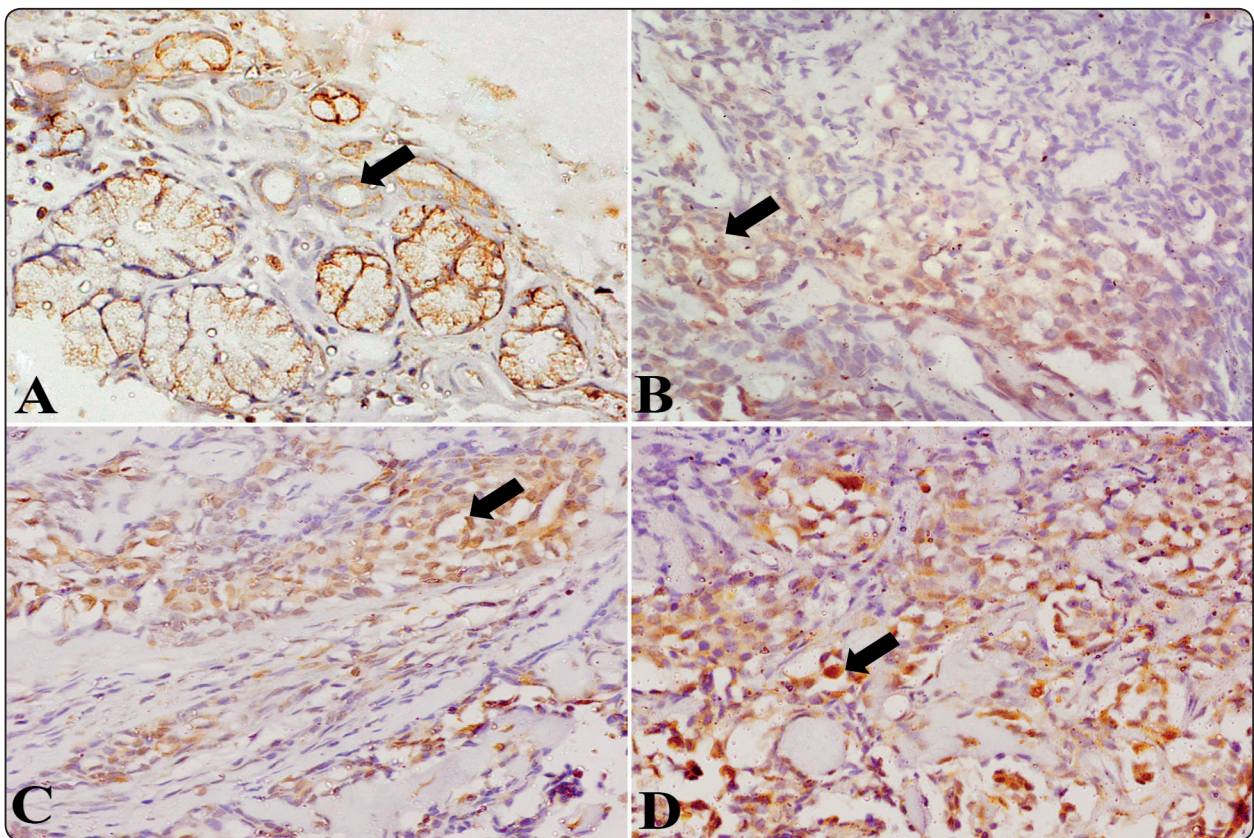


Fig. (1) Immunohistochemical staining of iNOS in PA. A) NSG and B), C), D) PA. Hematoxylen counter stain. In NSG, a cytoplasmic expression of iNOS observed mainly in the duct cells (arrow) (A). Positive cytoplasmic staining of iNOS appeared in tumor cells with various intensity score ranged from low expression in periphery of the lesion (B) to moderate expression (C) and high expression in center of the lesion (arrow) (D). X- 200

