

# Evaluation of serum levels and significance of soluble CD40 ligand in screening patients with hepatitis C virus-related hepatocellular carcinoma

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## تقييم المستوى المصلّي للجين CD40 الذواب وأهميته في تحري المرضى المصابين بسرطانة الخلايا الكبدية المرتبطة بفيروس التهاب الكبدى C

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**الخلاصة:** هدفت هذه الدراسة إلى تقييم الأهمية السريرية لـ sCD40L عند مرضى سرطانة الخلايا الكبدية المرتبطة بفيروس التهاب الكبدى C (HCV-HCC). فتمت مقايسة التركيز المصلّي لـ sCD40L و IL-10 التي تدور في الدم باستخدام مقايسة المتمز المناعي المرتبط بالإنزيم (ELISA) لدى 30 من مرضى سرطانة الخلايا الكبدية الإيجابية لفيروس التهاب الكبدى C، و30 من مرضى تشمع الكبد الإيجابييين لفيروس التهاب الكبدى C، و30 من المتطوعين الأصحاء من نفس الفئة العمرية سليبين لـ anti-HCV-Ab كفتة شاهدة. فأظهرت النتائج الخاصة بالمستوى المصلّي لـ sCD40L مستويات عالية ذات دلالة إحصائية عند مرضى سرطانة الخلايا الكبدية الإيجابييين لفيروس التهاب الكبدى C مقارنة مع مرضى تشمع الإيجابييين لفيروس التهاب الكبدى C والشواهد الأسوأ (P<0.001). وكان للمستوى المصلّي لـ sCD40L قيمة تشخيصية أعلى من AFP عند مرضى سرطانة الخلايا الكبدية. ولوحظ أن حساسية ونوعية sCD40L عالية مقارنة مع AFP (90%، 86.7%، 83%، و80% على التوالي). واكتُشف وجود علاقة طردية معتبرة بين المستويات المصلية لـ sCD40L و IL-10 (r=0.85 P<0.001)، و AFP (r=0.62 P<0.05) ومرحلة تطور (r=0.5 P<0.05). وفي الختام، يعتبر sCD40L أداة تشخيصية قيمة للتشخيص المبكر والتحرري عن فيروس التهاب الكبدى C وسرطانة الخلايا الكبدية، وكذلك للمتابعة الروتينية لمرضى تشمع الإيجابييين لفيروس التهاب الكبدى C. كما أن تقييم مستويات IL-10 في المصل عند مرضى التهاب الكبدى C قد يعتبر علامة تنبؤية محتملة لتقدم المرض.

**ABSTRACT** The study's objective was to evaluate the clinical significance of sCD40L in HCV-associated hepatocellular carcinoma (HCV-HCC) patients. Sera concentration of circulating sCD40L and IL-10 were assayed using ELISA in 30 HCV-positive patients with HCC, 30 HCV-positive patients with liver cirrhosis and 30 age-matched healthy volunteers with negative anti-HCV-Ab as a control group. Serum sCD40L showed statistically-significant high levels in HCV-HCC patients compared to HCV-cirrhotic patients and normal controls (P < 0.001). Serum sCD40L had higher diagnostic value in HCC patients compared with serum AFP. High sensitivity and specificity of sCD40L was observed compared to AFP (90%, 86.7% and 83% and 80% respectively). Significant positive correlation was detected between serum sCD40L and IL-10 (r = 0.85 P < 0.001), AFP (r = 0.62 P < 0.05) and tumour staging (r = 0.5 P < 0.05). The study concluded that sCD40L is a valuable diagnostic tool in early diagnosis and screening for HCV and HCC as well as routine follow up of HCV cirrhosis patients. Assessment of serum IL-10 levels in HCV patients may provide a possible predictive marker for disease progression.

