

Original Article

Detection of high levels of Survivin–immunoglobulin M immune complex in sera from hepatitis C virus infected patients with cirrhosis

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Aim: The identification and surveillance of patients with liver dysfunctions and the discovering of new disease biomarkers are needed in the clinical practice. The aim of this study was to investigate on Survivin–immunoglobulin (Ig)M immune complex (IC) as a potential biomarker of chronic liver diseases.

Methods: Serum levels of Survivin–IgM were measured using an enzyme-linked immunoassay that had been standardized and validated in our laboratory in 262 individuals, including healthy subjects and patients with chronic viral hepatitis, cirrhosis and hepatocellular carcinoma (HCC).

Results: Survivin–IgM IC was lower in healthy subjects (median, 99.39 AU/mL) than in patients with chronic viral hepatitis (median, 148.03 AU/mL; $P = 0.002$) or with cirrhosis (median, 371.00 AU/mL; $P < 0.001$). Among patients with cirrhosis, those with hepatitis C virus (HCV) infection showed the highest level of Survivin–IgM IC (median, 633.71 AU/mL; $P < 0.001$). The receiver–operator curve analysis revealed that

Survivin–IgM accurately distinguishes HCV correlated cirrhosis from chronic viral hepatitis (area under the curve [AUC], 0.738; sensitivity, 74.5%; specificity, 70.7%). A multivariate logistic regression model, including Survivin–IgM IC, aspartate aminotransferase (AST) and AST/alanine aminotransferase (ALT) ratio increased the prediction accuracy for the identification of the cirrhotic HCV patients (AUC, 0.818; sensitivity, 87.2%; specificity, 65.9%). Conversely, Survivin–IgM IC significantly decreased in HCC patients (median, 165.72 AU/mL; $P = 0.022$).

Conclusion: Our results suggest that Survivin–IgM immune complex may be used as a potential biomarker for liver damage, particularly for the identification of the HCV-related cirrhotic population.

Key words: disease biomarkers, hepatitis C, hepatocellular carcinoma, immune complexes, liver cirrhosis, Survivin

INTRODUCTION

LIVER CIRRHOSIS IS the final stage of repeated cycles of inflammation, necrosis and hepatocellular

regeneration that contribute to functional and structural alterations of the liver and is the strongest predisposing factor of hepatocellular carcinoma (HCC), the etiology of which includes mainly hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol abuse and then autoimmune and metabolic disorders.^{1,2} HCC is the sixth most common cancer in the world and the third most frequent oncological cause of death,³ and the prognosis is mostly poor; thus, it seems necessary to concentrate on achieving the earliest possible diagno-

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sis.^{4,5} For this reason, more effective surveillance strategies should be used to screen early occurrence targeted to the population at risk, and particularly to patients with chronic liver disease and cirrhosis.

The diagnosis of cirrhosis is usually based on the presence of a risk factor and is confirmed by physical examination, blood tests, imaging, and when more information is needed, diagnosis is often achieved by histological examination of samples obtained by liver biopsy.⁶⁻⁸ Therefore, from the clinical perspective, the most difficult challenge is the early detection of liver alteration, onset of the chronic disease, followed by the neoplastic transformation of cirrhosis. α -Fetoprotein (AFP), des- γ -carboxyprothrombin and *Lens culinaris* agglutinin-reactive fraction of AFP are the major HCC-associated biomarkers, but, due to their poor accuracy, many researchers, including our group, are focusing on the study of new biomarkers associated with the development of HCC,^{4,9-11} particularly to identify the onset of disease in patients with chronic liver disease.

The use of circulating antigen-immunoglobulin (IgM) immune complexes (IC) as disease biomarkers has been recently investigated for some tumors, particularly in liver cancer, and IC assessment provided a better diagnostic performance than the analysis of the corresponding free biomarker.¹²⁻¹⁶ In fact, it is known that IgM natural antibodies are considered an important component of the innate immunity with the binding capacity of a wide range of tumor antigens.¹⁷ Moreover, it is well established that natural IgM play an important role in the first line of defense against infectious agents, in the regulation of proliferation of immune cells and in the immunosurveillance against tumor cell growth.¹⁸

Survivin is the smallest member of inhibitors of apoptosis proteins and a regulator of cellular division that is primarily expressed in fetal and cancerous tissues, but not in normal developed tissues, modifications of which have been found in different tumors,¹⁹⁻²¹ including HCC, as well as in chronic liver diseases.²²⁻²⁵ Furthermore, at the present time, the critical role of Survivin in hepatocarcinogenesis has been established, and the inhibition of Survivin and other anti-apoptotic proteins are under evaluation for HCC therapy.^{26,27}

Thus, the aim of this study was to investigate the presence of circulating Survivin-IgM IC as potential biomarker of diverse phases of chronic liver diseases, including cirrhosis and HCC, by an appropriate enzyme-linked immunoassay (ELISA) that had been standardized and validated in our laboratory.

