RESEARCH ARTICLE

Serum Amyloid A is a Novel Prognostic Biomarker in Hepatocellular Carcinoma

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Abstract

Purpose: To investigate the prognostic value of serum amyloid A (SAA) in patients with hepatocellular carcinoma (HCC) undergoing surgery. Materials and Methods: Preoperative serum samples of 328 patients with HCC who underwent curative resection and of 47 patients with benign liver lesion were assayed. Serum levels of SAA were measured by enzyme-linked immunosorbent assay and its correlations with clinicopathological characteristics and survival were explored. Results: Levels of SAA were significantly higher in patients with HCC than those with benign liver lesion. There were strong correlations between preoperative serum SAA level and tumor size and more advanced BCLC stage. On univariate analysis, elevated SAA was associated with reduced disease-free survival and overall survival (*p*=0.001 and 0.03, respectively). Multivariate analyses showed that serum SAA level was an independent prognostic factor for overall survival (hazard ratio 2.80, *p*=0.01). Conclusions: High SAA serum level is a novel biomarker for the prognosis of HCC patients.

Keywords: Serum amyloid A - hepatocellular carcinoma - prognosis

Introduction

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer but the third leading cause of cancer-related death worldwide (Parkin et al., 2001). Although Hepatectomy and transplantation are effective methods for treatment, the prognosis of patients with HCC remains poor, mainly owing to a high incidence of tumor metastasis and recurrence (Ercolani et al., 2003; Forner et al., 2012). Early intervention with treatment is critical to obtain survival benefit. Therefore reliable markers to identify patients who are at high-risk for early death and recurrence would be necessary.

It is well established that cancer arises in chronically inflamed tissue. The fact that the majority of HCC patients have an underlying chronic inflammatory liver disease and liver cirrhosis is the main risk factor for HCC development (Gao et al., 2012). The chronic infection sustained by hepatitis viruses, as well as the production of cytokines and growth factors within the parenchyma and the surrounding extracellular matrix (ECM), lead to an complicated microenvironment. The acute phase response is a prominent nonspecific reaction of the organism to local or systemic disturbances caused by inflammation, tissue damage, immunological disorders or the presence of tumors. The link between acute-phase protein, particularly C-reactive protein (CRP) and increased risk of several human cancers has been clearly illustrated in many epidemiological studies (Toriola et al., 2013). Serum Amyloid A, produced by the hepatocytes, is another major positive acute-phase reactant, can act as a chemoattractant for immune cells such as monocytes, polymorphonuclear leukocytes, mast cells, and T lymphocytes to the site of inflammation (Badolato et al., 1994; Xu et al., 1995; Olsson et al., 1999). The induction of SAA is primarily regulated by inflammatory cytokines such as interleukin-1, interleukin-6, tumor necrosis factor α (TNF-α). Thus, SAA plays a key role in a wide range of inflammatory processes and provides a link among the immunity, inflammation and cancer. Furthermore, a number of studies has underscored that SAA is involved in tumorigenesis, apparently due to its capability to interact with ECM, which has a great influence on tumor initiation and development.

Serum SAA levels were increased in patients with lung cancer (Nel et al., 1984; Benson et al., 1986; Khan et al., 2004), prostate cancer (Kaneti et al., 1984), colorectal carcinoma (Glojnaric et al., 2001; Giessen et al., 2014) and renal cell carcinoma (Kimura et al., 2001; Mittal et al., 2012). In addition, a number of previous studies proposed a direct correlation between SAA concentrations and tumor stage that a higher percent of raised SAA levels was found in patients with more advanced disease (Weinstein et al., 1984; Biran et al., 1986; Chen et al., 2007; Liu et al., 2007). And other authors have suggested SAA work as an useful indicator for signs of distant metastases in

patients with RCC and LC (Ramankulov et al., 2008; Sung et al., 2011). Moreover, recent epidemiologic studies even suggest that elevated circulating levels of SAA, not only merely mark the presence of prevalent carcinoma, but also should possibly be related to an increased risk of future cancer in apparently healthy individuals (Sasazuki et al., 2010; Shiels et al., 2013). Most importantly, several researches have confirmed that SAA turned out to be an independent and effective factor in predicting survival of patients with different tumors (Kimura et al., 2001; Chan et al., 2007; Meng et al., 2014).

To our knowledge, few studies have investigated the SAA level in patients with HCC. A recent study (He et al., 2008) used proteomic technologies toward identifying serum biomarkers for HCC compared with normal and HBV. Three most discriminatory protein/peptide peaks were singled out and one of the three peaks was identified to be SAA. More importantly, there has never been any study to assess the relationship between serum SAA level and long-term survival in HCC patients. The aim of this study is to determine the preoperative circulating SAA levels in HCC patients and whether it could be a prognosis measurement to predict disease-free and overall survival in HCC patients undergoing hepatectomy.

