Journal of the Egyptian Nat. Cancer Inst., Vol. 19, No. 1, March: 48-60, 2007

Circulating AFP mRNA as a Possible Indicator of Hematogenous Spread of HCC Cells: A Possible Association with HBV Infection

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ABSTRACT

Background: Circulating alpha fetoprotein messenger RNA (AFP mRNA) has been proposed as a marker of hepatocellular carcinoma (HCC) cells disseminated into the circulation. The specificity of this molecular marker and its correlation with the main HCC clinico-pathological parameters remains controversial.

Aim: This case control pilot study has been undertaken to detect the expression of human AFP mRNA in the peripheral blood of patients with HCC and liver cirrhosis (LC), to evaluate its clinical implication and to clarify its relationship with some clinico-pathologic characteristics in HCC.

Methods: Peripheral blood (PB) samples were obtained from 32 patients with HCC (14 patients before treatment and 18 patients after treatment), 15 patients with LC and 10 normal donors. HCC patients with clinically evident extra-hepatic metastasis were excluded. AFP mRNA was amplified from the total RNA extracted from the whole blood by nested reverse transcription-polymerase chain reaction (RT-PCR).

Results: Circulating AFP mRNA was positive in 25% (8/32) of all HCC patients, 42.9% (6/14) of patients before treatment, 11.1% (2/18) of patients previously treated, and 13.3% (2/15) of patients with liver cirrhosis without HCC. Circulating AFP mRNA detection rate was significantly higher in untreated than in treated HCC patients (p=0.04). There was no significant difference among the other various patient groups (LC Vs HCC all: p=0.31; LC Vs untreated HCC; p=0.11; LC Vs treated HCC; p=1.0). Both patients who tested positive for circulating AFP mRNA in the treated HCC group were in the chemoembolization subgroup (2/8=25%). None (0/5=0%) of post resection as well as radiofrequency subgroups of patients tested positive for circulating AFP mRNA. None of the normal controls tested positive for circulating AFP mRNA. The only variable associated with the presence of circulating AFP mRNA in HCC patients was being hepatitis B positive: 87.5% (7/8) of AFP mRNA positive HCC patients had had clinical evidence of chronic hepatitis B virus (HBV) infection (p=0.01, OR=14, 95% CI=1.46-134.25). None of the other variables (age, sex, HCV infection, Child-Pugh score, liver function indices, serum AFP values and tumor size) was significantly related to the presence of AFP mRNA in HCC patients. No patient with chronic liver disease (CLD) due to HBV infection tested positive for circulating AFP mRNA.

Conclusion: Using nested RT-PCR assay, circulating AFP mRNA could be detected in HCC patients without clinical evidence of extra-hepatic metastasis as well as in patients with LC. Although the possibility of hematogenous spread of HCC cells cannot be excluded, AFP mRNA expression in blood does not necessarily distinguish between circulating HCC cells and noncancerous hepatocytes. Although this is a small sample size pilot study, our findings imply that HBV infection may be an important contributing factor to the hematogenous spread of HCC cells. HBV infection was strongly associated with AFP mRNA expression in blood of HCC patients. Thorough investigation of this association in a larger series with HBV infection is needed. Also, another larger treated HCC series is needed for the assessment of AFP mRNA expression in blood in relation to specified treatment approaches.

Key Words: *AFP mRNA – Hepatocellular carcinoma (HCC) – RT-PCR – Chronic liver disease (CLD).*

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy affecting approximately one million of people around the world every year. It represents the fifth most common cancer worldwide with an incidence equal to the death

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rate [1-3]. The incidence of HCC is low in the occidental world and high in Southeast Asia and sub-Saharan Africa. However, it has been rising during the last two decades in Europe, United States and Japan [3]. HCC primarily affects old people, reaching its highest prevalence among those aged 65 to 69 years old. Chronic infection by HBV is the most common cause of this neoplasm. Other important causes are cirrhosis, chronic viral hepatitis (hepatitis C virus, and hepatitis B plus D viruses), alcohol abuse, obesity, hemochromatosis, α-1 antitrypsin deficiency, and toxins similar to aflatoxin [1,3]. The major clinical risk factor for the development of HCC is liver cirrhosis (LC) since 70-90% of HCCs develop into cirrhotic liver [4].

The early diagnosis of HCC helps in improving the patients' prognosis and in the selection of appropriate treatment protocols. Treatment decisions and prognostic predictions in patients with HCC are commonly based on macroscopic tumor characteristics, detected by imaging studies (tumor size, number, lobar distribution, vascular invasion and extra-hepatic metastasis), and by the function quality of the non malignant liver. Although the diagnosis and treatment methods of HCC have been developing over the last 20 years, the recurrence and mortality of HCC is still high. One reason is that patients with HCC in the early state are asympatomatic, and most of them present in the late-stage, advanced, or micrometastasis state [5].

