The Value of Measurement of Circulating Tumour Cells in Hepatocellular Carcinoma

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Abstract: Objective: Liver cancer is the fifth most common cancer in men and the seventh in women. During the past 20 years, the incidence of HCC has tripled while the 5-year survival rate has remained below 12%. The presence of circulating tumour cells (CTCs) reflects the aggressive nature of the tumour during the development of the HCC. CTCs detection and identification can be used to estimate prognosis and may serve as an early marker to assess antitumor activity of treatment. CTCs are an interesting source of biological information in order to understand dissemination, drug resistance, and treatment-induced cell death. The aim is to estimate the CTCs (AFP mRNA & TGF- β1 mRNA) in the peripheral blood of patients with HCC as an early non invasive marker of HCC detection and prognosis. **Patients and methods:** The study was done on 100 patients, 58 patients with hepatocellular carcinoma (HCC) and 42 patients with liver cirrhosis (LC), and 20 healthy volunteers as a control group. Detailed clinical history and examination were carried out. Complete blood count, liver function test, serum Albumin, serum AFP, AFP mRNA, serum TGF-β1 and TGF-β1 mRNA were measured.Abdominal ultrasound was done for all studied subjects and CTscan Abdomen for those with HCC to determine the size and number of tumour. **Results**: The detection rate of AFP mRNA was 39.7%, 11.9% and5% in patients with HCC, LC and control subjects respectively with a significant expression in HCC patients compared to other groups. Also TGF-β1 mRNA expression was significantly high in HCC cases with detection rate 60.3%, 14.3% in HCC and LC respectively while it was not detected in the controls. Both CTC were correlated with Milan criteria.The serum levels of AFP and TGF-β1 was significantly higher in HCC patients. **Conclusion:** TGF-β1 mRNA is a more reliable marker for diagnosis of HCC and if combined with AFP mRNA yielded better prediction of HCC prognosis. Since HCC is among the cancers with worst prognosis, early diagnosis and treatment are essential for better outcome.
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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Major risk factors for the development of HCC include chronic infections with hepatitis B and hepatitis C viruses, alcohol consumption, and exposure to dietary alfatoxin B, hereditary liver disease or liver cirrhosis of any aetiology ***(Anzola, 2004).*** Moreover HCC is one of the most aggressive cancers and the treatment outcomes have remained generally poor. Patients who show progress over the terminal stage have a 1-year survival of less than 10% ***(Chiappini, 2012).*** Milan, Barcelona Clinic Liver Cancer, Cancer of the Liver Italian Program, and San Francisco criteria had determined the prognosis of patients based on the number and size of nodules, the metastasis and the state of liver function leading to the best choice for the patient ***(Caldwell & Park, 2009; Sanchez et al., 2009; Donato et al., 2010).*** Based on these data, the physician can choose resection, orthotopic liver transplantation (OLT), percutaneous ethanol injection or radiofrequency ablation, chemoembolisation, systemic chemotherapy and symptomatic therapy ***(Mendizabal & Reddy, 2009).*** At the time the HCC will be treated an important question that the physician has to face is the risk of recurrence and metastasis ***(Chiappini, 2012).*** Surgical resection is the most effective method for curing this disease, but a large number of cases are not adapted to surgery because of their intra hepatic or distant metastasis at time of diagnosis, so it is very important to detect HCC and the recurrence at its earlier stage.

Alpha fetoprotein (AFP) is a 70 KD glycoprotein, consisting of 591 amino acids and 4 % carbohydrate residues. It is encoded by a gene on chromosome 4q11 -q13.The serum AFP show high levels in newborns and then declines progressively below 10ng/ml in 300 days of life. An increase of serum AFP levels can be observed during pregnancy and in patients with mucovicidosis, acute hepatitis,

chronic hepatitis, cirrhosis and other cancers involving gastrointestinal, pancreatic, biliary, non-seminomatous germ-cell testicular and germ-cell ovarian ***(Mizejewski, 2001)***. Alpha-Fetoprotein (AFP) which expressed in the cytoplasm of HCC cells is a useful tumor marker for HCC, but although total AFP has been considered for a long time the ideal serological markers, for detecting HCC, the false-negative or -positive rate with AFP level alone may be as high as 40%, especially for its early diagnosis or the finding of small size HCC (<3 cm) ***(Yao et al., 2000)***. Even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients.

