Tolemerase Reverse Transcriptase Gene Expression as a Tumor Marker for Hepatocellular Carcinoma

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Abstract: Problem statement: Hepatocellular carcinoma will emerge as a major form of malignancy in the coming decades. The continuing high incidence of hepatocellular carcinoma, suggests that this disease will continue to represent a global health problem far into the future. Different genes encode for the various components of the human telomerase complex. These components include the human Telomerase RNA Component (hTERC) and the Telomerase Catalytic Subunit (hTERT). Correlation between Telomerase Reverse Transcriptase (hTERT) expression and telomerase activity has been reported in cancer patients. This work aimed to clarify the significance of human Telomerase Reverse Transcriptase (hTERT mRNA) as a potential molecular tumor marker for Hepatocellular Carcinoma (HCC). **Approach:** The current study included 25 patients of hepatocellular carcinoma (HCC), 30 patients with liver cirrhosis and 25 age and sex matched individuals with normal laboratory and Image findings as a control group. hTERT mRNA was measured in plasma by Real time PCR in all patients samples in comparison with normal healthy controls. **Results:** The expression of hTERT mRNA by relative unit was 129.10 ± 27.6 with range (67.72-69.6) Vs 5245.87 ± 2382.48 (2053-12232.90) Vs 92782.76 ± 16158 (61783.25-118596.47) for control Vs cirrhosis Vs HCC group respectively. The hTERT expression was significantly with 699 and 33 fold increase in HCC and cirrhosis groups correspondingly when compared to that of controls $p<0.05$. **Conclusion:** It was suggested that this procedure was highly discriminating between healthy subjects and cancer patients and strongly support the idea that a valuable diagnostic test for cancer might be developed using this genetic marker in plasma. However it needs to be combined with other markers in future studies to be more specific for liver cancer.

Key words: Hepatocellular carcinoma, Telomerase Catalytic Subunit (hTERT), telomerase reverse, real time PCR, tumor marker, gene expression, diagnostic test, global health problem, cancer patients, DNA-protein structures

INTRODUCTION

Hepatocellular Carcinoma (HCC) represents the fifth most common form of neoplastic diseases worldwide (accounting for > 5% of all human cancers) and the third most common cause of cancer-related death. It is also the ninth cause of cancer deaths in the united states (Altekruse *et al.*, 2009) and one of the fastest rising tumors as a result of chronic hepatitis B and C infection (Gupta and Misra, 2010). The world regions of highest incidence of hepatocellular carcinoma include Eastern Asia and Sub-Saharan Africa (Gupta and Misra, 2010). Hepatocellular carcinoma will emerge as a major form of malignant cancer in the coming decades. The continuing high incidence of hepatocellular carcinoma, suggests that this disease will continue to represent a global health problem far into the future (Bruix and Sherman, 2011). Considerable effort has been expended toward identification of noninvasive detection of hepatocellular carcinoma. The ideal biomarker for this type of application is one that can be detected with good sensitivity in a biological sample from the patient in a noninvasive manner. For hepatocellular carcinoma, blood represents the best source for detection of cancer-related biomarkers (Bruix and Sherman, 2011). Telomeres are specialized DNA-protein structures that cap the ends of linear chromosomes (Caterina *et al.*, 2011). The telomeric structure is crucial for protecting linear chromosomes from fusion by cellular DNA

