The Use of Serum Survivin and AFP-L3 and their Combination in Improving Hepatocellular Carcinoma Diagnosis Especially in Patients with Low Serum AFP

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Abstract

Background: Hepatocellular carcinoma (HCC) is responsible for about 4.7% of chronic liver disease in Egypt. Survivin is an essential element in apoptosis inhibition. Survivin is present in fetal and malignant adult tissue and is absent in non malignant tissues. A diagnostic and prognostic HCC marker is the alpha-fetoprotein (AFP) binding to Lens culinaris agglutinin (AFP-L3%) percentage. There is a need for HCC marker that is better than AFP especially in those low AFP.

Aim of Study: To detect the role of determination of serum Survivin gene, AFP-L3% and their combination in HCC diagnosis.

Subjects and Methods: Fifty HCC patients and 20 healthy control persons were subjected for measurement of serum AFP, survivin gene and AFP-L3%. Reverse transcription-polymerase chain reaction (RT-PCR) was used for detection of serum survivin gene. AFP-L3% was measured by a liquid-phase binding assay on the Wako LiBASys clinical auto analyzer.

Results: Serum Survivin gene was positive in 27 (54%) HCC cases and negative in 23 (46%) cases. AFP-L3% was detected in 36 (72%) HCC cases. The combined use of serum servivin gene and AFP-L3% were positive in 43 (86%) of HCC cases. In 22 HCC cases with serum AFP below 200 ng/ml serum servivin gene was detected in 9 (40.9%) cases but AFP-L3% was positive in 13 (59.9%) cases and the combined use of positive serum servivin and AFP-L3 were present in 18 cases (81.18%).

Conclusions: The combined use of serum survivin gene and AFP-L3% could have a valuable diagnostic role in HCC especially in those with low AFP.

Key Words: HCC – Survivin – AFP – AFP-L3 – Tumor markers.

Introduction

HEPATOCELLULAR carcinoma (HCC) is the third cause of cancer mortality worldwide. The

HCC incidence in Egypt had increased. HCC risk factors such as hepatitis C, B infection are present in Egypt Madden et al., [1]. Tumor markers are used for screening and early diagnosis of malignancies. Although, early HCC detection is still difficult because of absence of a suitable HCC marker that shows high sensitivity and specificity [2]. Survivin is essential in apoptosis inhibition. Survivin is expressed in most human neoplasms and is present in fetal and malignant adult tissue [3]. The inhibition of caspases are the main functions of survivin. This promotes mitosis and increases survival of cancer cells. It is responsible for resistance to chemotherapeutic drugs in HCC. The degree of survivin gene expression correlates with tumor aggressiveness and bad clinical outcome [4]. The improvement of AFP sensitivity can be increased by measuring the isoforms of AFP [5]. The percentage of AFP-L3 over the total AFP levels is used as a specific index for HCC. AFP-L3 could be used as a screening and diagnostic markers for HCC in high risk patients [6]. There is a need for an ideal tumor marker for HCC instead of depending on the AFP particularly in those with low serum level of AFP. The present study was designed to detect the value of measurement of serum Survivin gene and AFP-L3% and their combination in improving HCC diagnosis.

Patients and Methods

This study included 50 HCC patients and 20 healthy volunteers as a control. The diagnosis of HCC was done according to the American Association for the Study of Liver Diseases (AASLD) practice guidelines 2011 (one imaging technique (CT or MRI) showing the HCC radiological criteria (contrast uptake in the arterial phase and washout in the venous/late phase) of hepatic focal lesion

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more than one cm in a cirrhotic patient [7]. Those with past history of other malignancies and patients on chemotherapy whatever the etiology were excluded from this study. Samples of peripheral blood collected from HCC patients that had attended to National Liver Institute outpatient clinic-Menoufia University and another 20 samples from apparently healthy volunteer subjects with no viral hepatitis infection and HCC.

