

# Inducible Nitric Oxide Synthase and Survivin Messenger RNA Expression in Hepatocellular Carcinoma

Masahide Ikeguchi,<sup>1</sup> Tsuyoshi Ueta,  
Yoshiaki Yamane, Yasuaki Hirooka, and  
Nobuaki Kaibara

From the First Department of Surgery, Faculty of Medicine, Tottori University, Yonago 683-8504, Japan

## ABSTRACT

**Purpose:** Proliferative activity and suppression of apoptosis of cancer cells are important to tumor progression in hepatocellular carcinoma (HCC). Recently, the expressions of inducible nitric oxide synthase (iNOS) and survivin mRNA have been reported to correlate with suppression of apoptosis in some tumors. However, the clinical importance of expression of these genes in HCC progression remains unclear. In the present study, the correlation between the expression of iNOS and survivin mRNA and the occurrence of spontaneous apoptosis and proliferative activity of cancer cells and prognostic importance of expression of these genes in HCC were investigated.

**Experimental design:** Tissues were obtained by surgical resection of livers from 61 patients with HCC and 8 without HCC. Expressions of iNOS and survivin mRNA were evaluated using the reverse transcription-PCR in 61 tumors, 61 adjacent histologically noncancerous livers, and 8 normal livers. Apoptotic cancer cells and the proliferative activity of cancer cells were detected by immunohistochemistry.

**Results:** iNOS mRNA expression was detected in 34 of 61 (55.7%) HCCs, 19 of 61 (31.1%) noncancerous liver tissues adjacent to carcinoma, and none of the 8 normal livers. In addition, survivin mRNA was detected in 19 of 61 (31.1%) HCCs, none of 61 noncancerous liver tissues, and none of the 8 normal livers. iNOS mRNA expression did not correlate with the proliferative activity of cancer cells or with the occurrence of apoptosis in HCCs. In contrast, survivin mRNA expression strongly correlated with a high proliferative activity of cancer cells and a low apoptotic index. Disease-specific survivals did not differ between patients with iNOS-positive or -negative HCCs. Although, the disease-specific survival of patients with survivin-positive HCCs was significantly poorer than that of patients with survivin-negative HCCs.

**Conclusions:** These results indicate that iNOS may not correlate with cancer cell-proliferative activity or apoptosis; survivin, however, may not only suppress apoptosis but also accelerate cancer cell-proliferative activity and play an important role in tumor progression in HCC.

## INTRODUCTION

Surgical resection is the only proven cure for HCC.<sup>2</sup> However, >80% of patients with HCC who undergo hepatectomy develop new tumors in the residual livers within 2 years (1). Once a tumor has developed in the residual liver, the patient prognosis is poor. To achieve effective management of recurrence as soon as possible after hepatectomy, it is important to identify the patients with a high risk of cancer recurrence. A variety of clinicopathologic factors and biological markers has been investigated in HCC (2–4); these factors, however, have not yet been sufficiently defined in patients with a high risk of cancer recurrence.

The proliferative activity of cancer cells or regulation of programmed cell death (apoptosis) has been reported to be important to cancer recurrence in various cancers, including HCCs (5–7). Inhibition of apoptosis might confer a survival advantage on malignant cells harboring genetic alterations and thus promote neoplastic progression. Recently, the expressions of two genes (inducible NOS and survivin) are reported to correlate with suppression of apoptosis in cancer cells.

NO is a product of the conversion of L-arginine to L-citrulline by NOS (8). NO, a free radical with a short half-life, has a multitude of biological functions, including neuronal transmission, vasodilatation, and smooth muscle relaxation (9). NOS exists as three enzyme classes: the calcium-dependent endothelial (eNOS), the neuronal or brain isoforms (nNOS), and a calcium-independent inducible NOS (iNOS) (10). iNOS produces much larger amounts of NO and has been detected in many human tumors, such as breast cancer, melanoma, bladder cancer, and colorectal cancer (11–14). A significant positive correlation between tumor iNOS expression and tumor progression or poor patient survival has been reported from these studies. Moreover, recent studies have revealed that endogenous NO can exert an antiapoptotic function in different cancerous and noncancerous cell lines in response to proapoptotic stimuli (15–17).

