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• *LIVER CANCER* •

Expression and significance of new inhibitor of apoptosis protein survivin in hepatocellular carcinoma

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Abstract

AIM: To investigate expression and significance of inhibitor of apoptosis protein survivin in hepatocellular carcinoma (HCC).

METHODS: The expression of survivin and vascular endothelial growth factor (VEGF) was investigated in 38 cases of HCC tissues and 38 liver cirrhosis tissues by immunohistochemistry and Western blot. The relationship between the expression of survivin and clinicopathological factors of HCC was analyzed.

RESULTS: Survivin protein was detected in 23 (60.5%) of 38 HCCs and 3 (7.9%) of 38 liver cirrhosis tissues. In 23 cases of HCC which expressed survivin, the expression of VEGF was positive in 18 cases and slight positive or negative in 5 cases. While in 15 cases of HCC which did not express survivin, 12 cases did not express or slightly expressed, and 3 cases expressed VEGF. In liver cirrhosis tissues, the expression of VEGF was as follows: 24 cases were negative, 10 cases were weak positive and 4 cases were strong positive. The expression of survivin was coincident with the expression of VEGF in HCC (P<0.01). The expression of survivin in HCC had no relationship with the patients' age, gender, tumor size and differentiation level of HCC, while it was related to the metastasis of HCC. The protein quantitative analysis by Western blot also showed that overexpression of survivin in HCC was closely correlated to the expression of VEGF (P<0.01). Furthermore, stronger expression of survivin and VEGF was also found in patients with metastasis rather than in those with no metastasis (P<0.01).

CONCLUSION: Survivin plays a pivotal role in the metastasis of HCC, and it has some correlation with tumorigenesis. The expression of survivin in the primary lesion is very useful as an indicator for metastasis and prognosis of HCC. It could become a new target of gene therapy of HCC.

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Key words: Survivin; Vascular endothelial growth factor; Hepatocellular carcinoma

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INTRODUCTION

In organisms, cell death and cell cycle progression are two sides of the same coin, and these two different phenomena are regulated moderately to maintain the cellular homeostasis[1]. On the other hand, gene mutation could be accumulated and cell growth cycle could be prolonged, thus finally facilitating the formation of tumor. At present, it has been confirmed that P53 and bcl-2 family are critical to the regulation of cell apoptosis. Survivin protein (M_r 16 500) is a recently described member of inhibitor of apoptosis protein (IAP) family of antiapoptotic proteins, which may act simultaneously with the bcl-2 family proteins, but has a different apoptosis inhibitory mechanism[2]. Survivin is conserved across evolution with homologs found in both vertebrate and invertebrate animal species[3]. The tissue distribution of survivin has obvious cell selectivity. Some research has found that survivin is undetectable in terminally differentiated adult tissues, yet it is abundantly expressed in fetal tissues and in a variety of human tumors including lung, colo-rectal, breast, prostate, melanoma, pancreatic, and gastric carcinoma as well as in high-grade lymphomas and neuroblastomas[4-11]. However, the expression and implication of survivin in hepatocellular carcinoma are still unknown.

Vascular endothelial growth factor (VEGF) is considered to be the most primary factor prompting the angiogenesis in tumor tissue, which also holds the central position in the course of formation and metastasis of tumor. However, it is unclear how VEGF accelerates the metastasis of neoplasms.

To explore the role and mechanism of survivin in the progression of HCC and the relationship with VEGF, we adopted immunohistochemistry and Western-blot techniques to investigate the expression of survivin and VEGF in HCC.

MATERIALS AND METHODS

Patients and samples

A total of 38 cases of HCC was involved in this study. The

patients with HCC, who underwent potentially curative tumor resection at Hepatic Surgery Center of Tongji Hospital from 2001 to 2002, had received neither chemotherapy nor radiation therapy before surgery. Among them, there were 33 males and 5 females, and the mean age of the patients was 47.3 years (SD, 11.2 years; range, 18-76 years).

All the specimens were confirmed to be hepatocellular carcinoma by pathological diagnosis. Cell differentiation was graded by Edmondson-Steiner's criteria. Tumors with Edmondson-Steiner's grade I were regarded as moderate to well differentiation and those with grade II-IV were poorly differentiated. The criteria of metastasis included extrahepatic tissue or organ involvement; hilum or remote lymphoid nodule metastasis; tumor thrombus formation in the main portal trunk or hepatic vein or bile duct. Multifocal HCC was excluded from this study because it was a controversial issue[12].

