MEDICAL SCIENCE MONITOR

MEDICAL BIOCHEMISTRY

e-ISSN 1643-3750
© Med Sci Monit, 2015; 21: 3209-3215
DOI: 10.12659/MSM.895592

Received: 2015.08.09
Accepted: 2015.09.24
Published: 2015.10.23

Expression of P53 and HSP70 in Chronic Hepatitis, Liver Cirrhosis, and Early and Advanced Hepatocellular Carcinoma Tissues and Their Diagnostic Value in Hepatocellular Carcinoma: An Immunohistochemical Study

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Source of support: This study is supported by the grant of the Special Foundation for Young Scientists of Bureau of Health, Xinjiang Uygur Autonomous Region

Background: Tumor protein (P53) and heat shock protein 70 (HSP70) play key roles in chronic liver diseases. This study aimed to characterize P53 and HSP70 expression in chronic hepatitis (CH), liver cirrhosis (LC), early and advanced HCC, and to analyze their diagnostic value in hepatocellular carcinoma (HCC).

Material/Methods: Immunohistochemical staining was conducted to evaluate the expression of P53 and HSP70 in 200 human liver tissue specimens, with advanced HCC (n=80), early HCC (n=30), CH (n=30), LC (n=30), and Controls (n=30).

Results: P53 expression levels were lower in LC than those of HCC, but remained on par with those of CH and Controls. HSP70 expression levels were higher in HCC than those of LC, CH, and Controls. The sensitivity and specificity for HCC diagnosis were: 50.9% and 98.9% for P53, and 78.2 and 77.8% for HSP70, respectively. The sensitivity and specificity of different combinations were: 95.5% and 85.5% with either P53 or HSP70 being positive, and 33.6% and 98.9% if both were positive. Among the differentiation stages marked low, intermediate, and high in HCC, the P53 positive rate was higher in the low than in the intermediate, which was higher than that in the high. HSP70 positive rate was higher in the low and the intermediate than in the high, but no obvious changes were found between the low and the intermediate.

Conclusions: P53 and HSP70 could be potential biomarkers for HCC diagnosis, and proper combinations of these 2 markers could improve diagnostic accuracy.

MeSH Keywords: Carcinoma, Hepatocellular • Hepatitis, Chronic • HSP70 Heat-Shock Proteins • Liver Cirrhosis • Tumor Suppressor Protein p53

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/895592

2944 4 1 25

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Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1]. Multiple etiologies are related to HCC occurrence and development, including chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol consumption, and cirrhosis [2,3]. It has been reported that half of HCC in china occurs because of the high prevalence of HBV infection [4]. Persistent HBV-derived inflammations contribute to hepatic fibrosis/cirrhosis, which results in increased risks of HCC. The high morbidity and mortality of HCC are mainly attributable to late diagnosis; early detection of HCC is therefore the only way to reduce the effect of HCC [5]. The criterion standard for HCC diagnosis has been liver biopsy or surgical specimens. Currently, the conventional pathological diagnosis is confined to the morphological changes and limited histochemical stains. More specific and sensitive immunochemical biomarkers are thus urgently needed to bring about more alternatives [6].

Persistent chronic liver diseases evolve into liver cirrhosis and eventually HCC via a sustained and long process called the 'trilogy' of HCC development. Patients with chronic liver diseases, especially liver fibrosis or cirrhosis, are at high risk of HCC; therefore, it is very important to closely monitor the progression from liver diseases to malignant neoplasm [7].

HCC features a series of molecular changes, such as cyclase-associated protein 2 (CAP2), glypican-3 (GPC3) and IGF-1R, as well as heat-shock protein (HSP) family and P53. HSP70 and P53 are frequently reported molecules that are over-expressed in HCC biopsy, especially in advanced HCC [5]. Both of them are believed to be putative biomarkers of HCC, but the relationship between HSP70 and P53 expression in the process of chronic hepatitis (CH), LC, and early and advanced HCC has not been reported [8,9].

HSP70, a stress-induced gene in the tumor genesis that modulates cell apoptosis and proliferation, demonstrates an anti-apoptosis effect, which ensures the survival of cells and promotes tumor cell proliferation [10]. It has been reported that both the mRNA and protein levels of HSP70 increase markedly more in advanced HCC than in early HCC [11]. Serum HSP70 is up-regulated in both liver cirrhotic and HCC patients [10]. Moreover, HSP70 is seen in both overt and early HCC and is less likely to be positive in the regenerative nodule in hepatic cirrhotic liver, as determined by immunochemistry staining [12].

