

Evaluation of Tumor Necrosis Factor- α (TNF- α), soluble P-selectin (sP-Selectin), gamma-glutamyl transferase (GGT), glutathione S-transferase Pi (GST-Pi) and alpha-fetoprotein (AFP) in patients with hepatocellular carcinoma before and during chemotherapy

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ABSTRACT

Hepatocellular carcinoma (HCC) is an environmentally related cancer, with both viral and chemical carcinogens involved in multistage process. Up now, it is difficult to detect the asymptomatic precursor lesions in early stages of HCC. Therefore, the majority of HCC patients are not amenable to curative therapy as they are detected at late stages. To evaluate the significance of TNF- α , sP-selectin, GGT, GST-Pi and AFP in the diagnosis and follow up of HCC patients during chemotherapy with adriamycin, 45 subjects were studied (15 healthy volunteers as control group, 15 with benign liver diseases (SHF), and 15 HCC patients before and during chemotherapy (3 cycles of intravenous adriamycin). HCC patients had significantly higher serum level of TNF- α , sP-selectin, GGT, GST-Pi and AFP. Serum levels of GGT and GST-Pi were significantly higher in HCC patients with poorly differentiated tumors than in patients with well and moderately differentiated tumors. Treatment with adriamycin for three cycles forced a significant decrease in TNF- α , sP-selectin and GST-Pi. We conclude that GST-Pi is superior as a diagnostic and may be a prognostic marker in HCC patients. [Turk J Cancer 2005;35(1):5-11]

KEY WORDS:

TNF-alpha, chemotherapy, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly lethal human malignancy with a worldwide annual incidence of at least one million new cases and a male to female ratio of 4:1. While HCC is relatively uncommon in western countries, it is the most common cancer in African and Far East populations due to the hyperendemic hepatitis (1). In Egypt, HCC represents 7% of all cancer cases with 3:1 male to female ratio (National Cancer Institute Registry, 2001). Chronic hepatitis B and C viruses (HBV and HCV) infections with and without schistosomiasis, dietary aflatoxin B (AFB) and chemical carcinogen exposures, cigarette smoking and low consumption of vegetables are the main risk factors found to be associated with HCC development (1).

The contributions of cytokines to the development and progression of HCC is well documented (2). Cytokine production is thought to play a control role in regulating the recruitment of tumor associated inflammatory cells, in the induction of angiogenesis and in the direct modulation of tumor cell proliferation (3-5). Tumor necrosis factor- α (TNF- α) is a 17 KDa protein produced primarily by mononuclear phagocytes and lymphocytes (6). It has been reported that TNF- α is involved in the pathogenesis of a diversity of liver diseases including viral hepatitis and HCC (7). The immunomodulatory effect of TNF- α is achieved via binding

to specific cellular receptors (TNF-Rs); TNF-R55 and TNF-R75 (8). The TNF-TNFRs system has been implicated in a wide range of biological functions including cytotoxicity against tumors and virus-infected cells, direct antiviral and proinflammatory activities as well as stimulation of many immune effector cells (9). TNF- α plays a central role in up-regulating cell surface adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, thereby promoting adhesion of leukocytes to and subsequent transmigration through the endothelial barrier (10).

The expression of adhesion molecules, which lead to the interaction of particular sets of cells, may be the primary signal for the activation and extravasation of immune cells into the site of inflammation (11). P-selectin (CD62, GMP-140, PADGEM) belongs to the selectin family of adhesion molecules. P-selectin acts as a receptor that supports binding of leukocytes to activated platelets and endothelium. P-selectin-mediated adhesive interaction operates in conjunction with cell-cell interactions directed by related molecules and are likely to be important in both hemostatic and inflammatory processes (12). The physiologic role of P-selectin might be the mediation of initial leukocyte adhesion to activated endothelium during acute inflammation. It may work in concert with E-selectin to direct early, regionally specific adherence of neutrophils and monocytes at sites of acute inflammation. A soluble form of P-selectin found in serum and plasma has been described which might represent a proteolytic fragment or more likely a soluble splice variant lacking the transmembrane domain (13). P-selectin performs an important role in tumor formation and metastasis since malignant cells were shown to express receptors for P-selectin (14). Soluble adhesion molecules (sP-selectin) like their membrane bound counterparts may affect cell-cell interactions. Thus identification of soluble forms of these molecules, provide the potential for further clinical study of their role in the monitoring of the inflammatory and malignant diseases (15).

