

Promotor methylation: Does it affect response to therapy in chronic hepatitis C (G4) or fibrosis?

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ABSTRACT

Background and aim. DNA methylation plays a critical role in the control of important cellular processes. The present study assessed the impact of promoter methylation (PM) of some genes on the antiviral response to antiviral therapy and its relation to the presence of fibrosis in HCV-4 infected patients from Egypt. **Material and methods.** Clinical, laboratory and histopathological data of 53 HCV-4 infected patients who were subjected to combined antiviral therapy were collected; patients were classified according to their response to treatment and the fibrosis status. The methylation profiles of the studied groups were determined using the following genes: *APC*, *P14ARF*, *P73*, *DAPK*, *RASSF1A*, and *O6MGMT* in patients' plasma. **Results.** *O6MGMT* and *P73* showed the highest methylation frequencies (64.2 and 50.9%) while *P14* showed the lowest frequency (34%). Sustained virological response (SVR) was 54.7% with no significant difference in clinico-pathological or laboratory features between the studied groups. PM of *O6MGMT* was significantly higher in non-responders (p value 0.045) while *DAPK* showed high methylation levels in responders with no significance (p value: 0.09) and PM of *RASSF1A* was significantly related to mild fibrosis (p value: 0.019). No significant relations were reported between PM of any of the studied genes and patients' features. **Conclusion.** PM of some Tumor Suppressor genes increases in chronic active HCV-4. However, only *O6MGMT* can be used as a predictor of antiviral response and *RASSF1A* as a marker of marked fibrosis in this small set of patients. An extended study, including more patients is required to validate the results of this preliminary study.

Key words. HCV. Antiviral response. Promoter methylation.

INTRODUCTION

Hepatitis C virus (HCV) is the cause of a significant proportion of cases of chronic liver disease, hepatocellular carcinoma (HCC) and deaths from liver disease. Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV-4, which accounts for almost 90% of the cases.¹ Moreover a consistent increase of seropositivity for HCV antibodies with age was observed, with a peak level of 54.9% in all individuals for the age group 45-49 years.² The goal of treatment is to prevent complications of HCV infection, which is mainly achieved by elimination of the virus, predict-

ed by a sustained virological response (SVR).³ The main predictors of SVR are the IL28B (IFN k3) polymorphism, the HCV genotype, and the stage of fibrosis. Other predictors of response include baseline HCV RNA levels, the dose and duration of therapy, host factors e.g. body mass index, age, insulin resistance, gender, and the characteristics of liver disease e.g. the levels of alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), and the stage of fibrosis or co-infection with HIV or other hepatotropic virus.⁴

Epigenetic changes, including promoter methylation (PM) of several genes play a critical role in the control of cellular processes through switching genes on or off leading to differential expression of the genes, which determines the expression of proteins.⁵ Some studies have shown that the presence of hepatitis viruses, especially HCV, could play a role in accelerating the methylation process which is involved in HCC development, potentiate the progression of HCV related liver disease and affect its response to treatment.⁶

We have previously reported, in a case control study, a high frequency of PM of the APC, FHIT,

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p15, p16, and E-cadherin in tumor tissues and plasma obtained from 28 HCC patients from Egypt. The Promotor methylation frequency (PMF) ranged from 67.9% for p16 to 89.2% for p15 with a high concordance rate between plasma and tissue samples.⁷ In another study, we found that PM of a group of genes increases with disease progression from CH to HCC.⁸ In this study the PMF of p14, p73, RASSF1A, CDH1 and O6MGMT was significantly higher in HCC and their ANT whereas PMF of APC was higher in CH and we were able to suggest a panel of 4 genes (APC, p73, p14, O6MGMT) that can independently classify cases into HCC and CH with high sensitivity and specificity.⁹ Then, it may be valuable to assess whether PM of our previously tested genes contribute to fibrogenesis and response to antiviral treatment in chronic HCV-4 related liver disease.

Therefore the current study was conducted to clarify the contribution of PM to: the development of fibrosis and the response to antiviral therapy using some genes that proved to be significant in our previous studies (APC, P14ARF, P73, DAPK, RASSF1A and MGMT).

MATERIAL AND METHODS

Population samples

The study was conducted on 53 consecutive chronic HCV-4 patients from Egypt who were eligible for treatment with pegylated interferon α and ribavirin. All patients fulfilled the standards of care, inclusion and exclusion criteria for interferon therapy, which are applied on the national wide program controlled by the National Committee for Treatment of Viral Hepatitis. Informed consents were obtained from all the participants enrolled in the study, which was performed in accordance with the declaration of Helsinki, local and national laws (clinical trial NCT01758939).

