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Aberrant p16 methylation as an early diagnostic marker in blood of hepatocellular carcinoma patients

Arig Aly Seif, Heba Hassan Aly*, Doaa Mostafa Elzoghby, Ashraf Mohammed Elbreedy and Mohamed Lotfy

Abstract

Background: Alpha-fetoprotein (AFP) is currently used for serologic screening in hepatocellular carcinoma (HCC) but with low sensitivity ranging 41–65% with a high rate of false-negative and false-positive results. For the hypermethylation of the p16 inhibitor of cyclin-dependent kinase 4 (p16INK4A), a tumor suppressor gene results in the uncontrolled division of cells. This suggests that the loss of p16INK4A function due to promoter methylation may be an early event in HCC pathogenesis so the study aimed to assess aberrant p16INK4A gene methylation as an early diagnostic marker in HCC patients.

Results: Our study revealed a highly significant increase of p16INK4A methylation in patients versus controls (Fisher, 36.11; $p < 0.01$). P16INK4A methylation was detected in 86.6% (26/30) and none of the controls were methylated (100% specificity) compared to the low sensitivity of AFP 65.38% at a cutoff value of 28 ng/mL. Data revealed non-significant difference of p16INK4A methylation status between different HCC Barcelona stages (Fisher, 0.055; $p > 0.05$). While, AFP levels were statistically significantly higher in stages B and C (median = 243,400 ng/mL, respectively, when compared to stage A (median = 10 ng/mL) (H:16.667, $p < 0.01$)).

Conclusion: Early diagnosis of HCC can be achieved through the detection of p16INK4A gene methylation in chronic liver disease (CLD) patients with normal serum AFP especially in known cirrhotic patients that deteriorate clinically without apparent etiology.

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, and it accounts for 7% of all cancers and is considered the third cause of cancer-related deaths [1]. In Egypt, patients with hepatitis B- and C-related liver cirrhosis are at high risk of developing HCC. Exposure to aflatoxin is an additional risk factor for the development of HCC [2]. The prognosis of patients with HCC is poor when diagnosed at an advanced stage, but when diagnosed and treated at an early stage, the 5-year survival rate may reach up to 70–80% [3].

Although histopathological examination of tumor biopsy is considered the golden standard for diagnosis of HCC, it is considered an invasive technique with a high risk of seeding the tumor along the biopsy tract [4]. As regards serologic screening, alpha-fetoprotein (AFP) still represents the currently used test for HCC even though its low sensitivity ranges from 41–65% and high rate of false-negative and false-positive results [5]. This highlights the need for new more reliable non-invasive biomarkers with better sensitivity and specificity for early diagnosis of HCC [6].

Inactivation of tumor suppressor genes (TSG) and activation of oncogenes initiated by genetic and epigenetic changes may play an important role in carcinogenesis [7]. The p16 inhibitor of cyclin-dependent kinase 4 (p16INK4A) gene is a tumor suppressor, located on chromosome 9p21 and encodes the p16 protein, which binds selectively to cyclin-dependent kinase 4 (CDK4) to inhibit activation of the CDK4/cyclin D complex in the G1 phase of the cell cycle [8].

Reduced expression of the p16INK4A gene results in the uncontrolled division of cells. Several mechanisms that lead to p16INK4A inactivation had been described, including point mutations, homozygous deletions, and promoter hypermethylation [9]. Hypermethylation of

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p16INK4A has been detected frequently in human cancers including HCC [10]. Aberrant methylation of the p16INK4A promoter has also been reported in early preneoplastic lesions in the lung, colon, esophagus, and pancreas. These findings suggest that loss of p16INK4A function, often due to promoter methylation, may be an early event in the pathogenesis of various types of tumors [11]. Frequent promoter methylation of p16INK4A gene has been observed in the majority of HCCs in Chinese and Japanese populations [12]. However, the frequency of p16INK4A methylation in Egyptian HCCs has not been studied as extensively. In this view, our study aimed at assessing the possible role for p16INK4A gene methylation as an early predictor of HCC development either de novo on healthy individuals or on top of liver cirrhosis in Egyptian population compared to AFP as a currently used marker in clinical practice. Moreover, the study assessed the association between p16INK4A gene methylation and disease progression in HCC patients.

