### Disease Progression from Chronic Hepatitis C to Cirrhosis and Hepatocellular Carcinoma is Associated with Increasing DNA Promoter Methylation


**Abstract**

**Background:** Changes in DNA methylation patterns are believed to be early events in hepatocarcinogenesis. A better understanding of methylation states and how they correlate with disease progression will aid in finding potential strategies for early detection of HCC. The aim of our study was to analyze the methylation frequency of tumor suppressor genes, P14, P15, and P73, and a mismatch repair gene (O6MGMT) in HCV-related chronic liver disease and HCC to identify candidate epigenetic biomarkers for HCC prediction. **Materials and Methods:** 516 Egyptian patients with HCV-related liver disease were recruited from Kasr Alaini multidisciplinary HCC clinic from April 2010 to January 2012. Subjects were divided into 4 different clinically defined groups - HCC group (n=208), liver cirrhosis group (n=108), chronic hepatitis C group (n=100), and control group (n=100) - to analyze the methylation status of the target genes in patient plasma using EpiTect Methyl qPCR Array technology. Methylation was considered to be hypermethylated if >10% and/or intermediately methylated if >60%. **Results:** In our series, a significant difference in the hypermethylation status of all studied genes was noted within the different stages of chronic liver disease and ultimately HCC. Hypermethylation of the P14 gene was detected in 100/208 (48.1%), 52/108 (48.1%), 16/100 (16%) and 8/100 (8%) among HCC, liver cirrhosis, chronic hepatitis and control groups, respectively, with a statistically significant difference between the studied groups (p-value 0.008). We also detected P15 hypermethylation in 92/208 (44.2%), 36/108 (33.3%), 20/100 (20%) and 4/100 (4%) , respectively (p-value 0.006). In addition, hypermethylation of P73 was detected in 136/208 (65.4%), 72/108 (66.7%), 32/100 (32%) and 4/100 (4%) (p-value <0.001). Also, we detected O6MGMT hypermethylation in 84/208 (40.4%), 60/108 (55.3%), 20/100 (20%) and 4/100 (4%), respectively (p value <0.001. **Conclusions:** The epigenetic changes observed in this study indicate that HCC tumors exhibit specific DNA methylation signatures with potential clinical applications in diagnosis and prognosis. In addition, methylation frequency could be used to monitor whether a patient with chronic hepatitis C is likely to progress to liver cirrhosis or even HCC. We can conclude that methylation processes are not just early events in hepatocarcinogenesis but accumulate with progression to cancer.

**Keywords:** HCV - cirrhosis - hepatocellular carcinoma - tumor suppressor gene methylation - progression

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**Introduction**

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer death in the world (Iakova et al., 2011). Of the total new cancer cases reported each year, HCC represents 7.9% in men and 6.5% in women (Ferlay et al., 2010). Annually, more than 620000 new patients are diagnosed with this disease and 1-year survival rates remain less than 50% (Altekruse et al., 2009). In 70-90% of cases, HCC develops on a background of chronic liver disease, such as chronic inflammation or cirrhosis (Schütte et al., 2009). An increasing trend of incidence in hepatocellular carcinoma (HCC) has been recorded in most developed countries (Salhab and Canelo, 2011).

Chronic infection with hepatitis C virus (HCV) is...
considered a major risk for chronic liver failure. Recent epidemiological studies and data from the World Health Organization (WHO; www.who.int) estimated the global prevalence around 160-170 million of people chronically infected with HCV (Lavanchy, 2011). Chronic HCV infection is the second most common cause of all HCCs, and the leading etiology in Japan, Egypt, and within the USA (Yamashita et al., 2011). However, the carcinogenic processes leading to HCC development in HCV-cirrhosis cases are not yet well understood. It has been postulated that direct and indirect interactions between HCV-encoded proteins and host hepatic cells may contribute to the malignant process. In addition to this, the chronic inflammation scenario accompanied by immune-mediated destruction of infected hepatocytes, oxidative stress, virus-induced apoptosis, DNA damage leading to genomic heritable aberrations, and continuous hepatic regeneration processes may also be involved in promoting HCC development within the HCV-cirrhotic background (McGivern and Lemon, 2011).

