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Inducible nitric oxide synthase expression is related to angiogenesis, bcl-2 and cell proliferation in hepatocellular carcinoma*

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Abstract: In this study, we examined the expression of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) by immunohistochemical staining in 76 tissue sections collected from hepatocellular carcinoma (HCC) patients undergoing hepatectomy. Microvascular density (MVD) was determined by counting endothelial cells immunostained using anti-CD34 antibody. We performed DNA-flow cytometric analyses to elucidate the impact of iNOS and VEGF expression on the cell cycle of HCC. Most of the HCC cells that invaded stroma were markedly immunostained by iNOS antibody. The iNOS stain intensity of the liver tissue close to the tumor edge was stronger than that of HCC tissue, and the strongest was the hepatocytes closer to the tumor tissue. However, iNOS expression in 10 normal hepatic samples was undetectable. VEGF positive expression ratio was 84.8% in iNOS positive expression cases, and the ratio was 35.3% in negative cases. There was significant correlation ($P = 0.000$) between iNOS and VEGF expression. Moreover, iNOS expression was significantly associated with bcl-2 and MVD, but without p53 expression. DNA-flow cytometric analyses showed that combined expression of iNOS and VEGF had significant impact on the cell cycle in HCC. PI (Proliferating Index) and SPF (S-phase fraction) in the combined positive expression of iNOS and VEGF group was significantly higher than that in the combined negative group. The present findings suggested that iNOS expression was significantly associated with angiogenesis, bcl-2 and cell proliferation of HCC.

Key words: Hepatocellular carcinoma, Nitric oxide synthase, Angiogenesis, Bcl-2, Flow cytometric analyse
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INTRODUCTION

Nitric oxide (NO), a short half-life radical, is highly reactive, and is involved in many biological processes, It seems to play an important role in modulating angiogenesis and carcinogenesis. Nitric oxide synthase (NOS) is a key rate-limiting enzyme in NO synthesis, and its bioactivities directly determine the NO yields. The two different isoforms include constitutive NOS (cNOS) and inducible NOS (iNOS). Typically, iNOS is related to tumor immunology, and generates 100-1000-fold NO more than its constitutive counterparts (cNOS) involved in physiologic regulation (Edwards et al., 1996). Although increased iNOS expression had been demonstrated in colon, prostate, and bladder cancer (Swana et al., 1999), its exact function in the tumor biology is not yet clear.

Angiogenesis is a process in which new blood vessels develop from preexisting vessels. Growth, invasion, and metastasis of many cancers depend on angiogenesis. Solid tumors require neovascularization to growth beyond 1 mm^3 (Risau, 1997). HCC abundant in vascular, angiogenesis plays a crucial role in its development. Evidence suggested that the enhanced activity of NOS was closely correlated with tumor angiogenesis (Ambs et al., 1998); and that NO produced by iNOS inhibits apoptosis and promotes the survival of growth-arrested tumor cells via a bcl-2-mediated pathway (Dodd et

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al., 2000). However, there are only few articles describing the relationship between angiogenesis and the expression of iNOS in HCC.

In the present study, we examined the expression of inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), proteins bcl-2 and p53 by immunohistochemical staining in 76 tissue sections collected from hepatocellular carcinoma (HCC) patients undergoing hepatectomy. Meanwhile, microvascular density (MVD) was determined by counting endothelial cells immunostained using anti-CD34 antibody. Flow cytometry (FCM) was used to detect the cell cycle of HCC in 11 tissue sections. So the correlation of iNOS expression with angiogenesis, apoptosis-related protein and cell proliferation in HCC could be investigated.

MATERIALS AND METHODS

Materials

Seventy-six (63 males, 13 females) patients were selected from among those with HCC undergoing hepatectomy between 1999 and 2001 at the Second Affiliated Hospital, College of Medical Sciences, Zhejiang University (Hangzhou, China). Eligible patients had a histological diagnosis of HCC. Among those, 24 had paired tissues close to the tumor edge (no more than 1.5 centimeter) from the tumor tissue. Ten normal hepatic tissue samples collected from angiocavernoma patients served as control. The median age of the 76 eligible patients was 45.5 years (range, 27-82 years). The tissue samples were fixed in 10% neutral formalin and then embedded in paraffin. Serial sections 5 μm thick were prepared from paraffin blocks. The sections were performed using routine hematoxylin and eosin (H&E) staining, and then examined by immunohistochemical staining.

