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Research Paper

Expression of Survivin, p53 and its relationship with apoptosis, proliferation in hepatocellular carcinoma(HCC)

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Abstract

Objective:To investigate the expression of Survivin p53 and its relationship with apoptosis、proliferation in hepatocellular carcinoma (HCC). **Methods**:The expression of Survivin, p53 and the proliferation of tumor cells marked by proliferation cell nuclear antigen (PCNA) in 42 cases of HCC were assessed by immunohistochemical method. TUNEL was used to detect apoptosis. **Results**:Survivin protein was expressed in 30 of 42 cases of HCC(71.4%) and in 4 of 34 cases of adjacent cirrhosis tissues(11.8%). Expression of Survivin protein was negative in 10 cases of normal tissues. Survivin protein positive expression rate in HCC was significantly higher than adjacent cirrhosis tissues and normal tissues($P < 0.001$). The increased Survivin protein expression in cancer was significantly associated with histological grade($P = 0.003$), p53 protein($P = 0.013$), survival time($P < 0.05$) and the ratio of proliferative index to apoptotic index($P < 0.01$), but was not significantly correlated with age, sex, clinical stage, tumor size, metastasis, AFP, HBs-Ag($P > 0.05$). **Conclusion**:There is a marked increased expression of Survivin in HCC, which may play an important role in breaking the balance of proliferation and apoptosis of HCC cells. The correlation between Survivin and p53 expression in HCC indicates that cooperation between Survivin and p53 plays a certain role in occurrence and /or development of HCC.

Key words: Survivin; hepatocellular carcinoma; apoptosis; proliferation cell nuclear antigen; p53

INTRODUCTION

The disruption in the balance of cell proliferation and apoptosis is a common event in carcinogenesis and tumor growth, which also exits in HCC[1]. And this disturbance determines the rate of the cells growth. p53 is a classical antioncogene, which mutates at the rate of 55 percent in HCC. Survivin is a new inhibitor of apoptosis protein(IAP-family member) which suppresses cell apoptosis by the inhibition of effective caspase-7, caspase-3[2]. During mitosis the different sub cell of Survivin influences the pass cell cycle, so it is a co-regulator factor of cell proliferation and apoptosis[3]. Therefore it is essential to study the relationship of Survivin, p53, apoptosis and proliferation. It has been reported that Survivin expression is undetectable in most normal adult tissues, but is overexpressed in virtually every human tumors[4], including lung, prostate, colon, pancreas and stomach[5-9]. In the present study we investigated the expression and location of Survivin in HCC tissues with immunohistochemistry, detected apoptosis by Terminal deoxy-nucleotidyl transferase nick end labeling(TUNEL) and analyzed the relationship between Survivin, p53 protein expression, apoptosis and proliferation.

MATERIALS AND METHODS

Materials

42 specimens of HCC were obtained from surgical resections performed in the First Hospital of Xi' an Jiaotong University from 2000 to 2002, including 34 samples with adjacent cirrhosis tissues confirmed by pathology. Of the patients 34 were males and 8 were females, the mean age 40.36(range 29-72). All patients did not accept chemotherapy and radiotherapy before operation. According to Edmenson standard there were 15 cases of Ⅰ~Ⅱ grade and 27 of Ⅲ~Ⅳ grade. Clini-

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cal stage was conformed by TNM standard, including 12 case of 1~2 stage and 30 cases 3~4 stage. All specimens were fixed in PBS buffered formalin processed routinely, embedded in parsffin and cut into 4um-thick sections, which were placed on poly-l-lysine-coated slides for immunohistochemistry. Normal liver tissues specimens were collected from 10 cases of previously healthy men(dying accidentally) and performed using H&E staining as control group. Informed consent was obtained from all patients and family members.