As in other solid cancers, the hematogenous spread of HCC cells has been suggested as an important mechanism in HCC progression, both inside and outside the liver. According to this theory, the detection of HCC cells in the blood may be a good indicator of greater tumor invasiveness [6]. There have been correlative studies exploring the prognostic factors of HCC recurrence after curative resection. Among those clinico-pathological factors, characteristics representing pathological invasiveness are the most consistent indicator of long-term prognosis. Detection of circulating HCC cells might be a real feature representing cancer invasiveness [7].

Recurrent disease may develop from isolated tumor cells (ITCs), disseminated in regional lymph nodes, in the general circulation, or both. With regard to terminology, ITCs are different from micrometastasis [8]. ITCs are singly tumor cells, or small clusters, whereas a micrometastasis occurs when there has been sequestration and implantation of tumor cells in the organ involved. Thus micrometastasis can be diagnosed only with histologic examination.

The occurrence of tumor metastasis depends on the balance between selected properties of tumor cells and the reactivity of the host [9]. Whether micrometastasis occurs is determined by the ability of tumor emboli to survive in the circulation, attach firmly to the endothelium of distant organ capillaries, gain entrance to extravascular tissues, evade host defense mechanisms, and then grow into tumor colonies. Thus, a moderate number of circulating tumor cells is needed to form foci of micrometastasis [8]. Liotta and Stetler-Stevenson [10] reported that less than 0.01% of circulating tumor cells successfully establish metastatic colonies.

One of the difficulties in the management of HCC is its extensive characteristics including intrahepatic metastasis, venous invasion, and distant metastasis. Some patients with HCC who received curative resection or chemoembolization develop metastatic nodules shortly after these therapies. Furthermore orthotropic liver transplantation (OLT) for unresectable HCC yields disappointing results because of a high recurrence rate [11]. In these events, it is assumed that HCC cells have been disseminated in the systemic circulation and proliferated in an immunosuppressive environment during and after therapy. To improve the prognosis of HCC, it is important to detect such blood-borne HCC cells before therapy is carried out.

The International Union Against Cancer (UICC) recommends that finding of ITCs should be documented according to uniform criteria. Morphologic methods (in particular, immunocytochemistry) or non-morphologic methods (such as flowcytometry or the PCR) are available for detection of ITCs. These methods show considerable variation in their results. Immunocytochemistry is superior to conventional histology in being more sensitive in detecting ITCs, and its false-positive rate is lower than that of PCR [8]. The most sensitive technique is the identification of "tumor-specific" gene transcripts (m-RNA/circular DNA) from tumor

cells by means of PCR. The major problem is finding an adequate specific marker, which should obviously not be expressed in normal peripheral blood cells. AFP mRNA and albumin mRNA have been examined as surrogate markers for circulating HCC tumor cells. False-positive results may originate from illegitimate transcription, in nucleated blood cells, as shown for the albumin gene, or from pseudogenes, as shown for cytokeratin 18 and 19, or may simply be caused by contamination due to the high sensitivity of the method [12-14].

Reverse-transcriptase polymerase chain reaction (RT-PCR) is a sensitive and versatile method to amplify cDNA generated by reverse transcriptase from mRNA. It is very helpful in the qualification or quantification of the very small amount of RNA. However, RNA is less robust than DNA. After about 30 cycles of amplification, the yield visualized might not be clear and specific enough. Nested RT-PCR is developed to perform with one set of primers (the first round) and then with or without removal of reagents, to reamplify with an internally situated "nested" set of primers (the second round). This process ensures that all products nonspecifically amplified in the first round will not be amplified in the second round, producing a yield that is specific and enhanced. The increased sensitivity and specificity of nested RT-PCR were readily apparent in patients with minimal residual disease [15]. Comparison of the sensitivities of RT-PCR and nested RT-PCR revealed that the nested RT-PCR provides a 100-fold sensitivity [16]. Free mRNA is so fragile under conditions of abundant RNAase activity that the specific mRNA in blood will indicate the presence of intact cells providing such proteins just before the extraction of RNA, not circulating free mRNA [17].

AFP mRNA is generally accepted as a liver-specific and tumor-specific marker [18]. Many clinical studies have suggested that human AFP mRNA in peripheral blood can be used as a surrogate marker of circulating HCC cells and as a prognostic indicator in patients treated with ethanol injection and/or arterial embolization [19]. However, the possibility of a few AFP-producing virus-infected hepatocytes or degenerating hepatocytes cannot be excluded in the AFP mRNA positive results [20].

The current study has been designed: 1- To investigate the expression of AFP mRNA in the peripheral blood of patients with HCC and/or chronic liver disease (CLD) and its possible role as an indicator of hematogenous spread of HCC, and to 2- To clarify its correlation with some clinico-pathologic characteristics of HCC.

