ORIGINAL ARTICLE Egypt. J. Med. Hum. Genet. Vol. 10, No. 2, Nov. 2009

Hepatitis C virus infection and gene expression of hepatocellular carcinoma in Egyptian patients

Ola Sayed M. Ali[1], Mohammed Tarek M. Mansour[2], Hala T. El-Bassyouni[3], Sahar M. Abd El-Maksoud[1] and Rehab Refaat El-Awady[1]

[1]Biochemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, [2]Virology and Immunology, National Cancer Institute, Cairo University, [3]Clinical Genetics Department, National Research Center

ABSTRACT

Introduction: Gene expression profiling of hepatocellular carcinoma (HCC) is promising for refining the diagnosis and prognosis as well as identifying potential therapeutic targets.

Aim of the Study: Our study aimed to study the gene expression in 40 HCC patients infected with hepatitis C virus (HCV) using RT-PCR technique on surgical liver sample. Gene expression changes in HCV-positive group were compared with gene expression in HCV-negative group. Four genes were included in this study, AFP gene, CD10 gene, HGF gene and GRB2 gene. The expression of the four genes were slightly higher in HCV positive group than in HCV negative group, however, the difference between the two groups was non-significant. HGF gene was expressed in only 20% of HCC patients and GRB2 gene was expressed in 95% of HCC patients. AFP gene and CD10 gene were expressed in all patients.

Conclusion: AFP gene, CD10 gene and GRB2 gene play an important role as diagnostic markers of HCC.

Key Words:

Hepatocellular carcinoma, hepatitis C virus, AFP gene, CD10 gene, HGF gene and GRB2 gene.

Corresponding Author:

Hala El-Bassyouni
E-mail: halabassyouni@yahoo.com

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the fifth most common malignancy worldwide and one of the deadliest human cancers in the world.[1,2]

The prevalence of hepatitis C virus (HCV) infection in Egypt has been estimated to be around 15%[3]. The rising trend of HCC is more likely attributed to HCV infection[4]. It is more likely that HCC occurs against a background of inflammation and regeneration, associated with liver injury due to chronic hepatitis and this leads to fibrosis and cirrhosis.[5]

The most common finding on physical examination is an enlarged, irregular and nodular liver. Jaundice and abnormal findings of liver function tests may not be present until late in the course of the disease because of the functional reserve of the liver[6]. The HCC patients may have a genetic basis for their disease. Their predisposition could be iden-

tified through a detailed family history obtained through a genetic evaluation and those high risk families may benefit from genetic testing.[7]

The use of gene expression arrays as markers for genomic abnormalities is useful to characterize genomic changes responsible for pathogenesis in HCV[8]. Improved molecular characterization of HCC from gene expression profiling studies will undoubtedly improve the prediction of treatment responses, improve the selection of treatments for specific molecular subtypes of HCC and ultimately improve the clinical outcome of HCC patients.[9]

Type two cell surface metaloprotein (CD10) is a type II integral membrane protein known as neutral endopeptidase which functions to cleave small biologically active peptide at the amino terminus to hydrophobic residues within the peptide sequences.[10]

Serum alpha-fetoprotein (AFP) is one of the earliest markers for endodermal differentiation and a prognostic indicator of the response and survival of germ cell tumors[11]. Moreover, reference[12] concluded that AFP mRNA is a more reliable marker of metastasis compared to serum AFP.

Hepatocyte growth factor **(**HGF) plays key roles in the attenuation of disease progression as an intrinsic repair factor. In cases of liver damage, plasma HGF levels are likely to increase, not only because of the up-regulation of HGF, but also because of the lower clearance of HGF in the liver.[13]

Growth factor receptor-bound protein 2 (GRB2) act as an adaptor and is required during embyrogenesis for the differentiation of endodermal cells and formation of the epiblast.[14]

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene is utilized as a prototype for studies of genetic organization, expression and regulation. GAPDH is involved in apoptosis, age-related neurodegenerative disease, prostate cancer and viral pathogenesis.[15]

This study aims to identify diagnostic markers of HCC patients infected with hepatitis C virus (HCV).

