RESEARCH ARTICLE

Serum cytokine levels in patients with hepatocellular carcinoma

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ABSTRACT. The role played by the microenvironment in cancer induction, promotion and progression is crucial. Emerging evidence suggests that cytokines, chemokines and growth factors are major players in carcinogenesis. Therefore, a detailed understanding of factors and mechanisms associated with the processes leading from inflammation to cancer could improve the therapeutic strategies against this disease. We have used hepatocarcinoma as our model in this study. We evaluated the serum levels of 50 different cytokines, chemokines and growth factors in patients affected by HCC with chronic HCV-related hepatitis and liver cirrhosis using multiplex biometric ELISA-based immunoassay. Our data showed that some pro-inflammatory molecules were significantly up-regulated in these patients, and highlighted the complexity of the cytokine network in this disease. This work suggests the need to monitor these proteins in order to define a profile that could characterize patients with HCC or to help identify useful markers. This could lead to better definition of the disease state, and to an increased understanding of the relationships between chronic inflammation and cancer.

Keywords: hepatocarcinoma, HCV, cytokines, chemokines, cancer, growth factors

Over the past several years, there has been a renaissance of research into connections between inflammation and cancer [1, 2]. Inflammation is a physiological process, the inflammatory process being a crucial function of the innate immune system in its response to acute tissue damage, whether resulting from physical injury, ischemic injury, infection, exposure to toxins, or other types of trauma. It can play a role in tumor suppression by stimulating an antitumor immune response, but more often, under certain conditions, it appears to stimulate tumor development [3]. The intensity and nature of the inflammation could explain this apparent contradiction [1]. Inflammation may become chronic either because an inflammatory stimulus persists or because of dysregulation of the control mechanisms that normally turn the process off. Indeed, inflammation associated with cancer is similar to that seen with chronic inflammation, which includes the production of growth and angiogenic factors that stimulate tissue repair, factors that can also promote cancer cell survival, implantation, and growth [4]. Thus, the immune response can promote anticancer affects, or carcinogenesis and tumor growth [1, 2]. Many cancers arise from sites of infection, chronic irritation, and inflammation; thus, it is now clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, altering not only the metabolic needs of the tissue, but also fostering DNA and protein damage, proliferation, survival, mutagenesis, migration and metastasis of malignant cells [5, 6]. Indeed, all tumors in the presence of stromal and infiltrating inflammatory cells are facilitated and helped to maintain these metastatic processes [7]. Leukocytes, lymphocytes and other inflammatory cells are activated in this process and attracted to the inflamed site. Inflammation contributes to initiation by inducing the release of a variety of pro-inflammatory cytokines, chemokines, growth factors and inflammatory enzymes such as cyclo-oxygenases. These stimulate the vasculature to release inflammatory cells and factors into the tissue milieu, thereby causing oxidative damage, DNA mutations, and other changes in the microenvironment, making it more conducive to cell transformation, increased survival and proliferation [8]. We must not forget that many cytokines and chemokines are inducible by hypoxia, which is a major physiological difference between tumor and normal tissue [9]. An important aspect of the tumor microenvironment is the cytokine-mediated communication between the tumor and other cells. Cytokines and chemokines have many activities that permit cell-cell communication locally at the tissue, with an outcome determined by the cytokine concentration milieu and cell type [7].

Our study model is hepatocarcinoma (HCC), being a multi-state condition depending on a combination of many genetic, viral and environmental factors [10]. The

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pathway leading HCC generally begins with an acute hepatic insult that is associated with either chronic hepatitis C virus (HCV) or hepatitis B virus (HBV) infection [11], and progresses over decades. Fibrosis and cirrhosis are typically precursors of HCC [12].

In this paper, we examined serum levels of cytokines, chemokines and growth factors in HCC patients with HCV-related cirrhosis and, in particular, the role of pro-inflammatory cytokines in these patients.