## Évaluation des concentrations sériques et du rôle du ligand de CD40 soluble dans le dépistage des patients atteints d'un carcinome hépatocellulaire lié au virus de l'hépatite C

**RÉSUMÉ** L'objectif de l'étude était d'évaluer l'importance clinique du ligand de CD40 soluble (sCD40L) chez des patients atteints d'un carcinome hépatocellulaire (CHC) associé au virus de l'hépatite C (VHC). Les concentrations sériques de sCD40L circulant et d'interleukine 10 circulante ont été analysées à l'aide de la méthode immuno-enzymatique chez 30 patients positifs pour le VHC avec un CHC, chez 30 patients positifs pour le VHC avec une cirrhose du foie, et chez 30 volontaires d'âge correspondant en bonne santé avec des anticorps anti-VHC négatifs servant de groupe témoin. Les concentrations sériques de sCD40L ont montré des niveaux statistiquement élevés chez les patients atteints d'un CHC associé au VHC par rapport aux concentrations sériques d'AFP. Les concentrations sériques de sCD40L avaient une valeur diagnostique plus élevée chez les patients atteints d'un CHC que les concentrations sériques d'AFP. Une sensibilité élevée et la spécificité du sCD40L ont été observées par rapport à l'AFP (90 %, 86,7 % et 83 % et 80 % respectivement). Une corrélation positive significative a été détectée entre les concentrations sériques de sCD40L et d'IL-10 (r = 0,85 p < 0,001), l'AFP (r = 0,62 P < 0,05) et le stade de la tumeur (r = 0,5 p < 0,05). L'étude a conclu que le sCD40L est un outil précieux pour le diagnostic et le dépistage précoces de l'infection à VHC et du CHC, ainsi que pour le suivi de routine des patients cirrhotiques positifs pour le VHC. L'évaluation des concentrations sériques d'IL-10 chez les patients positifs pour le VHC pourrait fournir un possible marqueur prédictif pour l'évolution de la maladie.

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## Introduction

Primary liver cancer is the third leading cause of cancer-related mortality and the sixth most common cancer worldwide with ~750 000 new cases every year (1). Hepatocellular carcinoma (HCC) accounts for ~90% of primary liver cancers and remains the leading cause of death among patients with cirrhosis (2). Three-quarters of all the cases worldwide are attributed to chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) (3). The aetiology of HCC in Egypt indicates a higher prevalence of HCV than HBV (3). The risk of developing HCC in HCV-positive patients increases dramatically with severity of inflammation. This is most probably due to the influence of the inflammation, predominantly mediated by cytokines, on supporting the tumour microenvironment (4).

The poor prognosis of HCC is mostly linked to late diagnosis because few treatment strategies can be implemented in patients with advanced disease (4). Surgical resection is the only effective treatment; however, only a few patients are candidates for surgery. The ability to detect early HCC would increase the availability of surgery and improve patient survival (4).

Although many candidate molecular markers of HCC have been identified, such as  $\alpha$ -fetoprotein (AFP), glypican-3 and squamous cell carcinoma antigen-1, markers with the necessary sensitivity and specificity for early detection are still lacking. The most widely utilized blood-based biomarker is AFP, which is markedly elevated in patients with cirrhosis and/or exacerbated non-HCC chronic hepatitis (5). Moreover, there is a high false-negative rate of AFP in HCC patients with small or early-stage tumours (6,7). Thus, AFP still lacks adequate sensitivity and specificity for effective surveillance of HCC (5–7). Glypican-3 and squamous cell carcinoma antigen-1 are elevated in

many other tumours, so their role is still controversial.

The most sensitive diagnosis of HCC currently requires invasive imaging procedures such as endoscopic ultrasonography, which can lead to hepatic injury, and the accuracy of these procedures is highly operator dependent. Therefore, identification of reliable new markers with better performance for early detection of HCC would have a major impact on treatment outcome (4).

In the current study, we investigated the utility of soluble CD40 ligand (sCD40L) as a reliable marker for early detection of HCC and other neoplastic lesions in the liver. CD40L (also known as gp39 or CD154) is a trimeric, transmembrane protein of the tumour necrosis factor family that was originally identified on cells of the immune system (8). It was first identified in activated CD4+ T cells, mast cells, polymorphonuclear granulocytes, and natural killer cells (8). Subsequent studies revealed functional CD40L expression in a wide variety of cells, including endothelial cells, smooth muscle cells, macrophages and activated platelets (9,10). Membrane-bound CD40L is potentially cleaved into sCD40L, which has cytokine-like activity and is released into the circulation (11,12). Both membrane-bound CD40L and circulating sCD40L interact with CD40 protein that is expressed on vascular cells, resulting in several inflammatory and prothrombotic responses (13).

The significant platelet activation and inadequate T-cell reactivity in cancer patients indicates the greater likelihood of releasing or cleaving sCD40L from the platelets rather than T cells (14). Therefore, sCD40L can affect cancer development and progression by inducing thrombotic reactions and releasing angiogenesis-associated cytokines (14,15).

