Blood Ras-Association Domain Family 1 A Gene Methylation Status In Some Liver Diseases

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ABSTRACT: BACKGROUND: Hepatocellular carcinoma (HCC) is one of the most common human malignancies and its impact on mortality is significant and well documented. Biomarkers have been developed for early HCC detection, with serum α-fetoprotein (S.AFP) being the most widely used clinically, but with relatively low diagnostic sensitivity. Therefore new biomarkers are needed for early HCC detection to improve overall-survival rates. **METHODS:** Blood RASSF1A promoter methylation was evaluated using methylation specific PCR in patients with chronic liver diseases together with its potential use as a biomarker for detecting HCC in comparison to or in association with S.AFP. **RESULTS:** Blood RASSF1A promoter methylation was detected in 70% of HCC patients on top of hepatitis C virus- associated liver cirrhosis, 28.5% of hepatitis C virus-associated liver patients and 16.6% of bilharzial liver fibrosis patients. However none of the healthy control subjects showed blood RASSF1A promoter methylation. The sensitivity, specificity, PPV and NPP of blood RASSF1A promoter methylation for HCC diagnosis were 70%, 83.3%, 73.7% and 80.6% respectively. On the other hand the sensitivity, specificity, PPV and NPP of S.AFP with a cut off value of 33.6 for HCC diagnosis were 85%, 80%, 88.9% and 90.7% respectively. Moreover it was found that the combined use of RASSF1A promoter methylation status and S.AFP is better than S.AFP use alone in HCC prediction. **CONCLUSION:** RASSF1A promoter methylation plays an important role in the process of human hepatocarcinogenesis and is related to hepatic inflammation due to bilharziasis and viral hepatitis. Moreover it can be considered as an important biomarker for the diagnosis of HCC when combined with S.AFP.

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies and is a leading cause of cancer-related death worldwide[(1)]. In Africa, liver cancer has been ranked as the fourth common cancer, and most of liver cancers are HCC[(2)]. HCC incidence has doubled in Egypt in the past 10 years[(3)].Most HCC patients in African and Asian populations exhibit chronic hepatitis or cirrhosis caused by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)[(4)]. Of all the HCC cases, it is estimated that 66% are attributable to HBV and 42% are attributable to HCV, assuming that the relative risk of disease in both carriers is 20. However, in North Africa, infection with HBV is less common than in other regions. In Egypt, the prevalence of HCV infection is one of the highest in the world. HBV was found at high rates in Egypt, but after increase in HCV prevalence the rates of HBV declined[(2)]. In addition, HBV rates have declined after the introduction of the vaccine in 1992[(3)]. By contrast, the relative importance of the etiologic effect of schistosomiasis on liver cancer is still inconclusive. Schistosomiasis per se may cause the persistence of viremia due to reduced immunity or could play a minor role in the pathogenesis of HCC as a result of the copper sulfate sprayed in canals for snail control[(2)]. Therefore, schistosomiasis can be treated as a covariate in multivariable analysis for HCC.

Despite numerous advances in the treatment of HCC during the last decade, the 5-year survival rate remains <40% and late presentation remains an important obstacle to successful treatment. In fact, many HCC patients have already developed locally advanced disease or distant metastasis by the time of presentation. In this regard, biomarkers have been developed for early HCC detection, with serum α-fetoprotein (S.AFP) being the most widely used clinically[(5)]. However, its use in the early detection of HCC is limited, especially because about one-third of patients with HCC have normal levels of S.AFP. Serum AFP is a marker that has low sensitivity and high specificity[(6)]. Therefore, new biomarkers for early HCC detection are needed to improve overall-survival rates.

HCC might be related to genetic or epigenetic alterations. Hypermethylation of tumor suppressor genes is frequently observed in HCC[(7,8)]. Such

epigenetic changes are potential markers for detecting and monitoring HCC. Recently, methods for the detection of circulating hypermethylated DNA sequences were developed[9].

