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## **Authors:**

**Sara El –kateb email: [s.melkateb@gmail.com](mailto:s.melkateb@gmail.com)**

**Nehad Mosaad email: [nanymosaad@hotmail.com](mailto:nanymosaad@hotmail.com)**

**\*Marwa Sheta email: [shetamarwa@hotmail.com](mailto:shetamarwa@hotmail.com)**

**Mona Awad email: [dr.monaabdelkader@hotmail.com](mailto:dr.monaabdelkader@hotmail.com)**

**Iman Hamza email: [iman\\_hamza@hotmail.com](mailto:iman_hamza@hotmail.com)**

**Rasha Nazih email: [Rashanazzih@yahoo.com](mailto:Rashanazzih@yahoo.com)**

**Eman Awadallah email: [emainawad\\_28@yahoo.com](mailto:emainawad_28@yahoo.com)**

**\*Corresponding author:** Marwa Sheta, MD, Department of clinical and chemical pathology, faculty of medicine, Cairo University, Cairo, Egypt. **Email:** [shetamarwa@hotmail.com](mailto:shetamarwa@hotmail.com) Tel.: +20 1223563623

# Circulating Alpha Fetoprotein messenger-RNA as A Marker for Hepatocellular Carcinoma

Sarah El-Kateb<sup>1</sup>, Nehad Mosaad<sup>1</sup>, Marwa Sheta<sup>1</sup>, Mona A Awad<sup>2</sup>, Iman Hamza<sup>3</sup>,  
Rasha Nazih<sup>2</sup>, Eman Awadallah<sup>2</sup>

*1 Clinical and Chemical Pathology Department, Cairo University*

*2 Clinical and Chemical Pathology Department, National Research Centre*

*3 Endemic medicine Department, Cairo University*

## **Abstract:**

*Alfa-fetoprotein (AFP) mRNA is used as marker of hepatocellular carcinoma (HCC) cell dissemination as it represents the circulating tumor cells. The aim of this study is to investigate the role of serum AFP mRNA as a diagnostic non invasive marker for HCC and to assess its sensitivity and specificity among patients with HCC. **Methods:** Patients were classified as follows: Group I included 18 patients having HCC, Group II included 20 patients with HCV related cirrhosis; Group III included 20 apparently healthy volunteers. AFP mRNA was estimated using quantitative real-time PCR. **Results** within the HCC group, AFP mRNA was positive in 10 patients (55.5%) and not detected in any of the cirrhosis or control groups. At a cutoff value of 47 copies/ $\mu$ l, AFP mRNA yields a sensitivity, specificity, positive predictive value and negative predictive values of 55%, 100%, 100% and 71.4%, respectively. **Conclusion:** AFP mRNA is useful diagnostic marker of HCC. In patients where AFP determination is alone clueless in the diagnosis of HCC, the addition of AFP mRNA increases the sensitivity. Further studies are needed to validate AFP mRNA as a screening tool in cirrhosis patients, a predictor of response to treatment and a predictor of recurrence in HCC patients.*

## **Key words:**

**Hepatocellular carcinoma (HCC), Alpha-fetoprotein (AFP), AFP mRNA.**

## **Introduction**

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide. It is considered the fifth most common tumor. Also, it is the third cause of cancer-related deaths [1]. There is an overall increase in the relative frequency of liver related cancers in Egypt according to hospital-based studies, from 4% in 1993 to 7.3% in 2003 with more than 95% diagnosed as HCC [2].

AFP is considered the gold standard serum marker for HCC, but its role for testing people at risk of HCC is limited because of its low sensitivity. This is why the clinicians are in strong need for specific

biomarkers for HCC. So, research studies are focusing on the prognostic markers for early detection of the recurrence and help in the treatment options [3-5].

The aggressive nature of HCC is reflected by the presence of circulating tumor cells (CTCs) in the peripheral blood [6]. Presence of AFP mRNA in peripheral blood is considered a marker of HCC cell dissemination as it represents the CTCs [7]. Also, its disappearance from peripheral blood might be useful for monitoring response of patients to treatment. About (85%) of patients with highest metastatic recurrence and shorter recurrence free period have consistent AFP mRNA rates than the patients with negative post operative AFP mRNA [8].

