Telomerase Activity in Hepatocellular Carcinoma and Chronic Hepatitis C Virus.

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ABSTRACT

Serum telomerase activity and AFP were determined in patients with chronic hepatitis C and hepatocellular carcinoma to evaluate the use of telomerase activity in peripheral blood of patients using PCR-ELISA to perform highly sensitive, semi quantitative photometric enzyme detection to evaluate it as a prognostic tumor marker of the disease activity. The study was performed on 55 patients that classified clinically into three group: The first group included 15 healthy control subjects, the second group included 20 patients suffering from chronic hepatitis C and the third group included 20 patients with hepatocellular carcinoma (HCC). We measured AFP and hTERT mRNA levels in peripheral blood using quantitative real-time reverse transcription polymerase chain reaction. Increased telomerase activity was detected in 85% of HCC and 10% of chronic hepatitis cases but was not detected in controls. HCC is associated with increased telomerase activity in peripheral blood and the incidence of the increased telomerase activity is directly related to the degree of histological undifferentiating of HCC. So, we conclude that the increased telomerase activity in hepatocellular carcinoma is highly evident and it is directly related to the degree of histological undifferentiation of hepatocellular carcinoma. We also concluded that a positive telomerase activity existed in chronic hepatitis disease is evaluating telomerase as a more accurate and an early detector for hepatocarcinogenesis.

Key Words: Hepatocellular carcinoma, hepatitis C – telomerase-PCR.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worlds wide. The major etiologies and risk factors for the development of HCC are well defined and some of the multiple steps involved in hepatocarcinogenesis have been elucidated in recent years (Moradpour et al. 2005). The development of HCC is a multi step process associate with alteration in several genes and, depending also on external factors, such as infection by hepatitis B or C; chronic alcohol intake and exposure to certain chemicals (Richard. 2003). Among the many proposed prognostic factors for HCC are serum alphafetoprotein (AFP) levels, tumor size, node involvement, metastasis, (Park et al. 2008). Because HCC is a typical hypervascular tumor, invasive HCC is considered to be reflected in circulating tumor cells that have escaped from the tumor via blood vessels. Circulating tumor cells can be detected by identifying tumor-associated genes in peripheral blood (Funaki et al. 1997). Tumor-associated gene expression in peripheral blood reflects the presence of circulating HCC cells and could be a surrogate marker for HCC extrahepatic metastases. One of HCC-associated gene is human telomerase reverse transcriptase protein (hTERT), which is a component of telomerase. Telomerase is a ribonucleoprotein enzyme associated with cellular immortality, consists of human telomerase RNA component (hTERC), human telomerase protein 1 (hTEP1) and human telomerase reverse transcriptase (h TERT).They play an essential role in the stable maintenance of the eukaryotic chromosome within the cell by serving as specific binding sites for structural proteins. The proteins cap the ends of liner chromosomes, thus preventing and to end fusion and other events that are normally lethal to the cell. A progressive shortening of the chromosome ends with each replication cycle result from DNA polymerase’s inability to replicate linear DNA to its very ends. The maintenance of stable telomerase length in replicating cells is associated with the activation of telomerase is a ribonucleo protein reverse transcriptase enzyme, consisting of catalytic subunit (TERT), associated template RNA and structural proteins. The enzyme activity compensates for the loss of telomeric DNA by adding repeat sequences to the chromosome ends. As a result, telomerase acts as a reverse transcriptase that uses part of its intrinsic RNA component as a template for telomeric repeat synthesis (Ramirez et al. 2003). Numerous data on telomerase expression demonstrate the presence of telomerase activity in the vast majority of
different cancer types, but failed to detect telomerase activity in normal tissue (Lechel et al. 2004). The conclusions drawn from previous studies have been
based on a limited number of patients and in most cases, used qualitative or semi-quantitative measurements of AFP. In this study, we determined serum telomerase activity in patients with chronic hepatitis C and hepatocellular carcinoma to evaluate the use of telomerase activity in blood of patients as a prognostic tumor marker in HCC.

PATIENTS AND METHODS

The study was performed on newly diagnosis 55 patients before treatment (20 Patients with HCC were presenting to the outpatient clinic of Medical Oncology Unit of the National Cancer Institute, Cairo University. While (20) patients with chronic hepatitis C was recruited from Hepatology unit, Kaser El Ani, Cairo University and their ages range (30–60 years). Besides 15 healthy individuals were included as healthy control group, their ages ranged (27-50 years).