METHODS

Patients, healthy subjects and samples collection

THIS STUDY INCLUDED 262 serum samples with epidemiological characteristics listed in Table 1. Serum samples were collected at the Department of Internal Medicine and Liver Unit in Marino, Rome, by the Division of General Surgery and Transplantation of San Camillo Hospital in Rome, the Department of Medicine of the University of Padua and the Policlinic of Tor Vergata in Rome according to institutionally approved procedures. Before sample collection, all study participants gave full written informed consent authorizing their blood use for research purposes. The diagnosis of cirrhosis was obtained by clinical, biochemical, endoscopic and liver ultrasonography criteria. The diagnosis of HCC was defined according to interna-

Table 1 Clinical and epidemiological characteristics of patients and healthy subjects

	Healthy subjects	Chronic viral hepatitis	Cirrhosis	HCC
No. (total $n = 262$)	39	56	105	62
Age (years, mean \pm SD)	47.90 \pm 8.98	53.02 \pm 14.41	59.98 \pm 12.17	66.76 \pm 9.96
Sex (M/F)	18/21	34/22	68/37	48/14
AST (IU/L, median, IQR)	16 (13-20)	38 (27-60)	44 (30-71)	59 (38-99)
ALT (IU/L, median, IQR)	21 (18-32)	25 (19-46)	20 (12-23)	31 (26-37)
AST/ALT (median, IQR)		1.4 (1.0-2.2)	2.3 (1.4-3.9)	1.76 (1.0-3.5)
HCV (n /total, %)		41/56 (73)	47/105 (45)	37/62 (60)
Alcohol (n /total, %)			41/105 (39)	16/62 (26)
HBV (n /total, %)		15/56 (27)	12/105 (11)	7/62 (11)
Other† (n /total, %)			5/105 (5)	2/62 (3)

†Autoimmune and cryptogenic.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IQR, interquartile range.

tional guidelines (American Association for the Study of Liver Diseases/European Association for the Study of the Liver guidelines) and, when appropriate, the final diagnosis was confirmed by histopathological analysis on ultrasound-assisted fine-needle biopsy. Serum samples were prepared from each patient starting from 15 mL of venous blood, centrifuged at 1500 g for 20 min at room temperature (20–25°C) within 2 h of collection and stored at –80°C until use.

Purification of Survivin-IgM calibrator by gel filtration and preparation of standard curve

Pooled cirrhotic sera (100 µL) were analyzed using a gel filtration column BioSep EC S-4000 (Phenomenex, Macclesfield, Cheshire, UK) as previously described.¹² Fractions were collected every 30 s and immunoreactivity for Survivin-IgM was tested by ELISA, as described below. The higher expressing fractions were then collected and arbitrary units for mL (AU/mL) were assigned by mathematical software and serially diluted as reference standard in Survivin-IgM IC assay. Figure 1 shows linearity of the obtained reference standard; the highest point was 250 AU/mL, the lowest 3.91 AU/mL.

Survivin-IgM IC and free Survivin assays

The Survivin-IgM IC levels were determined in each serum sample as follows. Ninety-six-well ELISA plates were coated with 0.5 µg of the polyclonal rabbit antihu-

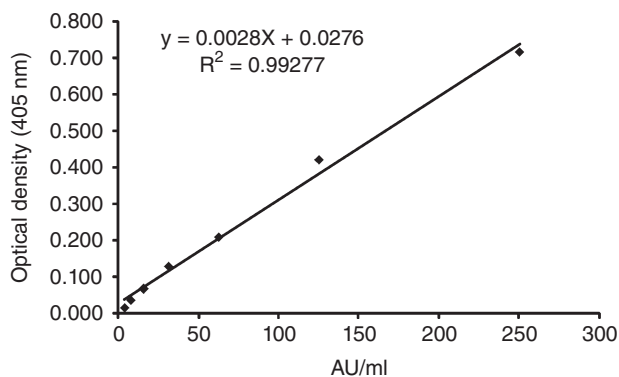


Figure 1 Standard calibration curve for quantification of circulating Survivin-immunoglobulin (Ig)M immune complex (IC). Optical density was measured by microplate spectrophotometer at the wavelength of 405 nm and the amount of Survivin-IgM IC was expressed in arbitrary units (AU/mL) by using a purified calibrator obtained from pooled sera of patients with cirrhosis. The line equation is shown and the R^2 value revealed a good fit of the line to the data.

man Survivin antibody (Novus Biologicals, Littleton, CO, USA) in 100 µL of phosphate-buffered saline (PBS), pH 7.2, per well at 4°C overnight and then blocked for 2 h with 3% bovine serum albumin (BSA) in PBS (all from Sigma, St Louis, MO, USA). After blocking, 100 µL of serially diluted reference standards and sera diluted 1:8 and 1:16 in PBS containing 1% BSA and 0.05% Tween-20 were incubated for 1 h at room temperature. The Survivin-IgM complexes were revealed using a polyclonal goat peroxidase-conjugated antihuman IgM at a concentration of 2 µg/mL and developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and hydrogen peroxide as substrates (all from Sigma). Optical density was measured by a microplate spectrophotometer at the wavelength of 405 nm (Labsystem Multiskan Bichromatic, Helsinki, Finland). The amount of Survivin-IgM IC was expressed in AU/mL by using a purified calibrator obtained from pooled sera of patients with cirrhosis, as described above. Each sample was tested in duplicate. To compare the concentration values of Survivin-IgM IC with free Survivin levels in sera, the Human Survivin Immunoassay (DuoSet; R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instruction.