Several apoptosis inhibitors related to the baculovirus inhibitor of apoptosis genes have recently been identified in the mouse, *Drosophila*, and humans (18). Survivin belongs to a family of inhibitors of apoptosis and has been shown to bind and inhibit the cell-death terminal effectors caspase-3 and -7, which

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<sup>1</sup> To whom requests for reprints should be addressed, at Department of Surgery I, Faculty of Medicine, Tottori University, 36-1 Nishi-cho, Yonago 683-8504, Japan. Phone: +81 859 34 8111; Fax: +81 859 34 8095; E-mail: masaike@grape.med.tottori-u.ac.jp.

<sup>2</sup> The abbreviations used are: HCC, hepatocellular carcinoma; NO, nitric oxide; NOS, nitric oxide synthase; RT-PCR, reverse transcription-PCR; AI, apoptotic index; LI, labeling index.

induce apoptosis in cells (19). In addition, survivin is expressed during the G<sub>2</sub>-M phase of the cell cycle. At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle. Disruption of survivin-microtubule interactions results in a loss of the antiapoptosis function of survivin, and during mitosis, caspase-3 activity increases (20). These results suggest that survivin not only inhibits apoptosis of cancer cells but also accelerates their proliferative activity.

However, the clinical importance of iNOS or survivin gene expression in HCC remains unclear. In the present study, we investigated whether iNOS mRNA or survivin mRNA expression correlates with tumor-cell proliferative activity and tumor-cell apoptosis and whether this mRNA expression is a good indicator of cancer recurrence in the residual liver in patients with HCC who have undergone hepatectomy.

## MATERIALS AND METHODS

**Cell Line.** The human HCC cell line, HepG2, was purchased from Riken Gene Bank (Tsukuba Science City, Japan). HepG2 was maintained in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS (Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

**Tissues.** We obtained tumors and adjacent noncancerous liver tissues from 61 patients with HCCs and eight normal liver tissues from 8 patients without HCCs and without liver cirrhosis (four hepatic injuries caused by traffic accidents, two hemangiomas, and two metastatic liver tumors of colorectal carcinoma). These 69 patients underwent hepatectomies between 1993 and 1999. Informed consent was obtained from all patients for subsequent use of their resected tissues. The present study conformed to the ethical standards of the World Medical Association Declaration of Helsinki. Tissue samples of ~1 g were collected immediately after liver resection. Noncancerous liver tissues were obtained from regions distant from the tumors. Half of the tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections (4- $\mu$ m thick) were prepared for H&E staining for histopathologic diagnosis and for immunohistochemical staining. The other half of the tissue was stored at -80°C until needed.

**Detection of iNOS and Survivin Transcripts.** Before starting the study, histopathologic examination confirmed that there were enough cancer cells in the tumor samples and that no cancer cells had contaminated the noncancerous liver tissues or normal liver tissues. Total RNA from HepG2 and tissues was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. Five micrograms of the total RNA from each sample were heated to 60°C in a water bath for 10 min and then cooled on ice for 2 min. cDNA was synthesized with 5  $\mu$ g of total RNA and 0.5  $\mu$ g of oligo(dT)<sub>15</sub> primer (Promega, Madison, WI) with Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The beads contained Moloney murine leukemia virus reverse transcriptase, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 7.5 mM DTT, 10 mM MgCl<sub>2</sub>, and 2.4 mM each deoxynucleotide triphosphate. Total volume was adjusted to 33  $\mu$ l with diethylpyrocarbonate water. The beads, with the reaction mixture, were incubated at 37°C in a

water bath for 60 min. A RT-PCR was performed using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) containing 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each deoxynucleotide triphosphate, and stabilizers. RT-PCR was performed with 5  $\mu$ l of cDNA and 50 pmol of each primer and was adjusted to 25  $\mu$ l with diethylpyrocarbonate water. The following primer pairs were used for amplification of iNOS: sense 5'-TGCCTGGCAAGCCCAAGGTCTATGTTTCAGGAC-3' and antisense 5'-GGTGCTGCTTGTAGGAGGTCAAGTAAAGGGC-3' (21), survivin: sense 5'-GGCATGGGTGCCCGACGTTG-3' and antisense 5'-CAGAGGCCTCAATCCATGGCA-3' (22), and for glyceraldehyde-3-phosphate dehydrogenase: sense 5'-CCACCCATGGCAAATTCATGGCA-3' and antisense 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (23). The amplification products of iNOS, survivin, and glyceraldehyde-3-phosphate dehydrogenase were 500, 439, and 600 bp in length. RT-PCR was performed by a DNA thermal cycler (Zymoreactor II; ATTO, Tokyo, Japan). The initial denaturation was performed at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and finally 72°C for 1 min. The PCR products were visualized on 2% agarose gels with ethidium bromide staining under UV transillumination.