Reagents

Anti-iNOS antibody used to detect inducible nitric oxide synthase (iNOS) was obtained from Santa-Cruz Pharmaceuticals Ltd (USA). Anti-bcl-2 and anti-p53 antibodies were from ZYMED Ltd (USA). Anti-VEGF, anti-CD34 antibody used to detect the MVD, and SP immunochemical test kit were from ManXin Ltd (Fuzhou, China). Propidium Iodide (PI) was purchased from Sigma Company (U.S.A).

Immunohistochemistry

Paraffin sections were deparaffined and hydrated by sequential immersion in xylene and graded alcohol solutions. The slides were then incubated in 3% H_2O_2 for 10 min at room temperature to block the endogenous peroxidase activity. Slides were treated with normal serum obtained from the same species in which the secondary antibody was developed for 30 min to block non-specific staining. Subsequently, slides were incubated with primary antibodies (anti-iNOS at 1:500 dilution in wet moist box for 60 min at 37℃, anti-bcl-2 and anti-p53 were at 1:500 dilution overnight at room temperature, and anti-CD34 was at 1:50 dilution for 60 min at room temperature). Then slides were treated with a biotin-conjugated secondary antibody for 10 min followed by incubation with peroxidase-conjugated streptavindin for 10 min at room temperature. All the above steps were followed by washing in TBS (0.01 mol/L, pH 7.4) for 3 times. Immunolabeling was detected using DAB as the chromogen, followed by washing and staining with Mayer's hematoxylin; positive and negative controls were included in every procedure.

Evaluation of iNOS, VEGF, bcl-2 and p53 expression

iNOS, VEGF and bcl-2 positive immunostaining resulted in the emergence of brownish-yellow granules. in the cytoplasm (Fig.1), and p53 were the brownish-yellow granules in the nuclelus. The degree of staining was categorized by the extent and intensity of the staining. The immunoreactive score was determined by the sum of extent and intensity as reported previously (Rahman et al., 2001). The intensity of staining was scored on a scale of 0 to 3, in which 0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining. The extent of positivity ("extent of distribution" of positive cells) was estimated on a scale of 0 to 4, in which 0 = negative, 1 = positive staining in 1-25% of cells; 2 = positive staining in 26% – 50%; 3 = positive staining in 51% – 75%; and 4 = positive staining in 76% – 100%. The combined staining score (extent + intensity) $\geqslant$ 3 was considered as positive staining.

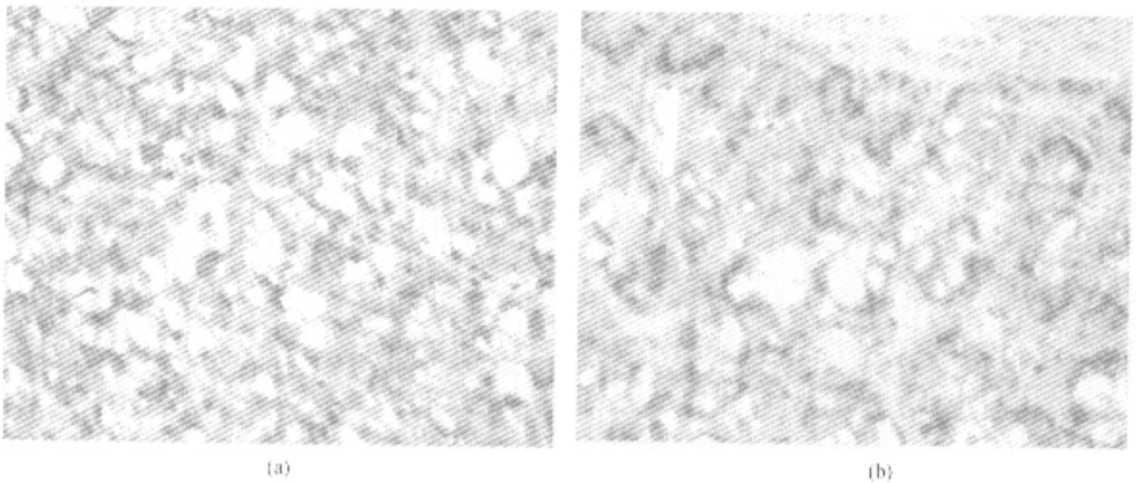


Fig.1 Respective section showing immunohistochemical expression of iNOS ((a), cytoplasm positive staining; magnification, ×400), and VEGF ((b), cytoplasm positive staining; magnification, ×400) in HCC

