Plasma albumin m RNA as anon invasive Marker to predict liver injury in chronic Hepatitis C and Hepatocellular carcinoma patients

Eglal Mariam Raymond Souaya[1], Eman Saleh El-Hadidi[2], Amr Ali Mohammed[1], Walaa Abdel Hameed Mahsoub[1]

[1] Ain Shams University, Faculty of Science, Department of Chemistry, Cairo, Egypt
[2] Ain Shams University, Faculty of Medicine, Department of Clinical Pathology, Cairo, Egypt

Abstract

Analysis of circulating nucleic acids in plasma, such as cell free RNA offers an avenue for non-invasive monitoring of a variety of physiological and pathological conditions. Because albumin is the most abundant protein in the body and is synthesized by the liver, the current study was designed to assess plasma albumin mRNA (ALB mRNA), as a non-invasive diagnostic marker of liver injury in chronic HCV (CHC) and hepatocellular carcinoma (HCC). The study included 50 patients, 20 patients had CHC and 20 were of HCC as well as 10 healthy control subjects. Patients were subjected to clinical examination, abdominal ultrasonography, CT for HCC cases and laboratory investigations including liver function tests, AFP and plasma albumin mRNA by Real Time-PCR.

Patients with CHC and HCC have a significant increase in their plasma ALB mRNA than controls; the higher level was in HCC cases.

ALB mRNA in plasma is liver specific; it is increased in liver disease suggesting liver pathology and may be more diagnostically sensitive than alpha-fetoprotein and Alanine amino transaminase (ALT) serum levels. Thus, future studies should assess if the plasma concentration of ALB mRNA may be used as therapy monitoring.

Keywords: Albumin mRNA, HCC, CHC.

1.Introduction

The clinical course of untreated hepatitis C virus (HCV) infection is highly variable with the majority of patients experiencing a slow fluctuating disease that may take 20 years or more for full expression. Approximately half of HCV patients develop chronic active hepatitis and this may progress to liver cirrhosis and hepatocellular carcinoma (HCC) ***(Pollicino et al., 2009).***

Egypt has the highest prevalence of hepatitis C virus (HCV) infection in the world. Screening of HCV during pregnancy is not as routinely done in Egypt compared with many other countries, although pregnancy is an important period where screening of HCV infection is important owing to low immunity, the possibility of vertical transmission and possible horizontal transmission to the baby or other household contacts at a later stage ***(Khamis et al., 2014).***

Hepatic fibrosis is the accumulation of extracellular matrix, or scar, in response to acute or chronic liver injury. Fibrogenesis represents a wound healing response to injury, and ultimately leads to cirrhosis. Both fibrosis and cirrhosis are the consequences of a sustained wound-healing response to chronic liver injury from a range of causes, including viral, autoimmune, drug induced, cholestatic and metabolic diseases ***(Rockey and Friedman, 2007).***

Corresponding Author: lulu4d@yahoo.com

The development of severe fibrosis and necroinflamatory changes in liver leads to cirrhosis and worsens prognosis by enhancing the risk of HCC. Chronic HCV varies greatly in its course and outcome. The presence of HCV RNA indicates that the patient has ongoing viral infection despite normal ALT levels ***(Berry et al., 2005).*** Serologic assays detect HCV antibodies that indicate present or previous infection, but they cannot discriminate acute from chronic or resolved infection. Occasionally immunocompromised patients, hemodialysis patients and patients with mixed cryoglobulinemia have false negative serology results and may require HCV-RNA listing for diagnosis ***(Richter et al., 2002).*** While needle biopsy is still the mainstay in diagnosing hepatic fibrosis, its days of dominance seem limited as laboratory technology and imaging studies improve ***(Thabet et al., 2011).***

The existence of extracellular mRNA in the circulation, i.e., plasma and other body fluids has been long known ***(Swaminathan et al., 2006).*** The extracellular mRNA is thought to be released into the circulation from intact and viable cells as well as necrotic cells ***(Fleischacker, 2006).*** The biological roles of circulating mRNA are still unclear, although its physiological significance has been investigated during the last several years.