SUBJECTS AND METHODS

This study included 40 HCC patients attending the National Cancer Institute - Cairo University, Egypt. Patients' clinical data including age, sex, similarly affected family members, liver function tests and the main clinicopathological features of the specimens are described in (Table 1).

Table 1: Percentage of demographic data of 40 HCC patients.

Variables	Number	Percentage
Sex:		
Males	35	87.5
Females	5	12.5
Similarly affected family members	15	37.5
Tumour grade:		
Grade I	9	22.5
Grade II	17	42.5
Grade III	14	35.0
Cirrhosis:		
Positive	29	72.5
Negative	11	27.5

Virological Studies:

HCV-Ab was detected by test kit (a solid phase enzyme linked immunosorbent assay (ELISA)) for the detection of antibodies to HCV in human serum (ADALTIS ITALIA S.P.A). HBsAg was determined by a non-competitive enzyme linked immunosorbent assay (ELISA), using plate coated with anti-HBs (ADALTIS ITALIA S.P.A). HCV viral sequence was detected by the extraction of HCV RNA from serum by the method described by[16]. Reverse transcribed into cDNA using RT mixture consisted of [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100 (Packard instrument Co., Downers Grove, III), 6 mM $MgCl_2$, 20 U of RNase inhibitor, 0.6 mM (each) deoxynucleotid triphosphate (dNTPs) (Sigma) and 25 ng of HCV-6 primer (5'ACC TCC 3')] and amplified by PCR performed in a 50 ul volume containing [2.5 u Taq polymerase (Perkin-Elmer Cetus), 50 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs (Sigma), 100 ng RB-6A (Sense primer 5'GTG AGG AAC TAC TGT CTT CAC G 3'[nt 47 to 68]), 100 ng RB-6B (antisense primer 5' ACT CGC AAG CAC CCT ATC AGG 3'[nt 292 to 312]), 200 U of superscript RTI (Gibco-BRL, Gaithersburg, MD)] and 12.5 ul of the reaction mixture. The RT-PCR products were detected by 2% agarose gel electrophoresis in 1x TBE buffer. The gel was stained with 5 ul of 10 mg /ml ethidium bromide. A positive result revealed a PCR product band at 265 bp using a size marker of the Φ X 174/Hae III DNA.

Molecular Studies:

Total cellular RNA was isolated from liver tissue using TRIZOL reagent, which maintained the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separated the solution into an aqueous phase and an organic phase. RNA remained exclusively in the aqueous phase whereas DNA and proteins were in the interphase and organic phase. After transfer of the aqueous phase, the RNA was recovered by precipitation with isopropyl alcohol and sodium acetate. The pellet was washed two times in 75% ethanol then stored at -80°C. RNA was resuspended at approximately 1 mg/ml in DEPC H_2O. The concentration and purity of RNA was determined in the concentrated sample spectrophotometrically by measuring the absorbance at 260 nm (A 260) and at 280 nm (A 280). The ratio A260: A280 of pure RNA should be in the range of 1.9: 2.1 Boom et al.[16]

Total cellular RNA was used in a RT-PCR reaction to detect the expression of genes included in this study. cDNA synthesis was performed using the cDNA synthesis kit (Clontech) according to the manufacturer's protocol. Briefly, 1 µg of total RNA was used in a 20 µl reaction mixture containing [5x reaction buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L $MgCl_2$), 10 mmol/L each dNTP, Moloney-murine leukemia virus reverse transcriptase (40 U/µl), RNase inhibitor (1 U) and an oligo (dT)18 primer]. The reaction mix was incubated at 42°C for 1 hour to synthesize the first strand cDNA and the reaction was then terminated at 94°C for 5 minutes and diluted to 100 µl with 80 µl of DEPC treated H_2O.

Subsequently, 5 to 10 µl of diluted reaction mixtures were subjected to 35 PCR cycles, using the AdvanTaq PCR

kit (Clontech) according to the manufacturer's suggested protocol.

PCR products were subjected to 2% agarose gel electrophoresis and were visualized under UV. Semiquantitation was done by comparing the intensities of the bands of the four genes with the intensity of GAPDH gene band using documentation camera.

Statistical Analysis:

of the results was performed using version 9 SPSS / PC computer programs (SPSS Inc. Chicago). All reported P-values were two-tailed, value <0.05 was considered significant.

