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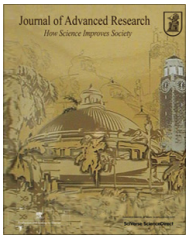

ORIGINAL ARTICLE

Methylation of multiple genes in hepatitis C virus associated hepatocellular carcinoma

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ABSTRACT

We studied promoter methylation (PM) of 11 genes in Peripheral Blood Lymphocytes (PBLs) and tissues of hepatitis C virus (HCV) associated hepatocellular carcinoma (HCC) and chronic hepatitis (CH) Egyptian patients. The present study included 31 HCC with their ANT, 38 CH and 13 normal hepatic tissue (NHT) samples. In all groups, PM of *APC*, *FHIT*, *p15*, *p73*, *p14*, *p16*, *DAPK1*, *CDH1*, *RARβ*, *RASSF1A*, O^6*MGMT* was assessed by methylation-specific PCR (MSP). *APC* and *O6-MGMT* protein expression was assessed by immunohistochemistry (IHC) in the studied HCC and CH (20 samples each) as well as in a different HCC and CH set for confirmation of MSP results. PM was associated with progression from CH to HCC. Most genes showed high methylation frequency (MF) and the methylation index (MI) increased with disease progression. MF of *p14*, *p73*, *RASSF1A*, *CDH1* and O^6*MGMT* was significantly higher in HCC and their ANT. MF of *APC* was higher in CH. We reported high concordance between MF in HCC and their ANT, MF in PBL and CH tissues as well as between PM and protein expression of *APC* and O^6*MGMT*. A panel of 4 genes (*APC*, *p73*, *p14*, O^6*MGMT*) classifies the cases independently into HCC and CH with high accuracy (89.9%), sensitivity (83.9%) and specificity (94.7%). HCV infection may contribute to hepatocarcinogenesis through enhancing PM of multiple genes. PM of *APC* occurs early in the cascade while PM of *p14*, *p73*, *RASSF1A*, *RARB*, *CDH1* and O^6*MGMT* are late changes. A panel of *APC*, *p73*, *p14*, *O6-MGMT* could be used in monitoring CH patients for early detection of HCC. Also, we

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found that, the methylation status is not significantly affected by whether the tissue was from the liver or PBL, indicating the possibility of use PBL as indicator to genetic profile instead of liver tissue regardless the stage of disease.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death [1]. It accounts for approximately 600,000 deaths per year [2] and it shows a wide geographical variation with low incidence areas in North America and Europe, and high incidence areas in Africa and Asia. In Egypt the incidence of HCC has doubled in the past 10 years, thus it is now the second most incident and lethal cancer in men after lung cancer [3]. The heavy burden of HCC parallels the high rates of HCV infection while hepatitis B virus (HBV) rates have declined after the introduction of the vaccine in 1992 [4,5]. Although it has been estimated that 80% of HCC occurs in cirrhotic livers, the exact molecular mechanisms underlying virus-associated hepatocarcinogenesis are still unclear.

Multiple genetic aberrations of oncogenes and tumor suppressor genes have been identified, which control hepatocytes proliferation, differentiation, maintenance of genomic integrity and death [6,7]. In addition, recent studies suggest aberrant DNA (PM) as an alternative mechanism of tumor pathogenesis because the hypermethylated promoters often lack transcriptional activity, which could result in gene inactivation [8]. DNA methylation refers to the addition of a methyl group to the cytosine residue in CpG dinucleotides. Normally, clustered CpG dinucleotides (CpG islands) are not methylated regardless of their transcriptional status, whereas in tumor cells, methylation of CpG islands in the promoter regions of many tumor suppressor genes (TSGs) and growth regulatory genes effectively silences those genes. Since different types of cancer show distinct DNA methylation profiles, it is possible to develop cancer- type specific methylation signatures [9]. The power of PM as a marker derives not only from its ability to be detected in a wide variety of samples, from fresh specimens to body fluids and archival paraffin-embedded tissues, but also from the defined localization of the lesion in promoter CpG islands of the genes. This could be an early important event in carcinogenesis and could also be of importance for treatment or prognostication [10]. DNA methylation profiles in Egypt has not been well studied, though it has the highest prevalence of HCV infection in the world with approximately 14% of the population infected, and seven million have chronic HCV induced liver disease [11].

We sought to assess DNA methylation patterns in Egyptian patients with HCV associated chronic hepatitis and HCC using a panel of genes that are commonly hypermethylated in other solid tumors (*p14*, *p15*, *p16*, *p73*, *APC*, *FHIT*, *DAPK1*, *CDH1*, *RARβ*, *RASSF1A*, and *O6MGMT*) in order to understand the role of epigenetic silencing in this patient population. The studied groups included 38 HCV/genotype-4-associated CH patients with matched PBL in 20 of them and 31 HCC cases with their ANT. Thirteen NHT obtained from healthy individuals, were used as a control group. The prognostic impact of aberrant PM was also assessed through correlations between methylation patterns and the clinic-pathological features of the studied patients.

Methodology

Study design

This prospective study encompassed three groups. The first group included 31 HCC cases, of which, 23 cases had enough adjacent normal tissue (ANT) samples to be assessed. The second group included: A) 20 cases of chronic CH patients with cirrhosis from which tissue samples and Peripheral Blood Lymphocytes (PBLs) were collected and 18 cases of asymptomatic carriers (ASC), from which tissue samples only were collected. The third group was a control group in which normal hepatic tissue (NHT) samples were obtained from 13 liver transplantation donors matched for age (±5 years) and sex.

HCC samples were obtained from patients who underwent surgical resection of their tumors at the National Cancer Institute (NCI), Cairo, Egypt. Whereas CH samples were obtained from the Endemic medicine department, Kasr Al-Aini School of Medicine, Cairo University. All cases were assessed for viral profile as a part of the routine clinical workup. All HCC and CH cases were positive for HCV/genotype-4 and negative for HBV by serological tests and/or HBV-DNA by real time PCR (qRT PCR). Histopathological diagnosis and grading of the HCC cases were done according to the World Health Organization (WHO) classification criteria [12] and staging was performed according to the American Joint Committee on Cancer [13]. Grading and staging of CH patients were performed according to the pathology activity index [14]. A written informed consent was obtained from each patient and the Institutional Review Boards of the National Cancer Institute and Kasr Al-Aini School of Medicine, Cairo University, reviewed the study protocol which was in accordance with the 2007 Declaration of Helsinki. All patients' characteristics were collected from the patients' records and illustrated in Table 1.

DNA extraction

DNA was extracted from PBL according to standard protocols (6). Briefly, equal volume of equilibrated phenol (pH 7.0–7.5) was added to samples and vortexed. The upper aqueous layer was removed and an equal volume of phenol/chloroform (1:1) was added and vortexed. The upper aqueous layer was removed again and an equal volume of chloroform/isoamyl alcohol (24:1) was added and vortexed. This was followed by the addition of 3 M Sodium acetate (pH 4.7–5.2), DNA precipitation by ice-cold ethanol and overnight incubation at −80 °C. The fluid was decanted and the DNA pellet was dissolved in sterile water. DNA was extracted from fresh tissue samples as previously described [15].

Table 1 Clinical features of the studied groups.