Methods

Subjects

A case-control study was conducted on 30 adult patients with hepatocellular carcinoma whose age ranged from 47 to 69 years. They were recruited from the HCC clinic at the Tropical Medicine Department. An informed written consent was obtained from each patient before participation in the study. The procedures applied in this study were approved by the *Ethical Committee of Human Experimentation of University*, and also the work has been carried out in accordance with The Code of Ethics of The World Medical Association of Helsinki for experiments in humans. The diagnosis of HCC was confirmed according to American Association of Study of Liver Disease (AASLD) guidelines [13]. They were further classified into early (stage A) and late stages (stages B and C), according to the tumor size and number of nodules, based on BCLC staging system [14]. In addition, a control group of 30 subjects were recruited and were further classified into group IIa including 15 patients with liver cirrhosis age ranged from 50 to 70 years old; the diagnosis was based on clinical picture, ultrasonography, and laboratory diagnosis including liver function tests and viral markers and group IIb that included 15 healthy subjects age- and sex-matched apparently healthy subjects whose age ranged from 37 to 60 years. All individuals included in this study were subjected to abdominal ultrasound, triphasic CT or MRI (for HCC patients only). Laboratory investigations including liver function tests including AST, ALT, albumin, total bilirubin, and direct bilirubin were performed on Beckman Unicell DX-C600 series (Beckman Instruments Inc., Scientific Instruments Division, Fullerton, CA92634-3100, USA) using the manufacturer's reagents.; PT and INR was performed on STA-Stago compact C.T. autoanalyzer using reagents supplied by Neoplastine CI plus (Diagonstica Stago, Inc. Five Century Drive. Parsippany, NJ 07054, USA), markers of chronic liver disease, e.g., markers of viral hepatitis and autoimmune hepatitis were performed on Cobas e 411 immunoassay autoanalyzer using a kit supplied by Roche Diagnostics (Roche Diagnostics Gmbh, Sandhofer Strasse 116, D-68305 Mannheim). Assay of serum alpha-fetoprotein by electrochemiluminescent immunometeric technique where the antigen (sample), a biotinylated monoclonal AFP specific antibody and a monoclonal AFP specific antibody labeled with a ruthenium complex react to form a sandwich complex. Streptavidin-coated microparticles were then added and the complex became bounded to the solid phase via the interaction of biotin and sterptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induced chemiluminescent emission which was proportionate to the concentration of AFP in the sample and measured by photomultiplier [15]. This was performed on Cobas e 411 immunoassay autoanalyzer using AFP kit supplied by Roche Diagnostics (Roche Diagnostics Gmbh, Sandhofer Strasse 116, D-68305 Mannheim) and finally, detection of the aberrant methylation status of p16 gene in the blood by methylation specific polymerase chain reaction (PCR).

Samples

Ten milliliters (10 mL) of venous blood was withdrawn from each subject under complete aseptic precautions and were handled as follows: 1.8 mL were poured into sodium citrated (3.2%) tubes in ratio 9:1(blood to citrate) for PT and INR assay and 2 mL were poured into K_3 EDTA tubes, centrifuged at 1000×*g* for 15 min; plasma was collected, aliquoted, and stored at −70 °C until used for the detection of aberrant methylation status of p16 gene. The remaining blood was placed in sterile vacutainers with a clot activator, and was left to clot for 30 min at 37 °C. Serum was then separated by centrifugation at 1000×*g* for 15 min and was used for immediate assay of routine liver function tests, markers of viral hepatitis and serum AFP.

Methods

Analytical method

Assay of p16INK4A gene methylation assay by methylation-specific PCR

DNA extraction DNA extraction from samples was performed using QIAamp DNA Blood Midi kit* according to the manufacturer's instructions (QIAGEN Incorporation, 28159 Avenuue, Stanford Valencia, CA91355, USA).

