Molecular Diagnosis of Hepatocellular Carcinoma in Egypt

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It is a prevalent cancer that often develops in patients with chronic hepatitis and cirrhosis in association with hepatitis B or C virus infection. Diagnostic modalities such as tumor markers, ultrasonography and CT have contributed to its earlier diagnosis but remain not sensitive enough. Telomeres are specializes structures at the ends of eukaryotic chromosomes and lead to their stabilization. Telomerase is a ribonucleoprotein enzyme responsible for replication of telomeres at chromosomal ends in most eukaryotes.Its RNA subunit provides the template for addition of (GGTTAG) repeats to chromosome ends .Human telomerase mRNA (hTERT- mRNA)has been identified in many cancers and claimed to be reactivated in HCC.

Aim: to investigate Human telomerase mRNA (hTERT- mRNA) in peripheral blood of hepatocellular carcinoma (HCC) and chronic liver diseases (CLD) patients,To correlate the level of Human telomerase mRNA (hTERT- mRNA) with alpha feto –protein (AFP),the traditional serological marker for HCC.

Patients and methods:The study was conducted on 60 patients selected from the National Liver Institute clinics and inpatients hepatology department. The patients were divided into group I (30 patients with CLD) and group II (30 patients diagnosed to have HCC). In addition to group III that comprised 20 apparantly healthy volunteers .All selected individuals were subjected to history taking ,thorough clinical examination, abdominal ultrasonography and routine laboratory investigations as liver function tests ,CBC, hepatitis viral markers , serum AFP and quantitative detection of m-RNA expression encoding for telomerase catalytic subunit hTERT by real time PCR measurement using 7500 Real Time PCR System(Applied Biosystems).

Results: A significant elevation in AFP level in HCC group when compared to CLD patients group or to control group ($p < 0.01$).The mean level of hTERT m-RNA expression in HCC patients group was significantly higher than both CLD patients group and controls ($p<0.01$). An hTERT m-RNA expression cut off level of 81.5 copies /ml showed prediction of HCC 100% sensitivity,95 % specificity, accuracy 99.6% ,standard error (SE) was 0.003 and confidence interval (CI) was (0.99-1.003).**Conclusion:** Real time assay of hTERT m-RNA in patients with CLD could be used as a satisfactory molecular marker for diagnosis of HCC. hTERT m-RNA is thought to be superior to AFP for early detection of HCC.

***Key words**: Diagnostics; HCC; AFP level; hTERT m-RNA*

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It is a prevalent cancer that often develop in patients with chronic hepatitis and cirrhosis in association with hepatitis B or C virus infection ***Santamaria (2007)*** [(1)].

Recurrence of HCC after resection still remains common and is considered the main cause of death after surgical resection. Recently developed diagnostic modalities, such as serum tumor markers, ultrasonography and CT, have contributed to its earlier diagnosis but they remain not sensitive enough ***Yu and Keefe (2003)*** [(2)].

Telomeres are specialized structures at the ends of the eukaryotic chromosomes and function in chromosome stabilization, positioning and replication ***Cairney and Keith (2008)***[(3)].

Telomerase is a ribonucleoprotein enzyme responsible in most eukaryotes for the complete replication of the telomeres at the chromosome ends. Its RNA subunit provides the template for the addition of the hexamer repeat (GGTTAG) to the chromosome end ***Karlseder et al (2002)***[(4)].

Clinically, telomerase activity can be a useful predictor for intrahepatic recurrences during the early period and after surgical resection of HCC ***Suda et al (1998)*** [(5)].

Telomerase activity has been found not only in germ line cells and stem cells, but also in various cancer specimens ***Shay and Wright (2002)*** [(6)] and almost in all human cancer including breast, bladder, ovary, prostate, colon, liver, stomach, brain and others ***Wu et al (2005)*** [(7)],However, the detailed relationship between telomerase activity and malignant progression of cancer has yet to be elucidated ***Miura et al (2005)*** [(8)].

