Serum heat shock protein 27 levels in patients with hepatocellular carcinoma

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Abstract Levels of serum heat shock protein 27 (sHsp27) have been studied in numerous cancer types, but their potential relevance in patients with hepatocellular carcinoma (HCC) is undetermined. Our aim was to compare sHsp27 levels in patients with HCC and HCC-free controls. Specifically, we recruited 71 patients with HCC (80 % with early tumour), 80 patients with chronic liver disease (59 with liver cirrhosis and 21 with chronic active hepatitis) and 42 healthy subjects. sHsp27 was measured by immuno-enzymatic assay. Results showed that sHsp27 levels were significantly ($p < 0.001$) higher in patients with HCC than in the other groups, particularly in those with hepatitis C virus (HCV)-related disease. In HCC patients, sHsp27 levels were not associated with prognostic risk factors, such as size/multiplicity of nodules and stage. In logistic regression analysis, performed in patients with liver disease, log-sHsp27 was associated with a significant age-adjusted 2.5-fold increased odds ratio of HCC and with a significant 4.4-

fold higher odds ratio of HCC in the subgroup with HCV-related liver disease. In receiver operating characteristic curve analysis, sensitivity and specificity of the best sHsp27 cut-off value (456.5 pg/ml) for differentiating patients with HCC from those with HCC-free chronic liver disease were 70 and 73 %, respectively. In conclusion, sHsp27 levels are enhanced in patients with HCC and may represent a candidate biomarker of HCC.

Keywords Heat shock protein 27 · Hepatocellular carcinoma · Liver cirrhosis · Hepatitis C virus

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related death (El-Serag and Rudolph 2007). In addition, HCC incidence has almost doubled in the past 20 years (Howe et al. 2001; El-Serag 2004 and 2007) and patient survival has improved minimally (El-Serag et al. 2001). There is, thus, an urgent need to identify new targets and candidate biomarkers for HCC.

Heat shock proteins (HSP) are abnormally expressed in a variety of cancer types (Ciocca and Calderwood 2005); they have been implicated in carcinogenesis (Calderwood et al. 2006) and are emerging as potential targets of therapy. Heat shock protein 27 (Hsp27) is a member of the small heat shock protein family and has important cytoprotective and anti-apoptotic activity (Welsh and Gaestel 1998). Hsp27 expression is enhanced in HCC in both experimental models and humans (Mairesse et al. 1990; Delhay et al. 1992; King

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et al. 2000; Lim et al. 2005). In addition, proteomic analysis of tissue specimens from patients with HCC has revealed that Hsp27 is one of the three most highly upregulated proteins in the tumour compared to the adjacent non-tumour tissue (Luk et al. 2006).

Hsp27 is also released into the circulation (De and Roach 2004; Gruden et al. 2008), but there are no data on serum Hsp27 (sHsp27) in patients with HCC. In this cross-sectional study, we investigated if sHsp27 levels are altered in subjects with newly diagnosed HCC with respect to HCC-free controls, either healthy or with chronic liver disease.

Material and methods

Subjects

The study was approved by the local Institution Review Board and written informed consent was obtained from each subject. Patients with newly diagnosed HCC ($n=71$) on underlying alcohol/HCV (HCV)/HBV-related clinically diagnosed liver cirrhosis were consecutively recruited at the Gastrohepatology Unit of the San Giovanni Battista Hospital of Turin. HCC patients were individually matched for age (± 5 years) and sex with 80 patients with chronic liver disease and no evidence of HCC. Specifically, 59 subjects with alcohol/HCV/HBV-related clinically diagnosed liver cirrhosis and 21 subjects with biopsy-proven chronic active viral hepatitis were enrolled. A group of 42 subjects scheduled for minor surgery, such as inguinal/umbilical hernia repair and hemorrhoidectomy, served as HCC- and chronic liver disease-free control group and was selected based on both age and sex group matching. Subjects with other conditions, which may alter sHsp27 (Vidyasagar et al. 2012), such as previous/concomitant other neoplasms, inflammatory and cardiovascular diseases, were excluded.

