ORIGINAL RESEARCH

Usefulness of Circulating Methylated p16 as a Noninvasive Molecular Biomarker for Hepatitis C-Related Hepatocellular Carcinoma with Normal Serum Alpha-Fetoprotein Levels

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Background: Screening of hepatocellular carcinoma (HCC) is challenged especially in patients with normal alpha-fetoprotein (AFP) levels. Aberrant p16 methylation has been implicated in HCC.

Objectives and Aims: This study aimed to assess serum methylated p16 (MP16) expression levels and to evaluate MP16 diagnostic performance in HCC detection among HCV-infected Egyptian patients with normal AFP levels.

Methods: MP16 levels were quantified using real-time PCR in 230 serum samples (30 healthy controls, 95 with HCV-HCC, 40 with chronic hepatitis C "CHC" and 65 with HCV cirrhosis). Diagnostic performance of MP16 for diagnosis of HCC was done using receiver operator characteristic curve analysis.

Results: Serum MP16 levels were significantly higher in HCC than CHC, cirrhosis, and healthy subjects and significantly higher in HCC with normal AFP levels than those with higher AFP. ROC curves revealed promising diagnostic performance for MP16 in discriminating HCC with normal AFP levels from non-HCC cases. This predictive ability improved by combining MP16 and AFP (AUC of 0.872 with 100% sensitivity, 76.5% specificity, 79.1% positive predictive value, 100% negative predictive value, and 87.5% accuracy).

Conclusion: MP16 can be a potential noninvasive molecular biomarker for HCC detection in patients with hepatic mass(es) and normal AFP levels especially in those where liver biopsy and radiological imaging cannot be done.

Keywords: methylated p16, hepatitis C virus, hepatocellular carcinoma, alpha-fetoprotein, quantitative real-time PCR

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent human malignancies and the third most common cause of death caused by cancer, globally.[1] Even though advances in the diagnostic and therapeutic modalities of HCC, there is still increasing in its incidence and lowering of the 5-year survival rate.[2] It has been revealed that different carcinogens activate or inhibit certain pathways during HCC development and progression. It is well known that risk factors as chronic viral hepatitis and cirrhosis are associated with HCC and it is also revealed that hepatitis C virus (HCV) infection accelerates the methylation process in HCC.[3]

Both genetic and epigenetic alterations are being increasingly identified for a better understanding of the patho-hepatic carcinogenesis.[4,5] Genetic alterations entail the irreversible DNA sequence changes, hence cumulative oncogene activation and tumour suppressor genes (TSGs) inactivation.[6] Furthermore, epigenetic alterations that denote the reversible and heritable modifications in gene expression without any DNA sequence alterations have been also concerned in HCC development and progression.[4] DNA methylation is one of the major epigenetic modifications in eukaryotic genome. It plays an essential role in the signalling pathways including apoptosis, cell proliferation, adherence, DNA repair, cell-cycle control, and during cancer development.[7] Moreover, aberrant DNA methylation includes genome-wide hypomethylation as well as promoter CpG island hypermethylation.[8] Methylation of the promoter CpG islands is present in the precursor lesions of a variety of cancers, including HCC and has been proved to be an early event in carcinogenesis where it inhibits the transcriptional initiation causing permanent silence of the downstream genes.[9] Multiple studies have documented certain cancer-related genes to be frequently methylated in HCC such as p16, CDKN2A, RASSF1A, and GSTP1.[10–12]

Many potential noninvasive biomarkers may help for the early detection of HCC in high-risk population hand in hand or even earlier to the currently available screening tests as alpha-fetoprotein (AFP) analysis and ultrasound.[10–14] These biomarkers include the detection of tumour-derived genetic and epigenetic alterations as point mutations, microsatellite instabilities, losses of heterozygosity and DNA hypermethylation in plasma or serum which identically reflects the tumour origin of altered cell-free DNA.[15]

Previous studies have reported the detection of frequencies or sequences of gene P16 methylation [p16 (INK4A)] which is a cyclin-dependent kinase inhibitor gene located on chromosome 9p21 in HCC patients.[6–18]

A third of HCC cases have normal AFP levels at the time of HCC diagnosis and usually remain low (<400–500 ng/mL), even with advanced HCC, in addition, a third of non-HCC cases have abnormal AFP.[13] Because of both false positives and false negatives, the accuracy of the AFP is challenged and its ongoing use for HCC screening test is still a matter of debate. So, we aimed to quantitatively estimate serum levels of methylated p16 (MP16) and to evaluate its diagnostic performance in the detection of HCC among HCV-infected patients, especially with normal serum AFP levels.