All patients and control groups were submitted to:

Laboratory investigations:

Under complete aseptic technique; 10ml of blood were obtained from each participant. These were samples used for estimations of CBC, AST, ALT, Bilirubin, albumin, creatinine and INR. Serological markers for viral hepatitis: (HBsAg, anti-HCV Ab) using ELISA supplied by Sorine-Italy. Serum Alpha fetoprotein was determined by kits from Bayer (Germany) using ACS: 180 (automated chemiluminenscene system). SerumAFP-L3% was measured by a liquid-phase binding assay on the Wako LiBASys clinical auto analyzer. Those with AFP-L3% above 10% were considered positive (having HCC) and those below 10% were considered negative (No HCC).

Molecular studies for survivin gene:

Survivin gene was measured by Reverse transcription polymerase chain reaction (RTPCR) and the test results are either positive or negative according to Bo et al., [8]. One mL of Peripheral blood samples were collected in EDTA, then nucleated cells were isolated for RNA extraction and PCR analysis by using QIA amp RNA extraction kit. Primers for Survivin and B actin amplification (Using RT-PCR kits: Qiagen (Catalog number: 12110007. Primer-1 (sense strand primer) for Survivin The survivin mRNA Genbank accession number is NM001168. It had the following sequence: 5'-ACCAGGTGAGAAGTGAGGGA-3'. primer-2 (Anti-sense strand primer) for Survivin 5'-AACAGTAGAGGAGCCAGGGA-3'. For B actin amplification, the B actin mRNA Genbank accession number is NM-001101 Primer-1 (sense strand primer). It had the following sequence: 5'TGACGGGGTCACCCACACTG3'. Primer-2 (Anti-sense strand primer): 5'CTAGAAGCA TTT-GCGGTGGA 3'. All reaction tubes incubated at 55ºC for 30 minutes for c-DNA synthesis at thermal cycler then, incubated at 95ºC for 15 minutes to inactivate the reverse transcriptase and to completely denaturate the template. For PCR amplification of Survivin, denaturation: 94ºC for 60 sec, annealing: 59ºC for 60sec, polymerization: 72ºC for 60sec, final extension 72ºC for 10min, this was

repeated for a total of 32 cycles. For PCR amplification of B- actin, denaturation: 94ºC for 60 sec. annealing: 55ºC for 60sec. polymerization: 72ºC for 1min, followed by a final extension cycle at 72ºC for 10min. Amplified material was stored at –20ºC until gel electrophoresis was performed using their different molecular weight (308bp for Survivin and 600bp for B-actin).

Radiological investigations:

Abdominal ultrasound was done and those with hepatic focal lesion were subjected to triphasic spiral CT or MRI and patients with HCC criteria mentioned before were selected and included in this study.

Statistical analysis:

Statistical analysis was performed using SPSS version 17.0 software. Results are expressed as mean $\pm$ standard deviation (SD), and the paired-sample t-test to analyze for each group. The results were considered statistically significant if the $p<0.05$, and highly significant if less than 0.001.

Results

Fifty HCC patients and 20 control persons were included in this study. Demographic data and clinicopathological features of the studied groups were shown in Tables (1,2). Fifty HCC patients, had ages ranged between 39-65 years with mean age 50±9 years. Twenty healthy controls, with ages ranged between 34-63 years with mean age 39 $\pm$ 10 years were included as regard to gender distribution between the two groups, in HCC group 35 (70%) of cases were males and 15 (30%) were females, and in control 13 (65%) were males. In case of serum AFP, there was a statistically significant difference in the different groups with ($p<0.001$), where the lowest serum AFP level was detected in the control group (2.59±1.63) and AFP in HCC group was (3625±1219) Table (2). Serum Survivin gene detected by RT PCR in 27 (54%) HCC cases and negative in 23(46%) cases. Survivin gene was not detected in any case of the control group Table (3), Fig (1). There was a statistically significant between the different groups in serum survivin gene and AFP-L3. The level of AFP-L3% above 10% was detected in 36 (72%) of HCC cases. The positivity of serum servivin gene and AFP-L3% were present in 43 (86%) HCC cases Table (3). In our study, there were 28 (56%) HCC patients with serum AFP >200ng/ml and 22 (44%) HCC patients with AFP ≤200ng/ml. In HCC cases with serum AFP above 200ng/ml, serum Servivin gene was detected in 18 (64.28%) cases but AFP-L3% above 10% was positive in 24 (85.71%) cases and the

combined positivity of serum servivin gene and AFP-L3 above 10% were positive in 25 cases (89. 28%) Table (4). In HCC cases with serum AFP below 200ng/ml serum servivin gene was detected in 9 (40.9%) cases but AFP-L3% was positive in 12 (54.54%) cases and the combined positivity of serum Servivin gene and AFP-L3 above 10% were positive in 18 cases (81.18%) Table (4).