Gamma-glutamyl transferase (GGT, Enzyme commission number 2.3.2.2.) is a membrane-bound ectoenzyme that catalyzes the degradation of glutathione and other γ -glutamyl compounds (16). Serum GGT activity is a sensitive marker of hepatobiliary disorders. It is generally accepted as the most sensitive marker of cholestasis and pancreatic disease or enzymatic induction by alcohol and drugs (17). Serum GGT levels are affected by several other factors

and a variety of clinical conditions; that measurement of serum total GGT lacks specificity, and its value in the differential diagnosis is limited because of great overlap in patients with benign or malignant liver diseases (18). GGT mediates the uptake of glutathione, by breaking down extracellular glutathione and making its amino acid component available to the cell (19).

Glutathione S-transferase-Pi (GST-Pi) is an acidic, 25 KDa dimeric protein. It is a detoxifying isoenzyme that catalyzes the nucleophilic addition of glutathione at electrophilic centers of wide variety of compounds, hence removal of such xenobiotics from the body becomes possible. The amount of GST-Pi in primary hepatomas are several ten-fold higher than in normal liver (1,20,21).

Alphafetoprotein (AFP) is a well-established cell differentiation and tumor marker. Activity transcribed by fetal and postnatal hepatocytes, AFP is almost undetectable in adult liver parenchymal cells, and its production can resume in the liver under pathologic conditions, such as primary hepatocellular carcinoma (HCC). An elevated AFP level is a sensitive marker for the diagnosis of HCC. However, higher false positivity and false negativity of the serum AFP marker also may be found in the diagnosis of HCC (22). An elevated concentration may be found in certain benign liver diseases [e.g. schistosomal hepatic fibrosis (SHF)]; 20% to 30% of patients with HCC had a negative AFP result, while some cases of chronic hepatitis and cirrhosis showed a positive AFP result (22).

The aim of the present study was to determine the levels of serum TNF- α , serum sP-selectin, serum GGT, plasma GST-Pi and serum AFP in patients with HCC before and after 3 cycles of single intravenous agent chemotherapy (adriamycin) to determine their value as tumor markers in HCC patients for diagnosis and follow up.

PATIENTS AND METHODS

This study comprised 45 subjects divided into 3 groups: Group I (control group), 15 normal healthy volunteers; Group II (benign group), 15 patients with SHF; Group III (malignant group), 15 patients with HCC. Patients were selected from the hospital and the Oncology Clinic of the Medical Research Institute, Alexandria University, in the period from April 2001 to October 2002.

The HCC patients were subjected to proper and full history recording, thorough clinical examination, routine

laboratory investigations including complete blood count (CBC) and liver function tests, ultrasonography (USG) of the liver, chest X-ray (when needed) and USG-guided needle liver biopsy to establish the pathological diagnosis.

Serum samples from the control and benign groups and the malignant group [before and during chemotherapy; i.e. after 3 cycles of single agent chemotherapy (50 mg adriamycin/m² of body surface area, given intravenously, every three weeks)] were used for determination of TNF- α , sP-selectin, GGT, GST-Pi and AFP (23).

Determination of serum TNF- α and sP-selectin was carried out using a solid phase sandwich enzyme immuno-sorbent assay (ELISA) kit (Immuno Tech, France for TNF- α and Bender MedSystems for sP-selectin). Briefly, serum samples were added to the wells of microtitre plate precoated with specific monoclonal antibodies for TNF- α and sP-selectin. After incubation at room temperature and washing of unbound antigen, the enzyme-linked polyclonal antibodies specific for TNF- α and sP-selectin were added to the wells. After washing to removal all unbound enzyme, the substrate was added to induce a colored reaction product. The color reaction was stopped and the intensity was measured at 450 nm. The intensity of this color is directly proportional to the concentration of TNF- α or sP-selectin present in the sample. Results are expressed as pg/ml for TNF- α and ng/ml for sP-selectin.

Determination of serum GGT was determined by the method of Rosalki and Tarlow (24), where the almost colorless L- γ -glutamyl-p-nitroaniline used as substrate from which the enzyme γ -glutamyl transferase liberates yellow p-nitroaniline so that the increase in color gives a measure of its activity, glycylglycine was used as glutamyl acceptor.