Materials and Methods
Study Design
This case–control study was done at Assiut University Hospital, Assiut, Egypt, from July 2018 to November 2019. The study was approved by the Local Ethics Committee of Assiut University Hospital and was conducted in accordance with the provisions of the Declaration of Helsinki. A written informed consent was obtained from all the participants before enrolment.

Study Population
The study group included 200 naïve chronic HCV-infected patients and they were divided into 95 patients with HCC and 105 patients with benign liver diseases "BLD" including 40 had chronic hepatitis C "CHC" and 65 had liver cirrhosis. Diagnosis of chronic liver disease was based on clinical, biochemical, and imaging findings. The severity of liver cirrhosis was measured by Child-Pugh and MELD scores.[19,20] The diagnosis of HCC was based on triphasic computed tomography scan according to the guidelines for the diagnosis and treatment of HCC.[21] Thirty volunteers who were sex- and age-matched, had negative markers for HBV and HCV infections, and apparently healthy based on clinical and laboratory examination were served as normal controls.

Those patients were consecutively selected from outpatient clinics and inpatient wards of The Tropical Medicine and Gastroenterology, and Internal Medicine Departments, Assiut University Hospital, Assiut, Egypt, while controls were selected randomly from outpatient clinic and the patients' relatives. Patients with evidence of non-HCV related liver diseases, HCV/HBV co-infection, HIV co-infection, hepatic metastasis, or receiving antiviral therapy were excluded.

Medical history and clinical examination were performed and measurement of liver function tests, serum creatinine, AFP, and serum methylated p16 (MP16) levels was undertaken.

Collection and Processing of Blood Specimens
Under aseptic precautions, 3 ml of blood was collected before therapy from each subject into plain polypropylene tube. Blood was allowed to clot in the tube and then centrifuged for 10 min at 4000 rpm. Sera were inspected to ensure it is clear and non-haemolyzed and were carefully transferred into plain polypropylene tubes. Part of collected serum was used for chemical investigations and

remaining part was stored at −20°C until further proces-
sing. Extraction of DNA from sera, bisulfite conversion,
and quantitative real-time PCR (qPCR) amplification were
done in the Molecular Unit, Laboratory of Clinical
Immunology, Assiut University Hospital, Egypt. The
laboratory specialist was blinded to patient diagnosis and
patient disease category.

Serum creatinine and liver function tests were done on
the automated chemistry analyzer Dimension RxL Max
(Siemens, USA), while serum α-fetoprotein was measured
using Architect AFP reagent kit (REF 3P36, Abbott,
Ireland) on Architect i1000 (Abbott, USA). This was per-
formed according to manufacturers' instructions.

DNA Extraction from Sera
DNA was extracted from serum using QIAamp DNA Mini
Kit (catalog no. 51304, Qiagen, Hilden, Germany) using
the blood and body fluids protocol according to manufac-
turer's instructions, a final elution volume of 50 µl was
used.

DNA Bisulfite Conversion
The principle of DNA bisulfite conversion is that treating
DNA with bisulfite would result in the conversion of
unmethylated cytosine residues into uracil. Methylated
cytosine residues, on the other hand, would remain
unchanged. Thus, the DNA sequences of methylated and
unmethylated genomic regions after bisulfite conversion
would differ and can be differentially amplified by
sequence-specific PCR primers. DNA Bisulfite conversion
was carried out using EpiTect Fast DNA Bisulfite Kit
(catalog no. 59824, Qiagen, Hilden, Germany) as follows:

- The bisulfite reaction mixture was prepared follow-
  ing the manufacturer's recommended setup using 25
  µl of DNA elutes.
- Reaction tubes were loaded into Veriti 96 well-
  thermal cycler (Applied Biosystems, USA) pro-
  grammed for the following cycling conditions: 2
  cycles (5 min denaturation at 95°C, then 20 min
  incubation at 60ºC) followed by a hold step at 20ºC.
- Cleanup of converted DNA was done according to
  manufacturer's instructions using carrier RNA in
  final concentration of 10 µg/mL in buffer BL.
- Bisulfite converted DNA was finally eluted using 12 µl
  of buffer BE.