Methods

Expression of Survivin, p53 and PCNA detection by Immunohistochemitry

Slides were deparaffinnized in xylene twice for 5 minutes respectively. Endogenous peroxidase activity was blocked with 3 percent hydrogen peroxidase for 20 minutes. Slides were heated in 0.01 mol/L citrate buffer (pH=6.0) at a high temperature in a microwave oven for 12 min for antigen retrieval. After being cooled to room temperature, the slides were incubated for 20 min in a blocking solution containing 10 percent normal goat serum in PBS(0.01 mol/L phosphate pH =7.4) and incubated with anti-human Survivin(1:100 Neomarker-4F7), p53(1:50 ZM-0408) and PCNA(1:50 ZM-0340) monoclonal antibody for 2 hours at 37℃. After rinsing, staining was performed with Elivision Plus two-step system(kit-9902, Dako, Carpiterry, CA). The 3.3-diaminobenzidine was used as chromogen for 3 mins. Slides were counterstaind for 2 mins with hematoxylin solution and differentiated for 3 s with hydrochlonic acid alcohol, then dehydrated and mounted by gum.

Detection of apoptosis

Apoptosis detection was performed using an in situ cell death detection kit. Ap(Boehring Mannheim, Germany). After a standard procedure of deparaffinization and rehydration, all specimens were heated by microwave in 0.05 mol/L citric acid solution for antigen retrieval and washed twice with PBS. The slides were incubated with Converter-Ap regent at 37℃. After washing the sections were counterstained with Fast Red. As positive control, tissue sections were pre-treated 60 min with 1ug/ml DNA-Se-Ⅰ. For negative control only TdT buffer without deoxynudeotidyl transferase was used.

Evaluation of results

The cells with brow-yellow granules in the cytoplasm were taken as Survivin positive cells, and the mean of 5 visual fields collected randomly on each slide was counted. If, the slide is regarded as positive, while the mean positive cells of 5 visual fields on each slid are less than 10 percent, the slide is regarded as negative. P53 protein and PCNA were expressed in the nucleus with brown-yellow granules. The judgment of positive standard is the same as Survivin protein. Proliferation activity was represented by PCNA label index(PCNA-LI). Randomly counting positive cells in 500cells, PCNA=positive cells/total cells × 100%. Apoptosis activity was represented by apoptosis label index (Apoptosis-LI). Counting method is the same as PCNA.

Statistical analysis

All data were analyzed with SPSS 11.0. χ^2 test was adopted to assess the difference Survivin expression between HCC, adjacent cirrhosis and normal liver tissues and the association between Survivin expression and clinicopathological indexes. *T* test was used to detect the relation between Survivin, p53 and AI, PI. $P < 0.05$ was considered statistically significant.

RESULTS

Survivin protein expression in HCC, adjacent cirrhosis and in normal tissuse

Survivin protein was expressed in the cytoplasm with brown-yellow granules(Fig. 1) Survivin protein was expressed in 30 of 42 cases of HCC(71.4%, Table 1) and in 4 of 34 cases of adjacent cirrhosis tissues (11.8%). Expression of Survivin protein was negative in 10 cases of normal tissues. protein positive expression rate in HCC was significantly higher than adjacent cirrhosis tissues and normal tissues($P < 0.001$).

Relationship between Survivin protein expression and clinicopathological indexes

The increased Survivin protein expression in cancer was significantly associated with histological grade ($P = 0.003$), but not with age, sex, tumor size, clinical stage, local metastasis, AFP and HBs-Ag($P > 0.05$) (Table 2).

Expression of PCNA,p53 and apoptosis in HCC

PCNA and p53 were stained in the nuclei of HCC cells with brown-yellow granules(Fig. 2). PCNA label index(PI) in HCC is 30.76%(Table 3). The color of apoptotic cells in HCC were stained blue, while non-apoptotic cells were pink through counterstained with Fast Red and eliminated necrosis cells through H&E stains. Apoptosis label index(AI) in HCC was 8.74%. The ratio of PI to AI in HCC was significantly higher than in adjacent cirrhosis tissues(3.72 ± 1.01 *vs.* 3.13 ± 1.26, $P < 0.05$). The positive rate of p53 protein expression in HCC was 40.5%(Fig. 3).

