El-shaarawy *et al.*
Egyptian Journal of Medical Human Genetics (2022) 23:48
https://doi.org/10.1186/s43042-022-00256-2

Egyptian Journal of Medical
Human Genetics

Relation of the methylation state of *RUNX3* and *p16* gene promoters with hepatocellular carcinoma in Egyptian patients

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Abstract

Background: Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy of adults. *RUNX3* and *p16* are tumor suppressor genes that may be inactivated by hypermethylation which is a key epigenetic mechanism that contributes to the initiation and progression of various types of human carcinomas including HCC. The aim of this study was to assess the association of hypermethylation of *RUNX3* and *p16* gene promoters with the incidence of HCC in Egyptian patients. The study included 120 subjects: 30 HCC patients, 30 patients with hepatitis C virus (HCV) without cirrhosis, 30 cirrhotic patients, and 30 healthy volunteers. Methylation-specific polymerase chain reaction (PCR) was done for detection of hypermethylated *p16* and *RUNX3*. Serum levels of liver enzymes and albumin were detected spectrophotometrically and alpha fetoprotein (AFP) was measured in serum by ELISA.

Results: Methylation of *RUNX3* and *p16* was detected in 25/30 (83.3%) and 26/30 (86.7%) of HCC patients, respectively. The methylation state of both *RUNX3* and *p16* genes was significantly higher in HCC patients compared to the control subjects ($P=0.016$, OR = 4.38) and ($P=0.014$, OR = 4.97), respectively. The methylation of both promoters was associated with higher AFP levels in the serum of all patients.

Conclusions: Hypermethylation of *RUNX3* and *p16* is significantly associated with the development of HCC and may be implicated in its pathogenesis.

Keywords: *RUNX3*, *p16*, Hepatocellular carcinoma, Methylation, Alpha fetoprotein

Background

Liver cancer is ranked as the fifth most common malignancy and the second responsible cause of cancer-related death around the world. Hepatocellular carcinoma (HCC) accounts for about 90% of primary liver malignancies [1]. In Egypt, chronic hepatitis C virus (HCV) is a major health burden and a major risk factor for HCC [2].

Alteration of DNA methylation at promoter regions has a recognized role during early evolution and development of human diseases, including cancers. It can be in the form of hypermethylation, hypomethylation, or loss of imprinting [3]. This alteration causes inactivation of tumor suppressor genes and activation of oncogenes by genetic and epigenetic mechanisms responsible for carcinogenesis [4]. DNA methylation occurs mostly at the 5′ of cytosine in CpG dinucleotides which are found in promoter regions [5].

RUNX Family Transcription Factor 3 (*RUNX3*) encoding gene is a member of the runt domain- containing family of transcription factors and one of the most critical genes that play a crucial role in the regulation of cellular processes as proliferation, apoptosis, angiogenesis, cellular adhesion and invasion [6]. *RUNX3* is located at chromosomal region 1p36–35, and is frequently deleted

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in different malignancies, such as colon, bladder, breast, lung, gastric, and liver cancers [7]. *RUNX3* promoter methylation (hypermethylation) has been found to play a critical role in hepatic epithelial tumorigenesis and epithelial-mesenchymal transition of HCC and is suggested to be helpful as a biomarker for early diagnosis of HCC [8].

The *p16* gene (known as cyclin-dependent kinase inhibitor 2A, CDKN2A) is a tumor suppressor gene, located on chromosome 9p21. It can inhibit the cyclin-dependent kinase 4 (CDK4)/cyclin D complex activation in the G1 phase of the cell cycle by encoding the *p16* protein, which selectively deactivates CDK4 [9]. Lack of *p16* gene expression may result in uncontrolled cellular division. Several mechanisms of *p16* inactivation had been recognized such as point mutations, homozygous deletions, and promoter hypermethylation [10]. *p16* hypermethylation may be involved in hepato-carcinogenesis from early stages and persistent hepatitis virus infection may play a role in the induction of *p16* promoter methylation in hepato-carcinogenesis [11].

