Decreased levels of nitric oxide production and nitric oxide synthase-2 expression are associated with the development and metastasis of hepatocellular carcinoma

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Abstract. Many studies have demonstrated the function of nitric oxide (NO) or nitric oxide synthase-2 (NOS-2) in cancer as pro-neoplastic or anti-neoplastic effectors, but the role of NO and NOS-2 in hepatocellular carcinoma (HCC) remains unclear. The aim of this study was to investigate the levels of NO production and NOS-2 expression in HCC and adjacent non-tumor liver tissues and to clarify whether the levels of NO/NOS-2 are related to the clinicopathological features of HCC. The levels of NO production were examined in tumor and adjacent non-tumor liver tissues of 30 patients with HCC. The expression of NOS-2 was detected by real-time polymerase chain reaction (RT-PCR) and immunohistochemical analysis in HCC and/or adjacent non-tumor liver tissues. Mutant p53 and proliferating cell nuclear antigen (PCNA) were also immunohistochemically investigated in liver tissues. The levels of NO in HCC were significantly lower compared to adjacent non-tumor liver tissues (P<0.001). The relative mRNA and protein expression levels of NOS-2 in HCC were also significantly lower compared to adjacent non-tumor liver tissues (P<0.01 for both). We found that the levels of NO in patients suffering from HCC metastasis were lower compared to those without metastasis (P<0.05) and NOS-2 expression was correlated with tumor diameter (P<0.05) and metastasis (P<0.05). In addition, mutant p53 protein was expressed in the majority of HCC samples and the proliferation rate of HCC was significantly higher than that of adjacent non-tumor liver tissues. These data indicate that decreased levels of NO/NOS-2 may partially contribute to overexpression of the mutant p53 protein and excessive proliferation; this may be a potential mechanism in the development and progression of HCC.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; EMT, epithelial to mesenchymal transition; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAB, diaminobenzidine; RT-PCR, real-time polymerase chain reaction

Key words: nitric oxide, nitric oxide synthases-2, p53, proliferation, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and deadly human tumor types. The majority of HCC cases occur in East and South-East Asia and in Middle and Western Africa, but HCC incidence rates are increasing in many parts of the world, including the United States and Central Europe in recent years (1-3). Surgical resection is the most effective treatment for HCC, but the tumor recurrence rate is approximately 70% at 5 years after resection (4). The exact molecular mechanisms responsible for HCC development have not yet been clarified. Therefore, searching for HCC-associated molecules may enable the identification of effective strategies for the chemoprevention and treatment of HCC.

Nitric oxide (NO) is generated by the oxidation of L-arginine under the catalytic activity of nitric oxide synthase (NOS). NO is a highly reactive radical that exerts a wide range of biological activities, including smooth muscle relaxation, inhibition of platelet aggregation and neurotransmission. There are three major isoforms of NOS: neuronal NOS (NOS-1), inducible NOS (NOS-2) and endothelial NOS (NOS-3). NOS-1 and NOS-3 are constitutively expressed at basal levels in various tissues, whereas NOS-2 is transcriptionally regulated and may be induced by various cytokines, such as tumor necrosis factor, interferon-γ and interleukin-1 (5). NOS-2 is expressed in various cell types and is capable of producing high amounts of NO.

Excessive NO production by NOS-2 has been demonstrated to be implicated in the pathogenesis of human malignant tumors, such as breast cancer (6), colon cancer (7),

melanoma (8) and lung cancer (9). A strong positive correlation between tumor NOS-2 expression and higher tumor grade or poorer patient survival has been reported in these studies. NO generated by NOS-2 may promote carcinogenesis through oxidative and nitrative DNA lesions, inducing mutations in the p53 gene (10). Studies in mice confirmed that the expression of mutant p53 protein may have a positive effect on cell growth and drive the development of various types of tumors (11). However, the functions of NO or NOS-2 in cancer development and progression remain controversial, with reports in the literature suggesting they are both pro-neoplastic and anti-neoplastic effectors. Recent evidence suggests that NO produced by NOS-2 can exert a negative effect on the regulation of tumor cell behavior and an antitumorigenic effect *in vitro* and *in vivo* (12-16). To investigate the role of NO/NOS-2 in hepatocarcinogenesis, the production of NO and the expression of NOS-2, mutant p53 protein and proliferating cell nuclear antigen (PCNA) were examined in HCC and non-tumor liver tissues.

