Circulating Transforming Growth Factor-β and Epidermal Growth Factor Receptor as Related to Virus Infection in Liver Carcinogenesis

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Abstract. *Background: The aim of our study was to improve the detection of HCC by measuring alpha-fetoprotein (AFP) in addition to other molecular markers by estimating the plasma concentration of transforming growth factor beta (TGF-β) and epidermal growth factor receptor (EGFR). In particular, the role of hepatitis C and B viruses (HCV and HBV) infection was evaluated with relation to TGF-β and EGFR plasma concentration. Materials and Methods: Eighty-five patients with liver disease, 54 with hepatocellular carcinoma (HCC), 16 with liver metastasis (LM), 15 with liver cirrhosis (LC) and 30 healthy volunteers were evaluated. AFP, TGF-β and EGFR were detected with enzyme-linked immunoassay (ELISA) in plasma of all study participants. Results: The mean values of TGF-β and EGFR in all patients were much higher than in control group, p<0.0001. In HCC patients the levels of TGF-β and EGFR were much higher than in LM and LC patients. Moreover, TGF-β and EGFR were significantly higher in the presence of both viruses or only in the presence of HCV, p=0.002. No decrease or increase of AFP was noted in these patients. Conclusion: Our data suggest the reliability of TGF-β and EGFR in detecting HCC, in particular when the carcinogenesis is affected by virus infection.*

In industrialized countries, an increase in hepatocellular carcinoma (HCC) incidence and mortality has been observed, being attributed to increased prevalence of hepatitis C virus (HCV) infection (1, 2). Chronic infection with hepatitis B (HBV) and hepatitis C virus (HCV) has been implicated in about 80% of HCC cases worldwide. HBV and HCV can be implicated in the development of HCC indirectly through induction of inflammation, necrosis and chronic hepatocellular regeneration, and directly by means of viral proteins, or, in the case of HBV, by creating insertional mutations by integration into the genome of hepatocytes (3). A number of HBV integrations have been shown to occur into or adjacent to genes that have important roles in oncogenesis. In contrast to HBV, HCV is an RNA virus that lacks a reverse-transcriptase enzyme and which cannot integrate into the host genome. Thus the molecular pathogenetic mechanisms by which HCV contributes to cell transformation remain unclear (4). Like other solid tumors, HCC is characterized by the accumulation of numerous genomic alterations. These include the progressive loss of liver-specific gene expression and the persistence of an inflammatory and promitogenic milieu orchestrated by a complex network of cytokines and growth factors (5).

Active research has identified a number of growth and signaling pathways involved in the support of cell proliferation and survival from the early stages of hepatocarcinogenesis, and in the autonomous growth and drug resistance of transformed HCC cells. Among these signaling systems, those controlled by trasforming growth factor beta (TGF-β) and the epidermal growth factor (EGF) family of growth factors are believed to play a prominent role (6, 7). Most of these growth factors, receptors and signaling cascades are part of tightly controlled regenerative and protective natural responses of the liver to acute tissue injury. TGF-β has been shown to play contradictory roles in liver development and carcinogenesis. On one hand, TGF-β secretion inhibits proliferation, suppresses transformation

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and induces apoptosis in HCC. On the other hand, its activation has been associated with the progression of hepatocarcinogenesis, and distribution of TGF-β signaling can deregulate apoptosis in HCC (8-10). Indeed, expression of TGF-β itself is often increased in HCC.

EGF receptor (EGFR) has emerged as a critical signaling hub capable of integrating and transducing a variety of signals from different sources that can have an impact on cancer progression. A role of EGFR and its ligands in liver regeneration and hepatoprotection during tissue injury has been clearly established, and accumulating observations support the notion that dysregulation of EGFR signaling participates in hepatocarcinogenesis (11). The interaction of the EGFR system with the inflammatory and pro-tumorigenic TGF-β may be of special relevance in liver carcinogenesis. TGF-β has been demonstrated to induce the expression of EGFR ligands such as EGF and TGF-α in isolated fetal rat hepatocytes through the activation of the inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) (12).

Interestingly, there is increasing evidence that hepatitis B (HPB) and C (HPC) viral proteins can directly interact with cellular components of growth factor signaling cascades. Such interactions can result in a direct oncogenic effect that may act independently or in cooperation with the hyperproliferative response triggered by chronic inflammation and hepatocellular death, and contribute to explain the enhanced risk of hepatocellular transformation observed upon chronic viral infection (13, 14).