**Immunohistochemistry.** The paraffin-embedded tissues (4- $\mu$ m thick) were dewaxed using xylene and transferred to alcohol, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. After washing with PBS, slides were incubated with the primary antibodies overnight at 4°C. A monoclonal antibody Ki-67 (MIB-1, diluted 1:50; Immunotech International, Marseille, France) was used for detection of proliferative activity of cancer cells. A polyclonal rabbit antisense-stranded DNA (diluted 1:200; DAKO Japan Co., Ltd., Kyoto, Japan) was used to detect apoptotic cells. A rabbit anti-iNOS polyclonal antibody (diluted to 1:2500; Chemicon International, Inc., Temecula, CA) and a rabbit antihuman survivin polyclonal antibody (diluted to 1:20; Alpha Diagnostic International, San Antonio, TX) were used to identify iNOS or survivin protein-expressing cells. After washing the slides with PBS, ENVISION+ (DAKO Japan Co. Ltd.) was applied as the secondary antibody for 30 min. The reaction products were visualized with diaminobenzidine as the chromogen, and the slides were counterstained with methyl green. To determine the average numbers of cancer cells with signs of proliferative activity or of apoptosis, 20 microscopic fields were monitored randomly in each sample, and between 1000 and 2000 cancer cells were examined by one independent observer (T.U.). The results were expressed as the Ki-67 LI (percentage of immunostained cancer cells) and the AI (percentage of immunostained apoptotic cancer cells).

**Patients.** The patients' clinical records and histopathologic diagnoses were fully reviewed. The subjects included 49 men and 12 women, and their age at the time of surgery was 61.2  $\pm$  11 years (mean  $\pm$  SD; median 64 years, ranging from 28 to 80 years). Histopathologic diagnoses of patients were made according to the guidelines for classification of primary liver cancer (24). The stages of the 61 patients were Stage I (*n* = 6), II (*n* = 16), III (*n* = 26), and IV (*n* = 13). None of the patients had received preoperative or postoperative chemotherapy. All patients were followed until August 2001. The mean follow-up

period for the 61 patients was 39.1 months (ranging from 2 to 97 months). Recurrence of HCC was detected in 36 patients. The sites of recurrence were identified by computed tomography and ultrasonography. After the detection of cancer recurrence, cannulation into the hepatic artery was performed from the right femoral artery, and transcatheter arterial embolization was performed in these 35 patients. Causes of death were determined from the clinical findings.

**Statistical Analysis.** The  $\chi^2$  and Fisher's exact probability tests were used to compare the distribution of individual variables among the patient groups. The differences between the numerical data of the two groups were evaluated using the Mann-Whitney U test. The differences in the numerical data among more than three groups were evaluated using the Kruskal-Wallis test. Survival rates were calculated using the Kaplan-Meier method. The Log-rank test was used for comparisons of two survival curves. A *P* of 0.05 was considered to be statistically significant.