SUBJECTS AND METHODS

Study population:

Forty-seven patients with HCC and/or CLD were included in the study. All patients were selected from the HCC clinic of the National Liver Institute, Menofeya University. 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. In addition, 10 apparently healthy individuals of comparable age and sex were included as a control group. AFP serum levels were measured in all patients. Modified Child's-Pugh score and the basic liver function tests (ALT, AST, ALP, GGT, total and direct bilirubin, total proteins and serum albumin) were used to assess liver function. All participants were divided into 4 groups: Group I were 10 healthy volunteers with normal liver function tests and no history or evidence of liver disease. Group II were non-tumor 15 patients with CLD. Group III were 14 untreated HCC patients; all blood samples were collected before any treatments. Group IV were 18 HCC patients after treatment (8 patients after treatment by chemoembolization, 5 patients after treatment by radiofrequency and 5 postresection patients). All patients included in the study were subjected to full history taking, thorough clinical examination and abdominal ultrasonography. The HCC diagnosis was made by abdominal ultrasonography, and Doppler study.

Serum determinations:

Liver function tests (LFTs) were determined (Integra Autoanalyser; Germany); then serum samples were stored at –20ºC until HBsAg (enzyme immunoassay, DiaSorin; Italy), HCV Ab (third generation ELISA procedure, DIA, PRO; Italy) and AFP (ACS: 180 SE Automated chemiluminescence System; Bayer, USA) were assayed.

RNA extraction and nested RT-PCR detection of AFP mRNA RNA extraction and RT-PCR:

Total RNA was isolated from fresh whole blood EDTA-treated samples. Reagents were provided by Purescript RNA Isolation Kit, Gentra; US. The RBCs were first lysed to facilitate their separation from the WBCs, then the RNA was extracted from the WBCs, AFP mRNA was detected by using Ready-to-go™ RT-PCR beads Kit, Amersham Bioscience Limited; GE Healthcare, UK. Each bead was optimized to allow the first strand cDNA synthesis and PCR reactions to proceed sequentially in a single-step reaction. Simply, RNA, first strand primer, and PCR primers were added to the RT-PCR bead reaction, incubated at 42ºC and cycled. When brought to a final volume of 50µl, each RT-PCR bead reaction contains ~2.0 units of Taq DNA polymerase, 10m M Tris HCl buffer, 60m M KCl, 1.5m M $MgCl_2$, 200µM of each dNTP, Moloney Murine Leukemia virus (M Mu LV) reverse transcriptase. The following primer sequences were used: For the first PCR: FPA1 primer: 5'-ATTCAGACTGCTGCAGCCAA-3' and FPA2 primer; 5'-GTGCTCATGT ACATGGGCCA-3'. For the nested PCR: FPI1 primer; 5'-GTTCCAGAACCTGTCACAAG-3' and FPI3 primer: 5'-CTTTGTTTGGAA GCATTCAACTGC-3' [21]. β-actin was amplified as an internal control in order to verify the integrity of the extracted RNA using the following primers; β-ACT1 primer; 6'-CTATTGGC AACGAGCGGTTC-3' and β-ACT2 primer; 5'-CTTAGGAGTGGGGGTGGCTT-3'. In the first RT-PCR reaction 10µL of extracted total RNA (template RNA) were reverse transcribed and amplified in a final volume of 50µL containing 1µL first strand primer, 1µL FPA1 primer, 1µL FPA2 primer and 37µL DEPC-treated water. AFP and β-actin reverse transcription and amplification of RNA were allowed by the following thermocycler program: 1- Incubation of the reaction mixture for one hour at 42ºC. 2- Initial denaturation at 94ºC for 5 minutes. 3- 35 PCR cycles of denaturation at 94ºC for 10 seconds, annealing at 58ºC for 10 seconds and extension at 72ºC for 30 seconds. 4- Final extension at 72ºC for 7 minutes.

Nested RT-PCR and detection of AFP mRNA:

A total of 10µL of the first RT-PCR product was transferred into a second tube; and it was amplified in a final volume of 50µL containing 25µL DyNAzyme™ II PCR Master Mix (FINNZYME OY; Finland), 1µL FPI 1 primer, 1µL FPI 3 primer, 13µL DEPC-treated water. The reaction was transferred to the thermal cycler. After an initial denaturation at 94ºC for 5 minutes, 35 amplification rounds were performed by denaturation at 94ºC for 10 seconds, annealing at 58ºC for 10 seconds and extension at 72ºC for 30 seconds followed by final extension at 72ºC for 7 minutes. The final product was detected on 2% agarose gel stained with ethidium bromide and visualized under a UV light source. The samples were considered positive when a sharp clear, distinct band was observed at the specific molecular weight 193bp for AFP mRNA (nested PCR) and 795bp for β-actin (RT-PCR).

Statistical analysis:

The results were analyzed using the Statistical Package of Social Sciences (SPSS) computer software program, version 10.0 (Chicago, IL, USA). Quantitative data were presented as mean ± SD for normally distributed data and as medians and percentiles for skewed data. Qualitative data were presented in the form of frequencies and percentages. For normally distributed parameters, differences among groups were tested by Student's *t* test and the one-way analysis of variance (ANOVA), while for skewed data Mann-Whitney rank sum test and Kruskal-Wills analysis of variance were used. For Qualitative data, differences among groups were tested using the Pearson's chi-square test (X^2) and the Fisher's Exact test. Odds Ratio (OR) and 95% confidence interval (CI) were calculated as a measure of association and statistical significance, respectively. All tests were two tailed and considered statistically significant at $p \leq 0.05$.