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repair processes and thus telomeres are essential for maintaining the integrity and stability of genomes. Dysfunctional telomeres, therefore, can lead to genomic instability, which is a common cause and hallmark of cancer and has also been proposed to contribute to aging.Telomerase is a large Ribonucleoprotein (RNP) complex that plays a major role in the maintenance of telomere DNA at the ends of linear chromosomes thus compensating for the continued erosion of telomeres with cell divisions (Caterina *et al*., 2011). Over 80% of human cancers show an activation of telomerase which stabilizes telomeres thus facilitating immortal proliferation of cells and making it a promising target for cancer therapy (Xianling *et al*., 2008). In human cancer the activation of telomerase often correlates with tumor progression. Telomerase is significantly activated in HCC and that in chronic liver diseases such as Liver Cirrhosis (LC) and Chronic Hepatitis (CH) but it is significantly lower than that in HCC because human Telomerase Reverse Transcriptase (hTERT) mRNA is up-regulated in the multistep process of hepatocarcinogenesis (Braicu *et al*., 2009). So the present study aimed to clarify the significance of human telomerase reverse transcriptase (hTERT mRNA) catalytic subunit as a tumor marker for detection of Hepatocellular Carcinoma (HCC).

MATERIALS AND METHODS

From patients who attending the Hepatology, Gastroenterology and Infectious Diseases, Benha University Hospital between January and August 2010, fifty five patients were consecutively recruited for participation in this study. The procedures used in this study followed the protocol approved by the local institutional review board. The research objectives were explained to each patient and consent was obtained. The selected patients categorized to HCC group: Included twenty five patients; 15 males and 10 females within the age range of 44-78 years, diagnosed to have Hepatocellular Carcinoma (HCC) depending on detection of focal hepatic lesions with typical radiological features of HCC by ultrasonography and Triphasic Contrast-enhanced Computed Tomography with or without Alpha fetoprotein level higher than 200ng mL^{-1}. Cirrhosis group: Included thirty patients with liver cirrhosis; 17males and 13 females within age range of 50-74 years and free of diagnostic criteria of HCC. All patients were classified to child A, B or C classes according to Child-Pugh classification (Pugh *et al*., 1973). Twenty five healthy volunteers 16 males and 9 females, within age range of 42-66 years and proved to be negative to HCV Ab, HBsAg were selected as control group (Fig. 1 and 2).

Sample collection: Five ml of anti-cubital venous blood were collected from each case and immediately put in a vaccutainer tube containing EDTA and centrifuged at 3500 rpm to separate plasma and then stored at -80°C for further processing (Table 1 and 2).

Total RNA extraction: Total RNA was extracted using Gene JET^{TM} RNA Purification kit, (Fermentas) following the manufacturer instructions and the standard protocol. Fifty µl of the eluted RNA was collected immediately, placed in ice or stored at -20°C for further processing.

Spectrophotometric quantification of RNA: To ensure significance, A260 readings should be greater than 0.15.An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity (Alhusseini *et al*., 2010), 10 µL of each RNA sample was diluted to 500 µl by D.W PH=7.0 (1:50 dilution).Measurement of the absorbance of the diluted sample in a 1ml cuvette (RNase free) at A260 and A280.Concentration of RNA sample was measured: = 44µg m L^{-1}×A260×dilution factor.A260 and A280 were taken by UV spectrophotometer (Optima SP-3000+, Japan). The ratio of the readings at 260 and 280nm (A260/A280) provides an estimate of the purity of RNA. Pure RNA has an A260/A280 ratio of 1.9-2.3

