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• CLINICAL RESEARCH •

# Relationship between inducible nitric oxide synthase expression and angiogenesis in primary gallbladder carcinoma tissue

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## Abstract

**AIM:** To explore the relationship between angiogenesis and biological behaviors of primary gallbladder carcinoma (PGBC), the relationship between the expression of inducible nitric oxide synthase (iNOS) and biological behaviors of PGBC and its relationship with the expression of iNOS and angiogenesis of PGBC.

**METHODS:** The expression of iNOS and micro-vessel density (MVD) were assessed by immunohistochemical method and image analysis system in 40 specimens of PGBC and in 8 specimens of normal gallbladder. The immunostaining results and related clinicopathologic materials were analyzed by statistical methods.

**RESULTS:** MVD in PGBC was significantly higher than that in normal gallbladder tissue (46±14 *vs* 14±6, $P<0.05$), and was not related with age, gender, tumor size and histological type. MVD of poorly and undifferentiated tumor tissues was higher than that of moderately-differentiated and well-differentiated tumor tissues (52±9 *vs* 43±9 *vs* 33±6, $P<0.01$). MVD of Nevin IV and V stages was higher than that of Nevin I, II and III stages (52±8 *vs* 37±13, $P<0.01$). MVD of cases with lymphatic or liver metastasis was significantly higher than that without liver metastasis (55±6 *vs* 42±10, $P<0.05$) or lymphatic metastasis (53±8 *vs* 38±8, $P<0.01$). The positive level index (PLI) of iNOS in PGBC was 0.435±0.134, and was not related with age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis (0.573±0.078 *vs* 0.367±0.064, $P<0.01$). The PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis (0.533±0.067 *vs* 0.424±0.084, $P<0.05$). There was a significant correlation between PLI of iNOS and MVD in PGBC ($P<0.05$).

**CONCLUSION:** Angiogenesis of PGBC is significantly related to the biological behaviors of PGBC. The expression of iNOS is related to the biological behaviors of PGBC. The detection of MVD and the expression of iNOS in PGBC can be used as parameters to determine the degree of malignancy and prognosis.

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## INTRODUCTION

PGBC is a kind of malignant neoplasm with poor prognosis, and accounts for 1.5% of digestive carcinomas[1]. It is difficult to be diagnosed in its early stage. Recent studies have shown its morbidity is gradually increasing[2]. Tumor growth is a multistage process and tumor angiogenesis is one of the key steps in tumor growth, infiltration and metastasis. In many tumors, the expression of iNOS is strong[3]. NO is synthesized from the amino acid L-arginine by iNOS and has many biological functions closely related with carcinogenesis and development of carcinomas, especially with tumor angiogenesis[4]. At present, the study about angiogenesis of PGBC is few and the relationship between angiogenesis and iNOS of PGBC has not been reported. In the present study the expressions of iNOS and MVD of 40 PGBCs and 8 normal gallbladders were investigated by immunohistochemistry and image analysis methods. The clinicopathologic indexes, the relationship between expression of iNOS and angiogenesis of PGBC and significance of the expression of iNOS were discussed.

## MATERIALS AND METHODS

### *Pathological materials*

Forty specimens of PGBC were collected between 1996 and 2002 in the Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University. No treatment was given before operation. Specimens were fixed with formaldehyde and embedded with paraffin. The histological grading of tumor was done on hematoxylin-eosin stained sections. There were 11 males and 29 females, their mean age of patients was 59 years old. Among the 35 adenocarcinomas, 31 belonged to differentiated adenocarcinomas, 2 mucinous adenocarcinomas and 2 undifferentiated adenocarcinomas. There were 2 adenosquamous carcinomas, 1 adenoacanthoma, 1 sarcoma carcinoma and 1 neuroendocrine carcinoma. Eighteen tumours had a size $\geqslant$ 3 cm, 22 had a size<3 cm. Seven were There were well differentiated carcinomas, 14 moderately differentiated, 16 poorly differentiated and 3 undifferentiated. Twenty cases had metastases in lymph nodes, 11 in liver. Eight normal gallbladders were studied as control.