RESULTS

Demographic information of enrolled groups is presented in Table (1), together with AFP serum levels and the RT-PCR detection results for AFP mRNA (Fig. 1). AFP mRNA was detected in blood of 8/32 (25%) HCC patients, 2/18 (11.1%) and 6/14 (42.9%) of treated and non-treated patients, respectively; and in 2/15 (13.3%) in patients with CLD; none of the controls tested positive for AFP mRNA (Fig. 2). Apart from a statistically significant different between treated and untreated HCC patients

(Fisher's Exact p=0.04), there were no statistically significant differences among the various study groups (CLD Vs all HCC patients p=0.307, CLD Vs treated HCC patients p=1.0, and CLD Vs untreated HCC patients p=0.109). In the treated HCC group, only 2 out of 8 patients (25%) in the chemoemolization subgroup tested positive for circulating AFP mRNA, meanwhile 0 out of 5 (0%) post resection patients and 0 out of 5 patients (0%) in the radiofrequency subgroup tested positive for circulating AFP mRNA (Table 2). As for tumor characteristics, the presence of circulating AFP mRNA was not significantly associated with the tumor size (indicated by the longest diameter of the tumor mass) in the untreated HCC group (t=2.82, p=0.098). Although serum AFP levels were significantly different among the various groups studied (Kruskal-Wallis=12.6; p=0.006), there was no statistically significant difference between patients with CLD and untreated HCC patients (Mann-Whitney-test; p=0.093).

Association of AFP mRNA with the HCC patients' clinical characteristics:

The HCC patients were redistributed into 2 groups: The 8 patients with suspected cell dissemination in the blood constituted the positive group, while the 24 patients in whom AFP mRNA was not detected constituted the negative group. As shown in Table (3), there were no statistically significant differences between the two groups in terms of age, sex, HCV infection, severity of cirrhosis, liver function tests or serum AFP levels; only HBV infection was strongly significantly associated with AFP mRNA positivity in blood (X^2=7.069, p=0.01, OR=14, 95% CI=1.46-134.25; Fig. 3). It is worth mentioning that group II (non-tumor CLD patients) included 5 patients with CLD due to HBV infection, all of whom were circulating AFP mRNA negative. Meanwhile, both patients who were AFP mRNA positive were HBsAg negative.

Table (1): Demographic information of enrolled groups together with serum AFP levels and PCR detection results.

	Controls n=10	CLD n=15	Untreated HCC n=14	Treated HCC n=18
Gender:				
M/F	4/6	11/4	9/5	11/7
Percentage (%)	40/60	73.3/26.7	64.3/35.7	61.1/38.9
Age (years):				
Range	39/53	22/76	42/72	42-69
Mean ± SD	53.3±4.92	59.1±14.1	57.6±8.6	55.1±7.37
Liver cirrhosis	0	15	14	18
Positive HBsAg	0	5	6	9
Positive HCV Ab	0	8	12	10
Child-Pugh score:				
A: No. (%)		1 (6.7)	4 (28.6)	2 (11.1)
B: No. (%)		3 (20.0)	3 (21.4)	9 (50.0)
C: No. (%)		11 (73.3)	7 (50.0)	7 (38.9)
Serum AFP (ng/ml):				
Range	4-30	12.5-141	4.4-4950	7.55-25650
Mean ± SD	27.5±17.1	51.9±42.6	2148±2737	3277±6525
Median	10.5	60.8	3280	6000
Detected PCR AFP mRNA:				
No.	0	2	6	2
(%)	0	13.3	42.9	11.1

Table (2): Frequency of positive circulating AFP mRNA in treated HCC patients.

	Resection n=5		Radiofrequency n=5		Chemoembolization n=8	
	No.	%	No.	%	No.	%
+ve AFP mRNA	0	0.0	0	0.0	2	25

Table (3): Characteristics of the HCC group, and comparison between HCC patients with (positive group) and without (negative group) AFP mRNA in blood.