CTCs can be detected by several methods either morphological methods such as immunocytochemistry and polymerase chain reaction (PCR) techniques as an example for non morphological methods [9]. Detection of tumor-specific gene transcripts (mRNA) by PCR is very sensitive as it detects 1 tumor cell among 107 normal mononuclear cells in peripheral blood [10].

In generally immune-cytochemistry is less sensitive than real-time PCR, but real-time PCR shows a higher rate of false positives. This can be assigned to different causes as primer selection, instability of mRNA in vitro, differences in laboratory techniques and time between sample collection and processing. Use of quantitative real-time PCR can improve the results of PCR techniques [11].

The combination of some highly sensitive and specific HCC biomarkers, especially in gene expression profiling and pathological features, appears to be a promising approach for early diagnosis and detection of HCC metastasis; it also helps for defining new diagnostic and therapeutic modalities and seems to be more practical up to present [12].

Objective: The aim of this study is to investigate the role of serum AFP mRNA as a diagnostic non invasive marker for HCC and to assess its sensitivity and specificity among HCC in patients.

### **Patients and methods**

In this cross sectional study; patients presenting to the Endemic Medicine Department, Kasr el Aini Hospital, Cairo University, during the study period June 2012- December 2012 were included as follows:

Group I included 18 patients diagnosed as having HCC. Group II included 20 patients with HCV related cirrhosis. Group III included 20 apparently healthy volunteers, age and sex matched, as a control group.

Patients were subjected to full clinical evaluation, routine lab tests, abdominal ultrasonography and estimation of the Child Pugh grade. They underwent estimation of serum Alpha Fetoprotein (AFP) and AFP mRNA.

Inclusion criteria for HCC patients:

Adults with proven HCC diagnosis, with negative history to previous or current treatment of HCC and without clinically evident extra-hepatic metastasis were included for the study.

Cirrhosis was diagnosed on clinical background (manifestations of liver cell failure), laboratory background (markers of cytolysis, cholestasis and synthetic function derangement) and imaging (morphological changes and signs of portal hypertension) [13].

Liver masses were diagnosed as HCC according to the Korean Liver Cancer Study Group (KLCSG) practice guidelines for the management of HCC [14]:

- AFP value >200ng/ml with a specific imaging pattern defined by intense contrast uptake during the arterial phase followed by contrast washout during venous or delayed phases in contrast enhanced study such as Computed Tomography (CT) scan or magnetic resonance imaging (MRI).
- AFP <200 ng/ml with two or more positive findings of dynamic contrast enhancement (CT or MRI).
- A tumor of 2 cm (or more) with typical characteristic of HCC in dynamic contrast enhancement CT or MRI regardless of the serum AFP levels.

Exclusion criteria:

Patients with hepatic masses indefinite for HCC, patients with other associated malignancies, those who received therapy for hepatocellular carcinoma lesion or recurrent HCC were excluded from the study. Also patients with proved metastasis (by CT chest or bone scan) were excluded as well. History of recent liver biopsy (earlier than 3months or less) was considered an exclusion criteria.

AFP mRNA assay

RNA extraction is done using Qiagen Kit (Cat No.52304 Blood Minikit) (50). Quantification of total RNA is determined by measuring the absorbance at 260nm (A260) in a spectrophotometer. To ensure significance, A260 readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per milliliter (A260=1 means 44 µg RNA/ml). RNA samples were diluted using RNase-free water. The buffer in which the RNA is diluted is used to zero the spectrophotometer. Reverse transcription polymerase chain reaction (RT-PCR) is done using High-Capacity cDNA Reverse Transcription Kit (Company name? Cat. No.4368814)[15]. Real-time PCR Method. The used system is "Applied Biosystems Step One™ Instrument". (Relative Standard Curve and Comparative CT Experiments: Getting Started Guide, Part Number: 4376785) [16].

## Statistical analysis

Statistical analysis was done using the Statistical Package of Social Sciences (SPSS) computer software program, version 15.0 (Chicago, IL, USA). Quantitative data were presented as mean  $\pm$  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 means were tested by Student's t test and the one-way analysis of variance (ANOVA). Correlation was done using correlation coefficient (r). For Qualitative data, differences among groups were tested using the Pearson's chi-square test ( $X^2$ ) and the Fisher's Exact test. ROC curve was plotted to determine the sensitivity and the specificity of AFP and AFP mRNA. All tests were considered statistically significant at  $p \leq 0.05$ .