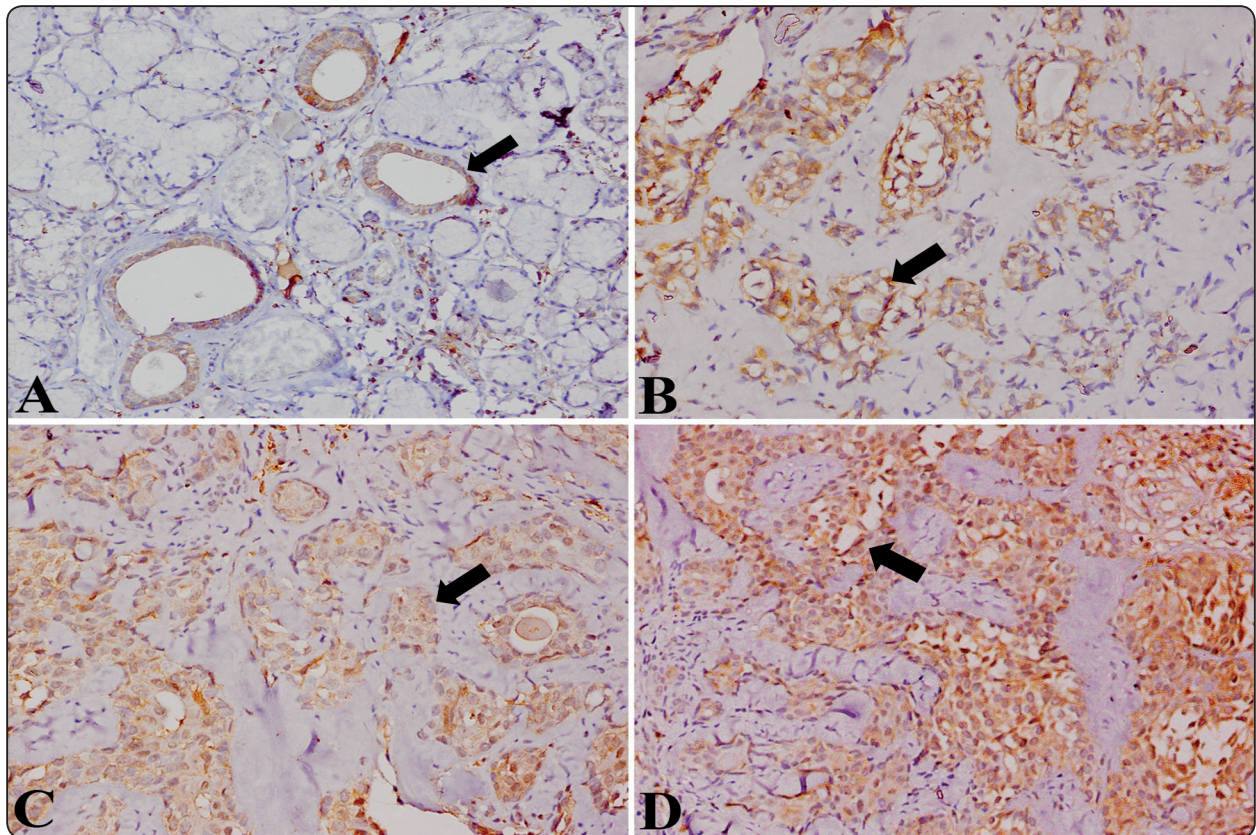


Fig. (2) COX- 2 Immunohistochemical staining in PA. A) NSG and B), C), D) PA. Hematoxylen counter stain. In NSG, the expression of COX-2 was a cytoplasmic brownish precipitate observed mainly in the duct cells (arrow) (A). The expression of COX-2 appeared as cytoplasmic brownish staining of the tumor cells. The expression score ranged from low expression in periphery of the lesion (B) to moderate expression (C) and high expression in center of the lesion (arrows) (D). X- 200.

TABLE (3) Expression scores of iNOS and COX-2 and PCNA-LI % in PA and NSG tissues

Expression Score	iNOS	COX-2	P- Value	PCNA-LI %		P- Value
				Center of the lesion	Periphery of the lesion	
Negative (-)	2	0	0.05*	12.3	15.4	0.01**
Low (+)	4	5				
Medium (++)	7	9				
High (+++)	5	4				

* Significant at 0.05 levels ($P \leq 0.05$)