Real-Time PCR Amplification
For relative quantitation of MP16, amplification of endo-
genous control (GAPDH) was performed to normalize
levels of methylated p16 towards differences in the
amount of DNA loaded into PCR reaction tubes. For
each sample, each of MP16 and GAPDH was amplified
in a separate PCR tube. All amplification/quantification
experiments included a No-Template Control (containing
all the components of the reaction except for the DNA
template) to detect carry-over contamination.

Real-time PCR amplification of methylated p16 was done
using custom methylated sequence-specific detection primers
(catalog no. 4304970, Applied Biosystems, USA), Taqman
probe (FAM/TAMRA; catalog no. 450025, Applied
Biosystems, USA), and EpiTectMethyLight PCR+ROX™
vial Kit (catalog no. 59496, Qiagen, Hilden, Germany). PCR
reaction mixture was prepared following manufacturer recom-
mendations: 10 µl of 2x Epitect Methy Light master mix (w/o
ROX), 0.4 µl of 50x ROX dye solution, 0.4 µM of each
primer, 0.2 µM probe, and 2.5 µl of bisulfite converted
DNA, in a final total volume of 20 µl per reaction. The
previously described[10,22,23] sequences of methylated p16 pri-
mers: 5́-TTATTAGAGGGTGGGGCGGATCGC-3́ and 5́-
GACCCCGAACCGCGACCGTAA-3́ and the previously
described[10] probe sequence: 5́-AGTAGTATGGAGTTTT
CGGTTGATTGGTTG-3́ were used.

GAPDH real-time PCR amplification was performed
using human GAPDH endogenous control primer/probe set
(VIC™/MGB probe, catalog no. 4326317E, Applied
Biosystems, USA) and TaqMan® Universal PCR Master
Mix II- no UNG (catalog no. 4440043, Applied Biosystems,
USA). PCR reaction mixture was prepared according to man-
ufacturer's instructions: 10 µl of 2x TaqMan Universal PCR
Master Mix, 1 µl of 20x GAPDH endogenous control primer/
probe, and 5 µl of DNA in a final total volume of 20 µl per
reaction.

PCR tubes were loaded into 7500 Fast Real-Time PCR
System (Applied Biosystems, USA) with initial denatura-
tion/activation step of 10 min at 95°C followed by 40
cycles (denaturation for 15 s at 95°C and annealing/exten-
sion for 60 s at 60ºC).

Interpretation of Results
In each run, raw data were analysed using the automated
setting (7500 Fast Software, Version 2.0.5) to assign baseline
and threshold. The cycle threshold (Ct) was determined

which is the fractional cycle number at which the fluorescence exceeds the given threshold.

The 2-ΔΔCt method[24] was used to calculate the relative quantitation of MP16 as follows:

1. For each patient/control subject, ΔCt was calculated as the difference between Ct of endogenous control (Ct GAPDH) and Ct of target MP16;

ΔCt sample = Ct MP16 of a sample – Ct GAPDH of the same sample.

1. The mean ΔCt of control subjects (ΔCt calibrator) was calculated as the difference between mean Ct of GAPDH assay of the control subjects and mean Ct of the MP16 assay of the control subjects.
2. For each sample (patient and control), the ΔΔCt of each target MP16 was calculated as the difference between ΔCt of this sample and ΔCt calibrator (mean ΔCt of control subjects);

ΔΔCt = ΔCt sample – ΔCt calibrator

1. Then, the equation 2–ΔΔCt was applied for each sample; Fold difference = 2–ΔΔCt

Accordingly, MP16 assay results were presented as fold changes relative to the mean of control group (healthy subjects) using the 2-ΔΔCt equation.

