# Cancer biomarker profiling in patients with chronic hepatitis C virus, liver cirrhosis and hepatocellular carcinoma

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Abstract. The detection and diagnosis of hepatocellular carcinoma (HCC) at an early stage may significantly affect the prognosis of HCC patients. Thus, it is necessary to always identify novel putative markers for improving diagnosis. Hepatocarcinogenesis correlates with pathological hepatic angiogenesis. However, each tumor-induced angiogenetic process is influenced by the microenvironment through several pro- and anti-angiogenic factors released from tumor cells, tumor-associated inflammatory cells and/or from the extracellular matrix, and modulated by various signal pathways. In this study, we evaluated the profiling of angiogenic factors using Bio-Plex Pro<sup>™</sup> Human Cancer Biomarker Panel 1, a 16-plex magnetic bead-based assay, in sera of patients with chronic hepatitis C (CHC) virus, liver cirrhosis (LC) and HCC. Our results demonstrated: i) high levels of hepatocyte growth factor (HGF) and prolactin only in LC and HCC patients, ii) high levels of soluble human epidermal growth factor receptor-2 (sHER-2/neu; ErbB-2), sIL-6Ra, leptin (LEP) and platelet endothelial cell adhesion molecule-1 (PECAM-1) in CHC, LC and HCC patients and iii) that sIL-6R correlated with the fibrosis stage in CHC patients, with Child-Pugh score in those patients with LC and with tumor size in those patients with HCC, confirming that this protein may be used as a predictor of liver damage and of inflammatory process leading to fibrosis, cirrhosis, and subsequently to cancer. Moreover, an interactomic study conducted using the Ingenuity Pathway Analysis (IPA) software proved the existence of a correlation between

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5 significant proteins [ErbB-2, sIL-6Ra, prolactin (PRL), HGF and LEP] which are involved in the same metabolic pathways.

# Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and the third most lethal cancer worldwide, accounting for 550,000 deaths annually. At present, Southern Italy has the highest rates of HCC in Europe (1). HCC is unique among cancers, occurring mostly in patients with a known risk factor; 90% of HCCs develop in the context of chronic liver inflammation and cirrhosis. Major risk factors for HCC include infection with hepatitis B (HBV) and C (HCV) viruses, alcoholic liver disease, and possibly non-alcoholic fatty liver disease. Less common causes include hereditary hemochromatosis,  $\alpha_1$ -antitrypsin deficiency, autoimmune hepatitis, certain types of porphyria and Wilson's disease (2). HBV and HCV viruses are the major cause of liver disease worldwide. Fortunately, the HBV vaccine has led to a substantial decline in the number of new cases of acute hepatitis B among children, adolescents and adults in wWestern countries since the mid-1980s (3). This success is not yet duplicable for HCV, where active or passive vaccination is not available.

Approximately 80% of newly infected patients develop a chronic infection, of which an estimated 10-20% develop cirrhosis and 1-5% advance to end-stage liver cancer over a period of 20-30 years (4). The management of patients at risk for developing HCC remains challenging. The detection and diagnosis of liver cancer at an early stage may improve the prognosis for such patients. An increased understanding of cancer biology and technological advances have enabled the identification of a multitude of pathological, genetic and molecular events that drive hepatocarcinogenesis, leading to the discovery of numerous potential biomarkers in this disease (5).

Tumor-induced angiogenesis is a pathophysiological condition that results from the aberrant deployment of normal angiogenesis. HCC is generally characterized as a hypervascular tumor of rapid growth with the pathological formation of new blood vessels (angiogenesis), a feature that has implications for investigative procedures applied for its detection (6). It is unclear whether or not angiogenesis merely represents a

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homeostatic mechanism aimed at ensuring an adequate oxygen and nutrient supply or one that exerts an additional pathogenetic role contributing to liver damage (7). Among features of the vasculature in the liver not found in other solid tissues, are the hepatic sinusoids, the characteristics of which include the presence of liver sinusoidal endothelial cells (LSECs) that possess distinctive fenestrations and pericytes, or hepatic stellate cells (HSCs). Angiogenesis in HCC depends on the same fundamental principles of activation, proliferation and migration of endothelial cells that occur in other tumors and diseases in which enhanced angiogenesis occurs (8).