Relative Quantitation of m RNA of the respective genes by real time PCR using SYBR green: The target gene sequences was the catalytic subunit of hTERT and the calibrator sample is healthy control. The reference gene (housekeeping gene) was GAPDH. RQ provides accurate comparison between the initial levels of template in each sample, without requiring the exact copy number of the template. Also the relative levels of samples can be determined without the use of standard curves. A singleplex reaction have been used in this study, we did one-step RT-PCR using QuantiTect® SYBR® Green RT-PCR master mix kit (Qiagen. GmbH). In ABI 7900 (Applied Biosystem.) the real time machine the prepared reaction components were done in 96 well PCR plate using real time cycler conditions of 48°C for 30 min, (Reverse transcription), 95°C, 15 min, (Initial denaturation) followed by 40 cycles of 94°C, 30 sec, 48°C, 1 min and 72°C, 1 min for Denaturation, Annealing, Extension steps respectively. Primer sequence of hTERT was 5'- CTT TTA TGT CAC GGA GAC CAC -3' (5'-3' sequence forward) and 5'-TAA TAC GAC TCA CTA TAT AGT CCA TGT TCA CAA TCG G -3' (5'-3' sequence reverse).
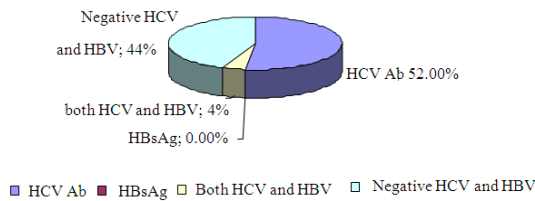
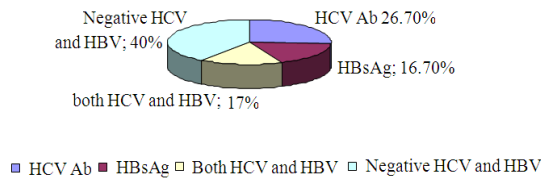Fig. 1: Percentage of viral hepatitis infection in HCC patients

Fig. 2: Percentage of viral hepatitis infection amonge cirrhotic patients

Primer sequence of glyceraldehyde phosphate dehydrogenase (GAPDH) as internal control (housekeeping gene) was 5' -TGA TGA CAT CAA GAA GGT GGT GAA G-3'(5'-3' sequence forward) and 5'-TCC TTG GAG GCC ATG TGG GCC AT-3' (5'-3' sequence reverse). The PCR primers were synthesized by (Operon, inc. Huntsville, Alabama Germany).

Data analysis: According to the RQ manager program ABI SDS software (ABI 7900), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale). The Threshold Cycle (CT) serves as a tool for calculation of the starting template amount in each sample (Fig. 5). Because the samples of control group and also samples of cirrhosis group are used as calibrators, the expression levels are set to 1. But because the gene expression levels were plotted as log10 values (log10 of 1 is 0), the expression level of the calibrator samples appear as 0 in the graph. (Alhusseini *et al*., 2010). Because the relative quantities of the hTERT gene are normalized against the relative quantities of the endogenous control (GAPDH) gene fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$ (Livak and Schmittgen, 2001).

Statistical analyses: The results were presented as means ± SD, Comparisons of categorical variables were made between cases and controls using chi-squared exact test. The Spearman's correlation coefficient was used to measure the closeness of a linear relationship between relative gene expression values. All analysis

was performed using the statistics package St. Social Sciences (SPSS) and Microsoft office Excel is used for data processing and data analysis. Differences are considered as statistically significant for a p value less than 0.05.

RESULTS

The expression of hTERT mRNA by relative unit was 129.10±27.6 with range (67.72-69.6) Vs 5245.87±2382.48 (2053-12232.90) Vs 92782.76±16158 (61783.25-118596.47) for control Vs cirrhosis Vs HCC group correspondingly Fig. 7. The expression was significantly with 699 and 33 fold increase in HCC when compared to control and cirrhosis correspondingly Fig. 8 and 9 p<0.05. As regards to tumors in HCC patients, eighteen HCC patients have a tumor size more than 5cm while 7 have it less than 5cm. It also shows that 13 patients have their focal lesion in the left lobe while 12 patients have their focal lesion in the right lobe. In addition 16 have a single focal lesion, 9 have multiple focal lesions and four patients have portal vein thrombosis. There is a statistically significant increase in hTERT mRNA level of HCC group as compared to both control and cirrhosis groups (P1<0.05and P2<0.05) correspondingly, however there is a slight increase in its level in cirrhosis patients as compared to controls but does not reach a significant level (P3>0.05).This work reported that there is no significant correlation between hTERT mRNA level and any laboratory finding in cirrhosis group. But in HCC group a statistically significant positive correlation was found between hTERT mRNA level on one hand and both AFP and tumor size on the other hand Fig. 3 and 4, however, no significant correlation between it and any other laboratory variable was reported. The area under the ROC curve of AFP was 0.945 Vs 0.969 of that under the curve of hTERT (Fig. 6).