Although previous studies have examined the role of *RUNX3* and *p16* gene promoters methylation in cancer progression, limited studies have assessed their role in HCC progression [12, 13] in Egypt and Arab countries. The aim of this study was to assess the state of hypermethylation of the *RUNX3* and *p16* gene promoters in Egyptian patients with HCC and other chronic liver diseases that are widely spread in Egypt as well (i.e. HCV and liver cirrhosis).

Methods

Study participants

The patients in this case control study were recruited from Tropical Medicine Department (outpatient, inpatient and ICU) at Zagazig University Hospital, Zagazig, Egypt. The study included 120 subjects divided into four groups (HCC, HCV without cirrhosis, HCV related cirrhosis, and apparently healthy controls) 30 subjects for each. The sample size required to investigate the association of *RUNX3* and *p16* gene promoters hypermethylation with the incidence of HCC in the current study was calculated using the formula described by Charan and Biswas [14] with a power of 80% and a margin of error of 0.05. HCC was diagnosed according to the criteria of the European Association for the Study of the Liver (EASL) [15, 16]. The diagnosis of HCC was confirmed by pathological examination or elevation of alpha-fetoprotein (AFP) > 400 ng/mL, combined with imaging examinations including magnetic resonance imaging (MRI) and/or computerized tomography (CT) [17].

Patients were investigated for anti-HCV antibodies using second generation enzyme linked immunosorbent assay (ELISA), with polymerase chain reaction (PCR) for HCV RNA positivity. Diagnose of liver cirrhosis was confirmed by combined clinical, laboratory and radiological findings. A questionnaire regarding the medical history including age at exposure to the virus and drug history was obtained. Clinical signs of portal hypertension and liver cell failure were evaluated.

Subjects who had history of any other causes of hepatitis, e.g. hepatitis B virus (HBV) or HCV co-infection with HBV, as well as other liver disorders, e.g. alcoholic liver disease or drug-related liver disease, and other causes of liver cirrhosis were excluded. Written consent was obtained from all participants, and the current study was approved by the Ethics Committee of Faculty of Pharmacy, Suez Canal University (Code 201807RH4).

Laboratory measurements

Peripheral venous blood (5 mL) was drawn after a 10 h fast and a portion was collected with EDTA anticoagulant and used for DNA extraction. The serum was separated from the remaining portion of the blood samples by centrifugation at 3000 rpm for 15 min. and used for assessment of: alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin spectrophotometrically (Spectrum, Egypt). AFP was assessed in the serum by Human alpha Fetoprotein ELISA Kit (Cat No. ab108838) (Abcam, UK).

Analysis of the methylation state of *RUNX3* and *p16* gene promoters

Analysis of methylation status was carried out on DNA extracted from whole anticoagulated blood using Wizard genomic DNA purification kit (Cat No. A1120) (Promega, USA) according to the manufacturer's instructions. Purity and concentration of the isolated DNA were tested by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA was stored at −20 °C. Genomic DNA (2 μg) was subjected to bisulfite modification with EpiTect Bisulfite conversion kit (QIAGEN, Germany) according to the protocol recommended by the manufacturer.

For assessment of the methylation state of each of *RUNX3* and *p16* genes, a 25 μL PCR reaction was performed for each sample; containing approximately 100 ng of the bisulfite modified DNA, 0.5 mM of each of the sense and anti-sense primers, 200 μM dNTPs, 1 × PCR buffer, 1.5 mM $MgCl_2$ and 1.25 U of the HotStarTaq DNA polymerase (QIAGEN, Germany). The conditions of the amplification reactions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles composed of: denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 4 min. The primers and annealing temperatures

specific for each of the methylated and unmethylated regions in both genes are presented in Table 1 [18, 19]. The amplification products were separated on 2.5% agarose gel stained with ethidium bromide, and visualized with ultraviolet illumine. Completely methylated and unmethylated human genomic DNA were used as positive and negative controls, respectively (Epi Tect® PCR control DNA, QIAGEN, Germany).