Materials and Methods

Patients and sample collection

The study population consisted of 328 patients with newly diagnosed HCC who underwent curative hepatectomy as an initial treatment in our hospital and 47 patients with benign liver lesion (focal nodular hyperplasia, cyst and hemangioma) between November 2010 and December 2011. Benign patients had normal liver function in this cohort relative to normal, age matched donors. The diagnosis of HCC was confirmed on the basis of CT, MRI and histopathology. Exclusion criteria were the patients with clinical evidence of infection or other chronic inflammatory conditions or who received prior intervention or died immediately after surgery (within 3 months). The study was approved by the Ethics Committee of the Zhongshan Hospital affiliated to Fudan University, and each patient provided informed consent to participate in the study. All blood samples were taken from every patient before operation. After centrifugation, sera were stored at -80°C until further use. Standard parameters of liver function and Alpha-Fetoprotein (AFP), C-reactive protein (CRP) levels were measured in the central laboratory of our hospital.

Follow-up

After the initial treatment phase, patients were carefully followed. Serum AFP levels, ultrasound and CT or MRI examinations were performed every 2-3 months after surgery to detect any intrahepatic recurrence or distant metastasis. Patients with confirmed recurrence received various treatment modalities. Overall survival (OS) was defined as the interval between surgery and time of either death or last follow-up. Disease free survival (DFS) was defined as the interval between surgery and time of recurrence. The last follow-up date for patients

still alive was July 2013. The median follow-up time was 23 months (range, 3-32 months)

Enzyme-linked immunosorbent assay

Sandwich ELISA kit (Human SAA immunoassay kit, Invitrogen, CA) was used to measure SAA levels in serum. The ELISA assay was performed according to the manufacturer's protocol and guideline.
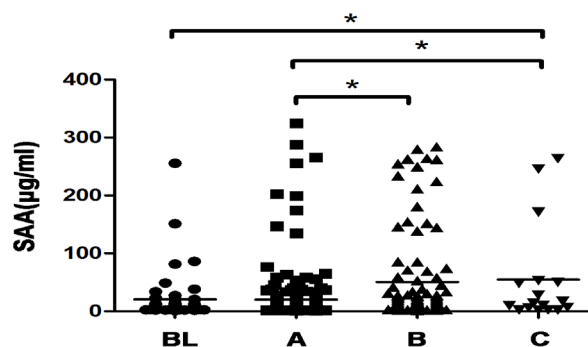

Figure 1. Serum Levels were Determined by ELISA in four Relevant Groups of Samples: Benign Liver Lesion(BL, n = 47), BCLC A (A, n = 126), BCLC B (B, n = 38), BCLC C (C, n =6). Bars indicate medians. Values represent means SD. P values represent the results of Mann-Whitney U tests. *$p<0.05$

Table 1. Correlation of Serum CRP and SAA with Clinicopathologic Characteristics in HCC

Categories	N	SAA		P value
	328	<7.5μg/ml	≥7.5μg/ml	
Age(yr)	≤56	83	69	0.38
	>56	87	89	
Gender	Male	27	24	0.88
	Female	143	134	
Liver cirrhosis	Absent	34	35	0.69
	Present	136	123	
HBsAg	Negative	30	34	0.41
	Positive	140	124	
ALT	<50	132	117	0.52
	≥50	38	41	
Total serum bilirubin	≤20	147	144	0.22
	>20	23	14	
Albumin	<40	54	45	0.55
	≥40	116	113	
AFP	≤20	77	74	0.83
	>20	93	84	
BCLC	A	126	87	0.001
	B	38	60	
	C	6	11	
Tumor size	<5cm	134	92	<0.001
	≥5cm	36	66	
Tumor number	Single	151	131	0.15
	Multiple	19	27	
Tumor capsule	Complete	116	91	0.05
	None	54	67	
Vascular invasion	Absent	134	121	0.69
	Present	36	37	
Edmondson grade	I-II	114	119	0.4
	III-IV	52	43	
CRP	<3	155	80	<0.001
	≥3	15	78	

*HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AFP, a-fetoprotein level; CRP: C-reactive protein

Statistic analysis

Statistical analyses were performed by SPSS16.0 statistical software. The results were analyzed using the Mann-Whitney U test for continuous variables, and the Chi-square test for categorical variables, as appropriate.