It is sometimes very difficult to make the distinction between tumors and falsely elevated AFP levels because of benign liver disease as it was found that AFP level can be elevated in patients with chronic liver disease such as HCV infection in absence of malignancy ***(Toyoda et al., 2004).*** So the concern is to use some additional serological markers in combination with AFP to improve its performance especially in terms of sensitivity. This necessitates the urgent needs to find sensitive markers for early diagnosis and monitoring of postoperative recurrence of HCC, and to give adequate treatment for HCC patients ***(Shi et al., 2004).***

Various types of cancer can be better understood by the utilizing of tumor biology markers, HCC is one of cancer that can benefit from tumor biomarkers. There are few markers specific to the HCC (tumor cells versus non tumor cells). These genetic markers of HCC or tumor specific protein can monitor carcinogenesis of hepatocytes and diagnose HCC at early stage of HCC development ***(Bae et al., 2005).***

In the field of biology of tumors, some expressions have been coined for the different types of circulating cellular elements. As the cancer cells in the circulation are an important source for HCC metastasis, the biomarkers that indicate the existence of malignant cells may be a useful predictor for HCC extra hepatic metastasis ***(Cillo etal., 2005)***. In the past few years the function of circulating tumor cells (CTCs) in the process of cancer metastasis has been under active investigation.

The term circulating tumour cells (CTC) defines specifically the tumour cells detected in blood or lymphatic vessels ***(Zieglschmid et al., 2005; Sch¨uler & D¨olken, 2006;)***.

CTCs also refer to cancer cells that are disengaged from the primary lesion and invade into blood circulation, they are also called disseminated tumour cells.The recurrence in the original site is probably due to returning of the surviving CTC from the blood stream and this process is known as tumour self - seeding. The presence of CTC reflects the aggressiveness nature of a solid tumour. The CTCs detection and identification can be used to estimate prognosis and may serve as an early marker to assess antitumour activity of treatment. In addition, CTC can be used to predict progression-free survival and overall survival. CTCs are an interesting source of biological information in order to understand dissemination, drug resistance and treatment-induced cell death ***(Riethdorf et al., 2008; Strijbos et al., 2008)***.

AFP mRNA in the peripheral blood detected by reverse transcriptase-PCR (RT-PCR) has been most extensively studied. AFP mRNA may be a valuable indicator of poor prognosis for HCC patients and its expression is correlated with portal thrombosis, nodules of tumour, tumour diameter and TNM stage ***(Ijichi et al., 2002; Gross-Goupil et al., 2003)***, and the result of meta -analysis of expression of AFP mRNA one week after surgery has also been shown to be correlated with the recurrence of HCC ***(Ding et al., 2005)***. However AFP m RNA was reported to be elevated in the peripheral blood of patients with chronic hepatitis B or cirrhosis ***(Aselman et al., 2001)***.

Hepatocellular carcinoma has shown to secret a lot of cytokines related to the development of tumour, like transforming growth factor-beta 1(TGF-β). TGF-β is a family of related proteins that regulate many cellular processes including growth, differentiation, extracellular matrix formation and immuno-suppression ***(Verrecchia & Mauviel, 2002)***. TGF-β1 is one of TGF-β isoforms (TGF-β1-5), that arrests the cell cycle in the G1 phase thereby eliciting inhibition of cell proliferation and triggering apoptosis ***(Scharf & Braulke, 2003)***.

Though a growth inhibitor, the over expression of hepatic TGF-β1 mRNA was found in HCC tissues and correlated with carcinogenesis, progression and prognosis of HCC ***(Ali et al., 2003)***. Invasive HCC cells secrete abundant active TGF-β1 in comparison to non invasive HCC cells suggesting that it plays an important role in HCC invasiveness. The sensitivity and specificity of circulating TGF β1 level were 90% and 94% for HCC diagnosis, and the combined detection of TGF-β1 and serum AFP could raise the detection rate of HCC up to 97% ***(Wang et al., 2007; Dong et al., 2008)***.So it might be used as diagnostic tool in HCC patients and may be an important target for new therapies.

Aim:

The aim is to estimate the expression of CTCs (AFP mRNA & TGF- β1 mRNA) in the peripheral blood of patients with HCC as an early non invasive marker of HCC and to detect their relevance to the prognostic parameters of HCC.

Patients and methods:

Hundred patients were selected from outpatient clinic and inpatient hepatology department of the National Liver Institute, Menoufiya University and Internal Medicine department, faculty of medicine, Cairo University in the period from May 2012 to April 2013.

The included patients were classified into 2 groups as following: **Group I** included 58 patients (38 males & 20 females) with confirmed HCC diagnosed by second generation contrast ultrasound or computed tomography scan showing a nodule larger than 2 cm with contrast uptake in the arterial phase and washout in the venous or late phases, or using two imaging techniques showing such radiological behaviour by nodules 1–2 cm in diameter with liver biopsy done once indicated ***(Bruix & Sherman, 2005)*** when possible.Their age ranged from 46 to 63 years. **Group II** included 42 patients (29 male & 13 females) with liver cirrhosis (LC) with no radiological evidence for presence of HCC. Their ages ranged from 41 to 57 years. Patients with clinically evident distant metastasis (detected by brain and chest CT scan or total-body bone scintigraphy) or simultaneous other malignant disorders were excluded from the study.