The diagnosis of HCC was based on the presence of nodules with typical appearance (i.e. hypervascular in the arterial phase with rapid wash out in the portal/venous phase) in one (nodule >2 cm) or two (1–2 cm) concordant dynamic imaging techniques (CT scan, contrast ultrasound, MRI with contrast) (Bruix and Sherman 2005). If findings were not characteristic or the vascular profile was not coincidental among techniques, the lesion was biopsied. Tumour staging was established using the American Liver Tumour Study Group modified TNM staging classification (American Liver Tumor Study Group 1998). The absence of HCC in patients recruited in the HCC-free chronic liver disease groups was confirmed by ultrasonography at recruitment and 6 months thereafter. Early tumour was defined as a single nodule ≤ 5 cm or ≤ 3 nodules each ≤ 3 cm. Aetiology of liver cirrhosis was confirmed by seropositivity for hepatitis

B surface antigen and/or serum antibody against hepatitis C virus (HCV), HCV RNA and/or positive history of daily alcohol intake of more than 40 g ethanol per day for more than 15 years.

sHsp27, α -fetoprotein and des-gamma-carboxy prothrombin measurements

Blood samples were taken from all subjects and serum aliquots stored at -20 °C until testing. α -Fetoprotein (α FP) was tested by immunometric assays using enhanced chemiluminescence (ARCHITECT, Abbott). Des-gamma-carboxy prothrombin (DCP) was measured by an enzyme-linked immunosorbent assay kit (Asserachrom PIVKA-II, Stago, France) using murine monoclonal anti-PIVKA-II antibodies. sHsp27 levels were measured by ELISA (Calbiochem Hsp27 ELISA kit, EMD Millipore Bioscience, USA) as we have previously described (Gruden et al. 2008). Briefly, 96-well plates were pre-coated with a mouse anti-human Hsp27, which is used as a capture antibody. Then, both Hsp27 standards and samples, together with a rabbit polyclonal detection antibody specific for human Hsp27, were simultaneously incubated in the pre-coated wells. After washing, a goat anti-rabbit IgG conjugated to horseradish peroxidase was added. Reaction was revealed by 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate and stopped with sulphuric acid. The absorbance was read at 450 nm and Hsp27 levels determined by comparing the absorbance of samples with the values obtained from the standard curve. Samples were coded and tested blindly in duplicate. The range of assay was 20–1,000 pg/ml and intra- and inter-assay coefficients of variation were 4.4 and 8.5 %, respectively.

Statistical analysis

Variables distributed normally were presented as mean and standard deviation (SD), whereas variables with skewed distribution (α FP, sHsp27) were analyzed after logarithmic transformation and results presented as median (25th–75th percentile). ANOVA and Student's *t* test were used for comparisons between groups, as appropriate. Pearson correlation was employed to assess linear correlations between log-sHsp27 and continuous variables and multiple linear regression analysis to assess their independent association with log-sHsp27. Logistic regression analysis was performed to establish if sHsp27 values, entered into models as continuous variable (log-sHsp27), were associated with HCC with respect to HCC-free chronic liver disease, independently of age, α FP and DCP. Receiver operating characteristic (ROC) curves were constructed using all possible cut-off values for α FP, DCP and sHsp27 in the diagnosis of HCC. The best cut-off values were the points on the ROC curve farthest from the diagonal lines, having the maximum

of the Youden index $J = \max[SE_i + SP_i - 1]$, where SE_i and SP_i are the sensitivity and specificity over all possible threshold values. The area under the ROC (AUROC) curves was calculated and compared. A 2-tailed p value of <0.05 was used to determine statistical significance. Analyses were performed using STATA/SPSS/MedCalc softwares.

Results

Patients' clinical characteristics

Table 1 summarised the characteristics of the patients included in the study. Patients and healthy control subjects [age 65.2 (7.3) years, male 69.0 %] had similar mean age and proportions of male. Among patients, hepatitis C was the most common etiologic factor. Most of the cirrhotic patients, with and without HCC, were in Child-Pugh class A. The majority of the HCC patients (80 %) had an early tumour. Indeed, 51 patients had a single nodule (≤ 2 cm, 12 patients; 2.1–5 cm, 38 patients, >5 cm, 1 patient), 15 patients had ≤ 3 nodules

(in seven of them, each nodule was ≤ 3 cm in size, but in one of them, there was vascular invasion) and 4 patients had more than three nodules. One patient had infiltrating HCC. As expected, both α FP and DCP levels were significantly higher in patients with HCC.

Levels of sHsp27

sHsp27 levels were measurable in all subjects with a right-skewed distribution of values. As shown in Fig. 1, sHsp27 values were significantly higher in patients with HCC than in the other groups ($p<0.001$). Patients with liver cirrhosis had also sHsp27 levels greater than those of controls ($p<0.01$), while there were no significant differences between healthy control subjects and patients with chronic active hepatitis.