DONORS AND METHODS

Patients

Twenty six patients (8 women, 18 men) with HCC were enrolled in the study. These patients had underlying HCV-related cirrhosis, and had Child's A cirrhosis in preoperative assessment. All patients had a potentially curative resection with tumor-free margins, macroscopically and microscopically. This choice was based upon on our interest in studying the differences in serum levels of cytokines between healthy subjects and patients in which chronic inflammation had promoted cancer. The clinical characteristics of these patients are listed in *table 1*. Their ages ranged from 57 to 84 years. In addition, 20 healthy subjects, matched for gender and age, were included as controls. The serum concentrations of about fifty cytokines, chemokines and growth factors were evaluated in all patients and healthy controls.

Table 1
Characteristics of HCC patients. We report the number of patients to which each parameter refers

	N°
Mean age (range), y	70 (57-84)
Age, y	
- < 70	11
- > 70	15
Gender	
- Male	18
- Female	8
Tumor number	
- 1	17
- 2	4
- 3	1
- Multifocal	4
Tumor invasion	
- T1	8
- T2	9
- T3	9
Tumor size, cm	
- < 2	3
- 2-5	5
- > 5	18
Metastases	
- Yes	9
- No	17
AFP, ng/dL	
- < 50	17
- > 50	9

BioPlex assay

Blood samples were collected from a peripheral vein and kept on ice. Serum was collected by centrifugation (3,000 rpm for 10 min at 4°C), aliquoted, and stored at - 80°C until analyzed. A multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used according to the manufacturer's instructions (Bioplex, Bio-Rad Lab., Inc., Hercules, CA, USA). Soluble molecules were measured using two commercially available panel:

1) 27-Plex panel: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, CXCL10, MCP-1, MIP-1α, MIP-1β, PDGF-ββ, RANTES, TNF-α, VEGF;

2) 21-Plex kit: IL-1α, IL-2ra, IL-3, IL-12p40, IL-16, IL-18, CCL27, CXCL1, CXCL9, CXCL12, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, β-NGF, SCF, SCGF-β, TNF-β, TRAIL.

Each experiment was performed in duplicate. Briefly, 30 μL serum samples were diluted 1:4 with suitable buffer and incubated with antibody-coupled beads. Complexes were first washed, then incubated both with biotinylated detection antibody, and, finally, with streptavidin-phycoerythrin prior to assessing cytokine concentration titers. Concentrated human recombinant cytokine was provided by the vendor (BioRad Lab., Inc.). A broad range, 1.951-32,000 pg/mL of standards, was used to establish standard curves to maximize the sensitivity and dynamic range of the assay. Cytokine and growth factor levels were determined using a Bio-Plex

Table 2
The U test and P values obtained for all significant molecules in HCC patients using the nonparametric Mann-Whitney U test (see Methods section)

	U-test	P-value	
CCL27	83	0.0016	**
CXCL1	106	0.0114	*
CXCL10	22	< 0.0001	***
CXCL12	103	0.009	**
CXCL9	5	< 0.0001	***
G-CSF	114.5	0.0132	*
GM-CSF	12	0.0117	*
IFN-α2	98	0.006	**
IFN-γ	99	0.0066	**
IL-10	28	0.0214	*
IL-12p40	60	0.0005	***
IL-1α	107	0.0123	*
IL-1β	89	0.001	**
IL-3	97	0.0056	**
IL-4	99.5	0.0068	**
IL-6	119	0.0183	*
IL-8	54	0.0005	***
M-CSF	89	0.0028	**
PDGF-ββ	65	0.0002	***
SCF	85	0.002	**
SCGF-β	60	0.0323	*
TNF-β	89	0.0028	**
β-NGF	88	0.0026	**

array reader (Luminex, Austin, TX). This instrument quantitates multiplex immunoassays in a 96-well format with very small fluid volumes. The analyte concentration was calculated using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software). A regression analysis was performed to derive an equation that was then used to predict the concentration of these proteins in serum samples.

