

Value of Hypermethylation (RASSF1A) Promoter Gene among Patients with Hepatocellular Carcinoma

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Received for publication December 15, 2019; Accepted May 22, 2020; Published online May 22, 2020.

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doi: 10.21608/aimj.2020.21008.1022

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies worldwide and one of the main causes of cancer-related mortality globally. Its incidence is increasing at alarming rates. The presence of cirrhosis is the major risk factor and this is largely due to chronic HCV and HBV infection. Serum alpha-fetoprotein (AFP) has insufficient sensitivity and specificity for detection of hepatocellular carcinoma (HCC). Aberrant hypermethylation of tumor suppressor genes e.g(RASSF1A) is one of the most frequent and early mechanisms involved in HCC development, so that it could help to select high-risk populations and thus to modulate the indications of screening procedures.

Objectives: To evaluate the frequency of tumor suppressor gene RASSF1A hypermethylation in whole blood from HCC patients.

Methods: The study included eighty subjects : 30 patients with HCC and elevated AFP. ; 30 with liver cirrhosis In addition, 20 healthy subjects were included as a control group. Clinical and radiological features (abdominal ultrasonography and/or abdominal triphasic computed tomography) were recorded. Liver function tests, complete blood cell count, and serum AFP were measured.. Detection of promoter methylation status of RASSF1A using methylation specific PCR.

Results: The obtained results showed a significant RASSF1A promoter hypermethylation in HCC subjects that was 83.3% in comparison to healthy control subjects as well as in comparison to subjects with non HCC chronic liver disease.

Conclusion: Detection of methylated RASSF1A promoter is useful marker for HCC screening in high risk vulnerable patients and early HCC diagnosis.

Keywords: Hepatocellular carcinoma (HCC); RASSF1A; Epigenetics; Hypermethylation; alpha-fetoprotein (AFP).

Disclosure: The authors have no financial interest to declare in relation to the content of this article. The Article Processing Charge was paid for by the authors.

Authorship: All authors have a substantial contribution to the article.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent and most common aggressive malignancies worldwide.¹

Although most HCCs originate from the accumulation of genetic and epigenetic abnormalities induced by various risk factors, underlying mechanisms of hepatocarcinogenesis are mainly involving cellular signaling pathways and its regulatory molecules.²

Aberrant DNA methylation is one of the most common epigenetic alteration characteristics of malignant cells.³

However, the use of tumor tissues to test for promoter methylation is invasive and thus impractical for HCC screening in high-risk populations.³

The RAS association domain family 1A gene (RASSF1A) is a tumor suppressor gene that is located in the 3p21.3 region and is an important member of the RAS signaling pathway.⁴

The RASSF1A gene has been concerned and studied intensively for its tumor suppression, and hypermethylation in the promoter region is suspected as the main mechanism of silencing that is observed widely in human malignancies, including HCC tissues.¹

The detection of the promoter hypermethylation of RASSF1A in peripheral blood DNA could be a valuable biomarker for early-stage diagnosis in populations at high risk of HCC.⁵

SUBJECTS AND METHODS

This study was performed on a total of 80 subjects, selected from Tropical Medicine Department at Theodor Bilharz Research Institute (T.B.R.I) Hospital. They were divided into following groups:

Group I (HCC): Comprised of 30 patients with HCC on top of cirrhotic liver with elevated serum AFP and typical imaging findings (abdominal ultrasound, triphasic CT).

Group II (LC): Comprised of 30 patients with liver cirrhosis (LC) on top of chronic hepatitis (CH), with normal serum AFP.

Group III (Control): It included 20 patients age and sex matched apparently normal healthy volunteers.

- Informed verbal consents were obtained from all subjects.
- This study was approved by local ethical committee.

All subjects were subjected to the following: Full History taking regarding age, smoking, schistosomiasis, HCV/HBV and diabetes, clinical examination for patients only and imaging techniques (abdominal ultrasonography and triphasic CT) for detection of liver cirrhosis, hepatic focal lesions (HFL), presence of ascites and hepatosplenomegaly and laboratory investigations:

Routine laboratory tests: complete blood count (CBC), was done on automated cell counter Beckman Coulter AcT Diff* and routine liver function tests and kidney function tests. They were all assayed by the automatic chemical autoanalyzer Beckman Coulter Synchron CX5 Pro.