Routinely processed formalin-fixed, paraffin-embedded blocks containing principal tumor were selected. Serial sections of 2-4 μm were prepared from the cut surface of blocks at the maximum cross-section of the tumor.

Immunohistochemical staining for survivin and VEGF

Immunohistochemical staining for survivin and VEGF antigen was carried out by the standard streptavidin-peroxidase-biotin technique (SP technique) using SP kit (Zhongshan Company, Beijing, China). Rabbit-anti-human survivin monoclonal antibody and rat-anti-human VEGF monoclonal antibody were obtained from Neomarkers Company. Experimental procedure was conducted according to the SP kit specification. Primary antibody was diluted at 1:200. Antigen retrieval was done by microwave citrate salt method. 3,3-diaminobenzidine and hematoxylin were used for color development and counterstaining respectively. Cells whose cytoplasm was dyed brown were regarded as positive ones. One case of stage III gastric cancer was stained intensively and reproducibly for survivin expression in >30% of tumor cells, and was used as a positive control throughout the study. Negative control slides processed without primary antibody were included for each staining. In brief, deparaffinized and rehydrated sections were bathed in a 10^{-3} mol/L sodium citrate buffer (pH 6.0) then the solution was put to a pressure cooker and boiled for 20 min while maintaining the pressure. After endogenous peroxidase was quenched in 3% hydrogen peroxide and blocked for 5 min, the sections were incubated overnight at 4 ℃ with primary polyclonal antibody at a 1:200 dilution. Biotinylated immunoglobulin and streptavidin conjugated to peroxidase were then added. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counterstaining. The mean percentage of positive tumor cells was determined in at least five areas at 200-fold magnification for survivin and 100-fold magnification for VEGF and scaled as the following: (0) <5%; (1) 5-25%; (2) 25-50%; (3) 50-75%; and (4) >75%. The intensity of survivin immunostaining was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. Because tumors showed heterogeneous staining, the dominant pattern was used for scoring. The scores indicating percentage of positive tumor cells and staining intensity were multiplied to produce a weighed score for each case. Cases with weighed scores <1 were defined as negative; cases with weighed scores >2 were defined as strongly positive and those in between were defined as positive.

Western blot

Proteins were extracted from tissues and cells by detergent lysis using NP-40 lysis buffer (0.2% NP-40, 100 mmol/L Tris-HCl, pH 8.0, 200 mmol/L NaCl, 0.01% SDS). Proteins were fixed quantitatively by ultraviolet spectrophotometer analysis. A total of 20 μg of the proteins of HCC tissue was fractionated on a 75 g/L polyacrylamide slab gel containing 1 g/L SDS and then transferred onto a nitrocellulose filter by electroblotting. The filter was incubated for at least 1 h in 10 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl, 0.05% Tween-20 and 50 g/L bovine serum albumin for survivin and in 10 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl, 0.05% Tween-20 and 50 g/L milk powder for VEGF to prevent nonspecific binding of antibodies. Then it was incubated with primary antibody (survivin, 1:1 000; VEGF 1:2 000) for 12 h, and with second antibodies for 1 h at room temperature in the same buffer. Respectively, the second antibodies of survivin were goat-anti-rabbit polyclonal antibody labeled by alkaline phosphatase and those of VEGF were goat-anti-rat polyclonal antibody labeled by horseradish peroxidase. VEGF was dyed by 0.01% DAB/H_2O_2 form 5 to 15 min and brown straps were viewed as positive results. Survivin was dyed by NBT/BCIP for 10 min or so and blue-black straps were positive. At the same time protein molecular weight marker (M_r 14 000-70 000) was used to identify destined straps. The expressed protein quantity was automatically analyzed by GIS gel image processing system (version 3.10, Tanon Technological Limited Company, Shanghai, China). The net area of strap represented the expressed protein quantity.

Statistical analysis

All statistical analyses were performed using the SPSS 10.0 J software package for Macintosh (SPSS, Inc., Chicago, IL). Variables associated with survivin expression were analyzed by the χ^2 test. The coincident expression of survivin and VEGF protein in HCC or liver cirrhosis tissues was analyzed by the paired t-test. The correlation between HCC metastasis and expression of survivin and VEGF protein was tested by bivariate correlation analysis. $P<0.05$ was considered significant and $P<0.01$ was considered remarkably significant.