A predetermined volume of 200 μL QIAGEN Protease was added to 2 mL plasma in a 15-mL centrifuge tube and mixed briefly. 2.4 mL Buffer AL was added and mixed thoroughly. All the solution was incubated at 70 °C for 10 min to reach a maximum lysis. Then 2 mL ethanol (96–100%) was added and mixed followed by additional vigorous shaking. All of the solutions were transferred onto the QIAamp Midi column placed in a 15-mL centrifuge tube, taking care not to moisten the rim of the spin column. Then, centrifugation was done at 3000 rpm for 3 min, after which the filtrate was discarded. Two milliliters of Buffer AW1 was added to the QIAamp Midi column and centrifuged at 5000 rpm for 1 minute. Then 2 mL Buffer AW2 was added to the QIAamp Midi column. Tubes were centrifuged at 5000 rpm for 15 min. The QIAamp Midi column was placed in a clean 15-mL centrifuge tube and the collection tube containing the filtrate was discarded. Three hundred microliters of Buffer AE was added directly onto the membrane of the QIAamp Midi column and incubated at room temperature for 5 min. Columns were then centrifuged at 5000 rpm for 2 min. For a maximum DNA elution, the elute containing the DNA was reloaded onto the membrane of the QIAamp Midi column and incubated at room temperature for 5 min. The centrifugation step was repeated. The filtered elute was stored at − 20 °C until the time for bisulfite modification step.

Bisulfite modification Bisulfite modification was done to the DNA extract using EpiTect Fast Bisulfite Conversion kit supplied by Qiagen. Complete conversion is followed by cleanup, with removal of bisulfite salts and chemicals used in the conversion process according to Dulaimi et al. [14] The bisulfite DNA conversion was performed using the following thermal cycler program: denaturation step at 95 °C for 5 min followed by incubation for 10 min at 60 °C then denaturation again at 95 °C for 5 min and finally, incubation was done for 10 min at 60 °C. The complete cycle should take approximately 30 min.

Amplification by PCR Amplification of modified DNA was done using EpiTect MSP and primers supplied by Qiagen, according to the manufacturer's instructions. Primer sequences for methylated forward primer (F): 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ as for reverse primer (R): 5′-GACCCCGAACCGCGACCGTAA-3′ and unmethylated p16 gene forward primer 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and reverse primer (R): 5′-CAACCCCAAACCACAACCATAA-3′.They were chosen according to He et al. [16].

Procedure of amplification

For all patients and controls, reaction mixtures were performed in duplicate, one reaction with methylated primers and the other with unmethylated primers, in a total volume of 25 μL containing: 12.5 μL EpiTect Master Mix 2x, 1 μL Primer F (methylated primers or unmethylated primers), 1 μL Primer R (methylated primers or unmethylated primers), 10 μL Template DNA, and 0.5 μL RNase-free water. Tubes were placed in a thermal cycler with the following cycling protocol: 30–40 cycles of initial heat activation at 95 °C for 10 min, DNA denaturation at 94 °C for 15 s and annealing at 56 °C for 30 s. These were followed by an extension step at 72 °C for 30 s and a final extension step for 10 min at 72 °C. In each run, a negative control tube was included to exclude contamination. It contained water instead of DNA template. The amplified products were stored at − 20 °C until the time of the detection step.

DNA detection by gel electrophoresis Amplified products of DNA samples were run on 2% agarose gel stained with ethidium bromide for 40 min at 100 V. A DNA molecular weight marker was also run in each gel to identify the site of bands (50 base pair ladder). The separated bands were visualized by ultraviolet transilluminator and photographed. Methylated samples gave bands at 150 bp in methylated lanes and unmethylated ones gave bands at 151 bp in the unmethylated lanes as shown in Fig. 1.

Figures 2 and 3 show agarose gel electrophoresis for the detection of aberrant p16INK4A methylation in HCC patients using methylation-specific polymerase chain reaction (MSP). Case numbers 4, 5, and 8 show positive p16 gene methylation, while case numbers 6 and 7 show negative p16 gene methylation.

Statistical Methods

Data analysis was done using IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013). Data were expressed descriptively as mean ($\overline{X}$) ± standard deviation (SD) for quantitative parametric data, median, and interquartile range (IQR) for quantitative non-parametric values and as a percentage for qualitative data. Comparison between more than two independent groups'means using analysis of variance (ANOVA) test for parametric data, and Kruskal-Wallis test for non-parametric data. Chi-square test was used for comparison between independent groups as regards the categorized data. Fisher's exact test was used instead when the expected frequency was less than 5. Receiver operator characteristic (ROC) curves were constructed and optimal cutoff values for serum AFP established by the best sensitivity and specificity where the right angle at the upper left corner is the best diagnostic threshold (cutoff) of the parameter being varied. p value > 0.05 was considered non-significant, p value < 0.05 was considered significant, and p < 0.01 was considered highly significant for all tests.