Special investigations: determination of serum HBsAg, and serum HCV Ab using ELISA method using kit purchased from Human (Cat. no. 51275) determination of serum AFP level was assayed by solid phase, chemiluminescent immunometric assay on automated analyzer-ADVIA centaur using kit supplied by Siemens Diagnostics and determination of RASSF1A methylation status using methylation specific polymerase chain reaction (MSP)⁶

Specimen collection and storage: ten ml venous blood were withdrawn from all subjects under complete aseptic conditions and then each was divided into 3 parts :

1) Six ml were collected on plain tubes , left for 10 minutes at room temperature to clot and then centrifuged at 800 xg (3000 rpm) for 5 minutes, then the separated serum was then divided into three separate aliquots .One aliquot used for routine biochemical laboratory tests,one for hepatitis (HBV,HCV) serological markers assay and the last one used for determination of serum AFP.

2) Two ml were collected in a sterile vacutainer containing ethylene diamine tetra-acetic acid "EDTA", which were used for DNA extraction. Samples were stored frozen at (-20°C) till the time of DNA extraction and RASSF1A methylation study.

3) Two ml were collected in a sterile vacutainer containing EDTA for complete blood picture assay .

N.B: Prothrombin Time (PT), Prothrombin Concentration (PC) and International Normalized Ratio (INR) values were collected from patients' data sheets.

Calculation of Child-Pugh score:

	1 point	2 points	3 points
Total bilirubin (mg/dl)	< 2	2-3	> 3
Serum albumin (g/dl)	> 3.5	2.8-3.5	< 2.8
INR	< 1.7	1.71-2.30	> 2.30
Ascites	None	Mild	Moderate to Severe
Hepatic encephalopathy	None	Grade I - II (or suppressed with medication)	Grade III - IV (or refractory)

Table 1: Clinical and laboratory measures of liver disease employed in Child-Pugh score

Point	Class	One year survival	Two year survival
5-6	A	100%	85%
7-9	B	81%	57%
10-15	C	45%	35%

Methodology:

Extraction of genomic DNA and estimation of RASSF1A methylation status using methylation specific PCR (MSP):

The test was done in 4 main steps: extraction of genomic DNA from EDTA anticoagulated whole blood, bisulfite modification of the extracted DNA, amplification of the modified extracted DNA and detection of PCR amplified products using 2% agarose gel electrophoresis and ultraviolet light transillumination.

Extraction of genomic DNA from peripheral blood leucocytes of EDTA anticoagulant done using QIAamp® DNA Blood Mini kit: by spin columns (Catalog no. 51104) supplied by QIAGEN*

Principle: with the QIAamp DNA Isolation method, genomic DNA is prepared from blood cells. The lysis is achieved by the incubation of whole blood in a lysis buffer in the presence of Proteinase K at 56°C. Appropriate conditions for the binding of DNA to the

specialized silica-based membrane in the spin columns are created by the addition of ethanol to the cell lysate. Contaminations are removed by washing with 2 different ethanol-based wash buffers. Pure genomic DNA is finally eluted with elution buffer.