Statistical Analysis

All statistical analyses were done by using Statistical Package for the Social Sciences (SPSS) for Windows version 16 (SPSS Inc., Chicago, IL, USA) and MedCalc program. The Kolmogorov–Smirnov Test of Normality was used to test the normality of data. The quantitative data were expressed as mean ± standard deviation (SD) or median and the range (minimum–maximum) for normally or abnormally distributed data, respectively. They were compared using Mann–Whitney U-test or The Kruskal–Wallis test for two or more groups of abnormally distributed data, respectively. The qualitative data were expressed as a percentage. Spearman's rank correlation coefficient (rho) was used to find correlations. The receiver operating characteristic curves (ROC) were plotted to measure the performance of MP16 (with and without AFP) in predicting the presence of HCC and to select its optimal cut-off value at which the sensitivity, specificity, positive

(PPV) and negative (NPV) predictive values, and overall accuracy could be calculated. All tests were two-tailed and P-values < 0.05 were considered significant.

Results
Characteristics of the Study Population

The present study was performed on 200 patients with HCV-related CLD (158 males and 42 females with mean age of 59.8 ± 7 years) and 30 healthy subjects (9 females and 21 males with a mean age of 55.5 ± 7.6 years). The baseline demographic and clinical data of the studied population are summarized in Table 1.

Expression of Methylated p16 in Serum of Patients and Healthy Controls

Serum MP16 expression was expressed in all analysed samples. There were no significant differences in serum MP16 levels with respect to gender and age among patients with HCC, BLD, and healthy controls (P > 0.05). Serum MP16 levels were significantly elevated in HCC cases as

Table 1 Baseline Demographic and Clinical Characteristics of Patients with HCV-Related Chronic Liver Disease

	Patients with HCC (n= 95)	Patients with BLD (n= 105)
Age (years, mean ± SD)	60.8 ± 6.1	58.6 ± 7.8
Sex (n, %)		
Male	77 (81.1%)	81 (77.1%)
Female	18 (18.9%)	24 (22.9%)
Severity of Hepatic Disease (n, %)		
CHC	–	40 (38.1%)
LC	95 (100%)	65 (61.9%)
Child-Pugh score (mean ± SD)	9.3 ± 2.5	9.6 ± 2.7
MELD score (mean ± SD)	16.7 ± 7.8	16.1 ± 6.5
AFP (n, %)		
≤500 ng/mL	38 (40.4%)	105 (100%)
>500 ng/mL	57 (59.6%)	-
Number of HCC (n, %)		
Single	40 (42.1%)	–
More than one	55 (57.9%)	
Size of HCC (n, %)		
≤5 cm	42 (44.2%)	–
>5 cm	53 (55.8%)	

Abbreviations: AFP, alpha-fetoprotein; BLD, benign liver disease; CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MELD, Model of end-stage liver disease; n, number; SD, standard deviation.

compared to BLD cases and normal controls (P < 0.001). Furthermore, MP16 levels were significantly higher in patients with BLD than normal group (P < 0.001). However, among BLD group, no significant difference in MP16 levels between chronic hepatitis C and liver cirrhosis patients [median; 108.3 (0.88–404.5) vs 142.54 (0.75–5.5x10^5); P = 0.365] (Table 2 and Figure 1).

Relationship Between Serum MP16 Expression and Clinical Characteristics in HCC Patients

No correlations were found between serum MP16 levels and age (rho = 0.110, P = 0.304), severity of liver cirrhosis based on Child-Pugh (rho = 0.042, P = 0.694) and MELD (rho = 0.059, P = 0.578) scores and AFP levels (rho = 0.240, P = 0.161). However, cases of HCC with normal AFP levels had significantly higher MP16 levels [median; 2091 (4.69–6.1×10^5) fold changes] when compared to HCC cases with higher AFP levels [median; 190.9 (1.07–1.5×10^6) fold changes, P = 0.028] and non-HCC cases [median; 142.54 (0.75–5.5×10^5) fold changes P = 0.004] as shown in Figure 2. In addition, serum MP16 levels were not associated with gender [median MP16 levels in males; 174.85 (0.75–1.1×10^6) fold changes vs those in females; 820.3 (1.07–5.5×10^5) fold changes, P = 0.111] and tumour characteristics; tumour size [median MP16 levels in those with HCC of ≤5 cm; 2091 (1.07–1.1×10^6) fold changes vs those with HCC > 5 cm; 2047 (0.91–8×10^5) fold changes, P = 0.111] and number [median MP16 levels in those with one lesion; 1962 (1.07–1.1×10^6) fold changes vs those with more than one lesion; 2202.3 (0.91–8×10^5) fold changes, P = 0.409].