Materials and methods

Tissue specimens. We obtained tumors and/or non-malignant liver tissues from 30 HCC patients who underwent hepatectomies in Tongji Hospital between 2010 and 2011. None of the patients had received chemotherapy or radiotherapy prior to surgery. Informed consent was obtained from all patients for subsequent use of their resected liver tissues. Tissue samples were collected immediately following liver resection. The non-malignant liver tissues were at least 2 cm in distance from the tumor margin. Half of the tissue was immediately frozen in liquid nitrogen and stored at -80˚C until use. Thirty patients had paired tumors and non-malignant liver tissues. The paired tumors and non-malignant specimens were not always available for HCC, due to limited tissue material, physical damage or necrosis in tissue. Part of the tissue (including 30 pair-matched tumors and non-malignant liver tissues) was immediately frozen in liquid nitrogen and stored at -80˚C until use for measurement of NO and NOS-2 mRNA. The other part of the tissue (including 28 pair-matched tumors and non-malignant tissues, 1 patient only had HCC tissue, 1 patient only had non-malignant tissue) was fixed in 4% paraformaldehyde and embedded in paraffin for histopathological diagnosis and immunohistochemical staining. The diagnoses were confirmed by histopathological study. Tumor staging was determined by the 6th edition of the Tumor-Node-Metastasis (TNM) Classification of the International Union Against Cancer. Table I shows the general clinicopathological features of the 30 patients with HCC. The evidence of metastasis included vascular invasion, particularly portal vein invasion and/or intrahepatic dissemination. Twenty-eight HCC patients showed markers of hepatitis B virus (HBV) infection, and 10 HCC patients had a history of alcohol abuse (mean alcohol consumption of 215.0±168.4 g/day; range 50-500 g/day). In 2 HCC cases, the underlying cause of liver disease remained unknown. No hepatitis C virus (HCV)-related HCC was found in this study. The present study was performed according to the guidelines of the ethics committee of the Tongji Hospital and approved in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

Table I. Clinicopathological characteristics of the 30 HCC patients.

Characteristics	Results
Gender	
Male	27
Female	3
Age (years)	
≤45	16
>45	14
Etiology	
HBV	18
HBV + alcohol	10
Unknown	2
Alpha-fetoprotein	
Normal	3
High	27
Tumor diameter (cm)	
≤5	3
>5	27
No. of tumors	
Single	24
Multiple	6
Pathological grade	
Well-differentiated	1
Well- to moderately differentiated	3
Moderately differentiated	15
Moderately to poorly differentiated	4
Poorly differentiated	7
Metastasis	
Yes	8
No	22
TNM	
I	17
II + III	13

HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

Measurement of NO production. Frozen tissue samples (100 mg) were mixed with 1.0 ml 0.01 M phosphate-buffered saline (PBS, pH 7.4) and incubated on ice and then homogenized. After centrifugation for 20 min at 12,000 rpm at 4˚C, the supernatants were transferred to fresh tubes. After the protein concentrations were assayed, NO levels were estimated by measuring the stable NO derivative, i.e. total nitrites, in tissue supernatant with a commercially available kit according to the manufacturer's instructions (Beyotime Biotech Inc., Haimen, Jiangsu, China). Briefly, 50 μl of supernatant was mixed with 100 μl of Griess reagent in a 96-well plate. Optical density was determined in a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 540 nm to form a standard curve (0-100 μM) derived from $NaNO_2$. Each experiment was performed in triplicate.

Real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from frozen tissue specimens (50-100 mg) using phenolchloroform and TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and the isolated RNA was dissolved in 0.1% diethylpyrocarbonate water for cDNA synthesis. RNA concentrations were examined by NanoDrop 2000 spectrophotometry (Thermo Scientific, Waltham, MA, USA). Reverse transcription was accomplished on 4 μg of total RNA using random primers Oligo$(dT)_{18}$ (Fermentas China, Shengzhen, China), dNTP Mix (Fermentas China) and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). PCR reactions were performed using Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). GAPDH was used as an internal control. The following gene-specific primers were used: GAPDH sense 5'-TCATTGACCTCAACTA CATGGTTT-3', and antisense 5'-GAAGATGGTGATGG GATTTC-3', yielding a 122-bp product; NOS-2 sense 5'-ACAAGCCTACCCCTCCAGAT-3', and antisense 5'-CCGGCCAGATGTTCCTCTA-3', yielding a 104-bp product (17). PCR assays were performed in triplicate on a ABI StepOne™ Real-time PCR System (Applied Biosystems, Foster City, CA, USA) running the cycling conditions: 5 min at 94˚C, followed by 45 cycles of 10 sec at 94˚C and 40 sec at 60˚C. Reaction specificity was detected by melting curve analysis, which was performed by heating the plate from 55 to 95˚C and measuring SYBR-Green I dissociation from the amplicons. The PCR products were visualized on 2% agarose gels with GoldView staining under UV transillumination. Relative mRNA levels of NOS-2 were calculated and expressed as $2^{-\Delta Ct}$, according to the formula ΔCt = Ct (NOS-2) - Ct (GAPDH) (18).