In general, it is known that tumor angiogenesis is influenced by the microenvironment and is modulated by several pro- and anti-angiogenic factors released from tumor cells, tumor-associated inflammatory cells, and/or from the extracellular matrix and by different signaling pathways. Mechanistically driven by tumor progression, these factors may be present in serum, reflecting the overall angiogenic activity of tumors (9). Since tumor progression and patient survival correlate with the serum levels of angiogenic factors (10) in several types of cancer such as HCC, they are thus ideal prognostic biomarker candidates of the chronic HCV infection process leading to cirrhosis and HCC.

In the present study, we discovered prognostic biomarkers for the chronic hepatitis C (CHC) virus infection process leading to liver cirrhosis (LC) and HCC by composite profiling of serum angiogenic factors using the Bio-Plex Pro<sup>™</sup> Human Cancer Biomarker Panel 1, a 16-plex unique blend of magnetic bead-based assays. The advantages of this multiplex approach include specimen conservation, limited sample handling, increased throughput and reduced labor costs.

### Patients and methods

*Patients*. In the current study, we enrolled 30 CHC patients (15 females and 15 males), 30 HCV-related LC patients (16 females and 14 males), 26 HCC patients (8 females and 18 males) and 20 healthy control subjects (11 females and 9 males). This was based upon our interest in studying the progression from chronic liver damage to cirrhosis and cancer. In particular, the severity of cirrhosis was defined by clinical diagnosis in the presence of liver functional insufficiency, ascites and encephalopathy (Child-Pugh score B and C) or by liver biopsies in the case of initial chronic hepatopathy without clear liver laboratory function tests of cirrhotic evolution (Child-Pugh score A). However, HCC patients had HCV-related cirrhosis, and a potentially curative resection with tumor-free margins, macro- and microscopically.

The clinical characteristics of all the study participants are listed in Table I. The patients with CHC, LC and HCC had higher serum transaminase (alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared to the control patients, as evaluated in the healthy donors. Moreover, patients with LC and cancer presented higher bilirubin and lower albumin levels and platelet counts (PLT) compared to the control patients. LC and HCC patients had normal  $\alpha$ -fetoprotein (AFP) levels and no clinical manifestations of cancer. This study was approved by the Ethics Committee of the National Cancer Institute 'G. Pascale Foundation' - Oncology Research Center of Mercogliano (CROM), Mercogliano, Italy and written informed consent was obtained from all participants.

Bio-Plex assay. Blood samples were collected from a peripheral vein and kept on ice. Serum was collected using centrifugation (3,000 rpm for 10 min at 4°C), aliquoted and stored at -80°C until analysis. A multiplex biometric enzyme-linked immunosorbent assay (ELISA)-based immunoassay containing dyed microspheres conjugated with a target protein-specific monoclonal antibody was used, according to the manufacturer's instructions (Bio-Plex; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The following soluble molecules were measured using the Bio-Plex Pro Human Cancer Biomarker Panel 1, a 16-plex multiplex immunoassay: sEGFR, fibroblast growth factor (FGF)-basic, follistatin, granulocyte-colony stimulating factor (G-CSF), soluble human epidermal growth factor receptor-2 (sHER-2/neu; ErbB-2), hepatocyte growth factor (HGF), sIL-6Ra, leptin (LEP), osteopontin, platelet-derived growth factor (PDGF)-AB/BB, platelet endothelial cell adhesion molecule-1 (PECAM-1), prolactin (PRL), stem cell factor (SCF), sTIE-2, soluble vascular endothelial growth factor receptor (sVEGFR)-1 and sVEGFR-2.

Each experiment was performed in duplicate using the procedure described in our previous studies (11-13). Serum levels of the proteins were determined using a Bio-Plex array reader (Luminex, Austin, TX, USA) that quantifies multiplex immunoassays in a 96-well plate with extremely small fluid volumes. The analyte concentration was calculated using a standard curve, with the software provided by the manufacturer (Bio-Plex Manager Software).

