Evaluation of Apoptotic Marker Bcl2, CD4+, Human Hepatocyte Growth Factor and Metalloproteinase-9 as Tumor Markers for Patients with Hepatocellular Carcinoma

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Abstract To examine the possible involvement of human B cell leukemia/lymphoma 2 (Bcl-2), CD4+ cells, hepatocyte growth factor (HGF), and metalloproteinase-9 (MMP-9), as biomarkers in early diagnosis of hepatocellular carcinoma (HCC), activities of these biomarkers in serum were demonstrated by the method of Enzyme Linked Immunosorbant Assay. Two groups of subjects (60 for each), were examined in this study; healthy controls and patients with HCC. The present results declare that, significant decrease in Bcl-2 ($p \leq 0.0001$), and CD 4+ ($p \leq 0.001$), while significant increase in HGF and MMP-9 ($p \leq 0.05$). These findings imply an influence of these biomarkers by the existence of hepatic carcinoma that might reflect the progression of disease and a distinction between the pathological mechanisms involved in hepatic carcinoma. Since, the serum MMP-9 activity was significantly varied between each stage of HCC. An individual

profile of the present investigated parameters was detected that might serve as an easy accessing serum marker to monitor the progression of hepatic cell disorders.

Keywords HCC · HGF · MMP-9 · CD4+ and Bcl-2

Introduction

Hepatocellular carcinoma (HCC), is the fifth most frequent human cancer, with the highest frequency found in sub-Saharan Africa and far eastern Asia, where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic and food is contaminated by Aflatoxin B1 [1]. Hepatocellular carcinoma incidence is rise in Europe and United States, due to increased incidence of hepatitis C virus infection, cirrhosis related to type II diabetes, and non-alcoholic steatohepatitis [1]. HCC is a rapid fatal disease, with a life expectancy of about 6 months from the time of the diagnosis. Therapies with pharmacological agents or alternative strategies do not improve substantially the prognosis of patients with unresectable HCC [1, 2]. This emphasizes the need to investigate the contribution of different biomarkers pathways to tumor development in different HCC, selected according to their clinical and pathological features, to identify novel biomarkers targets for early diagnosis, chemoprevention, and treatment. HCC therapy may take advantage from the identification of markers and targets specific for the different tumor stages. Several common morphological, biochemical and biological aberrations have been found between human and rodent preneoplastic and neoplastic liver lesions [3], indicating that, independently of the etiological factors, the basic mechanisms of HCC development are similar in different species. Therefore, strategies for the identification of new markers and targets should include the specific

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biomarkers changes in the early stages of liver carcinogenesis, relevant to the progression of preneoplastic lesions towards full malignancy. This may be done in rodent models, and must be followed by the functional evaluation of new markers and targets in human HCC [4].

The Multistep Process of Hepatic Carcinogenesis

Hepatic injury induced by risk factors causes necrosis followed by hepatocyte proliferation. Repeated cycles of necrosis-liver regeneration foster a chronic liver disease leading to cirrhosis. Cirrhosis is characterized by the formation of hyperplastic liver nodules, surrounded by collagen deposition and scarring of liver, and regenerative nodules. During this process different subtypes of foci of altered hepatocytes (FAH) develop in the liver, followed by low-grade dysplastic nodules (DNs), and then by high-grade DN, considered premalignant lesions, composed of small cells showing mild trabecular disarrays, increased nuclear-cytoplasmic ratio, and tendency to cytoplasmic basophilia. Subsequently, HCC develop which can be further classified into well differentiated, moderately differentiated and poorly differentiated tumors [4].

Angiogenesis is an essential process for both tumor development and metastasis. HCC is characterized by unusual hypervascularity, indicating the crucial role of angiogenesis in tumor development and the potential for therapeutic benefit by interfering with angiogenesis [5]. Many factors and regulatory pathways involved in angiogenesis of HCC, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, basic fibroblast growth factor, and their related pathways, have been described [6].

However, the molecular mechanisms orchestrating the dominant vascularization of HCC are largely unknown [6]. Thus, the present study aims to investigate some of these regulatory pathways including, apoptotic markers Bcl-2, CD4+ T cells, HGF and MMP-9 to demonstrate their role in the development of HCC and evaluating their possible application in serving as a serum biomarker.