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 20 (IBM, USA). For comparison between the four groups, data were presented as the means and standard deviation, one way analysis of variance (ANOVA) test followed by Tukey's HSD post-hoc test for multiple comparisons was used. Qualitative data of methylation/unmethylation of gene promoters were represented by frequency and relative percentage and chi-square test was used for testing the association of the qualitative data. Values of age and biochemical parameters in patients with methylated and unmethylated promoters were presented as median and interquartile range (IQR) and compared by the Mann–Whitney U test. In all analyses, P values < 0.05 were considered statistically significant.

Table 1 The primer sequences and annealing temperatures applied in the PCR reactions

Gene	Primers	Annealing temp (°C)
RUNX3		
Methylated	Sense: 5′-TTACGAGGGGCGGTCGTACGCGGG-3′ Antisense: 5′-AAAACGACCGACGCGAACGCCTCC-3′	69.1
Unmethylated	Sense: 5′- TTATGAGGGGTGGTTGTATGTGGG-3′ Antisense: 5′- AAAACAACCAACACAAACACCTCC-3′	61.8
p16		
Methylated	Sense: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ Antisense: 5′-GACCCCGAACCGCGACCGTAA-3′	65
Unmethylated	Sense: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ Antisense: 5′-CAACCCCAAACCACAACCATAA-3′	60

Results

General and biochemical parameters of the study groups are summarized in Table 2. Studied groups showed male percentage of 73.3%, 56.6%, 56.6% and 66.6% in healthy, HCV, cirrhotic and HCC groups, respectively. Patients in the HCC group were significantly older than the control subjects, the HCV non-cirrhotic patients and the cirrhotic patients. Serum ALT levels were significantly increased in HCV patients compared to the control subjects ($P < 0.001$), whereas albumin levels were significantly lower in HCV patients than in the control group ($P < 0.001$). Liver cirrhosis patients and HCC patients showed significantly higher serum levels of ALT, AST, and AFP compared to both the control group and the HCV patients ($P < 0.001$). Consistently, levels of albumin in the serum were significantly decreased in both the cirrhotic and the HCC groups compared to the control subjects and the HCV patients ($P < 0.001$). Moreover, post-hoc test revealed a significant increase of serum AST levels in the HCC patients compared to liver cirrhosis patients ($P < 0.001$). AFP levels in HCC patients were markedly higher than its levels in all other groups ($P < 0.001$) (Table 2).

Table 3 shows that methylation of *RUNX3* promoter was detected in 16/30 (53.3%) of the control subjects, 21/30 (70%) of both HCV patients and liver cirrhosis

Table 2 General and biochemical parameters in the study groups

	Control ($n = 30$)	HCV ($n = 30$)	Cirrhosis ($n = 30$)	HCC ($n = 30$)	F	P value
(Male/female)	(22/8)	(17/13)	(17/13)	(20/10)		
Age (years)	48.10 ± 6.67	48.80 ± 7.19	50.50 ± 4.24	55.60 ± 6.19*#	8.490	0.0001
ALT (U/L)	27.09 ± 2.05	48.88 ± 8.16*	61.95 ± 9.82*#	61.05 ± 7.86*#	149.389	0.0001
AST (U/L)	23.08 ± 3.14	30.94 ± 3.94	63.28 ± 15.46*#	82.52 ± 28.88*#$	100.277	0.0001
Albumin (g/dL)	4.50 ± 0.66	3.07 ± 0.52*	2.07 ± 0.68*#	1.61 ± 0.71*#	73.071	0.0001
AFP (ng/mL)	9.27 ± 1.13	13.13 ± 3.62	50.43 ± 9.15*#	483.72 ± 91.24*#$	897.126	0.0001

Data are expressed as mean ± SD and analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test

HCV hepatitis C virus, *HCC* hepatocellular carcinoma, *ALT* alanine amino-transferase, *AST* aspartate amino-transferase, *AFP* alpha fetoprotein

*Significantly different compared to the control subjects

Significantly different compared to the HCV non-cirrhosis patients

$ Significantly different compared to the liver cirrhosis patients. Differences were considered significantly different at $P < 0.05$