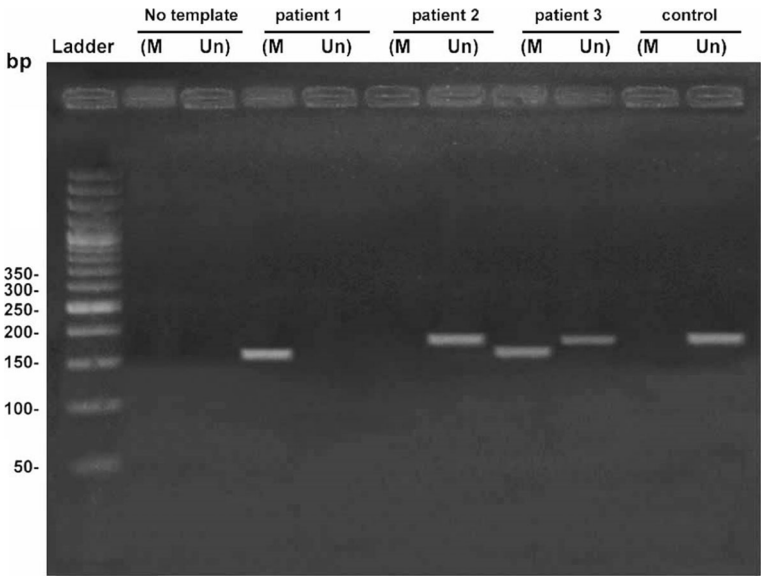

Fig. 1 MSP of p16INK4A gene: p16INK4A gene methylation pattern as viewed from left to right shows a 50-bp ladder as a molecular weight marker, a water control for contamination in PCR reaction, a methylated DNA band in patient 1, an unmethylated DNA band in patient 2, a heterozygous pattern (both methylated and unmethylated bands) in patients 3, and an unmethylated DNA band in a control sample

The minimal sample size estimated to detect statistically significant difference of p16INK4A methylation in HCC patients compared to control groups based on previous published data by Wong et al. [10] and Shiraz et al. [7] was 60 subjects enrolled with 30 cases and 30 controls

Results

Descriptive statistics of the three studied groups regarding the different studied parameters are shown in Table 1.

A statistical comparison between methylated and non-methylated HCC patients regarding different studied parameters using ANOVA test for parametric data and Kruskal-Wallis test for non-parametric data was done, and it revealed a statistically highly significant increase in AST, direct bilirubin, INR (H:21.5, H: 40.5, and F:33.2, respectively, $p < 0.01$) and highly significant increase in AFP while there is highly significant decrease in ALT, total bilirubin, and albumin (H:30.4, H:28.5, and H:28, respectively, $p < 0.01$) (Table 2).

A statistical comparison between HCC patients and liver cirrhosis patients regarding p16 INK4A methylation status using Fisher's exact test revealed a statistical highly significant increase in p16INK4A methylation in the HCC group compared to the liver cirrhosis group ($p < 0.01$) (Table 3).

Statistical comparison between HCC patients and healthy controls regarding p16INK4A methylation status using Fisher's exact test revealed a statistical highly significant increase in methylated p16INK4A in the HCC

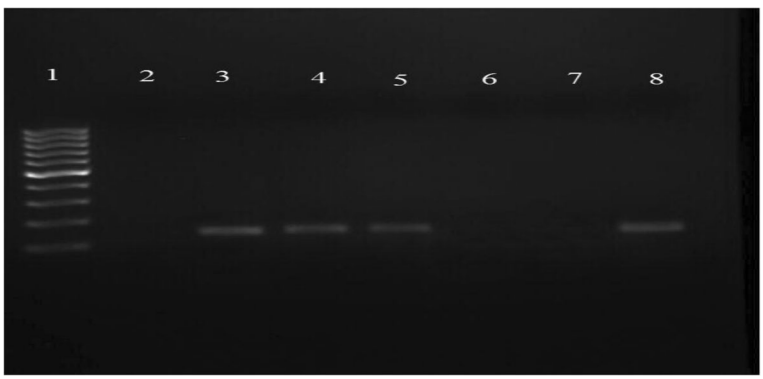

Fig. 2 Detection of products of MSP in HCC patients using methylated primer. Lane 1: 100 base pair ladder. Lane 2: water control for detection of contamination of PCR reaction. Lane 3: methylated positive control. Lanes 4 to 8: cases of HCC