We determined an optimal cutpoint of SAA for predicting survival using a statistical method called X-tile, Version 3.6.1 (Camp et al., 2004). These cut-off values were used for further categorical analyses (high or low levels). The disease-free survival (DFS) and overall survival (OS) were computed by using the Kaplan-Meier method and were calculated with the log-rank test. Factors that were statistically significant in the univariate analysis were subsequently evaluated using the multivariate Cox proportional hazard model with backward stepwise (likelihood ratio) entry. Two-tailed *P* values<0.05 were considered significant.

Results

Patient characteristics

There were 277 male patients and 51 female patients (mean age, 56 years; range, 29-85 years). A total of 56 patients (17%) developed recurrence, while 33 patients (10%) died during follow-up. The majority of patients had an associated viral infection, isolated hepatitis B virus (HBV) infection being the most frequent (80%). With regard to tumor differentiation according to Edmonson-Steiner stage, there were 233 (71%) stages I-II and 95 (29%) stages III-IV. The majority of patients were classified as early HCC according to the BCLC staging algorithm (BCLC A, 65%), with compensated liver function (Child-Turcotte-Pugh Class A, 100%).

Expression levels of SAA and the relationship with clinical characteristics of patients with HCC

The serum expression levels of SAA were significantly higher in HCC patients than those in liver benign lesions patients (Figure 1). With the X-tile for SAA, a cutoff point of 7.5 µg/ml was used to separate the patients into two groups. The association between serum SAA level and the clinicopathologic characteristics is showed in Table 1. There was no significant correlation between serum SAA concentration and patients' gender (*p*=0.76), age (*p*=0.38), hepatitis B surface antigen status (*p*=0.58), and underlying cirrhosis (*p*=0.89; Table 1). We also found that

Table 2. Results of the Univariate and Multivariate Analyses of the Overall and Disease-free Survival

Variables		Overall Survival			Disease-Free Survival		
		Univariate Analysis *P* value	Multivariate Analysis HR(95%CU)	*P* value	Univariate Analysis *P* value	Multivariate Analysis HR(95%CI)	*P* value
Age(yr)	<56	0.58		0.87			
	≥56						
Gender	Male	0.94		0.76			
	Female						
HBsAg	Negative	0.43		0.4			
	Positive						
Liver cirrhosis	Absent	1		0.69			
	Present						
ALT	<50	0.62		0.7			
	≥50						
Total serum bilirubin	≤20	0.64		0.86			
	>20						
Albumin	<40	0.38		0.89			
	≥40						
AFP	≤20	0.008	2.586(1.165-5.739)	0.02	0.03		
	>20						
BCLC	A	<0.001	4.072(2.486-6.668)	<0.001	<0.001	3.123(2.138-4.561)	<0.001
	B						
	C						
Tumor size	<5cm	<0.001	2.445(1.156-5.172)	0.019	<0.001	2.078(1.21-3.568)	0.008
	≥5cm						
Tumour number	Single	0.08		0.02			
	Multiple						
Tumour capsule	Complete	0.003		0.02			
	None						
Vascular invasion	Absent	<0.001	4.324(2.123-8.807)	<0.001	<0.001	2.711(1.566-4.692)	<0.001
	Present						
Edmondson grade	I-II	0.045		0.37			
	III-IV						
CRP	<3	<0.001		0.006			
	≥3						
SAA	<7.5	0.001	2.801(1.233-6.364)	0.014	0.03		
	≥7.5						

*SAA, Serum amyloid A; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AFP, a-fetoprotein level; CRP, C-reactive protein; HR, Hazard ratio; CI, confidence interval
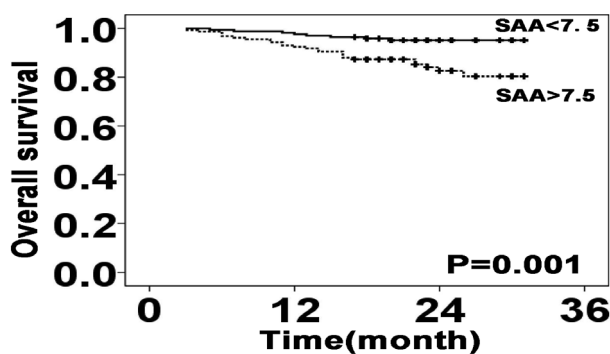
Figure 2. Kaplan-Meier Survival Curves for Patients with High or low SAA. There was a significant difference in OS between patients with high and low SAA
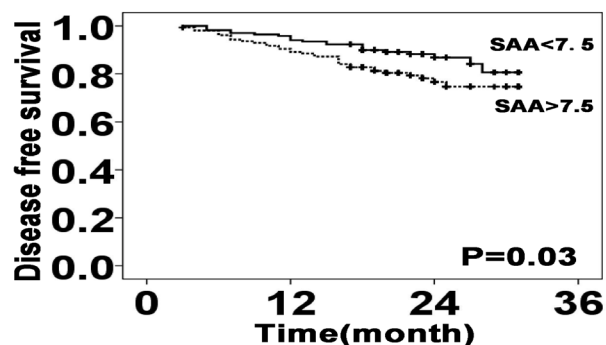
Figure 3. Kaplan-Meier Survival Curves for Patients with High or Low SAA. There was a significant difference in DFS between patients with high and low SAA