Data analysis and statistics. The non-parametric Mann-Whitney U test was used to evaluate differences between protein ratios in the patients and healthy controls. The T-test was used to compare the serum levels of these proteins evaluated in the different groups of patients. The correlations between the molecule concentrations and clinical/biochemical data were determined using the Pearson's correlation co-efficient. P<0.05 was considered to indicate a statistically significant difference. The statistical program Prism 4 (GraphPad Software, San Diego, CA, USA) was employed.

# **Results and Discussion**

*Comparison between patients and healthy donors.* The varying levels of the proteins present in the serum of CHC, LC and HCC patients compared to the healthy controls are presented in Table II (data not statistically significant, not shown). Higher levels of sHER-2/neu (ERbB-2), sIL-6Ra, LEP and PECAM-1 were secreted by all the patients, whereas higher levels of HGF and PRL were secreted only by LC and HCC patients.

HER-2/neu, also termed ErbB-2, is encoded by the ERBB2 gene. HER-2/neu is expressed in a variety of tissues of epithelial origin and plays a fundamental role in cellular proliferation and differentiation during fetal development. Previous studies have shown that the overexpression of this protein in HCC tissues plays a role in tumor invasion, metastasis and progression (9), underling that its amplification is not the primary mechanism in the development of liver tumors and contributes to one of the steps of multistage carcinogenesis (14).

Characteristics	CHC	LC	HCC	Control range
Age (years)	63.86	67.96	70	60.92
Gender (M/F)	15 M/15 F	14 M/16 F	18 M/8 F	9 M/11 F
AST (IU/l)	70.69	80.54	92.1	5-40
ALT (IU/l)	120.90	71.96	106.7	7-56
Total bilirubin (mg/dl)	0.91	1.70	1.68	0.20-1.30
Albumin (g/dl)	4.11	2.61	3.0	3.5-5
PLT (ml)	187,413	113,875	144,712	150,000-400,000
HCV-PCR RNA	Positive	Positive	Positive	
HCV genotype (no. of patients)	1 (18), 2 (12)	1 (22), 2 (8)	1 (15), 2 (11)	
AFP (ng/ml)	<10	<20	>20	
Child-Pugh score (no. of patients)		A (12), B (10), C (8)	A (10), B (10), C (6)	
Tumor number (no. of patients)		1 (17), 2 (4), 3 (1) >3 (4)		
Tumor invasion (no. of patients)		T1 (8), T2 (9), T3 (9)		
Tumor size (no. of patients)		<2 (3), 2-5 (5), >5 (18)		

Table I. Clinical characteristics of patients with chronic hepatitis C virus (CHC), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) with CHC-related hepatitis and LC.

We report the number of patients to which the parameters refer. For the clinical data, the mean value and the related control range, evaluated in healthy donors, are shown. M; male; F, female; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatits C virus; AFP,  $\alpha$ -fetoprotein.

Table II. Varying levels of proteins present in the serum of CHC, LC and HCC patients compared to the healthy controls.

Molecules	CHC vs. controls	LC vs. controls	HCC vs. controls
sHER-2/neu	0.0002°	0.00015°	0.0002°
HGF	0.0655	$0.0057^{b}$	0.0004°
sIL-6Ra	0.0011 <sup>b</sup>	0.0001°	0.0006°
Leptin	0.0130ª	0.0039 <sup>b</sup>	$0.0078^{b}$
PECAM-1	0.0001°	0.0003°	0.0008°
Prolactin	0.0622	0.0430ª	0.00199 <sup>b</sup>