The detection of circulating RNA offers certain advantages over the detection of circulating DNA ***(Fernandez-Mercado et al., 2015).*** First, if both plasma RNA and DNA were derived from the same cell population, the released RNA would likely be quantitatively more abundant than DNA. This is because multiple copies of RNA transcript may be present in each cell, depending on the gene's expression, whereas each cell contains only a single diploid genome equivalent of DNA. Second, some cancer researchers reported that a greater proportion of cancer cases were positive for the investigated plasma RNA markers than DNA markers ***(Anker et al., 2003).***

The analysis of circulating nucleic acids in plasma offers an avenue for non-invasive monitoring of a variety of physiological and pathological conditions ***(Chan et al., 2003; Lo and Chiu, 2007).***The plasma circulating mRNA in cancer patients ***(Miura et al., 2005)*** and pregnant women ***(Jhaveri and Swamy, 2014)*** has been detected and analyzed with respect to sensitivity and specificity. An increasing amount of evidence suggests that liberation of cell-free nucleic acids into plasma from organs or compartments is likely to be due to cell death ***(Thabet et al., 2011)***.

The detection and monitoring of hepatic injury or dysfunction of patients infected with hepatitis B and C are increasingly becoming important. Currently plasma aminotransferases such as ALT and aspartate aminotransferase (AST) are conventionally used to assess hepatic injury, however, it is well known that they lack specificity and sensitivity, and their levels vary in different populations and at different time points in an individual. Reliable and sensitive molecular markers have been awaited ***(Thabet et al., 2011)***

The analyses of cell free RNA species successfully detected in plasma have potential for use in disease assessment ***(Ng et al., 2003).*** There is much evidence to suggest that circulating DNA and RNA are released upon cell death ***(Jahr et al., 2001).*** Indeed, reports of previous studies have described the detection of *ALB* mRNA in peripheral whole blood and the peripheral mononuclear cell fraction of humans ***(Wong et al., 2000; Bastidas-ramirez et al., 2002).***

These studies have had a mixed level of success, however, with detection rates of blood *ALB* mRNA of >100% from patients with HCC ***(Anker &Stroun 2002),*** cirrhosis, or hepatitis and from healthy controls. The correlation of plasma *ALB* mRNA concentration with hepatic injury in rats have been reported, however, the studies in human is still lacking ***(Kudo et al., 2008).***

2.Materials and Methods

This study was conducted at Ain-Shams University Hospital on 20 patients with Chronic Hepatitis C (CHC) diagnosis based on PCR,20 patients with HCC (hepatocellular carcinoma)diagnosed by liver biopsy and 10 apparent healthy subjects (Healthy Control Group).

1.1 Patients' Groups (n = 40):

This group included fourtey patients attending the internal medicine departments at Ain Shams University Hospitals. Their diagnosis was based on the clinical picture, liver function tests for more than 6 months. They were further classified according to Child classification into two subgroups.

a) Group A (Child A patients) (n = 20):

This group includes twenty CHC Patients will be recruited from hepatology clinic and department at Ain Shams University Hospitals.

Diagnosis will be based on HCV Ab in blood. They were 15 males and 5 females whose ages ranged from 23 to 77 years.

b) Group B (Child B patients) (n = 20):

This group includes twenty patients with HCC (hepatocellularcarcinoma).

Diagnosis will be based on biopsy. They were16 males and 4 females whose ages ranged from 22 to 62 years.

1.2 Control Group (n = 10):

Ten apparently healthy subjects will be included in the study,Exclusion of liver disease will be done by history and normal liver function tests. They were 6 males and 4 females whose ages ranged from 31 to 73 years.

IBM SPSS statistics (V. 23.0, IBM Corp., USA, 2015) was used for data analysis. Date were expressed as Median Percentiles for quantitative non-parametric measures.

3.Results and Discussion

3.1Results

Results of the present study are shown in tables (1, 2, 3, 4 & 5) and figure (1).

Descriptive statistics of the various studied parameters in CHC group, HCC group and healthy control group are shown in (Table1).Median of ALT was18 IU/L, 42IU/Land 109 IU/Lin healthy control group, CHC and HCC, respectively. As regards ALP, it showed median 141.5 U/L, 245 U/L and295.5 U/L in healthy control group, CHC and HCC, respectively. Moreover, median of T.Bil was0.55 mg/dL, 1mg/dL and1.9mg/dL in healthy control group, CHC and HCC, respectively. Median of AFP was2.3IU/ml, 3.25IU/ml and17 IU/ml in healthy control group, CHC and HCC, respectively. Furthermore, median of Alb.mRNA was 1.415%, 0.58% and4.605% in healthy control group, CHC and HCC, respectively (Table 2).