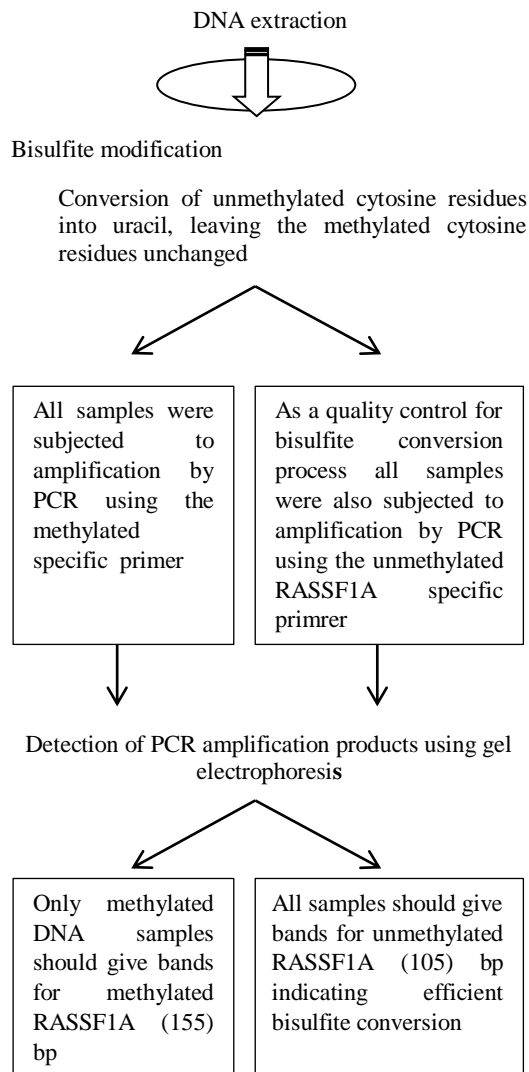


Fig. 1: Diagram of Extraction of genomic DNA and estimation of *RASSF1A* methylation status using methylation specific PCR (MSP)

Bisulfite modification of extracted DNA: this was done using EpiTect Bisulfite Kit (Catalog no. 59104) supplied by QIAGEN

Principle: the EpiTect Bisulfite Kit provides a fast and streamlined 6-hour procedure for efficient DNA conversion and purification of as little as 1 ng DNA. DNA fragmentation is prevented during the bisulfite conversion reaction by a unique DNA protect Buffer that contains a pH-indicator dye as a mixing control in reaction setup, allowing confirmation of the correct pH for cytosine conversion. Furthermore, the bisulfite thermal cycling program provides an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent

sulfonation and cytosine deamination, enabling high cytosine conversion rates of over 99%. Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure.

DNA amplification using the polymerase chain reaction (PCR): enzymatic amplification was performed using Dream Taq Green PCR Master Mix (2X) (Catalog no, K0171) (Thermo Fisher scientific, Fermentas, USA) and BIORAD thermal cycler(BIORAD).

Principle of PCR: it consists of repetitive cycles of DNA denaturation, primer annealing and extension by Tag DNA polymerase. Each cycle produces complementary DNA strands to which the primers bind before the polymerase affects another extension in the next cycle of amplification. Accordingly the products of each cycle are doubled, generating an exponential increase in the overall number of copies synthesized.

Reagents:

- 1) Dream Taq Green Master Mix (2X)
 - (2X) PCR Master Mix (2x1.25 ml) composition:
 - i)Taq DNA Polymerase (recombinant) in reaction buffer: 0.05 units/ul
 - ii) MgCl₂: 4 mM
 - iii)dNTPs (dATP, dCTP, dGTP, dTTP): 0.4 M of each.
 - Nuclease free deionized water (2x1.25 ml)

- 2) Primers: supplied by Fermentas.

Primers were reconstituted to obtain a concentration of each primer of 10 pmol /amplification

Methylated RASSF1A promoter;

RASSF1A-MS Sense: (5'-GGGTTTTGCGAGAGCGCG-3') and RASSF1A -MAS Anti-sense: (5'-GCTAACAAACGCGAACCG-3')

Unmethylated RASSF1A promoter;

RASSF1A-US Sense: (5'-GGTTTTTGAGAGTGTGT-TAG-3').
RASSF1A -UAS Anti-sense: (5'-CACTAACAAACACAAACC-3') .

The PCR Protocol:

- 1-All reactions were performed in a total volume of 25ul as (Yeo et al., 2005) ⁶ described. The following were pipetted with no delay on ice.
- 2-The PCR reaction mixture was mixed well by vortexing.
- 3-The PCR reaction tubes were closed and placed inside the heating block in the DNA thermal cycle , and the cap was tightly closed.
- 4-The computerized thermal cycler was programmed for the following conditions

- A. Initial denaturation step of 95°C for 12 minutes.
 B. 40 PCR cycles of amplification; each cycle consisted of
- Denaturation at 95°C for 45 seconds.
 - Annealing for 45 seconds at 60°C for methylated and at 56°C for the unmethylated primers.
 - Extension at 72°C for 1 minute.
- C. Extension step of 72°C for 10 min.
 D. Detection of PCR amplification products using 2% agarose gel electrophoresis and ultra-violet light transillumination.