Quantitation of MVD

Because of the immunoreactivity of CD34 showed slight heterogeneity within the same tumor, the five most highly vascularized areas were selected in × 100 magnification fields. The brownish-yellow microvessel number were counted at above five vascularized areas in × 200 magnification fields. The mean was value considered as MVD. Every positive immunostaining vascular endothelial cell (VEC) or VEC cluster that isolated from its neighbour microvessel was considered as an independent microvessel (Gallo et al., 1998)

DNA-flow cytometric analysis

Eleven samples were selected as subParaffin slices were cut into 5 mm squares and deparaffined, then hydrated. Tissues were treated with 0.1% protease, then the cellular fraction was separated through centrifugation. Cells were washed twice, resuspended in PBS and after staining with PI, were subjected to FCM analyis to obtain the histogram. Finally the fractions of cells in the G0/G1, S, and G2/M phases were obtained respectively using Multicycle program software, Version 3.0.

Statistical analysis

All statistical data were obtained by using SPSS statistical package (Version 10.0; SPSS Inc., Chicago, IL, USA). Significance differences between categorical variables were compared by the χ^2 test or Fisher's exact probability test when appropriate. Continuous variables were compared by the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

RESULTS

The distribution of the expression of iNOS, VEGF and CD34

The distribution of iNOS expression was either diffuse or local. Most HCC cells that invaded fibrous tissue were strongly positively stained (Fig.2 a). The iNOS stain intensity of the liver tissue at the tumor edge was stronger than that of HCC ($P = 0.000$), and the strongest was that of the hepatocytes close to tumor tissue (Fig.2 b). However, interestingly, VEGF positive expression was also strong close to tumor tissue (Fig.3 a). It was found that the new microvessel was either diffusely distributied or confined to the surrounding cancer nests in HCC cases (Fig. 3 b).

The expression of iNOS, VEGF, bcl-2 and p53 in HCC and the relationship between each other

Of the 76 HCC samples, iNOS expression was observed in 59 (77.6%), and VEGF was observed in 56 (73.7%). VEGF positive expression ratio was 84.8% in iNOS positive expression cases, and 35.3% in negative cases. The χ^2 test revealed significant correlation ($P = 0.000$) between iNOS and VEGF expression. iNOS expression was significantly associated with bcl-2 ($P = 0.01$), but not with p53 ($P =$

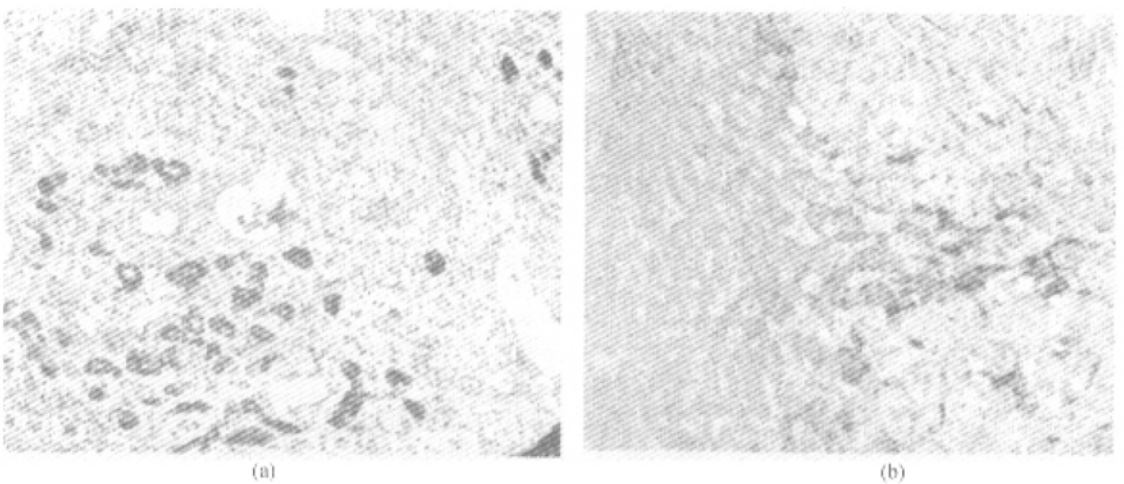
0.134) expression(Table 1).