### *Immunohistochemistry*

Tissue samples were fixed in 10g/L neutral-buffered formaldehyde, embedded in paraffin, sectioned (4 μm thick), and deparaffinized. Slides were immersed first in 3 mL/L $H_2O_2$ at room temperature for 10 min to get rid of the activity of endogenous peroxidase. Then slides were digested with 1 g/L trypsin for 10 min. The slides were washed with distilled water, soaked in PBS for 5 min, then put into microwave at 95 ℃ for 8 min to repair the antigen. Then slides were immersed in goat serum (1.5%) at room temperature for 40 min to block endogenous nonspecific binding sites. Immunostaining was performed with the primary rabbit polyclonal IgG specific for iNOS (dilution, 1:50; Santa Cruz Biotechnology, USA) at room temperature for 2 h. The primary rabbit polyclonal IgG specific for factor VIII-related antigen (dilution, 1:100; Sigma Biotechnology, USA) was used. Then a biotinylated secondary antirabbit antibody

(BOSHIDE Biotechnology, Wuhan, China) diluted 1:200 in PBS was applied on the sections for 20 min, followed by the streptavidin-biotin-peroxidase complex (dilution,1:200; BOSHIDE Biotechnology, Wuhan, China) for 20 min. The color was developed by diaminobenzidine (DAB). The sections were counter-stained with hematoxylin, dehydrated, made transparent, covered and observed. Primary antibody replaced by PBS was used as a negative control. For positive controls, hemangioma was stained for factor VIII-related antigen and gastric carcinoma for iNOS.

### *Determination of iNOS*

Under light microscope the positive cells were stained as brownish yellow in cytoplasm. Determination of iNOS was performed by an image analysis system (IBAS System, Kontron Eledtronik, Germany). According to guide system set operation was made for determination of optical density. The average optical density (AOD) of 100-200 iNOS positive cells was randomly measured with ×20 objective, and the average percentage of positive cells (APCP) was obtained by measuring positive cells and total tumor cells in random 10 high power fields (HPFs) with ×40 objective.The positive level index (PLI) was calculated according to the following formula: PLI= APCP×AOD.

### *Quantificantion of angiogenesis*

Determination of MVD was based upon the method reported by Marrogi *et al*[6]. An all-round observation was first made with ×10 objective, then five areas with the highest density of micro-vessels (hot spots) were selected, and the amount of micro-vessels was counted with ×20 objective (any endothelial cell or endothelial cluster close to tumor cells and connective tissue around tumor cells, which was stained brownish yellow were considered as a single, countable micro-vessel). MVD was counted from an average of five HPFs with ×20 objective (Figure 1).

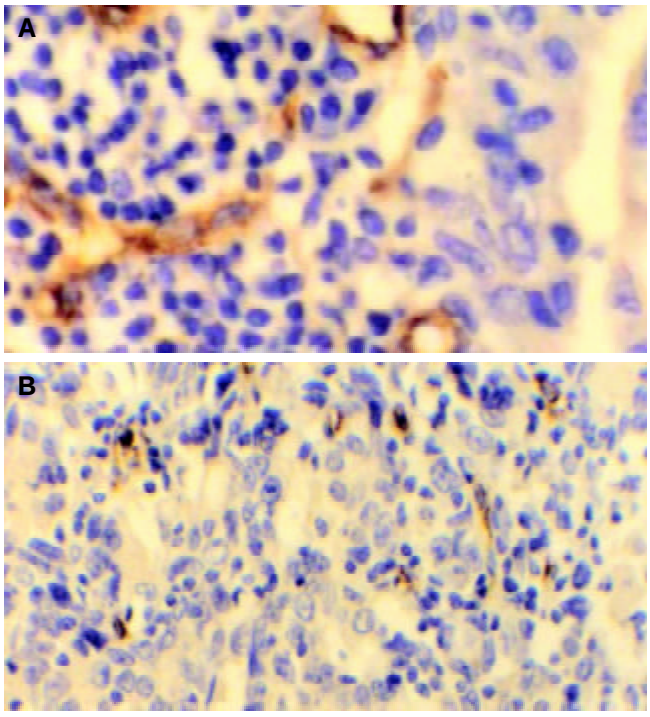


**Figure 1** Distribution of MVD in PGBC tissue, SABC ×200. A: high MVD, B: low MVD.

### *Statistical analysis*

SPSS for Windows (10.0 edition) was used for statistical analysis. All data were expressed as mean±SD. Statistical differences were evaluated using the unpaired *t* test, *F* test and *q* test. Analysis of linear correlation was used to discuss the relationship between expressions of iNOS and MVD. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### *MVD and PGBC*