RESULTS

This study was conducted on 80 subjects; divided into three groups:

Group I (HCC): Included 30 patients with HCC on top of cirrhotic liver with elevated serum AFP, they were 26 males and 4 females patients. Their ages ranged between 46 and 53.3 years with mean age (48.80±1.36) years.

Group II (LC): Included 30 patients with liver cirrhosis (LC) and normal serum AFP, they were 22 males and 8 females patients. Their ages ranged between 46 and 55 years, with mean age (49.7 ± 1.4) years.

Group III (Control): Included 20 -age and sex matched apparently healthy volunteers as the control group. They were 18 males and 2 females. Their ages ranged between 41.7 and 52.7 years, with mean age (47.6± 1.54) years.

	Group I (HCC) (n =30)	Group II (LC) (n =30)	Group III n =20)((Control)	P value
Age (years)	48.80±1.36	49.7 ± 1.4	47.6± 1.54	0.43
Gender Male , n=66 Female, n=14	26/30 (86.7%) 4/30 (13.3%) ¹	22/30 (73.3%) 8/30 (26.7%)	18/20 (90%) 2/20 (10%)	0.236
Smoking +ve , n=36 -ve, n=44	21/30 (70%) 9/30 (30%)	15/30 (50%) 15 /30 (50%)	0 / 20 (0%) 20/ 20 (100%)	0.000
Bilharziasis +ve, n=43 -ve, n=37	22/30 (73.3%) 8/30 (26.7%)	21 / 30 (70%) 9 / 30 (30%)	0 / 20 (0%) 20 / 20 (100%)	0.000
Diabetes +ve , n=16 -ve, n=64	10/30 (33.3%) 20/30 (66.7%)	6 /30 (20%) 24 /30 (80%)	0 / 20 (0%) 20 / 20 (100%)	0.016

*Data presented as number (%)

**Data presented as mean ± standard deviation

P value <0.05 is statistically significant

Table 2: Demographic data and principal characteristics of the studied groups.

Ascites	HCC (n=30)	Cirrhosis (n=30)	P value
Absent, n=24	13/30 (43.3%)	11/30 (36.7)	0.599
Mild-moderate, n=11	4/30 (13.4)	7/30 (23.3)	
Severe –refractory, n=25	13/30 (43.3)	12/30 (40)	

Table 3: Ascites findings among patients groups

	Group I (n =30)		
	N	%	
Size of tumor	< 5 cm	15/30	50%
	≥ 5 cm	15/30	50%
Portal vein	Patent	22/30	77.3%
	Thrombosed	8/30	26.6%

Table 4: Abdominal triphasic CT scan findings in group I (HCC) patients.