Data analysis and statistics

Data were analysed using the Bio-Plex Manager software version 3.0 (Bio-Rad Lab.). Sample concentrations were immediately interpolated from the standard curves. The single values were considered to be elevated when results were higher than the mean + 2 standard deviations (SD) of the controls. The nonparametric Mann-Whitney U test was used to evaluate differences between cytokine and growth factor ratios from patients with HCC and healthy individuals. It distinguished with one asterisk (*) values where $p < 0.05$, with two asterisks (**) values where $p < 0.01$, and with three asterisks (***) values where $p < 0.001$. The correlations between the cytokine levels and clinical data were determined using the Pearson correlation coefficient. A probability value of $p < 0.05$ was considered to be statistically significant. The statistical program Prism 4 (GraphPad Software, San Diego, CA, USA) was employed. The concentrations of

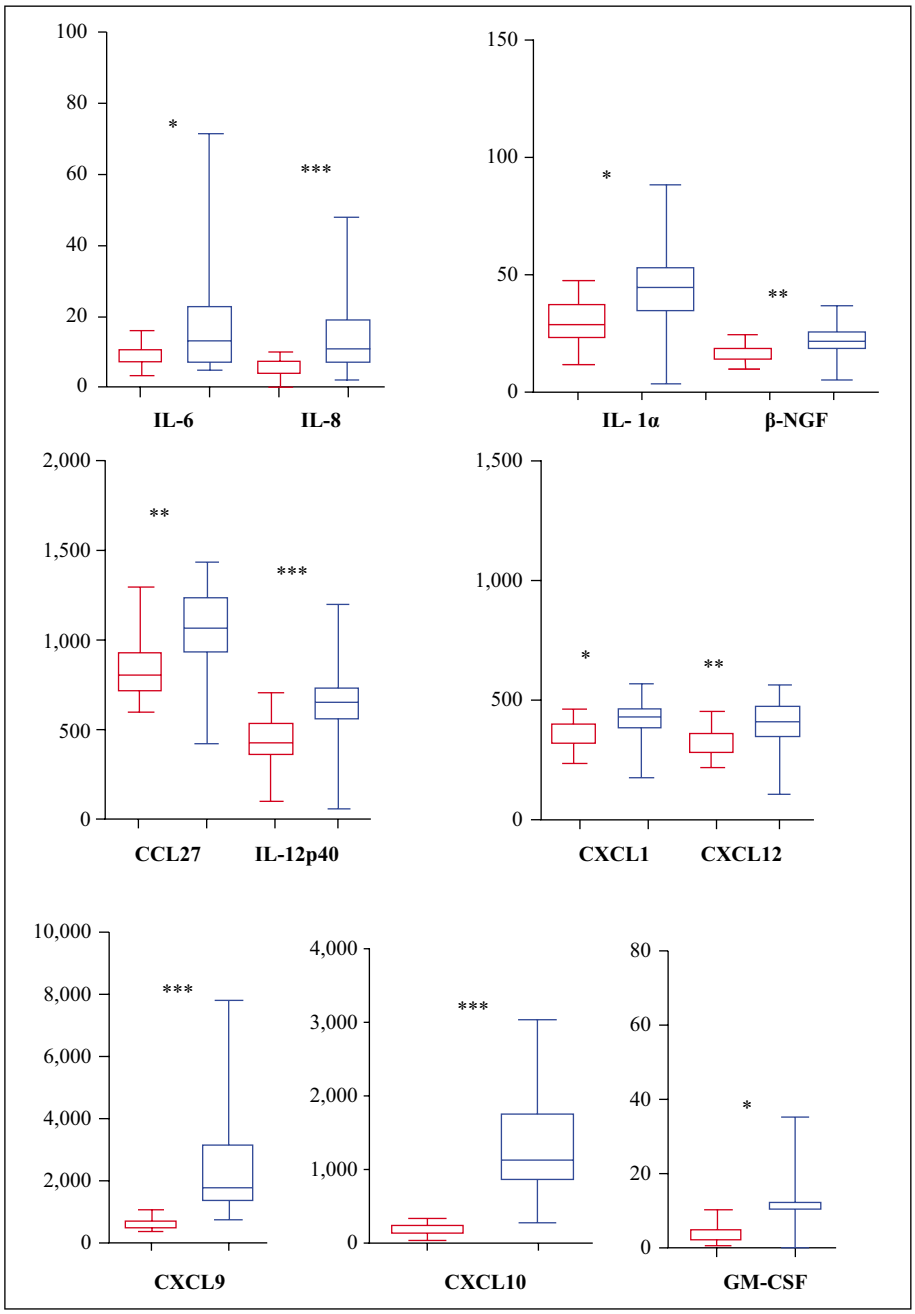

Figure 1
The pro-inflammatory cytokine levels from controls and HCC patients were plotted with red and blue box-and-whisker graphs, respectively. The boxes extend from the 25th to the 75th percentile, and the line in the middle is the median. The error bars extend down to the lowest value and up to the highest.

pro-inflammatory molecules (*i.e.* cytokines, chemokines and growth factors) were analyzed with the Cluster 3.0 program using a clustering algorithm applied by similarity metrics based on a Pearson correlation that builds a hierarchical structure among objects (molecules and patients) and shows a correlation [13]. The TreeView program was used for visualizing and browsing the clustered data.

RESULTS AND DISCUSSION

In *table 2* we reported the cytokines, chemokines and growth factors that showed statistically significant results; data that were not statistically significant were not reported.