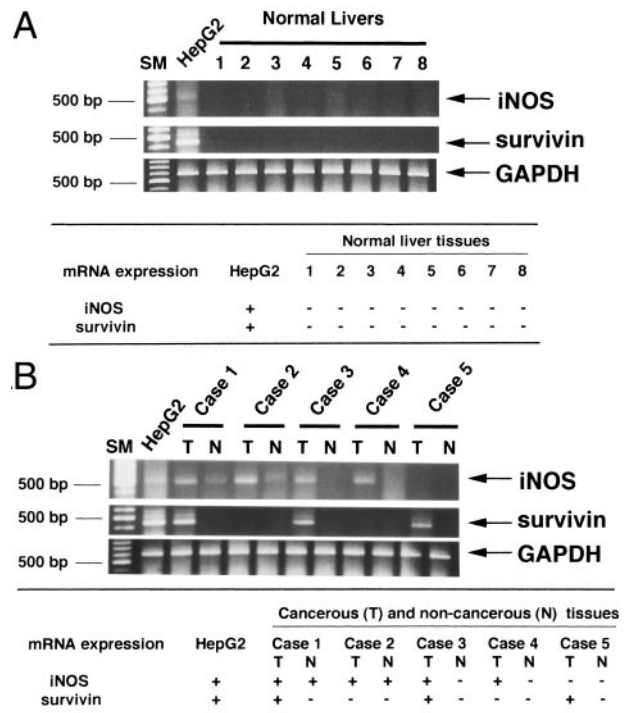
**RESULTS**

**iNOS and Survivin mRNA Expression in HCCs.** The HepG2 cell line was used as a positive control in subsequent experiments. iNOS mRNA expression was detected in 34 of 61 (55.7%) HCCs, 19 of 61 (31.1%) noncancerous liver tissues adjacent to carcinoma, and none of the 8 normal livers. In addition, survivin mRNA was detected in 19 of 61 (31.1%) HCCs, none of the 61 noncancerous liver tissues, and none of the 8 normal livers (Fig. 1). In 15 patients, iNOS mRNA was detected both in tumors and in noncancerous liver tissues. In 19 survivin-positive HCCs, coexpression with iNOS was observed in 17 HCCs (89.5%).

**Correlation between iNOS and Survivin mRNA Expression in HCC and Clinicopathologic Characteristics of the Patients.** Tumor iNOS expression did not correlate with the tumor stage and histological type of tumors (Table 1). iNOS mRNA expression was detected in noncancerous liver tissues in 19 patients. In these patients, no significant correlations between iNOS expression in noncancerous liver and the type of viral infection or degree of liver cirrhosis were observed (data not shown). In contrast, survivin mRNA expression was detected frequently in hepatitis C viral-infected HCCs, poorly differentiated HCCs, or stage III or IV HCCs (Table 1).

**Localization of iNOS and Survivin Proteins in HCCs.** Tumors with iNOS or survivin mRNA positive showed strong iNOS or survivin protein expression by immunostaining. iNOS protein was detected in the cytoplasm of cancer cells (Fig. 2A). Survivin protein was detected not only in the cytoplasm but also in nuclei of cancer cells (Fig. 2B).

**Relationship between iNOS and Survivin mRNA Expression and Proliferative Activity or Occurrence of Apoptosis in Cancer Cells.** The proliferative activity of cancer cells is described as Ki-67 LI in each case. The mean Ki-67 LI of 61 HCCs was 8.9% (range, 0–47.2%; median, 8%). The mean Ki-67 LI of poorly differentiated carcinoma (12.5%) was greater than that of well (4.1%) or moderately differentiated carcinoma (9%); however, this result was not significant (*P* = 0.1122 by Kruskal-Wallis test). The mean Ki-67 LIs of small did not differ (7.8%, tumor size < 3.4 cm) from that of large



**Fig. 1** A, RT-PCR products of eight normal livers. iNOS and survivin mRNA were not detected in normal livers. In B, iNOS mRNA expression was detected in tumors and noncancerous liver tissues (Cases 1 and 2). In contrast, survivin mRNA expression was detected in only tumors (Cases 1, 3, and 5).

tumors (10.1%, tumor size  $\geq$  3.4 cm; *P* = 0.876). The Ki-67 LI did not correlate with tumor stage (*P* = 0.5514). The mean Ki-67 LIs of stage I, II, III, and IV tumors were 7.1, 7, 8.1, and 13.6%, respectively. The mean AI of 61 HCCs was 2.5% (range, 0–14.5%; median, 1.7%). The mean AIs of well, moderately, and poorly differentiated carcinomas were 3.7, 2.2, and 2.6%, respectively. The mean AIs of small and large tumors were 2.8 and 2.1%. The mean AIs of stage I, II, III, and IV tumors were 4.4, 2.5, 2.1, and 2.2%, respectively. AIs did not correlate with tumor histology (*P* = 0.1738), tumor size (*P* = 0.2224), or tumor stage (*P* = 0.2971). Tumor iNOS mRNA expression did not correlate with either the Ki-67 LI or AI. In contrast, tumor survivin mRNA expression strongly correlated with a high proliferative activity of cancer cells and a low AI (Table 2).