	HCC patients (n=32)		AFP mRNA +ve HCC group (n=8)		AFP mRNA –ve HCC group (n=24)		*p*-value
Age (years)	56.2±7.8		54.3±9.3		56.6±7.52		0.589
Gender (M/F)	20/12		6/2		14/10		0.344
Cirrhosis	32	100	8	100%	24	100%	
HCV	22	68.8	6	75%	16	66.7%	1.000
HBV*	15	46.9	7	87.5%	8	33.3%	0.011*
HBV & HCV	12	37.5	5	62.5%	7	29.2%	0.512
T.Bilirubin (mg/dL)	6.1±8.6		10.38±16.25		4.72±3.57		0.727
D.Bilirubin (mg/dL)	3.1±4.8		4.0±9.0		2.84±2.62		0.098
AST (U/L)	145.2±74.9		116.1±88.5		106.9±100.3		0.794
ALT (U/L)	109.2±96.2		85.8±42.2		75.2±32.4		0.711
ALP (U/L)	145.2±74.9		139.9±74.7		161.2±78.3		0.305
GGT (U/L)	155.0±124.4		128.3±60.4		163.9±139.4		0.392
Albumin (g/dL)	2.99±0.66		2.92±0.6		3.0±0.7		0.513
Child-Pugh score:							
A	6	18.8	2	25	4	16.7	
B	12	37.5	1	12.5	11	45.8	0.240
C	14	43.8	5	62.5	9	37.5	
Serum AFP (ng/ml)	2782.6±5178.4		2912.2±5790.8		2394.1±2888.1		
Median	727.5		737.5		915.5		0.761

Data are expressed as mean ± SD.
*=Significantly different in the two groups.

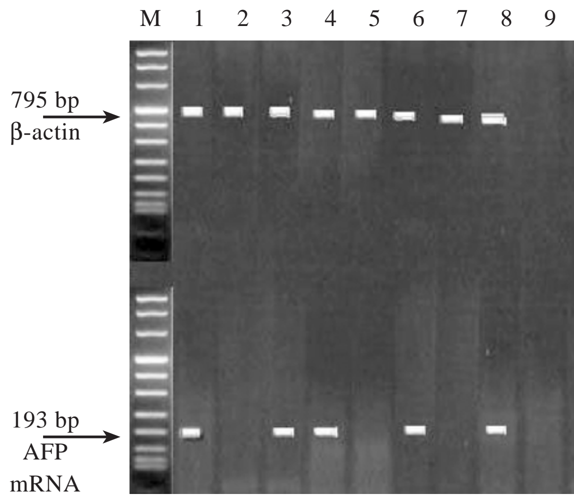

Fig. (1): Detection of AFP gene transcripts by nested RT-PCR in peripheral blood samples. Upper panel shows the result of amplification with β-actin specific primers; amplified product is 795bp. Lower panel shows the result of nested RT-PCR; amplified product is 193bp. M indicates molecular weight-marker. Lanes 1-7 correspond to patients' samples; patients 1,3,4 & 6 are AFP transcript positive; patients 2,5 & 7 are AFP transcript negative. Lane 8: Positive control (from Hep G2 cells). Lane 9: Negative control. PCR products were separated on 2% agarose gel.

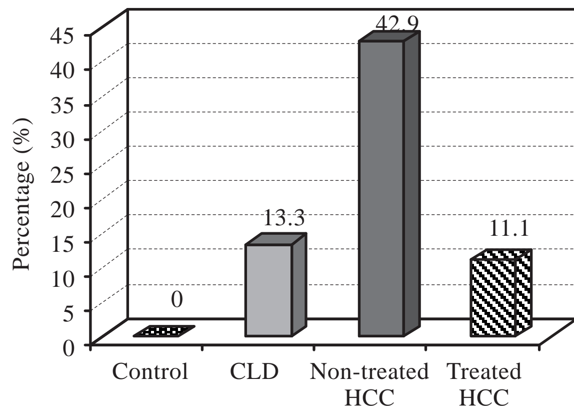

Fig. (2): Frequency of AFP mRNA positivity in the studied groups.

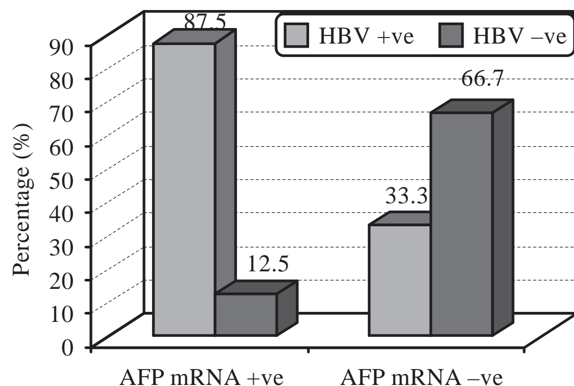

Fig. (3): Association between AFP mRNA positivity and HBV infection (X^2=7.069; *p*=0.01; OR=14; 95% CI=1.46-134.25).

