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Expression and clinical significance of TAp73α, p53, PCNA and apoptosis in hepatocellular carcinoma

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Abstract

AIM: To study the prognostic role of TAp73α, p53, proliferating cell nuclear antigen (PCNA) and apoptosis in patients with hepatocellular carcinoma (HCC) after surgical tumor ablation.

METHODS: Forty-seven human resected HCC tissues and 42 adjacent non-cancerous tissues were studied with 10 normal liver tissues as control group. TAp73α, p53, and PCNA were detected with Elivision immunohistochemistry. Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick-end labeling (TUNEL) method was used to detect the apoptosis cells. All clinical and pathological materials were analyzed by SPSS10.0 statistical package.

RESULTS: TAp73α overexpressed in HCC tissues (36.2%) when compared with adjacent non-cancerous tissues (2.38%, $P<0.005$) and normal liver tissues (0, $P<0.01$). Mutant type p53 (mt-p53) overexpressed in HCC tissues (38.3%) when contracted with adjacent non-cancerous tissues (16.7%, $P<0.05$) and normal liver tissues (0, $P<0.01$). Proliferation index (PI) level in HCC tissues was significantly higher than that in adjacent non-cancerous tissues (30.34%±4.46% *vs* 27.88%±5.89%, t, $P=0.028$). Apoptosis index (AI) level in HCC tissues was higher than that in adjacent non-cancerous tissues (8.62%±2.28% *vs* 7.38%±2.61%, t, $P=0.019$). Expression of TAp73α was associated with lymph node metastasis and mt-p53, with $r=0.407$ and 0.265, respectively. Expression of mt-p53 was associated with Edmondson's stage and AFP, with $r=0.295$ and -0.357, respectively. In Kaplan-Meier univariant analysis, TAp73α, AFP, TNM stage, portal vein invasion, liver membrane invasion and HBsAg correlated with prognosis (log rank, $P=0.039$, 0.012, 0.002, 0.000, 0.014, 0.007, respectively). Multivariant Cox regression analysis showed that TAp73α, AFP, TNM stage, portal vein invasion, liver membrane invasion and age were independent factors of prognosis.

CONCLUSION: These results suggest that TAp73α can be used as a prognostic indicator of patients with HCC undergoing surgical tumor ablation. AFP, TNM, portal vein invasion, liver membrane invasion and age also have a potency of predicting the prognosis of HCC.

Key words: HCC; TAp73α; PCNA; Apoptosis; p53; Prognosis; TUNEL

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INTRODUCTION

Primary liver cancer (PLC) is one of the most malignant tumors in China. It is often in the late stage when diagnosed, with a poor prognosis even through positive treatments[1,2]. Hepatocellular carcinoma (HCC) is the main pathological type of PLC, accounting for about 90% of all PLCs. Recently, the incidence of HCC has been found to be increasing particularly in males in countries such as Japan, Italy, France, Switzerland, UK and the USA[3,4]. It is urgent to find some biomarkers to predict the biological character of HCC. Although many biomarkers have been tried, such as AFP mRNA, circulating VEGF and PD-ECGF, human macrophage metalloelastase gene[5], p27[6], p53 mutation[7], expression of p73[8], telomerase activity[9], *etc.*, a routine biomarker for prediction of metastasis and recurrence is not yet available.

As it is a multi-step progress of the occurrence and development of HCC involving with multi-genes, it is impossible to predict the prognosis of HCC patients accurately by detecting only one biomarker. TAp73α and p53 play their roles in the development and invasion of HCC[10-13]. Cell proliferation and apoptosis are two different but associated processes in HCC[14]. Moreover, proliferation index (PI) and apoptosis index (AI) can reflect the biological characters of tumor cells[15]. So we try to predict the prognosis of HCC

patients more accurately by detecting TAp73α, p53, PI and AI together.

MATERIALS AND METHODS

Materials

Forty-seven human resected HCC tissues and 42 adjacent non-cancerous tissues were collected and studied in the First Hospital of Xi'an Jiaotong University from January 2001 to 2002 (clinicopathologic characteristics of HCC tissues are listed in Table 1). Ten normal liver tissues were used as control group acquired by autopsy from healthy adult male, who died from accidental death simultaneously.