GST-Pi was labeled with iodine-125 using the chloramine-T reaction by modification of the method of Hayes et al. (25). In brief, GST-Pi (Sigma Chemical Company Lts; St. Louis, MO 63178 USA, 10 μ l; 5 μ g) and 125I-labeled sodium iodide (MDS Nordion S.A; B-6220 Fleurus, Belgium, 5 μ l; 500 μ Ci, carrier free) were added to a reactivial (Pierce Co, USA), followed by chloramine-T (10 μ l; 16 μ g). After 30 seconds the reaction was stopped by cysteine (100 μ l; 56 μ g), potassium iodide (10 μ l; 100 μ g) and elution buffer (250 μ l). The contents of the reactivial were then equilibrated with the elution buffer and transferred to a (1.6 cm \pm 35 cm) gel filtration chromatographic column packed with sephadex G-25 (fine). The labeling reaction mixture was applied to the column and was eluted with the

elution buffer and the fractions with the highest both radioactivity (CPM) and protein content (Abs280, Lowry method) were used as the tracer for our improved RIA kit (26). The serum GST-Pi levels in different study groups were measured using a modified RIA procedure of Fan et al. (27) in which the first antibody was rabbit anti-human GST-Pi (DAKO A/S; produktionsvej 42, DK-2600 Glostrup, Denmark, diluted 1:25), the second antibody was swine anti-rabbit immunoglobulin antibody (DAKO A/S, diluted 1:10). GST-Pi was expressed as μ g/l.

Serum AFP was measured using a ready-to-use-IRMA (Immunoradiometric assay) kit (Diagnostic Products Corp. Los Angeles, U.S.A) (1). Results are expressed as IU/ml.

Statistical method

Data were subjected to an analysis of variance using the general model procedure (SAS Institute, 1994). Variables having a significant F-test ($p < 0.05$) were compared using the least significant difference (LSD) test (28).

RESULTS

Table 1 showed a significant elevation of serum level of TNF- α , sP-selectin, GGT, GST-Pi and AFP in malignant (HCC) group before chemotherapy compared to both the benign group (SHF) and the control group.

In follow up study for the malignant group, the levels of TNF- α , sP-selectin and GST-Pi showed a significant decrease after 3 cycles of single intravenous agent chemotherapy with adriamycin.

No correlation was reported between the studied parameters and tumor size in HCC patients group (Table 2).

In malignant group (Table 3), the serum levels of GGT and GST-Pi were significantly higher in HCC patients with poorly differentiated tumors than in patients with well and moderately differentiated tumors. While serum levels of TNF α , sP-selectin and AFP were insignificantly higher in HCC patients with poorly differentiated tumors than in well and moderately differentiated tumors.

DISCUSSION

The prognosis of HCC still remains dismal although many advances in its clinical study have been made. Therefore the main goal of the present study was to assess the

Table 1
Results of serum TNF- α , sP-selectin, GGT, GST-Pi and AFP in the control, benign (SHF) and malignant (HCC) groups before and after 3 cycles of chemotherapy

	Control	Benign (SHF)	Malignant (HCC)	
			Before chemotherapy	After 3 cycles of chemotherapy
Serum TNF- α (pg/ml)				
Min-Max	6.4-17.8	175.0-321.0	256.0-499.0	200.0-361.0
Mean \pm S.E.	12.4 \pm 0.98	248.3 \pm 12.1*	270.0 \pm 19.3*†	258.7 \pm 18.4*‡
Serum sP-selectin (ng/ml)				
Min-Max	149.0-264.0	190.0-285.0	260.0-350.0	210.0-290.0
Mean \pm S.E.	204.4 \pm 9.9	243.5 \pm 8.5*	306.1 \pm 7.2*†	251.4 \pm 7.6*‡
Serum GGT (U/l)				
Min-Max	4-12	29-48	79-1229	50-392
Mean \pm S.E.	8.6 \pm 0.7	39.0 \pm 1.2*	253.6 \pm 81.8*†	168.7 \pm 34.1*
Serum GST-Pi (μ g/l)				
Min-Max	0-35	0-48	33-155	18.5-87
Mean \pm S.E.	14.0 \pm 3.2	20.7 \pm 4.9*	62.7 \pm 6.3*†	43.2 \pm 5.5*†
Serum AFP (IU/ml)				
Min-Max	0.0-1.4	1.1-10.0	2.5-400.0	0.0-480.0
Mean \pm S.E.	0.91 \pm 0.22	3.2 \pm 0.69*	43.3 \pm 15.5*†	30.0 \pm 7.3*

S.E.: Standard error

*: Significant when compared with control group

†: Significant when compared with benign group

‡: Significant when compared with before chemotherapy

Table 2
The spearman correlation (r) and its P value for all studied parameters related to the tumor size in the HCC group

	TNF- α		sP-selectin		GGT		GST-Pi		AFP	
	r	p	r	p	R	p	r	p	r	p
Tumor size	0.253	0.481	0.198	0.548	-0.198	0.680	0.480	0.160	-0.038	0.926

Table 3
Relationship between the studied parameters and the pathological differentiation in the HCC patients group