The diagnosis of hepatocellular carcinoma was based on clinical picture ultrasonography; C T guided biopsy from the liver with histopathological examination.

Blood Sampling:
Ten ml venous blood samples obtained from patients with HCC, patients with chronic hepatitis C and healthy controls. The samples was divided into two parts, one for measuring telomerase activity and alpha-fetoprotein and second sample for the routine laboratory investigation (AST,ALT,ALP,Bilirubin (T&D),total protein and albumin.

Processing of blood samples and RNA extraction:
Blood samples were collected into EDTA-containing tubes and centrifuged at 2,000 rpm for 10 min at 4°C. Plasma was then carefully transferred to plain polypropylene tubes and theuffy coat was transferred to plain polypropylene tubes. All samples were stored at -80°C until RNA extraction.

RNA was extracted from 200 µL buffy coat using a QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was eluted in 30 µL RNase-free water and stored at -80°C until use.

Telomerase PCR ELISA:
The telomerase PCR ELISA developed by Boehringer Mannheim is an extension of original method described by (Kim et al. 1994), it allows highly specific amplification of telomerase mediated elongation products combined with non-radioactive detection following an ELISA protocol. The assay can be separated into the following steps:

Elongation-Amplification:
In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3 end of biotin- labeled synthetic P1-TS primer. In a second step, these elongation products were amplified by PCR using the primers P1-TS and P2, generated PCR products with telomerase specific 6 nucleotide increments.

Detection by ELISA:
An aliquot of the PCR product is denaturated and hybridized to a digoxigenin (DIG)- labeled, telomeric repeat specific detection probe. The resulting product is immobilized via the biotin labeled primers to steptavidin – coated microtiter plate. The detection probe and the hybridization conditions have been optimized for obtaining the highest specificity and sensitivity. The immobilized PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxides. Finally, the probe is visualized by virtue of peroxides metabolizing substrate TMB to form a colored reaction product.

Separation of mononuclear layer:
Mononuclear cells were separated in sterile laminar flow, 5 ml of blood collected on EDTA vacationer was layed on 5 ml ficoll hypaque gradient in a centrifuge tube, the tube was centrifuged at 1200 rpm for 20 minutes. The supernatant plasma was discarded then the mononuclear layer was separated in a sterile tube, wased 3 times in phosphate buffer saline (PBS) and separated mononuclear cells were resuspended in 1 ml (PBS) and the cells were counted by both haemocytometer and electronic counting (coulter STK-S Electronic Inc., USA). 2 X 105 cells per single reaction was transferred into fresh eppendorf tube centrifuged at 3000 rpm for 10 min. in refrigerated centrifuge at 4°C.

The supernatant was carefully removed and the cells resuspended in PBS and the centrifugation step was repeated again, the supernatant was removed and the pelleted cells were stored at –70°C until it was used.

Preparation of total protein extract from mononuclear cells:
Before running the PCR reaction, the frozen cell pellet was thawed on ice then 200 µ of pre-cooled lysis reagent provided with kit was added. The cell pellet was resuspended in lysis reagent by retropipetting at least 3 times and then incubated on ice for 30 min.

The sample was centrifuged at high speed for 20 min. at 4°C and protein concentration of the supernatant was removed and transferred to fresh tube to ensure that cellular debris of the pelleted cell is transferred.

Telomeric repeat amplification protocol (TRAP reaction):
PCR reaction: The 2x reaction mixture containing tris-buffer, telomerase substract, biotin labeled P1-Ts primer, nucleotides,
The washing buffer was removed carefully. 100µ TMB were washed 3 times with 250µ of washing buffer per well, the solution was removed completely and the MTP modules were transferred into PCR tubes containing 25 µ of reaction mixture and completed to final volume 50 µ by sterile water, all pipetting steps were done on ice.

PCR tubes were transferred to a thermal cycler, Perkin-Elmer 9600 and performed a combined primer elongation-amplification reaction.