RASSF1A (Ras association domain family 1 isoform A) is a recently discovered tumor suppressor gene located within 3 P21.3 locus. RASSF1A protein modulates multiple apoptotic and cell cycle checkpoint pathways which are commonly deregulated in cancer. It is mostly inactivated by transcriptional silencing of the gene by inappropriate promoter methylation in many cancers including HCC [10].

Our study was designed to evaluate the biochemical changes of blood RASSF1A promoter methylation in patients with chronic liver diseases including bilharziasis, HCV- associated liver cirrhosis as well as HCC on top of HCV- associated liver cirrhosis and its potential use as a marker for detecting HCC in comparison to or in association with S.AFP.

2. SUBJECTS AND METHODS

Our study was conducted on 56 subjects selected from internal medicine department, Benha University Hospitals. They were categorized into 1- *Control group*; consisted of 10 healthy volunteers 2- *Bilharzial liver fibrosis group*; consisted of 12 bilharzial liver fibrosis patients, free of HCV or HBV infection. *3- Liver cirrhosis group*; consisted of 14 HCV- associated liver cirrhosis patients, free from bilharziasis. 4- *HCC group*; consisted of 20 HCC patients on top of HCV- associated liver cirrhosis and free from bilharziasis. HCC was diagnosed by S.AFP, abdominal ultrasound & spiral CT with or without liver biopsy. Patients with hepatic metastasis due to other malignancies or with HBV infection were excluded from the study. Approval of the ethical committee was obtained and written informed consents were taken from all subjects of the study.

Sample collection and preparation:

Venous blood sample (10 ml) was withdrawn from patients and control. The blood samples were divided into two parts:

- The 1st part was left to clot, centrifuged and the sera were separated for measurement of S.AFP levels by enzyme linked fluorescent assay using VIDAS AFP kits supplied by bioMerieux sa,France, Bilharzial antibody titre by indirect haemagglutination using schistosomiasis fumouze kits supplied by Fumouze Diagnostics, France and HCV antibody test and hepatitis b surface antigen by immunochromatographic analysis using ACON® HCVone step test and ACON® HBsAg one step test respectively supplied by ACON Laboratories Inc..
- The 2nd part: was applied into EDTA containing vacutainer and stored at – 80°C for later detection of methylation status of RASSFIA gene by methylation specific PCR (MSP) technique.

A- DNA extraction:

100 µl of eluted DNA was extracted from 200 µl of anticoagulated blood using the "Axy prep blood genomic DNA mini prep kit" supplied by Axygen biosciences.

B- Bisulfite treatment (DNA modification):

Bisulfite modification of genomic DNA would convert unmethylated cytosine residues into uracil residues. Conversely, methylated cytosine residues would remain unmodified. Thus, methylated and unmethylated DNA sequences would be distinguishable by using sequence-specific PCR primers[11]. Bisulfite modification was conducted using the EZ DNA methylation-Gold™ kit. 10 µl of eluted modified DNA was obtained from 20µl of DNA sample. The eluted modified DNA was stored at-70°C as modified DNA is fragile like RNA for subsequent MSP**.**

C-Polymerase chain reaction (PCR):

Bisulfite-modified DNA was amplified using primers specific for the methylated sequence 5′-GTGTTAACGCGTTGCGTATC-3′ and 5′-AACCCCG CGAACTAAAAACGA-3′ together with primers specific for the unmethylated sequence 5′-TTTGGTTG GAGTGTGTTAATGTG-3′ and 5′-CAAACCCCACA AACTAAAAACAA-3′ [11].

Amplification of the methylated and the unmethylated sequences was done using the GeneAmp DNA Amplification Kit and AmpliTaq Gold polymerase (Applied Biosystems, Perkin-Elmer, Foster City, CA). The optimized thermal profile included initial denaturation at 95°C for 12 min., followed by 45 cycles of 95°C for 45 sec., 54°C for 45 sec., 72°C for 1 min., and a final extension at 72°C for 10 min..