Aim of the work

to investigate Human telomerase mRNA (hTERT- mRNA) in peripheral blood of hepatocellular carcinoma(HCC)and chronic liver diseases (CLD) patients,To correlate the level of Human telomerase mRNA (hTERT- mRNA) with alpha feto –protein (AFP),the traditional serological marker for HCC.

Patients and methods

The study was conducted on 60 patients selected from the National Liver Institute clinics and inpatients hepatology department.The patients were divided into group I (30 patients with CLD)and group II (30 patients diagnosed to have HCC).In addition to group III that comprised 20 apparantly healthy volunteers .All selected individuals were subjected to history taking ,thorough clinical examination, abdominal ultrasonography and routine laboratory investigations as liver function tests ,CBC, hepatitis viral markers , serum AFP

and quantitative detection of m-RNA expression encoding for telomerase catalytic subunit hTERT by real time PCR measurement using 7500 Real Time PCR System(Applied Biosystems).

Results

A significant elevation in AFP level in HCC group when compared to CLD patients group or to control group ($p < 0.01$).The mean level of hTERT m-RNA expression in HCC patients group was significantly higher than both CLD patients group and controls ($p<0.01$). An hTERT m-RNA expression cut off level of 81.5 copies /ml showed prediction of HCC 100% sensitivity,95 % specificity, accuracy 99.6% ,standard error (SE) was 0.003 and confidence interval (CI) was (0.99-1.003).

Table 1: Differences between studied groups regarding AFP and h-TERT

Variables	Groups	Mean ± SD	Kruskal Wallis test	p-value	LSD post hoc test p- value
AFP ng/ml	Control HCC Liver Cirrhosis	3.3±1.8 5271.2±9243.9 6.1±2.3	54.64	< 0.01	P1= < 0.001 P2= < 0.01 P3= < 0.001
h-TERT copies /ml	Control HCC Liver Cirrhosis	7.7±15.1 286.93±172.89 40.17±33.86	66.99	< 0.01	P1= < 0.001 P2= < 0.01 P3= < 0.001

There is highly statistical significant difference between all studied groups as regards AFP and h-TERT ($P < 0.01$).

Table 2: Spearman Correlation between h- TERT and all studied variables in Control group

		h-TERT
Age in years	R	0.263
	p- value	> 0.05
Total_bilirubin mg/dl	R	0.434
	p- value	< 0.05*
Direct_bilirubin mg/dl	R	0.186
	p- value	> 0.05
Total_protein g/dl	R	0.026
	p- value	> 0.05
Albumin g/dl	R	0.162
	p- value	> 0.05
AST U/L	R	0.204
	p- value	> 0.05
ALT U/L	R	0.279
	p- value	> 0.05
ALP U/L	R	0.449
	p- value	< 0.05*
GGT U/L	R	- 0.090
	p- value	> 0.05
PT %	R	0.007
	p- value	> 0.05
Hb gm/dl	R	0.040
	p- value	> 0.05
RBCs * 1000000	R	0.057
	p- value	> 0.05
WBCs * 1000	R	0.005
	p- value	> 0.05
Platelets * 1000	R	- 0.091
	p- value	> 0.05
Hct	R	0.058
	p- value	> 0.05
AFP ng/ml	R	- 0.297
	p- value	> 0.05

-There is positive correlation between T.bilirubin &h-TERT and also between alkaline phosphatase & h-TERT but no correlation between other statistical variables &h-TERT ($p > 0.05$).