## Results:

Group (I) (HCC group) is included 18 patients. Their ages are ranged from 53-67 years with a mean of  $59.4 \pm 4.5$ , 4 females (22.2 %) and 14 males (77.8%).

Group (II) (chronic liver disease group) is included 20 patients. Their ages are ranged from 48-63 years with a mean of  $56.3 \pm 4.4$ , 6 females (30%) and 14 males (70%).

Group (III)(control group) is included 20 apparently healthy controls. Their ages are ranged from 50-65 years with a mean of  $57.9 \pm 4.2$ , 8 females (42.1 %) and 12 males (57.9%).

All groups were comparable regarding their age and sex distribution ( $p=0.108$ ,  $0.435$  respectively). All patients in the HCC and chronic liver disease groups had HCV-related cirrhosis.

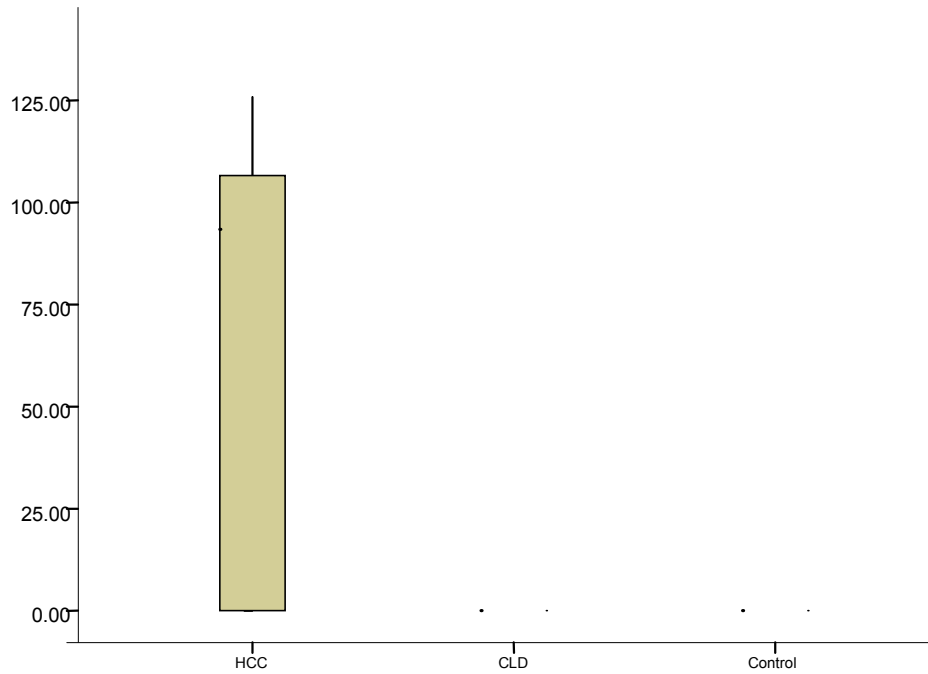
As regards the Child -Pugh grade; in the HCC group 9 patients were Child C, 9 patients were Child B and none of the patients was Child A (50%, 50%, 0% respectively). In the cirrhosis group 10 patients were Child C, 10 patients were Child B, and none were Child A (50%, 50%, 0% respectively).

Within the HCC group, 7 patients (38.9%) had single focal lesion, 4 patients (22.2%) had 2 focal lesions and 7 patients (38.9%) had multiple lesions. Portal vein thrombosis was absent in all patients, while hepatic vein invasion was detected in only one patient (5.6%). As regards the size of the focal hepatic lesions, 8 patients (44.4%) had tumor size  $< 5$ cm while 10 patients (55.6%) had tumor size  $> 5$ cm in diameter. As by the BCLC (Barcelona Clinic Liver Cancer) staging, the majority of cases were BCLC stage D (14 cases 83.3%) versus 4 cases with Stage C (16.7%).