P53 is the most studied tumor suppressor in a handful of cancers, including HCC [13]. Most human cancers are related to the abnormal expression and inactivation of P53 [14]. As a tumor-suppressor protein, P53 executes the function of DNA reparation, pro-apoptosis, and cell cycle arrest in tumor cells [15,16]. Mutation of P53 frequently occurs in HCC. Farazi et al. showed that reduced levels of P53 play an important role in the progression of chronic hepatic diseases to HCC by cooperating with telomere dysfunction [14]. Over-expression of P53 in liver, including both wild-type and its mutations, shows an increased predictability in HCC [16]. Due to the role of P53 in progression of CH to HCC, the expression level of P53 in the process of chronic liver diseases can be of help for monitoring and identification of HCC at an early stage [17,18].

Herein, we simultaneously detected the expression of HSP70 and P53 in CH, LC, and HCC in both early and advance stages. Furthermore, we analyzed the relationship between both predictors and their clinicopathologic features in advanced HCC.

Material and Methods

Patients and tissure samples

Patients pathologically diagnosed with advanced HCC (n=80), early HCC (n=30), LC (n=30), and CH (n=30), and patients with only liver hemangioma (Controls, n=30, distal tissues), in accordance with WHO criteria, were obtained from the First Affiliated Hospital of Xinjiang Medical University between 2009 and 2014. Clinical characteristics were collected by reviewing medical records and pathologic dates. All patients enrolled in the study signed consent forms and the study was approved by the Medical Ethics Committee of Xinjiang Medical University (No. 20140929-02). All resected surgical specimens were immediately fixed in formalin (10%) and embedded in paraffin; 4-μm-thick sections were cut serially and stained for P53 and HSP70.

Immunohistochemistry

Tissue sections were processed with xylene (10 min, 3 times) and graded alcohol for deparaffinization and hydration, after which sections were heated in 0.01 M citrate buffer solution (PH9.0) in microwave for 20 min for antigen retrieval. The sections were naturally cooled to room temperature (RT) before being treated with 3% H_2O_2 in methanol 15 min to block endogenous peroxidase activities. Sections were incubated overnight at 4°C with the primary antibodies against P53 (1: 100, mouse monoclonal antibody, Fuzhou Maixin Biotech, CN) and HSP70 (1: 200, mouse monoclonal antibody, SANTA CRUZ, USA), then they went through incubation 1 more time for 30 min with the secondary antibodies (ZSGB, China) at 37°C and stained with DAB solution. After counterstaining with hematoxylin, the sections were passed through graded ethanol and sealed.

The results of immunohistochemical processing for P53 (nucleic staining) and HSP70 (nucleocytoplasmic staining) were

