Possible role of C-erb B-2 and transforming growth factor β1 in malignant transformation of hepatocytes

Olfat Shaker[1], Ahmed Hashem[2], Lamia Mansour[3], Osama Taha[4]
[1]Departments of Medical Biochemistry, [2]Departments Tropical Medicine, [3]Departments of Microbiology and Immunology e [4]Departments of Clinical Oncology, Faculty of Medicine, University of Cairo, Egypt

ABSTRACT

To evaluate the expression of C-erb B-2 oncoprotein and transforming growth factor (TGF) β1 in polymorphonuclear cells and sera of chronic hepatitis C (HCV) and hepatocellular carcinoma (HCC) patients compared to healthy control subjects, thirty chronic HCV patients, thirty HCC patients and thirty healthy control subjects were enrolled in the study. Measurements of TGF-β1 and C-erb B-2 were done by reverse transcription-polymerase chain reaction and sandwich enzyme immunoassays. A significant overexpression of TGF-β1 and neu-oncoprotein C-erb B-2 was detected in HCV and HCC patients compared to control subjects. Also, TGF-β1 and C-erb B-2 concentrations were higher in HCC than in HCV patients ($P < 0.001$). The mean serum concentration of TGF-β1 was increased with increased degree of fibrosis ($P = 0.003$). Statistically significant differences ($P < 0.001$) in mean serum concentrations of C-erb B-2 were observed between grade I vs grade III and grade II vs grade III in HCC patients. It is suggested that TGF-β1 and C-erb B-2 may be associated with the malignant transformation of hepatocyte or the progression of HCV-associated HCC.

INTRODUCTION

Since the development of diagnostic tests for detection of hepatitis C (HCV) infection, it has become apparent that this infection is an increasing clinical and public health problem. The majority of these patients develop chronic hepatitis of varying degree and it is estimated that 20 to 30% will develop cirrhosis in the long term. Less than 5% will die from end-stage liver disease or hepatocellular carcinoma (HCC) but, given the magnitude of the problem, these numbers become very significant (1)

Some non-viral factors appear to play a role in development of fibrosis. The transforming growth factor β (TGF-β) is an important profibrogenic cytokine, having several polymorphisms in humans. Patients with a high producer polymorphism show slightly elevated mean fibrosis scores when compared with the low producer group (2). TGF-β are multifunctional polypeptides that have been suggested to influence tumor growth. They mediate their functions via specific cell receptors, i.e. type I ALK5 (TβR-I ALK5), type II (TβR-II) and type III (TβR-III) (3). TGF-β1 is involved in the regulation of liver cell proliferation and apoptosis, and escape of hepatoma cells from the growth restraining signals of TGF-β1 has been suggested to contribute to tumor development (4). TGF-β1 was found to positively correlate with the degree of liver insufficiency (according to Child- Pugh score) suggesting its use as a possible marker of hepatic fibrosis progression in cirrhotic patients (5). In patients with chronic B hepatitis, serum TGF-β1, hepatic collagenous and reticular fibres were found to be increased to the same degree, suggesting that TGF-β plays a decisive and crucial role in accelerating hepatic fibrosis (6).

HCC is one of the most prevalent fatal cancers in the world. Despite advances in early diagnosis and improvements in surgical techniques, the survival of patients with HCC, even after resection, is poor because of high incidence of recurrences. Therefore, the identification of prognostic factors may be helpful in the development of new treatment protocols (7). Hepatocarcinogenesis involves alterations in the action of proto-oncogenes, tumor suppressor genes and growth factors. One of the cellular oncogenes known to be important in the development of human malignancies is the neu (HER-2 or C-erb B-2) oncogene (8). Overexpression of the neu-oncogene has been implicated in experimental cellular transformation and tumorigenesis in a wide range of human cancer, including carcinoma of breast, ovary, gastrointestinal tract, kidney and liver (9,10). Over-expression of the C-erb B-2 oncogene can be detected immunologically in vivo by quantitation of the extracellular domain (ECD) of p185 in serum of human patients with cancer using the ELISA technique (11,12). In a study of human hepatocellular carcinogenesis, it was possible to detect increased serum concentrations of the p185 ECD in sera of individuals who developed cancer, 60 months before the clinical diagnosis (13). This suggested that serum neu-oncopeptide may be a useful biomarker for early detection of HCC. It was also reported that elevated serum concentrations of neu-oncopeptide correlated with the increased expression of this oncoprotein in tumor tissue, suggesting that the serum concentration of neu-oncoprotein can be used as an indicator of its tissue expression (14).