Table 3: Spearman Correlation between h- TERT and all studied variables in HCC group

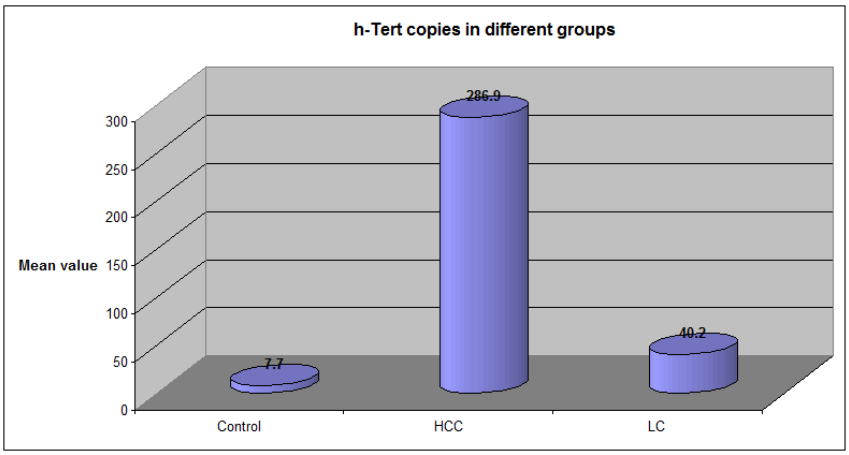
		h-TERT
Age in years	R	0.223
	p- value	> 0.05
Total_bilirubin mg/dl	R	0.224
	p- value	> 0.05
Direct_bilirubin mg/dl	R	0.166
	p- value	> 0.05
Total_protein g/dl	R	- 0.365
	p- value	< 0.05*
Albumin g/dl	R	- 0.405
	p- value	< 0.05*
AST U/L	R	0.052
	p- value	> 0.05
ALT U/L	R	- 0.018
	p- value	> 0.05
ALP U/L	R	- 0.045
	p- value	> 0.05
GGT U/L	R	0.126
	p- value	> 0.05
PT %	R	- 0.167
	p- value	> 0.05
Hb gm/dl	R	- 0.006
	p- value	> 0.05
RBCs * 1000000	R	- 0.025
	p- value	> 0.05
WBCs * 1000	R	0.103
	p- value	> 0.05
Platelets * 1000	R	- 0.273
	p- value	> 0.05
Hct	R	- 0.089
	p- value	> 0.05
AFP ng/ml	R	- 0.138
	p- value	> 0.05

-There is negative correlation between T.protein &h-TERT and also between albumin &hTERT but no correlation between other statistical variables &h-TERT ($p > 0.05$).

Table 4: Spearman Correlation between h- TERT and all studied variables in Liver Cirrhosis group

		H-tert
Age in years	R	0.197
	p- value	> 0.05
Total_bilirubin mg/dl	R	- 0.077
	p- value	> 0.05
Direct_bilirubin mg/dl	R	- 0.049
	p- value	> 0.05
Total_protein g/dl	R	- 0.031
	p- value	> 0.05
Albumin g/dl	R	0.259
	p- value	> 0.05
AST U/L	R	0.228
	p- value	> 0.05
ALT U/L	R	0.255
	p- value	> 0.05
ALP U/L	R	0.220
	p- value	> 0.05
GGT U/L	R	0.352
	p- value	> 0.05
PT %	R	0.032
	p- value	> 0.05
Hb gm/dl	R	0.131
	p- value	> 0.05
RBCs * 1000000	R	0.101
	p- value	> 0.05
WBCs * 1000	R	0.020
	p- value	> 0.05
Platelets * 1000	R	0.151
	p- value	> 0.05
Hct	R	0.098
	p- value	> 0.05
AFP ng/ml	R	0.091
	p- value	> 0.05

-There is no correlation between all studied statistical variables & h-TERT ($p > 0.05$).



Discussion

Human telomerase is a ribonucleic protein composed by the association of three structures: human telomerase RNA component (h-TERC); human telomerase-associated protein 1 (h-TEP1); human telomerase reverse transcriptase (h-TERT). h-TERT is the catalytic unit of the complex. Also, telomerase is expressed in embryonic cells, in most human cancer cells or immortal cell lines, but not in normal somatic cell lines or tissues ***Chiappini (2012)***[(9)]. For these reasons h-TERT was investigated as a marker of diagnosis and prognosis of HCC, but the results are controversal and appear that false-positive results can be observed because of lymphocytes, precancerous liver parenchymal cells, and micrometastasis maybe responsible ***Zhou et al (2006),Wang et al (2007)& Grizzi et al (2007)*** [(10)].