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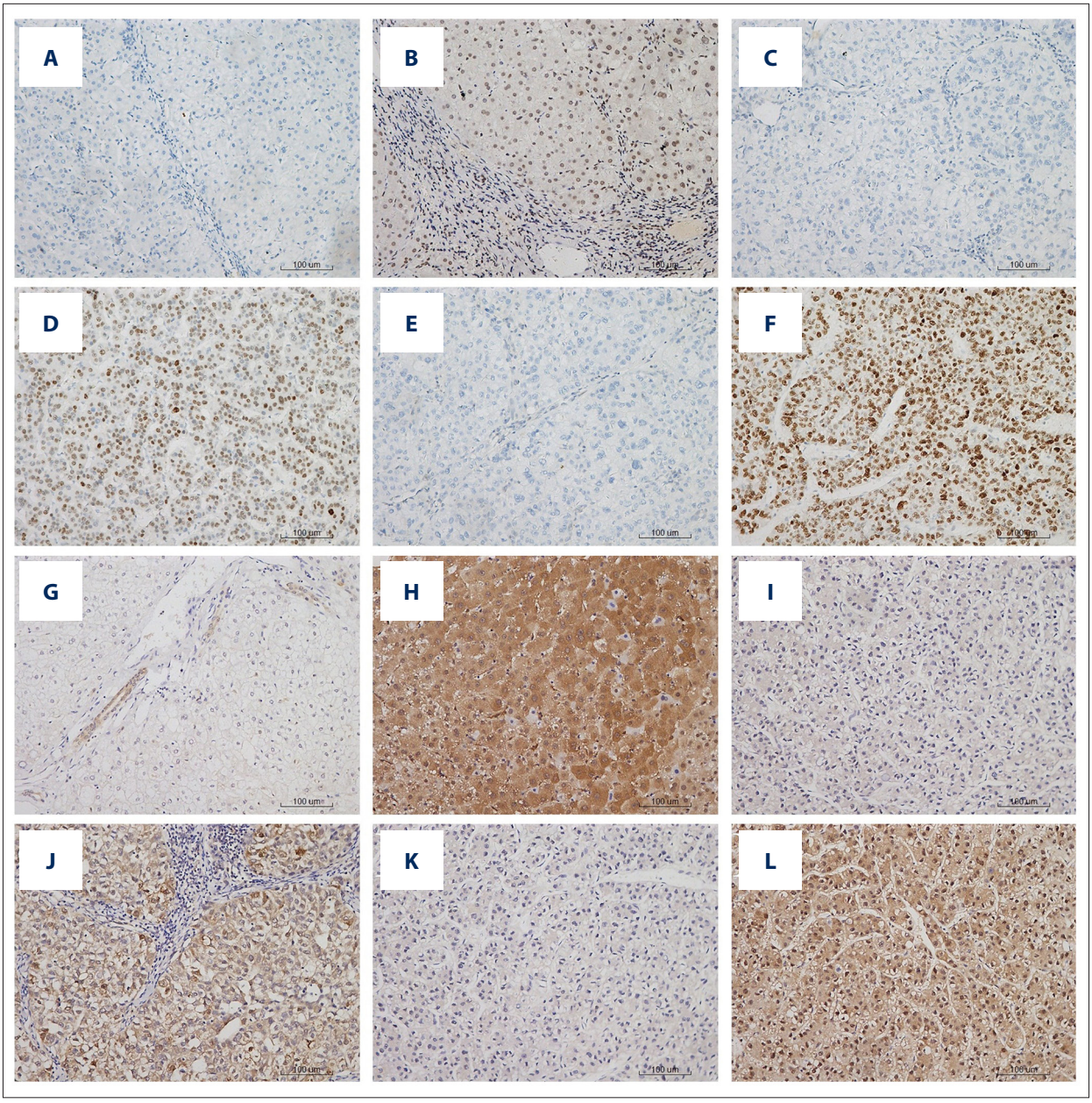

Figure 1. Immunohistochemical staining of P53 or HSP70 in LC, early HCC or advanced HCC (×200). (**A**) P53-negative staining in LC tissue, (**B**) P53-positive staining in LC tissue, (**C**) P53-negative staining in early HCC tissue, (**D**) P53-positive staining in early HCC tissue, (**E**) P53-negative staining in advanced HCC tissue, (**F**) HSP70-positive staining in advanced HCC tissue, (**G**) HSP70-negative staining in LC tissue, (**H**) HSP70-positive staining in LC tissue, (**I**) HSP70-negative staining in early HCC tissue, (**J**) HSP70-positive staining in early HCC tissue, (**K**) HSP70-negative staining in advanced HCC tissue, (**L**) HSP70-positive staining in advanced HCC tissue.

analyzed in blind fashion by 2 independent, experienced pathologists. Ten areas were selected randomly and the rating was done according to the methods reported by previous studies [19–21]. Staining density was graded: 1 (≤25% staining), 2 (26–75% staining), and 3 (≥76% staining). Staining intensity was evaluated: 1 for no positive staining, 2 for mild staining, and 3 for intense staining. The 2 grades of each specimen were then multiplied and categorized into negative (–) for scores <3 and positive (+) for scores ≥3.

Statistical analysis

Statistical analysis was performed with 1-way ANOVA using SPSS version 17.0 (SPSS, Chicago, USA). For the comparisons

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Table 1. The clinicopathologic characteristics of the Control, CH, LC, Early HCC and Advanced HCC.