RESULTS

The 40 HCC patients were categorized according to HCV infection into two groups: The first group include 23 patients with HCV positive, two of whom were coinfected with HCV and HBV. The second group includes 17 patients with HCV negative, five of whom were HBV positive. The age range was 25-62 years (39.3 ±8) and 35/40 were males with M/F ratio 7/1. Tumor tissues samples were obtained from the HCC patients to analyze the role of HCV infection in the genes expression profile of HCC.

In this study, four genes were selected to analyze the role of HCV infection in genes expression profile of HCC patients. These genes were *AFP, CD10, HGF* and *GRB2*. *GAPDH* gene was used as internal control. *AFP* gene and *CD10* gene were expressed in all HCC patients. *AFP* gene was highly expressed in 20%, moderately expressed in 22.5% and low expressed in 57.5% of HCC patients while the expression of *CD10* gene was high in 22.5%, moderate in 72.5% and low in 5% of HCC patients. Growth factor receptor-bound protein 2 *(GRB2)* gene was expressed in 95% of HCC patients, 90% of whom have low expression and 5% have moderate expression. *HGF* gene was expressed in only 20% of HCC patients and the expression was low (Figs. 1-5).

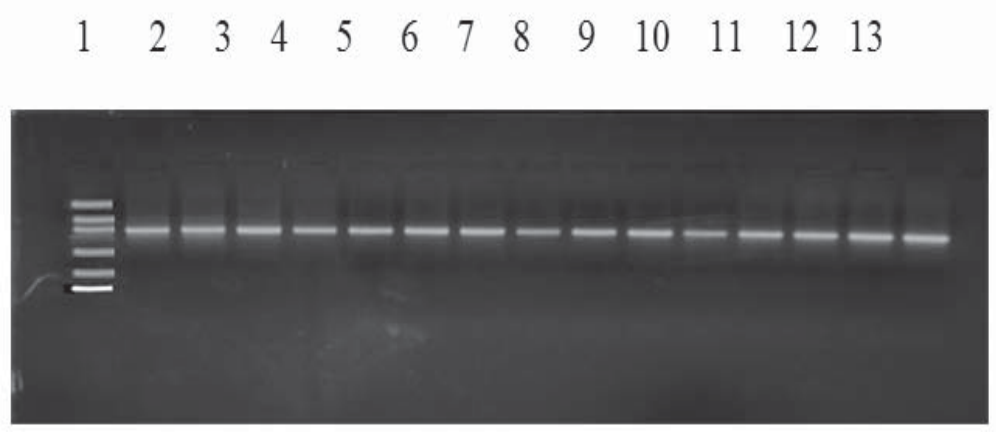

Fig. 1: RT -PCR product of *GAPDH*-gene as an endogenous control at 436 bp.

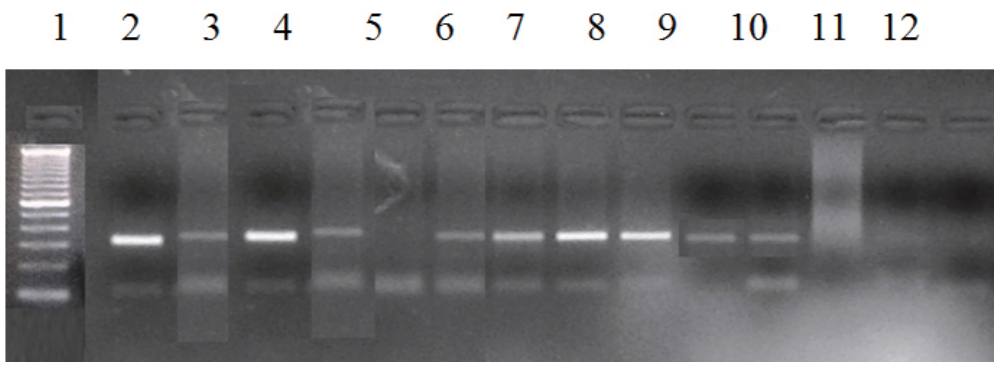

Fig. 2: RT-PCR products of AFP-gene expression in HCC patients using GAPDH as endogenous control.