For all patients height and weight were determined at baseline and body mass index (BMI) was calculated (weight in kilograms divided by height in meters squared). Laboratory investigations including complete liver profile, kidney function, Alfa-Fetoprotein (AFP), INR, and CBC were also done to justify suitability for therapy. HCV RNA was quantified in all patients' sera using quantitative real time PCR at baseline, after 12, 24, 48 and 72 week of anti-viral therapy. Histological examination was done on core needle biopsies to determine the grade of necro-inflammation and the stage of fibrosis ac-

ording to the Metavir scoring system prior to treatment. Clinical and laboratory follow up were done for every patient to report any possible adverse side effects and the response to treatment according to IFN treatment guidelines.

Detection of promoter methylation

High molecular weight DNA was extracted from patient's plasma samples collected before treatment, according to our previously published protocol.⁷ Briefly, an 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.⁷

The extracted DNA was subjected to bisulfite treatment using EZ DNA methylation kit which uses 300 ng of the extracted Nucleic acid. This was followed by MSP using the primer sequences and the methylation-specific PCR conditions illustrated in table 1. DNA methylation of CpG islands for p14, p73, APC, DAPK, RASSF1A and O6MGMT genes was determined using specific primers for methylated (M) and unmethylated (UM) DNA as previously described by.⁸ Negative control samples (without DNA) were included in each PCR set. PCR products were analyzed on 4% ethidium bromide-stained agarose gel and visualized under ultraviolet illumination. The methylation index (MI), defined as the ratio between the number of methylated genes and the total number of the studied genes for each sample was calculated for all patients.⁸

Statistical Analysis

This was done using Statistical Package for Social Sciences, Version 17.0 (SPSS, Inc., Chicago, III., USA) for Windows. Continuous variables were analyzed as mean values \pm standard deviation (SD) or median (range) as appropriate. Percentages were calculated for categorical data. For categorical variables, differences were analyzed with χ^2 (chi square) tests and Fisher's exact test when appropriate. Differences among continuous

Table 1. Primers sequences and conditions of the methylation specific PCR (MSP).

Gene	Primer	Annealing temperature °C
DAPK (M)	GGATAGTCGGATCGAGTTAACGTC CCCTCCCAAACGCCGA	60
DAPK (U)	GGAGGATAGTTGGATTGAGTTAATGTT CAAATCCCTCCCAAACACCAA	57
p73 (M)	GGACGTAGCGAAATCGGGGTTT ACCCCGAACATCGACGTCCG	59
p73 (U)	AGGGATGTAGTAAAATTGGGGTTT ATCACAACCCCAAACATCAACATCCA	60
O6-MGMT (M)	TTTCGACGTTTCGTAGGTTTTTCGC GCACTCTTCCGAAAACGAAAACG	55.2
O6-MGMT (U)	TTTGTGTTTTGATGTTTGTAGGTTTTTGT AACTCCCACTTTCCAAAAACAAAACA	57
p14 (M)	GTGTTAAAGGGCGCGTAGC AAAACCCTCACTCGCGACGA	54.9
p14 (U)	TTTTTGGTGTTAAAGGGTGGTGTAGT CACAAAACCCCTCACTCACAAACA	57.2
APC (M)	TATTGCGGAGTCCGGGTC TCAACGAACTCCCGACGA	62
APC (U)	GTGTTTTATTGTGGAGTGTGGGTT CCAATCAACAACTCCCAACAA	59.2
RASSF1A (M)	TTCGTCGTTTAGTTTGGATTTTG CCGATTAACCCGTACTTCG	54.4
RASSF1A (U)	TGTTGTTTAGTTTGGATTTTGG TACAACCCTTCCAAACACAC	52

variables with normal distribution were analyzed by Student's T-test; comparison between three groups was done using Kruskal-Wallis test (non parametric analogue for ANOVA). P value of ≤ 0.05 was considered statistically significant.

RESULTS

Clinical and epidemiological data: Baseline demographic and laboratory features of all patients enrolled in the study in addition to the stage of fibrosis are illustrated in table 2. Out of the 53 patients assessed, 47 (89%) were males and 6 (11%) were females. Their ages ranged from 35 to 45 with a mean of 39.2. Forty patients (75.5%) showed mild to moderate fibrosis (F1/F2) and 13 (24.5%) showed marked fibrosis.