Test \ Group	Group I (HCC) (n =30)	Group II (LC) (n =30)	Group III (Control) (n =20)	P value
Bilirubin total (mg/dl)*	2.15 (1 – 3.9)a	2.55 (1.3 – 5.1)a	0.6 (0.5 – 0.9)b	0.000
Bilirubin direct (mg/dl)*	0.65 (0.27- 1.5)a	1.15 (0.47 – 2.9)a	0.1 (0.025 – 0.1)b	0.000
AST (U/L)*	68 (46.75- 93.75) a	66 (46.75 – 98) a	18 (15 – 24.75) b	0.000
ALT (U/L)*	36.5 (19.5 – 51.25)a	36 (15 – 48.7) a	13 (10 – 19) b	0.000
Total protein (g/dl)*	6.6 (5.9 – 7.9) a	6.4 (5.9 – 6.9)a	7.2 (6.8 – 7.9)b	0.002
Albumin(g/dl)**	3.0 ± 0.7a	3.0 ± 0.8a	4 ± 0.3b	0.000
ALP (U/L)*	112 (74.7 – 163.5)a	100.5 (74 – 186) a	66 (48.5 – 79.5)b	0.000
Urea (mg/dl)*	33.6 (26 – 59.75)a	49.5 (28 – 107.25) a	26.9 (21.1 – 34.9)b	0.000
Creatinine (mg/dl)*	0.9 (0.8 – 1.4) a	1.2 (0.8 – 1.6) a	0.7 (0.6 – 0.8) b	0.000
AFP * (ng/ml)	525(206.7 – 2741.25)a	5.0 (3.75 – 8.0)b	3.5 (2.0 – 5.0)b	0.000
Hb (g/dl)**	11.5 ± 2.25 a	10.6 ± 2.5 a	14± 0.9 b	0.000
TLC* (x10³µl)	6.25 (4.95 – 7.87)	5.9 (3.8 – 8.1)	6.2 (4.67 – 8.0)	0.667
Platelets* (x10³ µl)	135 (83.7 – 179.2) a	127.5 (83.25 – 178.75) a	286 (197.25–341.5)b	0.000
INR*	1.3 (1.16 – 1.56)a	1.6 (1.3 – 1.87)b	1.03 (1.0 – 1.13)c	0.000

*Data expressed as Median , (25th % - 75 th %) percentiles .

**Data presented as mean ± SD.

** *P value ≤ 0.05 is statistically different

****Groups bearing same initials are statistically indifferent at P value 0.05, while groups sharing different initials are statistically different at P value 0.05 (by ANOVA testing).

Table 5: Comparison of laboratory tests among the studied groups.

Test \ Group	Group I (HCC) (n =30)	Group II (LC) (n =30)	Group III n =20)((Control)	P value
HCV Ab -ve , n=8 +ve, n=52	3/30 (10%) 27 /30 (90%)	5/30 (16.7%) 25/30 (83.3%)	20/20 (100%) 0/0	0.706
HBsAg -ve, n= 52 +ve, n=8	27/30 (90%) 3/30 (10%)	25/30 (83.3%) 5/30 (16.7%)	20/20 (100%) -	0.706
RASSF1A U, n= 45 M, n=35	5/30 (16.7%) 25/30 (83.3%)	22/30 (73.3%) 8/30 (26.7%)	18/20 (90%) 2 /20 (10%)	0.000

Table 6: Descriptive comparison between the studied groups as regard HCV -Ab, HBsAg, RASSF1A-Methylated and Unmethylated

	RASSF1A-U (n= 27)	RASSF1A -M (n= 33)	P value
Age (years)*	51.6 ± 5.8	47.3 ± 8.2	0.025
Gender**			
Male	22/ 27(81.5%)	26/33 (78.8%)	1.0
Female	5/27(18.5%)	7/ 33 (21.2%)	
AFP(ng/ml)***	6 (4 – 9)	350 (54.5 – 1171)	0.000
HCV Ab**			
-ve, n=8	4/ 27 (14.8%)	4/ 33 (12.1%)	1.0
+ve, n= 52	23/27 (85.2%)	29/33 (87.9%)	
HBsAg **			
-ve, n=52	23/27 (85.2%)	29/ 33 (87.9%)	1.0
+ve, n=8	4/27 (14.8)	4/ 33 (12.1)	

* Data presented as mean ± SD **Data presented as numbers (%)
 ***Data expressed as Median , (25th % - 75th%) percentiles- P Value ≤ 0.05, is statistically significant

Table 7: Relationship between RASSF1A-U/RASSF1A-M and clinicopathological parameters of all patients (n= 60) group I (HCC) and group II (LC).

	RASSF1A-U n= 45	RASSF1A-M n=35	P value	OR (95% CI)
Cases n=60	27/60 (60%)	33/60 (94.3%)	0.001	11 (2.34 – 51.67)
Control, n=20	18/20 (40%)	2/20 (5.7%)		

Table 8: Risk estimate of RASSF1A methylation status.