Table 1 The relationship of iNOS expression with other factors (VEGF, P53 and bcl-2) in HCC

Variables	Category	iNOS expression		χ^2	P
		Negative(%)	Positive(%)		
VEGF	Negative	11(64.7%)	9 (15.2%)	16.64	0.000#
	Positivea	6 (35.3%)	50(84.8%)		
P53	Negative	11(64.7%)	26(44.1%)	2.25	0.13
	Positive[a]	6(35.3%)	33(55.9%)		
Bcl-2	Negative	12(70.6%)	20(35.1%)	6.72	0.01*
	Positive	5(29.4%)	37 (64.9%)		

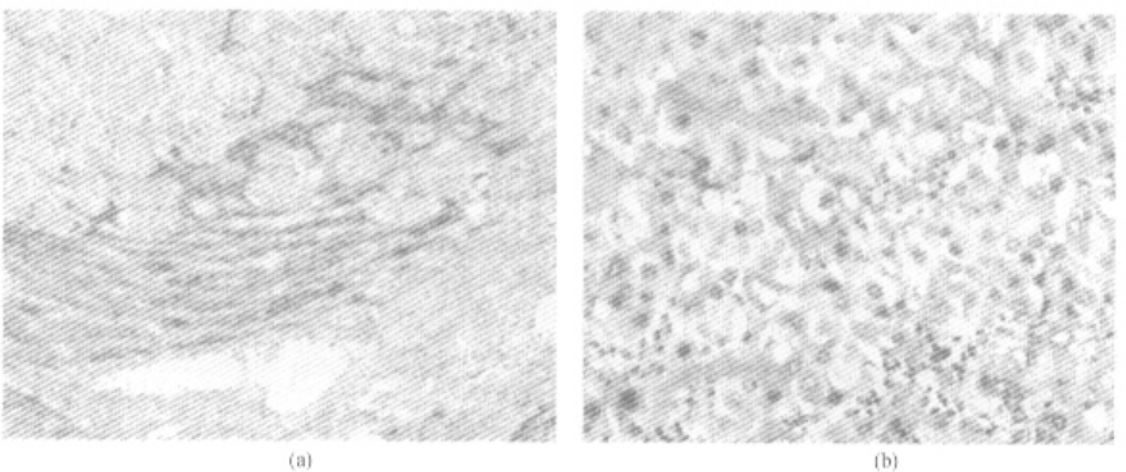
[a] Score ≥3 was considered as positive staining.

* There was significant correlation between iNOS and VEGF expressions, or and bcl-2 expressions, respectively.



(a) (b)

Fig.2 Respective section showing immunohistochemical expression of iNOS ((a), strongly positive staining; magnification, ×200) in HCC cells that invaded into fibrous tissue; and ((b), strongest staining; magnification, ×200) in hepatic tissue close to HCC



(a) (b)

Fig.3 Respective section showing immunohistochemical expression of VEGF ((a), strongly positive staining; magnification, ×200) in hepatic tissue close to HCC, and CD_{34} ((b), diffusely positive staining; magnification, ×400) of vascular endothelial cells in HCC