Table 2 Distribution of Serum Methylated P16 Levels Among Healthy Controls and Cases

	Cases (n)	Serum Methylated p16 Levels (Median & Range; Fold Changes)	p
Healthy controls	30	1.06 (0.57–1.69)	<0.001
Patients with HCV-related BLD	105	108.34 (0.75–5.5x10^5)[a]	
Patients with HCV-related HCC	95	760.08 (0.91–1.1x106)[b]	

Notes: [a]P < 0.001 compared with controls, [b]P = 0.037 compared with BLD.
Abbreviations: BLD, benign liver disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; n, number.

Diagnostic Performance of Methylated p16 for Prediction of HCC

As shown in Figure 2, ROC curves were developed to evaluate the diagnostic potential of MP16 as a noninvasive biomarker candidate. For diagnosis of HCC vs non-HCC cases, ROC curve analysis showed an area under the curve (AUC): 0.7 (95% CI: 0.580–0.772) with a sensitivity, specificity, PPV, NPV, and overall accuracy of 51.1%, 80.4%, 70.2%, 64.5%, 65% and 66.5%, respectively, at optimal cut-off value 464.65 fold changes (Figure 3A). With the combination of MP16 and AFP, the AUC was increased to 0.872 (95% CI: 0.742–0.951) with 100% sensitivity, 76.5% specificity, 79.1% PPV, 100% NPV, and 87.5% accuracy for discriminating HCC (Figure 3B). Furthermore, ROC analysis also showed that serum MP16 expression levels to distinguish patients with HCC and normal AFP levels from those without HCC had an AUC of 0.823 (95% CI: 0.694–0.942) with 55% sensitivity, 95.7% specificity, 75% PPV, 90.1% NPV, and 88% overall accuracy at optimal cut-off value of >824.14 (Figure 3C).

Discussion

Hypermethylation of gene promoters has been demonstrated as an early event in hepatocellular carcinogenesis.[25] In this study, we quantified the circulating MP16 levels using Rt-PCR in patients with HCV-related CLD for screening and early detection of HCC. These levels were significantly higher in patients with HCC as compared to patients with BLD or normal controls. Previous studies explored the detection of frequencies or sequences of gene P16 methylation in HCC, whereas higher frequencies of MP16 (65% and 96%) were observed in HCC cases.[9,16-18] While, Wong et al[16] showed that quantities of MP16 sequences were detected in peripheral circulation of 80% of HCC patients.

We found that MP16 levels were significantly higher in patients with BLD than normal controls but these levels were not significantly different between liver cirrhosis and CHC patients. These results were matched with Chu et al,[26] who informed that 17% of cirrhosis patients had serum DNA with aberrant p16 methylation. Furthermore, Narimatsu et al[17] had comparable results suggesting that hepatitis viruses may induce p16 methylation in liver tissues with chronic inflammation before the appearance of HCC. However, Wong et al[10] reported that p16 hypermethylation was not detected in the plasma/serum of patients with either liver cirrhosis or hepatitis. This discrepancy indicates that this correlation is still controversial.[27]
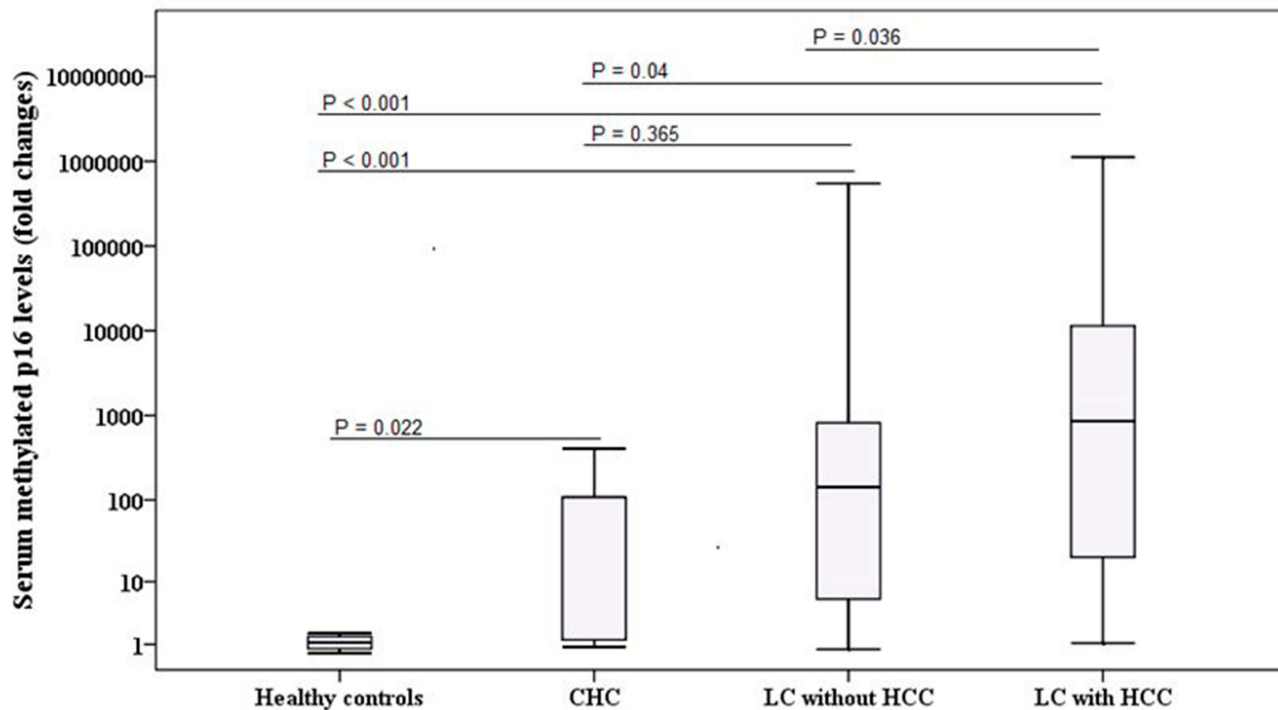
Figure 1 Comparison between serum methylated p16 levels among different groups (healthy controls and HCV-related chronic liver disease).
Abbreviations: CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