Immunohistochemical staining. Serial sections (5-μm thick) were prepared from paraffin blocks. The sections were deparaffinized in xylene and hydrated in graded alcohol, then incubated in 3% H_2O_2 in absolute methanol for 10 min to block endogenous peroxidase. They were heated by microwaving in citrate buffer (0.01 M, pH 6.0) for 3 min at 800 W, for 7 min at 640 W and for 3 min at 480 W. Slides were slowly cooled down to room temperature. After washing with PBS (0.01 M, pH 7.4), non-specific binding sites were blocked with 8% bovine serum albumin (BSA) and 2% horse serum for 20 min at room temperature. Slides were incubated with the following primary antibodies at 4˚C overnight: a rabbit anti-NOS-2 polyclonal antibody (Wuhan Boster Bio-engineering Co., Ltd., China) at 1:100 dilution, a mouse anti-p53 monoclonal antibody (Wuhan Boster Bio-engineering Co., Ltd.) at 1:100 dilution, and a mouse anti-PCNA monoclonal antibody (Wuhan Boster Bio-engineering Co., Ltd.) at 1:400 dilution. After washing the slides with PBS for 10 min, the antibody detection was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Immunoreactive cells were visualized with diaminobenzidine (DAB; Dako, Glostrup, Denmark) solution and then counterstained with hematoxylin. Finally, the sections were coved with neutral balsam. All slides were subjected to the same procedure under standardized conditions. Negative controls were performed by replacing the primary antibody with PBS.

The immunoreactive score of NOS-2 was assessed by the percentage of positively stained cells (8): 0, no positive staining; 1, 1-25% cells positive; 2, 26-50% cells positive; 3, 51-75% cells positive; 4, 76-100% cells positive. Specimens with scores ≥2 were labeled as 'positive'. The frequency of p53 and PCNA positively stained nuclei was expressed as a percentage of stained cell nuclei over the total number of cells counted, and 1,000 cells were observed in five or more random fields at a magnification of x200 to calculate the percentage of positive cells. 'p53 positive (+)' was defined as positive nuclear p53 staining ≥10%, whereas 'p53 negative (-)' was for cases whose positive nuclear p53 staining was <10%. The proliferation rate was shown as % PCNA-positive nuclei.

Statistical analysis. Quantitative data were expressed as the means ± standard deviation and qualitative data as the number of cases. The quantitative data were calculated using the Wilcoxon signed rank test for paired groups, unpaired groups were compared by Mann-Whitney U test. The qualitative data were compared using χ^2 test and Fisher's exact test. Correlation between factors was evaluated using the Spearman's rank correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 13.0 and GraphPad Prism version 5.0.

Results

Level of NO in HCC and pair-matched non-tumor liver tissues. The content of NO was 12.96±5.89 μmol/g protein in HCC, while it was 19.58±7.46 μmol/g protein in pair-matched non-tumor liver tissues, and the difference was significant ($P<0.001$; Fig. 1A). There was no significant difference in NO level in association with age, etiology, tumor number, differentiation and TNM stage (data not shown), but the patients suffering from HCC metastasis had lower NO levels than those without metastasis (9.11±3.48 μmol/g protein vs. 14.36±6.01 μmol/g protein; $P<0.05$; Fig. 1B).

NOS-2 mRNA expression in HCC and pair-matched non-tumor liver tissues. Comparing the relative expression of NOS-2 mRNA in HCC and pair-matched non-tumor liver tissues from 30 cases, HCC tissues showed much lower expression than non-tumor liver tissues ($P<0.01$; Fig. 1C).

NOS-2 protein expression in HCC and non-tumor liver tissues. The expression of NOS-2 was observed mainly in the hepatocytes or cancer cells, showing mainly cytoplasmic staining, with the positive cells distributed in both HCC and non-tumor liver tissues. The NOS-2 expression was not observed in inflammatory cells, bile duct epithelium and vascular endothelium. NOS-2 immunoreactive score was significantly higher in non-tumor liver than in HCC tissues ($P<0.01$; Table II and Fig. 2B). In addition, we found that NOS-2 expression was correlated with tumor diameter ($P<0.05$; Table III) and metastasis ($P<0.05$; Table III and Fig. 2F-H). There were no differences between the groups in terms of age, etiology, tumor number, differentiation and TNM staging (Table III).