The most common method used to detect overexpression of EGFR is immunohistochemical staining of paraffin-embedded specimens. Enzyme-linked immunoassay ELISA was recently developed and allows quantitative determination of the extracellular domain (ECD) of EGFR in the plasma/serum of cancer patients (15). A significantly elevated plasma level of EGFR in cancer patients compared with healthy controls was demonstrated in lung, colon and breast cancer (16-19). Determination of plasma levels of EGFR in patients with HCC may provide valuable information, considering the high expression of EGFR in HCC tissue, as well as the potential usefulness of plasma assay in the clinical setting. Increased EGFR expression might therefore be a strong prognostic feature in multiple tumor types, and the inhibition of its cellular actions may produce substantial therapeutic benefit. The primary marker for HCC is alpha-fetoprotein (AFP), a single polypeptide chain glycoprotein. Generally AFP shows acceptable sensitivity as a marker for HCC; however, AFP is not secreted in all cases of HCC and may be present at normal levels in as many as 40% of patients with early HCC (20, 21). The aim of this study was to verify a possible role for the detection of HCC by measuring molecular markers involved in the process of cancer invasion and metastasis by estimating the plasma concentration of circulating TGF-β and EGFR in peripheral blood of patients with HCC. In particular, the role of HCV and HBV infection was evaluated with relation to plasma concentrations of TGF-β and EGFR.

Materials and Methods

Patients. From June 2007 to December 2010, 85 patients, 63 male (74.1%) and 22 female (25.8%), aged from 45 to 87 years (median, 73 years) with liver disease were enrolled in this study at the Giovanni Paolo II National Cancer Institute (NCI) of Bari, Italy. Among the 85 patients, 54 (63.5%) had hepatocellular carcinoma (HCC), 16 (18.8%) had liver metastasis (LM) from other tumors and 15 (17.6%) had liver cirrhosis (LC). In regard to the 54 patients with HCC, 19 (35.18%) had antibody positivity for both HCV and HBV, 8 (14.81%) were positive only for HCV virus, and 27 (50%) were negative for both viruses. In our series, HBV infection was always associated with HCV presence. For all patients the clinicopathological characteristics (gender, age, etiology, underlying liver disease, total bilirubin, albumin, alanine, presence of metastasis) were evaluated. A control group was enrolled among donors (n=30) found to be healthy from laboratory data and imaging techniques. Their median age was 50 years (range, 40-70 years). Five milliliters of peripheral blood were collected from each participant in a vacutainer system with lithium-heparin. Samples were collected from each participant before any invasive procedures or therapy. Plasma from both patients and healthy donors was immediately separated from the cellular fraction by centrifugation at 2,500 r.p.m. (1,500 × g) for 10 min and frozen at –20˚C. A written consent should be obtained from all patients prior to enrolment in the study, and the Ethical Committee of the NCI approved the protocol which was in accord with the ethical guidelines of the 1975 Declaration of Helsinki.

TGF-β and EGFR ELISA assay. Plasma samples from the patients and the controls were assayed for the levels of TGF-β and EGFR extracellular domain (ECD) by a sandwich ELISA assay (Quantikine, Human TGF-β and Human EGFR Immunoassay; R&D Systems, Inc. Minneapolis, USA) according to the manufacturer's recommendations. The absorbance of the solution produced was measured at 490 nm. The absorbance is directly proportional to the amount of TGF-β/EGFR present in the sample. A standard curve was constructed by plotting the mean absorbance value measured for each standard versus its corresponding concentration. The minimal detection limit was 4.61 pg/mL for TGF-β and 0.014 ng/mL for EGFR.

AFP assay. AFP was tested by using commercially available immunometric assay (Architect AFP assay, Abbot Laboratories, North Chicago, IL, USA). The cut-off value of AFP for HCC was set at 20 ng/ml, the most commonly set value. The minimal detection limit of the AFP was 0.013 ng/mL.

Statistical analysis. The results are expressed as means±standard deviation. For the continuous variables data were analyzed by unpaired *t*-test, Mann-Whitney U-test and Kruskal-Wallis test, as well as analysis of variance (ANOVA). The correlation between TGF-β and EGFR was analyzed by Spearman correlation. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyzes were performed by NCSS-PASS (Statistical Analysis and sample Size – Power Analysis and Graphics) 2007 software.