D- Detection of amplified PCR product by Agarose gel electrophoresis:

The amplification products were analyzed by agarose gel electrophoresis in 2٪ agarose gel, and ethidium bromide staining and photographed by Polaroid camera under UV light.

STATISTICAL ANALYSIS

The collected data were computed and statistically analyzed using SPSS version 17 software. Suitable statistical techniques were calculated as mean, ±SD. ANOVA & Z tests were used as tests of significance. ROC curve was used to predict cutoff values of S.AFP with the optimum sensitivity and specificity of S.AFP & RASSF1A promoter

methylation for diagnosis of different liver diseases. In addition, logistic regression analysis was done to predict the equation that determines the probability of being HCC by S.AFP and RASSF1A promoter methylation.

Logit(Y) = $b_0 + b_1x_1 + b_2x_2 + \ldots\ldots$

(Probability >0.5 indicates that the test can predict the disease and the maximum of probability is being one).

Moreover spearman correlation coefficient was estimated to correlate the S.AFP and DNA methylation. P values less than 0.05 were considered significant.

3. RESULTS

The 56 subjects included in the study were 32 males and 24 females .The *control group* consisted of 4 males and 6 females with age ranging from 22 - 48 years and mean value of 35 ± 9.5, while the *bilharzial liver fibrosis group* consisted of 8 males and 4 females, with age ranging from 23 – 50 years and mean value of 32 ± 11.8. Moreover *the liver cirrhosis group* consisted of 7 males and 7 females, with age ranging from 47 – 80 years and mean value of 56.07 ± 9.2. In addition, *HCC group* consisted of 13 males and 7 females with age ranging from 40- 75 years and mean value of 59.35 ± 9.01.

S.AFP level was measured in the different study groups and it was found to be significantly elevated in the HCC group compared to the other study groups. Cut off values were determined for S.AFP in the different study groups (table 1) to determine the percentage of positive and negative S.AFP (Table 2). Also the percentage of positive and negative RASSF1A promoter methylation in the different study groups was determined (Table 2). Moreover our study showed that there was a positive significant correlation between S.AFP level and DNA methylation (r = 0.49, P<0.001).

Table (1): The mean, ± SD and cutoff values of S.AFP in the different study groups

Parameter / Group	S.AFP (ng/ml)					
	Mean ± SD	ANOVA test	P value	Post hoc (Bonferroni test)	P value	**Cut off value**
Control	3.45 ± 1.21	22.6	< 0.001	Control#HCC	< 0.001	4.55
Bilharzial liver fibrosis	5.30 ± 1.86			Fibrosis#HCC	< 0.001	6.4
Liver cirrhosis	31.42 ± 12.21			Cirrhosis#HCC	< 0.001	16.5
HCC	118.56 ± 67.30					33.5

Table (2): The percentage of positive and negative S.AFP and RASSF1A promoter methylation in the different study groups.

Parameter / Group	S.AFP							RASSF1A promoter methylation						
	Negative		**Positive**		**Z1 P**	**Z2 P**	**Z3 P**	**Negative**		**Positive**		**Z1 P**	**Z2 P**	**Z3 P**
	n	%	n	%				n	%	n	%			
Control (n=10)	8	80	2	20				10	100	0	0			
Bilharzial liver fibrosis (n=6)	8	66.7	4	33.3	0.68 >0.05	3.77 <0.001		10	83.3	2	16.7	1.36 >0.05	2.9 <0.001	
Liver cirrhosis (n=14)	1	7.1	13	92.9	3.6 <0.001	0.7 >0.05	3.2 <0.001	10	71.4	4	28.6	1.89 <0.05	2.4 <0.01	0.72 >0.05
HCC (n=20)	3	5	17	95	3.5 <0.001			6	30	14	70	3.6 <0.001		

n: number of cases Z1 versus control Z2 versus HCC Z3 versus bilharzial liver fibrosis

Table (3): Cut off values, sensitivity, specificity and predictive values of S.AFP and RASSF1A promoter methylation in the diagnosis of different liver diseases.

