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Genetic Markers for Early Detection of Cirrhotic-HCV and Non-Cirrhotic-HCV

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Abstract: The Egyptian Demographic Health Survey (EDHS), which was carried out in 2008, determined that Egypt has the highest HCV prevalence in the world. Individuals who suffer from chronic hepatitis C (CHC) are very susceptible to potentially fatal outcomes, including cirrhosis and hepatocellular carcinoma (HCC). Liquid biopsies, or the collection and examination of non-solid biological tissue such as blood, are a less intrusive diagnostic approach that may help with early tumor diagnosis. The methylation characteristics of tumor suppressor genes in DNA have been investigated as possible biomarkers for various forms of cancer for the past two decades. In that period, research has discovered that tumors are characterized by global DNA hypomethylation and localized hypermethylation, which frequently affects the promoter region of tumor suppressor genes. Ras Association Domain family 1A (RASSF1A) is one of the most well-known and studied biomarkers in liquid biopsies. Hypermethylation was associated with loss of RASSF1A expression.

Patients and methods: To evaluate the RASSF1A gene methylation percentage in patients with different liver disease stages, we collected fresh whole blood samples from 29 healthy subjects as control, 30 non-cirrhotic HCV patients, and 30 cirrhotic HCV patients. Demographic and clinical parameters were recorded, and the methylation status of the RASSF1A gene was detected using methylation sensitive restriction enzyme-quantitative polymerase chain reaction (MSRE- qPCR).

Results: The obtained results showed a significant RASSF1A promoter hypermethylation in non-cirrhotic HCV and cirrhotic HCV 31.0%, and 33.3% respectively, and got an overall predictive value of 75.3% to predict the incidence of cirrhotic HCV.

Conclusion: Detection of methylated RASSF1A status could be a valuable early diagnostic marker of Cirrhotic HCV patients.

keywords: RASSF1A; HCV; Cirrhotic HCV

1.Introduction

The 14th most prevalent cause of death in the world is liver cirrhosis and related chronic liver disorders, which dramatically raise death rates **(1,2)**. This denotes a more advanced phase of many liver conditions, including autoimmune diseases, alcohol use, non-alcoholic fatty liver disorder, and infections with hepatitis B and C **(1,3)**. HCV is a hepatotropic RNA virus that is a member of the Flaviviridae family. This virus is encapsulated and has a positive RNA strand that is 50 nm in size. Each of the six major genotypes of HCV differs from the others by at least 30% in terms of nucleotide sequence. A major selection mechanism for immune system evasion and medicine resistance is the population's genetic variability. The most common HCV genotype in Egypt is genotype 4 (92.5%), followed by genotype 1 (3.6%) **(4,5)**. Egypt accounts for 13% of all HCV infections worldwide, making

it the country with the highest prevalence of HCV in the world, according to WHO estimates.

The acute infection stage with Hepatitis C virus is asymptomatic with little visible symptoms. It starts appearing within 2 weeks to 6 months after initial exposure to the virus, 80% of them have no symptoms. Some reports show that 15 – 45% of people with acute HCV infection developed an immune response that clears the virus within 6 months of infection **(6)**, those cases who cleared HCV infection will still test positive on HCV antibody screening tests. About 20-40% of acute infections tend to resolve spontaneously **(7,8)**. More than 50% of all infections proceed to chronic hepatitis C, which is also silent until liver damage is severe enough to cause symptoms **(9,10)**. Chronic hepatitis C if left untreated can lead to death.

Cirrhosis and chronic liver disorders development is typically preceded by persistent inflammation, which causes a dramatic rise in death and morbidity rates; a case-fatality rate of up to 80% can occur within a year. Thus, chronic liver diseases pose a serious threat to global health and politics for all people, governments, and medical facilities **(1,11)**. The substantial disease burden of liver cancer has been highlighted by a recent large-scale study examining the incidence and death patterns of the disease **(12,13)**.

