ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (1): 66-72, 2008
ISSN 1682-296X

Blood Telomerase Activity and DNA Dielectric Properties in Human Hepatocellular Carcinoma and Chronic Liver Disease

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Abstract: The aim of the present study is to determine the diagnostic usefulness of the measurement of the telomerase activity and DNA electrical properties in peripheral blood from Hepatocellular Carcinoma (HCC) and chronic liver disease. In 10 patients with HCC, 13 with chronic hepatitis C and 7 healthy individuals, we measured peripheral blood telomerase activity by using the telomerase PCR ELISA assay. We examined its sensitivity and specificity in HCC, chronic HCV and its significance in comparison with other tumor markers. Telomerase activity was detected in peripheral blood of 80% hepatocellular carcinoma, 15% of chronic hepatitis C patients and absent in control group. A clear correlation between the incident of increased telomerase activity and that of elevated AFP is evident. The dielectric results indicated that the studied DNA has a dielectric dispersion in the frequency range used. The increase in the electrical conductivity and relaxation time for HCC and chronic disease could be attributed to the increased surface charge density of the DNA macromolecule. It is concluded that detection of telomerase activity in peripheral blood in combination with measurement dielectric dispersion of DNA could increase the accuracy diagnosis of HCC and improve the judgment on its prognosis.

Key words: Telomerase, diagnostic marker, dielectrics, α-fetoprotien, telomerase polymerase chain reaction ELISA assay, peripheral blood

INTRODUCTION

Telomeres are specialized structures at the terminal regions of eukaryotic chromosomes and are considered to be important in protecting and stabilizing the chromosomal ends (Blackburn and Szostac, 1984; Zakian, 1989; Blackburn, 1991). Human telomeric DNA is composed of tandem repeats of the TTAGGG sequence bound to an array of proteins (Blackburn, 2001; Chan and Blackburn, 2002; De Lange, 2002). These repeats are shortened at each cell division. Therefore normal human somatic cells have a limited proliferate capacity. In contrast, immortalized cells do not exhibit telomeric shortening due to the presence of telomerase (Shimojima *et al*., 2004). Telomerase is a ribonucleoprotein complex with transcriptase activity (Morin, 1989; Maser and Depinho, 2002), responsible for restoring telomere length in dividing cells (Allsopp *et al*., 1984; Bryan and Cech, 1999). High telomerase activity was detected in the majority of human tumors; whereas low or undetectable activity was found in differentiated cells of the homologous normal tissue (Kim *et al*., 1994; Broccoli *et al*., 1995; Wright *et al*., 1996). Therefore, detection of telomerase activity can be considered a biochemical signal for the presence of various cancer cells, the level of this enzyme activity may represent a new type of cancer marker (Dario *et al*., 2005). Recently, three components of the telomerase have been cloned; human telomerase RNA component (hTERC), which is used as a template in DNA replication (Feng *et al*., 1995); human telomerase reverse transcriptase (hTERT), a human telomerase catalytic sub unit (Nakamura *et al*., 1997) and human telomerase associated protein 1 (hTEP1) of unknown function (Harrington *et al*., 1997). It has been reported that hTERT mRNA expression is significantly correlated with telomerase activity (Zhang *et al*., 2001). These data strongly suggest that hTERT is probably the rate-limiting component for enzyme activity.

The delectability of telomerase activity in hepatocellular carcinoma (HCC) was reported

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suggesting its helpful role in the diagnosis of HCC (Tahara *et al*., 1995; Kinugasa *et al*., 1996; Kojima *et al*., 1997; Miura *et al*., 1997; Ohta *et al*., 1997; Nakashio *et al*., 1997; Suda *et al*., 1998; Hiroshige *et al*., 1999).

Dielectric properties of various biological materials have been previously investigated to get attractive information about their structural change under any internal or external effects (Polk and Postow, 1996; Ghannam *et al*., 2002).

The aim of the present investigation was to evaluate the peripheral blood telomerase activity in patients with chronic liver disease and in hepatocellular carcinoma for early detection of malignant changes. This way could open up new perspectives for use of telomerase as a simple minimally invasive blood test for diagnosis of hepatocellular carcinoma. Moreover, study of the DNA electrical properties may give us an understanding about DNA damage proteins mechanisms for both chronic hepatitis C virus and HCC patients.

