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 < 0.05) with a diagnostic value.

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 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

1Department of Public Health and Community Medicine (Correspondence to: S.M. Eltaher: sherefmoh@gmail.com; sherif.abdelmonem@fmed.bu.edu.eg). 2Medical Microbiology and Immunology Department. 3Internal Medicine Department. 4Hepatology and Gastroenterology Department, Faculty of Medicine, Benha University, Benha, Egypt.

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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 α-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, aliquotted and stored at −20 °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 χ² 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.

**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 0.05). 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 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