We saw that the HCC patients showed a different secretion profile of these proteins compared to healthy controls. Greater amounts of IL-1α, IL-3, IL-12p40, IL-6, IL-8, IL-10, CCL27, CXCL10, CXCL1, IFN-α2, M-CSF, GM-CSF, CXCL9, β-NGF, SCF, SCGF-β, CXCL12, TNF-β were secreted by the HCC patients.

No correlation was observed between serum levels and patients age/gender or between patients with a solitary tumour and those with multiple tumours.

In particular, we focused our attention on the pro-inflammatory molecules (IL-1α, IL-6, IL-8, IL-12p40, GM-CSF, CCL27, CXCL1, CXCL9, CXCL10, CXCL12, β-NGF). These were found to be significantly increased in HCC patients compared to healthy controls *(figure 1)*. The significantly increased serum levels of IL-6 and IL-8 found in our HCC patients are in agreement with data reported in other studies [14, 15]. IL-8 levels measured in HCC patients were found to be increased, and correlated significantly with large tumor size (> 5 cm) [14, 15]. This suggested that IL-8 may be involved in disease progression and might prove to be both a useful marker of tumor invasiveness and an independent prognostic factor for HCC patients [15]. In our work, IL-8 and IL-6 concentrations correlated significantly with large tumor size (p-value < 0.05 and R > 0.83). This result

A

	IL-8	GM-CSF	CXCL10	IL-1α	IL-12p40	CCL27	CXCL1	CXL9	β-NGF	CXCL12
IL-6	0,026	0,556	0,290	0,549	0,641	0,405	0,873	0,312	0,914	0,434
IL-8		0,900	0,266	0,297	0,772	0,146	0,246	0,638	0,552	0,154
GM-CSF			0,057	0,456	0,797	0,828	0,470	0,619	0,351	0,446
CXCL 10				0,806	0,876	0,888	0,795	0,337	0,620	0,557
IL-1α					< 0,0001	0,014	< 0,0001	0,062	< 0,0001	< 0,0001
IL-12p40						0,122	< 0,000	0,282	< 0,0001	0,000
CCL27							0,001	0,330	0,005	0,001
CXCL1								0,061	< 0,0001	< 0,0001
CXCL9									0,054	0,364
β-II GF										< 0,0001

