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## Telomerase as a tumor marker in hepatocellular carcinoma

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### ABSTRACT

This study was conducted on 25 healthy controls, 25 cirrhotic patients and 30 patients with HCC. All patients had been diagnosed with HCV-associated chronic liver disease. Methods: Serum PIVKA-II and AFP were measured by enzyme linked immunosorbent assay (ELISA), while telomerase activity in peripheral blood was estimated by polymerase chain reaction- enzyme- linked immunosorbent assay (PCR- ELISA method). Results: Mean telomerase activity, PIVKA-II and AFP levels were significantly higher in HCC patients as compared to both cirrhotic patients and controls, also a significant elevation in cirrhotic patients were found as compared to controls. Positive correlation was found between telomerase activity and size of hepatic focal lesions. Also, a positive correlation was found between both telomerase activity and PIVKA-II and between the pathological grades of HCC. In HCC the sensitivity/specificity (88.2/79.6) of telomerase activity was much higher than both PIVKA-II (80.5/69.3) and AFP (72.6/61.5). Conclusion: The usefulness of telomerase activity assay in HCC diagnosis and its superiority to other tumor marker were recorded. Therefore, telomerase activity is a novel, available detector and prognostic marker for HCC diagnosis.

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### KEYWORDS

Hepatocellular carcinoma;  
Telomerase;  
PIVKA-II;  
Telomerase PCR ELISA.

### INTRODUCTION

Hepatocellular carcinoma (HCC) is a major challenge in contemporary medicine. The incidence of HCC is increasing and it is becoming more and more significant both clinically and epidemiologically. Now HCC represents the fifth most common cancer in the world and the third most frequent cause of mortality amongst oncological patients<sup>1</sup>. It is responsible for more than 500 000 deaths with over 600 000 new cases yearly worldwide<sup>2</sup>. More than 95% of HCC patients present underlying hepatopathy in particular of viral etiology. The majority of the cases (>85%) have liver cirrhosis,

which masks symptoms of cancer progression. The clinical course of HCC is mostly asymptomatic. Suspected focal liver changes are often detected incidentally while monitoring the patient's condition during ultrasound (US) examination, and often are too large and too advanced for the tumor to be subjected to potentially effective and radical therapy<sup>3</sup>. So far, it is necessary to concentrate on the earliest possible diagnosis, particularly sensitive detection of resectable focal liver changes preferably when tumors are less than 2 cm in diameter<sup>4</sup>. The first serological assay for detection and clinical follow up of patients with HCC was alpha fetoprotein (AFP)<sup>5</sup>. Numerous data have proved that significantly higher

## Regular Paper

AFP serum levels accompany various liver diseases (viral hepatitis, liver cirrhosis, liver tumors: primarily HCC and hepatoblastoma, also metastasis in 5% -10% cases), which diminish its specificity as a golden standard serum marker for HCC<sup>6</sup>. Since the first description by Liebman et al. in 1984, prothrombin induced by vitamin K absence or antagonist -II (PIVKA -II), also referred to as des-gamma-carboxy prothrombin (DCP), has been found to be another useful tumor marker for HCC<sup>7</sup>. Recent study demonstrated that the diagnostic accuracy of PIVKA-II seemed to be higher compared to AFP<sup>8</sup>. It has been proved that significant concentrations of serum PIVKA-II in 50%—60% of all HCC patients, but in only 15%-30% of early HCC cases<sup>9</sup>. Clinical utility of PIVKA-II was further investigated in view of correlation between several factors such as tumor size, intrahepatic metastases, and histological activity of tumor tissue.<sup>10, 11</sup> These two markers (AFP and PIVKA-II) which are supposed to be produced independently by HCC may serve complementarily in the diagnosis of HCC.<sup>12,13</sup> Although the modalities such as ultrasonography and conventional tumor markers are important for detection of HCC, they are still not sensitive enough to detect HCC at the early stage.<sup>14</sup>