The incidence of cirrhosis and chronic liver disorders has not received as much attention in recent years as liver cancer reports. The majority of research on chronic liver diseases, including liver cirrhosis, focuses on determining the disease's pathological mechanism, prevalence, and severity concerning certain causes **(14–16)**. Geographical regions, genders, racial/ethnic groups, and socioeconomic classes all have distinct consequences of chronic liver disorders, which have evolved significantly over time. Although research has been done on the prevalence and mortality rates of chronic liver diseases and cirrhosis, they are old or fail to include incidence, mortality, gender, and age comprehensively to evaluate the disease burden of these conditions **(11,14–18)**.

The most extensively used serum biomarker for liver cancer is Alpha-fetoprotein (AFP), which plays an important role in liver cancer screening, diagnosis and assessment **(19)**. Its clinical applicability, however, has sparked **(20)**. The diagnostic power of AFP was determined by its cut-off level. A sensitivity of 64% and specificity of 91% were obtained with a cut-off of 20 ng/mL **(21),** while sensitivity of 17% and specificity of 99% were obtained with a cut-off of 400 ng/mL **(22),** Furthermore, patients with chronic viral hepatitis who do not have HCC may have an increased AFP during viral flares **(23)**.

Numerous human disorders are intimately linked to methylation-related changes in DNA, including imprinting loss, hypomethylation, and hypermethylation, such as cancer. The examination of the RNA sequence shows that promoter hypomethylation of oncogenic genes increases their expression levels, whereas promoter hypermethylation of tumor-suppressive genes lowers their protein levels. Certain tumor suppressor genes' expression is selectively suppressed by DNA hypermethylation at their promoter regions, which directly contributes to the carcinogenic processes of HCC brought on by HCV infection. Crucially, the primary mechanism for the inactivation of these genes is hypermethylation of the promoter CpG island. Previous investigations have also discovered that a number of other tumor suppressor genes, including the p53 tumor suppressor gene, are hypermethylated **(24–26)**.

It's been suggested that abnormal methylation of particular genes plays a key function in the hepatocyte's malignant transformation **(27,28)**. The ‘‘methyl" group is covalently attached to the 5-position of the cytosine residue by a series of enzymes known

as DNA methyltransferases (DNMTs), which include DNMT1, DNMT3A and DNMT3B **(29)**. Cytosine-guanosine (CG) motif in the context of methylation called CpG islands (CGIs), the "p" added to distinguish CG sites meant for methylation from other CG that means phosphate **(30)**. Recent findings provide more evidence in favor of the theory that hypermethylation of normally methylation-resistant regions develops early in phases of cancer formation and persists into later stages **(31)**.

The Ras Association Domain family 1A (RASSF1A) is a cell cycle-related tumor suppressor gene that controls cell division, colony formation, and apoptosis **(32,33)**. Two CpG islands, A and C modify the RASSF1 gene; CpG island A is located in RASSF1A's regulatory region. CpG islands A methylation has been identified in normal tissue and has no effect on gene expression. Hypermethylation, on the other hand, has been linked to the decrease of RASSF1A expression **(34)**. The methylation of these CpG islands is mediated by DNA methyltransferases (DMNTs) when DMNTs are mismanaged they cause RASSF1A to be epigenetically silenced in cancer cells. The CpG island in the RASSF1A promoter region is methylated as a result of this action and RASSF1A expression is lost as a result **(35)**. Loss of RASSF1A because of this process leads to the binding of RASSF1C to the RASSF1A effectors, which then tumor genesis starts **(36,37)**.

The majority of DNA methylation methods are based on the polymerase chain reaction (PCR) such as methylation sensitive restriction enzymes – quantitative polymerase chain reaction (MSRE-qPCR), they concentrate especially on the analysis of 5-methylcytosines in association with CpG dinucleotides **(38)**. A variety of clinical and molecular biology epigenetic studies can benefit from the use of MSRE-qPCR. DNA methylation patterns have been explored with restriction enzymes since the beginning of time and are being utilized today, often employing methylation-sensitive restriction enzymes (MSREs), when several targets/regions of interest must be addressed simultaneously and analytically sensitively **(39)**. Quantitative polymerase chain reaction (qPCR) can easily detect the amplification products, permitting an explanation of the targets of interest's methylation state. Additionally, a calibration curve can be used to

quantify the amount of methylation DNA in the examined genomic areas in either a relative or absolute manner **(40)**. When qPCR-based detection is combined with MSRE digestion, high-throughput PCR technologies are utilized to enable methylation testing of large study groups. There is one essential requirement for successful MSRE-qPCR methylation testing, to avoid false-positive results, Restrictions sites and the entirety of the enzymatically digested material must be taken into consideration while designing tests **(41)**. Because they often contain numerous amounts of MSRE cut-sites, CpG-rich regions are excellent candidates for MSRE-qPCR test design since they are often also potential locations for methylation changes. Furthermore, it is strongly advised to employ more than one **(42)**. We frequently employ a mix of MSREs, which enhances the possibility of the presence of suitable cut sites and full digestion and multiplexing possibilities for simultaneously targeting different regions.