Lane 1, represent 100 bp ladder; lane (2, 3, 4, 5, 7, 8, 9, 10, 11, 12) represented positive expression of AFP-gene expression among HCC patients groups at 300 bp; lane 6 represent negative normal control

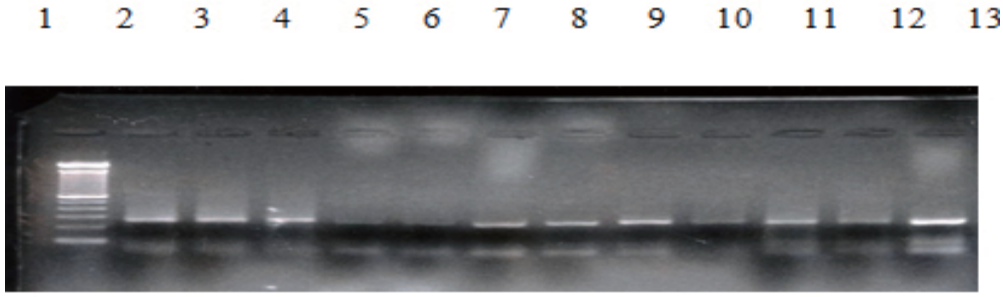

Fig. 3: RT-PCR products of *CD10*-gene expression in HCC patients using *GAPDH* as endogenous control.

Lane 1, represent 100 bp ladder; lane (2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13) represented positive expression of CD10-gene expression among HCC patients groups at 220 bp; lane 6 represent negative normal control.

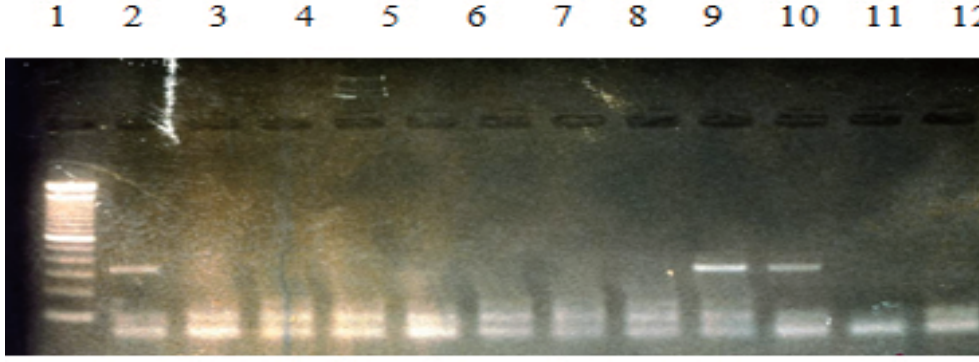

Fig. 4: RT-PCR products of *HGF*-gene expression in HCC patients using *GAPDH* as endogenous control.

Lane 1, represent 100 bp ladder; lane (2,10,11) represented positive expression of *HGF*-gene among HCC patients groups at 350 bp; lane (3-9, 12) represent negative expression of *HGF*-gene; lane 13 represent negative normal control.

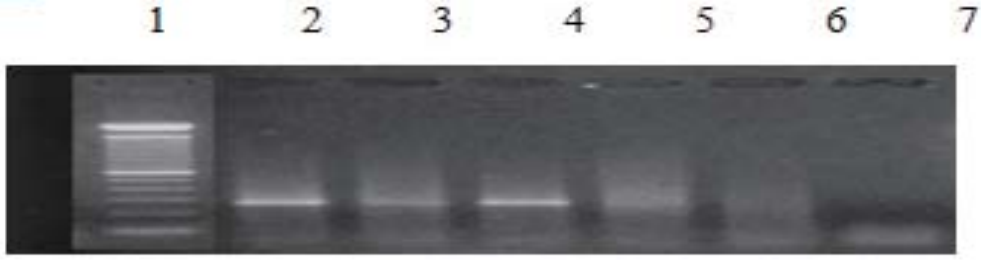

Fig. 5: RT-PCR products of GRB2-gene expression in HCC patients using GAPDH as endogenous control.

Lane 1, represent 100 bp ladder; lane (2,3,4) represented positive expression of *GRB2*-gene among HCC patients groups at 230 bp; lane (5, 6) represent negative expression of *GRB2*-gene; lane 7 represent negative normal control.