P-values obtained for the significant molecules in chronic HCV hepatitis (CHC), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) patients, with respect to healthy control subjects using the non-parametric Mann-Whitney U test. <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.0001. CHC, chronic hepatitis C virus; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

sIL-6Ra is a soluble form of the interleukin-6 receptor and is a ligand-binding protein that constitutes the extracellular part of the IL-6 receptor. It markedly prolongs the IL-6 plasma half-life, and, after having bound IL-6, can interact with membrane-bound gp130, thereby leading to the activation of the intracellular signaling pathway (15), acting as an agonist and stimulating a variety of cellular responses, including proliferation, differentiation and the activation of inflammatory processes (16). Additionally, through this mechanism, primary unresponsive cells expressing only gp130 and no gp80 can be activated through the sIL-6R/IL-6 complex. This process has been termed 'trans-signaling' (17). Recently, we reported that IL-6 levels were elevated in CHC, LC and HCC patients (11,12). In the literature, sIL-6R overexpression has also been observed in various pathological conditions such as liver diseases and HCC, indicating that serum IL-6 and its soluble receptor levels correlate with liver function impairment as well as the degree of liver fibrosis in patients with HCV infection (18). Studies using animal models have shown that transgenic mice expressing high levels of IL-6 and sIL-6R develop hepatic nodular hyperplasia and signs of sustained hepatocyte proliferation, suggesting that IL-6 and sIL-6R may provide the primary stimulus to cell proliferation and are involved in the development of HCC (19).

LEP is mainly produced by adipose tissues, detected in activated hepatic stellate cells and, although it serves as a regulatory mediator between the brain and the periphery through modulating the hypothalamic-pituitary-adrenal (HPA) axis, its circulating level is also regulated by hormones secreted by the HPA system, including corticosteroids, PRL, and insulin (20). Recently, we found elevated LEP levels in CHC and LC patients, suggesting that this protein is part of the immune response and host defense (13) during infection and inflammation, and acts as a paracrine modulator of hepatic fibrogenesis (21,22).

PECAM-1 is constitutively expressed in platelets, monocytes, neutrophils, natural killer (NK) and CD8 T cells. It is highly expressed in continuous endothelial cells at cell-cell borders, whereas its expression is weak in sinusoidal endothelial cells (SEC) (23). PECAM-1 plays a putative role in the inflammatory process and leukocyte-endothelial interaction, particularly in the transmigration of leukocytes through intercellular junctions, and has been implicated in cell survival and angiogenesis (24). Significantly higher PECAM-1 concentrations in patients with more advanced hepatitis and varying



Figure 1. Mean concentrations of statistically significant proteins in healthy control subjects and in patients with chronic hepatitis C virus (CHC), liver cirrhosis (LC) and hepatocellular carcinoma (HCC).

fibrotic levels suggest that this protein may reflect liver disease progression (23) and that serum PECAM-1 measurement may be useful in distinguishing between patients with or without fibrosis (25).

PRL is a polypeptide hormone secreted by the anterior pituitary gland, and plays a physiological role in breast development and lactation. When produced in excess it may lead to sterility, menorrhea and loss of libido (26). LC is associated with elevated levels of PRL. In fact, this is commonly attributed to an impaired hepatic metabolism of estrogens. In particular, LC is associated with profound endocrinological disturbances and this may explain why higher levels of this protein are found in LC, but not in CHC patients. Until recently, elevated PRL levels in LC patients were considered to be induced mainly by the ineffective elimination of hormones by the diseased liver. At present, the pathogenesis of disturbed hormonal function in LC is known to be more complex, since it usually involves disturbed secretion and feedback mechanisms (27). However, high PRL levels have also been found to correlate with the severity of liver disease, particularly in patients with ascites and hepatic encephalopathy (28).

Finally, in our previous studies (11-13), HGF was found significantly upregulated in LC and HCC patients but not in

patients with CHC. This protein is a multifunctional growth factor that regulates growth and cell motility. It exerts mitogenic effects on hepatocytes and epithelial cells, and plays diverse roles in organ development, tissue regeneration and tumor progression (29). Moreover, it has been implicated, along with IL-6, IL-8 and IL-1, in the hepatic stellate cell-activation pathway. Hence, we suggested that this growth factor could be used as an index of cellular growth and of the development of HCC in LC patients (11-13).