Widespread expression of CD40 in humans implies that its ligand has an

important role in cancer pathogenesis (16): inhibiting apoptosis; facilitating metastases (17); increasing epithelial cell proliferation, motility and invasion (18); and producing cytokines, such as interleukin (IL)-10, that modulate the anti-tumour response of T lymphocytes (13). Increased levels of IL-10 have been found in the plasma of patients with different histotypes of solid and haematopoietic tumours (19,20). IL-10 has been evaluated as a marker for HCC, however, it has not yet been validated for clinical use (21).

In the current study, we evaluated the clinical significance of sCD40L in patients with HCV-associated HCC compared with patients with liver cirrhosis as well as normal healthy controls. We also explored serum level of sCD40L as a novel potential marker for diagnosis and screening of HCC and validated it against the traditional marker AFP.

## Methods

### Patients

Sixty HCV-positive patients either attended or were admitted to the Department of Hepatology and Gastroenterology or Internal Medicine, Benha University Hospital, Egypt from October 2014 to March 2015. All procedures were performed in accordance with institutional guidelines using protocols approved by Benha Faculty of Medicine Patient Care and Ethics Committee. Written informed consent was obtained from patients and controls.

The study population was divided as follows: Group I, 30 HCV-positive patients with HCC aged 32–64 years; Group II, 30 HCV-positive patients with liver cirrhosis aged 34–58 years; and Group III, 30 healthy volunteers, negative for HCV antibody, aged 19–48 years old as a control group. Consideration of the socioeconomics of the patient groups was beyond the scope of this study. HCV-positive

patients received supportive treatment alone without any anti-HCV medication before or during the study.

We used triphasic computed tomography, and/or magnetic resonance imaging to detect characteristic focal lesions of HCC, with or without elevated AFP. Biopsied tumours were staged using the Okuda staging system for HCC (22).

### Serum samples

All patients and controls were subjected to thorough clinical examinations before 5-mL blood samples were collected by sterile venipuncture and allowed to clot. Serum samples were separated, aliquoted and stored at  $-20^{\circ}\text{C}$ . Comprehensive laboratory investigations were conducted including: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, albumin, hepatitis B surface antigen and

HCV antibody. AFP levels (0.3–1000 ng/mL) were assessed by AxSYM using microparticle enzyme immunoassay (MEIA) technology.

### Serum sCD40L and IL-10 assays

Circulating sCD40L and IL-10 were assessed using a commercial ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA). A quantitative sandwich enzyme immunoassay technique using a polyclonal antibody specific for CD40L or IL-10 was utilized with a minimum detection limit of 4.2 pg/mL for CD40L and 3.9 pg/mL for IL-10.

### Statistical analysis

The data were tabulated, coded and analysed using SPSS version 20. Qualitative data were presented as numbers and percentages, and quantitative data as mean and standard deviation. Analysis of variance (ANOVA) was

used to compare more than two groups of numerical data; post hoc analysis was done using Dunnett's test; inter-group comparison of categorical data was performed using the  $\chi^2$  test; and Pearson and Spearman rank correlation coefficients ( $r$ ) were used to correlate different parameters. Receiver operating characteristic curves were used to evaluate the diagnostic power of the different diagnostic tests.  $P < 0.05$  was considered statistically significant.

## Results

### Clinical and demographic data

Clinical and demographic data are presented in Table 1. There was no significant difference in gender between patients and controls ( $P > 0.05$ ). Age was significantly higher in Groups I and II compared to Group III ( $P < 0.001$ ). There was no significant difference