Table 3 Relation of methylation state of *RUNX3* and *p16* gene promoters with hepatitis C virus, liver cirrhosis, and hepatocellular carcinoma

	RUNX3		*P* value OR (95% CI)	*p16*		*P* value OR (95% CI)
	Methylated	Unmethylated		Methylated	Unmethylated	
Control (*n* = 30)	16 (53.3%)	14 (46.7%)		17 (56.7%)	13 (43.3%)	
HCV (*n* = 30)	21 (70%)	9 (30%)	0.187 2.04 (0.71 – 5.89)	15 (50%)	15 (50%)	0.605 0.76 (0.28 – 2.11)
Cirrhosis (*n* = 30)	21 (70%)	9 (30%)	0.187 2.04 (0.71 – 5.89)	23 (76.7%)	7 (23.3%)	0.105 2.51 (0.83 – 7.64)
HCC (*n* = 30)	25 (83.3%)	5 (16.7%)	0.016* 4.38 (1.32 – 14.50)	26 (86.7%)	4 (13.3%)	0.014* 4.97 (1.39 – 17.82)

Data are represented as N (%). Comparisons were performed by chi-square test

HCV hepatitis C virus, *HCC* hepatocellular carcinoma, *OR* odds ratio, *CI* confidence intervals

*Indicates significant difference at $P < 0.05$ compared to the control group

patients ($P = 0.187$, OR = 2.04) and in 25/30 (83.3%) of HCC patients ($P = 0.016$, OR = 4.38). Methylation of *p16* promoter was also detected in 17/30 (56.7%) of the control subjects, 15 (50%) ($P = 0.605$, OR = 0.76) of HCV non-cirrhotic patients, 23 (76.7%) ($P = 0.105$, OR = 2.51) of the liver cirrhosis patients, and in 26 (86.7%) ($P = 0.014$, OR = 4.97) of HCC patients.

The relation of the methylation state of both *RUNX3* and *p16* promoters with the biochemical hepatic markers in all 90 patients participating in the study was analyzed, where methylation of *RUNX3* promoter was associated with significantly higher AFP levels ($P = 0.044$) (Fig. 1). Similarly, AFP and AST serum levels were significantly higher in patients who had methylated *p16* promoter ($P = 0.015$ and $P = 0.001$, respectively) (Fig. 2).

Discussion

The majority of HCC cases have a known underlying etiology. Chronic viral hepatitis (B and C) are the most common causes of HCC as they cause liver cirrhosis which is a major risk factor for HCC. In fact, all forms of cirrhosis may induce tumor formation, but patients with chronic viral hepatitis are at greater risk as one from every three patients with liver cirrhosis will complicate with HCC during lifetime [20].

Recently, the molecular genetics of HCC development have been researched extensively. Aberrant DNA cytosine methylation is one of these molecular genetics, which is consistent with epigenetic changes in human cancers. Generally, cancer cells have lower DNA methylation level than in normal cells. In contrast, some loci tend to show DNA hypermethylation in cancer cells [21, 22]. In HCC, CpG island hypermethylation is frequently recognized at the promoter region of critical tumor suppressor genes. For example, suppressor of cytokine signalling 1 (SOCS-1) was found to be deactivated in HCC cases because of promoter hypermethylation [23]. Methylation of several genes has also been found not only in HCC but also in its precursor conditions such as chronic hepatitis and liver cirrhosis, indicating that these lesions are early events in the evolution of this aggressive cancer [24]. The role of epigenetics changes in diagnosis and progression of diseases potentiates their use as novel biomarkers and therapeutic targets as well. The purpose of the current study was to assess the relation of the methylation state of the *RUNX3* and *p16* gene promoters with chronic liver diseases in Egyptian patients.

The current study involved 90 chronic liver disease patients, including HCV but no cirrhotic, HCV related cirrhotic, and HCC patients. There was a significant correlation between age and incidence of HCC as age was significantly higher in HCC group in comparison to the control and HCV group. This matches with Fattovich et al. [25] who stated that older age at diagnosis is associated with increased incidence of HCC. The mean age of HCC group in the current study was 55.60 years in agreement with Shaker et al. [26] who found that the most frequent age category affected by HCC in Egypt was between 51 and 60 years. The current study found no relation of the age with *RUNX3* and *p16* promoter methylation in all patients (Figs. 1, 2).