Nuclear expression of mutant p53 protein in liver tissues. The nuclear expression of mutant p53 protein was detectable in 22 of 28 HCC tissues (78.6%, one case was not available due to

Table II. Expression of NOS-2 in HCC and adjacent non-tumor liver tissues.

	NOS-2 expression						
Tissue	0	1	2	3	4	Positive rate (%)	P-value
HCC (n=29)	11	4	9	5	0	48.3	0.0057[a]
Non-tumor (n=29)	0	5	10	11	3	82.8	

[a]HCC compared with non-tumor liver tissue. NOS-2, inducible NOS; HCC, hepatocellular carcinoma.

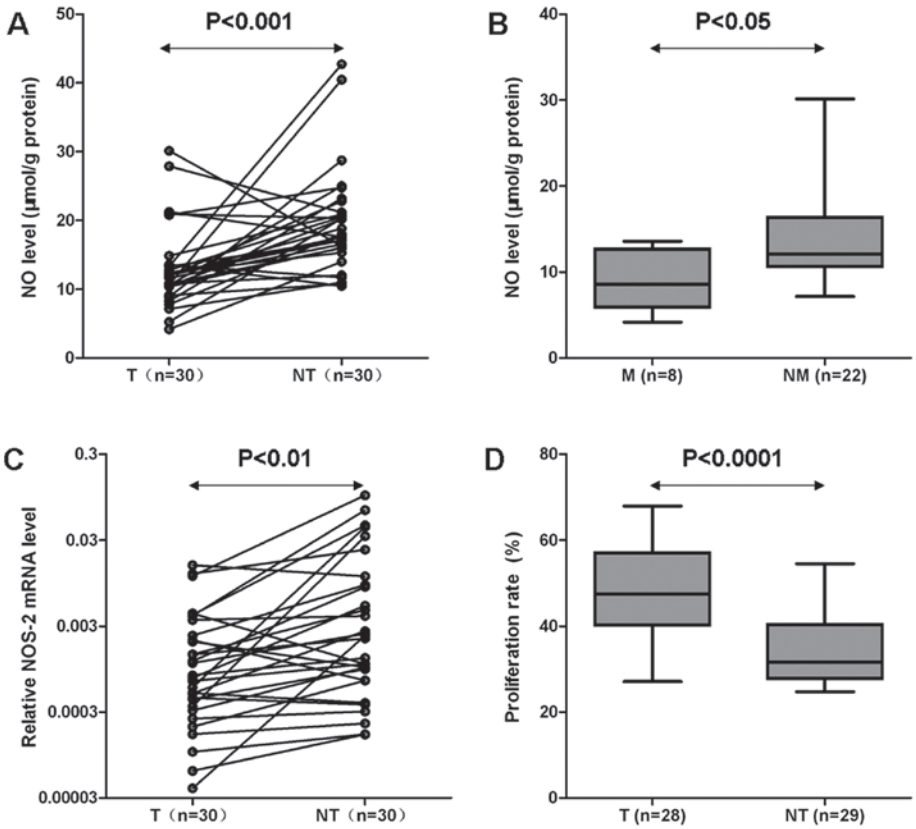

Figure 1. Comparison of NO level, relative NOS-2 mRNA expression and proliferation rate in liver tissues. (A) NO levels were compared in HCC and pair-matched non-tumor liver tissues. (B) NO levels were compared in HCC tissues with metastasis and those without metastasis. (C) Relative NOS-2 mRNA levels were analyzed in HCC and pair-matched non-tumor liver tissues by RT-PCR. (D) The proliferation rate was compared in HCC and non-tumor liver tissues. T, HCC tissue; NT, non-tumor liver tissue; M, metastasis; NM, no metastasis.

lack of material), whereas this was not found in any non-tumor liver tissue (Fig. 2C). Only well-differentiated (n=1), well- to moderately differentiated (n=1) and moderately differentiated (n=4) HCC showed negative expression of the mutant p53 protein. No significant differences were noted in mutant p53 expression in association with any clinicopathological features in HCC (data not shown). The positive expression of NOS-2 did not show a significant correlation with the expression rate of mutant p53 protein in HCC tissues.