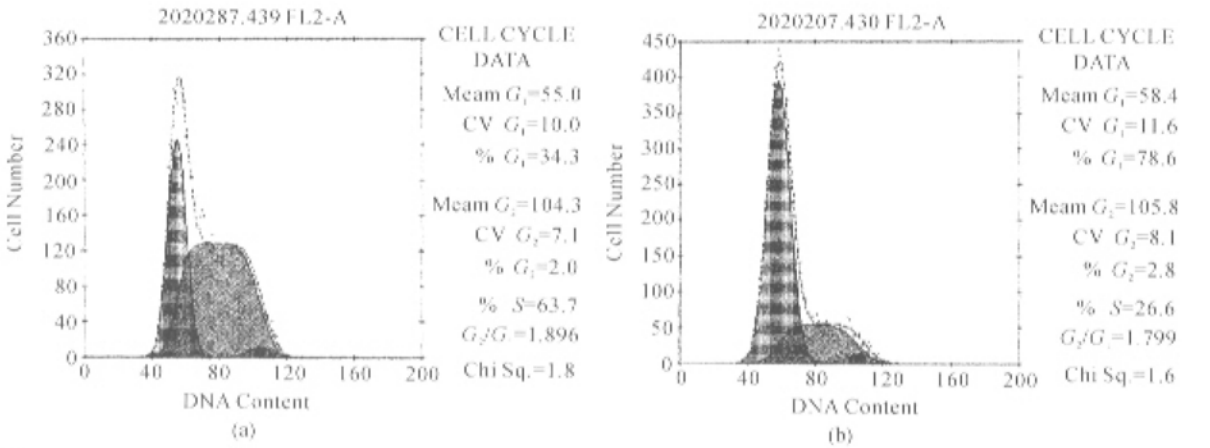


Fig.4 DNA-flow cytometric analyses ((a) the maximal fraction of cells in the sphase reached 63.7% in a case combined iNOS and VEGF positive expression, and (b) the minimal one was 26.2% in a combined negative expression case).

The relationships of MVD to the expression of iNOS and VEGF

In the cases that were combined negative for iNOS and VEGF, the mean of MVD was 20.15 ± 6.57 and 21.73 ± 8.11. Comparably, the mean of MVD was 28.94 ± 13.64 and 28.85 ± 13.82 in the cases that were combined positive for iNOS and VEGF. t test showed significant positive correlation ($P = 0.012$) between MVD and the combined positive expression of iNOS and VEGF (Table 2). This result indicated that the combined expression of iNOS and VEGF was correlated with MVD in hepatic tissues.

Table 2 The relationship of MVD with the expression of VEGF and iNOS

Variable	Group	MVD ($\bar{x} \pm s$)	cases	t	P
iNOS	Negative	20.15 ± 6.57	17	2.56	0.012
	Positive	28.94 ± 13.64	59		
VEGF	Negative	21.73 ± 8.11	20	2.17	0.033
	Positive	28.85 ± 13.82	56		

FCM analysis of the 11 HCC samples

DNA-flow cytometric analyses were performed in 11 cases in which iNOS and VEGF immunoreactivity was combined negative or positive. The finding indicated that the fraction of cells in the G_2/M and S phase in iNOS positive expression cases was higher than that in the negative cases. Of which, the maximal fraction of cells in S phase reached 63.7% in one iNOS positive expression case, and the minimal fraction of cells in S phase was 26.2% in one iNDS negative expression case (Fig 4). All the data were calculated using the following formula: PI (proliferation index) = $[S + G_2M]/[G_0G_1 + S + G_2M]$; SPF (S-phase fraction) = $S/[G_0G_1 + S + G_2M]$. t test showed that means of PI and SPF in iNOS positive expression cases was significantly higher compared to control respectively (Table 3).

Table 3 The relationship between iNOS expressionand cell proliferation in HCC

combined iNOS and VEGF expression	SPF(%) $\bar{x} \pm s$	PI(%) $\bar{x} \pm s$
Positive($n = 7$)	55.53 ± 6.60[a]	60.14 ± 6.97[b]
Negative($n = 4$)	38.53 ± 10.72	41.88 ± 10.59

[a] Significant difference compared to control ($t = 2.000$ $P = 0.024$)

[b] Significant difference compared to control ($t = 1.000$ $P = 0.014$)

DISCUSSION

Evidence suggested that NO produced by iNOS had dual bioactivity, indicating either its organism defense and anti-mutagenic activities while NO concentrations were appropriate, or its mutagenic and tumorigenic activities while there were too high concentrations of NO (Thompson et al., 1998). Another study indicated that NO is an important medium in the course of tumor progress. iNOS and cNOS exist in different tu-

mor tissues, and could promote tumor growth (Lala, 1998; Gallo et al., 1998).