Variables	CAH cases (38)		HCC cases (31)		*p* Value
	Mean	Range	Mean	Range	
Age (years)	40.0	(1–61)	57	(38–78)	< 0.0001
WBCs	5.9	(3.2–108)	5.4	(2.5–26.6)	< 0.0001
RBCs	14	(4.17–15.5)	4.2	(3.6–9.3)	
HG	14.7	(11.3–17)	13.1	(9.3–16.9)	< 0.0023
Platelets	202.5	(98–377)	195	(33–356)	< 0.0001
AST	48.5	(14–297)	67	(7–432)	
ALT	52	(4–209)	58	(10–480)	
Alk	90	(34–282)	96	(35–387)	
Albumin	4.3	(2.6–40)	3.3	(2.5–6.0)	
Total bilirubin	0.99	(0.28–23.4)	1.00	(0.20–4.1)	
Direct bilirubin	0.55	(0.1–12.1)	0.4	(0.2–2.6)	

Only significant *p* values are illustrated.

Bisulphate conversion and methylation-specific polymerase chain reaction (MSP)

The extracted DNA was subjected to bisulfate treatment followed by MSP using the primer sequences and the methylation-specific PCR conditions illustrated in Table 2. DNA methylation of CpG islands for *p14*, *p15*, *p16*, *p73*, *APC*, *FHIT*, *DAPK1*, *CDH1*, *RARβ*, *RASSF1A* and O^6*MGMT* genes was determined using specific primers for methylated (M) and unmethylated (UM) DNA [16]. Negative control samples without DNA were included in each set of PCR. PCR products were analyzed on 4% ethidium bromide-stained agarose gels and visualized under ultraviolet illumination (Fig. 1).

Immunohistochemistry

Protein expression of *APC* and O^6*MGMT* was assessed in 20 cases of HCC and 20 cases of CH which were assessed for PM by MSP as well as in a confirmatory set of 107 HCC, 52 CH cases and 40 NHT samples to confirm the results of the MSP using the tissue microarray (TMA) technique. Two (5 μm) thick sections were obtained from each TMA block on positive charged slides to be used for immunohistochemistry. Sections were deparaffinized, rehydratedin graded alcohols and the standard streptavidirin–biotin–peroxidase technique was performed [17] using the following antibodies: rabbit anti-human O^6*MGMT* (EPR-4397, Epitomics, USA 1:100) and the rabbit anti-human *APC* (EP-701Y, Epitomics, USA 1:50). Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer (pH 7.4) and then the primary antibody was applied and incubated overnight at 4 °C in a humidified chamber. After three washes in PBS, the secondary antibody and the avidin–biotin complex (ABC) were applied to slides with diaminobenzidine (DAB) as a chromogen and Mayer's hematoxylin as a counterstain. To evaluate the specificity of the antibodies, known positive and negative tissues were used as controls. Assessment was based on a cytoplasmic staining pattern for *APC* and on nuclear expression for O^6*MGMT*.

Statistical methods: The data comprised of information about the presence or absence of PM of the 11 genes in four distinct groups, HCC (31) *T*, CH with cirrhosis (20) C, ASC (18) A, and healthy controls (NHT, $n = 13$) B. In the HCC group, 23 HCC cases had data for the tumor and the ANT whereas the remaining eight cases, had data for the tumor and the corresponding PBL but not on ANT. In the CH group, data for tissues was paired with the corresponding PBL. We used one-way ANOVA test to detect differences in the available clinicopathological variables between disease states. For each of these variables, the corrected *p*-values were reported. Logistic regression with a random effect analysis (non-linear mixed model) was used to determine differences across categories within a group and methylation status controlling for the subject effect for CH and HCC groups. Logistic regression analysis (Proc Logistic) was used to determine differences in methylation status and we reported the interaction and the main effects. The interaction effects measure any synergistic or antagonistic effect of the methylation status (methylated versus unmethylated) and the disease site (normal or tumor and liver or PBL); disease state (healthy controls, ASC, CH with cirrhosis or HCC). All statistical tests were performed using the SAS software package (version 9.2, SAS, Cary, NC).

Results

Clinical findings

There was a significant difference (corrected for multiplicity) between the studied groups regarding age (*p*-value < 0.0001), HG (*p* value < 0.0023), platelets (*p* value < 0.0001) and WBCs (*p* value < 0.0001). In all cases, the HCC group was significantly different from CH patients with cirrhosis and the asymptomatic carrier groups as in Table 1.

Methylation index (MI)

Calculation of the MI (defined as the ratio between the number of methylated genes and the number of total genes analyzed for each sample) was done for all cases. The MI ranged from 0 to 0.55 in CH cases (average: 0.27), and from 0.27 to 0.90 in HCC (average: 0.36). The difference between both groups was statistically insignificant ($p > 0.05$).

DNA methylation in normal hepatic tissues

PM of the 11 tested genes was assessed in 13 NHT samples. None of the samples showed PM of *p15*, *p73*, *RARβ*, *RASS-*

Table 2 Primers sequences and conditions of the methylation specific PCR (MSP).

Gene	Primers	Annealing temperature (1C)	$MgCl_2$	Cycles
CDH1 (M)	TAATTAGCGGTACGGGGGGC CGAAAACAAACGCCGAATACG	59	4.5	32
CDH1 (U)	TTAGTTAATTAGTGGTATGGGGGGTGG ACCAAACAAAAACAAACACCAAATACA	59	4.5	32
DAPK (M)	GGATAGTCGGATCGAGTTAACGTC CCCTCCCAAACGCCGA	59	4.5	35
DAPK (U)	GGAGGATAGTTGGATTGAGTTAATGTT CAAATCCCTCCCAAACACCAA	59	4.5	35
p73 (M)	GGACGTAGCGAAATCGGGGTTC ACCCCGAACATCGACGTCCG	64	4.5	35
p73 (U)	AGGGGATGTAGTGAAATTGGGGTTT ATCACAACCCCAAACATCAACATCCA	60	4.5	35
O6O6-MGMT (M)	TTTCGACGTTCGTAGGTTTTCGC GCACTCTTCCGAAAACGAAACG	56	3.5	35
O6O6-MGMT (U)	TTTGTGTTTTGATGTTTGTAGGTTTTTGT AACTCCACACTCTTCCAAAAACAAAACA	57	4.5	35
p14 (M)	GTGTTAAAGGGCGGCGTAGC AAAACCCTCACTCGCGACGA	54	4.5	35
p14 (U)	TTTTTGGTGTTAAAGGGTGGTGTAGT CACAAAAACCCTCACTCACAACAA	56	4.5	35
p15 (M)	GCGTTCGTATTTTGCGGTT CGTACAATAACCGAACGACCGA	57	3.5	35
p15 (U)	TGTGATGTGTTTGTATTTTGTGGTT CCATACAATAACCAAACAACCAA	59	4.5	35
p16 (M)	TTATTAGAGGGTGGGGCGGATCGC CCACCTAAATCGACCTCCGACCG	68	1.5	33
p16 (U)	TTATTAGAGGGTGGGGTGGATTGT CCACCTAAATCAACCTCCAACCA	58	4.5	33
FHIT (M)	TTGGGGCGCGGGTTTGGGTTTTTACGC CGTAAACGACGCCGACCCCACTA	71–63	1.5	32
FHIT (U)	TTGGGGTGTGGGTTTGGGTTTTTATG CATAAACAACACCAACCCCACTA	64	1.5	33
APC (M)	TATTGCGGAGTGCGGGTC TCAACGAACTCCCGACGA	62	3.5	35
APC (U)	GTGTTTTATTGTGGAGTGTGGGTT CCAATCAACAAACTCCCAACAA	62	1.5	35
RASSF1A (M)	TTCGTCGTTTAGTTTGGATTTTG CCGATTAAACCCGTACTTCG	56	1.5	35
RASSF1A (U)	TGTTGTTTAGTTTGGATTTTGG TACAACCCTTCCCAACACAC	59	3.5	35
RARβ (M)	TCGAGAACGCGAGCGATTCG GACCAATCCAACCGAAACGA	62	1.5	35
RARβ (U)	TTGAGAATGTGAGTGAATTGA AACCAATCCAACCAAAACAA	59	1.5	35