This study was conducted to determine the gene expression of TGF-β1 and C-erb B-2 and their blood concentrations in patients with HCV and HCC as well as in healthy control subjects. The degree of fibrosis as well as the grades of HCC were also studied in an attempt to

assess the feasibility of using antibodies against neu-oncoprotein as immunotherapy for treatment of cancer.

SUBJECTS AND METHODS

This study enrolled 90 patients admitted to the Tropical Medicine Department, Kasr El-Aini Hospital, during 2005-2007. Blood samples were collected from 30 patients with chronic HCV and 30 patients with HCC. The HCV group was subclassified into mild, moderate, marked and no fibrosis. The HCC group was subclassified into grade I, II, and III. All HCV patients were positive for HCV antibodies as detected by EIA and immunoblotting techniques. Thirty blood samples from control subjects who were negative for anti-HCV antibodies were also included in this study.

C-erb quantitative ELISA

The Oncogene Research Products neu ELISA is a sandwich enzyme immunoassay, which utilizes a mouse monoclonal antibody for capture and a rabbit polyclonal antiserum for the detection of human neu protein. Both capture and detector reagents specifically bind to the ECD of neu protein. The colored reaction product is quantitated by spectrophotometry in human neu unit (HNU).

Human TGF-β1 immunoassay

The Quantikine TGF-β1 immunoassay (R&D Systems, Inc) employs the quantitative sandwich enzyme immunoassay technique. TGF-β1 soluble receptor type II, which binds TGF-β1, has been pre-coated onto a microplate. Before start, samples are activated to transform latent TGF-β1 to immuno reactive TGF-β1. Standards and samples are pipetted into the wells and any TGF-β1 present is bound by the immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. After washing to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF-β1 bound in the initial step.

RNA extraction

Total RNA was extracted from the polymorphonuclear cells by using the guanidinum thiocyanate phenol chloroform method of Chomezynski and Sacchi (15). Total RNA was reconstituted in 50 µL water and stored at –80 °C until use.

Primer sequences

Three sets of primers were used for detection of HCV, TGF-β1 and C-erb gene expression. The sequences of the primers are illustrated in Table 1.

Reverse transcription-polymerase chain reaction (RT-PCR) experiments

RT-PCR were done for detecting HCV-mRNA, TGF-β-mRNA and C-erb B-2-mRNA using the corresponding primer sequences as shown in Table 1.

RT-PCR for HCV

RT reactions were performed in a total volume of 25 µL containing 5 µL of total RNA and 50 pm of minus HCV specific primer (M1, Table 1). The material was incubated at 42 °C for 1 h, inactivated for 10 min and stored at -20 °C until use. A PCR mix containing the plus HCV specific primer (P1, Table 1) and *Taq* DNA polymerase was added to the RT mix and the first round of PCR was performed in a final volume of 50 µL at the following concentrations: 10 mmol Tris pH 8.3, 50 mmol KCl, 2 mmol $MgCl_2$, 0.2 mmol dNTPs, 2.5 units of *Taq* polymerase and 1 µmol of plus and minus primers (P1 and M1 outer primers). Nested PCR was performed in a final volume of 50 µL containing 5 µL of the reaction products of the first round of PCR at the following concentrations: 10 mmol Tris pH 8.3, 50 mmol KCl, 2 mmol $MgCl_2$, 0.2 mmol dNTPs, 2.5 units of *Taq* polymerase, and 1 µmol of plus and minus primes (P2 and M2 inner primers). The size of the target sequence amplified by the second round of PCR was 188 bp. Cycling conditions were as follows: hard denaturation at 94 °C for 3 min (1 cycle), denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min (30 cycles and 25 cycles for the outer and inner PCRs, respectively), and long extension at 72 °C for 7 min (1 cycle).