The development of HCC, as with other solid tumors, is believed to require the dysregulation of at least three biochemical pathways (proliferation, cell cycle, apoptosis/cell survival) within the cell (Whittaker et al., 2010). DNA methylation is one of the main epigenetic mechanisms and hypermethylation of CpG islands at tumor suppressor genes switches off these genes (Jung et al., 2012). Besides its importance as a gene inactivation mechanism, promoter CpG island hypermethylation is gaining attention as a potential tumor biomarker. In this respect, DNA methylation markers are being actively investigated to detect human cancers in blood, secretions, or exfoliated cytology specimens and to predict the risk of cancer progression and development (Herbst et al., 2011). For biomarkers to be most useful for early detection or screening, one would need to be able to detect the markers without biopsy, such as in the circulation (Chang et al., 2008).

This study was conducted as a confirmatory study to our previously published data by (Iyer et al., 2009) to address the possibility of using DNA promoter methylation in patient’s plasma as a simple method for early detection of HCC since DNA promoter methylation is better than measuring protein because of DNA stability.

Materials and Methods

Study design

This study was conducted on 416 HCV related chronic liver disease patients who were assigned into 208 HCC patients (group I) attending the multidisciplinary HCC clinic, Tropical Medicine Department, school of medicine and the National Cancer Institute (NCI) outpatient clinic, Cairo University. 108 patients with HCV related liver cirrhosis (group II) that were recruited from Endemic Medicine Department, school of medicine, Cairo University.100 patients with chronic hepatitis C (group III) were recruited from Kasr El-Aini, viral hepatitis center. In addition to 100 participants (group IV) who were enrolled as a control group and were seronegative for HCV and HBV and. The criteria for inclusion in the study groups were as follows: a) adult patients of both sexes (18 years or older) who were seropositive for HCV; b) patients with the spectrum of HCV related CLD (Chronic hepatitis and Liver cirrhosis) who are naïve to treatment; c) hepatocellular carcinoma patients with chronic hepatitis C who did not receive prior antiviral therapy or therapy for HCC. The criteria for exclusion in the study groups were as follows: a) patients with chronic HBV infection confirmed by PCR for HBV DNA; b) patients who received previous treatment for HCC or Antiviral therapy. All samples were obtained with a written informed consent from all patients and after the approval of the ethical committees of the NCI.

Collection of clinical specimens: 5 ml of venous blood were obtained and centrifuged and plasma was extracted. All plasma samples were stored at -80°C until used.

Genomic DNA extraction

DNA purification was extracted by using phenol/chloroform treatment. Briefly, equal volume of buffer equilibrated phenol (PH 7.0-7.5) was added to plasma samples and vortexed. The upper aqueous layer was removed with a “cut down” pipette tip, and an equal volume of phenol/chloroform (1:1) was then added to the aqueous supernatant and vortexed. The upper aqueous layer was removed again, and an equal volume of chloroform/isoamyl (24:1) was then added and vortexed. Sodium acetate (3M) (pH 4.7-5.2) was added in order to precipitate the DNA followed by 2 volumes of absolute ethanol and -20°C overnight incubation. After decantation of the liquid, the DNA pellet was recovered and dissolved in sterile water and stored at 4°C for further purification (Iyer et al., 2009).

DNA purification

DNA was done by using Wizard® DNA clean-Up system supplied by (promega, USA) and according to the manufacturer instructions. Briefly 1ml of Wizard DNA Clean-Up Resin was added to 50μl of DNA in 1.5ml microcentrifuge tube and was mixed by gently inverting several times. The Wizard DNA Clean-Up Resin containing the bound DNA was transferred into Syringe Barrel. Then, the syringe plunger was inserted and the slurry was gently pushed into the Minicolumn. The syringe was detached from the minicolumn and the column was washed by adding 2ml of 80% isopropanol. To dry the resin, the minicolumn was transferred to 1.5ml microcentrifuge tube to be centrifuged for 2 min. at maximum speed. The bound DNA was eluted in 50μl of TE buffer [10mM Tris-HCl (pH 7.6), 1Mm EDTA]. Finally, the purified DNA was stored at 4°C.