Group	Test	Cut off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	P value	95% confidence interval
Control (n=10)	**•S.AFP** **• RASSF1A Methylation**	≤ 4.55 –	80 100	95 52.5	80 34.5	95 100	96 73.8	< 0.001 <0.05	0-1 0.6-0.88
Bilharzial liver fibrosis (n=12)	**•S.AFP** **• RASSF1A Methylation**	6.4 –	66.7 83.3	70.5 59.1	23.5 21.7	93.9 96.3	78 62.1	<0.05 >0.05	0.66-0.91 0.4-0.84
Liver cirrhosis (n=14)	**•S.AFP** **• RASSF1A Methylation**	16.5 –	92.9 28.6	52.8 58.3	43.3 76	95 67.7	52.8 43.5	>0.05 >0.05	0.37-0.69 0.26-0.61
HCC (n=20)	**•S.AFP** **• RASSF1A Methylation**	33.5 –	85 70	80 83.3	73.9 73.7	88.9 80.6	90.7 76.7	< 0.001 < 0.01	0.79-1 0.62-0.91

According to the results of our study (table 3), bilharzial liver fibrosis is diagnosed with S.AFP level >6.4-16.5. In addition, liver cirrhosis is diagnosed with S.AFP level > 16.5- 33.5, while HCC is diagnosed with S.AFP level above 33.5. The sensitivity and specificity of S.AFP level as a diagnostic test for these diseases are shown in table (3). Also the sensitivity and specificity of RASSF1A promoter methylation as a diagnostic test for these diseases are shown in the same table.

According to the results of the logistic regression analysis:

●Logit (HCC) = -2.08 + 3.1 (S.AFP positive). So When S.AFP alone is positive the probability of the case to be HCC = 0.58.

●Logit (HCC) = -3.06 + 3.08 (S.AFP positive) + 2.4 (RASSF1A promoter methylation positive). So when both S.AFP and RASSF1A promoter methylation are positive, the probability of the case to be HCC = 0.82.

Also, according to the odds ratio calculated; it was found that individual with positive S.AFP is 21.7 times more likely to have HCC. Moreover, the individual with positive RASSF1A promoter methylation is 11.1 times more likely to have HCC (Table 4). The PCR products on the agarose gel are shown in figure (1).

Table (4): Odds ratios calculated for positive S.AFP and RASSF1A promoter methylation tests.

Test	Odds ratio	P value	95% confidence interval
Positive S.AFP	21.7	0.001	3.7-127.4
Positive RASSF1A methylation	11.1	0.007	1.9-64.3

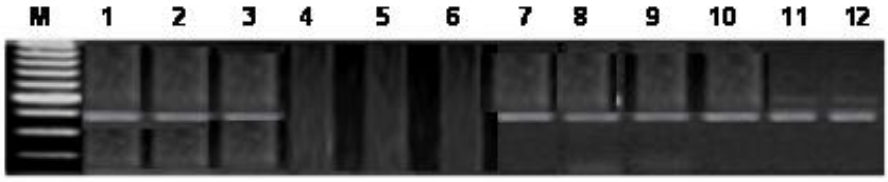

Figure (1): verification of the PCR products on agarose gel: Lane M shows PCR marker (100bp), lanes 1, 2, 3, 7, 8, 9, 10, 11 and 12 show bands of RASSF1A promoter methylation of **300 bp** length while lanes 4, 5 and 6 are negative for RASSF1A. Promoter methylation.

4. DISCUSSION

Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes results in transcriptional silencing of these genes and genomic instability. CpG hypermethylation acts as an alternative and/or complementary mechanism to gene mutations causing gene inactivation, and it is now recognized as an important mechanism in carcinogenesis. Although the mechanisms responsible for CpG island hypermethylation in cancer are poorly understood, it has been hypothesized that epigenetic silencing depends on activation of a number of proteins known as DNA methyltransferases that possess de novo methylation activity[12]. A growing number of genes have been reported to undergo CpG island hypermethylation in HCCs, which indicates the potential role of CpG island hypermethylation in hepatocarcinogenesis[13].