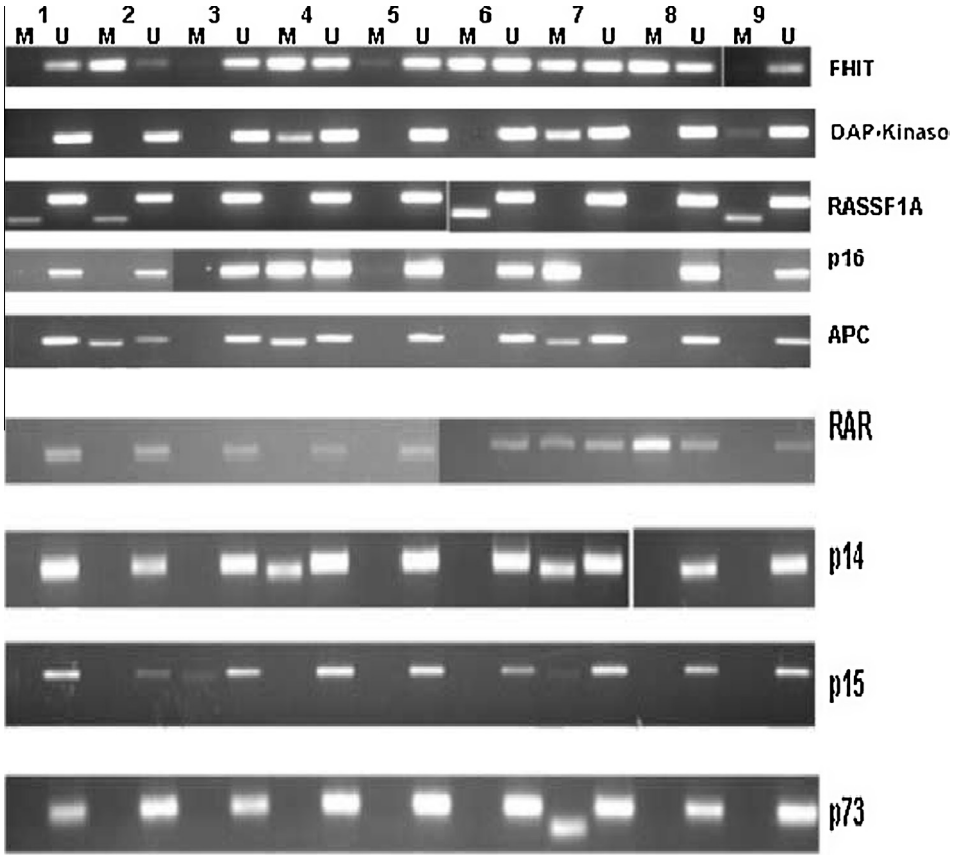


Fig. 1 Methylation-specific PCR analyses of nine representative HCC samples (labeled 1–9 on the top). Each gene is indicated on the right. Both methylated (M) and unmethylated (U) reactions were amplified for each bisulfite-treated DNA and run in a 4% agarose gel.

F1A or O^6*MGMT*. PM of the *p14* was detected in 46.2% of the cases followed by APC, which was methylated in 30.8% of the cases. A significant difference in methylation frequency (MF) between NHT and CH groups was reported for *APC*, *FHIT*, *DAPK* and *RASSF* genes as shown in Fig. 2 and Table 3.

Analysis of significant difference of DNA methylation within the CAH group

To understand aberrant DNA methylation of the selected 11 genes in CAH group ($n = 38$) to determine whether there are differences within CAH-tissues and CAH-PBL groups across the methylation profiles, data were analyzed according to b analysis approach (Generalized Linear Mixed Models) correcting for multiplicity using a Bonferroni adjustment. Our results as shown in Fig. 3A–K indicate that there are *no interaction* effects between methylation status and disease site among groups. This means that methylation status is not significantly affected by whether the tissue was from the liver or from the PBL. However, statistical values for *APC* (Fig. 3A; *p*-value = 0.03) and *p16* (Fig. 3F; *p*-value = 0.04) would be considered significant for the un-adjusted criteria. There are significant differences between methylated and unmethylated states for *APC*, *p14*, *p73*, *p16*, *DAPK1*, and *RASSF1A*. None of the genes were different across tissue and PBL groups, albeit APC had a *p*-value of 0.04. The interaction in APC (Fig. 3A) is evidenced by the change from 0.95 to 0.10 from methylated to unmethylated state for the chronic liver tissue and a smaller change of 0.80–0.40 from methylation to unmethylation for the PBL group.

Analysis of Significant difference of DNA methylation with HCC groups

To understand aberrant DNA methylation of the selected 11 genes in HCC, we followed the same technique of data analysis as with CAH group to determine DNA methylation status of genes in 31 HCCs and their adjacent non-cancerous tissues. We used a Bonferroni correction with 0.0045 (.05/11) as our cut-off for significance. Our results indicate that there are *no interactions* among the tissue sites and methylation status for any of the genes. As shown in Fig. 4A–K, there were differences in the methylation status for the genes *RASSF1A* (Fig. 4I), *FHIT* (Fig. 4B), *APC* (Fig. 4A), *p14* (Fig. 4E), *p73* (Fig. 4C), *RARβ* (Fig. 4H), O^6*MGMT* (Fig. 4J), and *DAPK1* (Fig. 4G). None of the genes showed much difference across the disease sites of cancerous and non-cancerous tissue though *p16* (Fig. 4F), it showed the smallest *p*-value of 0.072, which is not considered as significant by our criterion.

Analysis of DNA methylation status across the disease groups

Across group differences among the four groups enrolled (HCC, CAH, ASC, NHT) were analyzed using binary logistic

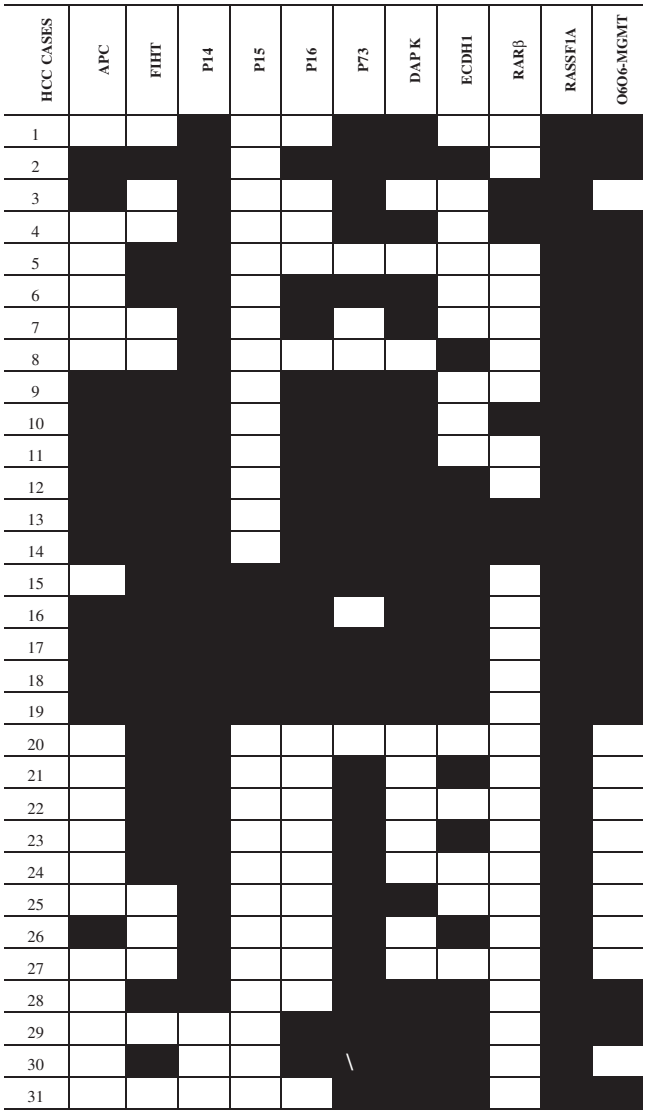


Fig. 2a Methylation of 11 genes in hepatocellular carcinoma patients. * Dark squares depict methylation and blank squares depict unmethylation.