Methods and Subjects

Chemicals

All chemical were of analytical grade and were purchased from Sigma Chemical Company, USA.

Methods

The informed consents were taken from the patients of our studied group according to guideline of the Medical Ethical

Committee of National Research Centre, Dokki, Giza. All the studied groups were subjected to full history report including personal history, complete present history, family history, social history and past history.

Clinical Examination and Subjects

This study included one group of healthy control subjects and other of patients with hepatocellular carcinoma. Samples were given from National Institute of Endemic Diseases Research and Liver, Cairo, Egypt. Each group consisted of 60 subjects. Blood was drawn by vein puncture into Venoject tubes in the morning after an overnight fast. Blood was centrifuged at 3000 rpm and serum was used for all the laboratory determinations including the apoptotic marker (Bcl-2), CD4+ T cells, HGF, and the activity of MMP-9. Plasma samples in all study subjects were collected at the time of diagnosis or before surgical resection. Samples were kept at 80 °C sensitivity of the assay was 3 pg/ml for the Bcl-2 and CD4+ T cells and was 125 pg/ml for HGF and MMP-9. Plasma concentration below these levels was considered non- detectable. All assays were performed independently by the laboratory personnel who did not have clinical information.

For healthy controls, blood samples were drawn during a medical checkup and were normal for blood biochemical and immunological examinations. Of the 60 HCC patients, 56 had undergone surgical resection and had a pathological proof for HCC; the diagnosis of the remaining 4 patients was established from characteristic imaging findings in ultra sonography, computed tomography scan and hepatic angiography along with progressively elevated serum AFP levels, according to the diagnostic criteria by the European Association for the Study of the Liver [7].

Determination of Serum Apoptotic Marker Human B-cell Leukemia/Lymphoma 2(Bcl2), CD4, HGF and MMP-9

The serum activity of human B-cell leukemia/lymphoma 2(Bcl2) was measured by Enzyme Linked Immunosorbant Assay (ELISA).

Statistical Analysis

The data were presented as mean $\pm$ SD of 120 subjects. The statistical significance of the means was determined by *T*-test (SPSS), Statistical Computer Program, where $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (***). Correlation between different biomarkers in the control and HCC groups was analyzed using Person's correlation analysis.

Results

The present result shows that, Bcl-2 significantly decreased ($p \leq 0.0001$), in serum of patients with HCC as compared to the normal healthy subjects with percentage decrease amounting to 54.64 %. Also, significant decrease was detected in CD4+ cells ($p \leq 0.001$), in patients with HCC (52.25 %), and in comparison with the normal control. However, significant increase was noticed in HGF and MMP-9 ($p \leq 0.05$), in serum of HCC patients with percentage increase reached to 110.65 and 152.04 %, respectively (Table 1; Fig. 1). In addition, significant correlation was noticed between different demonstrated biomarkers; Bcl-2, CD4+ cells, HGF and MMP-9 in HCC patients at $p \leq 0.01$ (Table 2).

Discussion

Most anticancer therapies kill cancer cells primarily by apoptosis. Inactivation of tumor cell apoptosis contributes to the development of cancer spreading and resistance to therapy [8]. It has been suggested that another response to therapeutic interventions can involve autophagy and autophagic cell death. Autophagy is activated in response to g-irradiation and various anticancer drugs [9], and can also be considered as a tumor suppressor mechanism since defects in autophagy profoundly perturb organism development. Beclin1, promotes carcinogenesis in mice, especially liver cancer [10]. The present result demonstrates significant decrease in Bcl-2 in patients with HCC as compared to the normal control subjects. It has been demonstrated that, hepatoma and primary HCC cells have lower autophagic activity than normal hepatocytes [11].