	RASSF1A-U n= 45	RASSF1A-M n=30	OR (95% CI)	P value
Cases n=30	5/30	25/30	45.0 (7.83 –258.48)	0.000
Control, n=20	18/20	2/20		

Table 9: Risk estimate among HCC (group I) and control (group III)

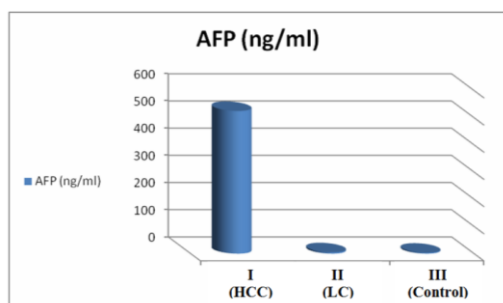


Fig. 2: AFP levels among the studied groups.

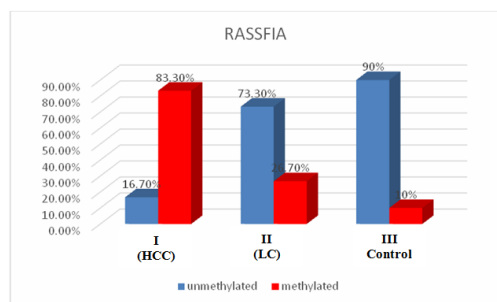
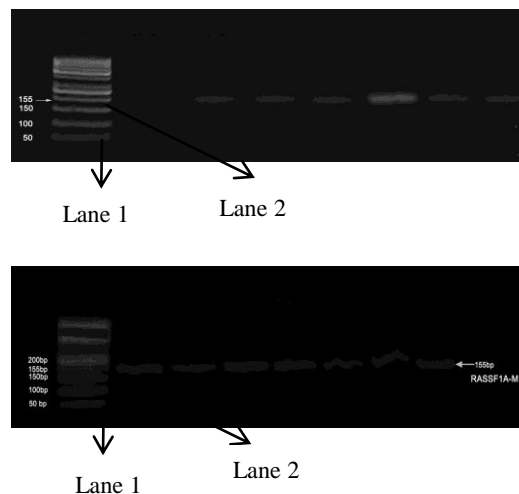
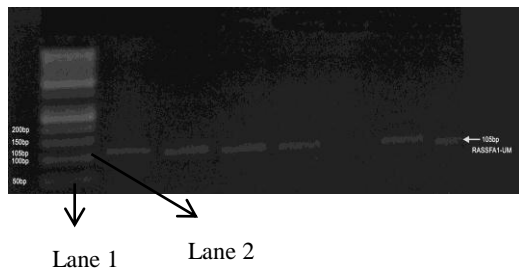


Fig 3: Distribution of RASSF1A -U, RASSF1A-M among studied groups.



Lane 1 : 50 bp ladder (PCR marker)
 Lane 2 :155 bp ladder of methylated RASSF1A -M.

Fig. 4: Amplified products of methylated RASSF1A on 2% agrose gel electrophoresis stained by ethidium bromide.



Lane 1 : 50 bp ladder (PCR marker)
Lane 2 :105 bp ladder of unmethylated RASSF1A-U.

Fig. 5: Amplified products of unmethylated RASSF1A on 2% agarose gel electrophoresis at 105 bp.

DISCUSSION

Hepatocellular carcinoma is one of the most common well-known aggressive malignancies worldwide. It ranks third among overall cancer-related mortality worldwide .

In Egypt, HCC is the second most frequent cancer among Egyptian males after bladder cancer and the sixth most frequent among Egyptian females. ^{9,10}

This rising incidence in Egypt may be due to high prevalence of HCV and its complications. ¹¹

The vast majority of HCC patients present at an advanced late stage of the disease at the first time to be diagnosed. ¹² Therefore, early detection of HCC among high risk population- remains a corner stone in increasing chances for better and early management of HCC patients. ¹³

Hepatocellular carcinoma is a multistep progressive and complex disease, which is associated with alteration in the molecular and cellular signaling pathways. Genetic and epigenetic changes are major and common molecular events that take place during HCC development and progression. Methylation status alteration of certain genes is the most common epigenetic mechanism involved in cancers development including HCC. ¹⁴

RASSF1A (tumor suppressor gene) is one of the genes that are silenced by promoter hypermethylation during early HCC development. So early detection of RASSF1A hypermethylation can be a useful marker for early diagnosis of HCC.