**Table 1 Demographic, clinical and laboratory characteristics of patients and controls**

Variable	Group I (n = 30)	Group II (n = 30)	Group III (n = 30)	P
<b>Age (years)<sup>a</sup></b>	59 (8.4)	53 (7.5)	30 (7.9)	<0.001
Gender (female/male)	18/12	15/15	10/20	>0.05
<b>Clinical picture</b>				
Hepatomegaly	6 (20%)	2 (6.7%)	-	>0.05
Splenomegaly	30 (100%)	25 (83.3%)	-	>0.05
Ascites	20 (66.7%)	18 (60%)	-	>0.05
<b>Serum levels<sup>a</sup></b>				
Albumin (mg/dL)	2.73 (0.42)	2.807 (0.36)	7.23 (0.06)	<0.001
ALT (U/L)	55.7 (19.9)	45.4 (11.7)	24.8 (3.6)	<0.001
AST (U/L)	60 (18.3)	48.2 (13.2)	20.4 (3.4)	<0.001
ALP (U/L)	246.8 (86.4)	213.6 (56.2)	67 (20.8)	<0.001
Total bilirubin (mg/dL)	3.68 (0.72)	1.71 (0.94)	0.9 (0.3)	<0.001
<b>Tumour number</b>				
Single/multiple	16/14	-	-	-
<b>Tumour stage</b>				
Stage I	10 (33.3%)	-	-	-
Stage II	14 (46.7%)	-	-	-
Stage III	6 (20%)	-	-	-
<b>Tumour size</b>				
<3 cm/>3 cm	22/8	-	-	-

<sup>a</sup>Data expressed as mean (standard deviation).

ALT = alanine aminotransferase; ALP = alkaline phosphatase; AST = aspartate aminotransferase.

between Groups I and II for hepatomegaly, splenomegaly and ascites (all  $P > 0.05$ ). Among patients with HCC, 16 (53.3%) had a single mass with defined borders. Tumour stage I, II and III was found in 10 (33.3%), 14 (46.7%) and 6 (20%) patients with HCC, respectively. Tumour size was  $< 3$  cm in 22 (73.3%) and  $> 3$  cm in 8 (26.7%) patients.

### Laboratory results

ALT, AST, ALP and total bilirubin were significantly higher in Group I than in Groups II and III (all  $P < 0.001$ ) (Table 1). Serum albumin was significantly higher in Group III than Groups I and II ( $P < 0.001$ ).

### Serum sCD40L, IL-10 and AFP levels

Serum sCD40L, IL-10 and AFP levels are presented in Table 2. Serum sCD40L level was significantly higher in

Group I than Groups II and III (one-way ANOVA,  $P < 0.001$ ). Serum sCD40L level was also significantly higher in Group II than Group III (post hoc test,  $P < 0.001$ ). Similarly, serum IL-10 level was significantly higher in Group I than Groups II and III (one-way ANOVA,  $P < 0.001$ ). Also, it was higher in Group II than Group III (post hoc test,  $P < 0.001$ ). Similar findings were observed for serum level of AFP.

To compare the diagnostic accuracy of serum sCD40L and AFP for HCC, receiver operating characteristic curves were generated and area under the curve (AUC) was calculated. Serum sCD40L exhibited superior diagnostic potential for HCC in comparison with serum AFP. AFP level had an AUC value of 0.860 with 83% sensitivity and 80% specificity at a cut-off of 273 ng/mL (Figure 1). In contrast, sCD40L level had a significantly higher AUC

value of 0.930 with 90% sensitivity and 86.7% specificity at a cut-off of 7305.5 pg/mL. Markedly high specificity and sensitivity, ~96.7% for both, were only possible when the two markers were combined, with an AUC value as high as 0.960.

### Correlation between sCD40L level and different parameters

sCD40L showed a significantly positive correlation with IL-10 ( $r = 0.85$ ;  $P < 0.001$ ), AFP ( $r = 0.62$ ;  $P < 0.05$ ) and tumour stage ( $r = 0.5$ ;  $P < 0.05$ ) (Table 3). No significant positive correlation was established between serum sCD40L level and tumour size, age, albumin, bilirubin, AST and ALT (Table 3, Figure 2). The correlations were calculated among all the groups for all variables, except for tumour size and stage, which were calculated for Group I only.