**iNOS and Survivin mRNA Expression and Prognosis of Patients with HCC.** No residual tumors were detected in 61 patients at hepatectomy. Thirty-two patients remained alive, and 22 patients died (17 died from HCC, and 5 died from liver failure caused by liver cirrhosis) in August 2001. Recurrence of HCC was detected in 36 of 61 patients (59%). Of these 36, 17 patients had died from HCC, and 19 patients were still living. In 34 patients with iNOS-positive HCC, 25 patients (73.5%) showed tumor recurrence, whereas tumor recurrence was detected in 11 of 27 (40.7%) patients with iNOS-negative HCC. The difference was significant (*P* = 0.0097). Moreover, tumor recurrence was detected in 16 of 19 (84.2%) patients with survivin-positive HCC and in 20 of 42 (47.6%) with survivin-



Table 1 iNOS and survivin mRNA expression in tumor and clinicopathological findings

Variables	No.	Positive cases with tumor iNOS and survivin expression (%)			
		iNOS positive	<i>P</i>	Survivin positive	<i>P</i>
Viral infection					
B	24	13 (54.2)	0.68	4 (16.7)	0.04
C	32	19 (59.4)		15 (46.9)	
B + C	1	0		0	
Non-B, non-C	4	2 (50)		0	
Histology					
Well	8	5 (62.5)	0.9181	1 (12.5)	0.0274
Moderate	42	23 (54.8)		11 (26.2)	
Poor	11	6 (54.5)		7 (63.6)	
Stage					
I	6	2 (33.3)	0.1499	0	0.0391
II	16	6 (37.5)		2 (12.5)	
III	26	17 (65.4)		12 (46.2)	
IV	13	9 (69.2)		5 (38.5)	

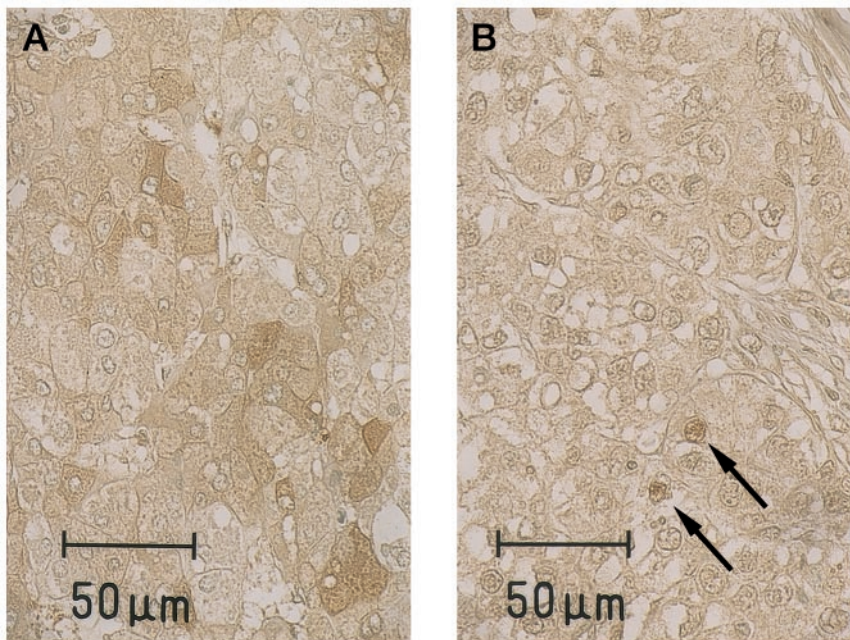


Fig. 2 Immunohistochemical detection of iNOS and survivin protein in HCC. In A, iNOS protein was detected in the cytoplasm of cancer cells ( $\times 100$ ). In B, Survivin protein was also detected in the cytoplasm, and in some cancer cells, it was detected in cancer cell nuclei (arrows,  $\times 100$ ).

negative HCC. This difference was also significant ( $P = 0.0071$ ). The disease-specific 5-year survival rate of 61 patients was 72.7%. The disease-specific 5-year survival rate of 34 iNOS-positive patients (66.7%) did not differ from that of 27 iNOS-negative patients (84.1%;  $P = 0.5404$ ). In contrast, the disease-specific 5-year survival rate of 19 survivin-positive patients (50.7%) was significantly poorer than that of 42 survivin-negative patients (86.1%;  $P = 0.0329$ ). Factors affecting tumor recurrence in the residual livers were analyzed with regard to five parameters (liver fibrosis, multiple cancers, venous involvement, iNOS mRNA expression, and survivin mRNA expression) using the multivariate logistic regression analysis (Table 3). Although there was not statistical significance, survivin mRNA expression in tumors was detected as a good risk factor regarding carcinoma recurrence in residual liver in HCC patients.