Hepatoma tissues synthesize and secrete valuable molecular markers such as AFP-beta1-mRNA, IGF-II-mRNA, telomerase, etc into blood. The analyses of these circulating hepatoma-specific biomarkers are useful to early diagnosis of HCC or monitoring metastasis or postoperative recurrence of HCC.<sup>15, 16</sup> Telomerase is a ribonucleoprotein enzyme composed of two key components: a catalytic component (human telomerase reverse transcriptase, hTERT) and a RNA template. It helps to stabilize telomere length in human stem cells, reproductive cells and cancer cells by adding TTAGGG repeats onto the telomeres using its intrinsic RNA as a template for reverse transcription, and it is a limiting component in telomerase activity.<sup>17,18</sup> During the early stages of carcinogenesis, cells undergo extensive proliferation until telomere length becomes critically shortened.<sup>19,20</sup> Telomere shortening is an early event in multistep hepatocarcinogenesis, occurring in preneoplastic lesions of dysplastic nodules.<sup>21</sup> Shortened telomeres have been reported to induce chromosomal instability in hepatocytes, especially important in viral-related hepatocarcinogenesis.<sup>22,23</sup> It has been suggested that detection of cancer-related gene expres-

sions in serum is very useful for diagnosis and follow-up of cancer patients. hTERT mRNA in serum was detected in breast cancer but not in benign diseases, suggesting that hTERT is available for cancer diagnosis.<sup>24</sup> Overexpression of telomerase is associated with HCC development, and its abnormality in liver tissues or in peripheral blood could be a useful marker for diagnosis and prognosis of HCC.<sup>25</sup> In the present study, we mainly focused on the comparison of telomerase activity in peripheral blood with serum AFP, and PIVKA-II to select the most reliable early diagnostic and prognostic markers in HCC. A correlation between AFP, PIVKA-II and telomerase activity and tumor size and histopathological grades was performed.

## PATIENTS AND METHODS

This study was conducted on 55 patients; 30 patients with biopsy proven hepatocellular carcinoma and 25 patients with biopsy proven liver cirrhosis. They were selected from attendants of the out and inpatients Libia Hospital. Twenty five healthy age and sex matched controls were enrolled in this study.

All cases were subjected to the following:

- I. Full history taking with special stress on jaundice, blood transfusion and bilharziasis.
- II. General and abdominal clinical examination.
- III. Ultrasonographic examination of the liver was done to assess its size and echo pattern in order to expose any pathology such as liver cirrhosis and to detect focal masses. The site and size of any detected mass were recorded. The portal tract as well as the portal and splenic veins were also measured.
- IV. Percutaneous liver needle biopsy was done for all patients. Patients not fit for biopsy were excluded from the study. The specimens were processed and embedded into paraffin blocks, cut at 5 um thick sections and stained with Hx and Eosin.
- V. Laboratory tests:
  - A) Routine laboratory tests including:
    - 1- Complete blood picture.
    - 2- Liver function tests (serum albumin, bilirubin, AST, ALT, Alkaline phosphatase and prothrombin time) were measured by conventional methods.
    - 3- Serological markers for hepatitis B (HbsAg and HbcAb) by ELISA of Boehringer

Mannheim and hepatitis C (HCV Ab) by Murex version 111.

B) Specific laboratory tests:

1. Determination of AFP level: by ELISA method (Chierigatti, 1990).<sup>26</sup>
2. Measurement of PIVKA-II level (Amiral et al, 1991)<sup>27</sup>: by Asserachro PIVKA-II ELISA Kit (Diagnostica Stago).
3. Assessment of telomerase activity: The protein extracts (2µl) from peripheral blood mononuclear cells (PBMCs), were analyzed by using Telomerase PCR ELISA (Telomerase PCR ELISA, Boehringer Mannheim).<sup>28,29</sup> In brief, 50 µl containing 25 µl reaction buffer, 2 µl of protein extract and 2 µl of primers telomeric repeats. The PCR condition was as follows: incubation for 30 min at 25°C for primer extension. The mixture was then 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 the final step). An aliquot of the PCR product was denatured, hybridized to a digoxigenin (DIG)-labelled telomeric repeat-specific probe and bound to a streptavidin-coated 96 well plate. Finally, the immobilized PCR product was detected with an anti-DIG-peroxidase antibody and visualized by tetramethyl benzidine substrate (colour reagent). The absorbance (A) of telomerase was measured at a wavelength of 450 nm (reference wavelength 620 nm) within 30 minutes of addition of the stop reagent. The cell extract was heated to 65°C for 10 minutes as a negative control.