Fig. 3 Detection of products of MSP in HCC patients using unmethylated primer. Lane 1: 100 base pair ladder. Lane 2: water control for detection of contamination of PCR reaction. Lane 3: methylated positive control. Lanes 4 to 8: cases of HCC

group compared to the healthy control group (Fisher, 36.111, $p < 0.01$) (Table 4).

Statistical comparison between different Barcelona stages in HCC patients regarding p16INK4A gene methylation status using Fisher's exact test revealed a non-statistically significant difference between the different stage subgroups ($p > 0.05$). On the other hand, statistical comparison between different Barcelona stages in HCC patients regarding AFP serum levels using Kruskal-Wallis test revealed a statistical highly significant increase in AFP among the different stage subgroups (Fisher, 0.055, $p < 0.01$) as shown in Tables 5 and 6, respectively.

At best chosen cutoff value > 28 ng/mL, serum AFP had a low 65.38% diagnostic sensitivity, 100% diagnostic specificity, 30.8% negative predictive value (NPV), 100% positive predictive value (PPV), and AUC = 0.875 for differentiation between patients of HCC and control groups (healthy and liver cirrhosis) as shown in Fig. 4.

Discussion

Hepatocellular carcinoma is ranked the third most frequent cause of cancer-related deaths worldwide with about 692,000 patients dying from the disease annually [17]. Chronic hepatitis and liver cirrhosis as a consequence of HBV and HCV infections are considered the most common predisposing factors of HCC [18].

Hepatocellular carcinoma is usually asymptomatic in its early stages and tends to be invasive intravascularly and intrabiliary. This makes early diagnosis of HCC crucial for a good prognosis.

The imaging-based diagnosis is relatively inaccurate for small tumors; ultrasound-guided fine needle biopsy can diagnose HCC accurately in about 90% of nodules, even nodules of a very small diameter [4]. The most widely used serological marker for the diagnosis of HCC is AFP. However, its sensitivity is limited (41–65%) especially in small well-differentiated HCC. In addition, false-positive rates were as high as 40% [13].

Hepatocarcinogenesis is a complex multistage process that requires, for the emergence of a fully malignant tumor, accumulation of genetic and epigenetic alterations causing inactivation of tumor suppressor genes and activation of oncogenes. The p16INK4A gene encodes a critical negative regulator of the cell cycle and is one of the most frequently inactivated tumor suppressor genes detected in various types of tumors [19].

Recent studies have found that the p16INK4A gene can be silenced epigenetically in many human neoplasms through the hypermethylation of its promoter region

Table 1 Descriptive statistics of the three studied groups regarding the different studied parameters

Parameter		HCC, $n = 30$	Liver cirrhosis, $n = 15$	Healthy control, $n = 15$
AST (IU/L)	Median (IQR)	62 (45–90)	44 (40–66)	22 (19–22)
ALT (IU/L)	Median (IQR)	40 (26–76)	39 (23–65)	23 (15–27)
Total bilirubin (mg/dL)	Median (IQR)	1.9(1.0–3.2)	1.4(0.9–2.3)	0.9(0.7–1.0)
Direct bilirubin (mg/dL)	Median (IQR)	1.0 (0.6–2.0)	0.9 (0.5–1.5)	0.1 (0.0–0.2)
Albumin (g/dL)	Mean ± SD	2.6 ± 0.2	2.7 ± 0.3	4.5 ± 0.2
INR	Median (IQR)	1.5 (1.2–1.7)	1.3 (1.1–1.6)	0.9 (0.8–1.0)
AFP (ng/mL)	Median (IQR)	220(35–706)	2.9 (1.2–4.1)	1.3 (1–3.3)

Table 2 Comparative statistics of the demographic and laboratory data in p16 gene methylated versus non-methylated HCC patients