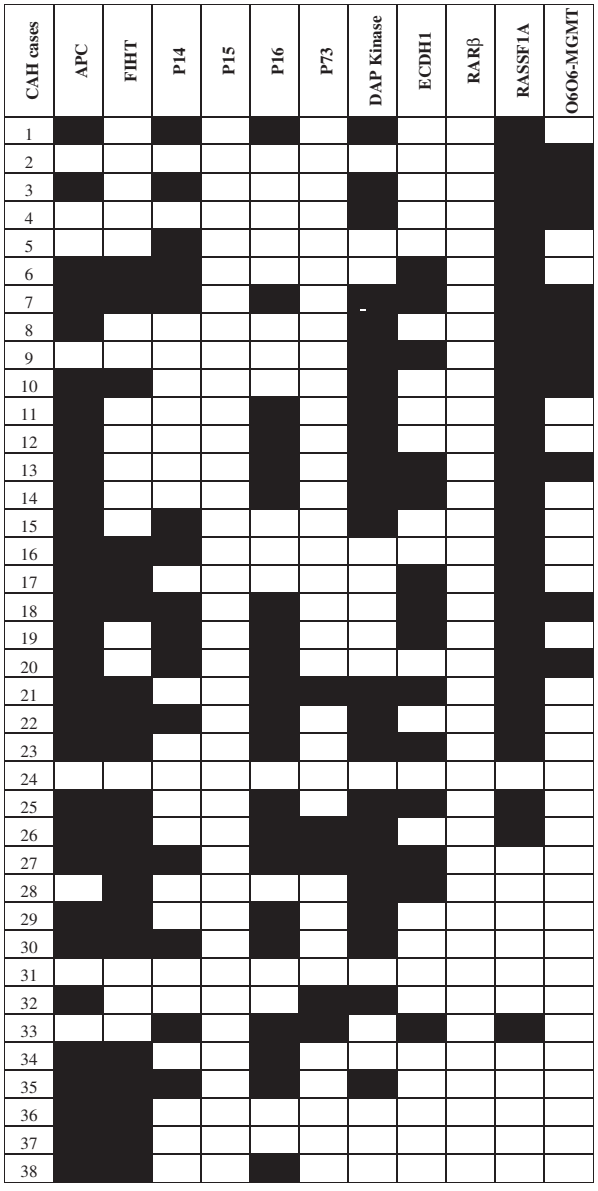


Fig. 2b Methylation of 11 genes in patients with chronic liver diseases. * Dark squares depict methylation and blank squares depict unmethylation.

regression in PROC LOGISTIC for each gene. Our results indicate that there is a significant interaction between disease state (groups) and DNA methylation of genes (Fig. 5A–K,). As shown in Fig. 4A, there is a significant group effect for *APC* (ASC group is different from HCC Group, *p*-value = 0.0006). As can be seen from the graph, the interaction is explained by the fact that there is a bigger difference between methylation and un-methylation for the CH group than any of the other groups especially the NHT. For *DAPK1* (Fig. 5G), there is a marginal group effect, not significant by our corrected level of *p* value = 0.004 (NHT is different fromHCC *p* value = 0.007) and *RARβ* (Fig. 5H) (NHT is different from-HCC Group *p*-value = 0.007). In contrast, there are significant methylation effects for *APC* (*p*-value <0.0001), *FHIT* (*p*-value <0.0001), *p15*, (*p*-value = 0.003), *p14* (*p*-value <0.0001), *DAPK1* (*p*-value <0.0001), *RARβ* (*p*-value <0.0001) and E-cadherin (*p*-value <0.0001).

Analysis of methylation coordination

Coordination of methylation at the 11 tested genes was analyzed by the Mann–Whitney U test through comparing the status of each gene (M or U) with the MI calculated with the remaining genes. A summary of methylation results and concordance tests of each locus in HCC patients is shown in Table 4 and Fig. 2. The combined effect of the studied methylated genes as biomarkers for diagnosis of HCC and CAH has also been studied. When all significant variables were entered into the stepwise logistic regression, only *APC*, *p73*, *p14*, O^6MGMT independently affected the classification of cases into HCC and CH as in Table 5. These four genes combined give an accuracy of 89.9%, sensitivity 83.9% and specificity 94.7%.

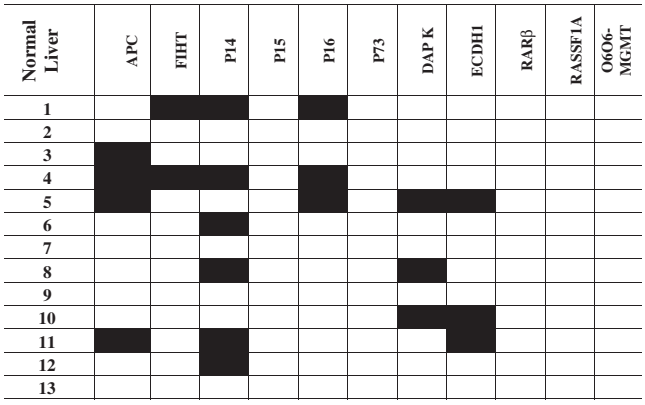


Fig. 2c Methylation of 11 genes in normal liver individuals. * Dark squares depict methylation and blank squares depict unmethylation.

Immunohistochemistry

Protein expression of two of *APC* and O^6*MGMT* was assessed in 20 NHT samples, 20 HCC and 20 CH tissues as well as in an additional set of samples including 40 NHT, 52 CH and 107 HCC tissue samples for confirmation of the methylation results. In the original set, cytoplasmic immunostaining for the *APC* protein was detected in 11 (55%) NHT, with loss of staining in 10 (50%) CH, and 15 (75%) HCC. As for the confirmatory set, we were able to detect cytoplasmic immunostaining for the *APC* protein in 20 (50%) NHT, with loss of staining in 30 (57.7%) CH, and 77 (72%) HCC tissues. On the other hand, nuclear immunostaining for O^6MGMT protein was detected in 13 (65%) NHT with loss of expression in 11(55%) CH and 16 (80%) HCC of the original set. While in the confirmatory set O6MGMT protein were lost in 26 (50%) CH and 70 (65.4%) HCC cases (Fig. 6).