P16 gene is an important tumour suppressor gene located on chromosome 9p21 and it is one of the most common altered genes observed in various human tumours.[28] Earlier studies demonstrated that p 16 (p16INK4A) gene methylation might play an imperative role in p16 gene inactivation which leads to p16-mediated cell-cycle control disruption and therefore participate in a role in hepatocarcinogenesis.[29,30] Several studies have indicated that these epigenetic changes might deceive "addict" cancer cells to signal transduction pathway changes during the early tumour stages.[31,32] The appearance of these serum/plasma tumour-derived DNA altered genes may be due to their release from the tumour during cell turnover, cellular necrosis, or apoptosis.[33]

In the current study, serum MP16 levels were not significantly influenced by the tumour characters (number and size) which was in accordance with earlier series.[18,34] However, Zhang et al[9] found that p16 methylation may be implicated in tumour progression.

Similar to previous studies,[34,35] we found no significant correlation between serum MP16 levels and AFP levels. However, MP16 levels were significantly higher in those with normal levels of AFP. Nevertheless, Wong et al[36] demonstrated that circulating MP l6 sequences were detected in all individuals with higher serum AFP of >45 µg/L, whereas they were found in 57% of cases who had lower AFP concentrations of <45µg/L. This indicates its importance in the screening of HCC in high-risk individuals with normal AFP levels.