Table 1 Kaplan-Meier for survival

Factor	*n*	%	*P*	Factor	*n*	%	*P*
Sex			0.910	Node metastasis			0.197
Female	9	19.15		Positive	8	17.02	
Male	38	80.85		Negative	39	82.98	
Age (yr)			0.258	PV invasion			0.000[b]
>45	29	61.70		Positive	7	14.89	
≤45	18	38.30		Negative	40	85.11	
HBsAg			0.007[b]	AI			0.938
Positive	44	93.62		<Mean	25	53.19	
Negative	3	6.38		>Mean	22	46.81	
AFP			0.012[a]	PI			0.052
<400 μg/L	23	48.94		<Mean	25	53.19	
>400 μg/L	24	51.06		>Mean	22	46.81	
Size			0.281	p53			0.402
≤5 cm	19	40.43		Negative	29	61.70	
>5 cm	28	59.57		Positive	18	38.30	
TNM stage			0.002[b]	TAp73α			0.039[a]
II	13	27.66		Negative	30	63.83	
III+IV	34	72.34		Positive	17	36.17	
Differentiation			0.566	Liver membrane invasion			0.014[a]
Well	39	82.98		Negative	28	59.57	
Poor	8	17.02		Positive	19	40.43	

[a]$P<0.05$; [b]$P<0.01$ *vs* control group.

Elivision IHC study of p53 , PCNA and TAp73α

Resected specimens were routinely fixed in 40 g/L formaldehyde solution immediately after the resection. Paraffin blocks were prepared as follows. The tumor tissues were sliced vertically at a length of 4 μm through the central site of the tissues. After paraffin was removed from the slice with alcohol and xylol, endogenous peroxidase activity was blocked with 30 mL/L hydrogen peroxide at room temperature for 5-10 min. The preparation was subsequently treated in a 600-W microwave oven for 5 min in 0.01 mol/L citric acid buffer (pH 6.0), repeated thrice. After blocking with goat serum, every section was added according Ab_1 [p53 (ZM-0407) and proliferating cell nuclear antigen (PCNA) (ZM-0213), Zymed Biotechnology, Zymed, CA] 50 μL (dilution rate 1:50), done at 37 ℃ for 1-2 h. Then staining was performed by Elivision™ plus two-step System (kit-9902, Dako, Carpinteria, CA). After treated with 3,3-diaminobenzidene (DAB) at room temperature for 7 min, every section was submitted to chromatin staining, dehydrated and mounted.

In this study, we used a-specific polyclonal p73 raised against a C-terminal peptide [poly-TAp73α (BA-1327), Wuhan Boster Biological Technology Co., Ltd, PRC] to detect TAp73α protein. After the hot plerosis of antigen, the preparation was added, antigen-plerosis, done at room temperature for 20 min and rinsed with PBS thrice for 3 min. Fifty microliters of X-Triton solution was subsequently applied, done at room temperature for 20 min. Following this, the preparation was rinsed with PBS thrice for 3 min; other steps were the same to detect p53 and PCNA.

The positive specimen of TAp73α and p53 protein was defined as nuclear was stained over 5%, while the plasma was not. Further, 5-25% was defined as (+), 25-50% as (++) and over 50% as (+++). As to PCNA, positive cell was defined as the nuclear was stained while the plasma not. We counted up to 500 cells to calculate PI.

TUNEL staining

Apoptosis cell was detected as followed. TUNEL kit was purchased from Roche Applied Science. After being dewaxed with alcohol and xylol, the preparation was reacted with 30 mL/L hydrogen peroxide to block endogenous peroxidase activity for 30 min, and rinsed with PBS. The preparation was subsequently reacted with proteolytic enzyme (0.5 μg/mL potassium cacodylate), and rinsed with deionized distilled water (DDW). Then the preparation was reacted with 50 μL TdT mixture at 37 ℃ for 1 h, which was combined with reagents 1 and 2, containing: 200 mmol/L potassium cacodylate; 25 mmol/L Tris-HCl (pH 6.5); 0.25 g/L bovine serum albumin; 1 mmol/L $COCl_2$; 5 μmol/L biotin-dUTP; and 100 U/mL TdT. After being rinsed with PBS, to the preparation 350 μL of reagent was added at 37 ℃ for 40 min and stained with BCIP/NBT. Every section was subsequently treated with DAB coloration substrate solution, rinsed with running water and added to a nuclear staining solution, after which it was dehydrated, cleared, and mounted. Apoptosis cells were stained as blue, while others were stained pink. We counted up to 500 cells to calculate AI.