Out of 53 patients studied, 29 showed SVR (54.7%) and 24 showed either no response or relapse (45.3%). None of patients was excluded from the treatment due to emergence of any side effects and no patient received $< 80\%$ of the therapeutic schedule. The demographic, laboratory and histopathological parameters of those patients are illustrated in table 3. No significant difference was observed between the two groups (responders and non-responders) regarding the age and sex, the

Table 2. Clinico-pathological features of the studied patients.

Variables	Patients n = 53
Age (years)	39.2 \pm 8.9
Sex M/F	47/6
BMI	27.2 \pm 4.2
Platelets/mm ³	197.7 \pm 64.8
Total bilirubin mg/dl	0.8 \pm 0.3
ALT IU/L	69.6 \pm 44.2
AFP ng/ml	7.2 \pm 10.4
HCV viral load IU/ml	371.785 \pm 762.2
Fibrosis as n (%):	
Mild to moderate (F1& F2)	40 (75.5%)
Marked (F3& F4)	13 (24.5%)

Data are represented by Mean \pm SD. AFP: α -fetoprotein. ALT: alanine aminotransferase. BMI: body mass index. F: female. HCV: hepatitis C virus. M: male.

hematological parameters, liver profile, HCV viral load or different fibrosis stages.

Promotor methylation frequency (PMF)

The PMFs of all studied genes are illustrated in table 4. The *O6MGMT* showed the highest PMF (64.2%) followed by *P73* (50.9%) and *APC* (49.1%) whereas *P14* showed the lowest PMF (34%). Out of

Table 3. Clinico-pathological features of the responder and non-responder patients

Variables	Responders (n = 29)	Non-responders (n = 24)	p-value
Age (years)	38.6 ± 8.8	39.8 ± 9.0	0.646
Sex M/F	26/3	21/3	1
BMI 26.3±3.8	28.3 ± 4.5		0.081
Platelets/mm ³	188.1± 63.6	209.3 ± 65.7	0.241
ALT IU/L	64.4 ± 47.4	75.9 ± 40.2	0.231
AFP ng/mL	5.3 ± 7.4	9.5 ± 12.9	0.15
HCV viral load IU/mL	500.000 ± 24.8	30.000 ± 300	0.368
Fibrosis as n (%):			
Mild to moderate (F1& F2)	23 (79.3%)	17 (70.8%)	0.264
Marked (F3& F4)	6(20.7%)	7 (29.2%)	

Data are represented by Mean ± SD. P-value > 0.05 is not significant. AFP: α -fetoprotein. ALT: alanine aminotransferase. BMI: body mass index. F: female. HCV: hepatitis C virus. M: male.

Table 4. Promotor Methylation Frequency of in the studied genes.

Studied genes	Methylated genes n (%)	Un-methylateds gene n (%)
O6 _{MGMT}	34 (64.2)	19 (35.8)
APC	26 (49.1)	27 (50.9)
RASSF1A	22 (41.5)	31 (58.5)
DAP-kinase	22 (41.5)	31 (58.5)
P73	27 (50.9)	26 (49.1)
P14	18 (34)	35 (66)

Numbers are represented as n (%).

the six genes assessed for PM, only RASSF1A gene methylation was significantly related to the presence of mild fibrosis (p value: 0.019, and O6_{MGMT} was significantly correlated with patient's response to therapy (p value: 0.045). On the other hand, PM of DAPK was higher in responders than in non responders, however, the difference between the two groups did not reach a statistically insignificant level (p value: 0.097) (Tables 5 and 6)(Figure 1).

Promotor methylation index (PMI)

There is no significant difference between IFN responders and IFN non-responders regarding the methylation index (2.76 ± 1.4 and 2.83 ± 1.46 ; $p = 0.851$) respectively as well as no significant difference in methylation index between mild fibrosis (F1 and F2) and marked fibrosis (F3 and F4) (Table 5).