Parameter		P16 gene non-methylated patients (No. = 4)	P16 gene methylated patients (No. = 26)	*H/F**	*p* value
AST (IU/L)	Median (IQR), range	46 (32.5–53.5), 20–60	68 (49–94), 23–242	21.5	< 0.01*
ALT (IU/L)	Median (IQR), range	42.5 (32.5–67.5), 25–90	40 (26–76), 10–135	30.4	< 0.01*
Total bilirubin (mg/dL)	Median (IQR), range	2.1 (1.15–8.8), 0.6–15.1	2 (1.3–3.4), 0.3–6.2	28.5	< 0.01*
Direct bilirubin (mg/dL)	Median (IQR), range	0.7 (0.5–4.95), 0.4–9.1	0.75 (0.5–1.3), 0.1–2.8	40.5	< 0.01*
Albumin (g/dL)	Mean ± SD, range	3.18 ± 0.76, 2.5–4.1	2.69 ± 0.65 1.5–3.7	64.6*	< 0.01*
INR	Mean ± SD, range	1.17 ± 0.18, 1.03–1.43	1.37 ± 0.24, 1–1.9	33.2*	< 0.01*
AFP (ng/mL)	Mean ± SD, range	5 (2.7–17.25), 1.9–28	131 (14–400), 2.3–3500	28.0	< 0.01*

H: Kruskal-Wallis test, *F*: ANOVA test
*$p < 0.01$, highly significant difference

[19]. Accumulating evidence proves that aberrant promoter methylation is well correlated with the transcriptional inactivation of certain tumor suppressor genes and it was found as a common molecular defect in neoplastic cells. The cause of this aberrant DNA methylation in cancer cells remains largely unknown.

In the course of searching for a better and more reliable diagnostic marker for HCC, many studies have identified aberrant promoter methylation as the chief mechanism underlying the inactivation of p16INK4A which was proposed as an early factor in the pathogenesis of HCC [20].

The aim of our study was to detect the presence of aberrant p16INK4A gene methylation in the blood of HCC patients, in an attempt to evaluate its role in hepatocarcinogenesis.

The present study showed a male predominance among HCC patients (90%).This was in agreement with Elmougy et al. [21] who explained their results by the fact that DNA synthetic activities are reportedly higher in male than in female cirrhotic tissue. Moreover, the high levels of 2-methoxyestradiol, a metabolite of estrogen, produced in the females' liver during their reproductive years has a protective effect against HCC [22].

Our results confirmed the presence of aberrant methylation of p16INK4A gene in a significant proportion of HCC patients as it was detected in 86.7% (26/30) of these cases. None of the serum samples neither from the cirrhotic patients nor from the healthy controls showed methylated p16INK4A sequence in their peripheral blood. These data are in agreement with the findings of Wong et al. [10] who demonstrated the presence of methylated p16INK4A sequences in the blood of 80% (24 of 30) of HCC patients but found no p16INK4A gene methylation neither in 30 non-HCC patients with chronic hepatitis/cirrhosis nor in the 30 healthy volunteers. In addition, Zhang et al. [23] investigated the presence of aberrant p16INK4A promoter hypermethylation in the serum of 50 HCC patients who provided blood samples before diagnosis and 50 healthy control subjects and revealed aberrant p16INK4A methylation in 44% (22 of 50) of serum samples from HCC patients taken 1 to 9 years before clinical diagnosis, and in 4% (2 of 50) of serum samples of healthy control subjects. In 2011, Shiraz et al. [7] investigated p16INK4A promoter methylation in 43 formalin-fixed tissues from patients with HCC and 20 normal specimens from liver graft donors as controls and detected aberrant p16INK4A methylation in 72% (31 of 43) of HCC patients while no aberrantly methylated p16INK4A sequence was detected in control specimens. Atta et al. [24] claimed that methylated p16INK4A gene in liver specimens was detected in 68% of HCC cases in comparison to 28% of chronic liver disease group. Similarly, a study done in 2016 by Tang et al. [25] revealed that p16 gene methylation rate in liver cirrhosis patients was significantly lower than in HCC patients ($p < 0.01$). Based on these data, p16INK4A gene methylation can be used as a valuable biomarker for early detection of HCC in high risk population.

Several explanations were given for aberrant p16 methylation being a predisposing factor for HCC. One of these is that aberrant methylation of at least one cytosine would significantly downregulate p16INK4A promoter activity with subsequent repression in its transcriptional activity.