Gene expression levels of hTERT mRNA for both cirrhotic and HCC samples are indicated by green bars. This color also indicates the samples in RQ. Because control samples are used as calibrators, the expression levels are set to one. But because the expression levels were blotted as log10 values (and the log 10 of 1 is 0), the expression level of the control samples appear as 0 in the graph Fig. 8. Because the relative quantities of the hTERT mRNA are normalized against the relative quantities of the GAPDH (endogenous control), the expression level of the endogenous control is 0; there are no bars for GAPDH. Because cirrhotic samples are used as calibrators, the expression levels are set to one. But because the expression levels were blotted as log10 values (and the log 10 of 1 is 0), the expression level of the cirrhotic samples appear as 0 in the graph Fig. 9.
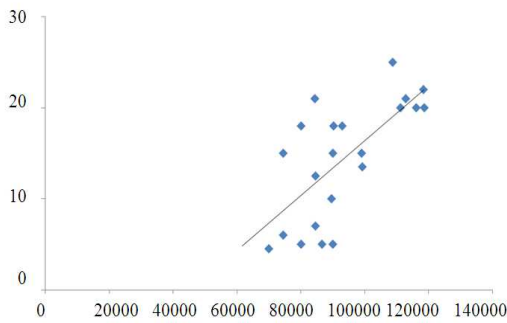
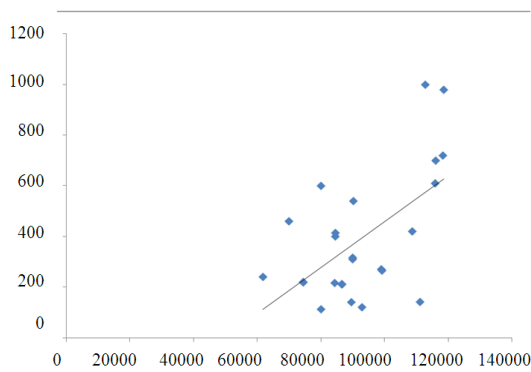Fig. 3: Correlation between hTERT mRNA and size of tumor

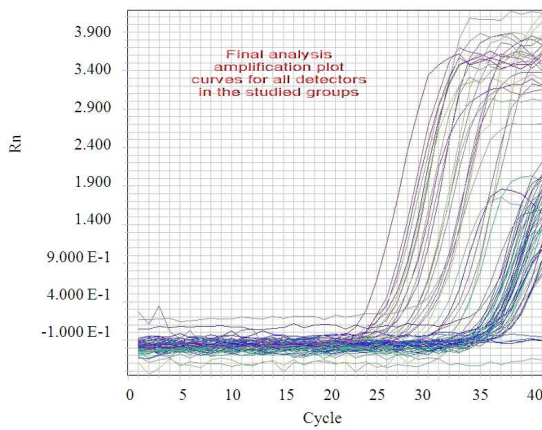Fig. 4: Correlation between hTERT mRNA and AFP in HCC patients

Fig. 5: Amplification plot curves for all detectors in the studied groups (Curves by ABI 7900 Real Time)
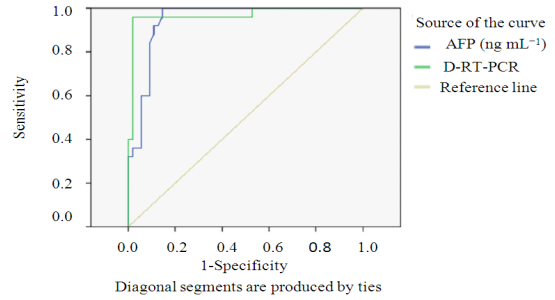