Despite elevated MP16 levels in HCC cases, MP16 had low performance in prediction of HCC, where AUC was 0.7 with 51.1% sensitivity and 80.4% specificity and 66.5% overall accuracy at the optimal cut-off value of 464.65-fold changes. Even though this performance got better with adding AFP (AUC was 0.872 with 100% sensitivity, 76.5% specificity, and 87.5% accuracy), the utility of using MP16 in the presence of high AFP for HCC diagnosis is questionable. On the other hand, in this study, about 40% of patients with HCC had normal AFP levels (<400–500 ng/mL) where their serum MP16 levels were significantly higher than patients without HCC. Furthermore, MP16 had a good diagnostic performance in discriminating HCC cases with normal AFP levels from non-HCC cases with an AUC of 0.823, high specificity and NPV (95.7% and 90.1%), and reasonable sensitivity and PPV (55% and 75%) at cut-off value of 824.14-fold changes suggesting their potential value for HCC detection. Owing to the previously mentioned findings, assessment of serum MP16 expression levels may be
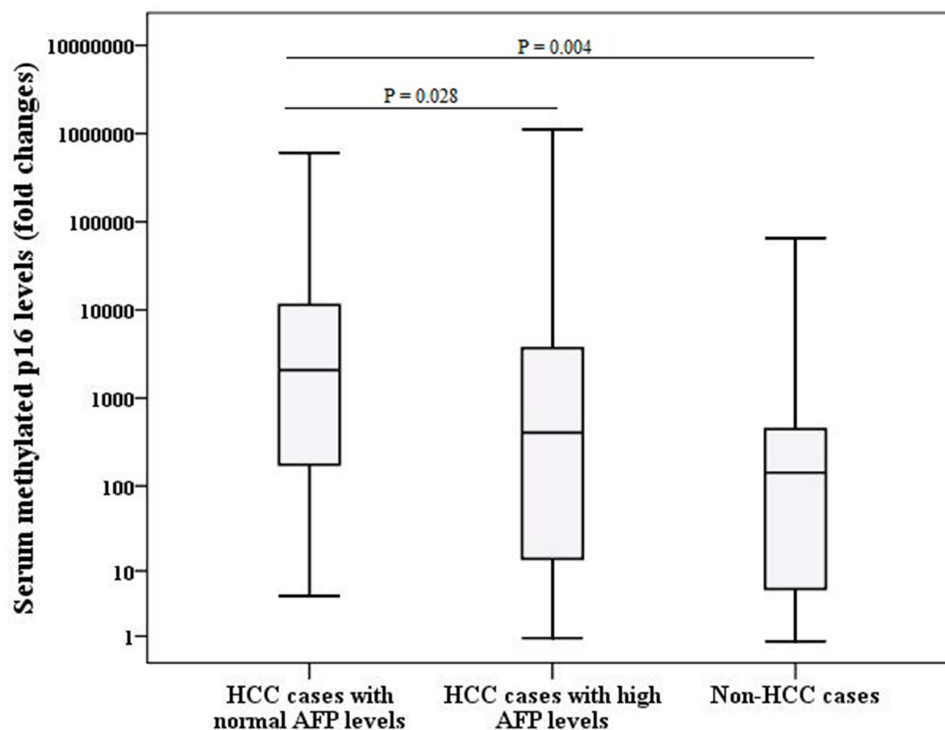
Figure 2 Comparison between serum methylated p16 levels among different groups (HCC cases and non-HCC cases).
Abbreviations: AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