Discussion

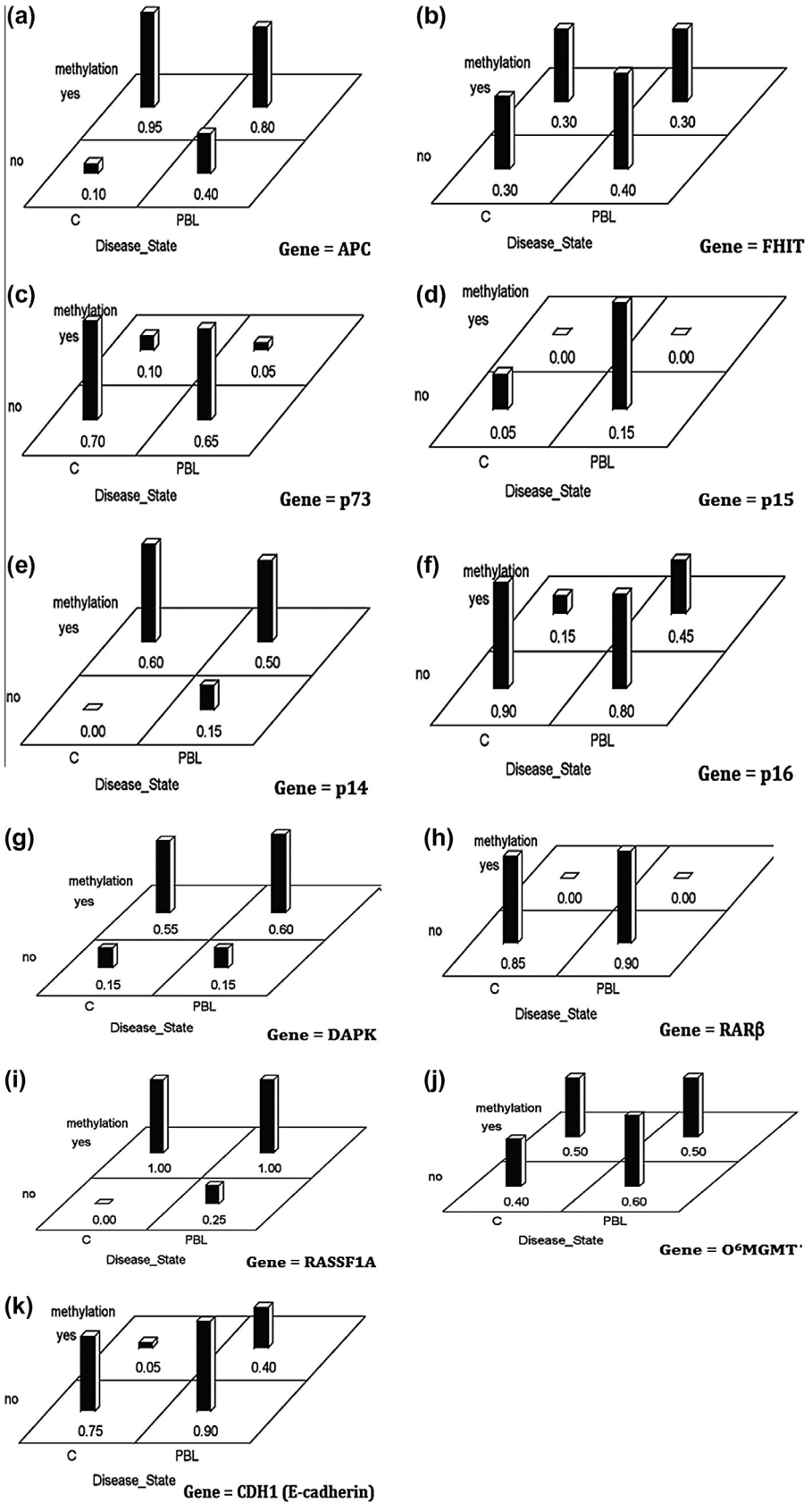
Changes in DNA methylation patterns of TSGs play a role in the development and progression of many tumor types. However, data regarding HCC show wide variability in the results that could be attributed to several factors including the underlying etiologic factor(s) [18,19]. Our study is the first to assess the role of DNA PM of a well selected panel of genes in clinical samples obtained from a cohort of patient population infected with HCV/genotype 4 in an attempt to understand their impact on disease progression.

We have previously reported a high methylation frequency of *APC*, *FHIT*, *CDH1* and *p16* in the plasma and tissues of 28 HBV and HCV-associated HCC patients from Egypt [15]. Therefore, we sought to confirm this data in a larger cohort of HCV- genotype 4 infected patients, including asymptomatic carriers, CH with cirrhosis and HCC using 11 genes that are commonly hypermethylated in several tumor types. We determined several differentially methylated genes both in liver tissues and PBL that represent the progression from NHT to CH and HCC in HCV genotype 4-infected persons. We also identified a panel of genes (*APC*, *p73*, *p14*, O^6*MGMT*) that can independently affect the classification of cases into HCC and CH with 89.9% accuracy, 83.9% sensitivity and 94.7% specificity.

Table 3 Methylation profile of the 11 genes in CAH, HCC and normal liver tissues.

Genes	Normal liver $N = 13$ (%)	Chronic hepatitis (CH)		Hepatocellular carcinoma HCC		*p*-Value*	
		(Tissue) (38) (%)	(PBL) (20) (%)	(HCC) (31) (%)	(ANT) (31) (%)	(CH and HCC)	(CH and ANT)
APC	4 (30.8)	33 (86.8)	16 (80)	13 (41.9)	14 (45.2)	< 0.001	< 0.001
FHIT	2 (15.4)	20 (52.6)	6 (30)	21 (67.7)	20 (64.5)	0.204	0.005
P15	0 (0)	0 (0)	0 (0)	5 (16.1)	5 (16.1)	0.010	_#
P73	0 (0)	8 (21.1)	1 (5.0)	26 (83.9)	23 (74.2)	< 0.001	< 0.001
P14	6 (46.2)	17 (44.7)	10 (50)	28 (90.3)	28 (90.3)	< 0.001	< 0.001
P16	3 (23.1)	15 (39.5)	9 (45)	14 (45.2)	19 (61.3)	0.634	0.390
DAPK	3 (23.1)	22 (57.9)	12 (60)	21 (67.7)	22 (71)	0.401	0.023
RARβ	0 (0)	0 (00)	0 (0)	5 (16.1)	3 (9.7)	0.015	_#
RASSF	0 (0)	26 (68.4)	20 (100)	31 (100)	31 (100)	0.001	< 0.001
O6O6-MGMT	0 (0)	10 (26.3)	10 (50.0)	21 (67.7)	20 (64.5)	< 0.001	< 0.001
CDH1	3 (23.1)	7 (18.4)	8 (40.0)	17 (54.8)	14 (45.2)	0.002	0.004

p-Values $\leqslant 0.05$ are considered significant.
Numbers are too small for a valid statistical analysis.



* CAH= C

Fig. 3 Differences across methylation profiles within CAH* cases between tissues and PBL.