As shown in Table 2, in patients with HCC, sHsp27 levels did not differ by sex, Child-Pugh class, tumour stage and size/number of nodules. However, patients with HCC on HCV-related cirrhosis had sHsp27 values significantly higher compared to those with HCC related to other aetiologies ($p=0.015$) and to those with HCV-related HCC-free chronic liver disease ($p<0.001$).

Table 1 Characteristics of the patients enrolled in the study

	Chronic liver diseases ($n=151$)		
	Hepatocellular carcinoma ($n=71$)	Cirrhosis ($n=59$)	Chronic active hepatitis ($n=21$)
Age (years)	67.2 $\pm$ 9.6	66.9 $\pm$ 8.9	62.0 $\pm$ 7.0
Males (%)	50 (70.4 %)	41 (69.5 %)	15 (71.4 %)
Aetiology (%)			
HCV	46 (64.8 %)	38 (64.4 %)	14 (66.7 %)
HBV	8 (11.3 %)	7 (11.9 %)	4 (19.0 %)
Alcohol	8 (11.3 %)	7 (11.9 %)	—
Combination	9 (12.7 %)	7 (11.9 %)	3 (14.3 %)
Child-Pugh (%)			
Class A	48 (67.6 %)	42 (71.2 %)	
Class B	21 (29.6 %)	15 (25.4 %)	
Class C	2 (2.8 %)	2 (3.4 %)	
Tumour stage ^a			
I	9 (12.6 %)		
II	46 (64.7 %)		
III	8 (11.3 %)		
IV	8 (11.3 %)		
No. of nodules			
1	51 (72.9 %)		
>1	19 (27.1 %)		
Nodule size			
≤ 2 cm	12		
>2 cm	59 ^b		
α FP (ng/ml)	34.2 (8.4–120.7)*	9.3 (4.0–22)	6.6 (4.0–9.2)
DCP (ng/ml)	5.7 (2.2–18.0)*	2.0 (2.0–2.6)	2.0 (2.0–2.0)

Data are expressed as mean $\pm$ SD, percentage or median (25th, 75th percentile)

α FP, fetoprotein, DCP des-gamma-carboxy prothrombin

* $p<0.0001$ (hepatocellular carcinoma vs cirrhosis and chronic active hepatitis)

^aTumour was staged accordingly to the American Liver Tumour Study Group modified TNM staging classification

^bOne patient had infiltrating HCC

Fig. 1 Box plot for serum Hsp27 values in the studied groups. The *box* indicates the 25th and 75th percentile of the data, and the *middle line*, the median. A line extends from the minimum to the maximum value, excluding outliers that are displayed as separate points. *CAH*, chronic active hepatitis; *CIR*, liver cirrhosis; *HCC*, hepatocellular carcinoma. $p < 0.001$, HCC vs others; $p < 0.01$, CIR vs controls

Univariate, multivariate and logistic analyses

In univariate analysis, patient sHsp27 levels were linearly correlated with age ($r=0.18$, $p=0.03$) and both serum α FP ($r=0.26$, $p=0.001$) and DCP ($r=0.27$, $p=0.0009$).

Table 2 Serum Hsp27 values by categorical variables in the 151 patients with chronic liver disease

	Hepatocellular carcinoma ($n=71$)		Cirrhosis or chronic active hepatitis ($n=80$)	
	Hsp27 (pg/ml)	p value	Hsp27 (pg/ml)	p value
Sex				
Women	861.3 (513.0–1368.0)	0.66	270.2 (164.3–391.8)	0.11
Men	764.7 (338.0–1375.0)		390.9 (236.2–562.0)	
Aetiology				
HCV	870.5 (477.0–1375.0)	0.015	315.3 (227.1–456.0)	0.34
HBV	316.5 (202.5–473.0)		361.6 (158.5–565.2)	
Alcohol	528.3 (306.5–824.0)		592.9 (385.6–915.5)	
Child-Pugh				
A	798.4 (397.0–1404.5)	0.98	348.7 (217.1–558.3)	0.43
B	793.5 (334.0–1137.0)		437.5 (265.4–594.6)	
Tumour stage				
I	602.7 (334.0–940.0)	0.38		
II	913.7 (478.0–1385.0)			
III	499.6 (267.0–1031.0)			
IV	751.2 (491.0–1490.0)			
Nodule number				
1	833.5 (405.0–1385.0)	0.42		
>1	665.5 (269.0–1164.0)			
Nodule size				
≤ 2 cm	768.9 (362.0–976.5)	0.94		
>2 cm	787.3 (405.0–1375.0)			

Data are median (25th, 75th percentile)

In multivariate analysis, sHsp27 values were linearly associated with both α FP and DCP values, independently of age. However, after further inclusion into the model of a dichotomous variable defining HCC (HCC = 1, HCC-free chronic liver disease = 0), the contribution of both α FP and DCP was no longer significant. In this model, HCC remained the only variable independently associated with sHsp27 levels ($\beta=0.36$, $p=0.0001$).