DISCUSSION

Aberrant methylation in the promoter regions of tumor suppressor genes (TSGs) is a crucial epige-

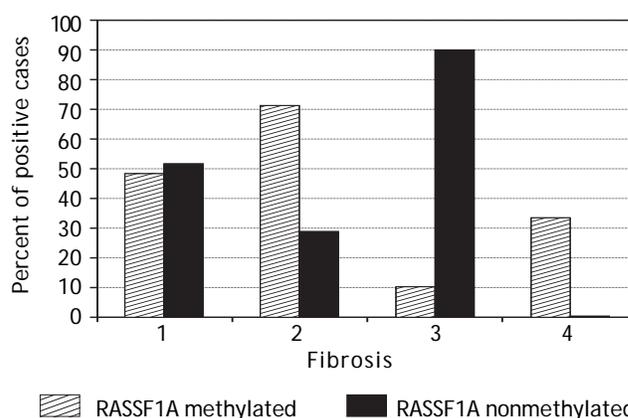


Figure 1. The significant relation between the methylation status of RASSF1A and fibrosis stages (p value: 0.019).

netic alterations that contribute to deregulation of many cellular processes leading finally to the initiation and progression of human cancers.^{9,10} Several studies revealed that different types of cancer, including HCC, show distinct DNA methylation profiles; suggesting the existence of cancer-type specific methylation signatures.^{11,12} Other studies have mentioned a possible HCV-induced HCC methylation profile.^{6,8} Such epigenetic defects have also been observed in non-cancerous liver tissues of HCC patients, which are usually show evidence of chronic inflammation.^{13,14}

Though Egypt has the highest prevalence of HCV infection in the world, DNA methylation profiles for HCV has not been well studied yet and there are only few studies in this context. In an early case control study by our group,⁷ we were able to detect a high frequency of APC, FHIT, p15, p16 and E-cadherin-PM (range 67.9-89.2%) in the plasma and tissues of 28 chronic HCV and/or HBV-associated

Table 5. The methylation status of the studied group in relation to IFN response.

Methylated gene	Responders N = 29	Non-Responders N = 24	p-value
O6MGMT	15 (51.7%)	19 (79.2%)	0.045*
APC	13 (44.8%)	13 (54.2%)	0.473
RASSF1A	11 (37.9%)	11 (45.8%)	0.540
DAP-kinase	14 (48.3%)	8 (33.3%)	0.097
P73	12 (41.4%)	15 (62.5%)	0.328
P14	9 (31%)	9 (37.5%)	0.930
Methylation Index	2.76 ± 1.41	2.83 ± 1.46	0.851

* P-value > 0.05 is not significant.

Table 6. Correlation between promoter methylation of the studied genes and degree of fibrosis.

Methylated gene	Mild Fibrosis (F1&F2) n = 40	Marked fibrosis (F3&F4) n = 13	p-value
O6MGMT	26 (65.0%)	8 (61.5%)	0.543
APC	20 (50.0%)	6 (46.2%)	0.687
RASSF1A	20 (50.0%)	2 (15.4%)	0.019*
DAP-kinase	18 (45.0%)	4 (30.8%)	0.366
P73	21 (52.5%)	6 (46.2%)	0.691
P14	15 (37.5%)	3 (23.1%)	0.340

P-value > 0.05 is not significant. * In spite of RASSF1A showed significant, the sample size was very small and further study need to confirm this Preliminary data.

HCC patients, with a high concordance for all studied genes. However, no significant association was found, in this study, between the methylation status of any gene and the presence of hepatitis virus infection. This was partially attributed to the small sample size in this study. Then, we assessed the contribution of methylation status to the development and progression of HCV-associated HCC and CH in Egyptian patients using a specific panel of genes (APC, FHIT, p15, p73, p14, p16, DAPK1, CDH1, RARb, RASSF1A, O6MGMT).⁸ We found that HCV infection may contribute to hepatocarcinogenesis through enhancing PM of certain genes. A panel of 4 genes (APC, p73, p14, O6MGMT) out of 11 tested genes successfully classified cases into HCC or CH with high accuracy (89.9%), sensitivity (83.9%) and specificity (94.7%).

A more extended confirmatory study, including 516 Egyptian patients with HCV-related liver disease (208 HCC, 108 liver cirrhosis, 100 CHC and 100 controls), was then performed to detect PM of P14, P15, P73 and Mismatch repair gene (O6MGMT) in patient's plasma by using EpiTect Methyl qPCR Array technology.¹⁵ This study provided evidence that PM of the studied genes is an early event in hepatocarcinogenesis and showed specific DNA methylation

signatures associated with the potential clinical applications in diagnosis and prognosis of HCC.¹⁵

The current study was then conducted to determine the impact of PM of our specified panel of genes on the degree of fibrosis in chronic HCV infected patients and their response to combined antiviral therapy.