Table 1
Primers used for reverse transcription-polymerase chain reactions (RT-PCR)

Primers	Sense	Sequence
P1	+	5’-GATGCACGGTCTACGACACCT-3’
M1	-	5’-AACTACTGTCTTCACGCAGAA-3’
P2	+	5’-GCGACCCAACACTACTCGGCT-3’
M2	-	5’-ATGGCGTTAGTATGAGTG-3’
TGF-β1	+	5'-CTTCAGCTGCACAGAGAAGAACTGC-3
TGF-β1	-	5'-CACGATCATGTTGGACAACTGCTCC-3'
C-erb B-2	+	5'-AGC TCT GCT ACC AGG ACA CG-3'
C-erb B-2	-	5'-TCA GGC TCT GAC AAT CCT CA-3

RT-PCR for TGF-β

Total RNA (5 µg) was reverse transcribed using RT-PCR Stratagene kit, according to the manufacturer's instructions. Then the reaction tube was placed at 42 °C for 1 h followed by heating to 95 °C for 5 min to inactivate the reverse transcriptase enzyme. For PCR, 3 µL cDNA was mixed with 5 µL reaction buffer (500 mmol/L KCl, 100 mmol/L Tris pH 8.3, 1.5 mmol/L $MgCl_2$, and 0.001% gelatin), 4 µL of 2.5 mmol/L dNTP´s mix, 0.4 µL (2 U) *Taq* polymerase, 37.6 µL water and 1 µL of each primer containing 0.4 µmol/L (Table 1). The reaction mixture was then subjected to 35 cycles of PCR amplification as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The PCR product yielded 298 bp fragment on 1.5% agarose gel electrophoresis.

RT-PCR for C-erb B-2

Total RNA (2 µg) was heated for 3 min at 65 °C and reverse transcribed in the reaction mixture containing 0.5 µg random hexamer, 0.5 mmol dNTPs, 1.5 mmol $MgCl_2$, 75 mmol Tris pH 7.5, 10 mmol dithiothreitol, 20 U RNasin (Promega), and 200 U MMLV reverse transcriptase (Promega) in a final volume of 20 µL for 60 min at 42 °C. 2 µL amounts of complementary DNA products were subjected to PCR amplification. PCR was carried out in a 50-µL final reaction containing 0.4 mmol of each primer (Table 1), 0.2 mmol dNTPs, 2 mmol $MgCl_2$, 50 mmol KCl, and 1 U *Taq* DNA polymerase (Roche Diagnostics) The amplification profile was: denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min over 35 cycles. PCR products were analyzed by 1% agarose gel electrophoresis using standard molecular markers.

Histopathological studies of liver

Liver needle biopsies from HCV and HCC patients were examined. HCC group was subclassified into 3 grades. The HCV group was also subclassified according to degree of fibrosis into mild, moderate, marked and no fibrosis, according to the scoring system of Knodell et al. (16). The Knodell score, also known as the histologic activity index (HAI), is composed of the summation of four individual scores representing periportal and/or bridging necrosis (scores from 0 to 10), intralobular degeneration and focal necrosis (scores from 0 to 4), portal inflammation (scores from 0 to 4), and fibrosis (scores from 0 to 4); the score ranges from 0 to 22. A decrease in the Knodell score is considered to represent histologic amelioration.

RESULTS

The demographic data of the studied subjects are shown in Table 2.