Relationship between the ratio of PI to AI in HCC and normal tissue

The ratio of PI to AI in HCC was significantly higher than in adjacent cirrhosis tissues(3.72 ± 1.01 *vs.* 3.13 ± 1.26, $P < 0.05$)

Relationship between Survivin protein expression in HCC and the ratio of PI to AI

The ratio of PI to AI in cases of positive Survivin

Table 1 Survivin expression in HCC, adjacent cirrhosis and normal tissuse

Item	Cases	Survivin expression		Positive rate
		+	-	
HCC	42	30	12	71.4%
Adjacent cirrhosis[a]	34	4	34	11.8%
Normal tissuse[a]	10	0	10	0

[a]$P < 0.001$, *vs*, HCC groupI wne.

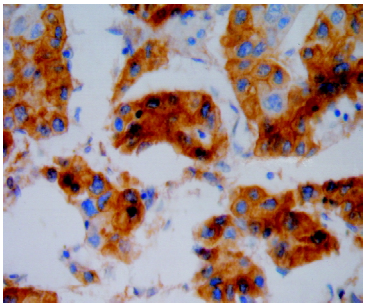

Fig.1 Expression of Survivin in HCC(Elivision, × 40)

Table 2 Relationship between Survivin and clinipathological indexes

Item	Cases	Survivin expression protein		*P value*
		+	-	
Age(yr)				
≤ 45	15	4	11(73.33)	1.000
> 45	27	8	19(70.37)	
Sex				
Male	34	11	23(67.65)	0.494
Female	8	1	7(87.50)	
Histological grade				
Ⅰ - Ⅱ	15	9	6(40.00)	0.003
Ⅲ - Ⅳ	27	3	24(88.89)	
Clinical stage				
1-2	12	4	8(66.67)	0.957
3-4	30	8	22(73.33)	
Tumor size				
≤ 50 mm	17	4	13(76.47)	0.551
> 50 mm	25	8	17(68.00)	
Metastasis				
-	28	8	20(71.43)	1.000
+	14	4	10(71.43)	
AFP(ug/L)				
< 400	19	9	10(52.63)	0.757
> 400	23	12	11(47.83)	
HBs-Ag				
-	3	2	1(33.33)	0.192
+	39	10	29(74.36)	

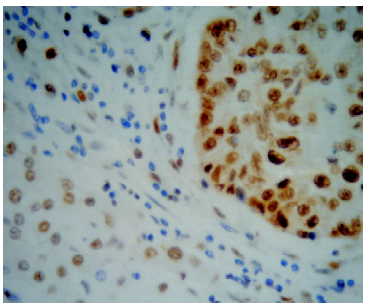

Fig. 2 Expression of PCNA in HCC(× 40)

expression in HCC was higher than that in negative Survivin expression in HCC. At the same time the cases of negative Survivin protein expression were longer than positive Survivin protein expression on survival rate for 1 year and survival time(Table 4).

The relationship between Survivin and p53 protein expression in HCC

The rate of Survivin protein expression in p53 negative in HCC tissues is significantly higher than that in p53 positive HCC tissues($P = 0.013$)(Table 5).

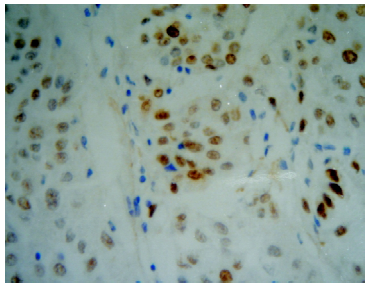

Fig. 3 Expression of P53 in HCC(× 40)

Table 3 Relationship between the ratio of PI to AI in HCC and adjacent cirrhosis

	HCC(%)	adjacent cirrhosis(%)	*P*
PI	30.76 ± 4.82	23.71 ± 3.67	
AI	8.74 ± 2.29	7.86 ± 2.17	
PI/AI(%)	3.72 ± 1.01	3.13 ± 1.26	0.019

Table 4 Relationship of Survivin with PI/AI and Prognosis

Index	Cases	PI/AI	1-year Surviving rate(%)	Survival time (year, x ± s)
Survivin				
+	26	4.03 ± 0.92[a]	34.62[b]	13.37 ± 8.95[b]
-	16	2.92 ± 0.73	81.25	20.00 ± 9.98

[a]$P < 0.01$, [b]$P < 0.05$ *vs*. Negative group.
PI: PCNA index; AI: apoptosis index.