Positive expression of PCNA in liver tissues. The proliferation rate in HCC (47.6±10.9%) was significantly higher than that in non-tumor liver tissues (34.1±8.2%, P<0.0001; Figs. 1D and 2D). There was no significant association between proliferation rate and any clinicopathological features in HCC (data not shown).

Discussion

NO is a simple, inorganic, free radical gas that exerts an important role in numerous physiological and pathophysiological conditions. However, the functions of NO in the development and progression of cancer remain controversial, with reports in the literature of it acting as a pro-neoplastic or an anti-neoplastic agent.

In the present study, we used *in vivo* measurement of NO to determine the role it may play in the development of HCC. We found the content of NO in HCC was significantly lower than in non-tumor liver tissues. This suggested that a systemic decrease in NOS expression may occur in HCC tissue. On the contrary, a previous study reported that NO biosynthesis was higher in tumor tissues obtained from primary human

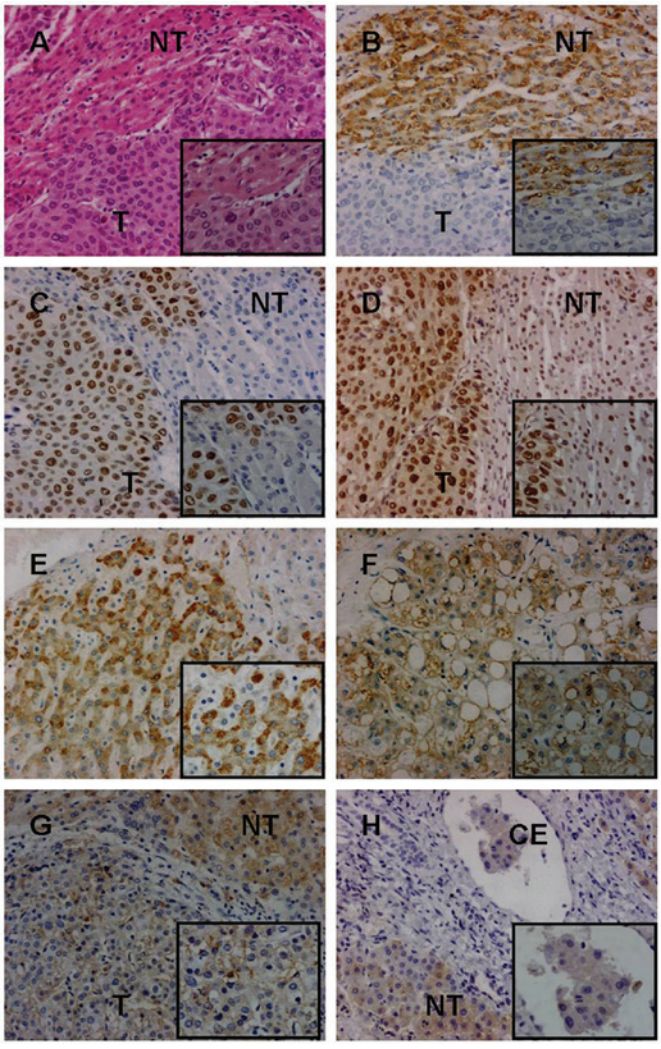

Figure 2. Immunohistochemical analysis of NOS-2, mutant p53 protein and PCNA expression in liver tissues. (A-D) Case no. 2100: (A) moderately differentiated HCC and adjacent non-tumor liver tissue were examined by H&E staining. (B) The immunoreactivity of NOS-2 in adjacent non-tumor liver tissue was significantly stronger than in HCC. (C) Mutant p53 protein was positively expressed in HCC, but was negative in adjacent non-tumor liver tissue. (D) The positive rate of PCNA was significantly greater in HCC than in adjacent non-tumor liver tissue. (E-F) Case no. 9073: (F) moderately differentiated HCC without metastasis (clear cell carcinoma subtype) and (E) non-tumor liver tissue (at least 2 cm in distance from the tumor margin); NOS-2 had significantly positive expression in HCC and non-tumor liver tissue. (G-H) Case no. 8894. (G) Moderately differentiated HCC (T, primary tumor) and adjacent non-tumor liver tissue (NT). (H) Pair-matched non- tumor liver tissue (NT, at least 2cm in distance from the primary tumor margin) and metastatic cancer embolus of portal vein (CE). The immunoreactivity of NOS-2 was significantly stronger in HCC without metastasis than in that with metastasis (F vs. G) and was also significantly stronger in primary tumor than in metastatic cancer embolus (G vs. H). (Original magnification, x200 and x400). T, HCC tissue; NT, non-tumor liver tissue; CE, cancer embolus; NOS-2, inducible NOS; HCC, hepatocellular carcinoma.