DISCUSSION

Hepatitis C virus may play a direct role in hepatic carcinogenesis through involvement of gene viral products in inducing liver cell proliferation.[17]

In this study, the age range was 25-62 years (39.3 ±8), 87.5% were males with M/F ratio 7:1. Szklaruk[6] showed that the peak age of incidence of HCC is 50-70 years, with a male predominance of 4:1. A number of factors, including genetic susceptibility androgenic steroids and higher body iron stores, may explain the male bias among HCC patients. Similarly affected family members were present in 37.5% of patients. Reference[18] reported that Liver cancers appear to be approximately 3-fold more frequent among patients with a history of similar cases in the family. This would support the existence of a genetic component in the familial aggregation of HCC.

In the present study, out of the 40 HCC patients 52.5% were HCV-Ab seropositive, 12.5% were HBV Ag seropositive, 5% were found coinfected with HCV and HBV and 30% were not infected with any of them. HCV-RNA was detected in all HCV-Ab seropositive HCC patients. These results confirm that HCC is strongly related to HCV and HBV infection. Other reports in Egypt showed a strong association of HCC with HCV and HCV-HBV double infection than with HBV alone.[19]

Hepatitis C virus mostly plays an indirect role in tumor development and appears to increase the risk of HCC by promoting fibrosis and cirrhosis. On the other hand, HCV may play a direct role in hepatic carcinogenesis through involvement of gene viral products in inducing liver cell proliferation.[17]

In this study, the first group included 23 HCV positive-HCC patients; all of them were cirrhotic. The second group included 17 HCV negative-HCC patients; only 35.3% of them were cir-

rhotic. This was in agreement with the study of[20] that showed that HCC rarely occurs in patients with HCV who do not have cirrhosis.

The HCV-positive group had higher significant levels of serum ALT and AST compared to HCV-negative group. Serum amino-transferase concentration is an indicator of hepatocellular damage. ALT is a more specific marker of hepatocellular injury because it occurs exclusively in the liver. There is a close correlation between multicentric hepatocarcinogenesis and sustained elevation of the serum ALT levels, which represent the inflammatory necrosis of hepatocytes.[21]

In the current study, the AFP serum level was normal in 25% of HCC patients, in abnormal level < 200 ng/ml in 55% of HCC patients and in abnormal level > 200 ng/ml in 20% of HCC patients. The diagnostic AFP level (> 200 ng/ml) was found in 28.9% of HCC cases in the study of.[4]

Alpha-fetoprotein (AFP) gene was expressed in all HCC patients and the expression was low in 57.5%, moderate in 22.5% and high in 20% of HCC patients. There was also a high correlation between mean AFP serum level and expression of AFP gene in the mRNA level. This coincides with the findings of[22] that showed that high AFP (> 200 ng/ml) was associated with AFP mRNA expression in HCC patients.

The CD10 gene was expressed at the mRNA level in all HCC patients included in the study. The expression was low in 5%, moderate in 72.5% and high in 22.5% of HCC patients. This was in agreement with the study of[10] that identified CD10 gene to be up regulated at the mRNA level in HCC samples as compared to non-neoplastic liver tissue with 40-fold increase in mRNA abundance in HCC samples. They also suggested that an increase in CD10 protein expression might be a diagnostic marker for HCC.

The hepatocyte growth factor (HGF) gene was low expressed at mRNA level in 20% of HCC patients included in the study. This coincides with the findings of[23] that showed that low levels of HGF mRNA were found in HCC patients.

However, in another study mRNA levels of HGF were markedly increased in HCC[24]. The different HGF mRNA levels found in different studies may be attributed to the different roles of HGF in the growth of HCC. Yang et al.[24] demonstrated that cell density determined the effect of HGF on the growth of HCC.

The expression of growth factor receptor-bound protein 2 (GRB2) gene at mRNA level was detected in 95% of HCC patients included in the study; 90% of HCC patients showed low expression and 5% of them showed moderate expression. This result is in agreement with the study of.[25]

CONCLUSION

AFP gene, *CD10* gene and *GRB2* gene play an important role as diagnostic markers of HCC. Further studies of genetic profile among Egyptian HCC patients should be done to elucidate the genetic basis of HCC.

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