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Original Article

Correlation of HSP70, P53 and Bmi-1 in hepatitis, cirrhosis and hepatocellular carcinoma

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Abstract: Objective: To investigate the correlation of HSP70, P53 and Bmi-1 in hepatitis, cirrhosis and hepatocellular carcinoma patients. Methods: Hepatitis (30 cases), cirrhosis (30 cases), early stage HCC (30 cases), and advanced stage HCC (80 cases) patients were recruited in this study, and we used immunohistochemistry S-P method to detect the protein expression levels of HSP70, P53 and Bmi-1, which were then correlated with clinical pathological parameters. Results: (1) HSP70, P53 and Bmi 1-expression levels were increased in early stage HCC and advanced HCC, compared to the hepatitis and cirrhosis patients. The result was statistically significant ($P < 0.05$); and P53 expression in advanced HCC patients was significantly higher than early stage HCC. (2) HSP70 expression level was positively correlated with P53 and Bmi-1 in advanced HCC, ($r_1 = 0.45$, $r_2 = 0.57$). P53 expression level was positively correlated with Bmi-1 in advanced HCC ($r_3 = 0.45$); (3) In advanced HCC patients, HSP70 and P53 expression is correlated with tumor differentiation grade ($P < 0.5$), regardless of the patient gender, age, tumor size, thrombus status; Bmi-1 expression level correlates with the TNM stage ($P < 0.5$), regardless of the patient gender, age, tumor size, thrombosis status or tumor differentiation grade. (4) Patients with positive P53, HSP70 and Bmi-1 would have poor prognosis, but the difference was not statistically significant. Conclusion: HSP70, P53 and Bmi-1 are expressed at higher levels in HCC patients. They are probably involved in the development and progression of HCC, and they are playing a synergestic role in the cell proliferation in HCC.

Keywords: HCC, IHC, HSP70, P53, Bmi-1

Introduction

Hepatocellular carcinoma, or HCC is the most common malignant tumor in gastrointestinal tract, with the third highest mortality rate in the world [1], and the incident rate is increasing every year. HCC is usually difficult to be first diagnosed, and its early stage metastasis has been commonly observed in the clinic. For this reason, most patients were first diagnosed at the mid or late stage of HCC, which will result in poor prognosis. Epidemiology and pathology studies have revealed HBV as well as HCV are closely related to the HCC [2]. They are also the main causes of cirrhosis. According to statistics, cirrhosis patients usually take average 7 years to progress into HCC. Hepatitis, cirrhosis, and HCC are the disease procession process. Heat shock protein 70, or HSP70 has been reported to be playing a role in the self-repair after damage. HSP70 and P53 are closely related with P53 in tumorgenesis, and HSP70 could regulate P53 under certain circumstances. Bmi-1is a transcription factor, and it has been shown abnormality in multiple tumors, and it's been believed to be one of the oncogenes. The published study focused on Bmi-1 and HSP70 or P53 is limited. And in our study, we aim to investigate the expression levels of these three genes in the hepatitis, cirrhosis and HCC patients, and correlate it with the disease progress and clinical pathology parameters.

Materials and methods

Clinical samples

Clinical samples were collected from pathology department of First Affiliated Hospital, Xinjiang Medical University by core needle biopsy or

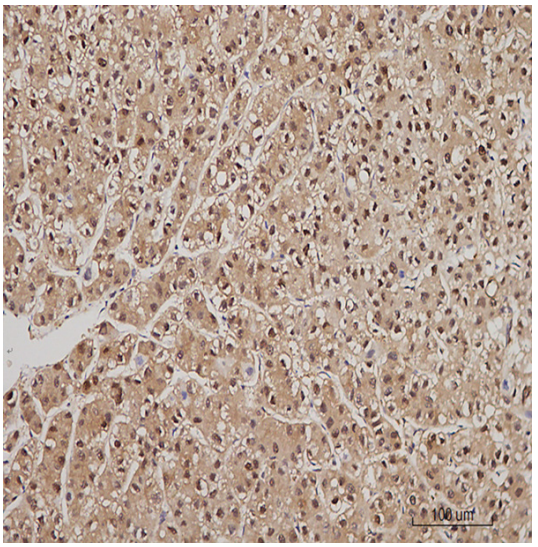

Figure 1. HSP70 positive staining in HCC (20×).