Our results regarding the correlation between PM of the tested genes and patients' response to antiviral therapy differ from previously published data in Western countries or USA. We found that only O6MGMT PM significantly affected patients' response to antiviral therapy (p value, 0.045), being significantly higher in non-responders than in responders. This is explainable since some previous studies have shown that; O6MGM plays an important role in cytoprotection through preventing DNA damage and triggering DNA repair mechanisms. Therefore, PM of O6MGM is frequently detected in chronic hepatitis patients.¹⁶

On the other hand, PM of DAPK showed a tendency to affect patients' response to treatment though this did not reach a statistically significant level (p = 0.097). This could be attributed, at least partially, to the small sample size. None of the other tested genes was significantly associated with response to antiviral treatment.

In the current study *O6MGMT* has the highest pretreatment PM frequency among HCV infected patients (64.2%) followed by *P73* (50.9%), *APC* (49.1%), *RASSF1A/DAP-kinase* (41.5%) and *P14* (34%). Our data in this regard is different from what has been previously reported by our group in chronic HCV and/or HBV-associated HCC⁸ or by Gioia, *et al.*¹⁷ These two studies showed that PM of the *RASSF1A* gene was the most frequently detected with progression from regenerative conditions to cirrhosis. The variability in results of different studies regarding the frequency of PM of the assessed genes could be attributed to several factors including: the differences in the CpG sites tested, environmental factors, HCV genotype present as well as geographical and racial differences. However, some previous reports have also shown significant association between *O6MGMT*-PM and HCV infection including the study of Matsukura, *et al.*¹⁸

The *p73* is the second most frequently methylated gene in our tested group. Data regarding PM of the *p73* gene in chronic active hepatitis patients are still immature and the few available reports in literature show a low PM frequency in CH patients.¹⁹ This contradicts with our results since PM of the *p73* was detected in 50.9% of the studied patients.

Data regarding PM of the *APC* gene varied significantly in different studies. In the current study, *APC*-PM was relatively high in CH patients (49.1%), confirming the data obtained from our previous study on the Egyptian population.⁸ However, Nomoto, *et al.*²⁰ reported a much lower frequency of *APC*-PM (21.6%) in CH patients with cirrhosis. One possible explanation for the difference between the results of the two studies could be attributed either to different HCV genotypes, the presence of fibrosis and/or environmental factors in Egyptian population.

Within our studied panel of genes, *p14* showed the lowest frequency of PM among HCV patients (34%). Similar findings were reported by Anzola,²¹ who showed that *p14* PM was associated with the pathogenesis of HCC and suggested that inactivation of *p14* through PM could be an important mechanism for HCV-induced HCC.^{8,21} To the best of our knowledge, data regarding the impact of PM on the response to antiviral therapy in HCV-associated CH patients are still preliminary. Thus, further studies including larger number of patients are still needed to evaluate and validate the already available small studies including ours.

Only few studies have addressed the association between gene methylation status and the develop-

ment of fibrosis in hepatitis patients. Murphy, *et al.*²² confirmed in their study the implication of methylation status of some genes in the progression of mild NAFLD (Non-alcoholic fatty liver disease) into advanced NAFLD, steato-hepatitis, fibrosis and carcinogenesis. Their study confirmed the presence of functionally relevant differences in the methylation patterns, which can distinguish a mild from an advanced disease. However no similar studies have been done in chronic hepatitis patients, except for the current study, which shows that only PM of the *RASSF1A* gene was significantly associated with mild fibrosis in the studied patients ($p = 0.0.019$). This provides an evidence for the role of an intact *RASSF1A* gene in the induction of fibrogenesis in chronic HCV patients.

We conclude that, promoter methylation of some genes increases in HCV genotype 4-associated chronic active hepatitis. However, only *O6MGMT* methylation can significantly affect patients' response to antiviral treatment, whereas *RASSF1A* is involved in the regulating the process of fibrogenesis and therefore, it could be helpful in predicting the stage of fibrosis or in the differentiation between mild and marked fibrosis in those patients.

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ABBREVIATIONS

- **AFP:** Alfa-Fetoprotein.
- **ALT:** Alanine aminotransferase.
- **BMI:** body mass index.
- **GGT:** gamma glutamyltransferase.
- **HCC:** hepatocellular carcinoma.
- **HCV:** hepatitis C virus.
- **HCVG4:** hepatitis C virus genotype 4.
- **M:** methylated DNA.
- **MI:** methylation index.
- **PMF:** The Promotor methylation frequency.
- **SVR:** Sustained virological response.
- **TSCs:** tumor suppressor genes.
- **UM:** unmethylated DNA.

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