Negative control of p53, TAp73α and PCNA were prepared by replacing Ab1 with PBS, while using the known positive specimens as the positive control. As to the detecting of apoptosis cell, negative control was prepared by omitting the TdT-labeled reagent solution and the positive control was prepared by pre-treating the specimens with DNAase I.

Follow-up method

All patients were followed up every 3 mo after the surgical tumor ablation. Survival length was defined as the time from the surgical ablation to death or the last day of our follow-up, 1st March 2004.

Statistical analysis

All the clinical and pathological data were put on computer and analyzed by SPSS 10.0 statistical packet. Results were examined using χ^2 test, exact probabilities in 2×2 table, Spearman rank correlation, log-rank and *t*-test. Statistical significance was defined by a *P*-value of less than 0.05 in two-sides.

RESULTS

Expression of protein TAp73α

In the cancer tissues, 17 specimens were positive (17/47, 36.2%), including 12+ and 5++ (Figure 1). In the adjacent non-cancerous tissues, only one of 42 was positive (2.38%);

while in the normal liver tissues none was positive. TAp73α in the cancer tissues was higher than that in the adjacent non-cancerous tissues ($P<0.005$) and normal liver tissues ($P<0.01$). Furthermore, TAp73α was associated with node metastasis ($r = 0.265$; $P = 0.032$) and p53 ($r = 0.407$; $P = 0.005$), but it showed no correlation with other clinicopathological characteristics.

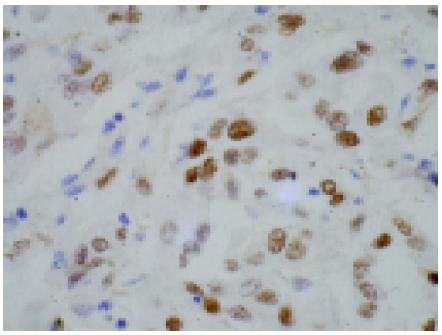

Figure 1 TAp73α protein in HCC, nuclei of tumor cells were stained with yellow or brown (×400).

Expression of protein p53

In the cancer tissues, 18 specimens were positive (18/47, 38.3%), including 8+, 5++ and 5+++ (Figure 2), while only 7 of 42 were positive (16.7%) in the adjacent non-cancerous tissues and none in the normal liver tissues. Expression of protein p53 in the cancer tissues was significantly higher than that in the adjacent non-cancerous tissues ($P = 0.023$) and normal liver tissues ($P<0.01$). p53 was associated with AFP ($r = -0.357$; $P = 0.014$) and the differentiation of tumor cells ($r = 0.295$; $P = 0.044$), but it showed no correlation with PI and AI.

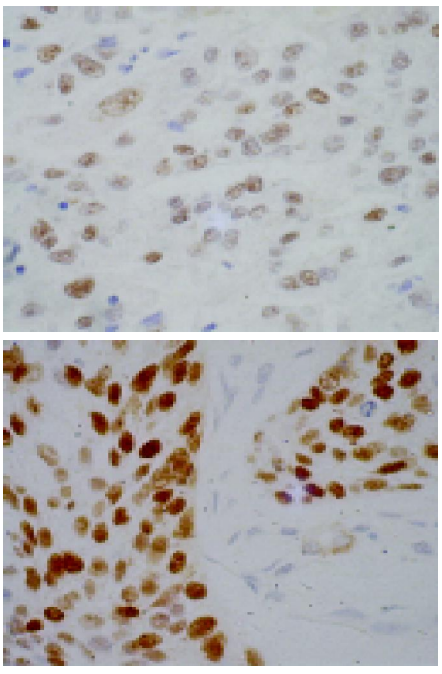

Figure 2 p53 protein in HCC, nuclei of tumor cells were stained with yellow or brown (×400).