the expression of SAA had no relation with the majority of tumor characteristics assessed such as tumor number, capsule and vascular invasion. In contrast, the patients with large tumor size and more advanced BCLC stage had a significantly higher serum SAA level. However, serum SAA levels in patients with BCLC stage A disease were not significantly different from those of benign controls, and the same result was revealed between the small size tumor (< 5cm) and benign control. Furthermore serum concentrations of SAA were found to correlate well with CRP.

The higher serum SAA status is associated with poor prognosis of HCC patients

The cumulative DFS and OS according to the serum SAA status are shown in Figure 2 and Figure 3. The cumulative DFS and OS rate of the patients in the low SAA group were significantly higher than that of the patients in the high SAA group. Table 2 shows the results of the univariate and multivariate analyses of the prognostic factors for the DFS and OS. Univariate analysis indicated that patients with higher SAA had significantly worse overall survival, with a hazard ratio of 3.3 (95% confidence interval [CI], 1.6-6.5, *p*=0.001). Other univariate predictors of OS included AFP, tumor size, capsule, vascular invasion, Edmonson grade, and BCLC stage. Multivariate survival analysis conducted by the Cox regression model showed that SAA remained as a significant unfavorable factors influencing the OS of HCC patients. As to DFS, univariate analysis showed that SAA, CRP, AFP, tumor size, number, capsule, vascular invasion, and BCLC stage were all worse predictors of

DFS for HCC patients. Tumor size, vascular invasion and BCLC stage, but not SAA were confirmed as independent prognostic markers of DFS.

Discussion

To our knowledge, this is the first study to date focusing on the relationship of serum SAA level with HCC patients and to evaluate SAA as a prognostic marker for HCC after hepatectomy. We observed significant associations between elevated concentrations of the inflammatory biomarkers SAA and reduced overall survival as well as reduced disease-free survival.

Elevated levels of SAA reflects a generalized host reaction that is either localized or systematic with regard to the initial stimulus. In chronic inflammation, however, which plays a critical role in tumor development, SAA levels increase substantially and indeed also increase in a number of neoplastic diseases. It has been observed in previous studies that the SAA level increases in patients with stomach, lung, renal, colorectal, breast and other forms of cancers (Biran et al., 1986; Glojnaric et al., 2001; Kimura et al., 2001; O'Hanlon et al., 2002; Cho et al., 2004; Chan et al., 2007; Farooqui et al., 2012). With regard to HCC, only one research confirmed the relation of circulating SAA with HCC, and verified a gradual increase of SAA levels in serum from normal to HBV and then to HCC, but cannot be regarded as a specific indicator for assessing HBV and HCC. In accordance with the above finding, we have demonstrated that patients with HCC have higher SAA concentrations than those patients with benign lesions. In this study, we further correlate the relationship between clinical characteristics and serum SAA level, which shows that SAA serum expression is not associated with any of the clinical characteristics assessed (such as age, histologic grade, tumor number, and vascular invasion), except tumor size and BCLC stage. These results have been proved in other malignant tumors that a concomitant rise in SAA has a positive correlation with the size of the primary tumor and in patients with more advanced stage (O'Hanlon et al., 2002; Sasazuki et al., 2010; Dowling et al., 2012), and further acted as an independent prognostic factor (Mittal et al., 2012). In addition, SAA was identified as a biomarker that discriminates disseminated and localized or regional diseases. Rosenthal (Rosenthal and Sullivan, 1979) found that the mean levels for various groups of tumors, including HCC, were significantly elevated in patients with distant metastases compared with those with localized disease. More importantly, the patients who had an initial significant elevation SAA concentration while no metastatic disease was detected of, were closely monitored for signs of metastatic disease during follow-up. While no patients without metastatic disease and with low SAA concentrations developed metastases during follow-up. These results led to the assumption that SAA might be regarded as a marker to monitor tumor progression and might have prognostic value for several types of malignancies. According to the previous studies, human hepatoblastoma HepG2 cell lines (Steel et al., 1996) and various hepatocellular carcinoma cell lines,