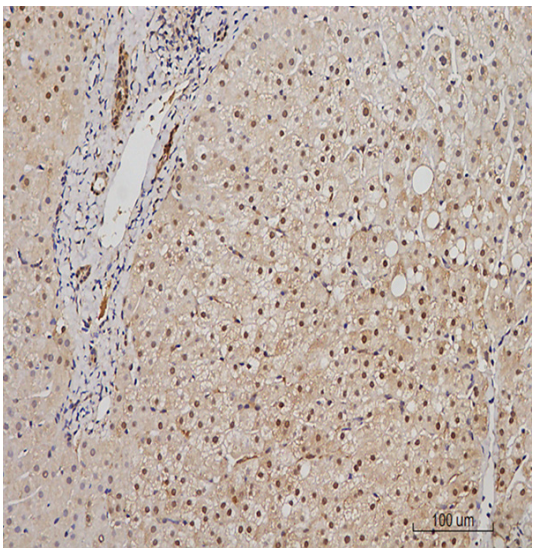

Figure 2. HSP70 positive staining in hepatitis (20×).

resection surgeries. 30 cases of hepatitis (12 male, 18 female, average 45 yrs old), 30 cases of cirrhosis patients (19 male, 11 female, average 52 yrs old), 30 cases of early stage HCC (20 male, 10 female, average 51 yrs old), and 80 cases of advanced HCC (63 male, 17 female, average 54 yrs old). The 30 cases of hepatitis petitions have been confirmed to be positive for virus marker by serology and IHC. Early stage HCC has the criteria of primary HCC with single nodule of diameter < 3 cm or two nodules of combined diameter < 3 cm.

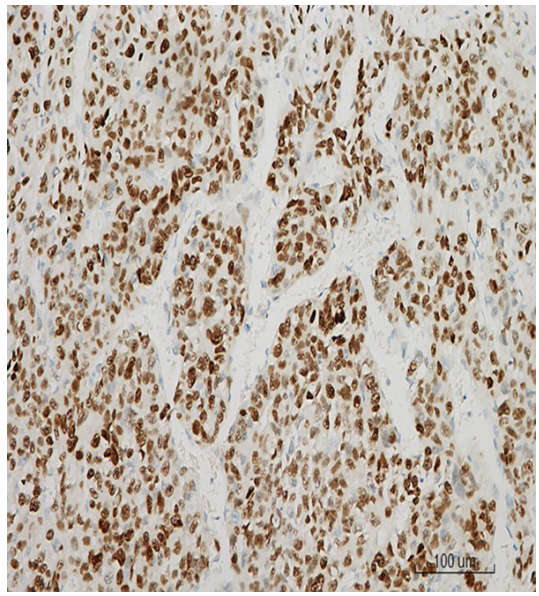

Figure 3. P53 positive staining in HCC (20×).

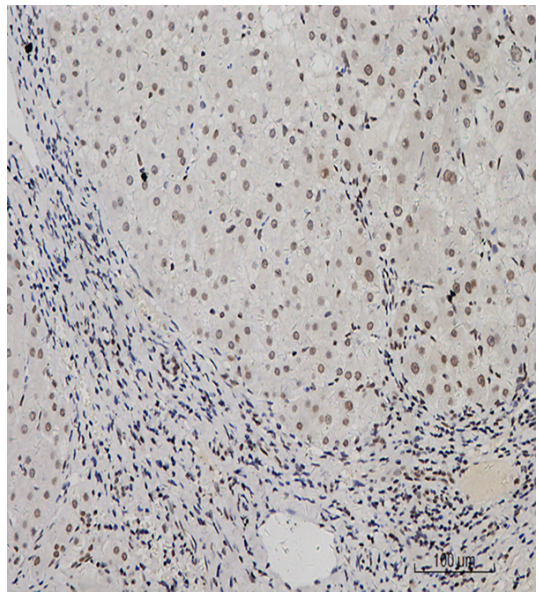

Figure 4. P53 positive staining in cirrhosis (20×).