In the current study, detection of methylation status of RASSF1A were assessed as biomarker for early detection of HCC among Egyptian patients with chronic liver diseases. This study was conducted on 80 subjects; 30 patients with HCC (group I), 30 patients with liver cirrhosis (LC) (group II) and 20 apparently healthy volunteers (group III) enrolled as a control group.

In the present study, In group I (HCC), the age of the patients ranged between 46 and 53.3 years with mean age (48.80 ±1.36) years., This is consistent with the results of Keddeas et al. (2011), ¹⁵ who found that the age of the patients with HCC ranged from 40 to 72 years

The present study showed a male predominance of patients: 26/30 (86.7%) while female patients were 4/30 (13.3 %) in group I (HCC) These finding was in agreement with Egyptian study done by Salama et al. (2003),¹⁶ who reported male predominance for HCC incidence. Also, these findings are consistent with the large-center study on Egyptian population by El-Zayadi et al. (2005) ¹⁰ who revealed that male patients were forming 85.4% while female patients were forming 14.6% among 1328 studied HCC patients .This finding may be explained by more exposure of males to risk factors , moreover sex hormones and other x-linked genetic factors may also, be considered Yeh and Chen (2010). ¹⁷ Also, in the study of El-Serag et al. (2007) ¹⁸ they reported that ; the rates of liver cancer among men are two to four times as high as the rates among women. Another explanation for male predominance in HCC by Chiu et al. (2012) ¹⁹ who demonstrated the role of HBx as a noncellular positive co-regulator for androgen receptors and this mechanism could explain the vulnerability of males to HBV infection and the subsequent development of cancer. A third one by Lui et al. (2000) ²⁰ , who suggested that, the low incidence and mortality of HCC found in females may have resulted from the high levels of 2-methoxyestradiol an estrogen metabolite produced in the liver during their reproductive years.

In this study smoking was significantly increased in group I (HCC) and in group II (LC) patient than group III (control) individuals. This coincides with a study by Koh et al. (2011) ²¹ , suggested that there is a strong evidence to date that tobacco smoking is a causal contributing factor for HCC development in humans.

In this study, past history of schistosomal infection was positive in 22/30 (73.3%) in group I (HCC) and 21/30 (70%) in group II (LC) and this come in agreement with Soliman et al. (2010) ²² who confirmed that hepatic viral infection and schistosomiasis were independent risk factors of HCC.

In this study, median serum alkaline phosphatase was higher in group I (HCC) L, in group II (LC), in comparison to group III (control).

High Alkaline phosphatase (ALP) in HCC may be explained by displacement of liver parenchyma by tumor. ²⁴

Chronic infections with HBV and/or HCV are the major etiological and risk factors for HCC worldwide, accounting for 80% of all HCC cases. ^{25,26}

Investigations in Egypt have shown the increasing importance of considering HCV infection as the

major risk factor in the etiology of liver cancer, estimated to account for 40–50% of cases, and the declining influence of HBV and HBV/ HCV infection (25% and 15%, respectively).¹¹

The mechanism of hepatocarcinogenesis by HBV was explained by Zhu et al., (2010)²⁷ who revealed that hepatitis B virus X protein (HBx) is a potential trigger of the epigenetic changes and can directly lead to cellular transformation, in addition to HCV encoded core proteins that cause disruption of regulatory pathways that control cell cycle.²⁸

In this study HCV prevalence in HCC group (I) was 27/30 (90%) and was 25/30 (83.3%) in LC group (II). These results are in agreement with El-Zayadi et al., (2005)¹⁰ who reported that HCV infection accounted for 86.9% of HCC cases during a single study over a decade for HCC in Egypt. Ahmed and colleagues (2010)²⁵ also reported a prevalence of HCV of 96% in HCC and 90% in LC.

In this study HBV prevalence in HCC group it was 3/30 (10%), while in LC group was 5/30 (16.7%), these results were in concordance to, Ahmed and colleagues (2010)²⁵ who reported a prevalence of HBV in HCC 16 % and in LC 10%.