Table (1): Demographic data of the studied groups.

	Control group	HCC group
Age:		
Mean ±SD	34–63	50±9
Range	39±10	39–65
Gender:		
Male	13 (65%)	35 (70%)
Female	7 (35%)	15 (30%)
DM	0 (0%)	9 (18%)
Hypertension	0 (0%)	5 (10%)
Smoking	0 (0%)	10 (20%)

Table (2): Control group versus HCC group in relation to different studied variables.

Studied variables	Control group (N=20) Mean ± SD	HCC group (N=50) Mean ± SD
Hb %	13.6±1.04	11.1±1.75
TLC	7756.7±1029.13	5937.2±3754.5
Platelets	267133.3±53772	148666.7±917114
AST (IU/ml)	18.4±4.07	98.06±52.9
ALT (IU/ml)	24.4±5.96	58.7±37.2
T.Bil (gm/dl)	0.68±0.13	4.03±6.07
Alb (g/dl)	4.28±0.37	2.81±0.82
Prothrombin concentration	92.1±6.42	63.2±18.6
HCV Ab positive	0%	48 (96%)
HBsAg positive	0%	2 (4%)
Portal vein diameter	10.07±18	14.8±1.7
AFP (ng/ml)	2.59±1.63	3625±1219

Table (3): Correlation between serum Survivin gene expression by RT-PCR and AFP-L3% in the studied groups.

Parameters	Controls (n=20)	HCC Cases (n=50)	*p*-value
Survivin:			
–ve	20 (100%)	23 (46%)	<0.001***
+ve	0 (0%)	27 (54%)	
AFP-L3%:			
≤ 10% (–ve)	0 (0%)	14 (28%)	<0.001***
>10% (+ve)	(0%)	36 (72%)	
Combined Survivin and AFP-L3%:			
–ve	20 (100%)	7 (14%)	<0.001***
+ve	0 (0.0%)	43 (86%)	

The combination was used in parallel .

Table (4): Survivn gene and AFP-L3% in relation to AFP level (>200 and ≤200ng/ml) in HCC group.

Parameters	AFP >200ng/ml (N=28) (56%) No.	AFP >200ng/ml (N=28) (56%) %	AFP ≤200 ng/ml (N=22) (46%) No.	AFP ≤200 ng/ml (N=22) (46%) %	*p*-value
Survivin gene:					
–ve	10	35.71	13	59.9	<0.09
+ve	18	64.3	9	40.90	
AFP-L3%:					
≤ 10% (–ve)	4	14.28	10	45.45	<0.01**
>10% (+ve)	24	85.71	12	54.54	
Combined Survivin and AFP-L3%:					
–ve	3	10.71	4	18.18	<0.68
+ve	25	89.28	18	81.18	

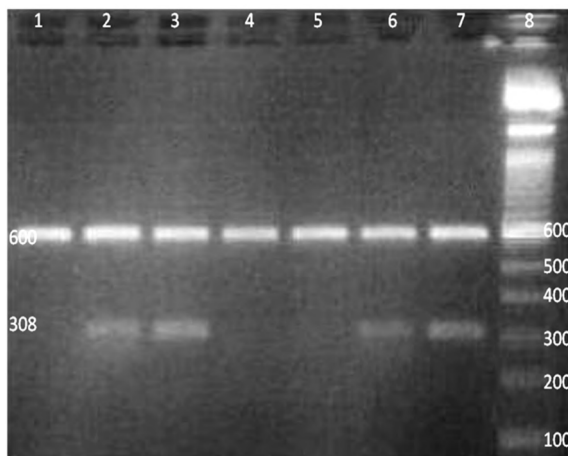FE=Fisher's Exact test. The combination was used in parallel.