The statistical comparison between the various studied groups as regards ALT, ALP and T.Bilin (Table 1). Our results showed a highly significant difference in patients with CHC

as compared to the healthy control group (P < 0.001respectively). Moreover, a highly significant difference was also found in patients with HCC as compared to the healthy control group (P < 0.001 respectively). Our study also revealed a highly significant difference in patients of HCC as compared to CHC patients and healthy control group (P < 0.001respectively).

Statistical comparison between the various studied groups as regards AFP and Alb.mRNA are shown in (table 2). AFP and Alb.mRNA showed no significant difference in CHC patients were compared to healthy control group (P > 0.05 respectively).On the other hand, a highly significant difference was found between HCC patients and the control group (P < 0.001 respectively). Results also revealed a highly significant difference between CHC group and HCC group (P < 0.001 respectively).

Correlation study between AFP and Alb.mRNA in healthy control, CHC and HCC groups revealed the absence of a significant correlation between both parameters (P > 0.05), these shown in (table 3).

The diagnostics performance of AFP and Alb.mRNA in discriminating HCC from CHC and control group is shown in (table 4).

As regards AFP, at a cut off 5.5 the diagnostic sensitivity was 80%, specificity 90%, negative predictive value was 87.1%, positive predictive value was 84.2% and efficacy was 86%. Meanwhile, Alb.mRNA at a cut off 1.879 showed a diagnostic sensitivity 90%, specificity 73.3%, negative predictive value 91.7%, positive predictive value 69.2% and efficacy 80%.

The addition of AFP at a cut off 5.5 to Alb.mRNA at a cut off 91.13 has shown an improvement in the diagnostic performance of Alb.mRNA to a sensitivity, specificity, positive predictive value, negative predictive value and efficacy of 100% respectively.

Table (1): Descriptive Statistics

	ALT (IU/L)			ALP (U/L)			T.Bil (mg/dl)		
	Median (IQR)	**P Value**	**Sig.**	**Median (IQR)**	**P Value**	**Sig.**	**Median (IQR)**	**P Value**	**Sig.**
Control	18 (16.25-23.75)	P < 0.001	HS	141.5 (105.25-174.75)	P <0.001	HS	0.55 (0.4-0.7)	P <0.001	HS
CHC	42 (35.5-58.5)			245 (192.25-300.25)			1 (0.825-1.2)		
Control	18 (16.25-23.75)	P < 0.001	HS	141.5 (105.25-174.75)	P <0.001	HS	200.55 (0.4-0.7)	P < 0.001	HS
HCC	109 (71.5-309)			295.5 (235-335.25)			1.9 (1.425-2.85)		
CHC	42 (35.5-58.5)	P < 0.001	HS	245 (192.25-300.25)	P >0.05	NS	1 (0.825-1.2)	P < 0.001	HS
HCC	109 (71.5-309)			295.5 (235-335.25)			1.9 (1.425-2.85)		
Control	18 (16.25-23.75)	P < 0.001	HS	141.5 (105.25-174.75)	P <0.001	HS	0.55 (0.4-0.7)	P < 0.001	HS
CHC	42 (35.5-58.5)			245 (192.25-300.25)			1 (0.825-1.2)		
HCC	109 (71.5-309)			295.5 (235-335.25)			1.9 (1.425-2.85)		