The reported median AFP values were  $13.6$  (2.8-904.7) ng/ml,  $3.8$  (2.7-13.6) ng/ml and  $2.2$  (1.5-3.5) ng/ml in each of HCC, cirrhosis and control groups respectively. These values showed a statistically significant difference between the HCC and control groups ( $p$  value =0.000), and between the cirrhosis and control groups ( $p$  value =0.002). On the other hand, there was no statistically significant between the HCC and the cirrhosis groups ( $p$  value=0.115).

## AFP mRNA

Copies/ $\mu$ l



Figure( 1). AFP mRNA levels among the studied groups.

As shown in (Fig 1), the median serum AFP mRNA in HCC patients was 93.4(0.00-107.5) copies/ $\mu$ l and it wasn't detected either in cirrhosis or control groups.

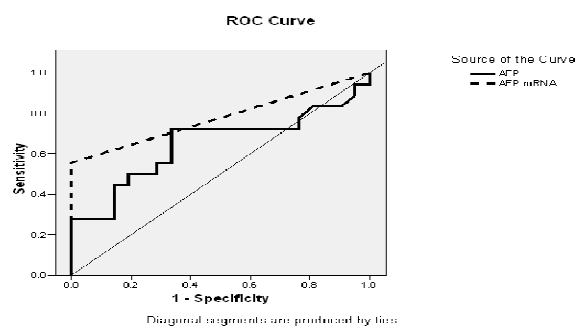


Figure 2.ROC curve for AFP mRNA in relation to AFP level.

By using the receiver operator characterizing (ROC) curve, to determine the specificity and sensitivity of AFP, the cutoff value of 10ng/ml yields a sensitivity, specificity, positive predictive value and negative predictive values of 55%, 28%, 62.5% and 63.6%, respectively. The maximum area under the curve (AUC) was 0.648 with 95% confidence interval from 0.463 to 0.833 for total AFP (Fig 2).

As for the AFP mRNA, the cutoff value of 47 copies/ $\mu$ l yields a sensitivity, specificity, positive predictive value and negative predictive values of 55%, 100%, 100% and 71.4%, respectively. The maximum area under the curve (AUC) was 0.778 with 95% confidence interval from 0.621 to 0.935 for AFP mRNA (Fig 2).

When any of the 2 markers is positive (AFP (ng/ml) or AFP mRNA at cutoff value of 10ng/ml and 47 copies/ $\mu$ l respectively), the sensitivity, specificity, positive predictive value and negative predictive values are 66%, 70%, 66% and 70%, respectively.

Table (1). Relation of AFP mRNA gene expression to demographic and Clinical and Ultrasonographic findings of HCC patients (n=18)

		AFP m-RNA		P
		-ve frequency (%) (Total: 8)	+ve frequency (%) Total (10)	
Gender	Male	5/8(62.5%)	9/10(90%)	0.275
	Female	3/8(37.5%)	1/10(10%)	
Ascites		8/8(100%)	10/10(100%)	-
Child grade	A	0 (0%)	0 (0%)	0.36
	B	3/8(37.5%)	6/10(60%)	
	C	5/8(62.5%)	4/10(40%)	
Tumor Number	1.00	5/8(62.5%)	2/10(20%)	0.096
	2.00	2/8(25%)	2/10(20%)	
	3.00	1/8 (12.5%)	6/10(60%)	
Tumor size	> 5 cm	3/8 (37.5%)	7/10(70%)	0.342
	< 5 cm	5/8 (62.5%)	3/10(30%)	
BCLC stage	Stage C	2/8 (25%)	2/10 (20%)	0.293
	Stage D	6/8 (75%)	8/10 (80%)	

Within the HCC group, AFP mRNA gene expression was positive in 10 patients (55.5%) and negative in 8 patients (45%). The relation of AFP mRNA gene expression to demographic and clinical data of HCC patients is shown in (Table 1).