Table 1 Levels of Bcl-2, CD4+, HGF and MMP-9 in controls and HCC subjects

Parameters	Groups	Mean $\pm$ deviation
Bcl-2	Cont	3.88 $\pm$ 0.50
	HCC	2.12 $\pm$ 0.28***
CD4 +	Cont	4.90 $\pm$ 0.67
	HCC	2.56 $\pm$ 0.23**
HGF	Cont	4.60 $\pm$ 0.67
	HCC	9.69 $\pm$ 0.94***
MMP-9	Cont	289.69 $\pm$ 24.32
	HCC	440.44 $\pm$ 65.48***

Data are expressed as mean $\pm$ SD of 120 subjects; control and HCC patients

Bcl-2, CD4+, HGF and MMP-9 are expressed in ng/ml

The statistical significance of the means was determined by T-test (SPSS), Statistical Computer Program, where, $p \leq 0.001$ (**) and $p \leq 0.0001$ (***)

Fig. 1 % change of different biomarkers in patients with HCC

Table 2 Person's correlations between different biomarkers

Parameters	Bcl-2	CD4	HGF	MMP-9
Bcl-2	1	0.935(**)	.985(**)	.945(**)
CD4		1	0.975(**)	0.946(**)
HGF			1	0.980(**)
MMP-9				1

** Correlation is significant at $p \leq 0.01$ level (2-tailed)

Acquired drug resistance to chemotherapy may cause resistance to cell-cycle arrest and autophagy [12]. COX-2 inhibitors may exert their anticancer activity by slowing down cell proliferation and inducing autophagy and cell-cycle arrest [13, 14]. Cell-cycle progression is promoted by a number of cyclin- dependent kinase which has been complex with regulatory proteins, cyclins, to drive the cell through the cycle. They are negatively regulated by cell-cycle inhibitory proteins cyclin-dependent kinase inhibitors (CKI) [13–15].

The cross talk between autophagy and apoptosis is partly mediated by the functional and physical interaction between Beclin1, an essential autophagy gene, or Bax, the best known pro-apoptotic factor, and Bcl-2, one of the programmatic apoptosis-inhibitory proteins [16]. When Bcl-2 interacts with calcineurin, it allows extracellular signal-regulated kinase (ERK) phosphorylation by Raf 1 and cell-cycle progression. Thus, Bcl-2 function seems to depend on its localization, since only endoplasmic reticulum- targeted Bcl-2 inhibits autophagy and only mitochondria-targeted Bcl-2 inhibits apoptosis [16]. Cytosolic Bcl-2 might actively maintain cell survival by favoring cell-cycle progression [8].

Among growth factors identified to be involved in HCC progression, the HGF/c-MET pathway has been demonstrated

to play a key role in growth, epithelial-mesenchymal transition, angiogenesis, invasiveness and metastasis in HCC cells [17]. c-MET has also been reported to be essential for the metastatic potential of HCC cells and represents as a powerful prognostic indicator for the early stage of cancer invasion and metastasis [17]. Our data indicates that, significant increase in HGF in HCC patients that might be due to forced expression of miR-198 abrogated HGF stimulated p44/42MAPK activation and subsequent cell migration and invasion by diminishing c-MET expression. It should be of note that expression of microRNA-198(miR-198) itself exerted no effect on cell migration and invasion, thus it should be considered as a conditional metastasis suppressor in HCC. Future efforts will be taken to determine whether miR-198 will impinge on other signaling pathways involved in progression of HCC [18].

Metastasis is the leading cause of death in patients with hepatocellular carcinoma and micro RNAs have been implicated to influence this process. Emerging evidence indicates that miR-198 is down-regulated in HCC as compared to normal liver parenchyma, but the functional roles of miR-198 in HCC cells remains unexplored. It was found that miR-198 directly targets c-MET via its 3' UTR. Forced expression of miR-198 decreased c-MET expression at both mRNA and protein levels and consequently diminished HGF induced phosphorylation of p44/42 MAPK in HCC cells. Forced expression of miR-198 inhibited HGF promotion of HCC cell migration and invasion in a c-MET dependent manner [18].