RASSF1A hypermethylation has been detected frequently in the tissues of different cancers including HCC[14]. Tissue RASSF1A promoter methylation has been documented in 85%[15], 100%[16], 93%[7] and 66.7%[13] of HCC. Moreover, it was detected slightly less frequently in the hepatitic / cirrhotic tissue adjacent to HCC, ranging from 70%[15] to 82.75%[16].On the other hand, absence of RASSF1A promoter methylation was detected in the non neoplastic hepatitic / cirrhotic tissue far from tumors[13,15], hepatitic tissue with or without cirrhosis in patients with absent HCC [13] and normal liver tissue in patients without HCC[15,16]. This indicates that RASSF1A promoter methylation occurs as an early event in hepatitis and cirrhosis before the development of HCC and is closely linked to hepatitis and cirrhosis in hepatocellular carcinoma patients. This suggests that it plays an important role in the human hepatocarcinogenesis. In addition, RASSF1A promoter methylation has been documented in different body fluids of different cancers including the serum in HCC which indicated its value for the early-stage diagnosis of tumors[14]. All of these data indicates that RASSF1A promoter methylation may play a role as a potential marker for cancer risk assessment and early detection of human HCC as like any ideal biomarker, it appears early in the course of disease and is detectable in biological samples that can be obtained noninvasively. This is in spite of the unclear exact mechanism of how the tumor DNA enters systemic circulation.

This study had evaluated RASSF1A promoter methylation in the blood of three types of chronic liver diseases including bilharziasis, HCV associated liver cirrhosis as well as HCC on top of HCV associated liver cirrhosis. Methylation was evaluated using MSP technique. MSP is the most widely used technique for studying the methylation of CpG islands of genes and is one of the most effective choices for investigating the methylation profile of these regions[17].

According to the results of our study, there was a significant elevation in the percentage of positive blood RASSF1A promoter methylation in the HCC group (70%) in comparison to the healthy control group (0%), bilharzial liver fibrosis group (16.6%) and the liver cirrhosis group (28.5%). RASSF1A promoter methylation was also significantly higher in the liver cirrhosis group compared to the healthy control group and non significantly higher compared to the bilharzial liver fibrosis group. Furthermore, RASSF1A promoter methylation was non-significantly higher in bilharzial liver fibrosis than in the control group.

RASSF1A promoter methylation was detected in the serum of 31%[14] and plasma of 44.1%[18] of HCC patients on top of HBV- associated liver cirrhosis compared to 79.3%[14] and 91.2%[18] in the corresponding cancerous tissue of the same patients using MSP. Moreover, serum RASSF1A hypermethylation was reported in 70% of HCV associated HCC and 80% of HBV associated HCC cases using MSP[19]. On the other hand, No RASSF1A methylation was detected in the serum[14] or plasma[18] or peripheral blood mononuclear cells[20] of 10 healthy blood donors in each study. However RASSF1A promoter methylation was reported in three out of 35 control subjects, but two or three of these subjects(as the authors did not clarify) had either hepatitis B virus or/and HCV infections; one subject had a history of smoking and alcohol drinking. They explained the hypermethylation in serum DNA from controls was perhaps due to hepatitis virus infection and chemical carcinogen exposure and that another possibility was that some normal controls have cryptogenic hepatic cirrhosis[19].