In logistic regression analyses performed in all patients with chronic liver disease with and without HCC ($n=151$), we found that each increment in log-Hsp27 was associated with 2.53-fold higher likelihood [95 % (CI) 1.65–3.91] of having HCC with respect to other chronic liver diseases. The strength of the association was not modified by adjustment for age [odds ratio (OR) 2.52 (95 % CI 1.63–3.90)] and only slightly reduced by further adjustment for α FP and DCP [OR 1.74 (95 % CI 1.12–2.72)]. In the subgroup with HCV-related disease ($n=98$), ORs for each unit of increment of log-Hsp27 were even greater [unadjusted: OR 4.39 (95 % CI 2.14–8.99); adjusted for age: OR 4.31 (95 % CI 2.09–8.87); adjusted for age, α FP and DCP: OR 2.60 (95 % CI 1.18–5.75)].

AUROC of sHsp27 in differentiating HCC from HCC-free chronic liver diseases

ROC curves were plotted to identify a cut-off value that would best distinguish HCC from HCC-free chronic liver

disease (Fig. 2). Table 3 shows that the optimal cut-off value for sHsp27 was 456.5 pg/ml. This yielded a sensitivity of 70 % and specificity of 73 %, with a highly statistically significant ($p=0.0001$) AUROC curve. Comparison of AUROC curves indicated a similar sensitivity and specificity of sHsp27, α FP and DCP for differentiating HCC from HCC-free chronic liver disease, but, in the subgroup of

Fig. 2 ROC curves comparing Hsp27 (blue), des-gamma-carboxy prothrombin (DCP, green) and α -fetoprotein (α FP, beige) in discriminating patients with and without hepatocarcinoma (HCC). Analysis was performed in all patients with chronic liver disease with and without HCC ($n=151$) (a) and in the subgroup of patients ($n=60$) with compensated HCV-related liver disease (b). Panel b: $p<0.05$, AUROC curve for Hsp27 vs AUROC curve for α FP

Table 3 Comparison of Hsp27, α FP and DCP performance in differentiating patients with hepatocellular carcinoma (HCC) from those with HCC-free chronic liver diseases by ROC curve analysis

	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUROC
All cohort ($n=151$)						
Hsp27	456.5	70 (58–81)	73 (61–82)	69	73	0.749
α FP	10.1	70 (58–81)	68 (56–78)	66	72	0.738
DCP	2.7	69 (57–80)	85 (75–92)	80	76	0.800
Child A and HCV-related chronic liver disease ($n=60$)						
Hsp27	646.5	65 (45–81)	93 (81–99)	87	78	0.836*
α FP	17.8	55 (36–73)	72 (56–85)	59	69	0.683
DCP	4.5	61 (42–78)	95 (84–99)	91	77	0.830

Data are percentage (95 % CI)

ROC receiver operating characteristic, α FP α -fetoprotein, DCP des-gamma-carboxy prothrombin, PPV positive predictive value, NPV negative predictive value, AUROC area under the ROC curve

* $p<0.05$, sHsp27 vs α FP

patients ($n=60$) with compensated (Child-Pugh A) HCV-related liver disease, sHsp27 performed significantly better than α FP.

Discussion

This cross-sectional study provides evidence that sHsp27 is a candidate biomarker for HCC, a leading cause of cancer-related death worldwide.

Circulating sHsp27 levels were significantly higher in patients with HCC than in healthy control subjects. Previous studies have reported elevated sHsp27 levels in patients with breast cancer (Fanelli et al. 1998; Banerjee et al. 2011), non-small cell lung cancer (Zimmermann et al. 2012) and both pancreatic and gastric adenocarcinoma (Melle et al. 2007; Huang et al. 2010); however, this is the first report of a rise in circulating sHsp27 levels in patients with HCC.