MATERIALS AND METHODS

Patients and samples: The study was conducted on 30 patients aged between 20-60 years. They were 19 males and 11 females. Patients in this study were chosen from Kasr EL-Aini Hospital, Cairo University. They were classified according to their pathological diagnosis and clinical data into 3 groups:-

1st Group: Seven patients (5 males and 2 females) with mean age of 42±12 years, as normal and healthy controls.

2nd Group: Thirteen patients (7 males and 6 females) with mean age of 50±10 years, suffering from chronic Hepatitis C Virus (HCV).

3rd Group: Ten patients (8 males and 2 females) with mean age of 50±10 years, suffering from hepatocellular carcinoma (HCC).

Patients and controls studied in this work were subjected to history taking and full clinical examination.

Blood sampling: Ten milliliter venous blood samples obtained from each patient with HCC, each patient with chronic hepatitis C and from each healthy control. The sample was divided into two parts, one for measuring telomerase activity and the second for the other laboratory investigations.

Sample preparation: Five milliliter of blood was collected on EDTA vacationer and lymphocytes were separated using Ficoll Hypaque gradient medium for separation of mononuclear cells and kept frozen at -70°C till performing the DNA extraction.

Cell counts: Cells were harvested and counted by both hemacytometer and electronic counting (Coulter Electronic Inc., USA)

Telomerase activity assays: Telomerase activity was assayed by using the telomerase polymerase (PCR) enzyme-linked immunoassay (ELISA) Kit supplied by Roche Diagnostics (Germany Cat. No. 2013789).

Briefly, 1 million cells were washed three times with cold Phosphate Buffered Saline (PBS) and lysed in 100 μL of pre-cooled lysis solution by incubating the suspension on ice for 30 min. Samples were microfuged and protein concentration of the supernatant were measured using the Bio-Rad protein assay Kit. Telomeric repeats were added to a biotin- labelled primer for 30 min at 25°C during the first reaction. The mixture was incubated at 94°C for 5 min to induce telomerase inactivation. The reaction mixture was then subjected to 30 PCR cycles at 94°C for 40 sec, 50°C for 40 sec and 72°C for 90 sec (72°C, 10 min for final step) in a DNA thermal cycler (Perkin-Elmer Co., Norwalk, CT.USA). An aliquot of the PCR product was denatured, hybridized to a digoxigenin (DIG)-labeled telomeric repeat-specific probe and bound to a streptavidin- coated 96 well plate. Finally, the immobilized PCR product was detected with an anti-digoxigenin- peroxidase antibody and visualized with a color reaction by using tetramethyl benzidine substrate. The absorbance of the samples was measured at a wavelength of 450 nm (with reference wavelength 620 nm) using a Dynatech MR 5000 Microplate reader (England) within 30 min of addition of stop reagent. This absorbance reading represents telomerase activity in the sample. Telomerase activity was determined in triplicate and negative as well as a positive control was run each time. A negative control was provided for each extract by heating inactivating the telomerase enzyme present in cell lysate at 95°C for 10 min prior to the PCR step.

Dielectric relaxation of DNA: DNA-U Kit supplied by Medical biological UNION (cat. No. P-4029-1 Novosibirsk, Russia) used to extract genomic DNA from blood sample.

The concentration of DNA for all samples estimated spectrophotometry, the absorbance (A) reading taken at 260 and 280 nm. The final concentration of the DNA diluted in sterilized water is 50 ng μL^{-1}. The ratios of (A_{260}/A_{280}) were calculated and get an average value of 1.85±0.12 which indicate a high purity extracted DNA.

DNA dielectric measurements were undertaken in the frequency range of 20 Hz to 100KHz using a WAYNE KERR precision component analyzer, model 6440 B (UK), connected with a conductivity celltype 19250-60

manufactured by Cole Palmer Co. The sample cell has two squared platinum black electrodes with cell constant $k = 1\ cm^{-1}$.

The measurements were performed at 20°C. The measured values of capacitance (C) and resistance R as a function in the frequency (f) were used to calculate the real (ε'), imaginary (ε '') parts of the complex permittivity ($\varepsilon^* = \varepsilon' - i\varepsilon''$), conductivity (σ) and relaxation time (τ) by means of the equations previously described in detail (Cole and Cole, 1941).