Characteristics	Control	CH	LC	Early HCC	Advanced HCC
Gender					
Male	11 (36.67%)	18 (60.00%)	23 (76.67%)	21 (70.00%)	63 (78.75%)
Female	19 (63.33%)	12 (40.00%)	7 (23.33%)	9 (30.00%)	17 (21.25%)
Age					
<55	22 (73.33%)	26 (86.67%)	17 (56.67%)	14 (46.67%)	46 (57.50%)
≥55	8 (26.67%)	4 (13.33%)	13 (43.33%)	16 (53.33%)	34 (42.50%)
Tumor size					
<3 cm	–	–	–	30 (100.00%)	–
≤3 and <6 cm	–	–	–	–	47 (58.75%)
≥6 cm	–	–	–	–	33 (41.25%)
Cancer embolus	–	–	–	6 (20.00%)	28 (35.00%)
Differentiation					
High	–	–	–	12 (40.00%)	21 (26.25%)
Middle	–	–	–	11 (36.67%)	28 (35.00%)
Low	–	–	–	7 (23.33%)	31 (38.75%)
HBs Ag	–	24 (80.00%)	16 (53.33%)	22 (73.33%)	63 (78.75%)
HCV Ab	–	2 (6.67%)	3 (10.00%)	4 (13.33%)	5 (6.25%)
Alcoholism	–	4 (13.33%)	11 (36.67%)	3 (10.00%)	8 (10.00%)
Cryptogenic	–	0 (0%)	0 (0%)	1 (3.33%)	4 (5.00%)

Control – patients with only liver hemangioma; CH – chronic hepatitis; LC – liver cirrhosis; HCC – hepatocellular carcinoma; HBs Ag – anti-hepatitis B surface antigen; HCV Ab – anti-hepatitis antibody.

of the data of 5 groups and data between clinicopathologic parameters and factors, the significance of differences was evaluated by Pearson's χ^2 test or Fisher's exact test. Differences were considered to be statistically significant when P value <0.05 and all P values are 2-tailed in all analyses.

Results

Immunoreactivity of P53 and HSP70 of the Controls, CH, LC, and early and advanced HCC was evaluated by immunohistochemical analysis. The expression of P53 appeared in the form of a nucleonic staining pattern, while the expression of HSP70 was localized in the nucleus and/or cytoplasm (Figure 1). Clinicopathologic characteristics of the groups are listed in Table 1.

As shown in Table 2, the positive expression rate of P53 in advanced HCC tissues was 60.0% (48/80), significantly higher than that in early HCC tissues (26.7%, 8/30, P=0.003), LC tissues (3.3%, 1/30, P<0.001), CH tissues, and tissues of Controls (0.0%, 0/30, P<0.001, both). The positive expression rate in early HCC tissues was 26.7% (8/30), significantly higher than that in LC tissues (3.3%, 1/30, P=0.026), CH tissues, and tissues of Controls (0.0%, 0/30, P=0.007, both).

The positive expression rate of HSP70 in advanced HCC (76.3%, 61/80) was significantly higher than that in LC tissues (43.3%, 13/30, P=0.003), CH tissues (23.3%, 7/30, P<0.001) and tissues of Controls (0.0%, 0/30, P<0.001). Meanwhile, the positive rate in early HCC tissues (83.3%, 25/30) was also significantly higher than that in LC tissues (43.3%, 13/30, P<0.001), CH tissues (23.3%, 7/30, P<0.001) and tissues of Controls (0.0%, 0/30, P<0.001). However, there was no significant statistical difference in the positive expression rate of HSP70 in early HCC and advanced HCC (P>0.5). Furthermore, the expression patterns of P53 in HCC tissues were significantly correlated with that of HSP70(r=0.684; P<0.001).

The Diagnostic accuracy and the combinations of the 2 positive markers are summarized in Table 3. The sensitivity, the specificity, and the positive and negative predictive values for

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Table 2. Immunohistochemical for expressions of P53 and HSP70 in Control, CH, LC, early HCC and advanced HCC tissues.

Tissues	P53		HSP70	
	Positive	Negative	Positive	Negative
Control	0	30	0	30
CH	0	30	7	23
LC	1	29	13	17
Early HCC	8	22	25	5
Advanced HCC	48	32	61	19

Control – patients with only liver hemangioma; CH – chronic hepatitis; LC – liver cirrhosis; HCC – hepatocellular carcinoma.