Validation tests and measurement of free IgM unspecific binding

Validation tests were performed by calculating repeatability (precision intra-assay) and reproducibility (precision interassay) of IgM IC measurement in the calibrator and samples. For samples, the intra-assay and interassay values of coefficient of variation were 7.15% and 6.03%, respectively; and for standard, the intra-assay and inter-assay values of coefficient of variation were 2.36% and 12.25%, respectively. To exclude specific reactivity due to free IgM, samples were tested in presence of unspecific human IgM. Ninety-six-well ELISA plates were coated with 0.5 µg of polyclonal rabbit antihuman Survivin antibody as described above. After blocking, 100 µL of serially diluted pools of sera, with and without IgM (Sigma) at a concentration of 150 µg/mL, were incubated for 1 h at room temperature. Then, Survivin-IgM IC were revealed as described above.

Statistical analysis

Differences between two independent groups were tested with the non-parametric Mann-Whitney *U*-test; for multiple comparison, the non-parametric Kruskal-Wallis test was used. The receiver-operator curves (ROC) and the respective areas under the curve (AUC)

with 95% confidence interval (CI) were used to assess the sensitivity and specificity of a single test or combined tests for the identification of cirrhosis. Multinomial logistic regression was used to identify interactivity between the markers. We compared the results acquired from the logistic regression with those from parallel testing, evaluated the possibility of improving the diagnostic specificity and sensitivity at the same time, and explored the optimized combination of markers to increase diagnostic accuracy for cirrhosis. Statistical analyses were done using the statistical software package R and SPSS statistical software version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Clinical and epidemiological characteristics of patients and healthy subjects

SURVIVIN-IMMUNOGLOBULIN M IC levels were evaluated by specific ELISA in sera from 262 subjects divided into four groups: healthy subjects ($n = 39$), drug-naïve patients with chronic viral hepatitis with no sign of cirrhosis ($n = 56$), patients with cirrhosis ($n = 105$) and HCC ($n = 62$) (Table 1). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) determination were performed and median comparison analysis by Kruskal–Wallis test showed significant AST, ALT and AST/ALT ratio modification among groups ($P < 0.001$). HCV infection, detected in 84 of 167 (50.3%) patients with cirrhosis or HCC, was the most representative etiology of advanced liver disease, followed by alcohol abuse (57/167, 34.1%), HBV infection (19/167, 11.4%) and others (autoimmune and cryptogenic, 7/167, 4.2%).

Modification of serological Survivin–IgM IC levels in patients affected by liver disease

Figure 2(a) reports the distribution of serum Survivin–IgM IC values in healthy subjects and patients from all groups, expressed as AU/mL. While healthy subjects showed low serum levels of Survivin–IgM IC (range, 0–323.45 AU/mL), increased levels of Survivin–IgM IC were found in sera from patients with chronic viral hepatitis (range, 48.30–5205.35 AU/mL). The majority of patients with cirrhosis showed high values of IC (range, 0–10 000 AU/mL). Surprisingly, the levels of Survivin–IgM IC in HCC patients were lower than those observed in patients with cirrhosis (range, 0–4000 AU/mL).

Median comparison analysis by Mann–Whitney *U*-test confirmed these results (Fig. 2b). Indeed, healthy

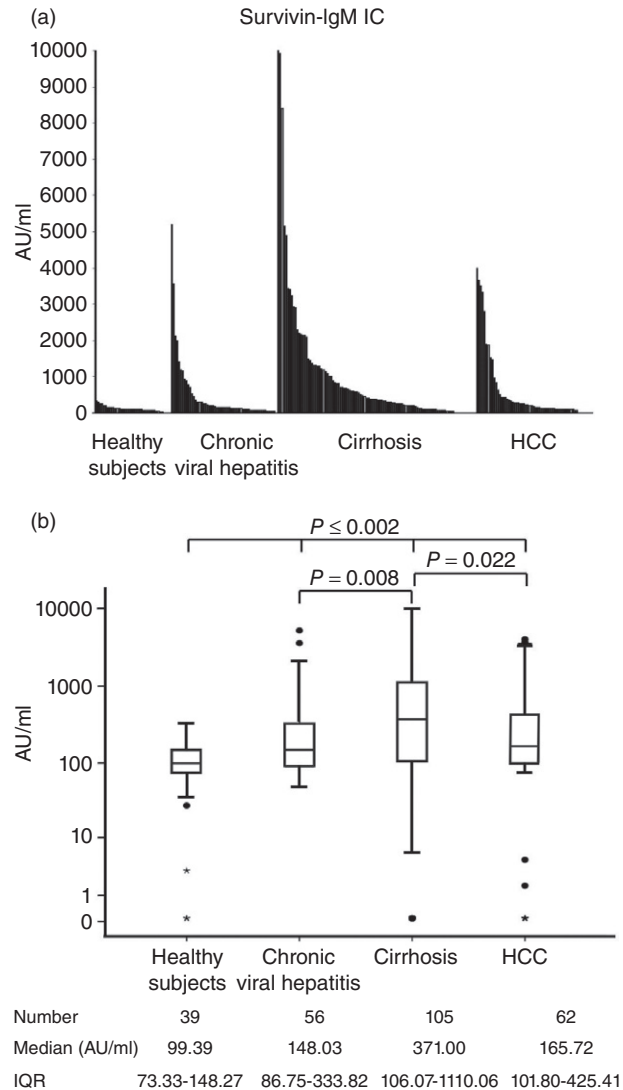


Figure 2 Evaluation of serological Survivin–immunoglobulin (Ig)M immune complex (IC). (a) Distribution of serum Survivin–IgM IC values in healthy controls and patients from all groups expressed as arbitrary units (AU/mL). The group of cirrhosis showed the highest levels of Survivin–IgM IC compared to all other groups. (b) Median comparison analysis demonstrated a significant difference among the groups. Data were represented by box plot; mild (black out) and extreme outliers (asterisks) are shown. Number of individuals, median values in AU/mL and interquartile ranges (IQR) are reported.