In this study we have determined whether the plasma levels of HGF, is increased in patients with HCC disease. HGF is produced in various organs of the body and is characterized as a multifunctional protein with various biological activities. Interestingly, although increased HGF levels in serum or tissue have been reported in patients with HCC, in other study [19], there was no significant difference of the HGF levels in patients with HCC, non-HCC tumor and chronic hepatitis group [7]. However, patients in these three groups did have a higher HGF levels as compared to normal subjects. It is well known that HCC is frequently associated with chronic hepatitis B or C infection and liver cirrhosis. A major function of a tumor marker is that the marker should be able to differentiate patients who truly have cancer from those who do not. HGF levels may also be elevated in liver disease-associated conditions other than HCC, suggesting measurement of HGF level is not helpful in differentiating HCC from other non-HCC conditions [7]. Consistent with our finding is that a previous study failed to support a direct relationship of HGF and human HCC and argued against the role of HGF in HCC [7]. Alternatively, the method to measure HGF level in blood could also confound the results. We have used plasma for HGF measurement because it has been reported that serum HGF level does not strictly reflect the blood

level due to the release from leukocytes during sample preparation (i.e., blood clotting), and plasma is more suitable for assay of blood HGF.

The present results clearly indicate significant decrease in CD4+ cells in patients with HCC. In spite of the severe prognosis in most HCC patients, increasing evidence suggests that immune response to HCC, in at least a fraction of the patients, may impact on the clinical evolution of the disease and even on survival. Early evidence indicated that small tumors with marked T cell infiltrate were associated with a lower recurrence rate and higher 5-year survival rate compared to a large set of tumors without such T cell infiltration [20]. In that study, most of the lymphocytes infiltrating the tumor tissue were identified by immune histochemistry as T cells with a predominance of the CD8+ over the CD4+ subset which demonstrates significant reduction [20]. These initial results were consistent with a protective effect against tumor growth T cell-mediated immune response directed to HCC-associated antigens. This hypothesis has been subsequently corroborated by the identification of antigen-specific T cells (at tumor site and in periphery), in HCC patients, with specificity for different classes of tumor-associated antigens including cancer testis, self antigens as AFP and SART-1, as well as viral epitopes [21].

By multivariate analysis the authors found that a high CD4+:CD8+ T-cell ratio had a predictive value greater than the Milan criteria [22], or tumor grade, and was equivalent to vascular invasion for predicting tumor recurrence. These results are consistent with the hypothesis that CD4+ HCC-specific T cells, with a “T Helper”, rather than with a “T regulatory” phenotype, may play a significant protective role against tumor recurrence, possibly by providing help for functional differentiation of HCC-specific CD8+ T cells [22]. In consistent with previous evidence, the lack of adequate CD4+ T cell help at tumor site may explain the defective CD8+ T cell maturation to effector and/or to effector memory stages. HCC were able to inhibit autologous CD8+ T cell proliferation. By considering also previous reports of Yang et al. [23], who concluded that Tregs can inhibit the activation of tumor-specific CD8+ T cells, thereby contributing to the uncontrolled growth of HCC cells. This is an important observation that, along with those already reported for other human tumors like melanoma, gastrointestinal and ovarian cancer, and non-Hodgkin's lymphomas [24], emphasizes the potential role of Tregs in determining the prognosis of cancer patients and, therefore, the crucial function of other different tumor cytotoxic CD8+ T cell subsets in limiting the growth and progression of the neoplastic disease [25]. It has become apparent that CD4+ CD25+ T-cells phenotypically similar to natural Treg cells block effective tumor immunity [26]. Although, several authors have reported increased number of CD4+ CD25+ Treg cells in the peripheral blood (PB) of cancer patients.

There are only a few studies that described an increase of CD4+ CD25+ Treg cells in tumor-infiltrating lymphocytes (TILs) or tumor-associated lymphocytes [27–29]. Woo et al. [28], has reported that CD4+ CD25+ T-cells are increased in the TILs of patients with lung cancer CD4+ CD25+ T-cells isolated from these tumors strongly inhibited the proliferation of autologous PB T-cells stimulated by anti-CD3/CD28. Unitt et al. [30], showed that CD4+ CD25+ Foxp3+ Treg cells were increased in HCC, and exceeded the proportion present in distant non-tumor tissues. The Treg cells isolated from HCC have been shown to suppress the proliferation of autologous CD8+ T-cells. A slight decrease in the proportion of circulating CD4+ CD25+ Treg cells was also observed in HCC patients when compared with healthy individuals. The decreased proportion of CD4+ CD25+ T-cells in the PB of patients with HCC could possibly result from increased migration of Treg cells to the marginal regions of the tumor.