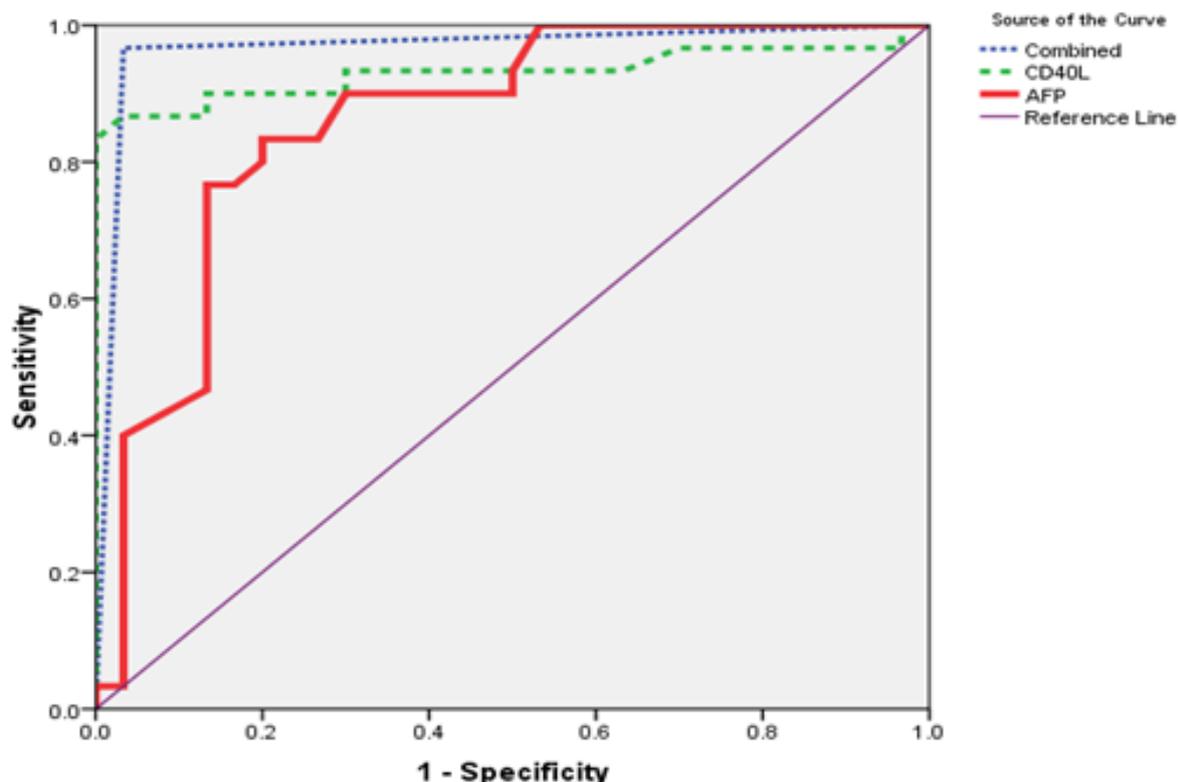
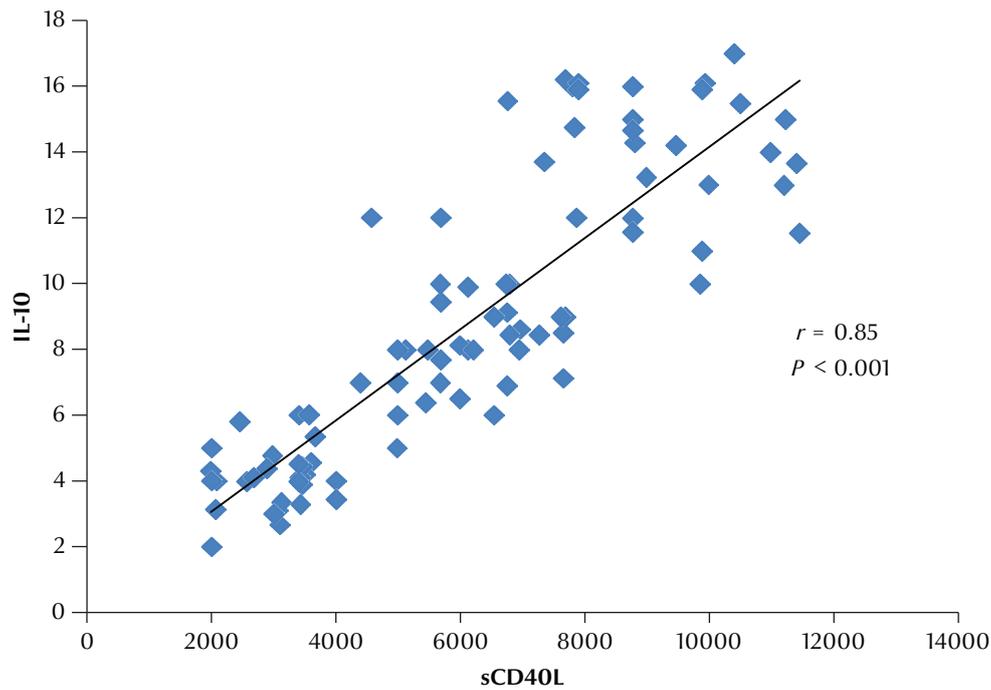


Figure 1 Diagnostic accuracy of sCD40L and AFP for hepatitis-C-virus-associated hepatocellular carcinoma. AUC was 0.860 and 0.930 for AFP and sCD40L, respectively. For the two combined markers AUC was 0.960. AFP =  $\alpha$ -fetoprotein; AUC = area under the curve; sCD40L = soluble CD40 ligand.