**Hybridization and ELISA procedure:**
For each sample 20 µ of denaturation reagent was transferred into a nuclease free uncoated microtitre plate (MTP) and 5µ of amplification product was added and incubated at room temperature 10 min.225µ per sample of hybridization buffer was added and mixed thoroughly. For every sample or control 100µ of the above mixture was transferred into wells of streptavidin precoated MTP modules supplied with Kit, covered with the self adhesive cover foil to prevent evaporation and incubated at 37ºC on a shaker (300 rpm) for 2 h. after incubation hybridization solution was completely removed and the wells were washed 3 times with 250µ of washing buffer (an appropriate volume of 10x washing buffer was diluted with autoclaved redist. Water (1: 100) and mixed thoroughly before use washing buffer was removed and 100 µ of Anti-DIG-POD working solution was added per well (an appropriate amount of reconstituted anti-DIG-POD was diluted by conjugate dilution on buffer to a final concentration of 10µ to each working concentration).

The MTP modules were covered with cover foil and incubated at room temperature for 30 min. on shaker after incubation, the solution was removed completely and the MTP modules were washed 3 times with 250µ of washing buffer per well, then the washing buffer was removed carefully. 100µ TMB substrate solution prewarmed to room temperature was added per well, covered, incubated for color development at room temperature for 10-20 min without removing the reacted substrate. 100 µl of stop reagent per well was added to stop color development. The addition of stop reagent causes the react POD substrate to change in color from blue to yellow and this is required to achieved maximal sensivity, the absorbance of the samples and controls were measured within 30 min. after addition of stop reagent using a Dynatech MR 5000 microplate ELISA reader at wave length of 450 nm against blank (with reference wavelength 620 nm).

**Serum alpha-fetoprotein:**
Serum alpha-phetoprotein (AFP) levels were measured by Abbott Axsyme (USA), using Microparticle Enzyme Immunoassay method (MEIA) (Schroff et al. 1985).

**Statistical methods:**
Results were expressed as mean ± SD and were analyzed by using the Student’s t test and ANOVA tests, as appropriate. Correlation between different parameters was performed using Pearson’s correlation test. P ≤ 0.05 was considered to be significant. All statistical procedures were performed using SPSS software, version 11 for Windows.

**RESULTS**

HCV antibodies and PCR for HCV – RNA were positive for all cases of the chronic hepatitis but were negative for all control subjects as shown in Table (1).

The mean serum alpha fetoprotein of chronic hepatitis group (8.43±0.56 ng/ml) was not significantly compared to that of control group (1.17 ±0.51 ng/ml). The mean serum alpha fetoprotein of HCC group (30.35 ± 30.66 ng/ml) was significantly higher compared to that of the control group (P<0.001) and that of chronic hepatitis (P<0.002). The mean telomerase activity of chronic hepatitis group (10.14 ± 5.71 units) was not significantly different compared to that of the control (2.82 ±0.39) (P > 0.5). The mean telomerase activity of HCC group (34.8 ± 24.8 units) was significantly higher compared to that of the control group (P<0.001) and that of chronic hepatitis C group (P<0.001).

**Correlation between telomerase activity and AFP:**
15 out of 17 HCC cases with increased telomerase activity showed elevated AFP value (i.e. 88.2% of HCC cases with increased telomerase activity) and 1 out of the 2 chronic hepatitis cases with increased telomerase activity showed elevated AFP values (i.e. 50 % of the chronic hepatitis cases with increased telomerase activity ). There was no direct relation established in this study between the relative telomerase activity and AFP in terms of value as shown in Figure (1).

The values of telomerase activity in peripheral blood from HCC group, chronic HCV group and healthy control group presented in Figure (2).

Table (2) showed that all cases of grade III HCC increased telomerase activity regardless of the relative values of this activity (i.e. 100% of grade III HCC). 4 of 5 cases of grade II HCC showed increased telomerase activity (i.e. 80% of grade II HCC) and 2 of 4 cases of grade I HCC (i.e. 50 % of grade I HCC) showed increase in telomerase activity. Thus, a direct relationship between the incidence of telomerase activity and the grade of HCC could be established in this study but again this relationship Was not established in terms of the relative telomerase activity values compared to the tumor grade.