Table I. *Association between trasforming growth factor beta (TGF-β) and epidermal growth factor receptor (EGFR) plasma concentrations and clinical characteristics of patients.*

	N (%)	TGF-β (pg/ml) Mean±SD	*P*-Value	EGFR (ng/ml) Mean±SD	*P*-Value
Control group	30	39.5±9.8	<0.0001[a]	58.5±8.22	<0.0001[a]
Patients	85	977±458		78.3±30.5	
Gender					
Male	63 (74.1)	989±440	0.6[a]	75±22.3	0.08[a]
Female	22 (25.8)	941±518		88±46.3	
Diagnosis					
HCC	54 (63.5)	1194±331	<0.0001[b]	86±31.8	<0.0001[b]
L M	16 (18.8)	421±184		63±15.4	
LC	15 (17.6)	419±204		48±9.2	
AFP					
<20 ng/ml	46 (61.3)	1155±355	0.1[a]	78±34.2	0.8[a]
≥20 ng/ml	29 (38.6)	1259±282		79±23.9	
Tumor differentiation					
Well (G1)	28 (50.9)	1157±381	0.1[b]	85±40.2	0.2[b]
Moderate (G2)	17 (30.9)	1304±215		93±19	
Poor (G3)	10 (18.1)	1042±354		73±15	

[a]*p*-Values were calculated with unpaired *t*-test; [b]*p*-values were calculated with ANOVA test. HCC: Hepatocellular carcinoma; LM: liver metastasis; LC: liver cirrhosis.

Table II. *Transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR) and alpha- fetoprotein (AFP) concentration in association with HCC viral infection.*

Concentration Mean±sd	HCV+/HBV+ (n=19)	HCV+/HBV– (n=8)	HCV–/HBV– (n=27)	*P*-value
TGF-β (pg/ml)	1323±328.2	1396±195.7	1044±302	0.02
EGFR (ng/ml)	110±36.2	100±11.2	64±11.8	0.0001
AFP (ng/ml)	34.2±36.8	23.9±19.4	32±35	0.7

P-values were calculate with ANOVA test. HBV: Hepatitis B virus. HCV: Hepatitis C virus.

Results

The mean values of TGF-β and EGFR for all patients were 977±458 pg/ml and 78.3±30.5 ng/ml respectively, both much higher with respect to those reported for control group 39.5±9.8 pg/ml for TGF-β ($p<0.0001$, *t*-test) and 58.5±8.22 ng/ml for EGFR ($p<0.0001$, *t*-test). Levels of TGF-β and EGFR plasma concentration were studied in relation to patient clinical and pathological characteristics (Table I). Significant differences were observed when considering the diagnosis, for HCC patients the plasma concentrations of TGF-β and EGFR were much higher with respect to that of LM patients and in those patients with LC: for TGF-β the plasma levels (pg/ml) were 1194±331 in HCC, 421±184 in LM and 419±204 in LC ($p<0.0001$, ANOVA), for EGFR plasma levels (ng/ml) were 86±31.8, 63±15.4, 48±9.2 respectively ($p<0.0001$, ANOVA). When HCC patients were divided into two groups according to serum AFP level, no significant difference in plasma TGF-β and EGFR were found between patients with AFP< 20ng/ml and those with AFP ≥20 ng/ml. TGF-β and EGFR did not relate to gender, age, tumor differentiation (unpaired *t*-test). In Table II were reported the plasma association of TGF-β and EGFR in HCC patients to viral infection. The levels of TGF-β and EGFR were higher in the presence of both viruses compared to when there was no viral infection: for TGF-β (1323±328.2 pg/ml in HBV+/HCV+ patients compared to 1044±302 pg/ml in HBV–/HCV– patients, $p=0.02$, ANOVA), for EGFR (110±36.2 ng/ml in HBV+/HCV+ patients compared to 64±11.8 ng/ml in HBV-/HCV- patients, $p<0.0001$, ANOVA). Positivity for TGF-β in HCC patients was defined level ≥1200 pg/ml (the median value) and for EGFR level ≥80 ng/ml (the median value). A total of 62.9% (N=34) of HCC patients were positive for TGF-β, of them 25 patients (73.3%) were positive for viral infection and 9 (26.7%) were negative for both virus. Positivity for EGFR was observed in 29 patients (53.7%), of whom 27 (91.1%) were positive for HCV/HBV infection and 2 (6.8%) were negative for both viruses. No association was noted for AFP with HCC viral infection. When the TGF-β plasma concentration and EGFR were compared as continuous variables, a significant direct correlation between the two markers was found (Spearman correlation $p=0.001$, r=0.5), Figure 1.