Table 5 Relationship between Survivin and p53

P53	Survivin			Positive rate
	-	+		
-	11	14	25	56.0%
+	1	16	17	94.1%
	12	30	42	71.4%

Survival analysis

The survival analysis was performed on 42 patients. Age, gender, tumor size, stage, grade, metastasis, AFP, Survivin were taken into account in the survival analysis. In unvaried analysis a significant correlation with short survival was found only for stage($P = 0.011$)(Fig. 4), metastasis($P = 0.002$)(Fig. 5), envelop invasion(0.023)(Fig. 6), and Survivin expression(0.010)(Fig. 7). Multivaried analysis was performed by Cox proportional hazard regression model, stage($P = 0.007$)

metastasis($P = 0.019$) and positive Survivin expression ($P = 0.015$) were significantly associated with shorter survival(Fig. 8). For other factors there were no significant correlations with survival time.

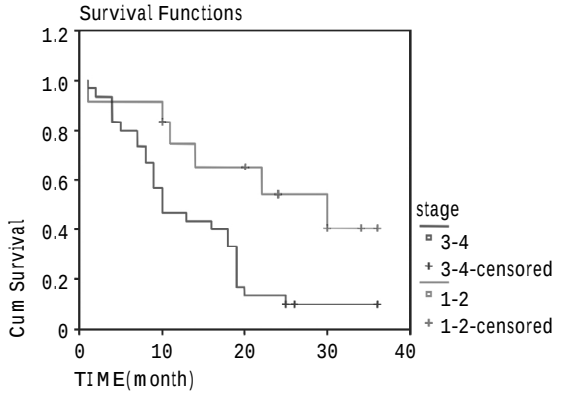

Fig. 4 Kaplan-Meier curves for survival of patients in stage 1 (1-2 stage) and stage 2(3-4 stage)

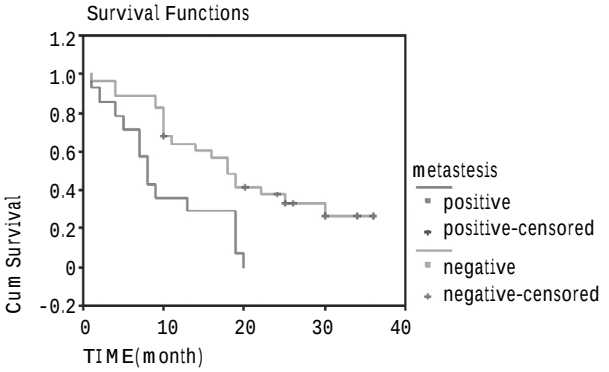

Fig. 5 Kaplan-Meier curves for survival of patients in positive and negative metastasis

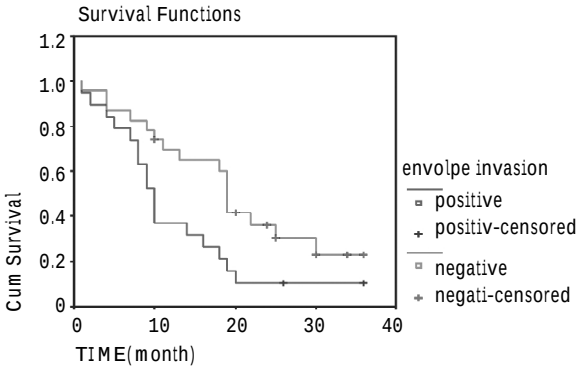

Fig. 6 Kaplan-Meier curves for survival of patients in positive envelop invasion and negative envelop invasion

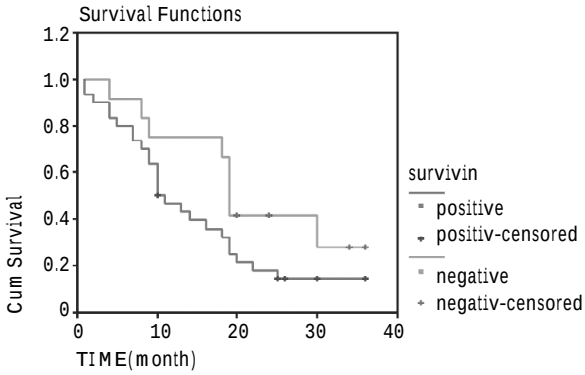

Fig. 7 Kaplan-Meier curves for survival of patients in positive Survivin expression and negative Survivin expression