The Milan criteria was defined as the presence of a tumour 5 cm or less in diameter in patients with single HCC or no more than 3 tumour nodules, each 3 cm or less in diameter, in patients with multiple tumours, and no extrahepatic metastasis, and no major hepatic vessel invasion ***(Mazzaferro et al., 1996).***

Twenty healthy volunteers (14 males and 6 females) were included in the study as a control group, age ranged from 35 to 59 years. Patients and controls were subjected to full history taking and thorough clinical examination. Informed written consent was obtained from all participants. The study protocol was reviewed and approved by the Ethics Committee of the National Liver Institute-Menoufia University.

Laboratory investigation

Liver function tests were determined using Integra 400 (Roche Germany). Prothrombin time was measured by fibrintimer (Dade Behring Germany). AFP was assayed using chemiluminescence technique by automated chemiluminescence system, ACS 180 of Chiron diagnostics (USA). Hepatitis viral marker including anti-HCV was detected by ELISA using Innogentic N.V. (Ghent – Belgium) and HBV by ELISA using Diasorin Kit (Diasorin SR, Italy). HCV RNA was measured by RT-PCR using the automated Cobas Amplicor system of Roche (Amplicor PCR, Roche Molecular Systems, and Germany).Complete blood count (CBC) was done using Sysmex automated cell counter.

Determination of TGF-β1 in serum samples:

Serum levels of TGF-β1 were measured by a commercially available quantitative enzyme-linked immunosorbent assay (ELISA) (Quantikine Human TGF-β1; R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription:

From each patient, 5 ml of peripheral blood was collected with trisodium citrate anticoagulant. Blood samples were prepared for measurement of total RNA using a Blood RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. To facilitate their separation from the PBMCs, then PBMCs were collected and washed three times in normal saline, pelleted by low-speed centrifugation then stored at -80 ºC for total RNA preparation.

Briefly, 5 mL of peripheral blood cells were mixed with 25 mL of reagent buffer erythrocyte lysis to facilitate their separation from the PBMCs, then PBMCs. They were cooled on ice for 15 minutes and centrifuged, and the cell pellets were collected. They were suspended with 1.35 mL buffer and applied into the reagent columns. They were washed 3 times with reagent buffer containing ethanol and eluted total RNA with RNase-free water. The blood RNA extract was stored at −80°C until used for AFP mRNA and TGF-β1 mRNA analysis.

Detection of TGF-β1mRNA by nested-PCR (Dong et al., 2008):

The two sets of primers were designed according to the TGF-β1 sequence and synthesized (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, China). The sequences of the two external primer pairs used for the initial PCR amplification were TGF-β1S-1 (sense), CTG CAA GAC TAT CGA CA TGG-3' (nt937-956) and TGF-β1AS-1 (anti-sense), 5'-AGA TAACCA CTC TGG CGA GT-3' (nt 1416-1435).

The sequences of the two internal primer pairs used for the second PCR amplification were TGF-β1S-2 (sense), 5'-AAT GGT GGA AAC CCA CAA CG-3 (nt 1177-1196) and TGF-β1AS-2 (anti-sense), 5'-GTATCG CCA GGA ATT GTT GC-3' (nt 1368-1387). For synthesis of cDNA, 2 μg of total RNAs was denatured in the presence of random hexamers (100 pmol/L, Promega) and reverse-transcriptase (Gibco, BRL) at 23 ºC for 10 minutes, 42 ºC for 60 minutes, and 95 ºC for 10 minutes, then on ice for 5 minutes, and stored at -20 ºC for PCR amplification. The PCR amplification consisted of initial denaturation at 94 ºC for 5 minutes, followed by 94 ºC for 25 seconds, 55 ºC for 30 seconds, and 72 ºC for 90 seconds for 30 cycles, the final product of

nested PCR was 159bp. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as control. The primer sequence for GAPDH was GAPDH-A1 (sense), 5'-ACC ACA GTCCAT GCC ATC AC-3' (nt 601-620) and GAPDH-AS1 (antisense), 5'-TCC ACC ACC CTG TTG CTG TA-3' (nt 1033-1052), and the PCR product was 452 bp. All PCR products were electrophoresed on 2% agarose gels with ethidium bromide staining and the fragment sizes were evaluated using PCR markers as molecular weight standards (Figure 1).