Table (2): Comparison between Albumin mRNA and AFP in Patients and Control Group

	Albumin mRNA (copies/ml)			AFP (IU/ml)		
	Median (IQR)	**P Value**	**Sig.**	**Median (IQR)**	**P Value**	**Sig.**
Control	1.4155 (0.206 – 2.926)	P > 0.05	NS	2.3 (1.8 – 3.6)	P > 0.05	NS
CHC	0.58 (0.25975 – 1.7085)			3.25 (2.22 – 5.475)		
Control	1.4155 (0.206 – 2.926)	P < 0.001	HS	2.3 (1.8 – 3.6)	P < 0.001	HS
HCC	4.605 (2.55 – 50.545)			17 (10 – 2135)		
CHC	0.58 (0.25975 – 1.7085)	P < 0.001	HS	3.25 (2.22 – 5.475)	P >0.05	NS
HCC	4.605 (2.55 – 50.545)			17 (10 – 2135)		
Control	1.4155 (0.206 – 2.926)			2.3 (1.8 – 3.6)		
CHC	0.58 (0.25975 – 1.7085)	P < 0.001	HS	3.25 (2.22 – 5.475)	P < 0.001	HS
HCC	4.605 (2.55 – 50.545)			17 (10 – 2135)		

Table (3): Correlation between Albumin mRNA Levels and Other Parameters:

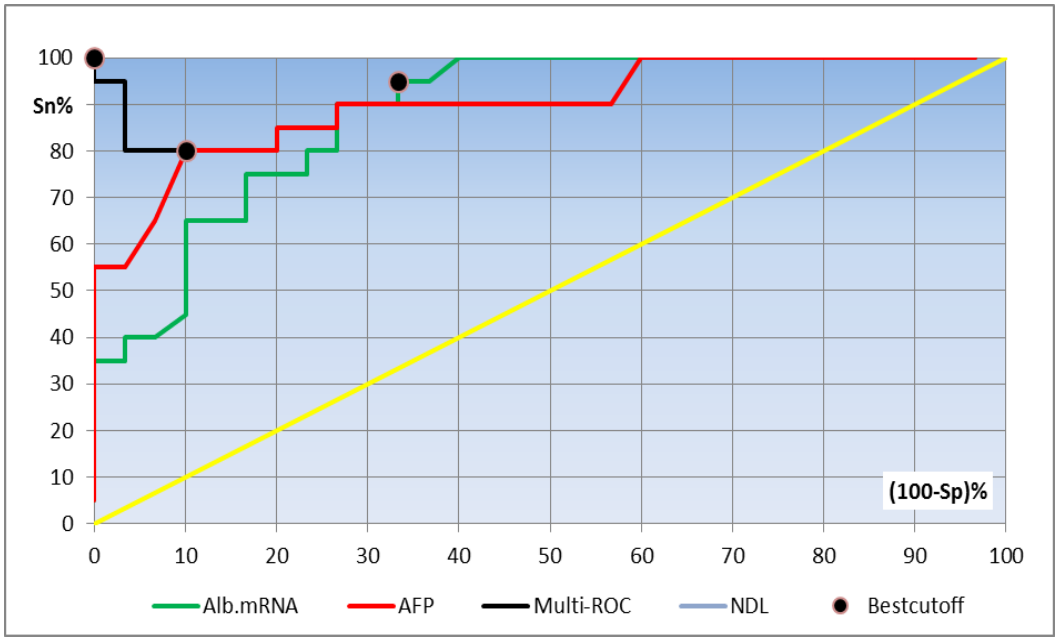
Variables	**Albumin mRNA**					
	CHC Patients			**HCC Patients**		
	r	**p**	**Sig.**	**r**	**p**	**Sig.**
ALT	-0.096	0.688	NS	0.053	0.826	NS
ALP	-0.007	0.977	NS	-0.152	0.523	NS
T.Bil	0.071	0.765	NS	0.411	0.072	NS
AFP	0.008	0.975	NS	-0.391	0.089	NS

Table (4): Diagnostic Performance of Albumin mRNA and AFP in Diagnosis of Hepatocellular Carcinoma:

Studied Variables	**Cut-off**	**Sensitivity**	**Specificity**
Albumin mRNA (copies/ml)	1.879	90%	73.3%
AFP (IU/ml)	5.5	80%	90%

Table (5): Multi ROC using AFP and Albumin mRNA in Diagnosis of Hepatocellular Carcinoma:

Studied Variables	**Sensitivity**	**Specificity**
Albumin mRNA (copies/ml) 91.13 & AFP (IU/ml) 5.5	100%	100%



AUC	
Alb.mRNA	0.922
AFP	0.864
Multi-ROC	1.000

Fig. (1): ROC curve analysis showing the diagnostic performance of Alb.mRNA, AFP and their combination for discriminating patients with HCC from non-HCC (CHC+Control)