Fig. (1): Detection of surviven gene expression, electrophoresis separation of survivin (308 bp) and single copy gene B-actin (600 bp) RT-PCR amplified fragments on 2% agarose gel. Lane 1 control case for surviven expression, Lane 2,3,6 and 7 HCC tumors positive for surviven expression, Lane 4 and 5 HCC tumors negative for surviven expression. Lane 8: 100 bp molecular weight marker.

Discussion

Cancer is the second leading cause of mortality after cardiovascular diseases Madden et al., [1]. Prognosis of HCC is worse; thus early diagnosis of HCC is precious especially in patients with chronic liver disease and cirrhosis Yang and Poon, [9]. Early detection of hepatocellular carcinoma is still difficult because markers of HCC had no acceptable sensitivity and specificity Elshemey et al., [10]. In our study, the prevalence of HCC in men is more than women with a male to female ratio of about 2:1. This finding is in accordance with Sherman, [11]. Exposures to risk factors are more common in males [12]. In the present study the mean age in HCC group was 50 ± 9 years. This matches with the known evidence that the incidence of HCC increases over the age of 45 and continues to increase till the seventies [11]. Survivin is one of the eight proteins involved in inhibition of apoptosis. It is detected in neoplastic adult tissues. Survivin is essential in tumour formation / progression, especially angiogenesis [13]. The Lens culinaris agglutinin-reactive fraction of alpha–fetoprotein (AFP-L3) is considered as a sensitive and a specific tumor marker for HCC. Moreover, it can be used an indicator of the prognosis of patients with HCC [14]. HCC is almost present in cirrhotic HCC patients with AFP-L3 above 10% even when imaging techniques cannot identify tumors [15]. The aim of this study is to detect the diagnostic value of combined measurement of serum Survivin gene and AFP-L3% in HCC diagnosis especially in patients with low serum AFP. In our study the mean serum AFP levels in control and HCC groups were 2.59 ± 1.63 and 3651 ± 1219ng/ml respectively with a statistically significant difference between the studied groups. This is in agreement with Leerapun et al., [14]. Serum AFP level above 200ng/ml was considered diagnostic for HCC in cirrhotic patients with hypervacular hepatic focal lesion on imaging according AASLD guidelines in 2005 Bruix and Sherman, [16]. In our series, AFP more than 200ng/ml was detected in 28 (56%) of HCC. AFP below 200ng/ml was present in 22 (44%) HCC cases. AFP could be normal in about 40% of HCC patients Bruix and Sherman, [16]. In the present study, serum survivin is positive in 27(54%) of HCC specimens. The survivin gene was expressed in a most of HCC tissues but not in liver cirrhosis tissues which indicates survivin gene may have a role in development and diagnosis of HCC. Chiu et al., [17] showed that Survivin is one of the pathogenic markers involved in HCC recurrence after living donor liver transplantation as Oncogenes is present in new graft and in the peripheral blood. Furthermore, Hung et al., [18] found