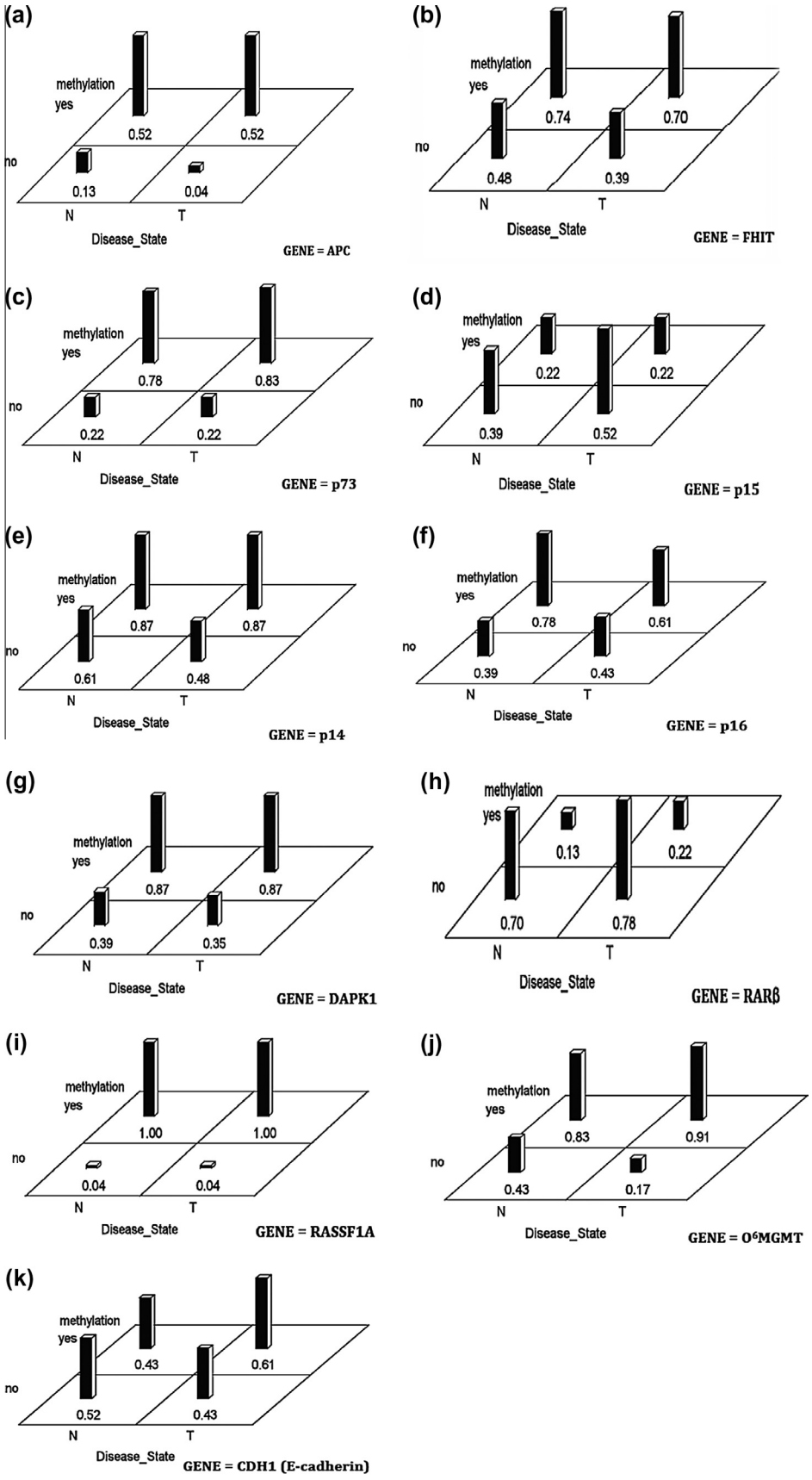


Fig. 4 Differences across methylation profiles between HCC* cases and their ANT# samples with 0.0045 as a cut-off for significance. * HCC = T. # ANT = N.

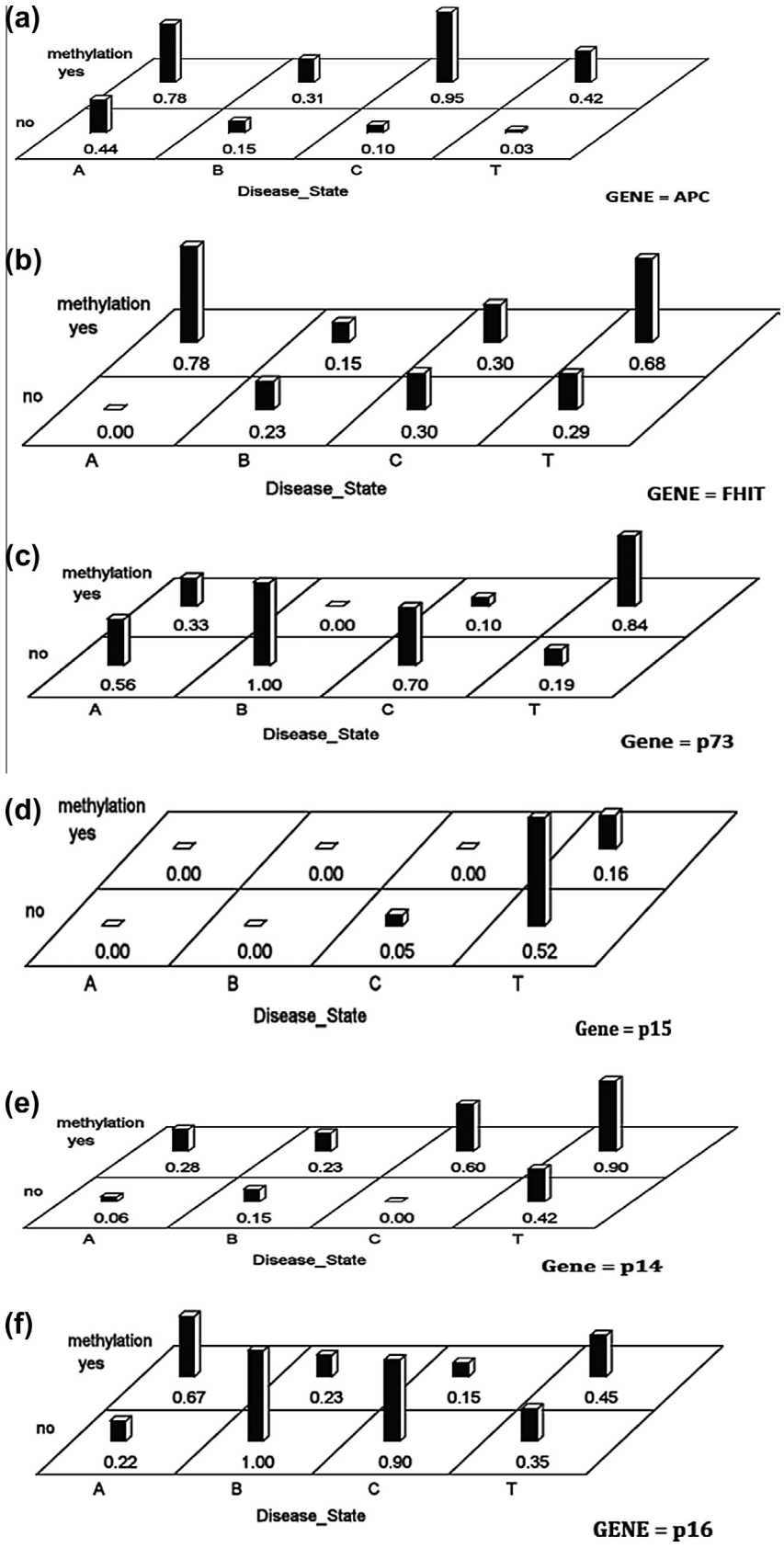


Fig. 5 Differences in the methylation frequency among the four studied groups. (T = HCC, C = CAH with cirrhosis, A = asymptomatic carrier and B = normal hepatic tissue).