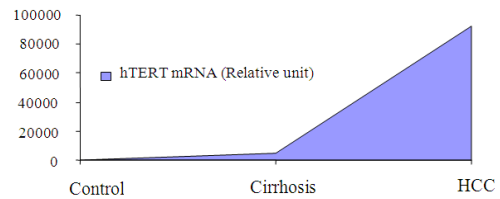Fig. 6: ROC curve for hTERT and AFP in patients

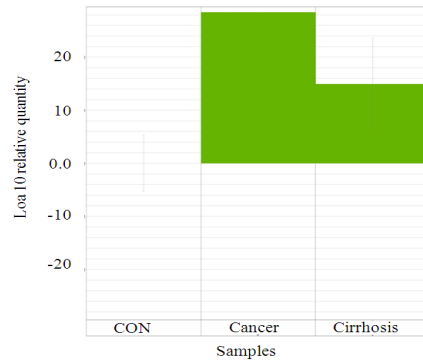Fig. 7: hTERT mRNA (Relative unit) amonge the three studied groups

Fig. 8: Gene expression levels of hTERT mRNA for both cirrhosis and HCC relative to controls (CON: Controls)
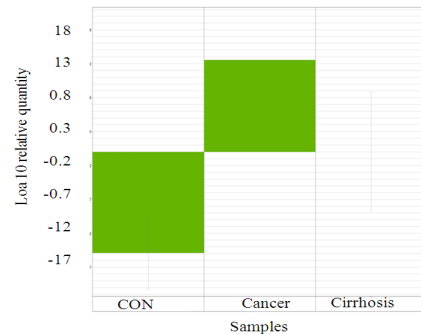

Fig. 9: Gene expression levels of hTERT mRNA for both control and HCC relative to cirrhosis

Table 1: Clinical profile of the studied groups

	Mean ± SD (Range)						
	Control n.=25	Cirrhosis n.= 30	HCC n.=25	F	P	p between groups	
Age (Year)	53.92±6.708 (42-66)	61.87±7.491 (48-74)	63.04±7.486 (44-78)	11.9	<0.05	*p1 <0.05 *p3 <0.05	p2 >0.05
HB (gm dL^{-1})	13.58±1.35 (11.4-16.0)	10.4±1.13 (8.1-11.9)	10.8±1.29 (8.2-13.0)	49.6	<0.001	*p1 <0.05 *p3 <0.05	p2 >0.05
RBCs (×106 uL^{-1})	4.76±0.527 (4-6)	4.11±0.591 (3-5)	3.47±0.30 (3-4)	47.7	<0.001	*p1 <0.05 *p3 <0.05	p2 >0.05
WBCS (×103 uL^{-1})	8.36±1.770 (5.5-11.2)	7.56±2.33 (4.0-10.6)	6.56±3.87 (3.8-23.8)	2.6	>0.05	*p1 <0.05 p3 >0.05	p2 >0.05
Platelets (×103 uL^{-1})	262.96±71.91 (170-406)	181.40±82.70 (92-310)	131.53±14.86 (106-176)	40.8	<0.001	*p1 <0.05 *p3 <0.05	p2>0.05
Liver and kidney functions							
ALT(U/L)	32.03±7.85 (20-46)	33±9.99 (13-44)	76.0±46.11 (20-152)	22.6	<0.001	*p1 <0.05 p3 >0.05	*p2 <0.05
s.Billirubin(mg dL^{-1})	0.424±0.198 (0.1-0.8)	5.24±3.23 (1.3-15.0)	6.56±2.125 (2.6-9.6)	49.2	<0.001	*p1 <0.05 *p3 <0.05	*p2 <0.05
s.albumin (gm dL^{-1})	4.27±0.53 (3.5-5.3)	1.82±0.58 (0.8-2.9)	1.56±0.54 (0.9-2.1)	54.9	<0.001	*P1 <0.05 P2 >0.05 *P3 <0.05	
s.creatinine(mg dL^{-1})	1.03±0.23 (0.6-1.4)	1.140±0.36 (0.6-2.0)	1.30±0.27 (1.0-2.4)	4.9	<0.05	*P1 <0.05 P2 >0.05 P3 >0.05	
				t-test	p		
P.T (seconds)		20.40±3.820 (14-27)	19.22±4.586 (12-25)	1.1	>0.05		
AFP (ng mL^{-1})		62.9±63.7 (24.8-125.2)	325.8±244.5 (112-1000)	48.1	<0.05*		