Reagents

Human HSP70 monoclonal antibody was purchased from SANTA CRU, with dilution factor of 1:200 in IHC assay. Human Bmi-1 monoclonal antibody was purchased from Thermo Fisher, with dilution factor of 1:800 in IHC assay. Human P53 monoclonal antibody was purchased from Fuzhou Maixin, with dilution factor of 1:100. Liver samples were fixed by 10%

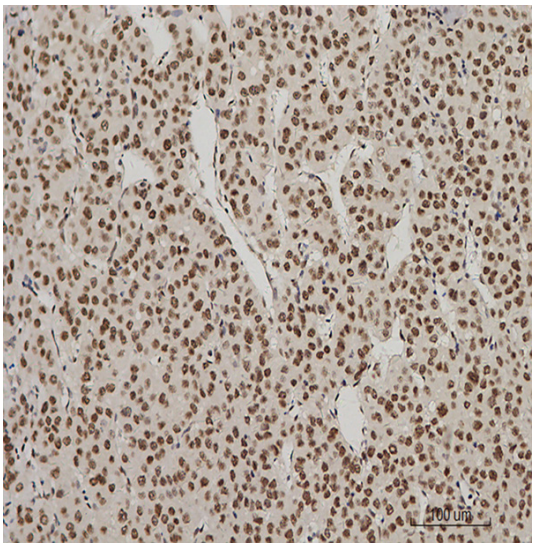

Figure 5. Bmi-1 positive staining in HCC (20×).

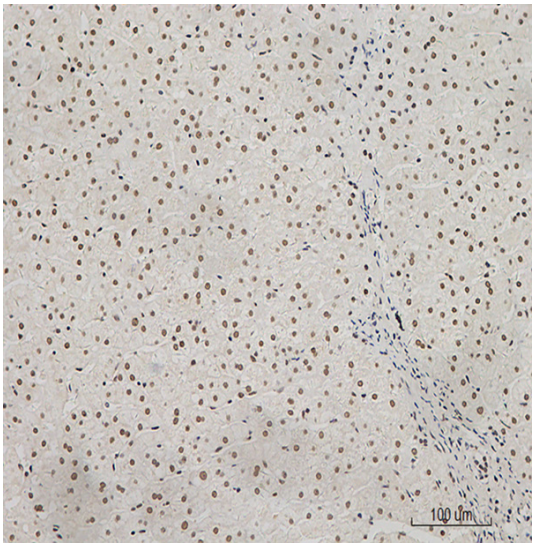

Figure 6. Bmi-1 positive staining in hepatitis (20×).

formaldehyde, followed by FFPE blocks preparation and sectioned at 4 um. The protocol of SP-IHC was followed in the following IHC assay, including antigen recovery by Tris/EDTA 9.0. PBS was included as negative control.

Data analysis

Nuclear and/or cytoplasm yellow or brown staining is regarded as HSP70 or Bmi-1 positive criteria, nuclear yellow or brown staining is regarded as P53 positive criteria. 100 cells from a total of 10 fields at 400 fold were randomly selected for analysis. The positive staining was classified into 3 grades: no expression 1, weak positive 2, strong positive 3. The tumor cell percentage is also classified into 3 grades: ≤ 25% grade 1, 26%~75% grade 2, ≥ 76% grade 3. The final score of each tissue section is to multiple the two scores (1-9). ≥ 3 will be positive, < 3 will be negative. The result was confirmed blindly by two independent pathologists.

Table 1. Expression summary of HSP70, P53 and Bmi-1 in hepatitis, cirrhosis, early and advanced stage HCC patients

	Case #	HSP70	P53	Bmi-1
Hepatitis	30	7	0	5
Cirrhosis	30	13	1	9
Early stage HCC	30	25	8	19
Advanced stage HCC	80	$61^{1*,\Delta}$	$48^{2*,\Delta}$	$58^{3*,\Delta}$

*vs. hepatitis, $\chi^2 1 = 25.88$, $\chi^2 2 = 31.95$, $\chi^2 3 = 27.79$, $P < 0.01$; $^{\Delta}$vs. cirrhosis, $\chi^2 1 = 10.74$, $\chi^2 2 = 28.36$, $\chi^2 3 = 16.55$, $P < 0.01$.