DISCUSSION

The early dissemination of cancer cells to secondary sites is the main cause of mortality of patients with solid tumors. With regard to HCC, hematogenous spread is the major route of recurrence [22]. Therefore, evaluation for circulating tumor cells has generated considerable interest because of its potential association with disease recurrence and poor prognosis. On the basis of previous reports, we postulated that the detection of AFP mRNA is a specific surrogate marker for ITCs and therefore a predictor for hematogenous spread of HCC cells [17,19,21]. Our goal was to show a correlation between the detection of AFP mRNA in the peripheral blood of patients with HCC and clinico-pathologic variables. Nested RT-PCR assay was applied for detecting AFP mRNA in the peripheral blood of all subjects included in the study. The positivity rate of AFP mRNA in patients with HCC was 25% (8/32), same as Lemoine et al. [23], and ~43% (6/14) if only the determinations before therapy were considered. Contrasting results, however, were reported by other investigators. For instant, Witzigman et al. [24] reported a 45% positivity rate of AFP mRNA in patients with HCC and ~28% when only determinations before therapy were considered. Variable results have been reported in the literature [25-29]. Interestingly, only 2/18 (11.1%) of treated HCC patients included in the study were tested positive or circulating AFP mRNA. Both of them had undergone transcatheter hepatic artery chemoembolization (TACE) therapy, representing 25% (2/8) of this subgroup. Comparably, Marubashi et al. [30] reported a 9.4% positivity rate of AFP mRNA in the blood of HCC patients after living donor liver transplantation (LDLT). Higher results have been reported in the literature. Witzigmann et al. [24] reported about 54% (7/13) AFP mRNA positivity rate after TACE therapy, and other authors [24,31,32], reported a high positivity rate (23%-54%) of AFP mRNA postoperatively after curative resection therapy. It is worth mentioning that none of our post resection patients or those who had undergone radiofrequency therapy tested positive for circulating AFP mRNA, a finding that needs a further thorough investigation.

The reason for discrepancy among various studies may be multifactorial. The limited number of tested patients (a pitfall in the present

work), and differences in patients (for example patients with versus patients without extrahepatic metastasis) and in the method of isolating circulating carcinoma cells may be contributory factors. Guo et al. [33] found that enriching circulating tumor cells by negative and positive immunomagnetic beads before RT-PCR could effectively increase the sensitivity and lower the contamination of leukocytes. Yao et al. [28] validated the feasibility of combining negative and positive immunomagnetic bead sorting and RT-PCR technique. Sampling error may be another reason for conflicting results during the detection of AFP-expressing cells in blood samples in the literature, as the conditions of blood sampling may not have been comparable. Differences in the exact method of obtaining peripheral blood have been postulated to be one of the reasons for the discrepancies between various reports. The high detection rate reported by Kienle et al. [18] in patients with benign and malignant diseases of the liver may be explained by the position of central line which is routinely placed in or just proximal to the right atrium, Normal liver cells expressing AFP are continuously shed into the hepatic veins and either undergo apoptosis or are filtered out of the blood system when passing capillary beds. Cell shedding may be enhanced by surgical manipulation and inflammatory conditions within the hepatic parenchyma. So, if blood is drawn from a central line in or just proximal to the right atrium, it most likely contains blood that has just left the liver without having passed through any capillary beds that may filter out circulating cells.

According to Louha et al. [34], not only liver surgery but also non surgical invasive managements such as needle liver biopsy or intervention therapies such as TACE, chemotherapy and ethanol ablation therapy, the increased shedding of either HCC cells or normal hepatocyte into circulation might contribute to the increased detection rate of AFP mRNA. This was also the reason why we excluded those who had just received intervention therapies from the current study. However, the best timing for postoperative blood sampling is still uncertain. Funaki et al. [20] and Okuda et al. [25] thought that ITCs transiently liberated during surgery would be destroyed within 7 days. In the quantitative analysis of Wong et al. [35], hematogenous dissemination of hepatocytes and tumor cells were removed within 8 weeks after operation, and HCC cells were cleared within 2-4 weeks. Jeng et al. [32] thought that 12 weeks were adequate to exclude the transient presence of HCC cells due to operative manipulation. In the present work, blood samples were obtained 1-2 years after operative manipulation and 3-6 months after intervention therapies.

Apart from a statistically significant difference between our untreated and treated HCC patients (p=0.04), the positive detection rates were not significantly different among various patient groups (CLD Vs all HCC patients, treated or untreated HCC patient groups). However, there is evidence in the literature that non-cancerous liver cells in the blood stream may produce AFP mRNA positivity, especially in patients with cirrhosis or active hepatitis [19]. Cillo et al. [27] reported a comparable AFP mRNA positivity in patients with HCC (40%) and in patients with nonmalignant liver cirrhosis or pancreatic or colon cancer (36%); 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 current study, AFP mRNA positive detection rate in patients with CLD was 13.3% (2/15); it is worth mentioning that all patients included in this group were cirrhotic. Comparably, Wu et al. [29] reported 16% positivity rate in patients with LC, and Yang et al. [36] reported 13.6% positivity rate in patients with hepatitis B and cirrhosis. Higher as well as lower results were reported by other investigators. Whereas Cillo et al. [27] and Yao et al. [28] reported a 50% (3/6) and 44.8% (7/16) positive detection rates in patients with LC, respectively, Witzigmann et al. [24] reported a 7.6% (3/39) positive detection rate in patients with benign liver disease. However, their three patients who where AFP mRNA positive had liver cirrhosis as well. Therefore, some authors suggested that AFP gene was not hepatoma specific, but rather a liver specific marker [16,18,37]. Conversely, all 20 of Jeng et al. [32] controls with CLD tested negative for AFP expression in peripheral blood, leading them to suggest that the presence of AFP mRNA was specific for patients with HCC.