Table (2). Correlation between AFP, AFP mRNA and studied parameters in the studied groups

		AFP m-RNA		P
		-ve frequency (%) (Total: 8)	+ve frequency (%) Total (10)	
Gender	Male	5/8(62.5%)	9/10(90%)	0.275
	Female	3/8(37.5%)	1/10(10%)	
Ascites		8/8(100%)	10/10(100%)	-
Child grade	A	0 (0%)	0 (0%)	0.36
	B	3/8(37.5%)	6/10(60%)	
	C	5/8(62.5%)	4/10(40%)	
Tumor Number	1.00	5/8(62.5%)	2/10(20%)	0.096
	2.00	2/8(25%)	2/10(20%)	
	3.00	1/8 (12.5%)	6/10(60%)	
Tumor size	> 5 cm	3/8 (37.5%)	7/10(70%)	0.342
	< 5 cm	5/8 (62.5%)	3/10(30%)	
BCLC stage	Stage C	2/8 (25%)	2/10 (20%)	0.293
	Stage D	6/8 (75%)	8/10 (80%)	

Table 2 shows correlation between AFP, AFP mRNA and studied parameters in different groups, there was a significant positive correlation between serum AFP and AFP mRNA ( $r=0.348$ ;  $p=.007$ ). Also, a statistically significant positive correlation could be estimated between AFP mRNA gene expression, age, serum bilirubin and INR. A statistically significant negative correlation could be estimated between AFP mRNA gene expression and albumin.

## Discussion

The current study has been designed to investigate the expression of AFP mRNA in the peripheral blood of patients with HCC and Cirrhosis and to clarify its correlation with some clinico-pathologic characteristics of HCC.

Real-time PCR assay was applied for detecting AFP mRNA in the peripheral blood of non metastatic HCC patients, in addition to the cirrhosis and control groups. Results showed a positivity rate of AFP mRNA in our HCC patients to be 55% [11],[20] without being detected in any of the cirrhosis or control groups with a median level of 93.4 copies/  $\mu$ l.



20-50% positive rate of pre-operative AFP mRNA is reported in several literature reports [6], [15]. 43% positivity rate is reported by Montaser et al., 2007[17] while 28% positivity rate reported by Yulianget al., 2011[18]. Also, Zhang et al., 2008[19] reported that AFP transcripts were detected in 29 (33.7%) of the 86 HCC patients and not detected in any peripheral blood specimens from 25 chronic liver disease (CLD) patients and 28 normal healthy volunteers. Also, Jeng et al., 2004 reported that AFP mRNA was negative in all 20 controls and CLD. According to these reports, authors suggested that AFP mRNA is specific for patients with HCC [20].

However, Yulianget al., 2011[18] found that 8 patients (28%) with HCC and only 1 patient (5%) with cirrhosis were positive AFP mRNA and non of healthy volunteers. In other researches the incidence of positivity of AFP mRNA reached up to 16% in non-HCC cirrhosis groups [21],[22]. Accordingly, AFP mRNA was considered not a tumor specific marker but a liver specific marker [23].

There are multiple factors for this variation in the AFP mRNA positivity rate among various studies. Presence versus absence of extra hepatic metastasis, the variable number of patients, sampling error and also the isolation technique of circulating carcinoma cells may be contributory factors. Different results may produce due to the difference in the PCR techniques (qualitative PCR vs quantitative PCR, nested RT-PCR vs RT-PCR, semi-quantitative PCR vs real-time PCR). Also, from a technical point, Galindo et al.[24] 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.

Also, the variation between various reports has been postulated referred to the different method of obtaining peripheral blood [18]. Liver cells expressing AFP are either filtered out of the blood system when passing capillary beds or undergo apoptosis. So, if patient has central line and the blood is drawn from it, it most likely contains blood with filter out circulating cells that has just left the liver without having passed through any capillary beds [17].

Increase detection rate of AFP mRNA might contribute to increase shedding of either normal hepatocytes or HCC cells. Intervention within the hepatic parenchyma such as needle liver biopsy, surgical manipulation transarterial chemoembolization (TACE), and ethanol therapy may enhance the cell shedding. That's why in the current study blood samples were drawn from a peripheral I.V line, patients who underwent therapeutic liver intervention or recent liver biopsy were excluded from the current study [20], [25], [26].

Still the possibility of a few AFP producing virus-infected hepatocytes or degenerating hepatocytes cannot be excluded in the AFP mRNA positive results, despite taking into consideration all the technical precautions to ensure proper estimation of AFP mRNA[25]. However, the lack of detection of AFP mRNA in our cirrhosis and control groups indicates that this inevitable error is probably eliminated in our work.