Detection of AFP-mRNA by Real-Time-PCR (Wang et al., 2007):

The cDNA was generated from 1 µg of total RNA using 20 pmol/L of each dNTP and 10 pmol/L oligo dT primers, in a 20-µL final reaction volume, at 42°C for 60 minutes, followed by heating at 99°C for 5 minutes. cDNA was amplified in the 20-µL PCR reaction mixture containing each dNTP (with dUTP instead of dTTP), 1 × PCR buffer, the specific primers, and magnesium chloride. For detection of AFP, 2 adjacent oligonucleotide probes were applied: the Light Cycler Red 640 fluorophore, hAFP-LCR; (5′-CTTGCACACAAAAGCCCACTCCA-3′) and the fluorophore labeled at the 3′ end with fluorescein, hAFP-FITC; (5′-TCGATCCCACTTTTCCAAGTT-3′) (Nihon Gene Research Laboratories, Sendai, Japan). The sense and antisense primers (kindly supplied by Dr. Hiroaki Nagano at Osaka University) for amplifications of AFP were as follows:5′-TGCAGCCAAAGTGAAGAGGGAAGA-3′)(hAFP-s)and5′-CATAGCGAGCAGCCCAAAGAAGAA-3′ (hAFP-As). RT-PCR amplification was carried out for one cycle of 95°C for 10 minutes, followed by 5 cycles of 95°C for 10 seconds, 62°C for 15 seconds, and 72°C for 15 seconds. After the final cycle, we used a 10 minutes extension period at 40°C. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, 2 adjacent oligonucleotide probes were hGAPDH-LCR; 5′-TTCCGTGTCCCCACTGCCAA-3′ and hGAPDH-FITC;5-GGAAGCTCACTGGCATGGC-3′. The sense and antisense primers for amplifications of GAPDH were as follows: 5′-GCCTCCTGCACCACCAACTG-3′(hGAPDH-S) and 5′-CGACGCCTGCTTCACCACCTTCT-3′ (hGAPDH-As). RT-PCR amplification was carried out for one cycle of 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 16 seconds. After the final cycle, we used a 10-minute extension period at 40°C. The result data were analyzed using the LighCycler analysis software (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer's description. The crossing point for the calculation of amplified PCR product was set the intersection of the best-fit line through the log-linear lesion and the noise band. The standard curve was a plot of the "crossing point" versus the copy number of DNA fragments constructed into the cloning vector.

Statistical analysis:

Data were statistically analyzed using SPSS computer program version 12.0. The mean ± SD were used to describe data. Mann Whitney test was used to compare non parametric data between two patient groups and ANOVA test to compare parametric data between more than two patient groups. The (X^2) test was used to compare the percentage means. The correlation studies were done employing the Pearson's correlation (r). $P>0.05$ is statistically non significant, $p<0.05$ and $p<0.01$ is statistically significant.

Results:

The demographic, associated risk factors and the patients' characteristics:

They were demonstrated in table (1). No statistically significant difference was detected between the studied groups as regard gender, age and smoking. Apart from a statistically significant difference in the non viral aetiology between HCC and LC groups ($p<0.05$), there were no statistically significant difference between them as regard other associated risk factors and Child-Pugh score. Child A class represents 6 (10.3%) patients in HCC group and 7 (16.7%) patients in cirrhosis group. While child B class represent 17 (29.3%) patients in HCC group and 12 (28.5%) patients in cirrhosis group. The majority of patients classified as Child C score as 35(60.4%) patients in HCC group and 23(54.8%) patients in cirrhosis group. As regard tumour characteristics 27(46.6%) patients were within Milan Criteria while 31(53.4%) patients were beyond the criteria. Tumour size was <3 cm in 20(34.5%) patients, while 38 (65.5%) patients had tumour size >3 cm. Also, 25(43.1%) patients had single tumour while multiple tumour was demonstrated in 33(56.9%) patients. Portal vein invasion was found in 27(46.6%) patients.

On the basis of AFP levels, among HCC group, 8(13.8%) patients have AFP <20 ng/ml, 15(25.9%) with AFP (20-100 ng/ml), and 35(60.3%), while in LC group, 19(45.2%) patients have AFP <20 ng/ml, 16(38.1%) with AFP (20-100 ng/ml), and 7(16.7%). As regard the hepatic profile statistical significant difference has been observed between HCC and LC patients namely ALP, GGT, the serum level of albumin and prothrombin concentration P value <0.05, <0.01, <0.05 and <0.05 respectively as shown in table (2). ***Representative RT-PCR product*** of TGF-β1 RNA of patients with HCC is shown in figure (1).

Table (1): Demographic data, associated risk-factors and patient characteristics in studied patients.

Parameters	HCC Group (N =58)	LC Group (N =42)
Gender (male%)	38(65.5%)	29(69%)
Age (X±SD)	54.73±8.21	48.51±7.42
Risk factors:		
Smoking	12 (20.7%)	10 (23.8%)
HCV only	31 (53.5%)	23 (54.8%)
HBV only	7 (12.1%)	3 (7.1%)
HCV & HBV	6 (10.3%)	4 (9.5%)
HCV & Bilharziasis	10 (17.2%)	5 (11.9%)
Non-viral aetiology	4 (6.9%)	7 (16.7%)
Serum AFP (ng/ml)		
<20	8 (13.8%)	19 (45.2%)
20-100	15 (25.9%)	16 (38.1%)
>100	35 (60.3%)	7 (16.7%)
Child-Pugh score:		
A	6 (10.3%)	7 (16.7%)
B	17 (29.3%)	12 (28.5%)
C	35 (60.4%)	23 (54.8%)
Milan Criteria:		
Within	27 (46.6%)	----------
Beyond	31 (53.4%)	
Tumor size:		
< 3cm	20 (34.5%)	----------
≥ 3cm	38 (65.5%)	
Tumor number:		
Single	25 (43.1%)	----------
Multiple	33 (56.9%)	
Portal vein invasion:		
Yes	27 (46.6%)	----------
No	31 (53.4%)	

Table (2): Comparison of laboratory parameters among studied groups.