The mRNA levels of both TGF-β1 and C-erb B-2 in polymorphonuclear cells were overexpressed in both HCC and HCV groups compared to controls (Table 3). A statistically significant difference in serum concentrations of TGF-β1 and C-erb β-2 were observed between HCV and HCC patients and also when each was compared with the control group (Table 4). The comparison of findings between Tables 3 and 4 shows that the higher gene expression in Table 3, the higher the serum concentration of proteins formulated by the expressed genes in Table 4.

In Table 5, the mean serum TGF-β1 concentrations in the HCV patient group are shown according to the degree of fibrosis. Increased degrees of fibrosis showed higher concentrations of TGF-β1 (F = 6.073, P = 0.003). Conversely, there was no dependence between the concentrations of C-erb B-2 and the degree of fibrosis (F = 2.796, P = 0.089).

In the HCC group, the mean serum TGF-β1 concentrations showed no statistical difference when different grades of disease were considered (Table 6). Conversely, a statistically significant difference in mean concentrations of C-erb B-2 was observed between HCC grade I and III (P <0.001) and grade II and III (P <0.001) whereas no statistically significant difference was found between HCC grade I and II.

DISCUSSION

Hepatocarcinogenesis is closely related to fibrosis. In the present work, TGF-β1 has been evaluated in patients with HCV and HCC. Upregulation of gene expression of TGF-β1 was found in both groups. These results are in agreement with those of Marek et al. (17), who demonstrated that both serum TGF-β1 concentrations and mRNA TGF-β1 expression in liver biopsy specimens may be useful as prognostic markers in patients with HCV undergoing antiviral therapy. At the same time, we found a quantitative increase in TGF-β1 expression in HCC compared to HCV patients. These results are in agreement with those of Sacco et al. (18), who reported that the rate of positive results of serum TGF-β1 concentrations in HCC patients (54%) was greater than in liver

Table 2
Demographic data of the studied groups

	HCV	HCC	Controls
Number of subjects	30	30	30
Males/Females	12/18	22/8	12/18
Mean age (±SD)	38.9 (±9.9)*	51.3 (±8.3)	41.3 (±8.4)

**Significantly different from other groups (P = 0.001).*

cirrhosis patients (26%) and healthy donors (3%). Our results are also in agreement with those of Kim et al. (19) and Pan et al. (20), who found a positive correlation between TGF-β1 and recurrence of HCC.

Veno et al. (21) found that TβR-II immunolocalization in liver tissues was significantly decreased in patients with HCC compared with that of patients with chronic hepatitis or liver cirrhosis. Their results indicated that human HCC has a reduced expression of type II TGF-β receptor for TGF-β1. This may provide a selective growth advantage to HCC to escape the inhibitory growth signals of TGF-β1 and may be linked with critical steps in the hepatoma cell growth.

TGF-β1 regulates diverse biologic activities including cell growth, cell death or apoptosis, cell differentiation and extracellular matrix synthesis. Elevated TGF-β1 concentrations in patients with chronic hepatitis and cirrhosis may have a role in the pathogenesis and chronicity of these diseases (22). In the present study, the concentrations of serum TGF-β1 were greater with increased degree of fibrosis. Our results agree with those of Flisiak et al. (5) and Liu et al. (6), who detected an elevation of serum TGF-β1 during hepatic pathological worsening, suggesting that TGF-β1 may be an important factor in accelerating liver fibrosis.

Relatively little is known about the biochemical mechanisms controlling proliferation and neoplastic transformation of HCC. Neu-oncoprotein overexpression in the tumour tissue has been found to correlate with the elevation of its serum concentrations (23). Thus, it seems likely that the source of increased serum neu-oncoprotein in such patients is their affected tumour tissue (24). The present study has found that serum C-erb B-2 concentrations in HCC and HCV patients were significantly higher than those of healthy controls, with highest concentrations found in HCC patients. These results are in agreement with Yu et al. (13), who demonstrated frequent overexpression of neu-oncoprotein in serum of HCC patients. Our present results disagree with those of Abdel-Rahman et al. (25), who detected increased serum concentrations of C-erb B-2 in HCC and HCV patients when compared to controls, but did not find any significant difference between HCC and HCV patients.