In the current study, the percentage of males was higher than females in all groups especially in HCC group that included 20 males out of 30 patients (66.7%). This agreed with El-Mougy et al. [27] who also detected male predominance among Egyptian HCC patients and explained it by the fact that DNA synthetic activities are usually higher in the cirrhotic tissue of the male than the female. Moreover, the high level of the estrogen metabolite 2-methoxyestradiol in females' liver apparently acts as a protective factor against HCC [28]. Darbari et al. [29] found that HCC occurs more frequently in men than in

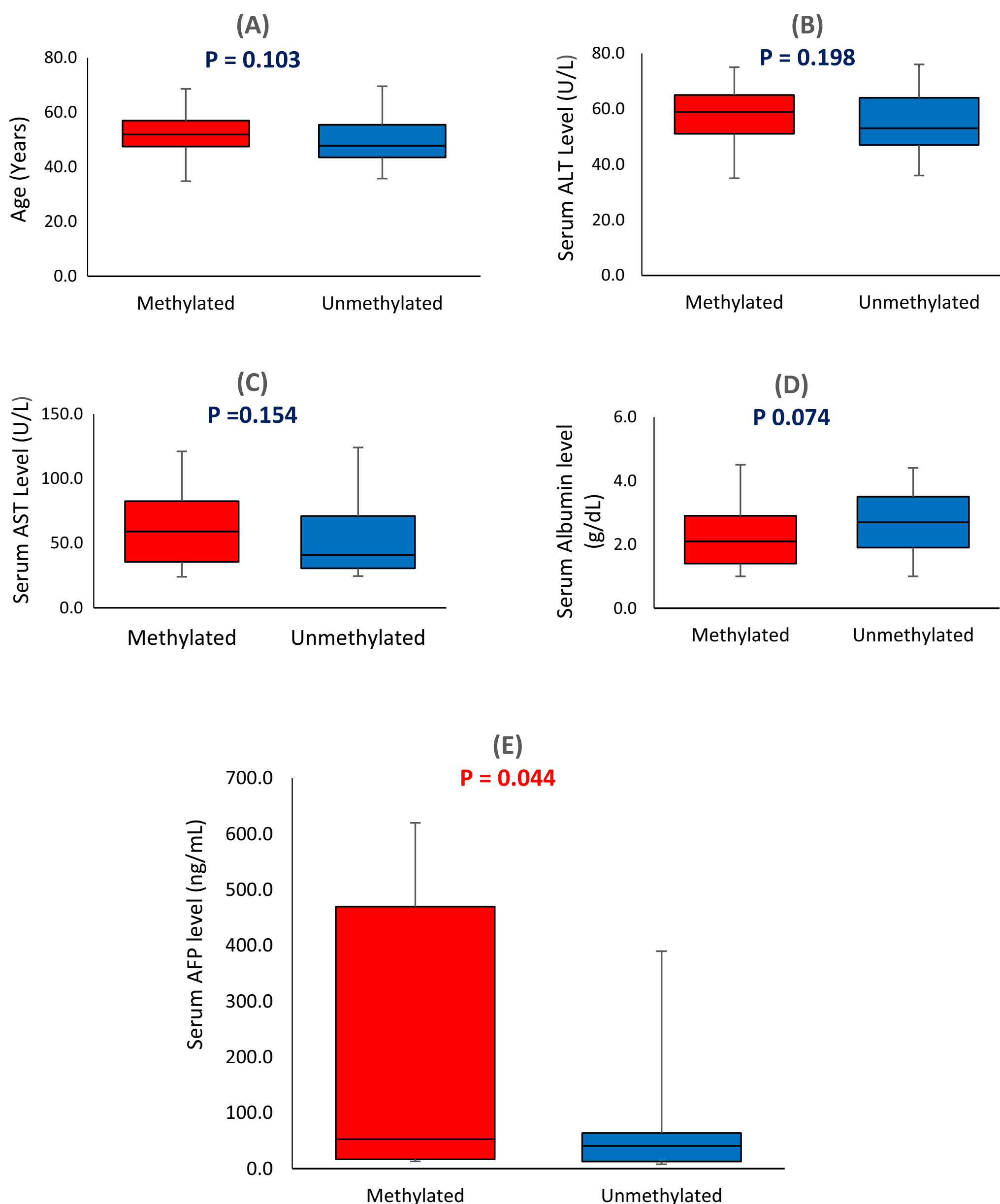

Fig. 1 Relation of *RUNX3* promoter methylation with age and biochemical parameters in all participant patients. **A** Age, **B** serum ALT levels, **C** serum AST levels, **D** serum albumin levels, and **E** serum AFP levels. Data are represented as median and interquartile range; and compared by the Mann–Whitney U test. The top, middle and bottom lines of the box plot indicate the 75th percentile, median and the 25th percentile values, respectively. Error bars demonstrate the maximal and minimal values. Differences were considered statistically significant at $P < 0.05$

women, regardless of the geographic location with the male:female ratios may reach up to 5:1 in some countries.

Regarding ALT and AST liver enzymes there was a stepwise elevation in the means of both enzymes in all groups of patients (HCV, cirrhotic and HCC) as compared to the control group. This is explained by the chronic persistent and progressive damage of liver cells with presence HCV as an injurious agent. It induces both hepatic inflammation and fibrosis. HCV protein expression promotes the mutation and malignant transformation of the infected cells which is an important risk factor in developing HCC in Egypt [30]. Our results revealed significant decrease of serum albumin in cirrhosis and HCC patients in agreement with Seif et al. [13]. This can be attributed to the deterioration of liver synthetic function as cirrhosis progresses [31].