The detection of AFP mRNA in peripheral blood does not reflect the whole expression of AFP gene, but represents the circulating tumor cells and the relationship between AFP and AFP mRNA in peripheral blood is conflicting [17]. The amounts of AFP synthesis and secretion and the level of AFP transcription may vary among HCC cells according to Feng et al., 2005[27]. In our study, we reported a significant positive correlation between serum AFP and AFP mRNA( $r=0.348$ ,  $p=0.007?$ ). Our results

come in agreement with several studies [10],[22],[30], [31]. While, Montaser et al 2007, and others [25],[26],[28],[29] failed to demonstrate such a correlation. This lack of correlation between AFP and AFP mRNA is explained by theoretically authors due to the difference in cellular origin of each marker, AFP mRNA exist within AFP producing cells only in the peripheral blood, while AFP is produced from HCC cells in the primary tumor, tumor recurrences and/or peripheral blood, so both markers may not be consistently related [25].

In the current study a statistically significant positive correlation could be estimated between AFP mRNA gene expression, age, serum bilirubin and INR. A statistically significant negative correlation could be estimated between AFP mRNA gene expression and albumin, raising a possible prognostic indication of AFP mRNA. On the other hand, there was no significant correlation with any of the liver enzymes (transaminases and cholestatic enzymes; ALT, AST, ALP and GGT). Feng et al., 2005[27] also found that the liver enzyme indices (ALT, AST,GGT and ALP) did not show an association with AFP mRNA in HCC patients. Cillo et al. reported that AFP mRNA correlated significantly with cholestatic indices (ALP and GGT) and nodule size [29], In the study by Montaser et al [17], only HBV infection associated significantly with AFP mRNA.

The relation between the level of AFP mRNA and the tumor size is conflicting. Some studies reported no significant relation [21],[32], while other studies proved significant correlation between severity of liver involvement by HCC indicated by tumor diameter or size and the frequency of positive AFP mRNA in blood [22],[29],[33].

In the current work there was no significant difference between the AFP mRNA positive and negative cases in terms of HCC characteristics (tumor number and size) and no significant correlation with the BCLC stage . The difference in study designs and the small number of our cases may explain this difference, for example Cillo et al [29] relied on diagnosing HCC by performing liver biopsy to all their patients, while

Chiappini [33] included patients with extra-hepatic metastasis, all of these may contributed to false elevation of AFP-mRNA.

The performance characteristics of AFP (at cutoff value of 10ng/ml) for diagnosing HCC patients showed a sensitivity, specificity, positive predictive value and negative predictive values of 55%, 28%, 62.5% and 63.6%, respectively. AFP mRNA at cutoff value of 47 Copies/ $\mu$ l yielded, comparable sensitivity 55% and increasing of specificity to 100%, positive and negative predictive values 100% and 71.4%, respectively. The maximum area under the curve (AUC) was 0.648 with 95% confidence interval (CI) from 0.463 to 0.833 for total AFP while the maximum area under the curve (AUC) was 0.778 with 95% confidence interval (CI) from 0.621 to 0.935 for AFP mRNA. When any of AFP or AFP-mRNA has been used sensitivity has increased to 66%, with a reported specificity of 70%. Norimasa et al., 2008 reported that the sensitivity, specificity, positive predictive value and negative predictive values of serum AFP in HCC detection were 69.3%,60.0%, 81.2% and 38.9% respectively and for AFP mRNA were 71.6% ,67.5%,69.5% and 74.1% respectively [28].

Finally, the present data provide circumstantial support for the hypothesis that AFP mRNA in blood reflects the presence of circulating HCC cells and therefore can be used as a useful diagnostic marker of HCC.

## Conclusion:

AFP mRNA is useful diagnostic marker of HCC. In patients where AFP determination is alone clueless in the diagnosis of HCC, the addition of AFP mRNA increases the sensitivity. Further studies are needed to validate AFP mRNA as a screening tool in cirrhosis patients, a predictor of response to treatment as well as a predictor of recurrence in HCC patients.

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