Through the stains by polyclonal IgG specific for factor VIII-related antigen, micro-vessels could be identified. The distribution of micro-vessels in PGBC was not even, which was irregular in morphology and differed greatly in quantity (Figure 1). MVD was the highest at the margin of tumor tissues. MVD (46±14) in PGBC was significantly higher than that (14±6) in normal gallbladder tissue ($P<0.05$). MVD in PGBC was not related with age, gender, tumor size and histological type. MVD in poorly and undifferentiated tumor tissues was higher than that of moderately differentiated and well-differentiated tumor tissues ($P<0.01$). MVD of Nevin IV and V stages was higher than that of Nevin I, II and III stages ($P<0.01$). MVD of cases with lymphatic or liver metastasis was significantly higher than that without liver metastasis ($P<0.05$) or lymphatic metastasis ($P<0.01$, Table 1).

### *iNOS and PGBC*

The expression of iNOS in normal gallbladder epithelial cells was negative and occasionally positive in stromal cells. In tissue of PGBC, iNOS was mainly expressed in cytoplasm and few in inflammatory cells around the tumor cells (Figure 2). The PLI of iNOS in 40 PGBCs was 0.435±0.134. It was not related with age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis ($P<0.01$). The PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis ($P<0.05$, Table 1).

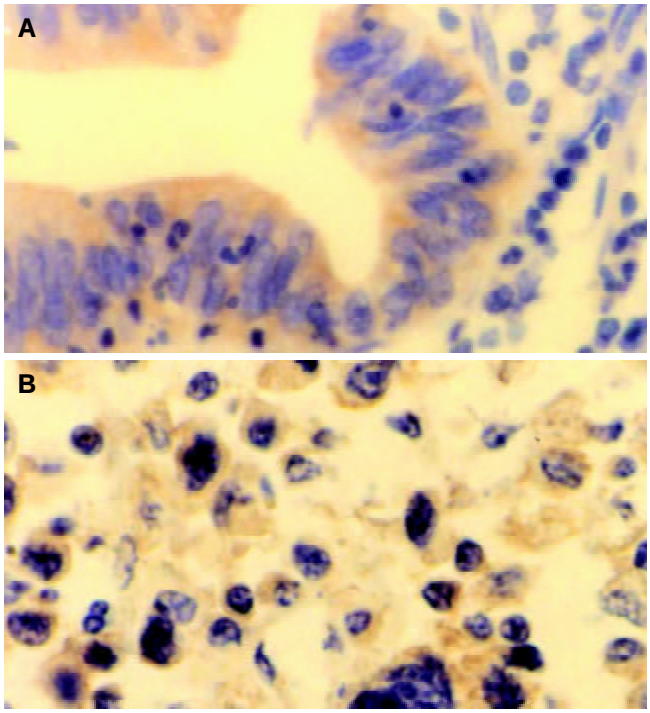


**Figure 2** Positive expression of iNOS in PGBC tissues SABC ×400. A: well-differentiated, B: poorly-differentiated.

### *iNOS and angiogenesis of PGBC*

By serial analysis of sections, high MVD was observed in

areas of high iNOS expression. Linear correlation analysis showed that PLI of iNOS was positively correlated with MVD ($r$=0.4021, $P$<0.05). This demonstrated that expression of iNOS could influence MVD and blood supply of tumor.