Table 2: Percentage of the clinical picture parameters among the studied groups

	Number (%)						
	Control n.= 25		Cirrhosis n.=30		HCC n.=25	X2	p
Female	9/25	(36.0%)	13/30 (43.3%)		10/25 (40.0%)	0.3	>0.05
Male	16/25	(64.0%)	17/30 (56.7%)		15/25 (60.0%)		
Clinical picture							
Abdominal pain	0%		14/30 (46.7%)		12/25 (48.0%)	0.1	P>0.05
Bleeding	0%		10/30 (33.3%)		10/25 (40.0%)	0.3	P>0.05
Disturbed consciousness	0%		10/30 (33.3%)		12/25 (48.0%)	1.2	P>0.05
Jaundice	0%		24/30 (80.0%)		18/25 (72.0%)	0.5	P>0.05
Enlarged liver	0%		0/30 (0.0%)		12/25 (48%)	17.6	<0.05*
Shrunken liver	0%		30/30 (100.0%)		13/25 (52%)		
Splenomegaly	0%		27/30 (90.0%)		24/25 (96.0%)	11.2	>0.05
Ascites	0%		30/30 (100%)		25/25 (100%)	1.2	>0.05

DISCUSSION

In the present study, the mRNA of the catalytic subunit of the telomerase enzyme (hTERT mRNA) was used as a target sequence for quantification, (Miura *et al*., 2006; Sirera *et al*., 2007) also used the same target. Sozzi *et al*. (2003) used hTERT gene itself as a target sequence for quantification of circulating DNA. Peter *et al*. (2010) found that quantification of mRNA of the catalytic subunit of the enzyme (TERT) was found to be superior to quantification of the whole gene encoding both TERT and telomerase RNA

component (TERC) because the regulatory units controlling TERC expression and its up regulation in tumor cells are much less defined. The expression of hTERT mRNA in plasma was measured by quantitative Real time PCR (RT-PCR).

Some previous studies (Dasi *et al*., 2006 and Sirera *et al*., 2007) reported using plasma samples while some studies reported using serum samples (Miura *et al*., 2006).Other studies used peripheral blood lymphocytes (Jang *et al*., 2008). In the current work, no significant sex difference was discovered when comparing the HCC, cirrhosis and control groups. As regards to age there was