Table III. Relationship between NOS-2 expression and clinicopathological features in HCC.

Variables	Positive (n=14)	Negative (n=15)	P-value
Age (years)[a]	45.4±8.9	44.9±12.5	0.7931
Etiology			0.1201
HBV	6	11	
HBV+ alcohol	7	3	
Unknown	0	2	
Tumor diameter (cm)[a]	6.5±3.0	9.4±3.8	0.0121
Tumor no.			0.6513
Single	12	11	
Multiple	2	4	
Pathological grade[b]			0.4497
Group I	10	8	
Group II	4	7	
Metastasis			0.0352
Yes	1	7	
No	13	8	
TNM staging			0.1394
I	10	6	
II + III	4	9	

[a]Data are expressed as the means ± SD; [b]HCC was subdivided into two groups according to differentiation degree; group I was defined as well-, well- to moderately and moderately differentiated and group II was defined as moderately to poorly and poorly differentiated. NOS-2, inducible NOS; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

breast cancers compared with benign lesions or normal breast tissue (6). Consistent with the biochemical observation, we found that the mRNA and protein expression of NOS-2 was lower in HCC than in non-tumor liver tissues. Our result was in agreement with that of another study, in which the authors also observed that NOS-2 immunoreactivity was significantly lower in tumor tissue than in the surrounding non-tumor liver tissues in HCV-positive HCC cases (19). These findings indicated that the decreased level of NO/NOS-2 may be associated with hepatocarcinogenesis. However, this is in contrast to the previous study, which demonstrated that the human colon tumors contained higher levels of NOS-2 mRNA than the surrounding normal tissues (7). The variation in NO production and NOS-2 expression in different types of tumor tissues suggested that NOS-2 may have a tissue-specific expression pattern in human tumors.

In the present study, it was of note that HCC with negative NOS-2 expression was more likely to have a large diameter and a greater propensity for metastasis. These findings implied that the deletion of NOS-2 could partially contribute to the growth and metastasis of HCC. This observation was consistent with a previous study in which NOS-2-null tumor cells that were injected subcutaneously grew much faster, and when these cells were injected intravenously (i.v.) there was more lung metastasis in NOS-2$^{-/-}$ mice than in NOS-2 wild-type mice (20). In addition, recent reports found that the high level of NO provided primarily by the NO donor could inhibit the metastatic cascade, including epithelial to mesenchymal transition (EMT), migration and invasion in multiple cancer cells. These results suggested that NO/NOS-2 may be an important mediator of an aggressive phenotype in HCC (13,14,21).

It was also of note that the immunoreactivity of NOS-2 was significantly higher in non-tumor liver tissues than in HCC tissues. The nuclear expression of mutant p53 protein was detectable in 78.6% of HCC tissues, whereas this was not found in any non-tumor liver tissues. This finding appears to be consistent with the hypothesis that low concentrations of NO may induce p53 alteration or mutation, which cause tumor cell resistance; however, at high concentrations, the DNA damage induced by NO increases wild-type p53, leading to programmed cell death (10). As a transcription factor, wild-type p53 protein has a crucial role in promoting apoptosis, senescence or protective cell cycle arrest and suppressing tumorigenesis. The p53 gene is one of the most frequently targeted for genetic alterations in many cancers, and is found to be mutated and accumulated in tumor tissues. Studies *in vitro* and *in vivo* have confirmed that the expression of mutant p53 protein can have a positive effect on cell growth and contribute to carcinogenesis (11,22). In our study, we found the mutant p53 protein was expressed in the majority of cases of HCC, and the proliferation rate of HCC tissue was significantly higher than that of adjacent non-tumor liver tissues. This implies that the expression of mutant p53 protein had a positive effect on cell proliferation and contributed to the development of HCC. Therefore, the decreased expression of NOS-2 in liver tissues may stimulate cell proliferation and promote hepatocarcinogenesis by inducing the overexpression of mutant p53 protein.

In conclusion, we have demonstrated that the production of NO and expression of NOS-2 in non-tumor liver tissues were higher than in HCC tissues. The decreased level of NO/NOS-2 in liver tissues may partly contribute to the mutant p53 protein overexpression and excessive cell proliferation, eventually leading to the development and progression of HCC.

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