3.2Discussion

There is much excitement about the possibility of developing blood-based tools for disease diagnosis and management through the analysis of circulating nucleic acids ***(Chan et al., 2003).*** Indeed, studies have reported the presence of circulating ALB mRNA, but with varying degrees of success ***(Wong et al., 2000).*** The liver being one of the largest organs of the body, suspect that RNA expressed by genes in the liver, such as ALB, should be detectable in the peripheral circulation because of cell death associated with typical cell turnover and/or with pathological damage ***(Lo and Chiu, 2007).***

Some researchers have suggested that Albumin mRNA in blood originates from malignant or non-malignant hepatocytes that have entered the peripheral circulation. For that, the present study was designed to evaluate the potential of circulating Albumin mRNAs as biomarkers of liver injury in chronic HCV and HCC in spite of normal ALT or AFP. The Real Time- PCR was used to quantify ALB mRNA in plasma of chronic HCV and HCC patients ***(Wong et al., 1997; Gion et al., 1998).***

The current study revealed that, A significant increase in ALB mRNA in plasma of chronic HCV and HCC patients more than controls, its level was significantly higher in HCC cases than in cases of chronic HCV. Our finding is in agreement of ***Chan et al. (2009)*** who demonstrated that, patients with HCC, cirrhosis, and active CHB (but not those with inactive hepatitis B carrier) had significantly higher plasma ALB mRNA concentrations than controls, and the ALB mRNA detected in plasma is liver specific. These data suggest that ALB mRNA

may be released into the plasma upon cell death, and therefore the concentration may correlate with the degree of cell death.

Serum aminotransferases have been the clinical standard for evaluating liver injury for the past 50-60 years. These tissue enzymes lack specificity, also tracking injury to other tissues. New technologies assessing tissue-specific messenger RNA (mRNA) release into blood should provide greater specificity and permit indirect assessment of gene expression status of injured tissue ***(Wetmore et al., 2010).***

A recent study by ***Chan et al. (2009)*** showed that, ROC curve analysis demonstrated plasma ALB mRNA measurement to be an attractive means for detecting the presence of liver pathology (91.9% of patients). In particular, for the HCC group, AFP was increased in only 48.6% of the cases, whereas the majority (91.4%) of these patients showed increased ALB mRNA concentrations.

The comparative analysis of circulating liver mRNAs with traditional serum transaminases and histopathology indicated that the circulating liver mRNAs were more specific and more sensitive biomarkers of liver injury ***(Wetmore et al., 2010).***

4.Conclusion

In summary, we conclude that, the measurement of plasma circulating albumin mRNA enabled sensitive and early detection of non-invasive diagnostic marker for hepatic injury in liver fibrosis and HCC compared with that of plasma ALT activity and serum AFP levels. Further studies are required, however, to investigate the clinical utility of this marker for assessing or managing such diseases. However, larger sample sets including long-term clinical data are urgently required for future studies.

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الملخص العربى

تقييم الحمض النووى الريبوزى الرسول كدلالة تشخيصية لمرضى فيرس س ومرضى سرطان الكبد

إجلال مريم ريموند صوايا١، إيمان صالح الحديدى٢، عمرو على محمد١، ولاء عبد الحميد محسوب١

١ جامعة عين شمس، كلية العلوم، قسم الكيمياء

٢ جامعة عين شمس، كلية الطب، قسم البثولوجية الاكلينيكية

التطور الاكلينيكى لمرضى فيروس الالتهاب الكبدى الوبائي (سي) بدون علاج يتغيربدرجة كبيرة مع غالبية المرضى فيعانون من التغير البطيء الذي قد يستغرق 20 سنة أو أكثر لظهور اعراض المرض كاملة. حوالي نصف المرضى الذين يصابون بالالتهاب الكبدي الوبائى (سي) المزمن النشط قد يتطورإلى تليف الكبد و سرطان الكبد.

التليف الكبدي هوتراكم النسيج الغشائى خارج الخلية نتيجة الى إصابة الكبد الحادة أو المزمنة.

وحيث ان التليف يمثل التئام الجروح نتيجة للإصابات ويؤدي في النهاية إلى تليف الكبد.