Table 3. Diagnostic accuracy for detection of hepatocellular carcinoma using one or two markers.

Markers	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR–
One marker						
P53	50.9	98.9	98.2	62.2	46.3	0.50
HSP70	78.2	77.8	81.1	74.5	3.53	0.28
Two markers						
Parallel	95.5	85.5	89.0	93.9	6.6	0.05
Serial	33.6	98.9	97.4	54.9	30.27	0.67

PPV – positive predictive value; NPV – negative predictive value; LR – likelihood ratio. Parallel – at least 1 positive. Serial – both 2 positive.

parallel combination were 95.5%, 85.5%, 89.0%, and 93.9%, respectively, with at least 2 markers being positive, and the sensitivity and negative predictive values of the parallel were comparatively higher. The 4 indicators for serial combination were 33.6%, 98.9%, 97.4%, and 54.9% respectively, with both markers being positive, and the specificity and positive predictive values of the serial combination were relatively higher. These results may indicate that proper combinations of the 2 predictors would help us reach a more reliable and accurate diagnosis of HCC, including early and advanced HCC, compared with use of only 1 marker.

The correlations between clinicopathologic characteristics and expression of P53 and HSP70 were assessed in the patients with advanced HCC, with 5 clinicopathologic parameters – tumor thrombus, tumor size, differentiation, age, and sex – as revealed in the present study (Table 4). As listed in Table 4, Pearson's χ^2 test or Fisher's exact showed that the differences in differentiation for P53 and HSP70 were statistically significant (P<0.001, both). The positive expression rates of P53 and HSP70 were both higher in patients with lower differentiation. In advanced HCC tissues, the positive expression rate of P53 in low-differentiation tissues was 90.03% (28/31), significantly higher than in moderately differentiated tissues (53.6%, 15/28, P=0.002) and highly differentiated tissues (23.8%, 5/21, P<0.001), and the positive rate of P53 in moderately differentiated tissues was also higher than in highly differentiated tissues (P=0.036). In addition, the positive rate of HSP70 in advanced HCC tissues of low differentiation (96.8%, 30/31) was higher than in intermediate differentiation tissues (78.6%, 22/28, P>0.05), but the differences were not statistically significant. The rate of low differentiation was significantly higher than in high differentiation tissues (42.9%, 9/21, P<0.001). Similarly, the rate of intermediate differentiation was higher than that of high differentiation (P=0.01). There was no significant statistical association between the 2 factors in terms of tumor thrombus, tumor size, age, or sex, suggesting that these 4 variables do not affect the expression of P53 and HSP70 in advanced HCC.

Discussion

HCC is a major cause of death in chronic liver diseases, especially in patients with liver cirrhosis. Preventing chronic hepatitis and/or liver cirrhosis from progressing to HCC is still an important challenge. Early diagnosis is an effective way to reduce the incidence of HCC. As HCC is closely related to liver cirrhosis, the monitoring of chronic liver diseases is of considerable importance. Morphological distinction of HCC among

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Table 4. The expression of P53 and HSP70 in human advanced hepatocellular carcinoma tissues with different clinicopathologic features.

Features	N.O patients	P53		HSP70	
		Positive	Negative	Positive	Negative
Gender					
Male	63	40	23	50	13
Female	17	8	9	11	6
Age					
<55	45	29	16	34	11
≥55	35	19	16	27	8
Tumor size					
<6 cm	47	28	19	38	9
≥6 cm	33	20	13	23	10
Tumor embolus					
Presence	18	13	5	14	4
Absence	62	35	27	47	15
Differentiation*					
High	21	5	16	9	12
Intermediate	28	15	13	22	6
Low	31	28	3	30	1

* Statistically significant differences among the high, moderate and low differentiation in expression of P53 ($P<0.001$) and HSP70 ($P<0.001$).

advanced chronic liver diseases and early neoplastic lesion still poses a number of problems. Therefore, more support is needed in the conventional pathological detection of HCC from chronic liver diseases, especially advanced hepatic cirrhosis.