Laboratory tests: In the study group the following laboratory parameters were analyzed:

Hepatitis C virus antibody by ELISA technique, quantitative detection of HCV RNA by PCR, alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum albumin (ALB), serum total protein (TP) and bilirubin.

Statistical analysis: Each value is expressed as mean and Standard Deviation (SD). The Mann-whitney U-test was used to determine the significant differences among semi-quantitative values of telomerase activity. One way analysis of variance was used to compare each variable in the different studied groups. For all statistical comparisons a p-value of <0.05 was considered significant.

RESULTS

Comparison of telomerase activity in peripheral blood from HCC, HCV and controls: Telomerase activity was observed in peripheral blood of 80% (8/10) of HCC group, 15% (2/13) of chronic HCV but not in control group. The values of telomerase activity in peripheral blood from HCC group, chronic HCV group and healthy control group presented in Fig. 1. The mean telomerase activity of patients with HCC group, chronic HCV group and of healthy controls group was 60.5±12.6 units, 0.95±0.20 units and 0.41±0.23 units, respectively. Telomerase activity (60.5±12.6 units) in the peripheral blood of HCC group was a highly significant difference when compared to the values obtained from chronic hepatitis C group and healthy controls group ($p<0.0001$). There was no significant difference in telomerase activity between the chronic hepatitis C group and healthy controls group ($p>0.05$).

Correlation between the histopathological staging of the HCC and peripheral telomerase activity: Of the 10 cases of HCC group, 3 cases were grade 3 HCC, 5 cases were grade 2 HCC and 2 cases were grade 1 HCC.

All cases of grade 3 HCC showed increase in the telomerase activity (i.e. 100% of grade 3 HCC), 4 of 5 cases of grade 2 HCC showed increase in the telomerase activity (i.e., 80% of grade 2) and 1 of 2 cases of grade 1 HCC (i.e., 50% of grade 1 HCC) showed increase in telomerase activity (Fig. 2). Thus a direct relationship between the incidences of telomerase activity and the grade of HCC could be established in this study.

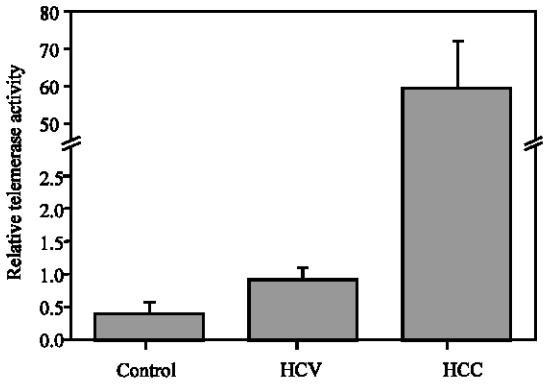

Fig. 1: The variation of telomerase activity in blood of control group, chronic hepatitis C (HCV) group and HCC group

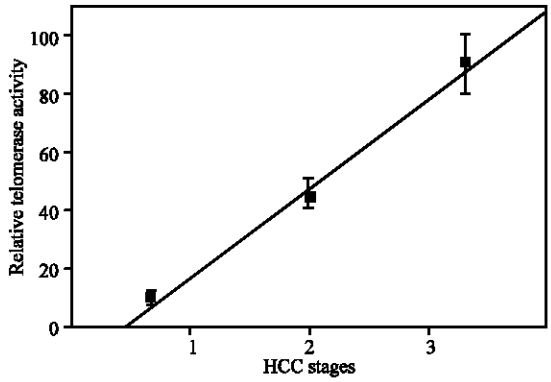

Fig. 2: Relationship between the histopathological staging of hepatocellular carcinoma (HCC) and peripheral telomerase activity

AFP (α-fetoprotien): The mean value of AFP (46.5±27.20 ng mL^{-1}) in the blood of HCC group was significantly higher than the values obtained for chronic HCV group and healthy controls ($p<0.001$). Also the mean value of AFP (8.54±2.5 ng mL^{-1}) in the blood of the chronic hepatitis C group was significantly higher than the value obtained for control group (0.797±0.99 ng mL^{-1}) ($p<0.001$).