Expression of PCNA and apoptosis

Positive cells of PCNA and apoptosis are seen in Figures 3 and 4, respectively. The mean value of PI in the cancer tissues was higher than that in the adjacent non-cancerous tissues (30.34%±4.46% *vs* 27.88%±5.89%; *t*, $P = 0.028$). PI in portal vein invasion subgroup was higher than that without portal vein invasion (33.43%±3.21% *vs* 29.82%±4.48%; *t*, $P = 0.048$). But PI showed no correlation with other clinicopathological characteristics.

AI in the HCC tissues was higher than that in the adjacent non-cancerous tissues (8.62%±2.28% *vs* 7.38%±2.61%; *t*, $P = 0.019$). AI in the late stage was higher than that in the early stage (7.46%±2.30% *vs* 9.06%±2.15%, *t*, $P = 0.030$), but it showed no correlation with other clinicopathological characters.

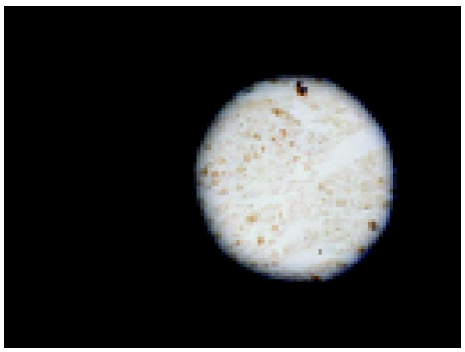

Figure 3 PCNA protein in HCC, nuclei of tumor cells were stained with brown (×400).

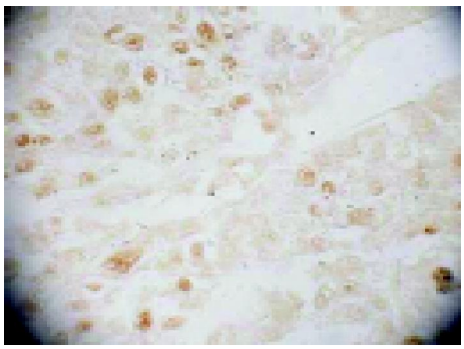

Figure 4 Apoptosis cells were stained in blue, while non-apoptosis cells were stained in pink (×400).

Survival analysis

We further classified the staining results of PI and AI into two subgroups, over or under the mean values in survival analysis. Until 1st March 2004, 35 patients died of HCC, 1 died of brain accident and 11 lived well. Survival time ranged from less than 1 mo to more than 34 mo (15.00±1.21 mo). In Kaplan-Meier univariant analysis, survival between different TAp73α, AFP, TNM stage, portal vein invasion, liver membrane invasion and HBsAg groups had statistically significant difference by log-rank test (Table 1).

As most patients in this study had positive HBsAg and

the degree of variation was almost 0, HBsAg was excluded in Cox regression analysis. It showed that TAp73α, AFP, TNM stage, portal vein invasion and liver membrane invasion were independent dangerous factors influencing the survival of HCC patients (P<0.05, Table 2).

Table 2 Variables in the equation method = backward stepwise (conditional LR)

	SE	Wald
AFP (>400 μg/L, <400 μg/L)	0.417	8.475
PV invasion (positive, negative)	0.519	5.684
AI (high, low)	0.380	3.495
TAp73α (positive, negative)	0.367	4.323
Liver membrane invasion (y, n)	0.367	8.434
Age (>45, ≤45)	0.392	4.360
TNM stage (II, III+IV)	0.477	6.180

DISCUSSION

p53, a transcription factor, connects to a specific base sequence, inhibits the expression of various genes, and plays a major role in regulating the cell cycle and preventing the malignant transformation of cells. In HCC, there is always p53 gene mutant or inactive[16], which leads to the abnormal growth of cells and ultimately the occurrence of cell conversion and carcinoma changes[17]. In spite of these important roles, the relationship between the pathological significance of p53 mutation and prognosis in HCC has not been fully established.