Parameters	HCC group (no=58)	LC group (no=42)	P-value
ALT (U/L)	67.2±19.3	59.4±20.8	>0.05
AST (U/L)	58.7±14.1	51.4±12.3	>0.05
ALP (U/L)	78.3±21.3	46.9±16.7	<0.05*
GGT (U/L)	87.1±14.2	42.7±10.6	<0.01*
T. bilirubin (mg/dL)	4.31±2.16	3.92±1.24	>0.05
Albumin (g/dL)	2.84±0.97	3.11±0.46	<0.05*
Prothrombin Conc. (%)	68.4±13.9	59.5±11.2	<0.05*
Platelets ($x10^3$/ul)	132.6±53.2	124.3±44.7	>0.05
TLC ($x10^3$/ul)	7.4±3.65	5.2±1.85	>0.05

***P*<0.05 is statistically significant, *p*>0.05 is statistically not significant.**

***The comparative analysis of circulating AFP mRNA, TGF- β1 mRNA and the serum level of AFP and TGF-β1 in the studied group is shown in* Table 3 & Figures 2&).**

The detection rate of AFP mRNA was 39.7%, 11.9% and5% in patients with HCC,LC and control subjects respectively with a significant expression in HCC patients compared to both controls (p<0.001) and LC patients (p<0.05).Also The detection rate of TGF-β1 mRNA was 60.3%, 14.3% in HCC and LC respectively with significant expression in the former group compared to the later (p <0.01) while no detected level was seen in the controls. The serum levels of AFP and TGF-β1 were

significantly higher in HCC patients compared with other groups as shown in table (3).

Regarding to the correlation between the presence of CTC and risk and prognostic markers of HCC

The presence of circulating AFP mRNA was correlated with Milan criteria, tumour size, portal vein invasion, and not correlated with age, sex, Child score or tumour number. The same pattern of correlation was observed with circulating TGF-β1 mRNA. In addition we also observed that TGF-β1 mRNA was correlated with the serum level of TGF-β1 however no significant correlation was observed between AFP mRNA and the serum level of AFP (Table 4).

Table (3): Comparison of AFP & TGF-β1 among studied groups.

Parameters	HCC group (N=58)	LC group (N=42)	Controls (N=20)	p-value
Serum AFP (ng/ml)				
Range	8.3 -9726	10.37-152	2.65-8.13	P^1<0.001; P^2<0.05; P^3<0.001
M±SD	4390±4298	62.1±49.6	5.2±2.3	
AFP mRNA (N %)	23 (39.7%)	5 (11.9%)	1 (5%)	P^1<0.001; P^2<0.05; P^3 < 0.05
Serum TGF-β1 (pg/ml)				
Range	103.2-146.7	42.6-76.2	37.2- 45.8	P^1<0.01; P^2>0.05; P^3 <0.01
M±SD	127.1 ± 18.2	59.4 ± 16.3	41.5 ± 4.9	
TGF-β1 mRNA (N %)	35 (60.3%)	6 (14.3%)	0 (-)	P^3<0.01

***P^1*: HCC vs. control, *P^2*: LC vs. control, *P^3*: HCC vs. LC (p<0.05 is statistically significant)**

Table (4): Correlation between AFP mRNA, TGF-β1 and prognostic parameters of HCC (N=58)

Parameters	AFP mRNA		TGF-β1 mRNA	
	r	p	r	p
Age	0.25	>0.05	0.15	>0.05
Sex	0.19	>0.05	0.22	>0.05
AFP serum level	0.32	>0.05	0.20	>0.05
TGF-β1 serum level	0.14	>0.05	0.61	<0.05*
Child score	0.26	>0.05	0.14	>0.05
Milan criteria	0.64	<0.01*	0.45	< 0.05*
Tumour size	0.57	<0.05*	0.42	< 0.05*
Tumour number	0.11	>0.05	0.26	> 0.05
Portal vein thrombosis	0.72	<0.001**	0.54	<0.05*