Table 2. Correlation of HSP70, P53 and Bmi-1 in advanced HCC patients

HSP70 expression	P53 expression*		Bmi-1 expression$^{\Delta}$	
	-	+	-	+
-	16	3	16	3
+	16	45	6	55

*HSP70 vs P53, $\chi^2 = 20.29$, $r = 0.45$; $^{\Delta}$HSP70 vs Bmi-1, $\chi^2 = 40.19$, $r = 0.57$; P53 vs Bmi-1, $\chi^2 = 22.11$, $r = 0.46$.

Follow up

The follow up was conducted in the following 8-72 months. Survival time is valid from the date of surgery. PFS is the time from surgery date to recurrence/metastasis date.

Statistics

SPSS17.0 was used for statistics. Correlation study was conducted using Spearman method, Kaplan-Meier method was used in survival analysis.

Results

Expression difference of HSP70, P53and Bmi-1 in liver disease progress

Nuclear and/or cytoplasm yellow or brown staining is regarded as HSP70 or Bmi-1 positive criteria, nuclear yellow or brown staining is regarded as P53 positive criteria (**Figures 1-6**).

Table 3. Correlation of HSP70, P31and Bmi-1 with the clinical pathology parameters in advanced stage HCC patients

Parameter	Case#	HSP70+	χ^2	P53+	χ^2	Bmi-1+	χ^2
Gender							
Male	63	50	1.589	40	1.506	48	3.533
Female	17	11		8		9	
AGE							
≤ 55	45	34	0.027	29	0.847	32	0.100
> 55	35	27		19		26	
Tumor size							
≤ 6 cm	47	38	1.332	28	0.009	33	0.299
> 6 cm	33	23		20		25	
Thrombosis							
+	18	14	0.030	13	1.446	15	1.367
-	62	47		35		43	
Differentiation grade							
Highly differentiated	13	6	7.788*	4	6.538*	8	0.940
Moderate differentiated	35	29		25		26	
Poorly differentiated	32	26		19		24	
LN metastasis							
Y	15	14	2.975	10	0.342	13	1.858
N	65	47		38		45	
TNM stage							
I+II Stage	46	30	0.204	23	4.510*	29	4.855*
III+IV Stage	34	31		25		29	

*P < *0.05*, statistically significant.

The positive rate of HSP70 in hepatitis, cirrhosis, early stage HCC and advanced stage HCC is 23% (7/30), 43% (13/30), 83% (25/30), 76% (61/80), respectively. The expression level in early stage HCC and advanced stage HCC is significantly higher than hepatitis and cirrhosis. Although early stage HCC is slightly higher than advanced HCC, but the difference has not reached statistical significance. Bmi-1 positive rate in hepatitis, cirrhosis, early stage HCC and advanced stage HCC is 16% (5/30), 30% (9/30), 63% (19/30), 72% (58/80), respectively. The expression level in early stage HCC and advanced stage HCC is significantly higher th an hepatitis and cirrhosis. And the expression level in advanced HCC is higher than early stage HCC, without statistical significance. The positive rate of P53 in hepatitis, cirrhosis, early stage HCC and advanced stage HCC is 0% (0/30), 3% (1/30), 26% (8/30), 60% (48/80), respectively. The expression level in early stage HCC and advanced stage HCC is significantly higher than hepatitis and cirrhosis. The expression level in advanced stage HCC is significantly higher than early stage HCC (**Table 1**).

Correlation study of HSP70, P53 and Bmi-1 in advanced stage HCC

In 80 cases of advanced stage HCC patients, 41 cases (51%) are positive for HSP70, P53 and Bmi-1. When correlation between any two of these genes is positively related (**Table 2**).