Table 3 Statistical comparison between HCC patients and liver cirrhosis patients regarding p16 methylation status

P16 methylation status	HCC, *n* = 30	Liver cirrhosis, *n* = 15	Fisher	*p* value
Methylated (%)	26/30 (86.7%)	0/15	36.111	< 0.01*
Unmethylated (%)	4/30 (13.3%)	15/15 (100%)		

*$p < 0.01$, highly significant difference

Table 4 Statistical comparison between HCC patients and healthy controls regarding p16 methylation status

P16 methylation status	HCC, *n* = 30	Healthy control, *n* = 15	Fisher	*p* value
Methylated (%)	26/30 (86.7%)	0/15	36.111	< 0.01*
Unmethylated (%)	4/30 (13.3%)	15/15 (100%)		

*$p < 0.01$, highly significant difference

Table 5 Statistical comparison between different Barcelona stages in HCC patients regarding p16 methylation status using Fisher's exact test

P16 methylation status	Stage			Fisher	*p* value
	A, *n* = 14	B, *n* = 7	C, *n* = 9		
Methylated	12/14 (85.7%)	6/7 (85.7%)	8/9 (88.9%)	0.055	> 0.05
Unmethylated	2/14 (14.3%)	1/7 (14.3%)	1/9 (11.1%)		

p > 0.05, non-significant difference

This occurs because gene methylation alters the interaction between RNA helicase A enzyme, an enzyme that facilitates the transcriptional activity of the gene, and the gene regulatory region [7]. Being a tumor suppressor gene, the inactivation of p16INK4A gene leads to excessive cell proliferation, accelerated cell cycles, and hence a premature entry into the S phase prior to the completion of DNA repair, resulting in tumorigenesis [25]. The previous explanation was verified by studies that assessed p16INK4A mRNA expression and its promoter CpG island methylation. Atta et al. [24] found that p16INK4A gene expression level was significantly lower in HCC patients who had methylated p16INK4A gene than HCC patients who had unmethylated p16INK4A gene. In addition, Hongmei et al. [26] detected hypermethylation of p16INK4A promoter gene with decreased protein expression in 79.5% (31/39) of HCC patients.

The present study also pointed to the possibility that p16INK4A methylation could be significantly related to chronic HCV infection. Our results detected the presence of significant methylation of p16INK4A gene in hepatitis virus C-associated HCC patients and its absence in healthy controls negative for hepatitis viruses. These observations suggest that the p16INK4A gene methylation may be induced by hepatitis virus in livers with chronic inflammation prior to tumorigenesis of HCC. Among the mechanisms that have been implicated in the pro-carcinogenic effect of HCV infection is the increased production of reactive oxygen species (ROS) in the liver which recruits DNA methyltransferase 1 (DNMT1) enzyme to the site of damaged chromatin inducing methylation of the promoter region of genes such as the p16INK4A gene. This mechanism is considered an early stage in hepatocarcinogenesis [27].

The absence of aberrant p16INK4A methylation in our pathological control group (liver cirrhosis patients) could be explained by the relatively small sample size and the diversity in the clinical course, as the frequency of aberrant promoter methylation increases in a stepwise fashion from chronic hepatitis to peak in HCC [24].

Table 6 Statistical comparison between different Barcelona stages in HCC patients regarding AFP serum levels using Kruskal-Wallis test

Parameter		Stage			*H*	*p* value
		A	B	C		
AFP	Median (IQR)	10 (3.8–17)	243 (120–500)	400 (203–600)	16.667	< 0.01*