### SAMPLE

Under aseptic conditions, fasting venous blood sample (12 ml) was withdrawn by clean venipuncture from the antecubital vein: 2 ml on EDTA for blood picture; 3 ml to separate serum for routine laboratory tests on the collection day and aliquot of serum was stored at -20 °C for AFP level measurement; 2 ml on citrate to

separate citrated plasma for PIVKA-II level measurement; 5 ml on heparin (for anticoagulant) and 2.5 ml of ficoll to be centrifuged at 2000 /min for 20 minutes at 4 °C, peripheral blood mononuclear cells (PBMCs) were collected from the ficoll/plasma interface. The cells were then washed three times in normal saline and pelleted by using low-speed centrifugation for 10 minutes. The samples were immediately aliquoted and stored at -80 °C for determination of telomerase activity.

### STATISTICAL ANALYSIS

Data were processed using SPSS version 17 software program. Means and standard deviations were computed for clinical data. T-test analysis was done between means of two groups. Analysis of variance was done between measured of variables more than two groups and receiver-operator characteristic (ROC) curve used. *P* value equal to or less than 0.05 was considered the threshold for significant.

### RESULTS

Results were summarized, statistically analyzed and tabulated in TABLES (1-5), and graphically presented in Figures (1-3). Thirty hepatocellular carcinoma patients, 25 cirrhotic patients and 25 control cases were included in this study. The clinical data of all patients were illustrated in TABLE 1. Mean telomerase activity, PIVKA-II and AFP levels were significantly higher in HCC patients as compared to both cirrhotic patients and controls with a significant elevation in cirrhotic patients as compared to controls (TABLE 2, Figure 1). The mean telomerase activity and PIVKA-II values were found to be significantly higher in poorly differentiated grade III HCC compared to moderately differentiated grade II HCC and well differentiated grade I HCC ((TABLE 4, Figure 1 & 3). As regards the size of the focal lesions, it was found that telomerase activity was significantly higher in patients with lesions 5-10 cm than in those with lesions below 5 cm. By using correlative studies, statistical significant correlation could be found between the mean telomerase activity in relation to the size of the hepatic focal lesions (TABLE 5). No correlation was found between telomerase activity, PIVKA-II and AFP in the studied groups. In HCC, the sensitivity/

## Regular Paper

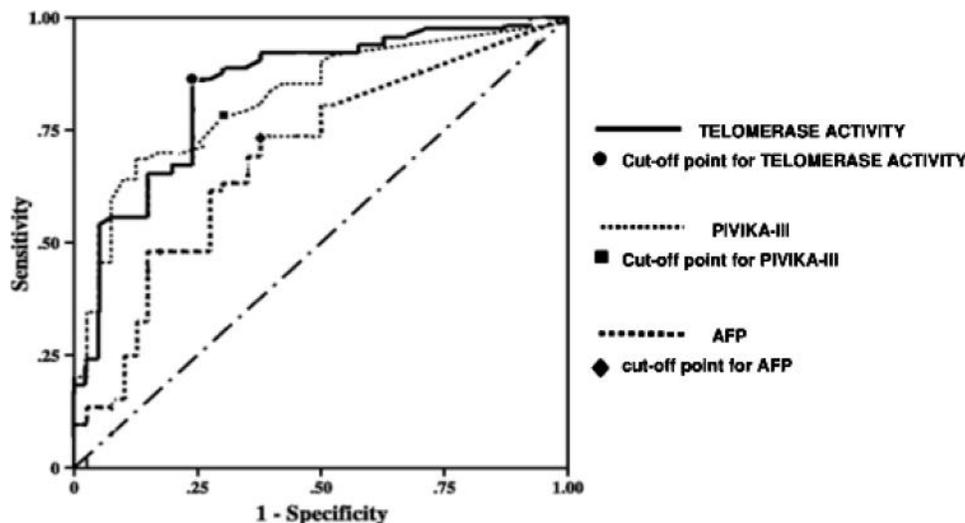
specificity (88.2/79.6) of telomerase activity was much higher than both PIVKA-II (80.5/69.3) and AFP (72.6/61.5). Combination of AFP and PIVKA-II yielded increasing sensitivity/specificity 82.6%/77.6% and accuracy 87.8%, while both AFP and telomerase increasing sensitivity (90.4%), specificity (82.3%) and accuracy (89.9%) (Figure 3 & TABLE 3).