Correlation of HSP70, P53 and Bmi-1 with clinical pathology parameters in advanced stage HCC

In advanced stage HCC patients, HSP70 and P53 are not correlated with the patient gender, age, tumor size, thrombosis status, but they are correlated with the tumor differentiation grade ($P < 0.05$). On the other hand, P53 expression level is correlated with TNM stage ($P < 0.05$) Similarly, Bmi-1 expression level is not correlated with patient gender, age, tumor size, throm-

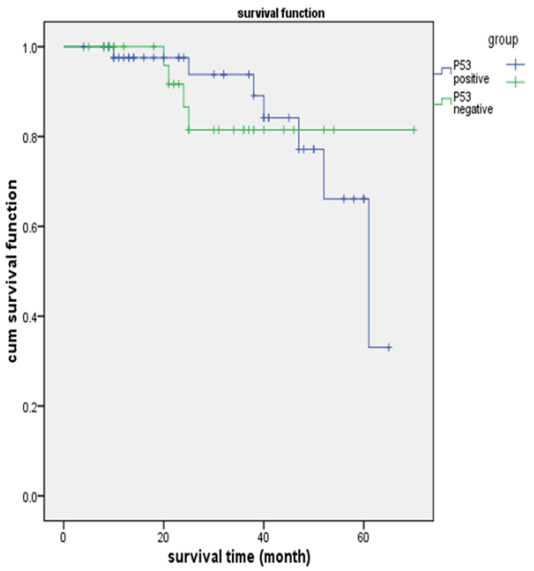

Figure 7. Survival curve of patients with different P53 levels.

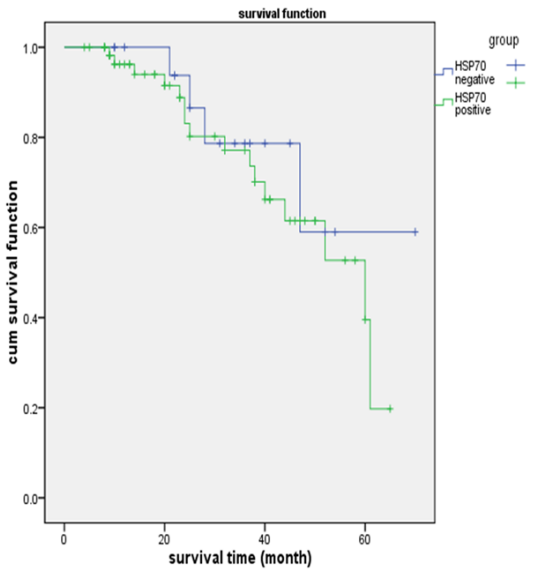

Figure 8. Survival curve of patients with different HSP70 levels.

bosis status or tumor differentiation grade, but it is correlated with patient TNM stage ($P < 0.05$, **Table 3**).

Correlation study of the expression levels of HSP70, P53 and Bmi-1 with prognosis in advanced stage HCC patients

In the 80 cases of advanced stage HCC patients, after 8-72 months follow up, 69 cases were completed with detailed follow up data, and 11 cases dropped out from this study.

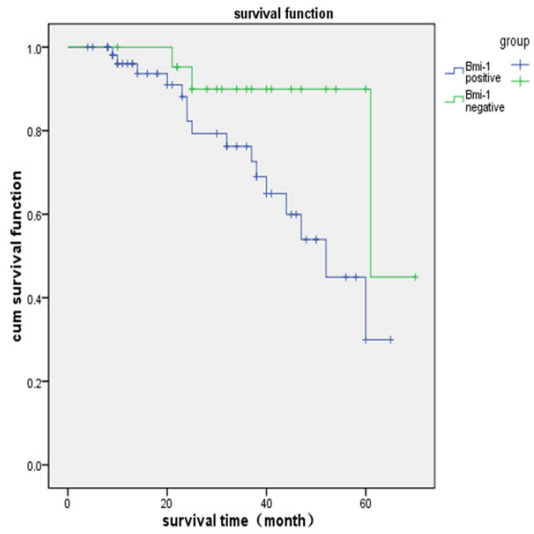

Figure 9. Survival curve of patients with different Bmi-1 levels.