In the present study,hTERT was detected in the peripheral blood of the patients of the three groups by 286.9 copies /ml in HCC group ,40.2 copies/ml in CLD group and 7.7 copies/ml in control group.

The present study showed no significant difference in age and gender among the studied groups ($p > 0.05$) indicating the homogenicity of the samples distribution in each group. Also, no significant difference in HCV infection among the studied groups ($p > 0.05$), however, HBs Ag was significantly frequent in HCC group compared to LC ($p<0.01$).

However ,no statistically significant difference was detected among liver enzymes (AST,ALT,GGTand ALP) and PT % in the two studied groups (HCC and liver cirrhosis groups) ($P>0.05$)

There were significant decrease in hemoglobin level ($p <0.01$), RBCs count ($p < 0.01$) and platelet counts ($p <0.01$) were illustrated in the diseased groups compared to control group.

The present work showed a significant elevation in AFP level in HCC group when compared to LC ($p < 0.001$) or control groups ($p < 0.001$). Also AFP levels in LC group were significantly higher than the control group ($p < 0.05$).

Data against these were reported by ***Joyce et al (2007)*** [11] who stated that AFP had the best performance of all markers for all stages include stage zero HCC, the detection of which is the main goal of a surveillance program, while ***Deng et al (2007)*** [12] reported less performance of AFP in early stages and small size HCC (less than 3 cm).

Also, data against these were reported in a study by ***Kong et al (2009)*** [13]; the presence of AFP was not a useful prognostic marker for HCC.
In the present work, a significant differences in h-TERT mRNA expression were detected between LC and controls ($p <0.01$), which coincides with results reported by ***Miura et al (2007)&Tatsuma et al (2000)*** [14] and explained by normal hepatocytes that may express negligible amount of h-TERT mRNA and inflamed hepatocytes still express more weakly than hepatocellular carcinoma cells ***Miura et al (2003)*** [15].
However , no significant correlation ($p>0.05$) was discovered between h-TERT mRNA expression and each of age, CBC results and liver function tests in liver cirrhosis group and control group ($P> 0.05$). While, among HCC patients, a significant negative correlation ($p<0.05$) was discovered between h-TERT mRNA expression and each of total protein and serum albumin.
Against these results, ***Murashima et al, (2006)*** [16] reported in a study carried out on HCV chronic patients receiving interferon therapy ; in patients with high AFP levels, both platelet counts and serum albumin were significantly lower ($p = 0.044$ and 0.002 respectively). They attributed this to the increase in AFP with the advance in the liver disease.
The present work showed that h-TERT mRNA at a cut-off level of 81.5 copies/ml, prediction of HCC showed a sensitivity of 100%, specificity of 95%, diagnostic accuracy of 99.6% and AUC 0.99.

In a previous Egyptian study, ***Attia et al., (2008)*** [17] showed that h-TERT mRNA give a sensitivity of 77.3%, specificity 96.8% and diagnostic accuracy 83% at a cut-off level of 112.5 copies/ml.
Furthermore,***Miura et al,(2010)*** [18] could detect serum h-TERT mRNA expression even in HCC patients with less than 10 mm moderate-differentiated tumor, indicating that h-TERT are upregulated during rapid proliferation of tumor at the early phase of oncogenesis and differentiation.

Conclusion

Our results support the suggestion that quantification of circulating h-TERT mRNA expression is clinically useful for the early detection of HCC.

Furthermore, h-TERT mRNA is superior to conventional tumor markers in the diagnosis and recurrence of HCC at the early stage.

In the future, another large-scale study will be required to confirm the value of h-TERT mRNA for monitoring HCC and the feasibility for its detection even on a primary care level.

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