Funaki et al. [20] reported that 100% of patients with recurrent HCC were positive for circulating AFP mRNA. Moreover, their AFP

mRNA-negative, recurrence-free patients remained recurrence-free during a period of 22 months, whereas 57% of AFP mRNA-positive, recurrence-free or preoperative patients developed a clinically detectable recurrence from 2 to 16 months after analysis. In all patients who already recurred or had recurred later; recurrence was detected in the liver; only 2 of them had an additional lung recurrence. They stated that anchoring-free non-cancerous hepatocytes cannot possibly live long. Moreover, the AFP mRNA positive rate among their non-cancerous patients was zero, findings suggesting a high possibility of hematogenous prevalence of HCC. Thinking of the limited life of anchoring-free HCC cells, it seems more likely that these cells may be continuously or intermittently provided by some secondary foci arising either from hematogenous spreading, intra-hepatic metastasis or multi-centric carcinogenesis. At this secondary site, they may keep growing to make a "recurrence" under preferential environments or surviving and watching for the chance to rally in the unwilling environments. In this sense, the remaining AFP mRNA-positive, yet recurrence-free patients may be thought well as a high risk group for recurrence. On multivariate analysis, Kamiyama et al. [38] found that positive AFP mRNA was the most important risk factor for postoperative recurrence and disease-free survival after curative hepatic resection. Repetitive analysis at certain intervals for prolonged periods and ongoing quantitative analysis of AFP mRNA instead of the routine analysis of AFP may provide further information on the up-to-date status of these patients.

Would the PCR techniques (nested RT-PCR Vs RT-PCR, qualitative PCR Vs quantitative PCR, semi-quantitative PCR Vs real-time PCR) influence the sensitivity or specificity of positive results? Most reports used nested RT-PCR with a good sensitivity, but the results still had a wide range. Few studies [35,37] used semi-quantitative RT-PCR to investigate the dynamic change of circulatory HCC cells. The advent of real-time PCR in recent years makes the quantification of DNA/cDNA product easy work. Further investigation using real-time RT-PCR to add more information about quantity of circulating cancer cells should give us a more complete understanding. Wong and coworkers [37] quantified the level of AFP mRNA in liver cells and found that HCC cells had a much higher level than nonmalignant cells. They concluded that the actual level of expression was useful for diagnosis. Recently, Morimoto et al. [39] demonstrated that using real-time PCR to measure the AFP mRNA level in blood, but not bone marrow, could be useful for predicting postoperative tumor recurrence.

Circulating hepatocytes positive for AFP mRNA have rarely been reported in healthy persons [40,41]. In agreement with the majority of other investigators [17,24,29,31], none of the healthy controls included in the current study tested positive for circulating AFP mRNA, although some control subjects had elevated serum AFP values, same as Liu et al., report [26]. Some authors had previously shown that 100% of HCC patients with extra-hepatic metastasis were AFP mRNA positive [20,26,28]. In this study, we deliberately excluded patients with clinically evident extra-hepatic metastasis because we were more interested in focusing on a phase of HCC progression with still a potential for cancer treatment. Despite this restrictive element criterion, a considerable proportion (25%) of our HCC population expressed AFP mRNA in blood, enabling us to further analyze the correlation between this molecular marker and the corresponding clinical data in order to identify their relative influence on AFP mRNA molecular detection in the blood. Therefore, the 32 HCC patients were further redistributed into two groups: The 8 patients with suspected cell dissemination in the blood formed the positive group, while the other 24 patients in whom AFP mRNA was not detected formed the negative group.

In accordance with a previous report [27], no statistically significant differences were detected between the two groups in terms of demographic characteristics (age and sex), severity of cirrhosis and HCV infection. On the other hand, the presence of AFP mRNA in the blood correlated significantly with cholestatic indices (ALP and GGT) and nodule size in the study of Cillo et al. [27], while only HBV infection associated significantly with AFP mRNA in our HCC patients. In accordance with the results of the present work, Yao et al. [28] found that liver function indices (ALT, AST, GGT and ALP) did not show an association with AFP mRNA in HCC patients.

AFP is an oncofetal protein, and its expression is shown mainly by hepatocytes and endoderm cells of the visceral yolk sac in the early stages of fetal liver development, but repressed soon after birth. Reactivation of AFP gene expression is shown during massive liver necrosis and in hepatic carcinogenesis. The results of Ping et al. [42] showed that the AFP mRNA in HCC tissues was specially expressed by HCC cells and mutated hepatocytes, and its transcription levels were positively correlated with the presence of cirrhosis. In the current work, and in accordance with a previous report [27], the expression of AFP mRNA in blood of patients with HCC was not related to severity of cirrhosis (assessed by Child-Pugh score of liver function reserve).