In this study, group I (HCC) demonstrated hypermethylation in RASSF1A promoter gene in : 25/30 (83.3%) of them, in addition RASSF1A-Methylation was detected in 8/30 (26.7%) of group II (LC) patients and ,in 2/20 (10%) of the subjects of the group III (control), which had denoted that the prevalence of hypermethylated RASSF1A was significantly higher in HCC in comparison to LC and control group.

Also, a statistically significant difference was detected among the studied groups enrolled in this study ,regarding RASSF1A- Unmethylated: 5/30 (16.7%) , 22/30 (73.3%) and 18/20 (90%) in group I (HCC), group II (LC), and group III (control), respectively, and P=0.000.

These findings were consistent with the findings of other studies such as a study done by Chan et al. (2008)²⁹ who revealed that hypermethylated RASSF1A was found in 93% HCC patients, 58% HBV carriers, and 8% of normal volunteers.

Mohamed et al. (2013)³⁰ also supported the current study results, where they found that the prevalence of hypermethylated RASSF1A gene in their study was 90% (36/40), 62.5% (25/40) , and 10% (2/20), in HCC, LC and control groups respectively .

In addition to the study of Lambert et al. (2011)³¹ who found a high frequency of aberrant hypermethylation of RASSF1A in HCC tumors as compared to normal liver tissue, Zhang et al. (2013)³² showed that the frequency of RASSF1A methylation in 48 HCC tissues was 100%, which greatly supports the present study results but was higher than ours. This finding could be explained and supported by a study conducted by Philipp et al ., (2014)³³ in which

they found that circulating free DNA may result from the formation of circulating tumor cells or DNA fragments generated by tumor cell necrosis and apoptosis.

This hypothesis implies that serum RASSF1A methylation may originate in circulating tumor cells, which then leads to tumor metastasis Dong et al., (2015).¹⁵

On the other hand the current study results are somewhat higher than an Egyptian study done by Azab et al, (2011)³⁴ where blood RASSF1A promoter hypermethylation was detected in 70% of HCC group, 28.5% of HCV-associated liver patients and was not detected in any of the healthy control subjects.

The other study showed less prevalence of hypermethylated RASSF1A than the present study did. They revealed the presence of hypermethylated RASSF1A in 42.5% and 70 % of HCC patients.⁶

This may be due to the difference in sample size and the technique they used is highly sensitive combination of methylation-sensitive restriction-enzyme digestion and real-time PCR detection.

It is noteworthy that the present study denoted that in HCC (group I) patients, the prevalence of hypermethylated RASSF1A was significantly increased in patients with HCV 27/30 (90%) to HBV patients 3/30 (10%) in the same group (P<0.05).

Also, the current study revealed that; in LC (group II) patients RASSF1A hypermethylation, was significantly increased in patients with HCV 25/30 (83.3%) than HBV patients 5/30 (16.7%).

These findings are in accordance with Mohamed et al. (2013)³⁰ who stated that chronic HCV patients had insignificantly higher levels of hypermethylated RASSF1A than the controls. The levels were significantly higher in patients with HCC compared to the controls (p=0.0001) and chronic HCV patients (p=0.001).

Egypt has possibly the highest HCV prevalence worldwide, estimated among the general population to be around 14-20%. Investigations in Egypt have shown the increasing importance of HCV infection in the etiology of liver cancer, estimated to account for 40-50% of cases, and the declining influence of HBV and HBV/HCV infection.⁹

Egyptian studies revealed that Egypt is of intermediate endemicity for HBV, having a prevalence of 2–8%, (El- Serag et al., 2007)¹⁸, while the overall prevalence of anti-HCV antibodies in Egypt was found to be 20%.⁹

In a study done by Guo et al., (2011)³⁵ they reported that the hepatitis C virus core (HCVc) could up-regulate the methylation status of the RASSF1A promoter.

CONCLUSION

Detection of methylated RASSF1A could be of value for early detection of ongoing hepatocarcinogenesis as hypermethylated RASSF1A was significantly higher in HCC in comparison to LC and control group 83.3%, 76.7 % and 10%.

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