The study used immunocytochemistry to measure the expression pattern of HSP70 and P53 in different stages of chronic hepatic diseases and their correlation with clinicopathological parameters. Findings derived are: (1) P53 obviously increased its presence in HCC more than in chronic hepatitis and cirrhosis, which can be used to detect early-stage HCC; (2) HSP70 could clearly distinguish HCC from chronic hepatitis/cirrhosis, but failed to distinguish between early and advanced HCC; (3) P53 and HSP70 are correlated with malignancy and are highly expressed in poorly differentiated tumors.

Furthermore, our study found that the parallel combination of P53 and HSP70 could increase the sensitivity at the expense of specificity loss, which was the opposite of the series combination. Therefore, proper combination of P53 and HSP70, in parallel or in series, could increase the specificity and sensitivity at the same time, which would help reach a more reliable and accurate diagnosis of HCC, including early and advanced HCC, rather than use of only 1 marker.

P53 is related to more than half of human cancers [22]. Mutation and sequential inactivation of P53 are characteristics of HCC [23]. Our study found the expression levels of P53 were consistent with previous reports, and specific expressions of P53 in tumors demonstrated no expression in chronic hepatitis, 3.3% (1/30) being positive in liver cirrhosis; and 26.7% and 60.0% in early and advanced HCC, respectively. There was a significant difference between early and advanced HCC, indicating that P53 is sensitive enough to distinguish early HCC from the other stages. Our data showed that P53 had no obvious relationship with sex, age, tumor size, or cancer embolus of patients with advanced HCC. Moreover, P53 was correlated with the differentiation levels of HCC, with well-differentiated HCC showing a lower P53 level.

HSP70, together with the other 2 biomarkers – GPC3 and glutamine synthetase (GS) – has demonstrated utility in detecting early and grade 1 HCC in the dysplastic nodules of cirrhosis [24,25]. Other investigations also showed highly expressed levels of HSP70 in HCC [12]. The positive rate of HSP70 in this study was found to gradually increase in the progression of chronic liver diseases: 23.3% (7/30) in hepatitis, 43.3% (13/30) in liver cirrhosis, and 83.3% (25/30) in early-stage HCC. There was no significant change of HSP70 between hepatitis and cirrhosis ($P>0.05$). The early and advanced HCC (76.3%, 61/80) both

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possessed a higher level of HSP70 (P>0.05). Similar to P53, there was no obvious relationship between HSP70 and patient sex, age, tumor size, and cancer embolus. However, it displayed a significant correlation with the differentiation, indicating HSP70 as a potential prognostic indicator for malignancy. We found HSP70 was more positively expressed in lower-differentiated tumors.

HCC diagnosis at an early stage is crucially important. A series of tumor biomarkers have recently emerged as important diagnostic indicators for HCC. Our results showed that P53 was highly expressed in neoplastic tissues and was almost only expressed in HCC, while HSP70 could be detected in both non-neoplastic (CH and LC) and neoplastic liver tissues. Kamal et al. reported that by combining 3 different biomarkers (CGP3, HSP70, and GS), the diagnostic accuracy could be increased [9]. Furthermore, our study found that the parallel combination of P53 and HSP70 could increase the sensitivity at the expense of specificity loss, which was the opposite of the series combination. Therefore, proper combination of P53 and HSP70, in parallel or in series, could increase the specificity and sensitivity at the same time, which would help reach a more reliable and accurate diagnosis of HCC, including early and advanced HCC, rather than use of only 1 marker.

The current study has a few limitations. Firstly, we only enrolled the patients in Xinjiang area of China in 1 center, which limits applicability of the conclusion that combination of P53 and HSP70 could improve the diagnosis of HCC. Secondly, the sample size is small, and for clinical application of P53 and HSP70 to diagnose HCC, a cohort with more cases from multiple centers should be included. Secondly, we only used immunohistochemistry staining in this study, but ELISA and Western blot should be used to detect the protein levels. More patients will be enrolled in our next study, and we will also follow up these patients to analyze the relationship between survival time and these 2 bio-markers.

Conclusions

In conclusion, this study shows that P53 could be used to distinguish early HCC from advanced HCC, but HSP70 cannot. The proper combination of these 2 well-known markers of HCC is a more sensitive and accurate alternative to monitor the progression of chronic liver diseases and diagnosis HCC at an early stage, through dynamic observation of P53 and HSP70 in different stages of chronic liver diseases.

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