RESULTS

Immunohistochemical staining

Twenty-three cases (60.5%) of 38 HCCs expressed survivin protein, among them, 8 cases strongly expressed, 12 cases positively expressed, and 3 cases slightly expressed. Survivin protein was detected in only 3 (7.9%) of 38 liver cirrhosis. The relationship between the expression of survivin and clinicopathological factors of HCC is shown in Table 1. In 23 cases of HCC which expressed survivin, the expression of VEGF was positive in 18 cases and slightly positive or negative in 5 cases. In 15 cases which did not express survivin

Table 1 Relationship of the expression of survivin and clinicopathological factors of HCC

	n	Survivin		*P*
		Positive (*n*)	Percent (%)	
Tissue				<0.01
Cirrhosis	38	3	7.9	
HCC	38	23	60.5	
Age (yr)				>0.05
<60	31	19	61.3	
≥60	7	4	57.1	
Sex				>0.05
Male	33	21	63.6	
Female	5	2	40.0	
Size (cm)				>0.05
≥5	17	12	70.6	
<5	21	11	52.4	
Differentiation				>0.05
Moderate to well	16	9	56.3	
Poor	22	14	63.6	
Metastasis				<0.05
Positive	14	12	85.7	
Negative	24	11	45.8	

protein, 12 cases of HCC did not express or slightly expressed VEGF and 3 cases expressed VEGF. In liver cirrhosis tissue, the expression of VEGF was negative in 24 cases, weak positive in 10 cases and strong positive in 4 cases. The staining of both survivin and VEGF was mainly localized in cytoplasm.

By statistical analysis, there was a remarkable difference in survivin expression between the tumor tissue and liver cirrhosis tissues ($P<0.01$). The expression of survivin in HCC had no significant relation with the patients' age, gender, tumor size and differentiation level of HCC, while it was related to the metastasis of HCC ($P<0.05$). Furthermore, a high expression of survivin was coincident with the expression of VEGF in HCC ($P<0.01$) (Table 2 and Figures 1 and 2).

Western blot

By analysis with GIS gel image processing system, the values of net area indicating the expressed protein quantity are as shown in Tables 3 and 4, Figures 3 and 4.

DISCUSSION

It has been identified that bcl-2 family and IAPs family have close relations with cell apoptosis. So far six members of IAPs family (NIAP, c-IAP1, c-IAP2, XIAP, Survivin and Bruce) have been cloned[13]. IAPs family expresses extensively among many species, which are of homology in their

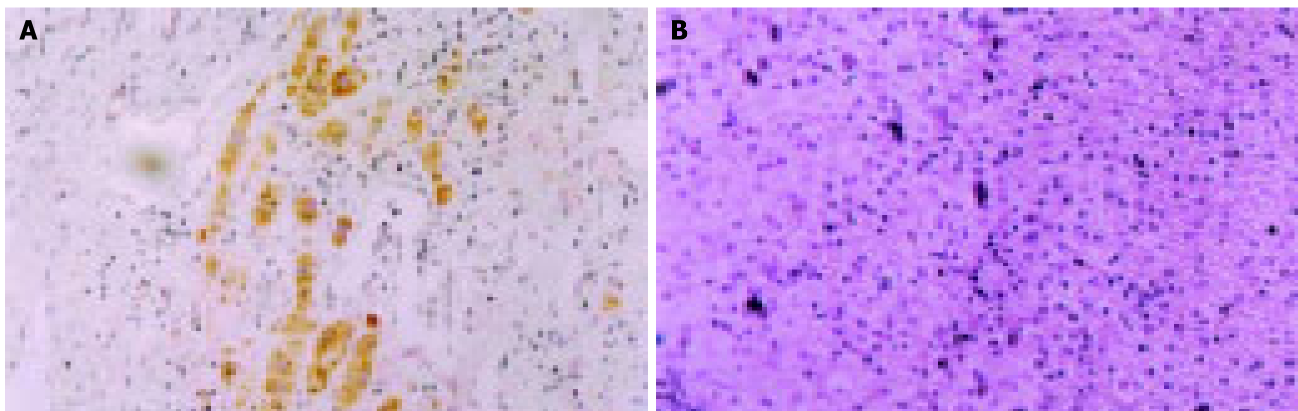

Figure 1 Expression of survivin in HCC and liver cirrhosis tissues (SP method, ×200). **A:** The brown granules in the cytoplasm indicate survivin protein in liver cancer cells; **B:** Survivin protein is not detected in liver cirrhosis tissues.