The exact role of neu-oncoprotein overexpression in hepatocarcinogenesis remains to be defined. The elevated expression of neu-oncoprotein may simply reflect

Table 3
Mean ±SD concentrations (µg/mL) of the mRNAs of TGF-β1 and C-erb B-2 in the polymorphonuclear cells of studied subjects

	HCV	HCC	Controls
TGF-β1	284.6 ± 23.5	543.7 ± 211.6	127.9 ± 15.6
Compared to controls	P <0.001	P <0.001	
Compared to HCC	P <0.001		
C-erb B-2	1658.7 ± 423.5	2344.7 ± 973.5	679.7 ± 176.4
Compared to controls	P <0.001	P <0.001	
Compared to HCC	P <0.05		

Table 4
Mean ±SD serum concentrations of TGF-β1 and C-erb B-2 in the studied groups

	HCV	HCC	Controls
TGF-β1, ng/L	404 ± 186.3	631 ± 222.5	49.5 ± 9.6
Compared to controls	P <0.001	P <0.001	
Compared to HCC	P <0.001		
C-erb B-2, HNU/mL	2339 ± 615	3756 ± 1178	1620 ± 263
Compared to controls	P <0.001	P <0.001	
Compared to HCC	P <0.05		

Table 5
Mean ±SD serum concentrations of TGF-β1 and C-erb B-2 in the HCV group according to the degree of fibrosis

	No of cases	TGF-β1, ng/L	C-erb B-2, HNU/mL
No fibrosis	10	253 ± 167	2373 ± 606
Mild fibrosis	8	377 ± 225	785 ± 146
Moderate fibrosis	7	450 ± 153	2750 ± 541
Marked fibrosis	5	686 ± 210	3456 ± 1488

Table 6
Mean ±SD serum concentrations of TGF-β1 and C-erb B-2 in HCC patients according to the disease grade

	No of cases	TGF-β1, ng/L	C-erb B-2, HNU/mL
Grade I	10	595 ± 196	2914 ± 555
Grade II	9	670 ± 210	2802 ± 422
Grade III	11	632 ± 257	5302 ± 2365

the increased cell proliferation in preneoplastic liver tissue or in association with the cellular degeneration and necrosis that are characteristic of chronic liver disease. Alternatively, the increased expression may result from exposure to carcinogens in the course of hepatocarcinogenesis. In a study (7), there was no significant association between HER-2/neu overexpression and clinicopathological parameters. The authors concluded that there is a low frequency of HER-2/neu overexpression/amplification in HCC. Also, they suggested that there appears to be no role for HER-2/neu as a prognostic marker and no benefit of anti-HER-2/neu trastuzumab treatment in patients with HCC (7).