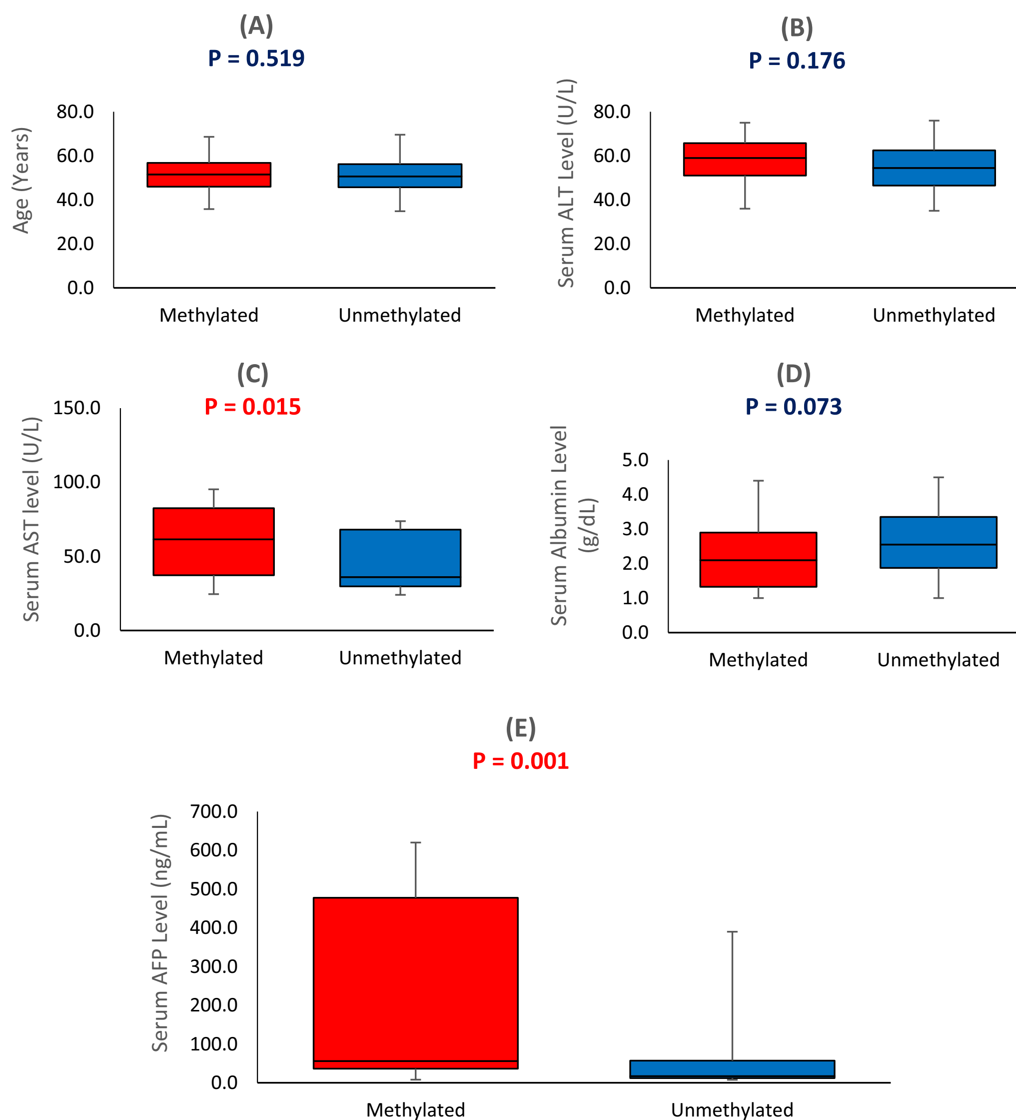

Fig. 2 Relation of *p16* promoter methylation with age and biochemical parameters in all participant patients. **A** Age, **B** serum ALT levels, **C** serum AST levels, **D** serum albumin levels, and **E** serum AFP levels. Data are represented as median and interquartile range; and compared by the Mann–Whitney U test. The top, middle and bottom lines of the box plot indicate the 75th percentile, median and the 25th percentile values, respectively. Error bars demonstrate the maximal and minimal values. Differences were considered statistically significant at $P < 0.05$

Serum levels of AFP in this study were significantly increased in liver cirrhosis patients compared to both the control and HCV groups, but HCC patients had almost tenfold higher levels of AFP in comparison to the cirrhosis group. These findings are consistent with previous reports [12, 27]. AFP was first recognized as an HCC marker more than 40 years ago and has since been described to detect preclinical HCC [17]. In chronic liver disease, sustained increase in serum AFP was considered as one of the risk factors for HCC. In liver cirrhotic patients, increased serum AFP level may reflect sudden onset of viral hepatitis with deterioration of liver disease and HCC progression [32].