Hypermethylated RASSF1A sequences were detected in the sera of 93% of HCC patients mostly on top of HBV infection(89%), 58% of HBV carriers, and 8% of the healthy volunteers using real-time PCR after digestion with a methylation-sensitive restriction enzyme. The sensitivity of this technique is higher than MSP [5]. This explains the higher RASSF1A methylation frequency in HCC patients (93%) to approach that of tissues detected by other studies[7,15,16,18]. It also explains the higher RASSF1A methylation frequency in HBV carriers and the presence of RASSF1A methylation in 8% of the healthy volunteers. These results are higher than our study and the previous studies using MSP[14,18,19] or using bisulfite sequencing and PCR-RFLP[20]. The lower sensitivity of MSP or bisulfite sequencing and PCR-RFLP is due to substantial degradation of DNA (up to 96%) caused by the bisulfite conversion step. However with using real-time PCR after digestion with

a methylation-sensitive restriction enzyme, there is specific degradation of unmethylated sequence by the methylation-sensitive restriction enzyme, in contrast to the nondiscriminatory degradation of both methylated and unmethylated DNA with bisulfite conversion[21].

The absence of RASSF1A methylation in the peripheral blood mononuclear cells[20] indicates that the source of RASSF1A methylation in the blood of the HCC, liver cirrhosis and bilharzial liver fibrosis groups using MSP in our study is not from the peripheral blood mononuclear cells but from diffusion from the liver tissue into the blood.

The development and progression of HCC is a multistep process whereby the normal hepatocytes undergo inflammation, fibrosis by the hepatitis virus or other stimuli, followed by liver cirrhosis, which then progresses to HCC or dysplastic nodule and subsequent HCC[13].

RASSF1A promoter methylation in our HCV- associated liver cirrhosis and in HCC on top of HCV- associated liver cirrhosis can be explained by increased DNA methyltransferase mRNA expression that have been observed to occur early in the HCC tissues and in liver tissues showing hepatitis [22,23] . Persistent inflammatory stimulation caused by chronic hepatitis and cirrhosis results in aberrant hypermethylation that with its continuation finally lead to HCC, as it has been reported that inflammatory proliferative diseases such as ulcerative colitis[24], Barrett's esophagitis[25], and Epstein-Barr virus-associated gastritis[26] are strongly related to aberrant hypermethylation of various CpG islands. Also several studies reported frequent silencing of multiple genes by CPG island methylation, including RASSF1A and other genes in hepatitis B virus associated HCC which accumulate during the pathogenesis of human HCC[20]. It was reported that there were no associations between the methylation status of *RASSF1A* and other tumour suppressor genes and the type of hepatitis virus[13]. Moreover, the status of promoter methylation of RASSF1A and other tumour suppressor genes were significantly correlated with the viral infections in the background liver parenchyma[27]. In addition, HBV- or HCV-positive HCCs showed more frequent hypermethylation of CpG islands than virus-negative ones. Furthermore, there is possibility that the regeneration process that is characteristic of chronic liver disease may be associated with aberrant methylation that may involve tumour suppressor genes[20].

It is possible that the detection of blood RASSF1A promoter methylation in the HCV associated -liver cirrhosis cases in our study is indicator that these cases are going to develop HCC and that this methylation is a step towards HCC development. This is evidenced by the presence of RASSF1A promoter methylation in the hepatitic / cirrhotic tissue adjacent to HCC[15,16] and its absence in the hepatitic / cirrhotic tissue far from the tumour or in patients without HCC[13]. So, it is recommended to follow up these cases more frequently to find out if they are going to develop HCC and the time of occurrence of HCC in comparison to the liver cirrhosis cases without RASSF1A promoter methylation. Also, it is recommended to quantitate those cases of HCV associated -liver cirrhosis with RASSF1A methylation to follow up these cases by quantitative measurement of RASSF1A methylation to find out if they exceed the cut off value for HCC reported by Allen and associates [5] . This is to ensure the usefulness of the use of RASSF1A promoter methylation as a predictor of the future development of HCC in cases of HCV associated liver cirrhosis and thus its usefulness as a potential molecular biomarker for cancer risk assessment in the precancerous lesions.