B

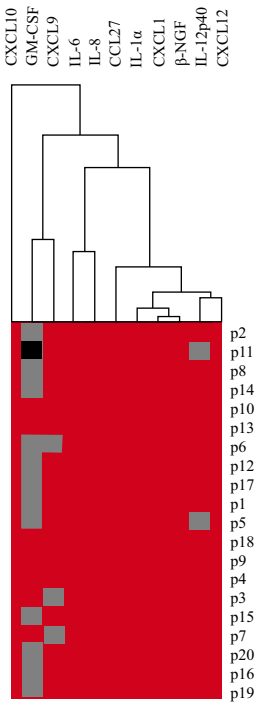

Figure 2

Correlations between the pro-inflammatory cytokines, chemokines and growth factors that were statistically significant in the comparison between HCC patients and controls: **A)** a p-value evaluated between the cytokine concentrations by using the Pearson correlation and **B)** protein hierarchy assessed by a computational clustering analysis. More specifically, the length of branches indicates, in inverse proportion, the similarity of protein concentrations, and the scale of protein intensity is indicated by the different colors: over-expressed cytokines, chemokines and growth factors in red, lower values in grey, and values equal to zero in black.

also confirms the clinical significance of IL-6 as a prognostic factor of cancer and, in particular, its association with the development of HCC [16-18].
Moreover, IL-12 has a strong anti-angiogenic activity and stimulates the production of CXCL10 [19]. This could explain the fact that both these proteins were found to be over-expressed in our HCC patients. However, all five chemokines (CXCL1, CXCL9, CXCL10, CXCL12, CCL27) are involved in the angiogenesis and inflammatory processes. CXCL1 has roles in angiogenesis, inflammation and tumor genesis and is involved in melanoma pathogenesis [20]. CXCL9 is closely related to CXCL10, and increased levels of both were detected in serum from HCC patients and in culture supernatants from HCC cell lines [21]. In our work, CXCL10 levels correlated both with any tumour size and with transaminase levels. This suggests that it could be used as marker of liver inflammation status and cancer progression. CXCL12 is induced by pro-inflammatory stimuli and plays a role both in pathogenesis by promoting tumor growth and malignancy, and in the HCC metastatic network by recruiting endothelial cell tumor progenitors [21-23]. CCL27 is involved in skin inflammation processes because it is associated with memory T lymphocytes homing; no role for them in cancer has yet been reported in the literature [24]. GM-CSF induces activation, differentiation, growth of granulocyte and monocyte precursors, and is involved in inflammatory processes *via* macrophage activation and proliferation [25]. The over-expression of GM-CSF in HCC patients may be due to cancer cell growth [26]. Recently, some papers have suggested that β-NGF is involved in cancer growth and metastasis [27]. In particular, in 2007, β-NGF was detected in diseased liver tissues, and has been suggested to be involved in chronic inflammation leading to cancer [28].
Moreover, we evaluated correlations between the concentrations of over-expressed pro-inflammatory molecules measured in our patients. The data showed that β-NGF correlated with IL-1α, IL-12p40, CCL27, CXCL1 and CXCL12 *(figure 2A)*. This is confirmed by the related computational clustering analysis which shows that the molecules cluster in two groups, as demonstrated by branches joining them; β-NGF is grouped with the proteins indicated above *(figure 2B*). Therefore, we suggest that a panel composed of β-NGF and these five proteins may be useful for diagnostic/prognostic purposes.
In conclusion, our work represents a preliminary screening study that identifies candidate cytokines, chemokines and growth factors that may be involved in the chronic inflammation processes versus HCC, and in its progression. Further studies will consider:
– the inclusion of more patients and their monitoring at different times in order to define which molecules might used as prognostic tools;
– the involvement of chronic hepatitis C patients without HCC, as controls, in order to distinguish between cytokines, chemokines and growth factors induced by HCV-induced chronic inflammation, and those secreted by hepatocarcinoma;
– the validation of these results using other experimental methods.

Disclosure. *None of the authors has any conflict of interest to disclose.*

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