In the present study, *RUNX3* promotor methylation rate was significantly higher in HCC group relative to the control group [*P* value: 0.016; OR (95% CI): 4.38 (1.32–14.50)]. Furthermore, *RUNX3* hypermethylation was significantly correlated with higher serum levels of AFP in all patients. This confirms the suggestions that serum hypermethylation of *RUNX3* could be considered as a sensitive HCC marker [33] and may represent a new screening marker for liver cancer patients [34].

Earlier studies have revealed that RUNX family members play important roles in both normal developmental processes and carcinogenesis. *RUNX3* inactivation by gene silencing via promoter hypermethylation is frequently involved in primary human gastric cancers [35]. A previous study reported that the methylation of six CpG loci in the *RUNX3* gene was significantly different among HCC patients, high risk HCC patients, and healthy individuals, indicating that those *RUNX3* gene loci might be involved in the epigenetic development of HCC. They suggested that, the abnormal methylation of *RUNX3* in plasma may provide a new screening marker for HCC. However, the mentioned study found no significant correlation between the methylation of *RUNX3* gene and the clinical feature of HCC patients [36]. Recently, in a study that involved 207 cases of HCV-related liver cirrhosis, 193 HCC patients and 53 healthy controls, methylation of *RUNX3* was found to be significantly higher in the HCC group compared to both cirrhosis and healthy groups [12]. It was suggested that promoter hypermethylation of *RUNX3* gene may be integrated in the early events of HCC development [37].