no statistically significant difference between cirrhosis andHCC groups. However there was a statistically significant increase in age of both HCC andcirrhosis groups as compared to control groups correspondingly. This age difference is in agreement with (Elizabeth *et al*., 2008) who reported an incidence of 64.9% of HCC cases in Egypt between the age of 50 and 69 years which may be explained by that liver cancer are generally attributed to HCV and chronic HCV most probably lead to carcinogenesis after 10-30 years following infection or may be due to late diagnosis in Egypt (Shariff *et al*., 2011). The higher increase of hTERT mRNA level in HCC patients were in agreement with (Braicu *et al*., 2009; Roessler *et al*., 2007; Miura *et al*., 2010). Although telomerase per se is not carcinogenic, it plays a direct role in oncogenesis by allowing the precancerous cells to proliferate continuously and become immortal, which can be explained by the positive correlation between telomerase activity and tumors of different histological origins and types (Cortez-Gonzalez and Zanetti, 2007). Over 80% of human cancers show an activation of telomerase which stabilizes telomeres thus facilitating immortal proliferation of cells. The activation of telomerase is a general phenomenon in different types of human cancer. In human cancer the activation of telomerase often correlates with tumor progression. Premalignant tumors show no or very little telomerase activity (Xianling *et al*., 2008). On the other hand, there was a slight increase in expression of hTERT mRNA level in cirrhosis patients as compared to controls but does not reach a significant level, a finding that coincides with the results reported by Miura *et al*. (2010) who attributed this to the fact that normal hepatocytes may express a negligible amount of hTERT-mRNA and that inflamed hepatocytes may still express more weakly than HCC cells. Shimojima *et al*. (2004) reported a sensitivity of 100%, but their study measured hTERT-mRNA in HCC tissue and not in the peripheral blood, which might indicate that the locally expressed hTERT-mRNA may be a more sensitive predictor of HCC in tissue than in peripheral blood, but still blood samples are much more easily obtained for monitoring HCC vulnerable patients than tissue biopsies. Our results were contradictory to multiple studies that failed to explain low hTERT-mRNA in some HCC cases, Cavin *et al*. (2003) reported that some HCC cases had low hTERT-mRNA, because of cirrhosis which may be the underlying cause for HCC, causes a high level of TGF-β, which promotes apoptosis of immortalized hepatocytes. Nittis *et al*. (2008) suggested that immortal hepatocytes may acquire an "Alternative Lengthening of Telomerase" (ALT) mechanism for maintenance of their chromosomal stability. We can give an explanation about these reports by previous studies that their samples were HCC tissues and the low telomerase enzymatic activity in tissue samples taken from necrotic malignant tissue possibly due to variability in the collected sample and the used technique. In the current work, no significant correlation was reported between CBC results, liver enzymes, serum albumin, serum creatinine or prothrombin time and hTERT mRNA expression in neither HCC or cirrhosis groups. Also, Kim *et al*. (2006) found no significant correlation between hTERT mRNA and any of the clinico-pathological variables in cirrhosis and HCC patients. For the HCC group, the hTERT-mRNA expression levels were positively correlated with AFP and tumor size but not with site or number of the focal lesion. These findings are in agreement with (Miura *et al*., 2007; Braicu *et al*., 2009; Miura *et al*., 2010).However, Stravitz *et al*.(2008) reported that the enzyme level was significantly associated with tumor size but not AFP level.

Our findings revealed that hTERT-mRNA is a better marker than AFP as Wu *et al*. (2005) suggest that AFP sensitivity is lower in certain races (e.g., African Americans) and Cortez-Gonzalez and Zanetti (2007) found that AFP is significantly decreased in cirrhotic patients receiving interferon therapy for HCV. (Farinati *et al*., 2006) reported that the usefulness of AFP testing for HCC diagnosis is seriously questioned because AFP monitoring misses many HCC cases and inappropriately arouses suspicion of malignancy in many cirrhotic patients. So, it has limited utility in differentiating HCC from benign hepatic disorders for its high false positive and false negative rates and also patients with acute viral exacerbation but not HCC may have remarkably elevated AFP levels. Therefore new biomarkers with better sensitivity and specificity than AFP to complement the imaging diagnosis are needed (Stravitz *et al*., 2008). So quantitative analysis of plasma hTERT mRNA expression was superior to AFP for the purpose of the early detection of HCC, because hTERT mRNA was detectable in HCC patients with normal AFP levels.

CONCLUSION

HTERT mRNA expression in hepatocellular carcinoma patients showed significant elevation in comparison to healthy controls and also cirrhotic patients. So, it might be a diagnostic tumor marker for HCC.A valuable diagnostic test for HCC might be developed using this genetic marker in plasma in combination with other tumor markers. hTERT mRNA

was found to be superior to AFP as a tumor marker for HCC. hTERT mRNA level was significantly correlated with size of the focal lesions among HCC cases. So, it could be a prognostic tool for morbidity in these patients.

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