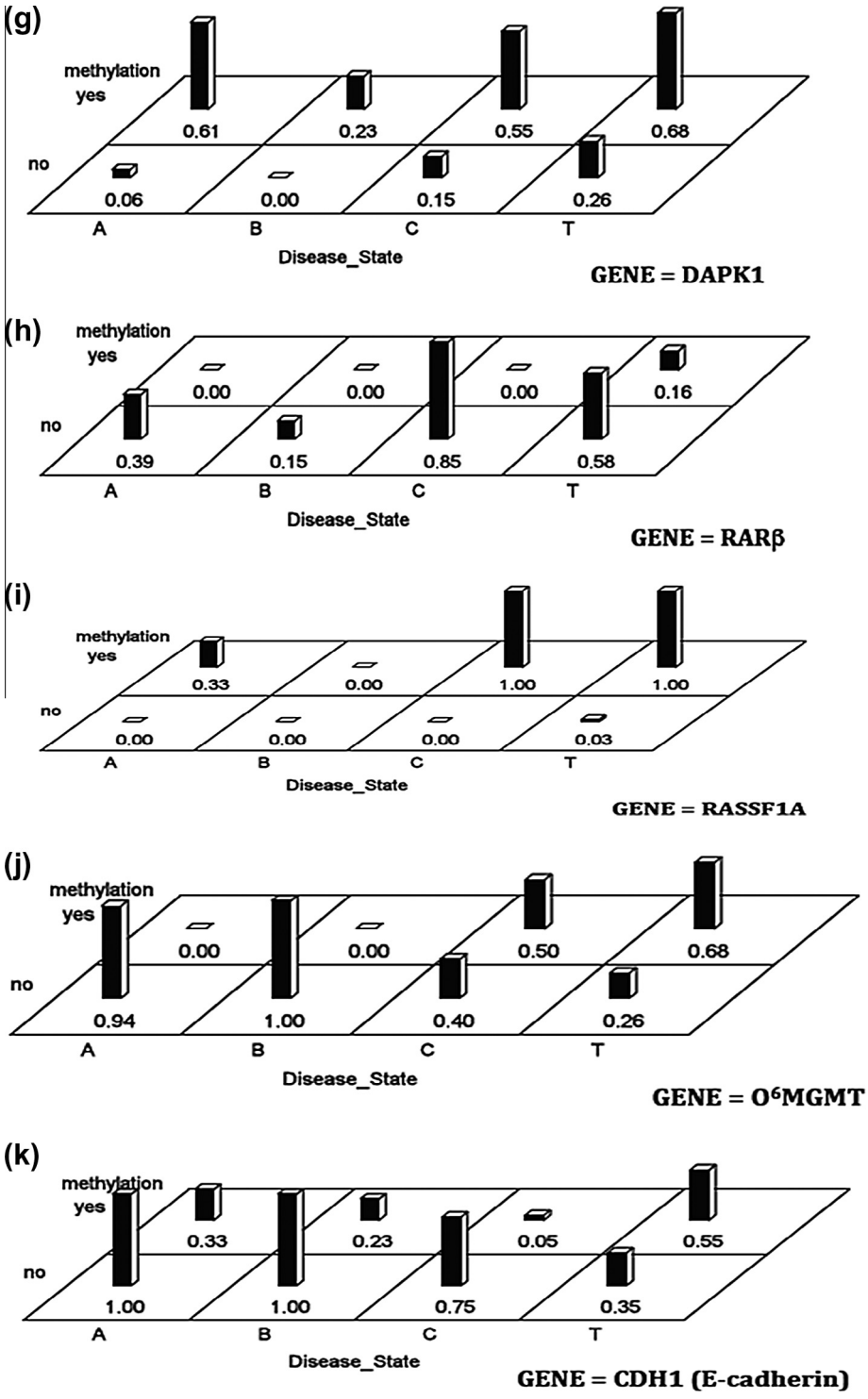


Fig. 5 (*continued*)

A high methylation frequency was reported for all studied genes, except for *p15*, in the PBL and tissues with increasing MI as the disease progresses. Our data regarding the *p15* gene confirms our previous study where *p15* methylation was reported in 14.2% only of HCC cases [15]. Within the studied groups, the methylation frequency of *p14*, *p73*, *RASSF1A* and O^6*MGMT* was significantly higher in HCC and their ANT compared to CH and the NHT samples whereas PM of *APC* was significantly higher in CH patients. This applied to PBL and tissues except for *RASSF1A* and O^6*MGMT*, where the difference in methylation frequency in PBL was statistically insignificant. The high methylation frequency reported here confirms the results of some previous studies including that of Archer [20] who found a high methylation frequency of their studied genes in HCV-associated HCC compared to HBV-associated cases or to NHT. They concluded that the virus

Table 4 Summary of methylation specific PCR results and concordance tests of each locus in HCC samples.

Factor	Concordance	Kappa[#]	*p*-Value[*]
	n = 31 *n* (%)		
APC	28 (90.3)	0.803	<0.001
FHIT	24 (77.4)	0.497	0.006
P15	31 (100.0)	1.000	<0.001
P73	18 (58.1)	−0.248	0.150
P14	31 (100.0)	1.000	<0.001
P16	24 (77.4)	0.558	0.001
DAPK	22 (71.0)	0.318	0.076
RARβ	27 (87.1)	0.431	0.012
RASSF	31 (100.0)	–	–
O6O6-MGMT	26 (83.9)	0.640	<0.001
CDH1	22 (71.0)	0.425	0.016

– Numbers are too small for a valid statistical analysis.
[#] Kappa measure of agreement.
[*] *p*-Values $\leqslant$ 0.05 are considered significant.

Table 5 Stepwise logistic regression for HCC.

Parameter	Regression estimate	*p*-Value	Odds ratio	95% CI for OR	
APC	−3.606	0.003	0.027	0.003	0.287
p73	3.671	0.001	39.302	4.752	325.017
P14	3.638	0.009	38.014	2.492	579.829
O6-MGMT	2.589	0.014	13.311	1.685	105.132

may induce accelerated hypermethylation even in the early stages of infection, which can subsequently lead to tumor development.

RASSF1A, a candidate TSG, frequently shows hypermethylation and loss of heterozygosity with consequent gene silencing in several human cancers [21]. In HCC, PM of *RASSF1A* gene was reported in 78–95% of the studied cases [22–24]. Our results are comparable to these studies since *RASSF1A* methylation was detected in all HCC and in 68.4% CH cases (second only to *APC*). Our results are also comparable to Gioia et al. [25] who reported an increase in *RASSF1A* methylation with progression from regenerative conditions (cirrhosis) to hepatocellular nodules and HCC as well as with Chan et al. [26] who reported *RASSF1A* methylation in the blood and tissues of HCC patients.

Within the identified panel of genes that independently affected the classification of cases into HCC and CH in this study, *p14* showed a high methylation frequency in HCC cases. Our data confirms those of Anzola et al. [27] and Yang et al. [28] who demonstrated that *p14* PM is associated with the pathogenesis of HCC and suggested that inactivation of *p14* through PM could be an important mechanism for HCV-induced HCC. The fact that we were able to detect PM of the *p14* gene in NHT as well as in CH with almost the same frequency suggests that it might be an early event during the cascade of HCV-induced HCC. In contrast, *p16*PM did not show a similar profile suggesting that *p14* and *p16* are regulated by different promoters [29].

Our results show an increasing frequency of *p16* PM from NHT to HCC which is in agreement with the some previous studies of Vivekanandan and Torbenson [30].

Similar to *p14*, O^6*MGMT* plays an important role in cytoprotection by preventing DNA damage and triggering DNA repair mechanisms [31]. Because O^6*MGMT* methylation is a hallmark of specific cancers, it is perhaps not surprising to find a consistent PM in O^6*MGMT* in both CH and HCC tissues. Our results show a significant increase in the frequency of O^6*MGMT*PM from CH (26%) to HCC (67.7%) providing an evidence that this gene could differentiate between CH and HCC. Literature reviews also revealed varied frequencies of O^6*MGMT* PM in HCC ranging from 0% [32], to 22–39% [33]. The variability in the results could be attributed to several factors including the sensitivity of the PCR, the primer sequences and the differences in CpG sites, the etiological factors contributing to HCC and the geographical differences. The significant association reported here between O^6*MGMT* hypermethylation and HCV infection is also comparable to previously published data [22,33].