This increase in circulating sHsp27 is likely due to enhanced Hsp27 both expressed and released by tumour cells. Indeed, previous studies in both experimental and human HCC have shown Hsp27 overexpression in tissue from HCC (Mairesse et al. 1990; Delhay et al. 1992; King et al. 2000; Lim et al. 2005). In addition, it has been recently demonstrated that human breast tumour cells, expressing elevated levels of intracellular Hsp27, can secrete high levels of Hsp27 in the surrounding microenvironment (Banerjee et al. 2011). In our study, tumour Hsp27 expression was not assessed because liver biopsies are rarely performed for diagnostic purposes in patients with HCC and their use in research is restricted by ethical reasons. Therefore, the relationship between Hsp27 expression

within the tumour and serum levels of Hsp27 remains to be explored.

In HCC patients, sHsp27 levels were not correlated to prognostic risk factors, such as nodule size/multiplicity and HCC stage. However, most of our patients had early HCC; therefore, we cannot exclude the possibility that the low number of patients with advance HCC might have reduced our power to assess statistical significant associations. On the other hand, the rise of sHsp27 in early HCC emphasises the potential clinical relevance of our finding and also raises the possibility of a role of sHsp27 in carcinogenesis. In keeping with this, a previous study has shown positive immunoreactivity for Hsp27 in 10.5 % of dysplastic nodules and 100 % of early HCC and no correlation between Hsp27 expression and prognostic factors, such as tumour grading, nodule size and stage (Lim et al. 2005). At variance, King et al. reported that Hsp27 expression correlates with both HCC histological grade and patient survival (King et al. 2000).

To control for the potential confounding effect of underlying chronic liver disease, we have included in our study control groups of patients with chronic liver disease, either chronic active hepatitis or liver cirrhosis, which is the most common underlying liver disease in patients with HCC. Comparisons showed that sHsp27 values were significantly greater in patients with HCC than in those with either liver cirrhosis or chronic active hepatitis. In logistic regression analyses, each increment in sHsp27 was associated with a 2.5-fold higher likelihood of having HCC with respect to other chronic liver diseases, independently of age. These findings are of clinical relevance as they suggest that sHsp27 may help in identifying patients with HCC among subjects with chronic liver disease.

To further assess the potential relevance of sHsp27 as a candidate circulating biomarker of HCC, we have also performed ROC curve analysis. Using the best cut-off value of 456.5 pg/ml, sensitivity and specificity of sHsp27 for the presence of HCC were 70 and 73 %, respectively, confirming the ability of sHsp27 to effectively discriminate between patients with HCC and those with HCC-free chronic liver disease. However, the performance of sHsp27 was comparable to that of α FP and DCP, which are no longer recommended for HCC screening (Bruix and Sherman 2005); therefore, further studies are required to explore the potential clinical application of our findings and to assess prospectively if sHsp27 levels can detect preclinical HCC in cirrhotic patients.

Among patients with HCC, sHsp27 levels were particularly elevated in subjects with underlying HCV-related cirrhosis, and in logistic analysis, the independent association between sHsp27 and the risk of HCC was much stronger in the subgroup of patients with HCV-related chronic liver disease. The underlying mechanism is unknown; however, Hsp27 interacts at intracellular level with NS5A, a non-structural HCV protein important in both the control of virus replication and

prevention of apoptosis (Choi et al. 2004), suggesting the possibility that Hsp27 may play a role in tumorigenesis specifically in patients with HCV-related cirrhosis.

Of interest, in the subgroup of patients with compensated HCV-related chronic liver disease, ROC curve analysis showed that sHsp27 performed significantly better than α FP. This may be important as patients with compensated cirrhosis are the ideal target population for HCC surveillance and HCV is the most common cause of cirrhosis-related HCC in the Western countries. However, subgroup analysis should be taken with caution as results may also be due to chance.

There is increasing evidence that HSP released by cells are biologically active (van Eden et al. 2005), and a recent study has shown that Hsp27, present in breast tumour cell culture supernatant, can induce T cell tolerance (Banerjee et al. 2011). This raises the hypothesis that Hsp27, released by HCC and detected in serum, may also contribute to tumour growth and invasiveness besides being a marker of the disease, but further studies are required to explore this attractive possibility.

In conclusion, our study demonstrates that sHsp27 levels are enhanced in patients with HCC, particularly in those with HCV-related underlying liver cirrhosis. Future prospective studies examining large cohorts of patients with chronic liver diseases are needed to confirm the clinical relevance of sHsp27 in patients with HCC.

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Conflict of interest The authors have no conflict of interest to declare.

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