2. Materials and methods

The protocol for the study was authorized by the Egyptian Liver Research Institute and hospital- Sherbeen- Daqahlia, and the Faculty of Science, Mansoura University.

Fresh whole blood samples are collected from 89 participants split up into three cohorts:

Group I: Included 29 controls. There were 7 males (24.1%) and 22 females (75.9%) with a median age of 41 years.

Group II: Included 30 non-cirrhotic-HCV. Anti-HCV-abs were detected in 100% of the patients group. There were 20 males (66.7%) and 10 females (33.3%) with a median age of 43 years.

Group III: Included 30 cirrhotic-HCV. Anti-HCV-abs were detected in 100% of the patients group. With a median age of 58 years, there were 23 men (76.7%) and 7 women (23.3%) among them.

All clinical and demographic parameters were analyzed via automated systems. The RASSF1A gene methylation detection is done through the following steps:

1. DNA Purification from Whole Blood (spin protocol): The QIAamp DNA Blood Mini Kit (Cat. no. 51104) supplied by **QIAGEN** is a quick and simple way to purify total DNA for PCR and Southern blotting. There are four phases in the QIAamp DNA purification technique (lyse, bind, wash, elute). The method is meant to prevent cross-contamination between samples and to allow safe handling of

potentially infectious samples. The kit contains QIAamp Mini Spin Columns, collection tubes (2 ml), buffer AL, buffer AW1, buffer AW2, buffer AE, QIAGEN protease,

protease solvent, and proteinase K.

2. Methylation sensitive restriction enzyme-quantitative polymerase chain reaction (MSRE-qPCR): A novel technological advancement that makes it possible to quickly and precisely profile each gene's CpG island DNA methylation is the EpiTect Methyl II PCR Kit (Cat. No. 335002) according to **(43–45)**. This method is based on detecting the remaining input DNA after cleavage with methylation-dependent restriction enzymes. These enzymes digest methylated and unmethylated DNA separately. The enzyme reaction and the qPCR master mix are combined immediately upon digestion and quantified by real-time PCR using primers flanking the promoter region (RASSF1A gene in our study). After the cycling program has been completed, export and/or copy-paste the threshold cycle C_T values from the instrument software to a blank Microsoft Excel spreadsheet (Microsoft Excel-based data analysis template) which you can obtain from *Geneglobe.qiagen.com/product-groups/epitect-methyl-ii-pcr-arrays*. Gene methylation status is represented as unmethylated percentage (UM) and methylated percentage (M) fraction of input DNA.

The SPSS 26.0 program was used for all statistical analyses (IBM, Armonk, NY). When applicable, the Mann-Whitney U test or the Kruskal-Wallis test (for non-normal distribution samples) was used. For every numerical variable, the median range was determined. Categorical data is expressed using percentages. Differences were considered statistically significant for *p- values* less than 0.05.

Optimum cutoff values for the gene and determination of the sensitivity and specificity using receiver-operating characteristics (ROC) curve. The area under ROC curve (AUC-ROC) is used to detect if the test is useful or not, if ranges between 0.9-1 it's a very useful test, if ranges between 0.7-0.8 it's a fair test, if was between 0.5-0.6 it failed test.

3. Results and Discussion

The demographic data of all study groups are represented at **Table 1**, In the non-cirrhotic HCV group, patients' ages varied from 33 to 58 years old, with a 43-year-old median age, while the cirrhotic HCV group's age ranged between 55 and 67 years with a median age of 58 years. This agrees with the findings of **(46)** who discovered that the age of non-cirrhotic HCV, and cirrhotic HCV ranged between 25–85, and 30–86 respectively, while **(47)** verified that the cirrhotic HCV patients' ages varied from 27 to 65 years.