RUNX3 acts as a tumor suppressor and its inactivation can lead to tumor initiation and progression [38]. Decreased *RUNX3* expression has been linked to cell cycle deregulation, inhibition of apoptosis and enhancement of angiogenesis [39]. *RUNX3* is an important component of the transforming growth factor-beta signaling (TGF-β) pathway. *RUNX3* is required for the TGF-β-mediated growth arrest, where it may be involved in induction of CDK inhibitors and/or the Smad-mediated repression of the proto-oncogene c-Myc promoter [40], by interacting with Smads through their C-terminal segment and recruiting Smads to subnuclear sites of active transcription to exert their biological control [41].

In the current study, *p16* promotor methylation was significantly higher in HCC group compared to the control group [*P* value: 0.014; OR (95% CI) 4.97 (1.39–17.82)]. Hypermethylation of *p16* was significantly associated with higher serum levels of AST and AFP in all patients. These data are in agreement with the findings of a recent study conducted on 30 adult patients with HCC versus 30 controls divided into 15 with liver cirrhosis and 15 healthy subjects, where 86.7% of HCC patients had aberrant methylation of *p16* gene. In 2017 a meta-analysis was done including collected studies on 3105 HCCs and 808 non-tumor controls (chronic hepatitis and liver cirrhosis), and found that *p16* promoter methylation was significantly higher in HCC than in non-HCC patients [42]. Therefore, aberrant *p16* methylation was suggested as an early diagnostic marker in blood of hepatocellular carcinoma patients [13].

Suggested explanations for aberrant *p16* methylation as a predisposing factor for HCC is that aberrant methylation of at least 1 cytosine can significantly inactivate *p16* promoter with subsequent inhibition of its transcriptional activity. This happens due to alteration of the interaction between RNA helicase A enzyme, which facilitates the transcriptional function of the gene, and its regulatory region [4]. *p16* is a CDK inhibitor that plays a key role in cell cycle regulation [43]. The loss of *p16* expression through methylation has revealed its function as a tumor suppressor gene, suggesting frequent inactivation of this gene in human tumors [44]. As a tumor suppressor gene, the inactivation of *p16* gene results in excessive cell proliferation, accelerated cell cycles and a premature entry into the S phase before DNA complete repair, resulting in tumorigenesis [45].

Conclusions

The current study reported a significant correlation of *RUNX3* and *p16* gene promoters' hypermethylation with HCC in Egyptian patients. Methylation of both genes was linked with increased AFP serum levels in chronic liver disease patients. The findings of the current study emphasize the previous reports of the association of *RUNX3* and *p16* gene promoters hypermethylation with HCC in different ethnicities; suggesting their potential viability as biomarkers for early detection of HCC. However, the findings of the current study are limited by the small sample size and the significant age difference between HCC patients and the control subjects.

Abbreviations

AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CDK4: Cyclin-dependent kinase 4; CT: Computerized tomography; EASL: European Association for the Study of the Liver; ELISA: Enzyme linked immunosorbent assay; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; MRI: Magnetic resonance imaging; PCR: Polymerase chain reaction; *RUNX3*: Runt-related transcription factor 3; SOC-1: Suppressor of cytokine signalling 1; TNM: Tumor node metastasis; TGF-β: Transforming growth factor-beta signaling.

Acknowledgements

Not applicable.

Authors' contributions

FE substantively revised the manuscript. RHM and DMA designed the study. MIR collected the samples and was responsible for diagnosis of the cases. MMA and RHM performed the laboratory work. ETM performed the statistical analysis. MMA and ETM wrote the manuscript. All authors read and approved the final manuscript.

Funding

No funds, grants, or other support was received.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Written consent was obtained from all participants. The current study was conducted following the principles of Declaration of Helsinki, and approved by the Ethics Committee of Faculty of Pharmacy, Suez Canal University (Code 201807RH4).

Consent for publication
Not applicable.

Competing interests
He authors declare that they have no competing interests.

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Received: 15 June 2021 Accepted: 9 February 2022
Published online: 06 March 2022

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