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Table 1: HCV antibodies and PCR for HCV RNA in control group, chronic hepatitis C group and hepatocellular carcinoma (HCC) group.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group</th>
<th>Chronic HCV group</th>
<th>HCC group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patient</td>
<td>15</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.4</td>
<td>46.13</td>
<td>52.2</td>
</tr>
<tr>
<td>sex (M/F)</td>
<td>9/6</td>
<td>12/8</td>
<td>14/6</td>
</tr>
<tr>
<td>Viral serological marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV Ab</td>
<td>Negative</td>
<td>20 positive</td>
<td>20 positive</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>15 Negative</td>
<td>20 positive</td>
<td>20 positive</td>
</tr>
</tbody>
</table>

HCV Ab = hepatitis C virus antibodies, PCR = polymerase chain reaction, RNA = Ribonucleic

Table 2: Comparative study of results of telomerase activity in relation to histopathology.

<table>
<thead>
<tr>
<th>Histopathology Grade</th>
<th>No. of cases</th>
<th>Telomerase activity</th>
<th>Percent of telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>positive</td>
</tr>
<tr>
<td>Grade I</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Grade II</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Grade III</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

DISCUSSION

Telomerase are nucleoprotein structures located at the end of eukaryotic chromosomes that contain protein-bound, simple repeat units of a nucleotide sequence (Rhyu 1995). Telomerase protect chromosomes from shortening and unraveling during each replication cycle. It has been suggested that telomerase protect chromosome ends, because damaged chromosomes lacking telomeres undergo fusion, re-arrangement and translocation (Blackburn 1991). The expression of telomerase activity is important for cell proliferation, senescence, immortalization and carcinogenesis. Based on the fact that telomerase activity is present in the vast majority of different types of Cancer. (Shay and Wright 1996 and Soldateschi et al. 2005).

This study aims to clarify the role of telomerase in liver diseases, mainly in chronic hepatitis and hepatocellular carcinoma (HCC). Chronic viral hepatitis is characterized by varying degrees of liver cell necrosis and in flamanation. The diseases have the potential to progress to cirrhosis and even to hepatocellular carcinoma. Hepatitis B and C are the most common causes of chronic hepatitis. Together they account for more than 75 percent of the cases in the world (Coleman 1996).

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and is observed characteristically as a complication of chronic liver disease (Kong et al. 2009). The relationship between telomerase activity in various type of malignancies as a whole and in hepatocellular carcinomas in particular has been the subject of intensified studies and debate over the past few years.

Patients and controls studied in this work were subjected to history taking, clinical and laboratory assessment as well as
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measuring serum alpha fetoprotein and telomerase activity in peripheral blood using PCR-ELISA. The results revealed that 85% of HCC group and 10% of chronic hepatitis group showed increased telomerase activity in peripheral blood but not in control group.

Furthermore the study revealed that 100% of grade III HCC, 80% of grade II HCC and 50% of grade I HCC showed increased telomerase activity in peripheral blood. A number of previous studies have been performed to detect telomerase activity in hepatic tissues biopsied from HCC and chronic liver disease but very limited number of studies were performed to detect telomerase activity in the peripheral blood of cancer patients in general and HCC in particular. Tahara et al. (1995); Nouso et al. (1996); Kojima et al. (1997); Miura et al. (1997); Ohta et al. (1997); Harada et al. (2001), examined telomerase activity in hepatic tissues using telomeric repeat amplification protocol (TRAP). Telomerase activity was detected in 85% of HCC cases. Chronic liver disease showed a lower incidence of telomerase showed no telomerase activity in their liver tissues.

Kojima et al. (1997) examined 46 tissue samples (26 HCC and 20 normal) for telomerase activity using telomeric repeat amplification protocol (TRAP).

Telomerase activity was detected in 85% of HCC specimens. In contrast it was weekly detected in 9% of non neoplastic length in chronic liver disease, HCC and normal liver by southern blot hybridization method. The telomere length in cirrhotic liver tissues were significantly shorter then tended in normal liver. In chronic hepatitis they also tended to be shorter than normal. The telomere length in HCC specimens showed a wide variability in their length with short and long ones. Tahara et al. (1995); Nouso et al. (1996); Kojima et al. (1997); Miura et al. (1997); Ohta et al. (1997) and Shimada et al. (2000), all reached the same final results which also parallel to the results in this study. The conclusion was that telomerase activity is increased in hepatocellular carcinomas and very weak or absent activity in chronic hepatitis cases.