There is a male predominance shown in our study groups which are 66.7% male and 33.3% female in non-cirrhotic HCV, 76.7% male, and 23.3% female in cirrhotic HCV. These findings were in agreement with **(46)** who reported male predominance for non-cirrhotic HCV and cirrhotic HCV as 60%, and 52.1% respectively. Also, these findings are consistent with **(47)** who revealed that male patients were forming 63.5% of cirrhotic HCV.

Egypt has shown that HCV infection is thought to be the primary risk factor for liver cancer, accounting for 40–50% of cases **(48)**. In our study, we find that the HCV prevalence in non-cirrhotic HCV was 100%, and in cirrhotic HCV 100%. These results are in agreement with **(49)** that reported a prevalence of HCV by 90% in cirrhotic patients. All clinical parameters were analyzed and calculate the median percentiles for the non-normal distributed samples across groups of the study using the Kruskal-Wallis test at **Table 2**.

Table (1): Demographic characteristics of patients groups compared with control group

	Control (n=29)	**Non cirrhotic-HCV (n=30)**	**Cirrhotic-HCV (n=30)**	***P- value***
Age Median (25th % - 75 th %)	41 (34-45)	43 (33-58)	58 (55-67)	0.000**
Gender				
Male	7 (24.1%)	20 (66.7%)	23 (76.7%)	0.000**
Female	22 (75.9%)	10 (33.3%)	7 (23.3%)	
Anti–HCV Abs	-ve (100%)	+ve (100%)	+ve (100%)	0.000**

p-value <0.05 is statistically significant.

**: Highly significant value

There is a low elevation of alpha-fetoprotein (AFP) in the non-cirrhotic group

and moderate elevation in the cirrhotic group. The laboratory parameters show elevated rates of alanine transaminase (ALT), aspartate transaminase (AST), bilirubin, and international normalized ratio (INR) among all study groups in agreement with **(50,51)** which confirmed that the release of AST and ALT is caused by inflammation and hepatocyte damage. The high levels of PT/INR ratio may be due to the decrease in clotting factors production. There is a decrease in albumin and platelets levels as in **(46,47)**. A highly significant increase in AFP in all study groups that consistent with **(52)**.

The methylation and unmethylation percentage of the Ras Association Domain family 1A (RASSF1A) gene across the groups were calculated using the Kruskal-Wallis test, there are **Table 3** . RASSF1A methylation levels increased gradually as the disease progressed, reaching noticeably greater levels in tumor tissues. We notice a low elevation rate of methylated RASSF1A in non-cirrhotic HCV and moderate in cirrhotic HCV respectively 31.0% (9/29), 33.3% (10/30) while 17.2% (5/29) in the control group.

These results were consistent with the other studies such as a study done by **(53)** was found that the prevalence of hypermethylated RASSF1A gene was 90% (36/40) in HCC, 62.5% (25/40) cirrhotic HCV, and 10% (2/20) in the control group.

Egyptian study was done by **(54)** gives lower results than ours, significant hypermethylation of the blood RASSF1A promoter was seen in

28.5% of liver patients with HCV and was not found in any of the control group. The difference is regarded to the use of methylation-specific PCR, which is less sensitive than MSRE-qPCR.

The control group in our study display higher methylation levels than other studies such as **(53)**. Also in **(55)** using methylation-specific PCR, they found 6% of methylated RASSF1A in control DNA specimens. Our findings also agree with the findings of **(56)** who used real-time PCR and detected methylated RASSF1A in 8% of the healthy participants. Our study's comparatively greater levels might be because

the dominance of the female gender in our control subjects while in those studies were males.

A relationship between RASSFIA gene methylation status and the demographic and laboratory parameters of patients groups and control group shown in **Table 4** using the nonparametric correlations of Kruskal-Wallis test. The only significant result was between albumin and RASSFIA methylation (P=0.001).