**TABLE 1 : Clinical, sonographic, hispathological data of cirrhosis and HCC groups.**

	Cirrhosis	HCC
Number	25	30
HCVAb positive	14	19
HBsAg, HCVAb positive	6	7

**TABLE 3 : Accuracy of AFP, PIVKA, Telomerase and combined markers in HCC group**

	AFP	PIVKA-II	Telomerase	AFP+ PIVKA-II	AFP+ Telomerase
Cut off value for HCC	>10ng/ml	>1.3 ng/ml	>0.7 unit	Combined	Combined
Sensitivity	80.5%	88.2%	82.6%	90.4%	92.6%
Specificity	61.5%	69.3%	79.6%	77.6%	82.3%
Over all accuracy	69.8%	70.3%	73.5%	87.8%	90.1%



**Figure 2 : Comparison of telomerase activity in peripheral blood from HCC, cirrhosis and normal controls. Arbitrary cut-off level = 0.7 units**

## DISCUSSION

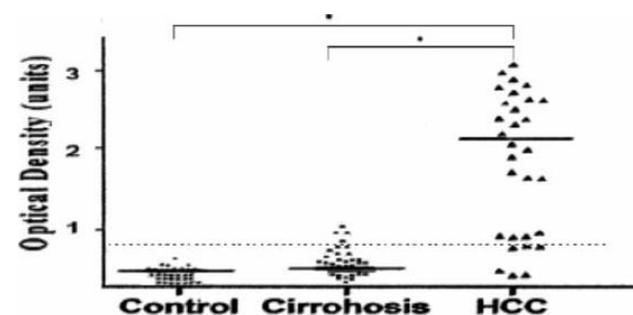
Hepatocellular carcinoma (HCC) is the most common form of primary hepatic malignancy. Although recent advances in imaging diagnosis such as real time ultrasonography, computed tomography and magnetic

**TABLE 2 : Tumor markers: AFP, PIVKA-II and Telomerase in control, cirrhosis and HCC groups**

	AFP (ng/ml)	PIVKA-II (ng/ml)	Telomerase Optical density (units)
Control (n=25)	3.05±1.2	1.26±0.5	0.39±0.14
Cirrhosis (n=25)	13.5±5.9	1.44±1.07	0.43±0.26
HCC (n=30)	98.8±95.42ab	2.29±1.03ab	2.08±0.98ab

**TABLE 4 : Tumor markers in patients with different grades of HCC**

Variables	Grade I N=10	Grade II N=12	Grade III N=8
AFP mean±SD (ng/ml)	83.55±8.69	95.25±10.50	116.80±25.6
PIVKA-II mean±SD (ng/ml)	1.68±0.09	2.19±0.06a	2.68±0.08ab
Telomerase mean±SD unit of activity	1.08±0.059	1.65±0.086a	2.38±0.095ab