**Figure 2** Correlation between sCD40L and IL-10. There was a positive correlation between serum sCD40L and IL-10 among the study groups. IL-10 = interleukin-10; sCD40L = soluble CD40 ligand.

## Discussion

Several studies have suggested the essential role of binding sCD40L to CD40 protein in tumour development, propagation, tumour microenvironmental and metastasis (23,24). However, little is known about the behaviour of platelet-derived sCD40L after becoming detached from the cell surface and its downstream effects in cancer patients.

To explore the potential role of serum sCD40L as a diagnostic and prognostic marker for HCC, we evaluated sCD40L levels in patients with HCV-positive HCC. To validate its role, we

compared HCV-positive patients with liver cirrhosis and a control group of HCV-negative healthy volunteers with intact liver margins and no evidence of any cirrhotic cells. To the best of our knowledge, this is the first study to validate serum sCD40L as a potential diagnostic and prognostic marker for HCC.

The markedly elevated level of sCD40L in HCV-HCC patients compared to healthy individuals indicates the potential of using serum sCD40L as a marker for early detection of HCC. In addition, it underlines the presumed role of sCD40L in many molecular events including carcinogenesis, angiogenesis

and immunosuppression (25). Similarly, the increase in serum sCD40L in HCV-associated HCC relative to its level in HCV-positive patients with liver cirrhosis reflects the direct correlation between elevated serum sCD40L and progression of HCC. The increase in serum sCD40L in HCV-positive patients with liver cirrhosis compared with the HCV-negative control group indicates the potential of serum sCD40L as a molecular marker for minimally invasive, early detection of HCC. The main advantage of this technique is the ability to detect early changes in sCD40 in contrast to the traditional late morphological remodelling.

**Table 2** Serum levels of sCD40L, AFP and IL-10

Variable	Group I (n = 30)	Group II (n = 30)	Group III (n = 30)	P
Serum sCD40L (pg/mL)	9462 (2385) <sup>b</sup>	6956 (1754) <sup>a</sup>	3280 (938) <sup>a,b</sup>	<0.001
Serum AFP (ng/mL)	459.6 (54.9) <sup>b</sup>	7.8 (2.2) <sup>a</sup>	3.47 (1.58) <sup>a,b</sup>	<0.001
Serum IL-10 (pg/mL)	13.86 (3.58) <sup>b</sup>	8.63 (2.57) <sup>a</sup>	4.37 (1.53) <sup>a,b</sup>	<0.001

<sup>a</sup> Significantly different from Group I (HCV-related HCC).

<sup>b</sup> Significantly different Group II (HCV-related cirrhosis).

AFP =  $\alpha$ -fetoprotein; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; IL-10 = interleukin-10; sCD40L = soluble CD40 ligand.

**Table 3 Correlation between sCD40L and different variables**

Variable	r	P
IL-10 <sup>a</sup>	0.85	<0.001
AFP <sup>a</sup>	0.62	<0.05
Tumour stage <sup>b</sup>	0.5	<0.05
Tumour size <sup>b</sup>	-0.1	>0.05
Age <sup>a</sup>	0.09	>0.05
Albumin <sup>a</sup>	0.13	>0.05
Bilirubin <sup>a</sup>	0.16	>0.05
AST <sup>a</sup>	0.14	>0.05
ALT <sup>a</sup>	0.13	>0.05

<sup>a</sup> Pearson correlation.

<sup>b</sup> Spearman rank correlation.

AFP =  $\alpha$ -fetoprotein; ALT = alanine aminotransferase; AST = aspartate aminotransferase; IL-10 = interleukin-10; sCD40L = soluble CD40 ligand.

As in previous studies, serum AFP showed a less significant increase in patients with HCV-associated HCC compared with HCV-positive patients with cirrhosis and the HCV-negative control group (26,27).