Because of the short $T_{1/2}$ of wide-type p53 (wt-p53) protein, p53 protein detected by immunohistochemistry (IHC) is almost mutant-type p53 (mt-p53) protein. In this study, 38.3% HCC tissues expressed mt-p53 protein, higher than that in the adjacent non-cancerous tissues and normal liver tissues, indicating that p53 mutation is frequent in HCC. Furthermore, the different level of mt-p53 in HCC correlated with the differentiation of tumor cells[18-20], that is, with the increasing of p53 level, the differentiation of tumor cells becomes poorer. It can be regarded that p53 level can reflect the differentiation of tumor cells. In this study mt-p53 did not show its correlation with the prognosis. So, we think, though mt-p53 is frequent in HCC, correlating with the occurrence and development of HCC, and can be used as an index of tumor cell differentiation, it is not an ideal index of prognosis.

p73 gene is a new family member of p53, it shares 63% identity with the DNA-binding region of p53 including the conservation of all DNA contact residues, 38% identity with the tetramerization domain, and 29% identity with the transactivation domain[21]. It plays an important role in several cancers, such as lung cancer, colorectal cancer and bladder cancer[22-25].

In this study, TAp73α overexpressed in HCC tissues, indicating that TAp73α may play its role in regulating occurrence and development of HCC. Similarly, Zemel and Pan found that p73 protein highly expressed and accumulated in HCC tissues, and overexpression of p73 may in some way be associated with the pathogenesis of HCC[26,27].

In 1999, Tannapfel found that p73 expression status was statistically significantly related to prognosis (P<0.0001). Patients with p73-positive tumors had a poorer prognosis than those with p73-negative carcinomas. Multivariate Cox survival analysis identified the age of the patient, p73 expression status, co-existing cirrhosis, and Edmondson grade as independent prognostic factors. The protein p73 is overexpressed by a subset of HCCs and could serve as a useful indicator of prognosis in patients with this disease[8]. In this study, the expression of p73 also influences the prognosis of HCC patients. Patients who overexpressed p73 had a poorer prognosis than that did not. In the Cox regression, p73 protein expression was still an independent factor of prognosis of HCC patients. Besides, the expression of p73 correlated with node metastasis, which indicated a late stage and poor prognosis[8,28]. In some reports, p73 can induce apoptosis by p53-dependent and p53-independent way[29,30], but in this study p73 does not show any relationship with AI.

There is not only overproliferation of tumor cells, but also over apoptosis of tumor cells[14]. But as PI overcomes AI, cancer develops in some degree. PCNA can be used as the index of proliferation and be detected by IHC[15]. Nakano *et al*[18], find that the poorer the differentiation of the tumor cells, the higher the PI is (P<0.001). In this study, though there was no correlation between PI and differentiation, PI in the cancer tissues was higher than that in the adjacent non-cancerous tissues, indicating a tendency of higher proliferating potency in tumor cells (P = 0.028). Moreover, PI correlated with portal vein invasion, indicating that patients with portal vein invasion had a worse biological character than that did not. Soini[31] finds that patients with a higher AI have a better survival than patients with a lower one after HCC tissue resection. In this study, AI in the HCC tissues was higher than that in the adjacent non-cancerous tissues (P = 0.019). And AI in late stage was higher than that in early stage. But as there are more proliferating cells than apoptosis ones, cancer has a tendency of being advanced. So we suggest that AI may be an index of late stage.

In this study, Kaplan-Meier univariant analysis showed that patients in stage II had a better prognosis than patients in III and IV, indicating that TNM stage is one of the useful independent indices of prognosis[32]. The status of liver membrane and portal vein invasion also influences the survival of HCC patients[10,33].

Besides pathological characters, AFP is a most useful index of prognosis of HCC patients. In Kaplan-Meier univariant analysis, AFP correlated with prognosis significantly (P<0.05), and in the Cox regression analysis, AFP was also an independent index of prognosis.

Together, these data suggest that TAp73α can be used as a prognostic indicator for patients with HCC undergoing surgical tumor ablation; AFP, TNM stage, portal vein invasion and liver membrane invasion also have a potency of predicting the prognosis of HCC.

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