The results of our study showed that the PPV of RASSF1A methylation in cases of HCC was 73.7% and in cases of liver cirrhosis was 76%. This supports the use of RASSF1A methylation as a powerful marker in the diagnosis of both liver cirrhosis and HCC and thus differentiating these diseases from other liver diseases. . However the PPV of RASSF1A methylation in the diagnosis of bilharzial liver fibrosis is low indicating that that it is a weak biomarker for its diagnosis.

As regards schistosomal liver fibrosis, it was reported that the liver injury produced by schistosomal egg-induced inflammatory response is mild or limited compared to the necro-inflammatory reaction produced by the HCV. Also, the liver mesenchymal cells (myofibroblasts) involved in fibrogenesis were increased in both schistosomal periportal fibrosis and HCV-induced cirrhosis than in normal liver, but higher in HCV-induced cirrhosis[28]. This indicates that the regenerative process is stronger in response to the more inflammatory reaction produced by HCV infection. So the stronger inflammatory reaction and thus the higher regenerative process in response to HCV infection may explain the higher RASSF1A promoter methylation in HCV- associated liver cirrhosis group and HCC group than in schistosomal liver fibrosis group. Also the limited inflammatory response to schistosoma eggs may explain the non significant mild increase of RASSF1A methylation in the schistosomal liver fibrosis group in comparison to the control group.

To our knowledge, there are no previous studies conducted to assess RASSF1A promoter methylation in schistosomal liver patients. However, it was found that mice infested with schistosoma mansoni have promutagenic methylation damage in liver, but

not in kidney, spleen or bladder[29]. Also remnants of schistosomal eggs were found in the severe granulomatous reaction present in a well-differentiated hepatocellular carcinoma that had developed in a chimpanzee devoid of hepatitis B or C markers[30]. On the other hand, it was stated that in bladder cancers, schistosoma-associated tumors had more genes methylated than non-Schistosoma tumors and they suggest that schistosomal involvement in bladder cancers associates with a greater degree of epigenetic changes in the urothelium [31]. However, infection with schistosoma mansoni is not classified as being carcinogenic to humans, while infection with schistosoma haematobium is carcinogenic to humans, but schistosoma mansoni may still be linked to hepatocellular carcinoma through potentiating the effects of hepatitis B virus and hepatitis C virus on the liver[32]. Moreover a study supported the rapid progressive course of HCV infection in the presence of bilharzial infection in Egypt leading to rapid development and aggressive course of cirrhosis and higher incidence of HCC[33].

The presence of RASSF1A methylation in 16.6% of bilharzial liver fibrosis, which is classified as non carcinogenic to humans may contradict the possibility that the detection of blood RASSF1A promoter methylation in the HCV associated liver cirrhosis cases in our study is indicator that these cases are going to develop HCC..However, we suggest that continuous limited inflammation and regeneration in the bilharzial liver fibrosis without viral hepatitis may result in limited RASSF1A methylation unable to conduct the development of HCC. Moreover, when schistosoma mansoni infection is combined with HCV infection that causes further methylation, HCC may develop. Therefore quantification of blood and tissue RASSF1A methylation in bilharzial liver fibrosis with and without HCV infection is recommended.

As regards S.AFP, our results showed that its level was significantly higher in the HCC group compared to the other groups. Also, the percentage of positive S.AFP according to our cut off values was significantly higher in the HCC group (95%) compared to the control (20%) and bilharzial liver fibrosis (33.3%) groups and in the liver cirrhosis group (92.9%) compared to the control group and bilharzial liver fibrosis groups.

AFP is a fetal-specific glycoprotein, synthesized primarily by the embryonic liver, by cells of the vitelline sac and by the fetal intestinal tract in the first trimester of pregnancy. The expression of AFP is repressed within a few weeks after birth. Pathologically, patients with chronic liver disease, particularly those associated with a high degree of hepatocyte regeneration, can express AFP in the absence of cancer. Also, S.AFP is elevated in hepatocarcinogenesis, embryonic carcinomas and in gastric and lung cancer. S.AFP is not elevated in all patients with HCC. Up to 42% of patients with HCC present with S.AFP levels within normal values[34].