that the extent of HCC malignancy is strongly associated with survivin expression. They suggest that analyzing survivin expression in HCC tumors before treatment can identify HCC patients who would benefit from molecular targeting- survivin agents [19]. In the present study, AFP-L3%>10% increased significantly in HCC group compared to control and considered as diagnostic for HCC. The level of AFP-L3 above 10% was detected in 38 (76%) of HCC cases. AFP-L3% above 10% in HCC patients with AFP values less than 200ng/ml was present in 12 cases and this allows the confident diagnosis of an additional 24% of HCC cases. This is in agreement with Leerapun et al., as this allowed the additional diagnosis of 10% of HCC cases in their study using AFP-L3 more than 35%. In an attempt to enhance the HCC detection rate, we investigated the use of combined markers. The combined use of Serum servivin gene and AFP-L3% were present in 43 cases (86%) of HCC cases. In 22 HCC cases with serum AFP below 200ng/ml, serum Servivin gene was detected in 9 (40.9%) cases but AFP-L3% more than 10% was positive in 12 (54.54%) cases. So in our study, the determination of combined serum AFP-L3% and survivin gene improved HCC diagnostic value. Studies suggested the combined use of more than one tumor marker for HCC allowed the improvement in HCC diagnosis. For example, the use of combining AFP, AFP-L3, and PIVKA-II improve the diagnostic accuracy of HCC in cirrhotic patients compared with using each marker alone [20]. Hann et al., [21] reported that the combined use of AFP, AFP-L3% and DCP in HCC diagnosis is associated with increased diagnostic value mainly in individuals with negative imaging results. Finally it could be concluded that the combined use of positive serum AFP-L3% and survivin gene were detected in 86% of HCC cases However in those with serum AFP below 200ng/ml, they were present in 81.1% of HCC cases. Thus the combined use of serum Survivin gene and AFP-L3% as tumor marker for HCC is better than the use of one marker alone particularly in those with low serum AFP and in conclusion imaging techniques as CT, MRI, further research studies are required to detect the impact of different treatment modalities for HCC on serum survivrin gene and AFP-L3%.

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أستخدام قياس جين سرفيفين ونسبة الفا فيتوبروتين -ل٣
بالدم المتلازمين فى تشخيص مرضى سرطان الكبد
الذين لديهم مستوى متدنى من
الفا فيتوبروتين أقل من ٢٠٠ نانو جرام/مل

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يمثل سرطان الخلايا الكبدية ٤.٧٪ من أمراض الكبد فى مصر، وجين سرفيفين هو عنصر مهم فى منع الموت الخلوى المبرمج وموجود فى الخلية الجينية والخلية الخبيثة ولا يتواجد فى الخلية الغير خبيثة ويعتبر الفا فيتوبروتين من أحد المؤشرات التشخيصية والتنبؤية بأورام الكبد وفى الوقت الحالى يوجد حاجة لوجود مؤشر تنبؤى عن سرطان الخلايا الكبدية أفضل من الأعتماد على الفا فيتوبروتين وخصوصاً فى المرضى الذين لديهم الفا فيتوبروتين بمستوى متدنى. يهدف البحث إلى قياس مستوى تشخيص سرطان الكبد بإستخدام جين السرفيفين ونسبة الفا فيتوبروتين-ل٣ بالدم المتلازمين أو كلا على حدى.

وأشتملت هذه الدراسة على خمسين مريض مصابون بسرطان الكبد، وعشرون شخصاً أصحاء ويعتبرون كمجموعة ضابطة. وتم أستبعاد المرضى المصابون بسرطان آخر غير سرطان الكبد، والمرضى الذين يتناولون علاج كيماوى لأى سبب من الأسباب وخضع هؤلاء إلى قياس الفا فيتوبروتين وجين السرفيفين الفا فيتوبروتين -ل٣ بالمصل تبين من هذه الدراسة وجود زيادة ذو دلالة احصائية فى مستوى كل من الفا فيتوبروتين وجين السرفيفين ونسبة الفا فيتوبروتين -ل٣ فى مرضى سرطان الكبد، كان التعبير الجينى للجين سرفيفين بالمصل (٥٤٪) من الحالات، بينما الفا فيتوبروتين -ل٣ بنسبة أكثر من ١٠٪ كان إيجابياً فى ٧٢٪ من الحالات، فى حالات سرطان الكبد وجد أن إيجابية جين السرفيفين بالمصل والفا فيتوبروتين -ل٣ بنسبة أكثر من ١٠٪أحدهم أو كلاهما مجتمعين موجودين فى ٨٦٪ من حالات سرطان الكبد، و لقدوجد أيضاً ايجابيين فى ٨١٪ من الحالات التى يوجد بها الفا فيتو بروتين أقل من ٢٠ نانو جرام/مللى. بناء عليه يمكن القول بأن أستخدام أكثر من نوع من دلائل الأورام المذكورة سابقاً لهم قيمة تشخيصية عالية لأورام الكبد السرطانية وخصوصاً أولئك الذين نسبة الفا فيتو بروتين بقى متدنية أقل من ٢٠٠ نانوجرام/مللى.