***P*<0.05 & **p< 0.01 is statistically significant, *p*>0.05 is statistically not significant.**

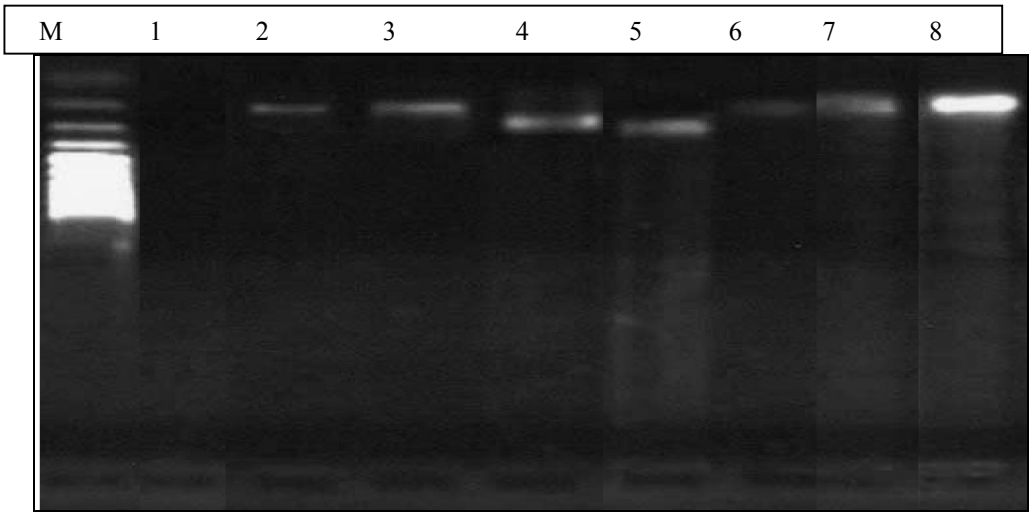

Figure (1) shows PCR product of TGF-β1 m RNA, lane M = molecular marker, 100 bp DNA ladder; lanes 1, 2, 6: negative cases, lanes 3,4, 5, 7: positive cases, lane 8: positive control.

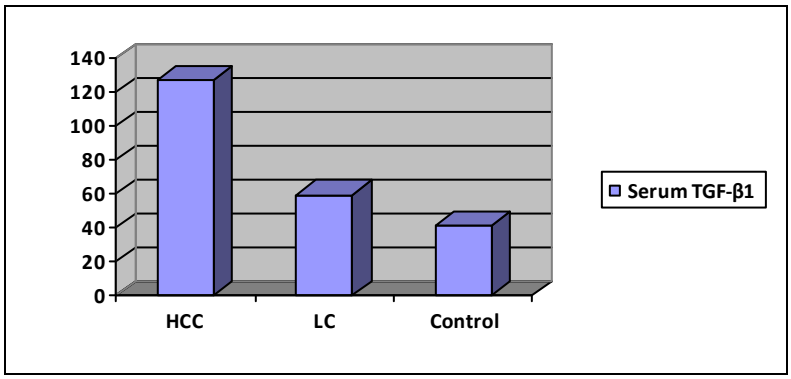

Figure (2) Shows the serum levels of TGF-β1 in studied groups

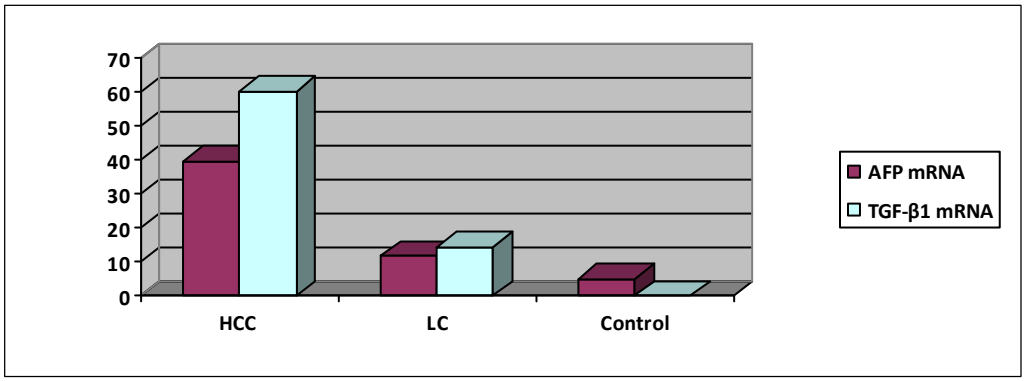

Figure (3) Shows the percentage positive cases of AFP mRNA & TGF-β1 mRNA in studied groups

Discussion:

Hepatocarcinogenesis is a complex multistate process usually occurring after many years of chronic exposure to several mitogenic and mutagenic environments precipitating random genetic alterations. HCC ranks high among the most common and fatal cancers in world and it develops from chronic liver diseases specially hepatitis C –virus and hepatitis B virus related disease and is responsible for significant morbidity and mortality ***(Hussain & El-Serag, 2009).*** The early diagnosis of HCC helps in the patients' prognosis and selection of appropriate treatments. Presence and frequency of circulating tumour cells (CTCs) in bloodstream of patients with epithelial cancers (carcinomas) is an important intermediate step in cancer metastasis and provides valuable insights associated with disease stage and treatment evaluations ***(Hayes etal., 2006)***. More importantly, because primary tumour sampling may not reflect the actual metastatic conditions, CTCs also serve as a representative surrogate tumour biomarker for real-time monitoring of disease status and tailoring personalized therapy ***(Punnoose et al., 2010)***. Because haematogenous spread is the major route of HCC recurrence, detection of circulating tumour cells (CTCs) undoubtedly has important clinical significance in predicting recurrence and monitoring therapeutic efficiency in HCC patients ***(Xu et al., 2011)***. Many researches had been focused on the improvement in the detecting ways and equipment ***(Allard et al., 2004; Guo et al., 2004)*** and the screening of high risk HCC patients ***(Chalasani et al., 1999; Gao et al., 2004).*** In our study we estimated the expression of CTCs in terms of AFP mRNA & TGF- β1 mRNA using RT-PCR technique as well as AFP and TGF- β1 in the peripheral blood of patients with HCC and we compared the results with those of liver cirrhosis. We also analysed the relationship between such CTCs and the different prognostic parameters of HCC.

In this study, we observed that in spite of good radiological selection for patients with HCC 39.7% of them have serum level of AFP less than 100 ng/ml as there were 13.8% (8 patients) with AFP <20 ng/ml and 25.9% (15 patients) with AFP ranges between 20-100 ng/ml. However only 60.3% (35 patients) with HCC have serum level of AFP >100 ng/ml, which means that sensitivity of AFP in diagnosis of HCC only 60.3% which coincide with recent studies showed that AFP determination lacks adequate sensitivity and specificity for effective surveillance and for diagnosis ***(Singal et al., 2009; Lok et al., 2010)***. On the other hand AFP can be elevated in patients with chronic liver disease with high degree

of hepatocytes degeneration in absence of malignancy ***(Toyoda et al., 2004)*** as seen in our results which showed that in patients with LC, AFP serum level were <20 ng/ml in 45.2% (19 patients), those with level ranging between 20- 100 ng/ml were 38.1% (16 patients) while 16.7% (7 patients) had AFP level more than 100 ng/ml, for these reasons some serological markers used in combination with AFP to improve the performance especially in term of sensitivity are needed. We observed that the positive rate of AFP m RNA in patients with HCC was 39.7% (23/58), in LC was 11.9% (5/42) and in the control was 5% (1/20) which demonstrates the significant higher expression rate in HCC patients compared to LC group and controls, p value is <0.05 and <0.001 respectively.

Variable results have been demonstrated in the literature. ***Ijichi et al. (2002)*** demonstrated preoperative positivity rate (36%) of AFP mRNA in their HCC patient. ***Lemoine et al. (1997)*** found AFP mRNA was detected in 11 (17%) of the 64 HCC patients while ***Montaser et al. (2007)*** mentioned the positive rate in 8 (25%) out of 32 and non of their control tested positive however ***Witzigman et al. (2002)*** reported 28% of the patients with HCC and 3% of the control patients were tested positive for AFP mRNA. Higher level of expression in HCC patients was reported by ***Yan et al. (2011)*** where the positive expression rates of AFP mRNA in the peripheral blood samples of HCC, hepatitis B, and cirrhosis patients were 56%, 5%, and 10%, respectively with non detected rate in their controls. The reason for discrepancy among various studies may be multifactorial. The limited number of tested patients, the differences in patients characteristics, the feasibility RT-PCR technique in addition to Sampling error may be reasons for conflicting results ***(Yao et al., 2005).***

The relationship between serum AFP and AFP mRNA was conflicting; in the present study we did not find any significant correlation between AFP mRNA and serum levels of AFP in HCC patients. Similarly studies from other investigators ***(Lemoine et al., 1997; Witzigmann et al., 2002; Cillo et al., 2005; Yao et al., 2005; Montaser et al., 2007)*** showed no correlation was found between these two markers. Contradictory to our results other researches had been able to identify a significant association between serum AFP and AFP mRNA ***(Komeda, 1995; Louha et al., 1997; Matsumura et al., 1994)***. The detection of AFP mRNA in peripheral blood represents the circulating tumour cells but it does not reflect the whole expression of APF gene ***(Lazarevich, 2000)***, therefore, the secreted AFP protein level does not necessarily agree with mRNA results and seems useful in this meaning.

In the current study we excluded patients with metastasis liver cancer or associated tumours because it has been reported that non hepatic circulating malignant cells derived from epithelial cancer such as colon and pancreatic cancer could express AFP mRNA as a consequence of a loss of cellular differentiation in the bloodstream ***(Cillo et al., 2005)***. Also The relevance of AFP mRNA as a marker of circulating tumor cells is controversial because these cells have not been further characterized, and it has been shown that they may correspond to normal circulating hepatocyte ***(Witzigman et al., 2002; Marubishi et al., 2006)*** as shown in our study where 5 % of the controls had AFP mRNA in their peripheral blood and supported by others,so we assessed the circulating level of serum TGF-β1 and TGF- β1 mRNA assuming it is a better indicator for HCC.