REFERENCES

1. Clouston AD, Jonsson JR, Macdonald GA, et al. Steatosis and chronic hepatitis C: analysis of fibrosis and stellate cell activation. J Hepatol 2001;34:314-20.
2. Powell EE, Edwards-Smith CJ, Hay JL, et al. Host genetic features influence disease progression in chronic hepatitis C patients. J Hepatol 2000;828-33.
3. Abou-Shady M, Baer HU, Friess H, et al. Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. Am J Surg 1999;177:209-15.
4. Buenemann CL, Willy C, Buchmann A, et al. Transforming growth factor-beta 1 induced Smad signaling, cell cycle arrest and apoptosis in hepatoma cells. Carcinogenesis 2001;22:447-52.
5. Flisiak R, Pytel-Krolezuk B, Prokopowiez D. Circulating transforming growth factor-beta (1) as an indicator of hepatic function impairment in liver cirrhosis. Cytokine 2000;12:677-81.
6. Liu F, Li B, Nan Y. The effect of serum TGF β1 of patients with chronic hepatitis B in liver fibrosis transformation. Zhonghua Gan Zang Bing Zazhai 1999;7:196-8.
7. Xian ZH, Zhang SH, Cong WM, et al. Overexpression/amplification of HER-2/neu is uncommon in hepatocellular carcinoma. J Clin Pathol 2005;58:500-3.
8. Prss MF, Pike MC, Chazin VR. HER-2 neu expression in nod-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res 1993;53:4960-70.
9. Hudziak RM, Schlessinger, Ullrich A. Increase expression of the putative growth factor receptor P185 Her2 cause transformation and tumorigenesis of NIH3T3 cells. Proc Natl Acad Sci VSA 1987;84:7159-63.
10. King CR, Kraus MH, Di Fiore PP, et al. Implications of C-erb B-2 overexpression for basic science and clinical medicine. Semin Cancer Biol 1990;1:329-37.
11. Carney W, Hamer P, Petit D. Detection and quantitation of the human neuoncoprotein. J Tumor Marker Oncol 1991;6:53-72.
12. Jiin-Chyuan L, Ming-Whei Y, Chen-Jen C. Serum C-erb B-2 oncopeptide in hepatocellular carcinogenesis. Med Sci Res 1993;21:305-7.
13. Yn M-W, Chen CT, Luo JC, et al. Correlation of chronic hepatitis B virus infection and cigarette smoking with elevated expression of neuoncoprotein in the development of hepatocellular carcinoma. Cancer Res 1994:54:5106-10.
14. Breuer B, Luo JC, De Vivo I. Detection of elevated C-erb B-2 oncopeptide in the serum and tissue in breast cancer. Med Sci Res 1993:21:383-4.
15. Chomkczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform method. Anal Biochem 1987;162:156-60.
16. Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431-5.
17. Marek B, Kajdaniuk D, Mazurek U, et al. TGF-beta1 mRNA expression in liver biopsy specimens and TGF-beta1 serum levels in patients with chronic hepatitis C before and after antiviral therapy. J Clin Pharm Ther 2005;30:271-7.
18. Sacco R, Leuci D, Tortorella C, et al. Transforming growth factor beta-1 and soluble Fas serum levels in hepatocellular carcinoma. Cytokine 2000;12:811-4.
19. Kim HG, Chung YH, Song BC, et al. Expression of transforming growth factor-beta 1 in chronic hepatitis and hepatocellular carcinoma associated with hepatitis C virus infection. Korean J Intern Med 2000;15:165-70.
20. Pan Z, Yang B, Liu Y. TGF beta subtypes in hepatocellular carcinoma. Zhonghua Yi Xue Za Zhi 1998;78:595-7.
21. Veno T, Hashimoto O, Kimura R, et al. Relation of type II transforming growth factor-beta receptor to hepatic fibrosis and hepatocellular carcinoma. Int J Oncol 2001;18:49-55.
22. Kirmaz C, Terzioglu E, Topalak O, et al. Serum transforming growth factor-beta 1 (TGF-beta 1) in patients with cirrhosis, chronic hepatitis B and chronic hepatitis C. Eur Cytokine Netw 2004;15:112-6.
23. Mori S, Mori Y, Mukaiyama T. In vitro and in vivo release of soluble C erb B-2 protein from human carcinoma cells. Jpn J Cancer Res 1990;81:489-94.
24. Brandt-Rauf PW, Luo J, Carney WP. Detection of increased amounts of extracellular domain of the C erb B-2 oncoprotein in serum during pulmonary carcinogenesis in humans. Int J Cancer 1994;56:383-6.
25. Abdel-Rahman NZ, Abeer AB, Sabry MS, et al. Hepatitis C virus genotyping in relation to neu-oncoprotein overexpression and the development of hepatocellular carcinoma. J Med Microbiol 2000;49:89-95.