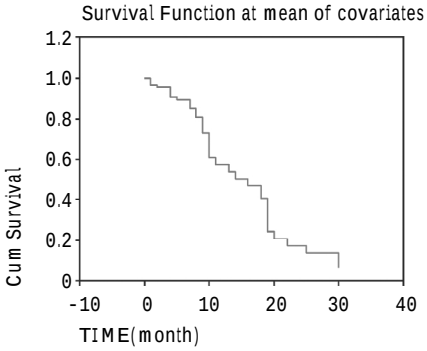

Fig. 8 Cox proportional hazard regression model for sex, age, type, HBs-Ag, grade, stage, size, metastasis and Survivin

DISCUSSION

A coordination and balance between cell proliferation and apoptosis is very crucial for normal development and tissue-size homeostasis in the adult. Cancer results when clones of mutated cells survive and proliferate inappropriately causing disruption. In HCC both the apoptotic rate and the proliferative activity increased with decreased tumor differentiation and with increasing tumor size and stage($P < 0.01$). However, the apoptotic rate was always less than the proliferative activity and their ratio tends to decrease in higher tumor grade, size, stage and p53-positive tumors($P < 0.001$)[2]. Apoptosis play a very important role in each phase of HCC cells growth and differentiation. The maladjustment of ration of PI to AI is commonly caused by regulation turbulence of cell apoptosis. Survivin is a new member of the inhibitor of apoptosis family of anti-apoptotic proteins. Many investigations have been done about its function, but few studies were made about its expression and significant in HCC. We used immunohistochemical method to detect Survivin protein expression in 42 cases of HCC tissues, 34 cases of adjacent cirrhosis tissues and 10 cases of normal liver tissues. The result is similar to other investigations on Survivin protein expression in other tumor tissues performed preciously[10-14]. Statistic analysis indicated that the positive rate of Survivin protein expression in HCC is higher than in adjacent cirrhosis tissues and normal liver tissues.

And what's more Survivin protein expression in lower differentiation HCC tissues is significantly higher than that in higher differentiation HCC tissues. The survival time of positive Survivin expression is significantly longer than that of negative Survivin expression. All these results indicated that higher Survivin expression in HCC tissues may destroy the balance between cell proliferation and apoptosis. But at the same time high Survivin expression is not related to age, sex, tumor size, clinical stage, and local metastasis, showing that the occurrence and development of HCC is a process involving multiple factors such as; stage and more heredity character changing, chromosome aberration oncogene activation、antioncogene inactivity、growth factor and its receptor abnormality. Therefore Survivin only has a role on some aspects of HCC occurrence and development. More investigation are needed to make sure about how Survivin exerts its effects in complicated network of cell apoptosis and its relationship with regulator factors. At the same time our investigation found that higher expression of Survivin in HCC tissues while no expression in normal liver tissues made target therapy possible. The anti-cancer property of Survivin T34A(Thr34 → Ala) has been recently tested in preclinical xenograft models of melanoma tumor formation and suppressed growth of existing tumors by 60-70% in a reaction associated with loss of proliferating cells and tumor cell apoptosis *in vivo*[15]. p53 is a classical antioncogene involving many kinds of cell function. The inactivity of p53 is a common event in human malignant tumors[16]. There is a very high rate of p53 point mutation in HCC, which is related to decreased differentiation, increasing tumor size and poor prognosis[17]. Some investigations found that over-expression of Survivin and loss of wild-type p53 existed in many tumors. Wild-p53 repressed Survivin expression at both mRNA and protein level. Recent analyses revealed that the expression of wild-p53 was associated with strong repression of Survivin promoter in various cell types. Our investigation applies immunohistochemical method to detect p53 protein expression in HCC tissues and analyzed the relevance between p53 and Survivin expression. The result showed positive expression of Survivin in mutant p53(mtp53) negative expression cases was significantly lower than that in mtp53 positive expression cases. The correlation between Survivin and p53 expression in HCC indicates that cooperation between Survivin and P53 plays a certain role in occurrence and /or development of HCC.

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