In this study we found that the serum level of TGF- β1 was significantly higher in HCC group than LC and controls, the mean ±SD was 127.1±18.2, 59.4±16.3 and 41.5±4.9 with p value <0.01 respectively while no significant difference was detected in its level between LC group and controls. The study of ***Giannellie et al. (2002)*** support our results as they found higher levels of TGF-β1 serum concentrations and stronger expression of TGF- β1 in HCC tissues.

Regarding TGF-β1 mRNA the comparative analysis of its detection rate yield that it was positive in 35 patients of HCC with expression rate 60.3% which was significantly high compared to LC patients where it was detected in 6 patients with detection rate 14.3% ($p<0.01$),while it was not detected in the controls. This means that the amplification of TGF-β1 mRNA by means of PCR is a sensitive method for detection of HCC in peripheral blood. We also found a significant correlation between the TGF-β1 m RNA and the serum level of TGF-β1 ($r = 0.61$; $p < 0.05$)

Our results are an accordance with those obtained with ***Dong et al. (2008)*** whose found that the levels of circulating TGF-β1 and TGF-β1 m RNA were significantly higher in the HCC patients than any other group of patients and they concluded that the sensitivity and specificity of circulating TGF-β1 level were 90% and 94% for HCC diagnosis and recommend that combined detection of TGF- β1 and serum AFP could raise the detection rate of HCC up to 97%. Also ***Giannellie and his co-workers (2002)*** found higher levels of TGF- β1 serum concentrations and stronger expression of TGF- β1 in HCC tissues.In this study we analysed the relationship between the studied CTCs and the different prognostic parameters of HCC and we found that the disease extent as classified by the Milan criteria.,the

tumour size and portal vein thrombosis were positively correlated with circulating AFP mRNA (r= 0.64, p<0.01; r= 0.57, p<0.05 & r= 0.72, p<0.001 respectively). While no significant correlation had been observed with age, sex, Child score, tumour number, AFP or TGF-β1 serum level. Also the presence of circulating TGF-β1 mRNA was correlated with Milan criteria, tumour size, portal vein thrombosis (r= 0.45, p<0.05; r= 0.42, p<0.05; r= 0.54, p<0.05 respectively). It was also correlated with the serum level of TGF-β1.

As with our results regarding AFP mRNA, other previous studies reported a significant correlation between the frequency of positive AFP mRNA in blood and severity of liver involvement by HCC indicated by tumor diameter (***Liu Y et al., 2002; Yang et al., 2005)*** or tumor size **(*Cillo et al., 2005).** However, ***Lemoine et al. (1997) and Wu et al. (2006)*** showed that, the presence of AFP mRNA in blood was not related to the severity of HCC, indicated by tumor diameter. ***Ping et al. (1999)*** experiments approved the AFP gene expression in tissue samples from patients with HCC had no relationship with tumor mass size, capsule status and differentiation.

Our results regarding TGF-β1 mRNA are supported by ***Xuz et al. (2011) and Okumoto et al. (2004)*** whose found a close relationship between high expression of CTC and development, metastasis,and progression of HCC while ***Dong et al. (2008)*** reported no significant correlation between TGF- β1 expression and size and number of tumours. Also in agreement with our results ***Yao et al. (2006)*** found no significant correlation between TGF-β1 expression and AFP levels however in contradictory to our results they mentioned no significant correlation was found between TGF-β1 and the tumour size.

In addition it was previously thought that portal vein tumour thrombus would mainly cause intrahepatic metastases ***(Toyosaka et al., 1996)***. In our study, the significant correlation of the CTCs with the presence of portal vein thrombosis suggests that portal vein tumour thrombus may also be a source of systemic spread of CTCs ***(Xu et al., 2011).***

In conclusion:

Since HCC is among the cancers with worst prognosis, early diagnosis and treatment are essential for effective treatment. CTCs are an interesting source of biological information for tumour activity seeing that obtaining fresh tissue biopsy which is often technically challenging and causes great inconvenience to patients, ‘‘liquid biopsy’’ for CTCs detection can be carried out routinely in patients due to accessibility and ease of blood collection. AFP mRNA and TGF-β1 mRNA could be detected in the blood of HCC patients without clinical evidence of extra hepatic metastasis. TGF-β1 mRNA is a more reliable marker for diagnosis of HCC and if combined with AFP mRNA yielded better prediction of HCC prognosis and it might be interesting to assess the disease consequences of including more than one CTC markers in the scoring system to select HCC patients for liver transplantation and their follow up.

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