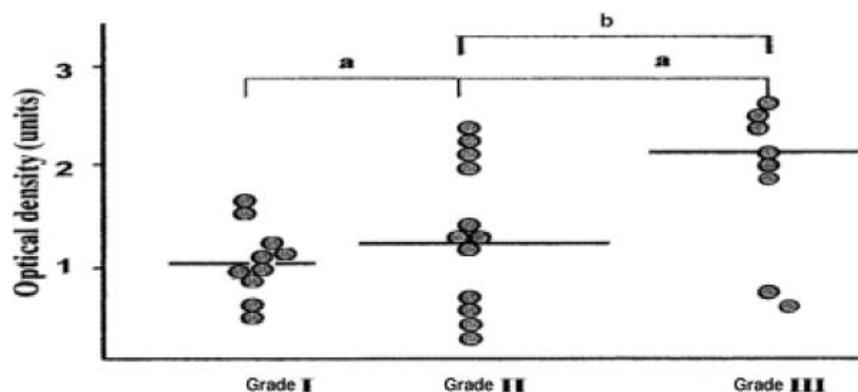


**Figure 1: AFP, PIVKA-II and Telomerase in control, cirrhosis and HCC groups**

resonance imaging have changed the diagnostic strategy for early diagnosis of HCC, determination of tumor markers for HCC at regular intervals is still a common practice. Nowadays it is believed that early HCC diagnosis is presently considered feasible in 30%-60% of the cases in the developed countries. Tumors smaller than 2 cm in diameter represented < 5% of cases in

1990s, whereas now they represent up to 30% of cases in Japan.<sup>4</sup> Significantly more effective surveillance strat-

egies lead to earlier HCC detection and earlier qualification for effectively curative radical surgery, with very



**Figure 3 :** Receiver-operator characteristic curve analysis curves were representing, AFP, PIVKA-III and telomerase respectively obtained by importing quantified raw data and the sensitivity/specificity values were calculated. Each line has a cutoff point for a marker.

good postoperative survival rates.<sup>9</sup>

The current study enrolled 30 patients with biopsy proven hepatocellular carcinoma and 25 patients with biopsy proven liver cirrhosis and 25 healthy age and sex matched controls. We mainly focused on the comparison of telomerase activity in peripheral blood with serum AFP, and PIVKA-II to select the most reliable early diagnostic and prognostic markers in HCC. A correlation between AFP, PIVKA-II and telomerase activity and tumor size and histopathological grades was performed.

It has been confirmed on numerous occasions that AFP serum concentration increases in parallel with HCC tumor size. For this reason AFP has to be considered 'the golden standard' for HCC serum

Since the first description by Liebman et al. in 1984, prothrombin induced by vitamin K absence or antagonist -II (PIVKA -II), also referred to as des-gamma-carboxy prothrombin (DCP), has been found to be another useful tumor marker for HCC. It was demonstrated that there is excessive synthesis of prothrombin precursors by human HCC tissue and that it might be a contributing factor to the production of DCP by HCC.<sup>7</sup> Several case control studies have shown sensitivities of PIVKA-II of 28% to 89% and specificities of 87% to 96% in the diagnosis of HCC.<sup>34-36, 37, 38</sup> In some studies, PIVKA-II was more sensitive than AFP,<sup>36,37,38</sup> whereas, in other studies, AFP was more sensitive.<sup>34,35</sup> A recent study of 1377 HCC patients and 355 non- HCC controls with chronic hepatitis or cirrhosis showed that the accuracy of PIVKA-II was inferior to AFP, particularly for small tumors.<sup>39</sup>

Various factors may influence the performance of HCC biomarkers, including ethnicity, cutoff value, patient demographics, cause of underlying liver disease, presence of cirrhosis, tumor stage and tumor biology and so on.<sup>40,41</sup> markers. However, the usefulness of AFP testing for the population at risk should be seriously questioned. AFP diagnostic values for this assay are undoubtedly poor. AFP specificity varies from about 76% to 96% and increases with elevated cut-off value. Simultaneous sensitivity decrease much more from about 25% for potentially respectable of less than 3 cm in diameter to about 50% for lesions of >3 cm in diameter.<sup>30, 31</sup> 20%-30% AFP sensitivity coincides with cut- off >100 µg/L, which means that 70%-80% of liver tumors, normally respectable are non-detectable and unfortunately do not undergo treatment or are subjected to it too late.<sup>32</sup> Therefore new biomarkers with better sensitivity and specificity than AFP to complement the imaging diagnosis are needed.<sup>33</sup>