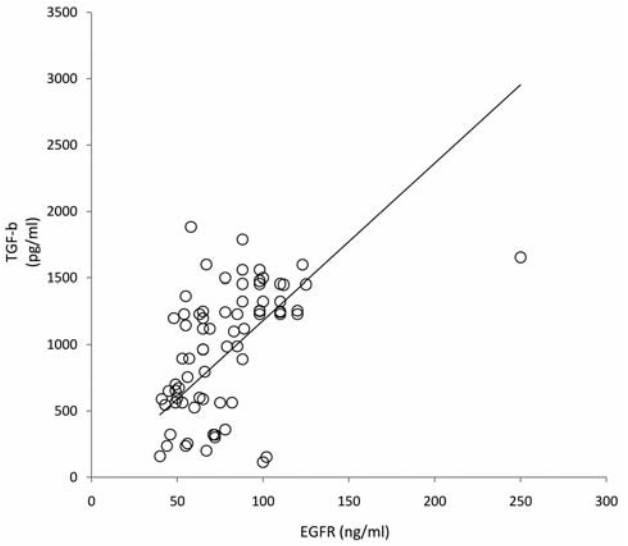

Figure 1. *Scatter plot showing correlation between circulating transforming growth factor beta (TGF-β) and epidermal growth factor receptor (EGFR). (Spearman correlation r=0.51, p=0.001).*

Discussion

The aim of our study was to examine the correlation between circulating plasma levels of TGF-β and EGFR in patients with HCC in relation to viral infection. This study is the first to examine the plasma EGFR concentrations in HCC patients. The mean levels of TGF-β and EGFR were significantly more elevated in HCC patients than in healthy controls ($p<0.0001$). These data are in accordance with that of a previous study which clearly demonstrated that plasma levels of TGF-β in patients with HCC were significantly higher than those found in healthy donors and that plasma levels decreased progressively in tumor-free patients during follow-up, while increasing in those in whom a recurrence occurred (22-24). These date confirm the results obtained from numerous studies that TGF-β plays a crucial role in the process leading to initiation of hepatocellular fibrogenesis (25, 26). TGF-β, is a potent cytokine involved in many functions such as epithelial mesenchymal transition, tissue morphogenesis, angiogenesis, and hence tumor progression, invasion and metastasis (27). In liver, TGF-β has a profibrotic activity, and is involved in the pathogenesis of liver fibrosis, cirrhosis and HCC (28). During the last few years, data have accumulated suggesting that TGF-β actions may be modulated by other growth factors or cytokines (29). In fact, in our study, a direct correlation was shown between circulating plasma levels of TGF-β and EGFR. EGF is an important survival signal for TGF-β-induced apoptosis in hepatocytes. Indeed, some autocrine signals, such as EGFR ligands, might protect liver tumor cells from TGF-β-induced apoptosis (30). Inhibiting EGFR greatly increases apoptosis induced by TGF-β in such cells. Caja *et al*. suggested that in HCC, inhibition of EGFR would be a useful therapeutic target since it would not only result in attenuation of tumor cell proliferation, but it would also enhance pro-apoptotic signaling by TGF-β, as the levels of this cytokine are elevated in HCC (31). In hepatoma cells, this dual response to TGF-β might convert this cytokine to a pro-tumorigenic factor in hepatocarcinogenesis (32). Furthermore, recent genomic profiling studies have revealed that many inflammation-related genes are involved in virus-related hepatocarcinogenesis hypothesizing the central role of inflammation in the development of HCC. HBV or HCV infection can result in hepatocyte inflammation and high regenerative potential (33, 34). For this reason, in this study, circulating plasma TGF-β and EGFR have been analyzed in primary liver tumors with and without virus infection. Significantly, higher plasma levels of TGF-β and EGFR were observed when virus infection, both HBV and/or HCV were present. In the present study, the high positive rate of TGF-β and EGFR plasma levels in patients with HCC HCV+/HBV+, suggesting that HBV protein and HCV core protein may stimulate the expression of TGF-β and EGFR. This confirms that the presence of virus increases risk for HCC. Hepatocytes produce fibrogenic mediators after viral infection. For example, HCV patients have an increased expression of TGF-β signaling proteins (TGF-βRII and ligands) throughout the liver tissue (35). It would seem that the HCV core protein is taken up by hepatocytes and can directly induce the expression of TGF-β. The increase in serum TGF-β in HCC patients was repeatedly reported, HCV may enhance the increase of this growth factor. Moreover, in HCV patients who respond to interferon-alpha or ribavirin treatment, TGF-β levels decrease (36). Our data suggested that these factors could be more useful than or associated with the AFP test in the diagnosis of HCC and that the direct correlation between EGFR and TGF-β plasma levels suggest that the inhibition of EGFR would be a useful therapeutic target in HCC prevention and treatment. Therefore there is the possibility that the determination of plasma EGFR and TGF-β is useful for the prediction of prognosis of follow-up after treatment in those patients with HCC correlated to virus infection.

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