In this study, iNOS positive staining was observed in 77.6% of tumor samples obtained from 76 patients with HCC. Comparatively, iNOS expression was not observed in 10 normal hepatic samples. The iNOS stain intensity of tissue close to tumor edge was stronger than that of HCC. These results were consistent with recent findings that there was high iNOS content in the bladder tumor tissue, but not in the benign tissue (Klotz et al., 1999). Rahman, et al. found that iNOS expression was significantly higher in the surrounding liver in cirrhotic patients, and suggested that iNOS expression might be one of the phenotypical changes associated with the carcinogenic process in the cirrhotic liver (Rahman et al., 2001).

Recent studies showed that NO controls angiogenesis by modulating the activity of angiogenic factors (such as vascular endothelial growth factor) released by tumor cells (Ziche et al., 1994; 1997; Gallo et al., 1998) showed that NO played a central role in the angiogenic cascade by demonstrating that VEGF, released as a purified protein or produced by tumor cells, required a functioning NO/cyclic guanosinemonophsophate (cGMP) pathway within the endothelial compartment to promote neovascular growth. Dulak et al. (2000) also reported that VEGF expression was up-regulated by NO. In this study, a higher level expression of iNOS and VEGF was observed in HCC than in normal hepatic tissue. Which suggested that iNOS expression might play an important role for angiogenesis by up-regulating VEGF in HCC.

Tumor vascularization is a vital process for the progression of all solid tumors from a small, localized focus to an enlarging tumor with the capability to metastasize (Folkman et al., 1990). The authors experimental study showed that NO could stimulate the proliferation and migration of endothelial cell growth. NO inhibitor could reduce tumor vascularization in animal xenograft models. Initial studies by Gallo et al. (1998) demonstrated the enhanced NOS activity was accompanied by increasing tumor MVD in head and neck cancer. These findings were also supported by Ziche et al. (1997). In this study, the findings indicated that cases with combined positive expression for iNOS and VEGF had a higher MVD than cases with combined negative expression. In 10 normal hepatic tissues, there was lesser than HCC did, and most of the microvessels were concentrate on the portal area. This result was also consistent with the above reports and suggested that monitoring MVD may be a reference index for clinical diagnosis of HCC.

In this study, we also examined the expression of the apoptosis-related proteins p53 and bcl-2 in HCC. The findings showed that there was positive correlation between iNOS expression and bcl-2 expression, but not with p53 expression. This is consistent with the report that NO produced by iNOS inhibited apoptosis and promoted the survival of growth-arrested tumor cells via a bcl-2-mediated pathway (Dodd et al., 2000). Another report showed that regulation of bcl-2 levels might be critical in angiogenesis in vivo (Shigeki et al., 2001). Pidgeon et al. (2001) also showed that VEGF could upregulate bcl-2 and inhibit apoptosis in human and murine mammary adenocarcinoma cells. Our findings showed that iNOS positive expression might be closely correlated not only to angiogenesis but also to bcl-2 expression of HCC, and that these could be partially attributable to inhibiting tumour cell apoptosis.

To elucidate the impact of iNOS and VEGF expression on the cell cycle of HCC, we perform DNA-flow cytometric analyses in 11 cases in which iNOS and VEGF immunoreactivity was combined negative or positive. The finding indicated that the fraction of cells in the G_2M and S phase of the cell cycle in combined positive iNOS and VEGF positive expression cases was markedly higher than that in negative cases. This observation suggested that overexpression of iNOS and VEGF was associated with cell proliferation in HCC; and that iNOS and VEGF could participate in the regulatory mechanism of alteration of cell cycle in HCC.

CONCLUSIONS

All of these finding indicated that the expression of iNOS is closely related to angiogenesis bcl-2 and cell proliferation of HCC. Further research in this direction may elucidate the roles of iNOS in the development of HCC, which may form the bases for future anti-angiogenesis thera-

peutic strategies.

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