restricted for cases with hepatic mass(es) and normal AFP levels as a potential noninvasive biomarker for HCC diagnosis especially in cirrhotic patients where liver biopsy (eg, those with severely impaired prothrombin time or low platelet count) and/or radiological imaging (eg, renal impairment, high cost, or less availability) cannot be done.
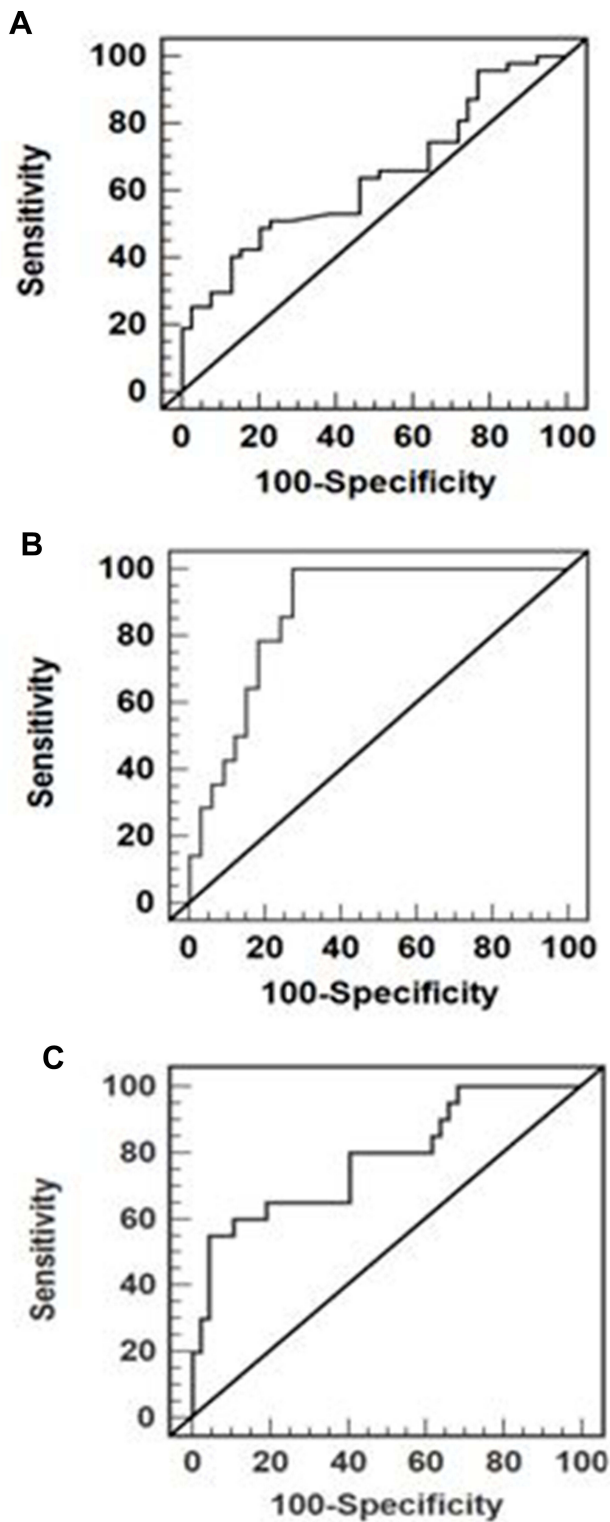
Many resources have been triggered for the development of different epigenetic therapeutic approaches because of their reversible alterations. They have emerged as attractive targets for therapeutic intervention.[37] These epigenetic drugs are able to reexpress silenced genes, either by demethylation of methylated promoter regions (demethylating agents) or by histone acetylation.[38] Several compounds have been preclinically tested and showed promising results in other cancers and HCC as 5-azacytidine (Vidaza), 5-aza-dC (decitabine), MS-275, and Valproic Acid.[39] Therefore, we recommend performing quantitative analysis of methylated gene p16 in large cohorts with liver cirrhosis especially for high-risk patients who had suggestive clinical manifestations of HCC as loss of weight, repeatedly hepatic encephalopathy without evidence of predisposing factors or rapid accumulating ascites even without focal lesion(s) in imaging. Furthermore, those who had MP16 levels > 464.65-fold changes should be subjected for meticulous laboratory and careful imaging follow-up for early HCC detection and hence a trial of epigenetic therapeutic interventions.

To our knowledge, this is one of the first studies to evaluate quantitatively MP16 expression and to evaluate its diagnostic performance in the detection of HCC among HCV-infected patients especially in with normal AFP in our community. However, some limitations as small sample-sized and a single-centre study were considered. The quantitative assessment of MP16 in liver tissue and its correlation with serum levels was not accessible. This is a preliminary study so, phased validation with large-scale samples is needed to confirm these findings, to demonstrate whether it can be incorporated into routine clinical practice in patients with hepatic masses and normal AFP and early hepatocarcinogenesis detection and to assess its ability of monitoring tumour progression, response to treatment and recurrence.

Conclusion

Serum MP16 expression levels were higher in HCC than non-HCC patients with HCV-related chronic liver disease. Furthermore, MP16 may be a potential molecular biomarker for diagnosis of HCC in HCV-infected patients with

hepatic mass(es) and normal AFP levels with high specificity and reasonable sensitivity especially in those where liver biopsy and radiological imaging cannot be done. Understanding roles of MP16 in modulating the signalling pathways for HCC might be helpful in lowering the incidence and progression of HCC through epigenetic therapeutic interventions.

Acknowledgment

Disclosure

The authors declare that they have no competing interests.

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Figure 3 Area under the receiver operating characteristic curves (AUC) of (**A**) methylated p16 [AUC= 0.7; 95% CI (0.580–0.772)] for predicting HCC versus non-HCC, (**B**) combination of methylated p16 and AFP [AUC= 0.872; 95% CI (0.742–0.951)] for predicting HCC versus non-HCC, and (**C**) methylated p16 [AUC= 0.823; 95% CI (0.694–0.942)] for predicting HCC in patients with hepatic mass and normal AFP versus non-HCC cases.
Abbreviations: AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

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