subjects showed a median value of Survivin–IgM IC of 99.39 AU/mL (interquartile range [IQR], 73.33–148.27), significantly lower than that of patients with chronic viral hepatitis (148.03 AU/mL; IQR, 86.75–333.82; $P = 0.002$). The patients with cirrhosis showed the highest median value (371.00 AU/mL; IQR, 106.07–

1110.06; $P < 0.001$ vs healthy subjects; $P = 0.008$ vs chronic viral hepatitis). The median value of Survivin-IgM IC (165.72 AU/mL; IQR, 101.80–425.41) in the sera of HCC patients, although higher than that observed in healthy subjects ($P < 0.001$), was significantly lower than that observed in cirrhotic patients ($P = 0.022$), and similar to patients with chronic viral hepatitis. These results highlight a characteristic modification of Survivin-IgM in the presence of cirrhosis.

Detection of high levels of serological Survivin-IgM IC in HCV positive patients with cirrhosis

In order to evaluate the levels of serum Survivin-IgM IC according to the etiologies of liver disease, the enrolled patients were initially divided into the following categories: HCV positive ($n = 125$), HBV positive ($n = 34$), alcohol ($n = 57$) or others etiologies (autoimmune and cryptogenic) ($n = 7$). Among the four categories, HCV positive patients showed the highest median levels of Survivin-IgM IC (data not shown). Thus, patients were divided as HCV positive (HCV⁺) ($n = 125$) and HCV negative (HCV⁻) ($n = 98$), including all the other etiologies. Figure 3 reports the distribution of Survivin-IgM IC values as AU/mL in patients with chronic hepatitis C ($n = 41$), HCV⁺ patients with cirrhosis ($n = 47$) and HCV⁺ patients with HCC ($n = 37$) (Fig. 3a,b) or in patients with chronic hepatitis B ($n = 15$), HCV⁻ patients with cirrhosis ($n = 58$) and HCV⁻ patients with HCC ($n = 25$) (Fig. 3c,d). As shown in Figure 3(a), in patients with chronic hepatitis C without cirrhosis Survivin-IgM IC was detected within a range of 48.30–5205.35 AU/mL; HCV⁺ patients with cirrhosis showed a higher reactivity of IC with values ranging 0–10 000 AU/mL, while HCV⁺ patients with HCC presented lower values of IC when compared with the cirrhotic group (range, 0–4000 AU/mL).

In contrast, as shown in Figure 3(c), all HCV⁻ patients showed lower levels of Survivin-IgM IC when compared to the corresponding HCV⁺ group (range: chronic hepatitis B, 51.68–2123.97 AU/mL; cirrhosis, HCV⁻, 0–3436.28 AU/mL; HCV⁻ HCC, 0–1903.35 AU/mL).

Statistical analysis demonstrated that HCV⁺ patients with cirrhosis showed the highest median value of Survivin-IgM IC (633.71 AU/mL; IQR, 304.60–2084.07) ($P < 0.001$ vs healthy subjects and chronic viral hepatitis C) (Fig. 3b). Sera from patients with chronic hepatitis C showed a median value of 155.93 AU/mL (IQR, 81.61–488.70) significantly higher than healthy subjects (99.39 AU/mL, $P = 0.002$). Even when samples were divided for different etiologies,

HCV⁺ patients with HCC showed a lower Survivin-IgM IC median value of 225.66 AU/mL (IQR, 98.08–461.11) when compared with those with cirrhosis ($P = 0.002$), but significantly higher than healthy subjects ($P = 0.001$).

Compared to the HCV carriers, the HCV⁻ patients showed lower levels of Survivin-IgM IC in all the groups considered for the study (Fig. 3d), with significant difference between HCV⁺ and HCV⁻ cirrhotic patients ($P < 0.001$). In the HCV⁻ patients, non-significant differences of median levels among hepatitis B, cirrhosis or HCC groups were detected (chronic hepatitis B: 122.61 AU/mL; IQR, 93.41–255.96; cirrhosis: 249.52 AU/mL; IQR, 79.68–681.36; HCC: 137.97 AU/mL; IQR, 104.77–403.68). However, HCV⁻ patients with cirrhosis or HCC showed Survivin-IgM IC median values that were significantly higher than healthy subjects ($P = 0.003$ and $P = 0.001$ respectively; chronic hepatitis B, $P = 0.107$) (Fig. 3d).