Our results also show a significant difference in the methylation frequency of *APC* and *CDH1* between CH and HCC cases where *APC* was more frequent in the first group and *CDH1* in the second group. Methylation of *APC* and *CDH1* genes has been previously reported by Yang et al. [28] who demonstrated that PM of *APC* and *CDH1* are more frequent in HBV- and HCV-positive HCC than in HBV- and HCV-negative cases. PM of these genes was also reported by other investigators [34]. Nomoto et al. [32] reported *APC* PM in 88.2% of the NHT compared to 21.6% in chronic hepatitis with cirrhosis and 82.4% in HCC. They claimed that loss of *APC* in cirrhotic and inflammatory cases could possibly be attributed to the presence of inflammatory cells and fibroblasts. In contrast, we reported a high methylation frequency of the *APC*PM in CH patients, both in blood and tissues. The difference between our results and those of Nomoto et al. [32] could be attributed to (a) their smaller sample size (19 cases); (b) samples of CH and cirrhosis were obtained from the HCC cases and not from separate patients or (c) a possibly different etiology since viral infection was not mentioned in their study.

Finally, PM of the*p73* was also reported in 83.9% of the HCC cases assessed in the current study compared to 21.1% of CH and none of the NHT. Thus *p73* PM could be used to differentiate between CH and HCC cases even in patient's blood. PM of the *p73* was reported in some previous studies on HCC and CH [34].

Conclusion

We conclude that aberrant DNA PM of multiple cancer-related genes is associated with different stages of disease progression from hepatitis to HCC since PM of *p73*, *p14*, *O6-MGMT* was associated with HCC whereas aberrant PM of *APC* was more common in CH. *APC* PM could be used as a maker for early detection of HCV-induced chronic active hepatitis. In our study the (*APC*, *p73*, *p14*, *O6-MGMT)* panel independently affected the classification of cases into HCC and CH with high accuracy (89.9%), sensitivity (83.9%) and specificity (94.7%). Moreover, detection of PM of certain genes in PBL is a highly sensitive and specific, noninvasive indicates that blood could be used, as efficiently as tissue biopsies, to assess PM which could help in the follow-up of chronic hepatitis patients and possibly for early detection of HCC, especially when using well-selected panel.

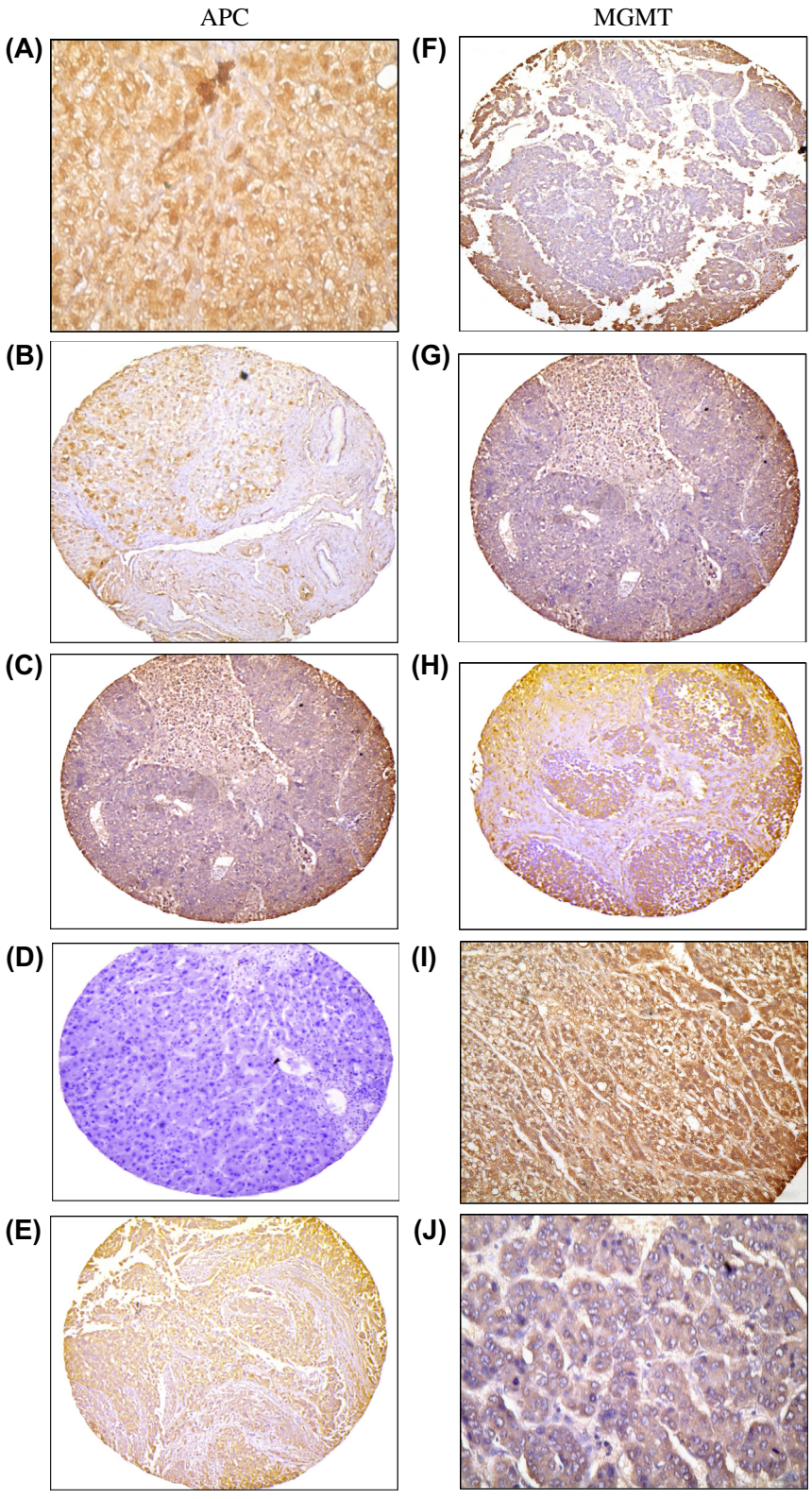


Fig. 6 (A) Normal hepatic tissue sample showing positive cytoplasmic immunostaining for APC (X200) B: A case of HCV induced chronic hepatitis showing mild focal cytoplasmic immunostaining for APC (X100). C: A case of HCV induced chronic hepatitis with cirrhosis negative for APC (X100). D: A case of HCV-associated HCC negative for APC (X100). E: A case of HCV-associated HCC with positive cytoplasmic immunostaining for APC (X40). (F) Normal hepatic tissue negative for MGMT (X100). (G): A case of HCV-induced chronic hepatitis with cirrhosis negative for MGMT (X100). (H) A case of HCV-induced chronic hepatitis with cirrhosis positive for MGMT immunostaining (X100). (I) A case of HCV-induced HCC with marked cytoplasmic immunostaining for MGMT(X200). (J) A case of HCV-induced HCC showing faint cytoplasmic immunostaining for MGMT(X200).

To study the combined effect of the different markers on the diagnosis of HCC compared to CAH. All significant variables were entered into the stepwise logistic regression. The above variables are the ones which independently affects the classification of cases into HCC and CAH. These four variables combined will give an accuracy of 89.9%, sensitivity 83.9% and specificity 94.7%.

Conflict of interest

The author has declared no conflict of interest.

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