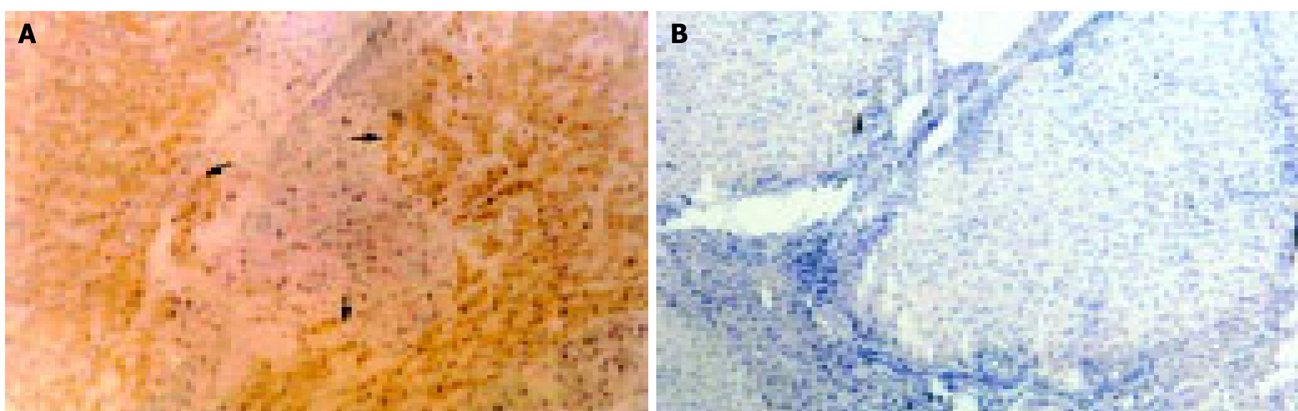

Figure 2 Expression of VEGF in HCC and liver cirrhosis tissues (SP method, ×100). **A:** The brown granules in the cytoplasm indicate VEGF expression in HCC; **B:** Expression of VEGF was weak positive in some liver cirrhosis tissues.

Table 2 Expression of survivin and VEGF protein in HCC tissues

	VEGF		P
	Positive	Negative	
Survivin			<0.01
Positive	18	5	
Negative	3	12	

Table 3 Coincident expression of survivin and VEGF protein in HCC or liver cirrhosis tissues

	HCC group (n = 38)	Cirrhosis group (n = 38)	P
Survivin	3229.3±1383.2	562.6±226.5	<0.01
VEGF	1134.8±862.2	445.4±322.6	

Table 4 Correlation between HCC metastasis and expression of survivin and VEGF protein

	Metastasis group (n = 14)	Non-metastasis group (n = 24)	P
Survivin	3841.4±1061.2	1253.8±725.3	<0.01
VEGF	1388.6±652.4	1081.3±773.5	<0.01

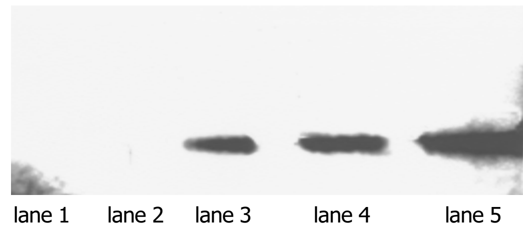

Figure 3 Expression of survivin in HCC and liver cirrhosis tissues. Lanes 1 and 2: liver cirrhosis tissues; lanes 3-5: HCC tissues. M_r of survivin: 16 500.

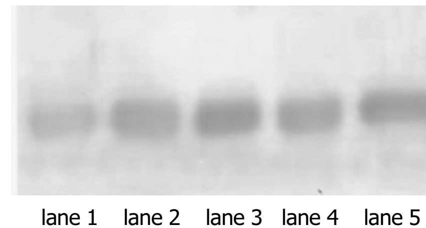

Figure 4 Expression of VEGF in HCC and liver cirrhosis tissues. Lanes 1 and 2: liver cirrhosis tissues; lanes 3-5: HCC tissues. M_r of VEGF: 21 000.

structures and the abilities of inhibiting apoptosis[14]. Survivin is the smallest member among the IAPs family. Altieri of Yale University utilized EPR-1 (effector cell protease receptor-1) cDNA to screen and clone survivin from human GenBank in 1997[3]. It has been suggested (but not proven) that EPR-1 may act as a natural anti-sense to survivin in cells[15].