Finally, to better define the increasing level of Survivin-IgM IC in HCV⁺ patients with cirrhosis, these were further divided on the basis of Child-Pugh score (Fig. 4). Significant difference of Survivin-IgM IC level median values between diverse Child score were found only in HCV⁺ patients, with higher levels in Child B + C compared to Child A (Child A, 690.30 AU/mL; Child B + C, 1280.55 AU/mL; $P = 0.008$). Once more, significant higher levels of Survivin-IgM IC were detected in HCV⁺ patients, when compared to HCV⁻ patient within the same Child-Pugh score (Child A, $P = 0.010$; Child B + C, $P = 0.003$).

Overall, results showed that circulating Survivin-IgM IC is progressively higher in HCV infected patients with chronic hepatitis and cirrhosis, when compared to healthy subjects, suggesting a specific increase of Survivin-IgM in HCV carriers with cirrhosis. Moreover, Survivin-IgM IC is significantly lower in HCC patients, suggesting a specific modification towards HCC development.

Analysis of diagnostic accuracy of Survivin-IgM alone or in combination with transaminase variation

Results showed significant modification of serum Survivin-IgM IC in HCV⁺ patients; thus, we wanted to evaluate the diagnostic accuracy of this marker for the detection of cirrhosis determined by the analysis of the ROC and the respective AUC; we also investigated the optimum cut-off value, by maximizing the sum of sensitivity and specificity, for diagnosis in HCV⁺ cirrhotic patients based on Survivin-IgM IC levels in the

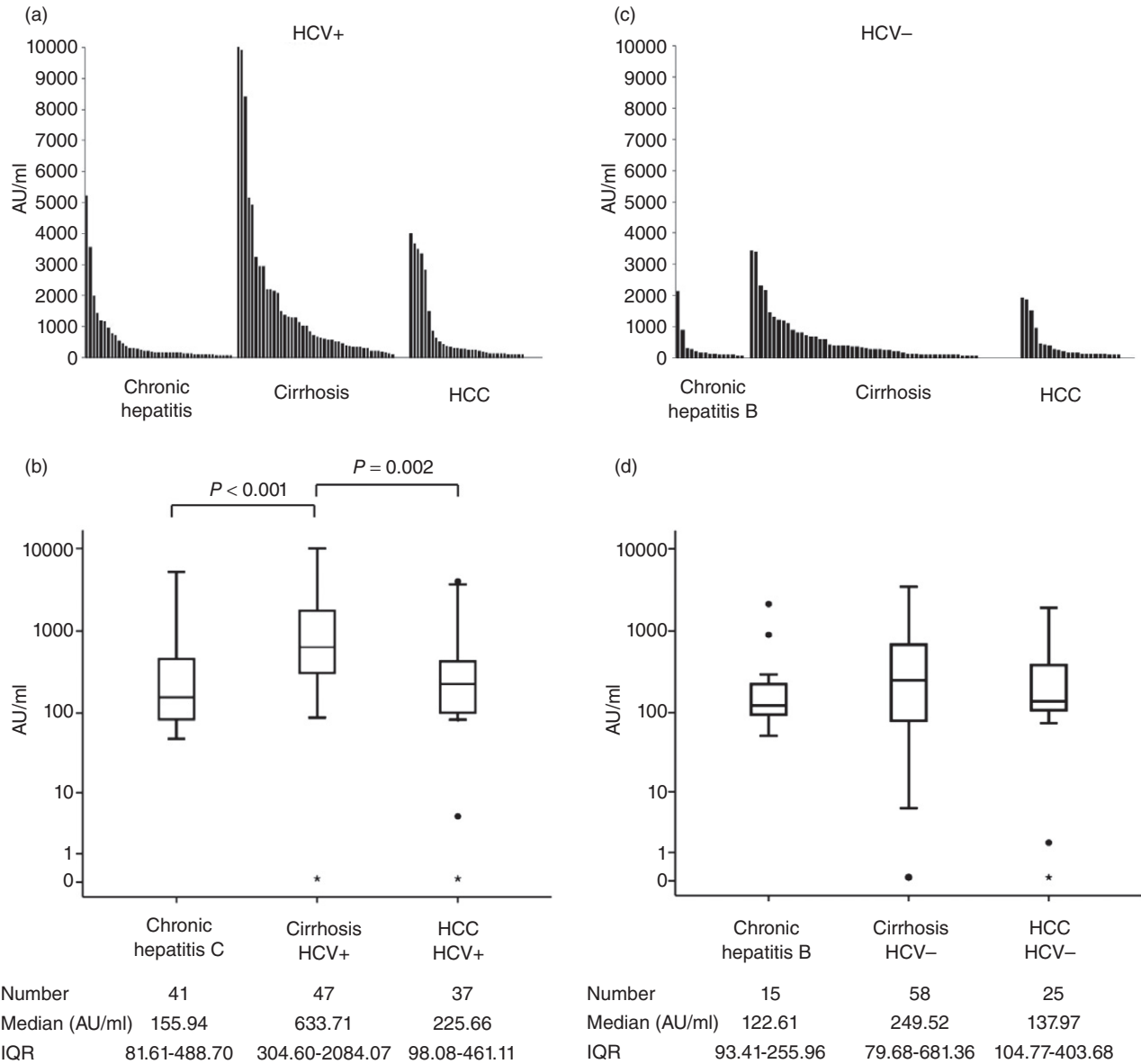


Figure 3 Detection of high levels of Survivin-immunoglobulin (Ig)M immune complex (IC) in hepatitis C virus positive (HCV⁺) patients with cirrhosis. (a) Distribution of Survivin-IgM values as AU/mL in patients with chronic viral hepatitis, cirrhosis and hepatocellular carcinoma (HCC) infected by HCV. (b) Median comparison analysis demonstrated that HCV⁺ patients with cirrhosis showed the highest levels of IC when compared to patients with chronic hepatitis C or HCC. (c) Distribution of Survivin-IgM IC values as AU/mL in patients not infected by HCV. (d) Hepatitis C virus negative (HCV⁻) patients showed lower levels of IC when compared to the corresponding HCV⁺ group, with significant difference in cirrhosis ($P < 0.001$); non-significant differences of median values among hepatitis, cirrhosis or HCC groups were detected.