## DISCUSSION

Clear evidence that iNOS mRNA or protein expression is correlated with carcinogenic processes in the cirrhotic liver has not been demonstrated previously. However, in the present study, we found that the percentage of detectable iNOS mRNA expression significantly increased from 0% of normal controls to 31.1% of noncancerous livers adjacent to HCCs and to 55.7% of HCCs ( $P = 0.0012$ ). The same result was reported in colorectal cancer (25). The overproduction of NO in malignant tissue by iNOS may inhibit immune defense mechanism (26), and NO may increase tumor blood flow and promote angiogenesis (27). These findings indicate that aberrant iNOS expression may be one of the phenotypical changes associated with carcinogenic processes in the cirrhotic liver. Moreover, Salvucci *et*

Table 2 Correlation between proliferation index (Ki-67 LI) and apoptotic index (AI) of HCCs and tumor iNOS and survivin mRNA expression

mRNA expression in HCCs		No.	Ki-67 LI <sup>a</sup> (% , mean $\pm$ SE)	P	AI <sup>a</sup> (% , mean $\pm$ SE)	P
iNOS	Positive	34	10.3 $\pm$ 1.8	0.334	2.4 $\pm$ 0.5	0.2167
	Negative	27	7.1 $\pm$ 1.2		2.5 $\pm$ 0.5	
Survivin	Positive	19	15.5 $\pm$ 2.5	0.0001	1.0 $\pm$ 0.3	0.0002
	Negative	42	5.9 $\pm$ 0.9		3.1 $\pm$ 0.5	

<sup>a</sup> Ki-67 LI, percentage of Ki-67-positive cancer cells; AI, percentage of apoptotic cancer cells.

Table 3 Factors affecting tumor recurrence analyzed by multivariate logistic regression analysis

Variable	$\chi^2$	P
Liver cirrhosis (cirrhosis vs. fibrosis)	2.227	0.1356
Multiple cancers (present vs. absent)	0.984	0.3213
Venous involvement (positive vs. negative)	2.265	0.1323
iNOS mRNA expression (positive vs. negative)	1.35	0.2452
Survivin mRNA expression (positive vs. negative)	2.934	0.0867

*al.* (17) have reported that inhibition of endogenous iNOS activity induces a significant increase in the number of apoptotic cells in human melanomas. This finding indicates that endogenous iNOS expression may exert an antiapoptotic role in transformed cells and may correlate with tumor progression. However, we found no significant correlation between iNOS mRNA expression and tumor stages or tumor differentiation in HCC; moreover, iNOS mRNA expression did not correlate with the proliferative activity of cancer cells or with the occurrence of apoptosis in cancer cells. From our results, we conclude that iNOS mRNA or protein expression may correlate with carcinogenesis of cirrhotic livers but may not play an important role in tumor progression in HCC.

Strong survivin expression has been reported in several fetal tissues, whereas survivin transcripts have been detected only in the thymus and placenta of adult tissues (28). However, in the most common human cancers, survivin expression is again turned on. Survivin is known to be a member of suppressors of apoptosis (18, 19). Recently, Ambrosini *et al.* (29) have reported that survivin antisense oligonucleotide-transfected HeLa cells showed decreased cell proliferation as compared with induced vector control transfectants. In addition, Ito *et al.* (22) have reported that HCC cell lines transfected with survivin show a significant decrease in cells in the G<sub>0</sub>-G<sub>1</sub> phase and an increase in cells in the S and G<sub>2</sub>-M phase. These findings suggest that the re-expression of survivin mRNA may correlate not only with reduced apoptotic cell death but also with increased proliferative activity of cancer cells. In the present study, we found that the expression of survivin mRNA was significantly associated with a low incidence of apoptosis and with a high proliferative activity of cancer cells in HCC. Moreover, we found that the risk of death attributable to recurrent cancer in patients with HCC significantly increases in cases with survivin mRNA-positive tumors than in those with survivin mRNA-negative tumors. The same results have been observed in colorectal carcinoma (30) and in non-small cell lung cancer (31). These results indicate that survivin mRNA expression in tumors is a good indicator of poor prognosis in patients with various carcinomas.

Even when curative hepatectomy is performed, a considerable number of patients with HCC develop new tumors in the residual liver. iNOS mRNA expression may play an important role in HCC carcinogenesis, but it may not correlate with tumor progression in HCC, whereas survivin mRNA re-expression may provide information regarding the status of patients with a high risk of cancer recurrence in HCC.

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