The carcinogenesis of HCC is a multifactorial multistep and complex process. Omata and Yoshida [43] stated that HBV infection is merely a carcinogenic factor and is not related to the growth, infiltration and metastasis of HCC. Cillo et al. [27] found no significant association between HBV infection and AFP mRNA positivity in the blood of HCC patients as well. On the other hand, Ping et al. [42] suggested that the transcriptional activation of AFP in HCC tissues may be related to HBV infection. In the present work, 87.5% (7/8) of the positive group had chronic HBV infection with various grades of cirrhosis, and the only characteristic that was positively highly significantly associated with the presence of AFP mRNA in our HCC patients was HBV infection (p=0.01; OR=14). On this basis, HBV infection can be interpreted not only as a major risk factor for the development of HCC, but also as a pathological mechanism facilitating HCC progression. We cannot distinguish AFP mRNA originating from HCC cells from that originating from normal hepatocytes that could be released into the blood because of HBV infection and LC [43]. However, in our non-tumor CLD group the 2 AFP mRNA positive patients were HBsAg negative, whereas 5 patients with CLD due to HBV infection tested negative for circulating AFP mRNA. Therefore, our findings seem to suggest, at least in part, that HBV infection may be an important contributing factor to the hematogenous spread of HCC cells. In the study of Yang et al. [36], 80% of HCC patients developed cirrhosis after HBV infection. Muguti et al. [44] reported that the serum AFP level of HCC patients with serum HBsAg was higher than that of patients without HBsAg. They suggested that serum AFP levels had significant differences among different HBsAg titers. Lee et al. [45] also found that most patients with serum HBsAg had high serum AFP levels.

Histochemical studies have shown the presence of AFP in malignant hepatocytes [46], and serum AFP concentration rapidly return to normal after radical resection of HCC indicating that malignant hepatocytes are responsible for the production of AFP. Although AFP is a useful marker for HCC that greatly affects diagnosis, the evaluation of therapy, and therapeutic policy, it is sometimes very difficult to make the distinction between tumors and slightly falsely elevated AFP levels of benign liver diseases [47,48]. In the present work, serum AFP determinations were not significantly different between cirrhotic patients and those with untreated HCC.

The relationship between serum AFP and AFP mRNA was conflicting; in the present study we did not find a significant correlation between AFP mRNA and serum levels of AFP in HCC patients. Likewise studies from many other investigators [20,23-25,27,28], no correlation was found between these two markers. In contrast, other researches have been able to identify a significant association between serum AFP and AFP mRNA [17,19,34]. Elevation of the serum-secreted AFP protein level is not consistent with the presence of AFP-producing cells in the blood. Secreted AFP may originate from HCC cells in the primary tumor, those in some clinically occult recurrence and/or those in the blood. On the other hand, vulnerable mRNA may well be considered to exist within AFP-producing cells present in the peripheral blood. Therefore, the secreted AFP protein level does not necessarily agree with mRNA results and seems useful in this meaning [20].

In agreement with Lemoine et al. [23] and Wu et al. [29], the presence of AFP mRNA in blood was not related to the severity of HCC, indicated by tumor diameter. Ping et al. [42] experiments certified the AFP gene re-expression in tissue samples from patients with HCC had no relationship with tumor mass size, capsule status and differentiation. However, some 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 **[26,36]** or tumor size **[27]**.

In conclusion, using nested RT-PCR assay, AFP mRNA could be detected in the blood of HCC patients without clinical evidence of extra-hepatic metastasis as well as in patients with LC. Although the possibility of hematogenous spread of HCC cells in such patients cannot be excluded, qualitative measurement of AFP mRNA is too inaccurate to warrant clinical decisions, whether diagnostic or therapeutic. A much interesting result is the highly significant association between the presence of circulating AFP mRNA and HBV infection in HCC patients, a finding suggesting HBV infection as a contributing factor to HCC progression. None of the other clinico-pathologic variables (age, sex, HCV infection, Child-Pugh score, liver function indices, serum AFP values and tumor diameter) was significantly related to the presence of AFP mRNA in HCC patients. False positive results will be unavoidable if qualitative AFP mRNA determination is used as the single marker for the detection of circulating HCC cells. Advances in medical techniques are warranted for both the early detection and therapeutic evaluation of HCC. Using the appropriate single tumor marker or combination markers-two markers assay with a liver specific marker or a HCC specific marker-may improve the effectiveness in screening of HCC patients. If validated-in screening or as a predictor of disease metastasis or recurrence-this method would provide a powerful complement to routine histopathologic analysis of HCC. The association between HBV infection and the hematogenous spread of HCC and/or liver cells-as detected by AFP mRNA-needs to be investigated. New large series with HBV infection are needed. The finding that 25% in the chemoemblization subgroup of our treated HCC patients tested positive for circulating AFP mRNA compared to 0% AFP mRNA expression in blood of both post resection and radiofrequency treated patients needs a further thorough investigation. A large series with long-term follow-up, and quantitative analysis of AFP mRNA using real-time RT-PCR instead of the routine analysis of AFP protein may provide further information on the up-to-date status of HCC patients as well as on the efficacy of the different therapeutic modalities.

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