chronic HCV population with high risk of developing cirrhosis. Moreover, Survivin-IgM diagnostic accuracy was compared with other markers used to evaluate hepatic dysfunction such as AST, ALT and AST/ALT ratio, either alone or in combination (Fig. 5). ROC showed

that the optimal cut-off calculated for Survivin-IgM IC was 310.5 AU/mL (AUC, 0.738; 95% CI, 0.630–0.846; sensitivity, 74.5%; specificity, 70.7%), for AST the optimal cut-off point was 48.50 U/L (AUC, 0.646; 95% CI, 0.528–0.764; sensitivity, 72.3%; specificity, 61.0%),

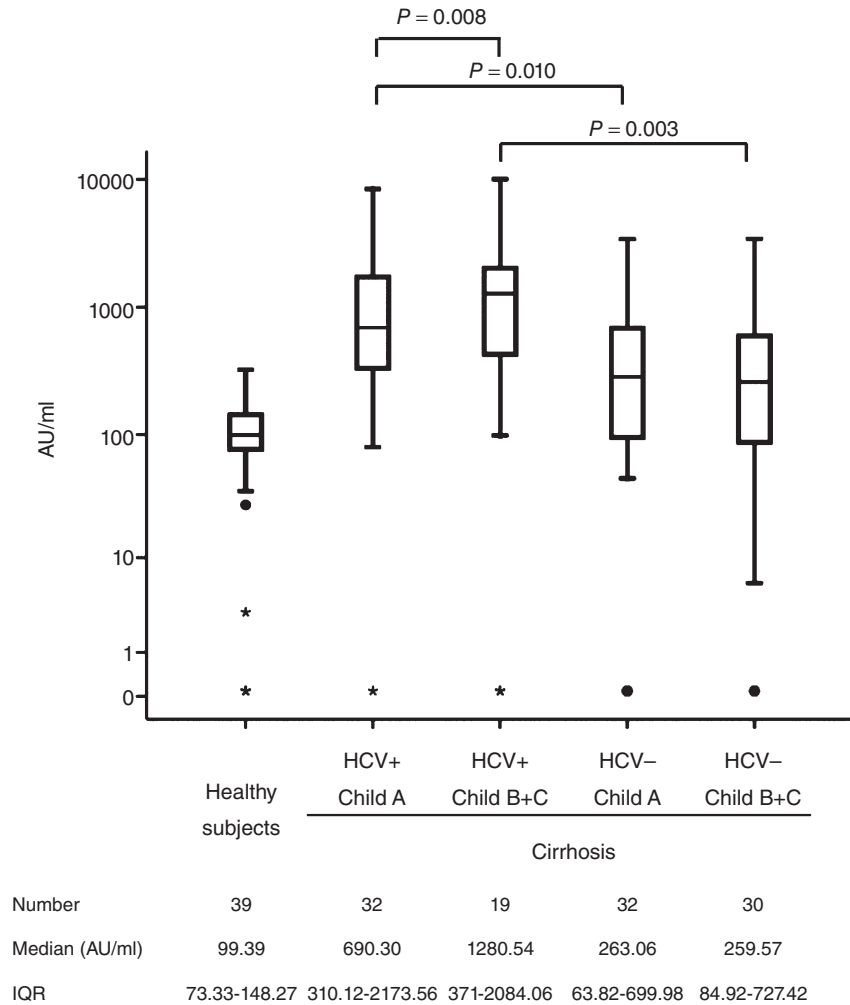


Figure 4 Levels of circulating Survivin-immunoglobulin (IgM) immune complex (IC) in cirrhotic patients with different values of Child-Pugh score. Significant difference of Survivin-IgM IC level median values between diverse Child score were found only in hepatitis C virus positive (HCV+) cirrhotic patients, with higher levels in Child B + C compared to Child A. Significant higher levels of Survivin-IgM IC were detected in HCV+ patients, when compared to HCV- patient within the same Child-Pugh score.

for ALT was 13.50 U/L (AUC, 0.628; 95% CI, 0.511–0.747; sensitivity, 38.3%; specificity, 92.7%) and for the AST/ALT ratio the optimal cut-off point was 3.39 (AUC, 0.737; 95% CI, 0.629–0.844; sensitivity, 57.4%; specificity, 92.7%). To correctly classified HCV+ patients with cirrhosis, Survivin-IgM IC and AST showed relatively higher sensitivity, while higher specificity was showed by AST/ALT ratio and ALT.