The results of our study showed that the PPV of S.AFP (with a cut off value 33.5 ng/ml) in cases of HCC was 73.9%. This supports the use of S.AFP as a powerful marker in the diagnosis of HCC. However the PPV of S.AFP in the diagnosis of bilharzial liver fibrosis and liver cirrhosis are low indicating that it is weak biomarker for diagnosis of these diseases. The high sensitivity, specificity and NPV of S.AFP (with a cut off value 33.5 ng/ml) in our study which were 85%, 80% and 88.9% respectively, indicates the importance of S.AFP at this cut off value in the diagnosis of HCC.

In accordance to our study, a study revealed the diagnostic importance of S.AFP in differentiating HCC from other liver diseases as they reported that the PPV of S.AFP in HCC was high (60%) [35].

A group of scientists analyzed five studies detecting the sensitivity and specificity of S.AFP for detecting HCC in patients with HCV. They reported that by using the most commonly reported cutoff value of a positive test result for HCC (AFP level > 20 ng/ml), the ranges of test characteristics were as follows; sensitivity, 41% to 65%; specificity, 80% to 94%; positive likelihood ratios, 3.1 to 6.8; and negative likelihood ratios, 0.4 to 0.6. Four of the 5 studies reported sensitivity and specificity for S.AFP cutoff value higher than 200 ng/ml, a value that is frequently reported to be specific for the diagnosis of hepatocellular carcinoma. The range of specificities for hepatocellular carcinoma was very high at this cutoff value (99% to 100%), but the sensitivity was very low (20% to 45%) [36].

However, a study reported that the best discriminating AFP value between HCC and chronic liver disease was 16 ng/ml. Using S.AFP level of 20 ng/ml (the upper normal range) as the cut-off yielded equivalent sensitivity (60.0% *vs.* 62.4%) and specificity (90.6% *vs.* 89.4%). The PPV and NPV were 84.6% and 69.7%, respectively. In non-infected patients with either HCV or HBV the PPV was 100% and the NPV ranged from 59.0 to 73.0% [37].

Also, another study revealed that the best discriminant cut-off value of S.AFP between liver cirrhosis and HCC on top of liver cirrhosis was 30 ng/ml which approaches that of our study. The etiology of liver cirrhosis and HCC in this study was mostly HCV (more than 70%), and less frequently non viral cause, HBV or combined HCV and HBV respectively. At this cut-off value 65% sensitivity, 89% specificity, 74% PPV and 79% NPV were determined. This cut-off

value was more useful in detecting non-viral HCC, because PPV to diagnose non viral HCC was significantly (94%) higher than in viral HCC (70%). In the non-viral diseases PPV reached 100% for S.AFP levels of 100 ng/ml, while in the viral diseases PPV was 100% when S.AFP was greater than 400 ng/ml. There were no significant differences in specificity, sensitivity or NPV between viral and non-viral liver diseases[38].

Our study had revealed a positive significant correlation between the serum concentrations of AFP and RASSF1A methylation status. This disagrees with another study that reported no statistically significant correlation of plasma methylation with S. AFP levels[18]. However a significant association between methylation status of tissue RASSF1A, and S.AFP level was detected before [39].

The results of our study showed that combined use of RASSF1A promoter methylation and S.AFP is better than the use of S.AFP alone in the prediction of HCC. This result agrees with a study which indicated the usefulness of combined measurement of S. AFP and RASSF1A levels in HCC diagnosis than the measurement of S.AFP alone. It reported that the diagnostic sensitivity and specificity were 77% and 89%, respectively, for combined AFP and RASSF1A levels analysis compared with 65% and 87%, respectively, for AFP measurement alone[5].

In conclusion, RASSF1A methylation plays an important role in the process of human hepatocarcinogenesis and is an important biomarker for the diagnosis of HCC when combined with S.AFP.

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