The expression of survivin depends on cell cycle. Hela blocked tumor cells in G1, S, G2/M phase separately to detect survivin mRNA amount. Survivin mRNA was not detectable in G1 phase, increased 6.2 times in S phase and elevated 40 times in G2/M phase. Therefore, the expression of survivin was closely related to cell proliferation. Survivin can suppress apoptosis of NIH3T3 cell induced by Taxol and 293 cell induced by Fas and Bax[16]. Survivin inhibits apoptosis mainly through targeting the terminal effectors caspase-3 and -7 activity in apoptotic protease cascade reaction[17,18]. These caspases operate in the distal portions of apoptotic protease cascades, functioning as effectors rather than initiators of apoptosis. Survivin is characterized by a unique structure with a single BIR and no zinc-binding domain known as the RING finger, thus survivin cannot bind caspase-3 directly. Survivin is expressed in the G2-M phase of the cell cycle in a cell cycle-regulated manner and associates with microtubules of the mitotic spindle by the coiled spirals zone[19-21]. Survivins are also called chromosomal passenger proteins: they associate with inner centromere regions during prophase, but subsequently relocalize to the midzone of the central spindle and concentrate at the midbody[22]. Survivin has the double function of controlling spindle checkpoint and apoptotic checkpoint. The over-expression of survivin in neoplasms may obliterate this apoptotic checkpoint and allow aberrant progression of transformed cells through mitosis. The disruption of survivin-microtubule interaction results in loss of antiapoptosis function of survivin and increases caspase-3 activity during mitosis[21].

The results revealed that survivin was highly expressed in HCC and seldom detected in liver cirrhosis tissues. The expression of survivin had no association with the patients' age, gender, tumor size and differentiation level of HCC. It suggests that survivin may play some role in tumorigenesis of HCC. Simultaneously, in our series, we found that high expression of survivin was significantly correlated to VEGF in HCC. More importantly, stronger expression of survivin and VEGF was found in patients with metastasis than in those without metastasis. Consequently the expression of survivin seems to be involved in metastatic capacity of HCC.

As we know, VEGF is considered to be the most cardinal vascular growth factor prompting angiogenesis in tumor tissue. The mechanism of liver cancer cell shift induced by VEGF is closely related to increased proliferation ability of liver cells. Emerging studies have implicated a marked induction of survivin by VEGF in vascular endothelial cells, which can facilitate tumor metastasis by controlling apoptosis during angiogenesis. VEGF binding to VEGF-R2 activates the phosphatidylinositol 3-kinase (PI3K) survival pathway, resulting in phosphorylation and activation of the serine/threonine kinase protein kinase B (PKB/Akt). VEGF induces the expression of several anti-apoptotic effector molecules in Ecs by PKB/Akt pathway, including bcl-2, A1, and two members of the IAP family, X-linked IAP (XIAP), and survivin[23-26]. VEGF-dependent upregulation of survivin could be prevented by cell cycle arrest in the G1 and S phases and allows for the maintenance of the microtubule network to inhibit apoptosis of endothelial cells[27-30]. Our results demonstrate that the expression of survivin is consistent with that of VEGF in HCC and both are closely correlated to infiltration and metastasis of HCC. This is similar to findings in endothelial cells. As such, we assume that VEGF probably promotes the expression of survivin in HCC tissues, then the latter inhibits apoptosis of hepatocarcinoma cells and enhances tumor cell ability of infiltration and invasion,

thus accelerating tumor metastasis. To clarify the interaction mechanism between survivin and VEGF in HCC, further studies are needed.

The cancer-specific expression of survivin, coupled with its importance in inhibiting cell death and in regulating cell division, makes it a useful diagnostic marker of cancer and a potential target for cancer treatment[31]. Recently some studies have set about to evaluate the possibility of targeting survivin function *in vivo* as an anticancer strategy in which it is shown that inhibition of survivin could effectively inhibit *de novo* tumor formation and progression[32,33]. In the light of the effect of survivin on the progression of HCC, it is possible that inhibiting the function of survivin can be a new treatment of HCC.

In conclusion, inhibitor of apoptosis protein survivin plays a pivotal role in the metastasis of HCC, and it has some correlation with tumorigenesis. It is associated with the progression of HCC as a late event in tumorigenesis. The expression of survivin in the primary lesion can be an indicator for metastasis and prognosis of HCC. It could become a new target of gene therapy of HCC.

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