Within the 69 cases, 21 mortalities (30.43%) were reported, and 15 cases were reported with recurrence or metastasis (21.74%), and the rest 33 cases are survived without tumor progression (47.83%).

The survival data of the 80 cases of advanced stage HCC patients have been analyzed using Kaplan-Meier method. The data suggests that P53, HSP70 and Bmi-1 positive patients would have worse prognosis, but it has not reached statistical significance (**Figures 7-9**).

Discussion

HCC is one of the most commonly seen malignant tumors in China, which is a cancer type that involved multiple stages, and multiple causing factors. With the development of medical science, the early diagnosis of HCC has been greatly improved. The model of HBV infection-hepatitis-cirrhosis-early stage HCC-advanced stage HCC is one of the ideal disease models. It has been suggested that different molecular pathways have been involved in the different stages of tumorgenesis, and these pathways are usually associated with the on and off of some of the key genes, which is playing a crucial role in tumor development.

HSP proteins were firstly discovered in fruit flies. They are a class of protein that ubiquitously distributed in organisms from prokaryotic organisms to eukaryotic organisms. HSPs are playing important roles in cell development,

metabolism, and maintenance of cellular homeostasis. Among all the HSPs, HSP70 is one of the most important and most conserved members. Most studies focusing on this protein is related to the cancer cell proliferation and anti-apoptosis [3]. It has been reported HSP70 mRNA level is upregulated in early stage HCC patients, suggesting the important role of HSP70 in early stage HCC. P53 is a tumor suppressor gene, which encodes a protein of 53 KDa. P53 mutation is associated with tumorgenesis in many cancer types [5]. Patients with P53 mutations usually have poor prognosis. Cui et al. have reported that the expression level of HSP70 and P53 are closely linked in HCC, and hypothesized that HSP70 and P53 are associated with each other, which might mediate the degradation of mutated P53.

In our studies, the expression level of HSP70 is significantly higher in the early and advanced stage HCC patients (83% and 76%, respectively), compared to the hepatitis and cirrhosis patients. This suggests HSP70 is overexpressed in HCC patients, and the fact that HSP70 has higher expression level in the early stage HCC than advanced stage HCC, is consistent with the published data. This indicates HSP70 is an important factor in early stage HCC. It has been suggested that HSP70 abnormality could be used as a marker and indicator of early diagnosis of HCC [4]. Our data indicates, although the expression level of HSP70 is higher in the early stage HCC than advanced stage HCC, but the difference is not statistically significant. With the analysis of the clinical pathology parameters of advanced stage HCC, HSP70 was found to be irrelevant with the patient gender, age, tumor size, thrombosis status, LN metastasis or TNM stage, but it is correlated with tumor differentiation grade. The data suggests the positive rate of HSP70 in highly differentiated HCC is 46% (6/13), in moderate differentiated HCC is 82% (29/35), in poorly differentiated HCC is 81% (26/32). The positive rate of HSP70 in moderate and poorly differentiated HCC is significantly higher than highly differentiated HCC. This data indicates the poorly differentiated HCC, or more malignant tumor, will have worse prognosis. It is suggested to use HSP70 as a key marker for tumor differentiation grade and patient prognosis.

In our studies, early stage HCC, or small HCC, has the criteria of primary HCC with single nodule of diameter < 3 cm or two nodules of diameter < 3 cm. There's usually no symptom in early stage HCC, thus HCC patients are diagnosed when the disease progressed into late stage. We found HSP70 expression level is upregulated in early stage HCC, which is significantly higher than hepatitis and cirrhosis, indicating this protein could potentially be the diagnosis marker for early stage HCC, either through serum marker monitoring or other non-invasive approaches. Meanwhile, HSP70 could also be a potential indicator to differentiate benign liver diseases (i.e. hepatitis, cirrhosis) and HCC, especially in small core needle biopsy samples. Under the circumstances when it is difficult to differentiate the highly differentiated HCC, HSP70 IHC assay could be useful as a reference to help with diagnosis.