H, Kruskal-Wallis test
*$p < 0.01$, highly significant difference

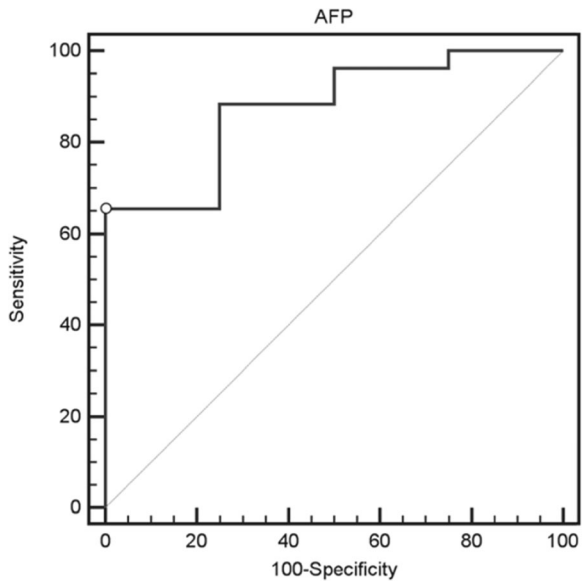

Fig. 4 ROC curve shows the best cut off point for serum AFP for differentiation of HCC patients from control groups (healthy and liver cirrhosis)

Concerning AFP serum levels, it was found to be significantly higher in the methylated group of patients than in the non-methylated one. This can be explained by the effect of p16INK4A gene methylation on AFP expression. As renewed AFP expression occurs only when the differentiated liver exits G_0 and enters a state of resumed cellular proliferation as a consequence of hepatic carcinogenesis. p16INK4A gene methylation and subsequent loss of expression lead to progressive inactivation of the cell cycle regulatory genes and hence continued cell proliferation. Therefore, functional inactivation of p16INK4A gene mediated by promoter methylation may be required for the aberrant expression of AFP during hepatocarcinogenesis [28].

HCC patients were classified according to the BCLC staging system and AFP serum levels were studied among the different HCC subgroups. Our data revealed that AFP is significantly higher in stages B and C (median = 243 and 400 ng/ml, respectively) when compared to stage A (median = 10 ng/mL). These results are in agreement with those of Shingaki et al. [29] and El-Gezawy et al. [30] who reported that AFP was significantly higher in late stages of HCC when compared to the early stage of the tumor. Hence, AFP has a lower prediction capability for HCC diagnosis in the early stages of the tumor.

On studying the p16INK4A methylation status in the different subgroups of HCC patients, our data revealed a

non-significant difference of p16INK4A methylation status between different Barcelona stages of HCC patients. These results agree with those of Shiraz et al. [7] and Elmougy et al. [21] who found no association between p16INK4A methylation status and tumor grade. This indicates that methylation has a negative effect on gene expression both in the early stages of the tumor and during its progression. Hence, the assessment of p16INK4A methylation status may play an important role as an early marker in low-stage HCCs and risk assessment in high-risk populations and provide clues to develop potential prevention strategies for the subset of HCCs that develop through the epigenetic pathway.

Conclusion

Detection of p16INK4A gene methylation is highly recommended in chronic liver disease patients especially in known cirrhotic patients that deteriorate rapidly without any apparent etiology. It is also recommended to add blood p16INK4A methylation assessment to the current standard tests for the diagnosis of HCC as a new diagnostic and screening tool. This, in turn, could greatly improve the ability to identify such patients and thus could allow them to benefit from earlier treatment.

Recommendations

It is recommended to add p16 methylation assessment to the current standard tests for the diagnosis of HCC as a new diagnostic and screening tool. This, in turn, could greatly improve the ability to identify such patients and thus could allow them to benefit from earlier treatment.

Abbreviations

AASLD: American Association of Study of Liver Disease; AFP: Alpha-fetoprotein; BCLC: Barcelona-Clinic Liver Cancer; CDK4: Cyclin-dependent kinase 4; CpG : Cytosine phosphate guanine; CT: Computed tomography; Ct: Threshold cycle; DNMT1: DNA methyltransferase 1 enzyme; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; ROC: Receiver-operating characteristic; ROS: Reactive oxygen species; RT-PCR: Reverse transcriptase PCR; TSG: Tumor suppressor genes

Acknowledgments

Not applicable.

Authors' contributions

AS drafted the manuscript. HA carried out the molecular genetic studies, participated in the sequence alignment, and participated in the study design. DE carried out the molecular genetic studies and participated in the sequence alignment. AE participated in the study design. ML performed statistical analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available with the corresponding author.

Ethics approval and consent to participate

The ethical approval was taken from the Faculty of Medicine, Ain Shams University, research ethics committee FMASU MD 127/201 and written consent to participate is available upon request with the corresponding author.

Consent for publication

Consent for publication is not applicable.

Competing interests

The authors declare that they have no competing interests.

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