**Table 1** Relationship between MVD, PLI and clinicopathologic indexes

| Group | | *n* | MVD | PLI |
|---|---|---|---|---|
| Age | <58yr | 17 | 43±14 | 0.340±0.048 |
| | ⩾58yr | 23 | 45±11 | 0.371±0.061 |
| Male | | 11 | 46±11 | 0.376±0.056 |
| Female | | 29 | 44±11 | 0.421±0.073 |
| Size | ⩾3 cm | 18 | 42±11 | 0.365±0.077 |
| | <3 cm | 22 | 46±10 | 0.434±0.097 |
| Adenocarcinoma | | 35 | 45±12 | 0.473±0.086 |
| Adeno-squamocarcinoma | | 2 | 41±3 | 0.391±0.131 |
| Others | | 3 | 47±6 | 0.447±0.025 |
| Differentiation well | | 7 | 33±6 | 0.397±0.089 |
| Moderate | | 14 | 43±9[a] | 0.413±0.072 |
| Poor & Non | | 19 | 52±9[ac] | 0.453±0.113 |
| Nevin stages I, II, III | | 18 | 37±13 | 0.410±0.092 |
| IV, V | | 22 | 52±8[b] | 0.459±0.088 |
| Lymphatic Metastasis | + | 20 | 53±8 | 0.573±0.078 |
| | - | 20 | 38±8[d] | 0.367±0.064[f] |
| Liver Metastasis | + | 11 | 55±6 | 0.533±0.067 |
| | - | 29 | 42±10[e] | 0.424±0.084[g] |

[a]$P$<0.05, *vs* Well. [c]$P$<0.05, *vs* Moderate. [b]$P$<0.01, *vs* I, II, III Stages. [d,f]$P$<0.01, *vs* Positive. [e,g]$P$<0.05, *vs* Positive.

## DISCUSSION

The formation of new blood vessels known as angiogenesis induced by tumor cells is a critical determinant of tumor progression[5-10]. Unlike normal blood vessels, tumor blood vessels are not mature vessels which are chaotic, irregular and leaky. Studies showed that proliferation, infiltration and metastasis of solid tumor were closely related with tumor angiogenesis[11-16]. In our study we found that the distribution of micro-vessels in PGBC was not even, but irregular in morphology and its quantity differed greatly. MVD was the highest at the margin of tumor tissues. In tumor tissues MVD was higher than that in normal tissues. Our study showed that MVD in PGBC was significantly higher than that in normal gallbladder tissue. Research on mammary cancer showed that MVD was increased in patients with distant metastasis compared with those without distant metastasis[17]. The risk of metastasis would increase by 159% per increase of 10 micro-vessels[18]. This study found that MVD in cases with lymphatic or liver metastasis was significantly higher than that without liver or lymphatic metastasis, which was consistent with other researchers[19-22]. It is generally believed that invasion of tumor cells from tumor tissue with rich vasculature increases and the risk of lymphatic metastasis is increased by invasion of veno-lymphatic anastomosis and lymphatic vessels accompanying blood capillaries.

Calcium-independent iNOS was expressed in macrophages, neutrophils, hepatocytes, cardiac myocytes, chondrocytes, and many other cell types[23-26]. It was mainly induced by cytokines and could generate locally high concentrations of NO for a prolonged period of time and play a variety of regulatory functions *in vivo*[27,28]. In many studies, iNOS positivity was predominantly found in tumor cells[29,30], but in another study a relatively high iNOS immunoreactivity was noted in stromal cells[31]. Our study showed that in PGBC iNOS was mainly expressed in cytoplasm of tumor cells and in few inflammatory cells around the tumor cells. Vakkala *et al*[32] showed that iNOS positivity was observed in mammary cancer cells in 46.5% *in situ* carcinomas and 58.8% invasive carcinomas. Expression of iNOS was related with differentiation of carcinomas *in situ*. In this study PLI of iNOS was not related to age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis and the PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis. The effects of NO could be tumor promoting or tumor suppressing. High concentrations of NO (umol/L) could be cytotoxic, whereas low concentration (nmol/g or pmol/g) might even protect some cell types from damage and apoptosis[33]. During the initiation of tumor growth, natural killer cells and macrophages could kill tumor cells by a NO-mediated mechanism[34]. However, NO might also suppress antitumor effect, promote tumor angiogenesis and blood flow in tumor neovasculature, and enhance tumor growth, invasion, and metastasis[35]. Jenkins *et al*[36] engineered gene of iNOS into adenocarcinoma cell line DLD-1 to get iNOS-19 subclone which generated NO continuously, and found that in nude mice tumors from iNOS-19 subclone cells grew faster than those derived from wild-type cells and were much markedly vascularized and had a stronger ability to invade. Our conclusion is that NO produced by iNOS in PGBC is in low concentration and can promote tumor angiogenesis, invasion and metastasis of PGBC.

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