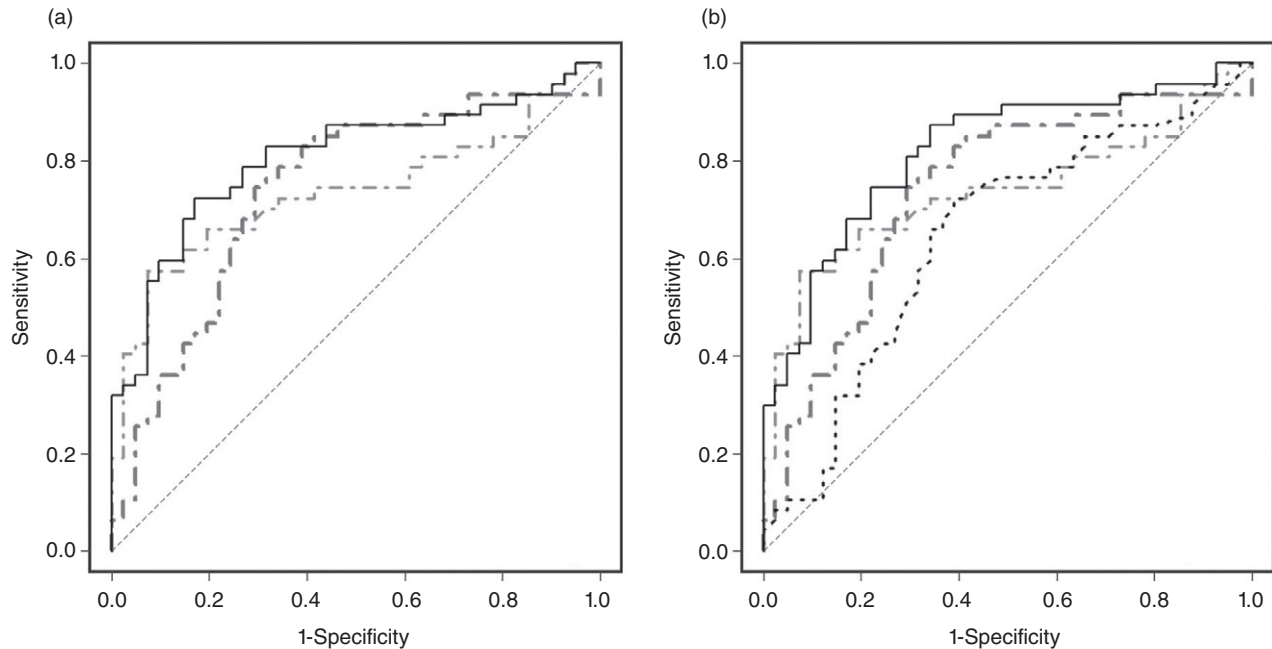
As shown by the increase of AUC, two optimal combinations were found in Survivin-IgM plus AST/ALT ratio and Survivin-IgM plus AST/ALT plus AST. The ROC analysis based on the predicted probability of Survivin-IgM plus AST/ALT ratio (AUC, 0.804; 95% CI, 0.706–0.881) provided a cut-off value of 0.53, with a sensitivity of 72.3% and the specificity of 82.9% (Fig. 5a), while the prediction probability of 0.36 was taken as the cut-off value for diagnosis for Survivin-IgM plus AST/ALT plus AST (AUC, 0.818; 95% CI, 0.722–

0.893), with a sensitivity and specificity of 87.2% and 65.9%, respectively (Fig. 5b).

The calculated ROC area and the value of sensitivity for Survivin-IgM IC confirmed a good diagnostic performance of this assay for the identification of HCV patients with cirrhosis. Moreover, the combination with other markers of hepatic dysfunction enhanced the diagnostic sensitivity.

DISCUSSION

THE AIM OF this study was to evaluate the role of serum Survivin-IgM as potential biomarkers of diverse phases of chronic liver diseases. The results demonstrated that Survivin-IgM IC values were significantly higher in cirrhotic patients chronically infected with HCV compared to chronic viral hepatitis, suggesting that Survivin-IgM could be a potential novel biomarker to



	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Survivin-IgM IC	0.738 (0.630-0.846)	74.5%	70.7%	74.5%	70.7%
AST/ALT ratio	0.737 (0.629-0.844)	57.4%	92.7%	90.0%	65.5%
AST	0.646 (0.528-0.764)	72.3%	61.0%	68.0%	65.8%
ALT	0.628 (0.511-0.747)	38.3%	92.7%	85.7%	56.7%
Logistic (Survivin-IgM IC + AST/ALT ratio)	0.804 (0.706-0.881)	72.3%	82.9%	82.9%	72.3%
Logistic (Survivin-IgM IC + AST/ALT ratio + AST)	0.818 (0.722-0.893)	87.2%	65.9%	74.5%	81.8%

AUC=area under curve. PPV=positive predicted value. NPV=negative predicted value

Figure 5 Diagnostic accuracy of Survivin-immunoglobulin (Ig)M immune complex (IC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT ratio either alone or in combination to identify cirrhosis in hepatitis C virus (HCV) patients. (a) Receiver-operator curve (ROC) for Survivin-IgM, AST/ALT ratio or both. (b) ROC for Survivin-IgM, AST/ALT ratio, AST either alone or in combination. The analysis of the ROC and the respective areas under the curve (AUC) showed that the detection of Survivin-IgM has the high ability to well discriminate cirrhosis from chronic hepatitis C and that the combination with AST and/or AST/ALT ratio enhanced the diagnostic accuracy. ---, AST/ALT ratio; - - -, Survivin-IgM IC; —, AST/ALT ratio + Survivin-IgM IC; ····, AST; —·—, AST/ALT ratio + Survivin-IgM IC + AST.

monitor transition of chronic HCV hepatitis toward more severe liver disease.