Table 5 shows the predictive ability of methylation analysis for discriminating patients groups from control. A clinical test's sensitivity is its capacity to accurately identify patients who have the condition, whereas its specificity is its ability to accurately identify patients who do not have the disease. When a test is positive, the positive predictive value (PPV) calculates the subject's likelihood of having the disease; when the test is negative, the negative predictive value (NPV) calculates the subject's likelihood of not having the condition.

Table (2): Some laboratory parameters of patients groups compared with control group.

	Control(n=29)	**Non-cirrhotic-HCV (n=30)**	**Cirrhotic-HCV (n=30)**	***P- value***
AFP(ng/ml)	2.10(1.61-2.85)	3.85(2.77-5.17)	7.90(3.78-20.15)	0.00**
ALT (U/L)	18.00(12.00-22.50)	36.50(19.97-70.19)	42.00(31.75-55.50)	0.00**
AST (U/L)	18.00(16.50-22.00)	37.50(23.90-49.35)	53.00(35.75-65.50)	0.00**
Albumin (g/dl)	4.60(4.30-4.80)	4.52(4.17-4.73)	3.60(3.20-4.18)	0.00**
T-Bilirubin (mg/dl)	0.50(0.34-0.70)	0.60(0.40-0.96)	1.30(0.80-1.62)	0.00**
D-Bilirubin (mg/dl)	0.18(0.10-0.20)	0.22(0.15-0.40)	0.53(0.30-0.82)	0.00**
Creatinine (mg/dl)	0.73(0.67-0.90)	0.70(0.61-0.90)	0.90(0.72-1.00)	0.026*
Hb(g/dl)	12.69(11.97-14.31)	14.38(12.62-15.17)	13.34(12.10-15.10)	0.083
WBCs	7.23(5.86-9.50)	6.30(4.78-7.85)	5.34(3.89-6.78)	0.009*
PLT(x10^3 µl)	261.00(192.0-310.5)	204.5(176.5-280.0)	116.0(78.75-166.5)	0.00**
INR	1.01(1.01-1.01)	1.05(1.01-1.11)	1.08(1.01-1.26)	0.00**

Table (3): RASSF1A gene methylation and unmethylation status across groups

RASSF1A	Control (n=29)	Non cirrhotic-HCV(n=29)	Cirrhotic-HCV(n=30)	*P- value*
Unmethylation state (n=70)	n=24 82.8%	n= 20 69.0%	n= 20 66.7%	0.00**
methylation state (n=47)	n=5 17.2%	n=9 31.0%	n=10 33.3%	0.00**

Table (4): Correlation of methylation levels and the demographic and laboratory parameters

	Methylation	
	Pearson coefficient (r)	***P- value***
Age	0.116	0.213
AFP	-0.074	0.426
ALT	-0.093	0.317
AST	0.014	0.883
Albumin	-0.292	0.001*
Creatinine	0.015	0.870

	Methylation	
	Pearson coefficient (r)	***P- value***
T-Bilirubin	-0.023	0.807
D-Bilirubin	-0.054	0.564
Hb	-0.122	0.189
WBCs	-0.018	0.846
PLT	-0.144	0.120
INR	0.102	0.273

Table (5): Diagnostic accuracy of methylation analysis for discriminating patients groups from control.

	AUC	*P value*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
RASSF1A	0.757	0.000*	76.7	73.9	50.0	90.3	75.3

By using Youden index method in cooperation with receiver operating characteristic (ROC), our study reveals that methylation of RASSF1A gene shows an early diagnostic ability in discriminating the patients groups (non-cirrhotic HCV and cirrhotic HCV) from the control by area under ROC curve (AUROC) of 0.757 within a cut-off point of 56.17 we got 76.7% sensitivity and 73.9% specificity, PPV 50.0% and NPV 90.3% and a predictive accuracy of 75.3%. When compare our results with the study by **(57)** , they got AUROC of 0.840 and a lower sensitivity result than ours 72.2% with PPV of 96.4% and a higher specificity result of 95.1% with NPV of 68.4% these differences may be due to the different cut-off selection also they isolated the DNA from plasma, not whole blood as we did. patients groups from control.

In Conclusion, the detection of methylation status for the RASSF1A gene extracted from Whole blood could be a valuable early diagnostic marker of Cirrhotic –

HCV and non–cirrhotic - HCV using MSRE-qPCR, with an overall predictive value of 75%.

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