P53 has been extensively studied in the oncology field. It has been reported the loss of function mutation of tumor suppressor gene P53, is the key event in the tumorgenesis in liver. In HBV patients, P53 positivity is a risk factor for liver fibrosis [7]. It has also been reported that P53, together with other factors, could be indicators for HCC recurrence [8]. Our data suggests, P53 has higher expression level in early and advanced stage HCC, compared to hepatitis and cirrhosis. When comparing the positive rate in early (26%) and advanced stage HCC (60), P53 is significantly higher in advanced stage HCC, implying P53 is one of the changing factors in advanced HCC. Our data indicates that in the 30 cases of hepatitis and 30 cases of cirrhosiss liver samples, only 1 cirrhosis case showed P53 positive staining, and P53 is more specific to the tumor tissues in the development of HCC in the disease cascade from HBV infection to hepatitis and cirrhosiss, which then lead to early and advanced stage HCC. This implies P53 may play a role in the tumorgenesis of hepatocytes. In addition, our data demonstrates P53 is not correlated with patient gender, age, tumor size, thrombosis status, or lymph node metastasis status, but it is correlated with tumor TNM stage. P53 is 31% positive (4/13) in highly differentiated HCC, 71% positive (25/35) in moderate differentiated HCC, and 59% positive (19/32) in poorly differentiated HCC. The positive rate in poorly and moderate differentiated HCC is signify higher than highly differentiated HCC, which may imply P53 could be a disease progression marker for prognosis prediction. This role is similar to HSP70 as discussed previously.

Tumor progression is a multi-stage and multi-step process, which involves many genes simultaneously. It has been reported in breast cancer or oral cancer patients with P53 antibody positive in the serum, HSP70-P53 conjugates were found in the tumor tissue, which could be associated with oncogenesis process. In our data of advanced stage HCC patients, 41 cases (51%) showed positive staining for both HSP70 and P53, and these two proteins are positively correlated with each other after further analysis. This may suggest HSP70 and P53 are interacting with each other and affecting tumor cell proliferation and apoptosis, but more detailed study need to be done to further elucidate their roles. Cui et al. proposed the interaction of HSP70 and P53 could mediate the degradation of mutant P53, which induce the immune response to P53. In HCC tissues, HSP70-P53 complex could compromise the normal P53 function, and promote tumor progression [9].

Bmi family members are crucial to embryo development, stem cell maintenance and tumor progression, among which Bmi-1 is one of the regulators. There're studies showing Bmi-1 could regulate tumor cell proliferation, which is an important role in tumorgenesis. In our studies on Bmi-1, we found its expression level is higher in early and advanced stage HCC, in comparison to hepatitis and cirrhosis [10, 11]. This high level of Bmi-1 in HCC tissue, suggests its correlation with tumorgenesis. It has also been shown that the upregulation of Bmi-1 in HCC tissue is relevant to the malignant process of hepatic stem cells [12]. There are also report showing Bmi-1 is associated with pathological grading of HCC and overall survival, and it could be a risk factor in HCC [13]. In our studies, we found Bmi-1 is not correlated with patient gender, age, tumor size, thrombosis status, or tumor differentiation grade in advanced stage HCC patients, but Bmi-1 is associated with tumor TNM stage. In addition, our experiment also demonstrated Bmi-1 is positively correlated with HSP70 and P53 expression. There're 41 cases (51%) showed positive for all three markers, indicating they might have a synergistic role in tumor progression.

We also used Kaplan-Meier method to analyze the clinical data of 80 cases advanced stage HCC patients. The analysis shows HSP70, P53 and Bmi-1 positive patients would have worse prognosis compared to patients with negative expression, but this difference has not reached statistically significance. This could probably due to the small sample size of the study. HSP70, P53 and Bmi-1 expression levels could be risk factors to evaluate HCC progression.

In summary, HSP70, P53 and Bmi-1 are expressed at higher levels in HCC, suggesting their roles in HCC progression. HCC progression involves modulation of many genes, and investigations focus on HCC specific biomarkers has been the hot spot in this field. Our study would provide evidence for HCC early diagnosis, and improve patient prognosis, as well as provide insight to HCC target therapy.

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Disclosure of conflict of interest

None.

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