Previous studies reported the detection of IgG class autoantibodies to Survivin in sera of patients with

chronic hepatitis or HCC.^{28,29} Yagihashi *et al.* found elevated antibodies only in seven of 37 patients with chronic hepatitis C (19%); no analysis of cirrhotic patients was performed, but interestingly, among HCC

group, significantly higher levels of Survivin autoantibodies were detected in HCV⁺ patients when compared to HBV⁺.²⁸ Otherwise, Zhang *et al.* reported the detection of serological Survivin autoantibodies only in HCC with an incidence of 11.3%.²⁹ In our study, using a cut-off of 310.5 AU/mL, Survivin-IgM IC was positive in 74.5% of HCV⁺ cirrhotic patients, with a specificity of 70.7%. The combination of Survivin-IgM with AST/ALT ratio, a marker with lower sensitivity but higher specificity, enhanced diagnostic accuracy as demonstrated by AUC increase with a sensitivity of 72.3% and a specificity of 82.9%. Moreover, a triple combination of Survivin-IgM IC, AST/ALT ratio and AST values further increased the prediction accuracy (sensitivity, 87.2%; specificity, 65.9%).

Concerning the identification of the free protein, a study reported an increase of free Survivin in serum of patients with chronic HCV, when compared to a healthy group; however, median levels of Survivin were very low and no difference among viral hepatitis and presence of cirrhosis was assessed.³⁰ Nevertheless, free Survivin protein was undetectable in all sera tested in the present study (data not shown). It could be reasonable that the Survivin molecules circulating as free form or immune complexes were below the detection limit of the commercial assay; moreover, the steric hindrance of IgM (900 kDa) could cover the binding sites on Survivin surface (16 kDa) recognized by the antibodies used for capture and revelation phases of the assay. On the other hand, our detection method reveals Survivin-IgM using a polyclonal rabbit antihuman Survivin as capture antibody in the solid phase, and an enzyme-conjugated antihuman IgM as detector which may have multiple binding sites on the pentameric structure of IgM, thus enhancing the signal detection for each Survivin molecule in the IC.

The molecular basis for the overexpression of Survivin in cancer has been intensely investigated. Survivin reactivity is typically observed in the vast majority of tumor cells and its high expression in liver cells and during hepatocarcinogenesis has been previously studied.²²⁻²⁴ However, several papers describe diverse expression, cellular localization and prognostic significance of Survivin in cirrhotic liver and HCC.³¹⁻³⁸ Actually, compared to healthy subject, Survivin-IgM IC was found significantly higher in all patients with liver diseases included in the study, suggesting a modification correlated to liver dysfunction; moreover, recent papers reported a link of Survivin to liver regeneration,^{39,40} that extensively occurs after liver damage. However, the Survivin-IgM IC values were exceptionally high in HCV-related cirrhosis, and,

unexpectedly, those of HCV⁺ HCC patients were lower compared to those detected in cirrhotic patients. The high values of IC in the presence of HCV may be due to a specific activity of this virus to transactivate Survivin expression and to induce immune disorders. Indeed, it was demonstrated that *in vitro* HCV infection of hepatoma cell or NS5A viral protein transfection are able to enhance Survivin expression.^{41,42} Moreover, it has been suggested that HCV binding to CD81 on B lymphocytes *in vivo* and the polyclonal proliferation of naïve B cells is a key factor for the development of HCV-associated B-lymphocyte disorders.⁴³ Thus, B cells from HCV patients would be more sensitive to autoantigen stimulation, which would in turn facilitate the production of autoantibodies associated with cryoglobulinemia.⁴⁴⁻⁴⁶ All these evidences could explain in part the higher levels of Survivin-IgM IC in sera of patients with HCV. However, at the present time, we are not able to explain the nature of the lower levels of IC in the presence of HCC and a longitudinal study could clarify the individual modulation of the IC levels in the progression of the disease.

In the complex, the results extended the occurrence of biomarker-IgM complexes in chronic liver diseases with the first description of the presence of Survivin-IgM IC in the sera of patients with chronic hepatitis, with cirrhosis and with HCC.

Moreover, we underline the detection of high levels of Survivin-IgM IC during cirrhosis in HCV⁺ patients. When Survivin-IgM IC were combined with AST and AST/ALT ratio, further increase of diagnostic accuracy was achieved for the identification of HCV⁺ cirrhotic patients. Furthermore, the low reactivity in high-risk non-cirrhotic HCV⁺ patients suggests that Survivin-IgM could be a potential biomarker to evaluate the progression of chronic HCV to cirrhosis by means of a non-invasive, rapid and inexpensive method. In addition, even if higher than in healthy subjects, this biomarker was found to be lower in sera from HCC patients compared to cirrhosis. Follow-up studies are in progress to validate Survivin-IgM modification during the onset of cirrhosis and to monitor the downregulation of Survivin-IgM IC with the progression of tumoral disease.

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