** Significant at 0.01 levels ($P \leq 0.01$)

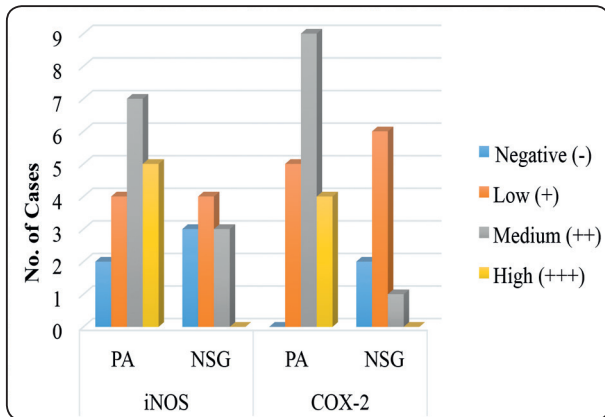
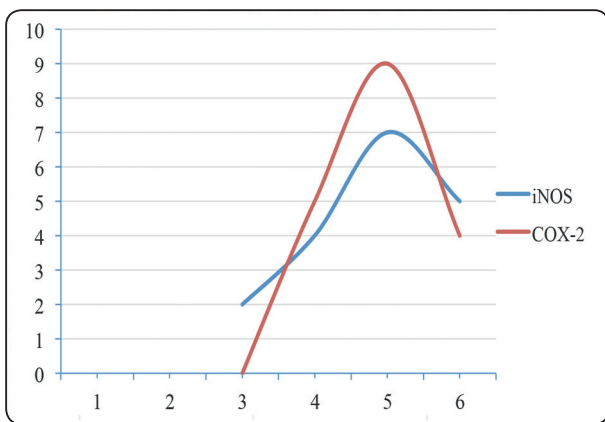


Chart 1: Bar chart showing the expression scores of iNOS and COX-2 in PA and NSG tissues



Graph 2: showing the correlation between Cox-2 and iNOS expression scores in PA

PCNA expression and PCNA-labeling index

In NSG, the expression of PCNA was a nuclear brownish precipitate observed only in the duct cells (Fig 3A). In PA, positive expression of PCNA was observed in the ductal cells and myoepithelial cells distributed mainly at the periphery of the lesion (Fig 3B). The mean PCNA-LI of PA was ranged between 12.4 % at the center of the tumor to 15.3 % at the peripheries. The staining scores of both COX-2 and iNOS showed significant positive correlation with PCNA-LI ($P \leq 0.01$) (table 3).

DISCUSSION

In this study, we explored immunolocalization of iNOS, COX-2, and PCNA in PA of salivary glands of oral cavity to determine their distribution and localization pattern within the tumor and correlation. Both COX-2 and iNOS were immunolocalized in ductal cells of NSG, whereas, in PA it was observed in ductal cells surround the ducts and in cell nests as well as in myoepithelial cells of tumor tissues. These results are in accordance with the previous reports, that the expression of iNOS and COX-2 were observed in both ductal and myoepithelial cells (21, 23, 28-30). However, in another study myoepithelial cells but not the ductal cells were suggested to be responsible for widespread expression of iNOS in PA (28).