such as Hep3B, HuH-7, and NPLC/PRF/5, constitutively synthesized SAA in steady state and were capable to accumulate increased level of SAA in response to various inflammatory mediators at the RNA and protein levels (Ganapathi et al., 1988; Thorn et al., 2003; Thorn et al., 2004). Moreover, immunohistochemistry analysis showed that the SAA protein expression only accumulates in the hepatocellular carcinoma cell without reinforcement of the staining in inflammatory cells, nor in hepatocytes around (Bioulac-Sage et al., 2007). Combined the above findings with that SAA level was closely associated with the tumor size, not the several parameters of liver function, we put forward a hypothesis that the higher circulating SAA was mostly attributed to the production of hepatocellular carcinoma cell, not the normal hepatocytes, and was probably a long term consequence of the local chronic inflammatory interactions between the tumor cells and surrounding microenviroment not the acute-phase or systemic induction.

CRP, another nonspecific, acute-phase, hepatic proteins, plays a key role in a wide range of inflammatory processes and tumor development. Recent studies have showed that the preoperative elevated serum CRP level is an independent and significant predicative indicator of poor prognosis and early recurrence in patients with HCC (Hashimoto et al., 2005; Sieghart et al., 2013). However, SAA is superior to CRP for detecting inflammation resulting from viral infections, kidney transplantation, Crohn's disease, and ulcerative colitis (Yamada, 1999), and therefore SAA is probably more useful for detecting slight elevations in systemic or chronic inflammation. In addition, our results showed that there was a high correlation between CRP and SAA in HCC, so we had the intention to investigate the prognosis power of SAA in DFS and OS for HCC compared with CRP. The results showed that the DFS and OS in the high SAA group were significantly worse than those in the low SAA group (Fig 2), the same as that of CRP. Although the multivariate analyses demonstrated that both SAA and CRP were not independent prognostic factors for the DFS, while the serum SAA level was an independent prognostic factor for the OS (Table 2). Based on the analysis above we come to make a conclusion that as compared to the currently widely used CRP, SAA is a more powerful prognosis marker in HCC. In a recent study (Chan et al., 2007), the mean SAA concentration increased significantly in patients with tumor recurrence but did not change in patients without recurrence after curative gastric cancer resection during follow-up. This finding will perform as a strong support that serum SAA level is not only a reliable tool in predicting survival of patients with HCC, but also might be a valuable method of detecting recurrence and metastasis for postoperative follow-up in future clinical trials.

The mechanism by which SAA is related to HCC prognosis is unclear. Accumulating evidence supports the role of SAA in human malignancies is that SAA has a major influence on alterations in ECM composition and its degradation. It seems that SAA interacts specifically with ECM-linked glycoprotein moieties, inhibiting the adhesion of tumor cells to the ECM. Furthermore, SAA may modulate adhesion of tumor cells to platelets

and enhance plasminogen activation, both of which are involved in ECM degradation and tissue remodeling processes. Indeed, another important approach to SAA influencing ECM, has been verified by an increasing number of studies, is that it can stimulate the production of MMPs (Stix et al., 2001; Lee et al., 2005). This partially explains the value of SAA level as a monitor of metastatic dissemination in several solid tumor and the relationship with prognosis. Alternatively, a potential role of SAA lies in its chemoattractant properties on immune cells such as monocytes, neutrophils, and T-cells (Bjçrkman et al., 2008), associated with cell survival or apoptosis, angiogenesis, suppression of anti-tumor immune responses. Recently, it has been reported that SAA can mediate the production of reactive oxygen species in primary human neutrophils. All these above indicated SAA, whether it is produced systemically or in the tumor microenvironment, closely links cancer, inflammation and immunity.

Our study has several limitations. First, the whole median follow-up period of 23 months (range, 3-32 months), may not be long enough to evaluate 5-year survival and recurrence. Second, because the patients enrolled in our study were treated surgically, further clinical trials are needed to test our findings in HCC patients undergoing various treatments, to further verify the prognosis efficacy of SAA .

In conclusion, this study demonstrates for the first time that serum SAA expression is up-regulated in the majority of HCC compared with liver benign lesions and associated with tumor progression. Our data also provides the first evidence that SAA is a novel valuable prognostic biomarker of HCC patients. Further investigation is essential to explore the molecular mechanism of SAA involved in the pathogenesis and progression of HCC.

Acknowledgements

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