	TNF- α	SP-selectin	GGT	GST-Pi	AFP
WD (n = 3)	258.7 \pm 19.0	299.9 \pm 13.9	96.8 \pm 7.4	33.5 \pm 0.4	13.8 \pm 2.8
MD (n = 7)	364.9 \pm 38.5	311.5 \pm 7.6	194.3 \pm 17.4	62.6 \pm 9.3	15.5 \pm 3.3
PD (n = 5)	365.1 \pm 36.0	313.4 \pm 13.9	234.5 \pm 4.5	125.7 \pm 17.2	72.1 \pm 29.4
	NS	NS	S	S	NS

WD: Well differentiated

MD: Moderately differentiated

PD: Poorly differentiated

S: Significant

NS: Non-significant

serum levels of TNF- α , sP-selectin, GGT, GST-Pi and AFP in HCC and SHF groups, this may offer an opportunity to understand the mechanism of the pathogenesis of HCC in relation to these parameters and severity of the disease.

In our study, the level of all studied parameters were significantly increased in HCC patients before chemotherapy compared to SHF group and control group and this revealed that TNF- α , sP-selectin, GGT, GST-Pi and AFP levels may have diagnostic role in HCC patients.

The levels of TNF- α , sP-selectin and GST-Pi were significantly decreased after 3 cycles of treatment with adriamycin compared to patients before treatment. The noticeable reduction in all parameters during treatment mirrored the response to chemotherapy and may be due to the direct effect of adriamycin on cells or hepatic retention or both. This may indicate the validity of these parameters in the follow up of HCC patients treated with adriamycin.

According to the pathologic differentiation of the HCC group it was found that GGT and GST-Pi were significantly increased, while TNF- α , sP-selectin and AFP levels were insignificantly increased in patients with poorly differentiated tumors than those with well and moderately differentiated tumors and that revealed a good correlation between the levels of both GGT and GST-Pi and pathological differentiation.

The actual stimulus for increased TNF- α production in patients is not clear. It is generally accepted that hepatitis B and/or C viruses are major causes of chronic liver disease such as chronic hepatitis and cirrhosis as well as HCC (29). Larrea et al. (30), have shown that hepatitis infection is associated with increased transcriptional expression of the TNF- α gene in the liver with high serum levels of TNF- α . Several reports postulated that the production of TNF- α in the liver take place not only in the non-parenchymal cells but also in hepatocytes (30). In addition, Chisari (31) observed that in hepatitis B and/or C infected patients the cytotoxic T-lymphocytes (CTLs) control the viral replication not only by direct cytotoxic activity of infected cells but also via the production of IFN- γ and TNF- α . It seems also possible that oxidative distress might be involved in the induction of TNF- α in HCC or SHF patients (32). Another possibility related to the increased levels of TNF- α in patients is its release from malignant cells or from other non-malignant cells (as T-cells or macrophages upon their activation) (33).

In our study, elevated levels of sP-selectin in both benign and malignant groups may be attributed to many factors. Some of these factors were studied in this work such as TNF- α which induces the expression of P-selectin, affects the release of P-selectin from cells and seems to be important for hepatic injury in HCC or SHF patients. Also

elevated levels of serum sP-selectin may indicate the activation and/or damage of platelets and endothelial cells in these patients groups. However, it is not known whether the sP-selectin is secreted rather than shed from platelets or endothelial cells (34). The increased levels of TNF- α and sP-selectin in the HCC patients more than in SHF patients can attribute to the more intense inflammation and severity of the disease which may be occurring in HCC patients.

In our study, the possibility of diagnosis and follow up values of GST-Pi were confirmed by observations of both Fan et al. (27) and Niitsu et al. (21) The presence of a significant direct strong correlation between the GST-Pi and GGT levels and the pathological differentiation in HCC patients and the absence of correlation between both GGT and GST-Pi and the tumor size in the same patients can be interpreted in that the tumor production of GST-Pi and GGT is related to the biological characteristics of the tumor cells but not to the tumor size.

The diagnostic value of serum AFP that was reported in this study was confirmed by observations of Abd El-Moneim et al. (35) and Chen and Sung (36); on the other hand, these results were encountered by the observation of Chen et al. (22). Our results of the non-usefulness of using serum AFP to follow up HCC patients was confirmed by observation of Johnson and Williams (37). The absent correlations we found between the serum AFP level and both the pathological differentiation and the tumor size in HCC patients were typically confirmed by observation of Chen et al (22).

In conclusion, all the studied parameters may be used in HCC diagnosis, while only serum TNF- α , sP-selectin and GST-Pi are valuable in monitoring chemotherapy. A study including larger number of cases and different therapeutic modalities is mandatory. In addition, serum GGT and GST-Pi would correlate with HCC grades.

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