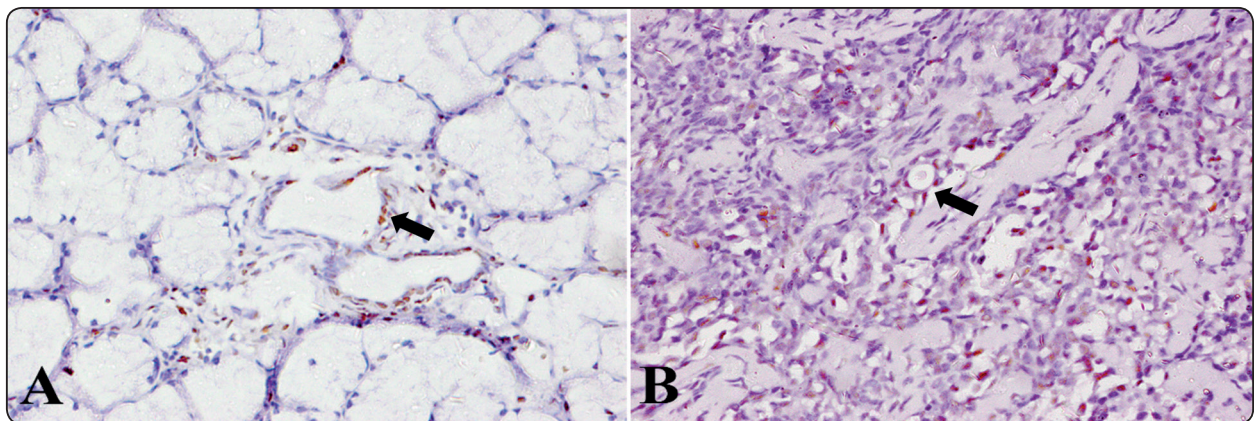


Fig. (3) Immunohistochemical staining of PCNA in A) NSG, B) PA. Hematoxylin counter stain. PCNA positive staining is observed in few cells of NSG (A) and in many tumor cells of PA (B) (arrow). X; 400

The intensity of expressions were enhanced in PA when compared with the NSG tissues supporting the previous findings^(21,23,30,31). The most noticeable finding was the expression patterns of COX-2 and iNOS in PA, which varied according to the area of the tumor. In the center of the lesion, the expression was sporadic and the score was low to medium while in tissues near the margins of the lesion it was widely distributed, strong and upregulated and the expression score was high. Added to that the PCNA proliferative index was also higher in PA tumor tissues near the margins. These findings suggest that the tumor cells near the periphery of PA have more tumorous, aggressiveness and proliferative activity, than the tumor cells in the center of the lesion.

These findings can be explained by the previous report regarding the expression of both COX-2 and iNOS in other tumors of the body such as colorectal tumors⁽³²⁾, in which the expression was heterogeneous and upregulated in the invasive front than center area of the tumor tissues suggesting that the periphery of the tumor is the most aggressive portion.

In our previous researches, we revealed that COX-2 expression increased in salivary gland carcinoma when compared with PA and there was a correlation between its expression and the degree of malignancy⁽²³⁾. Moreover, iNOS expression was significantly increased in high-grade mucoepidermoid carcinoma when compared with low and intermediate grade mucoepidermoid carcinomas⁽²⁴⁾ and that PCNA proliferative index was increased with the increased of malignancy⁽²³⁾. Previous reports demonstrated that the increased expression of COX-2 is one of early events of malignant transformation of PA⁽³¹⁾.

Since previous studies revealed that, there is a link between expression of COX-2 and iNOS and reported that NO can stimulate formation of prostaglandin through the activation of COX-2 and prostaglandins may affect iNOS expression,

furthermore, in colorectal cancer COX-2 activation induce angiogenesis mediated by NO through increase production of VEGF⁽³³⁻³⁵⁾. Therefore, iNOS could be one of early events of malignant transformation of PA and need further elucidation in recent researches.

In the current study, expression of iNOS was significantly correlated with COX-2, and that both molecules are co-localized in PA which in accordance with the previous findings and suggestion that they have an important role in progression of many epithelial tumors including salivary gland tumors^(8-12,24,32). Moreover, correlation between the expression of both investigated molecules and proliferative index of PCNA was in accordance with the previous reports^(23,27,32).

COX-2 and iNOS have been identified to have essential roles in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis^(1,2). A sustainable NO concentrations produced by iNOS in response to pro-inflammatory agents which has antiapoptotic effects induced by TNF- α and Fas-ligands blocking Caspase-3 like activation⁽¹⁾.

Peroxyinitrites free radical is produced when endogenous NO react with superoxide anions which lead to tissue damage⁽³⁵⁾. Furthermore, COX-2 produces prostaglandins from arachidonic acids stimulate proliferation of epithelial cells, which support the previous reports that COX-2 overexpression suppress apoptosis⁽²⁾. The previous studies reported that COX-2 inhibitors prevent the progression of several epithelial cancers of animals and human by blocking the production of VEGF, reduce tumor angiogenesis and suppress vascular endothelial cells migration^(38,40).

In conclusion, iNOS and COX-2 enzymes are localized and upregulated in PA. Their localization pattern and distribution are not the same in all areas of tumor tissues. They are more expressed, widely distributed in tissues near the tumor margins than the center area and they are positively correlated

with each other and with the proliferative activity of the tumor cells. These findings suggest that both enzymes are colocalized, and act concomitantly during tumor progression. Further study is recommended to clarify the genetic background and relation between the expressions of both enzymes and their role in malignant transformation of PA.

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