Park et al. (1998), examined telomerase activity in 82 liver tissues were obtained from 24 chronic viral hepatitis patients, 34 cirrhosis patients and 24 patients with hepatocellular carcinomas. Telomeric repeat amplification protocol (TRAP). Telomerase was strongly activity in 79% of the hepatocellular carcinomas, while weakly in 8% of the chronic hepatitis tissues and 24% of the cirrhosis tissues. These results suggest that telomerase activity is one of the critical steps of hepatocarcino-genesis. Miura et al. (2003), measured serum human telomerase reverse transcriptase mRNA (hTERT) as a novel tumor marker for hepatocellular carcinoma (HCC). They measured serum hTERT mRNA by using the real-time quantitative reverse transcription – PCR. They examined its sensitivity and specificity in HCC diagnosis, clinical significance in comparison with other tumour markers and its correlations with the clinical variables by using multivariable using multivariate analysis. They found that serum hTERT mRNA showed higher values in patients with HCC than those with chronic liver disease. hTERT mRNA expression was shown to be independently correlated with clinical variables such as tumor size, number and degree of differentiation (P < 0.001). The sensitivity specificity of hTERT mRNA and AFP mRNA in HCC diagnosis were 88.2 / 70% for hTERT and 71.6% / 67.5% for AFP, respectively. hTERT mRNA proved to be superior to AFP mRNA in HCC diagnosis.

Tatsuma et al. (2000), investigated telomerase activity in peripheral blood from 20 HCC patients, 20 chronic liver disease patients and 20 healthy controls. In their study used the PCR-ELISA technique to evaluate the rate of incidence of telomerase activity. The positive rate of telomerase was 88% for the HCC and 25% for chronic hepatitis in peripheral blood. These incidences of telomerase activity in chronic hepatitis cases were 25% in their study compared to 10% in present study. In HCC cases was 88% in their study compared to 85% in present study.

Thereby all of the studies revealed similar results to those of the present study as regards the frequency of increased telomerase activity in hepatocellular carcinoma cases but there was a significant variability between the results of telomerase activity in chronic hepatitis cases in the current study compared to the most of other studies. Such findings may be related to that several tumor cell lines keep their telomere length without telomerase activity (Bryan et al. 1995 and Bryan et al., 1997). In addition, as telomerase is a fragile ribonucleo protein, a lack of telomerase activity may occur due to degradation of essential telomerase templating RNA before sampling or during storage.

All the results of the previous studies also showed a direct relationship between the increased incidence of telomerase activity and the histopathological grading of HCC, which also coincides with the results concluded in this study showing increased telomerase activity in 100% of grade III HCC, 80% of grade II HCC and 50% of grade I HCC. This is also agreement with Tahara et al. (1995) who concluded that telomerase activity was increased in 100% of poorly differentiated HCC samples, 86% of moderately differentiated HCC and 71% of well differentiated HCC after examining 33 HCC nodule samples. hepatitis cases and its relation to hepatocarcinogenesis. One of the crucial parameters in our
study was alpha fetoprotein levels. Its considered to be the most important tumor marker for hepatocellular carcinoma at the present time. Alpha-fetoprotein levels might be increased in cases of chronic hepatitis and cirrhosis due injury of the hepatocytes (McMahon and London 1991). In our study a clear correlation between the incidence of increased telomerase activity and that of elevated AFP is apparent but the same correlation between the values of both tests was not evident. As AFP was elevated in 75% of HCC cases and 5% of chronic hepatitis C cases compared to increased telomerase activity in 85% in HCC cases and 10% of chronic hepatitis C cases. AFP was less sensitive but more specific to HCC than telomerase activity.

CONCLUSION

We concluded from the present study that the incidence of increased telomerase activity in hepatocellular carcinoma is highly evident and it is directly related to the degree of histological undifferentiation of hepatocellular carcinoma. We also concluded that a positive telomerase activity existed in chronic hepatitis disease, the incidence and the biological value of which is debatable and awaits further assessment.

Finally, we recommend a wider scale study involving a larger number of patients with HCC, chronic hepatitis and hepatic cirrhosis. Also a long term follow up of these patients might give us an idea about the prognostic value of telomerase and survival rate in those who show increased telomerase activity, thus evaluating telomerase as a more accurate and an early detector of hepatocarcinogenesis. Furthermore studies should be carried out targeting telomerase for anticancer therapy.

REFERENCES


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