It was found that, the adult human liver contains large numbers of lymphocytes with a unique phenotypic distribution compared with the PB and other organs, and have distinctive features in the immune system. This may be related to TGF- β induces native CD4+ cells to become CD25+ regulatory cells that have properties indistinguishable from natural CD4+ CD25+ Treg cells [30]. These CD4+ CD25+ Treg cells prevented CD8+ T-cells from proliferating in response to alloantigens and from becoming cytotoxic effector cells [26].

Concerning, degradation of the extracellular matrix (ECM), is thought to be a prerequisite for cancer invasion and metastasis which strongly affects the prognosis of cancer patients [31]. Recent investigations have indicated that the matrix metalloproteinase (MMP) family plays important roles in the invasion of cancer cells by digesting the ECM, and tumor cells with highly invasive characteristics have been shown to secrete large quantities of these proteolytic enzymes [32]. It was found that, MMP-2 and MMP-9 mRNA expression were more positively correlated with liver metastasis, [32]. Hepatocellular carcinoma is considered as a common malignancy in oriental countries, the invasive ability of HCC can be characterized by the infiltration of cancer cells into the fibrous tumor capsule. Moreover, approximately 90 % of HCC cases are associated with chronic liver disease in which the ECM is markedly enriched. These observations led us to speculate that the MMP family may be involved in determining the invasion potential of HCC [31].

The present results reveal significant increase in MMP-9 in patients with HCC. Previous studies have demonstrated that MMP-9 is involved in the invasion and metastatic behavior of carcinomas [33–35]. It is commonly accepted that most MMP family are secreted in latent forms and are activated by various factors, including several serine proteinases such as trypsin, plasmin, and elastases in addition

to acid exposure. These serine proteinases can convert Pro MMP-1, proMMP-3, and proMMP-9 from inactive latent to active forms. However, proMMP-2 cannot be activated by serine proteinases and it has been suggested that proMMP-2 may be activated on the surface of the cancer cells by some membrane-associated activator [31].

Recent studies indicated that, the invasion potential of HCC is closely correlated with the expression of MMP-9 mRNA but not with that of MMP-2 mRNA [32]. However, this observation does not necessarily exclude the possibility of MMP-2 involvement [32]. Furthermore, suggestive evidence that MMP-2 was preferentially observed at the invading border of the tumorous tissue [32]. Based on these observations, MT1-MMP mRNA was expressed in the tumorous portions of HCC in all cases, while expression in the non-tumorous portions was minimal [32]. With respect to clinical- pathological variables, the intensity of MT1-MMP mRNA expression in the tumor tissue was positively correlated with cancer infiltration into the tumor capsule, but was not correlated with portal involvement, patient age, patient sex, distribution, size of the tumor, the grade of cell differentiation, nor the survival period after hepatectomy [32]. Yamamoto et al. [36], reported that MT1-MMP mRNA expression correlated with capsular infiltration in HCC using Northern blot and gelatin zymography. Collectively, these observations suggested that MT1-MMP is involved in the ability to invade, most notably the capsule infiltration of cancer cells. However, MT1-MMP is not exclusively responsible for invasion potential, because even tumors without a high expression of MT1-MMP sometimes infiltrate the tumor capsules [36]. Previously it was found that MMP-9 also participated in the invasion of the tumor capsule [37]. Indeed, tumors with capsular invasion which did not have high expression of MT1-MMP mRNA exhibited high expression of MMP-9 mRNA. So, high expression of the MT1-MMP gene in HCC cells suggested the possible involvement of MT1-MMP in the invasion potential of HCC. Also, MT1-MMP and MMP-2 may cooperate in the process of cancer cell invasion, although the exact mechanism of MMP-2 activation by MT1-MMP remains to be resolved. This evidence of MT1-MMP involvement may provide a novel strategy against the invasion and metastasis of HCC [33].

Thus, it could be concluded that, Bcl2, CD4+ cells, HGF and MMP-9 might be applied in serving as a serum biomarkers for early diagnosis and implicated to demonstrate the grade of cell differentiation in HCC patients.

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