Comparison between patients with CHC, LC and HCC. We compared the mean concentrations of these 6 molecules in 3 patient groups using the Student's t-test. As shown in Fig. 1, the concentrations of sHER-2/neu, sIL-6Ra, LEP and PECAM-1 were higher (P<0.05) in patients with LC compared to those with CHC, while the concentrations of sHER-2/neu, HGF, sIL-6Ra, LEP, PECAM-1 and PRL were higher in HCC patients compared to those with LC. Hence, the expression of these 4 molecules, i.e., sHER-2/neu, sIL-6Ra, LEP and PECAM-1, tends to increase in the progression of chronic inflammation leading to LC and HCC. Consequently, their evaluation may be used for prognostic studies and therapy guidance.



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Figure 2. Ingenuity pathway analysis (IPA) of statistically significant molecules. The graph shows the closely associated network for significant cytokines, presented in Table II (highlighted with green and cyan symbols), and other molecules (highlighted with white symbols), obtained using the IPA software.

Of note, HGF and PRL levels varied significantly in LC patients compared to the healthy controls and CHC patients, while their concentrations in HCC patients were higher compared to patients with LC. This suggests that the levels of HGF and PRL may have increased during the progression of chronic inflammation to LC and cancer. Therefore, we suggest that HGF and PRL may represent the degree of the carcinogenic state in the liver of chronic inflammation and LC patients, and may potentially be used for predicting the progression to HCC in patients with chronic HCV-related liver disease.

The serum levels of the significant proteins in CHC, LC and HCC patients were then compared with clinical/biochemical data using Pearson's correlation co-efficient. sIL-6Ra showed a significant correlation (P<0.01) with the fibrotic stage in CHC patients, with Child-Pugh score in patients with LC and with tumor size in patients with HCC. These results confirm that sIL-6Ra may be used as a predictor of liver damage and of inflammatory processes leading to fibrosis, cirrhosis and subsequently, to cancer.

Interactomic analysis. The abovementioned molecules were analyzed using the Ingenuity Pathway Analysis (IPA) software version 7.1 (Ingenuity Systems, Inc., Redwood City, CA, USA) that has created a network on the basis of associated functions and data mining from experimental studies reported in the literature (Fig. 2). This graph presents 2 hub genes, signal transducer and activator of transcription (STAT3) and estrogen receptor 1 (ESR1).

In particular, STAT3 is activated in response to various cytokines and growth factors, including IL-6 and LEP. In fact, the binding of LEP or IL-6 to their receptors triggers the signal transduction through the stimulation of JAK2-STAT3 pathway (30,31) and induces the phosphorylation of its tyrosine 705. However, phosphorylated STAT3 dimerizes and translocates to the nucleus, where it regulates gene transcription. Thus, it can be hypothesized that in these patients, the higher level of LEP, IL-6 and its receptor may induce increased STAT3 expression and consequently, an increase in angiotensinogen (AGT) and chemokine (C-C motif) ligand 2 (CCL2) levels.

ESR1 is associated with ERbB-2 and PRL receptor (PRLR) at the plasma membrane (32). Notwithstanding, ERbB-2 is frequently co-activated with EGF and c-Met that binds HGF. In particular, this growth factor regulates cell growth, cell motility and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor that is highly expressed in the liver (33). These data prove the existence of a correlation between 5 statistically significant proteins, ErbB-2, sIL-6Ra, PRL, HGF and LEP.

In conclusion, in the present study, we evaluated the analytical performance of the Bio-Plex Pro Human Cancer Biomarker Panel 1, a 16-plex unique blend of magnetic bead-based assays, in the serum of CHC, LC and HCC patients for the composite profiling of angiogenic factors. Our results demonstrate that: i) high levels of HGF and PRL in LC and HCC patients represent the degree of the carcinogenic state in the liver of chronic inflammation, ii) high levels of sHER-2/neu, sIL-6Ra, LEP and PECAM-1 in CHC, LC and HCC patients suggest that these 4 proteins are markers of the chronic inflammation progression that leads to LC and HCC and iii) sIL-6Ra correlates with the fibrosis stage in CHC patients, with the Child-Pugh score in patients with LC, and with tumor size in patients with HCC, confirming that this protein may be used as a predictor of liver damage and inflammatory process leading to liver fibrosis and cirrhosis and subsequently, to cancer.

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