In the present study, both AFP and PIVKA-II showed statistically significant higher levels in HCC patients compared to cirrhotic and control groups.. In HCC, the sensitivity/specificity of PIVKA-II (80.5/69.3) and accuracy (70.3%) were higher than AFP (72.6/61.5, 69.8%). Combination of both AFP and PIVKA-II yielded increasing sensitivity/specificity 82.6%/77.6% and accuracy 87.8%. In agreement with the current study, has been postulated that PIVKA-II is a useful marker for detecting HCC, especially in small HCC and may have correlations with known staging systems, especially in combination with AFP.<sup>42, 43</sup> In contrary to our findings, recent study reported that AFP

## Regular Paper

was more sensitive than PIVKA-II and AFP-L3% for the diagnosis of early stage HCC at a new cutoff of 10.9 ng/ml<sup>44</sup> while others concluded that PIVKA-II was not superior to AFP in the early detection of HCC and that neither AFP alone, PIVKA alone, nor the combination of both was sufficiently accurate to be used for early HCC diagnosis and monitoring recurrence after curative resection.<sup>45</sup>

Hepatoma tissues synthesize and secrete valuable molecular markers such as AFP-beta1-mRNA, IGF-II-mRNA, Telomerase, etc into blood. The analyses of these circulating hepatoma-specific biomarkers are useful to early diagnosis and monitoring metastasis or post-operative recurrence of HCC.<sup>15, 16</sup> Telomerase is a ribonucleoprotein enzyme composed of two key components: a catalytic component (human telomerase reverse transcriptase, hTERT) and an RNA template.<sup>25</sup> In this assay, telomerase activity showed statistically significant higher levels in HCC patients compared to cirrhotic and control groups.. In HCC, the sensitivity/specificity (88.2/79.6) was much higher than both PIVKA-II (80.5/69.3) and AFP (72.6/61.5). Combination of both AFP and telomerase is increasing sensitivity (90.4%), specificity (82.3%) and accuracy (89.9%). The mean telomerase activity was found to be significantly higher in poorly differentiated HCC compared to moderately differentiated HCC and well differentiated HCC. As regards the size of the focal lesions, it was found that telomerase activity was significantly higher in patients with lesions 5-10 cm in diameter than in those with lesions below 5 cm. By using correlative studies, statistical significant correlation could be found between the mean telomerase activity in relation to the size of the hepatic focal lesions. No correlation was found between telomerase activity, PIVKA-II and AFP in the studied groups. To some extent, these results are consistent with a previous studies which concluded that although the measurement of telomerase activity in peripheral blood is a useful and potential approach to establish a practical diagnostic/predictive marker of HCC, it would be imprudent to state that telomerase activity is a specific molecular marker for circulating malignant hepatoma cells.<sup>46, 47</sup> Other recent studies reported that in HCC, serum hTERT mRNA showed higher sensitivity/specificity values than AFP, PIVKA-II and AFP mRNA. There was a positive correlation of hTERT mRNA between tumor tissue and serum and this proved that se-

rum hTERT mRNA is derived from tumor hepatoma cells.<sup>48</sup> In a study done by Bong et al., on 49 HCC patients showed long telomere, high telomerase activity and positive correlation with hTERT mRNA and more advanced tumor stage.<sup>49</sup>

In conclusion, in HCC patient's telomerase activity, is more specific marker and is related more closely to the degree of histologic differentiation and tumor size of HCC than AFP and PIVKA-II. Therefore, telomerase activity might be a useful tumor marker in early diagnosis of HCC and the monitoring of recurrence after curative resection. We recommend testing of AFP with telomerase, as in combination they showed higher overall accuracy.

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