كلا من التليف والتشمع هي النتائج المترتبة على التئام الجروح كنتيجة لاصابة الكبد المزمنة لاسباب عديدة بما في ذلك الفيروسات، المناعة الذاتية، تاثير الادوية، الركود الصفراوي.

ان حدوث التليف الشديد والالتهابات في الكبد يؤدي إلى تليف الكبد والاصابة بسرطان الكبد. فيرس سى المزمن يختلف كثيرا في مساره ونتائجه. وجود الحمض النووى الريبوزى لفيرس سى يشير إلى أن المريض يعاني من عدوى فيروسية مستمرة على الرغم من ان مستويات انزيمات الكبد طبيعية. هناك فحوصات مصلية للكشف عن الأجسام المضادة التي تشير إلى الإصابة بفيروس **HCV** الحالي أو السابق، لكنها لاتستطيع أن تميز الحاد منها والمزمن ولا تستطيع حل المشكلة . أحيانا مرضى نقص المناعة، والمرضى الذين يخضعون لغسيل الكلى والمرضى الذين يعانون **cryoglobulinemia** مختلط يكون لها نتائج مصلية سلبية كاذبة، قد تتطلب الحمض النووى الريبوزى لفيرس سى للتشخيص. وحيث ان اخذ عينة من الكبد لا تزال هى الدعامة الأساسية في تشخيص التليف الكبدي، فان عصرها يبدو انه محدود فى ظل تحسن تكنولوجيا التصوير والمعامل.

وجود الحمض النووى الريبوزى الرسول خارج الخلية في الدورة الدموية، أي البلازما وسوائل الجسم الأخرى كان معروفا منذ فترة طويلة. و يعتقد ان الحمض النووى الريبوزى الرسول خارج الخلية يخرج إلى الدورة الدموية من الخلايا السليمة و كذلك الخلايا الميتة.

الأدوار البيولوجية لدورة الحمض النووى الريبوزى الرسول لاتزال غيرواضحة،على الرغم من أنه تم تحقيق أهميتها الفسيولوجية خلال السنوات القليلة الماضية.

كشف دورة الحمض النووى الريبوزى يقدم مزايا معينة على كشف دورة الحمض النووي. أولا، إذاتم خروج كل من الحمض النووى الريبوزى والحمض النووي من نفس الخلية، فان كمية الحمض النووي الريبوزى من المرجح أن تكون الكمية أكثر وفرة من الحمض النووي. وذلك لأن عدة نسخ من الحمض النووى الريبوزى قد تكون موجودة في كل خلية، اعتمادا على التعبير الجينى، في حين أن كل خلية تحتوي على نسخة واحدة فقط من الحمض النووي. ثانيا، ذكرت بعض الابحاث أن نسبة أكبر من حالات السرطان كانت إيجابية لدلالات الحمض النووى الريبوزى اكثر من الحمض النووي. وعلى ذلك فان تحليل الأحماض النووية المنتشرة في البلازما يوفر وسيلة لرصد مجموعة متنوعة من الظروف الفسيولوجية والمرضية بدون تدخل جراحى .

كشف ورصد إصابة أو خلل الكبد في المرضى المصابين بالتهاب الكبد بى وسى أصبحت ذات أهمية متزايدة. وحاليا تستخدم انزيمات الكبد مثل (**ALT**، **AST**) لتقييم الإصابة الكبدية، ومع ذلك، فمن المعروف جيدا أنها تفتقر إلى الدقة والحساسية، وتختلف مستوياتها في مختلف الافراد.

فان كشف انواع خلايا الحمض النووى الريبوزى الحرة بنجاح في البلازما اعطى لديهم إمكانية استخدامها في تقييم المرض. هناك الكثير من الأدلة التي تشير إلى أن الحمض النووى والحمض النووى الريبوزى يتم خروجهم من الخلية بعد موتها. في الحقيقة فقد وصفت تقارير الدراسات السابقة الكشف عن البومين الحمض النووى الريبوزى الرسول وجوده في الدم.

وقد كان لهذه الدراسات على مستويات مختلفة من النجاح، ولكن مع معدلات الكشف عن البومين الحمض النووى الريبوزى الرسول فى اكثر من 100٪ من المرضى الذين يعانون من سرطان الكبد، تليف الكبد، والتهاب الكبد أو من الاصحاء.