Correlation between telomerase activity and AFP: AFP was elevated in 60% of HCC cases compared to increased telomerase activity in 80% of HCC cases. AFP was less

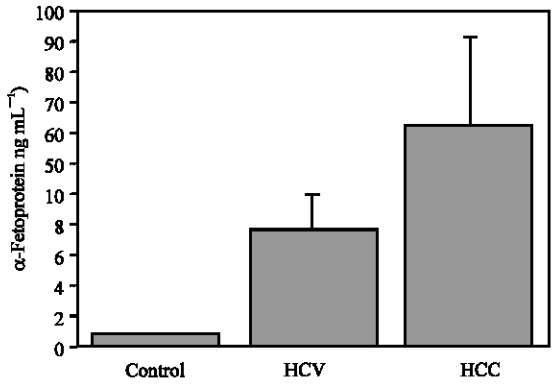

Fig. 3: The variation of the AFP levels in the blood of control group, chronic hepatitis C group (HCV) and HCC group

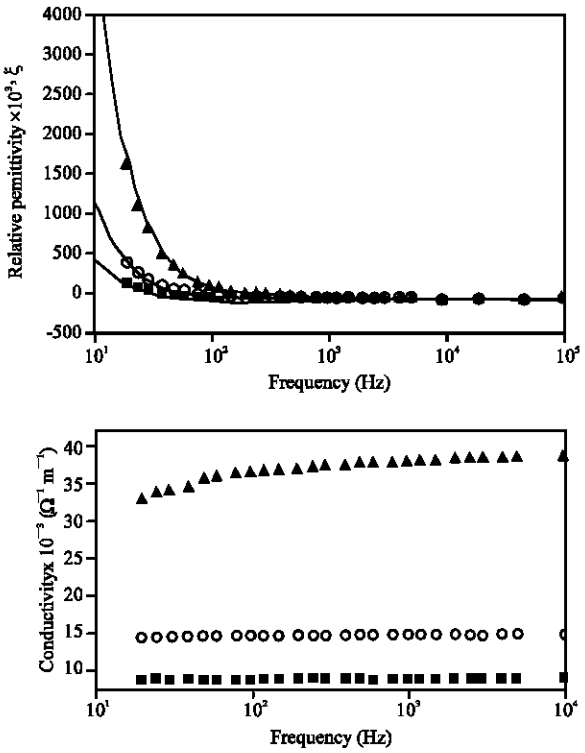

Fig. 4: Relative permittivity ε' (upper) and electrical conductivity σ (lower) as a function of the applied frequency in the range of 20 Hz to 100 kHz for DNA suspension of healthy persons (control, ■), chronic hepatitis C virus (HCV, o) and hepatocellular carcinoma (HCC, ▲) patients

sensitive to HCC than telomerase activity. Telomerase have been indicated to be more valuable than AFP in predicting the prognosis. There was no direct relation established in this study between the relative telomerase activity and the AFP in terms of values (Fig. 3).

Dielectric properties: There are relatively significant increase in both values of ε' and σ for the studied patient groups when compared with those of healthy group. The relaxation time for DNA molecules of the studied groups were calculated and gives the values 1.1, 1.3 and 1.8±0.05 for healthy, HCV and HCC groups, respectively (Fig. 4).

DISCUSSION

Telomerase activity has been detected in a wide variety of cancers, but not in most normal cells and tissues (Shimojima, 2004). According to previous works, telomerase activity in peripheral blood was found in 88% of hepatocellular carcinoma and in 25% of chronic hepatitis diseases (Hisatomi *et al*., 2000; Tatsuma *et al*., 2000). In the present study, telomerase activity in peripheral blood was detected in 80% of hepatocellular carcinoma patients and in 15% of chronic hepatitis C patients, but not in control group. Although there has been several reports regarding the positivety of telomerase activity in HCC (Tahara *et al*., 1995; Kinugasa *et al*., 1996; Kojima *et al*., 1997; Miura *et al*., 1997; Ohta *et al*., 1997; Nakashio *et al*., 1997; Suda *et al*., 1998), telomerase activity in peripheral blood of cancer patient in general and HCC patient in particular has been studied in a very limited number. The present results revealed that 100% of grade 3 HCC, 80% of grade 2 HCC and 50% of grade 1 HCC showed increased telomerase activity in peripheral blood. These results suggest that the assay of the telomerase activity level is useful for HCC diagnosis. The presence of telomerase activity was also reported in about 9% up to 30% of chronic liver diseases (Tahara *et al*., 1995; Kinugasa *et al*., 1996; Kojima *et al*., 1997; Miura *et al*., 1997; Ohta *et al*., 1997).