The superior selectivity of sCD40L as a marker was demonstrated by the AUC value of 0.930, compared to 0.860 for AFP. This not only reflects the superiority of sCD40L in detecting HCC, but more importantly, in differentiating HCV-positive cancer patients from HCV-positive cancer-free patients. This attribute makes sCD40L a marker of choice in cases in which traditionally used serum AFP cannot distinguish between cancer patients and cancer-free individuals. For instance, it has been reported that some patients with HCC present without any elevation of serum AFP level (28). In contrast, cancer-free patients with liver cirrhosis show elevated serum AFP level regardless of the absence of any clinical and molecular evidence of cancer cells. This allows serum sCD40L to overcome the limitations of AFP as a selective biomarker in individuals in whom it is not possible to discern adequately HCC.

Our results suggest that sCD40L and AFP should be measured simultaneously to improve diagnostic accuracy, especially in the early stage of HCC or

in cases with small tumour size, and with a significant increase in AUC of 0.960.

The positive correlation between serum sCD40L and tumour staging in patients with HCV-positive HCC reflects the potential of using serum sCD40L as a noninvasive tool for determining HCC prognosis and curability. However, its reliability as marker that stands alone without the need for liver biopsy needs to be further investigated.

For some individuals, it can take up to 40 years for chronic hepatitis C to develop into cirrhosis (29). This may depend on the underlying cause and other factors, including patient response rate to disease progression and decline in immune function with ageing. This long-term development of cirrhosis may explain the older age of patients in Groups I and II relative to individuals in the control Group III.

The ALT and AST levels differed greatly among all three groups and it was not possible to establish any correlation with sCD40L level. The observed results might have been due to the modest elevation in the enzyme levels, usually less than 2–3 times the normal range, observed in patients with acute-over-chronic cirrhosis. Some patients even showed a dramatic decrease in aminotransferase level, with laboratory evidence of hepatic failure.

It is evident that the metabolic functions of the liver decline dramatically and directly with cirrhosis development. Albumin is extensively synthesized in the liver, therefore, major decreases in its levels are predictable in patients with liver disease, especially with acute-over-chronic cirrhosis. This could explain the lack of a significant correlation between serum sCD40L and albumin, mainly in patients in Groups I and II.

The lack of correlation between serum sCD40L and bilirubin resulted from variation in its levels from normal in cases of compensated cirrhosis and some cases of HCC to elevated levels as cirrhosis progressed.

Significantly elevated levels of serum IL-10 were observed in patients with HCV-positive HCC and HCV-positive cirrhosis compared with cancer-free individuals. Similar significant elevation of serum IL-10 has also been reported in patients with different types of chronic hepatitis C and various stages of cirrhosis (30,31). Level of IL-10 was mostly prominent in patients with severe hepatic lesions and development of HCC, which contributed to immunosuppression by inhibiting antigen presentation, cytokine production, macrophage activation and antigen-specific T-cell proliferation. All these factors help tumour cells to escape host immune surveillance and potentiate them to metastasize.

In this study, significant elevation of serum IL-10 level in patients with HCV-positive HCC compared to HCV-positive cirrhosis supports the direct correlation between IL-10 serum level and clinical outcome. Therefore, serum IL-10 may serve as a predictive marker for HCC progression in HCV-positive patients. It has been suggested that IL-10 level serves as complementary tumour marker and contributes to differential diagnosis of HCC (31). In addition, it is reported that IL-10 is produced vigorously by some tumour cells (32,33). High circulating and local levels of IL-10 might allow tumour cells

to elude host defence mechanisms and affect the metastatic potential of the tumour. The positive correlation between IL-10 and sCD40L in all the groups in our study suggests that the higher level of sCD40L seen in HCC patients has an immunosuppressive effect through induction of IL-10. This is in accordance with previous results indicating that sCD40L induces expansion of regulatory T cells and production of IL-10,

which can affect both HCC development and metastasis (25,34).

In conclusion, we detected significantly elevated levels of sCD40L in patients with HCV-positive HCC. We provide fundamental clinical evidence of the utility of sCD40L as a diagnostic tool with the potential to replace AFP in everyday diagnosis and screening of HCC and routine follow-up of patients with HCV cirrhosis. Our data indicate the potential of using serum IL-10 levels

in HCV-positive patients as a predictive marker for disease progression. The full utility of this approach needs further studies with larger numbers of patients with age- and sex-matched controls. We also need prolonged follow-up of patients with HCC and cirrhosis to clarify the role of sCD40L in the early diagnosis and improvement of prognosis of HCC

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