The telomerase activity in peripheral blood was detected in 15% of chronic hepatitis C patients in the current study, suggesting the presence of weak telomerase activity in chronic liver hepatitis C patients.

The rate of telomere DNA shortening is regulated by telomerase expression and activity. Therefore, telomere shortening limits the proliferative capacity of primary human cells and restrains the regenerative capacity of organ systems in the course of chronic diseases and aging (Sato *et al*., 2004; Li *et al*., 2004).

AFP is a diagnostic marker for HCC, but its significance in the early diagnosis of HCC is obscure because of the low positive rate (Qin and Tang, 2002). In the present study, the clinical significance of telomerase activity and AFP level in HCC patients was analyzed. AFP was elevated in 60% of HCC cases compared to increased

telomerase activity in 80% of HCC cases, AFP have no satisfactory accuracy in detecting HCC or prefiguring the prognosis when used alone.

Telomerase activity is not necessarily positive in all the well-established HCC cases as already reported (Tahara *et al*., 1995; Kinugasa *et al*., 1996; Kojima *et al*., 1997; Miura *et al*., 1997; Ohta *et al*., 1997; Nakashio *et al*., 1997; Suda *et al*., 1998).

It was shown that several tumor cell lines keep their telomere length without the telomerase activity, so there must be some other mechanisms for restoring telomere length besides telomerase activity, as for example, chromosomal recombination (Bryan *et al*., 1995, 1997).

In addition, as telomerase is a fragile ribonucleoprotein, it must be always kept in mind that a lack of telomerase activity may occur due to degradation of essential telomerase templating RNA before sampling or during storage (Feng *et al*., 1995).

In this study, we have demonstrated that higher levels of telomerase activity were detectable in majority of HCC group in contrast to its lower levels or absence in a majority of chronic HCV group.

The dielectric properties of normal DNA, DNA of HCV patients and DNA of HCC patients shown in figure 4 indicated that electric conductivity and permittivity have much higher values in DNA of HCC patients than normal DNA. These results are in agreement with Surowiec *et al*. (1988), who reported that both permittivity and conductivity of breast carcinoma tissues (20-100 MHZ) were higher than those of normal breast tissues. The dielectric results indicate that the studied DNA have a dielectric dispersion in the frequency range used (20 Hz-100 kHZ). This behavior was identified as anomalous frequency dispersion and it was found for different biological materials (Polk and Postow, 1996; Ghannam *et al*., 2002). Therefore the increased value of permittivity and conductivity for DNA of HCC and HCV patients, as compared with normal DNA of healthy persons, indicate large increase of the surface charge density of the DNA macromolecules which resulted from the formation of highly active molecular species. This speculation is supported by the defined increase in the relaxation time τ of the patient groups which indicates high increase in the molecular size of formation of new cluster cells.

Moreover, since the dipole moment is directly proportional to the relaxation time, the increase in τ will cause an increase in the value of the dipole moment of the studied patients groups. Consequently a higher electric conduction is expected as a direct response of the highly surface charge density of the formed clusters cells growth. The results of the telomerase activity in Fig. 1 is in agreement with the above data which suggest that the dielectric measurements of DNA could also be used as available prognostic indicator for hepatocellular carcinoma (HCC).

In conclusion, the expressions of telomerase are different in different stages of HCC patients and telomerase activities can be detected in peripheral blood of HCC patients. The telomerase could be a useful molecular marker for the diagnosis of HCC. Dielectric properties of DNA may be used as an effective method for detecting malignant tumors and it could be valuable supplementary to ultrasonography and computer tomography in the diagnosis of HCC. Using the appropriate single or combination of tumor markers may improve the effectiveness in screening HCC patients.

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