Methylation PCR

The restriction digestions were performed using the EpiTect Methy D NA Restriction Kit provide by (Qiagen, Germany) (custom array, cat. no. 335451) which was especially designed for our reach study. In which a reaction mix without enzymes was prepared from 1μg genomic DNA, 26μl of 5× Restriction Digestion Buffer, and RNase-DNase free water to make the final volume 120μl. Four digestion reactions (Mo, Ms, Md, and Msd) were set up.
Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences, Version 17.0 (SPSS, Inc., Chicago, III, USA) for Windows. Continuous variables were analyzed as mean values ± standard deviation (SD). Rates and proportions were calculated for categorical data. For categorical variables, differences were analyzed with χ² tests and Fisher’s exact test when appropriate. Differences among the four groups (HCC, liver Cirrhosis, Chronic HCV & control subjects) regarding continuous variables with normal distribution were analyzed with Univariate ANOVA and Bonferroni post hoc test and those not normally distributed were analyzed by Kruskals wallis test (non parametric analogue for ANOVA). The p value of <0.05 was considered statistically significant.

Results

Clinical features

The clinical and laboratory data of the different studied groups are shown in Table 1. There was a statistically significant difference among the studied groups regarding age, gender, hemoglobin percentage, WBC count, platelet count, serum bilirubin, ALT, AST, ALP, serum albumin concentration, prothrombin concentration, and AFP; p value<0.001.

Analysis of methylated genes

According to the manufacturer’s instructions, our studied genes (P14, P15, P73 and O6MGMT) are considered methylated if>10% and/or intermediately methylated if>60% as shown in Table 2. The methylation frequency of P14 gene was detected in 100/208 (48.1%), 52/108 (48.1%), 16/100 (16%) and 8/100 (8%) among HCC, liver cirrhosis, chronic hepatitis and control groups respectively. Out of them 32/208 (15.4%), 16/108 (14.8%) and 8/100 (8%) were intermediately methylated among HCC, liver cirrhosis and chronic hepatitis C groups respectively with a statistically significant difference between the studied groups (p value=0.008) as shown in Table 2, Figure 1A and 2A.

In addition, HCC group was significantly different.
from chronic hepatitis group (p value=0.035) and from control group (p value=0.004). Also Cirrhosis group was significantly different from chronic hepatitis group (P value=0.050) and from control group (p value=0.004) as shown in Table (3).

The methylation frequency of P15 gene was detected in 92/208 (44.2%), 36/108 (33.3%), 20/100 (20%) and 4/100 (4%) among HCC, liver cirrhosis, chronic hepatitis and control groups respectively. Out of them 32/208 (15.4%), 20/108 (18.5%), 8/100 (8%) and 4/100 (4%) were intermediately methylated among HCC, liver cirrhosis, chronic hepatitis and control groups respectively with a statistically significant difference between the studied groups (p value=0.006) as shown in Table 2, Figure (1B, 2B). In addition, HCC group was significantly different from control group (p value<0.001) and cirrhosis group was significantly different from control group (p value=0.024) as shown in Table 3.

The methylation frequency of O6MGMT gene was detected in 84/208 (40.4%), 60/108 (55.6%), 20/100 (20%) and 4/100 (4%) among HCC, liver cirrhosis, chronic hepatitis and control groups respectively. Out of them 48/208 (23.1%) and 36/108 (33.3%) were intermediately methylated among HCC and liver cirrhosis groups respectively with a statistically significant difference between the studied groups (p value<0.001) as shown in Table 2, Figure (1C, 2C). In addition, HCC group was significantly different from chronic hepatitis group (p value=0.016) and from control group (p value=0.003). Cirrhosis group was significantly different from chronic hepatitis group (p value=0.002) and from control group (p value<0.001) as shown in Table 3.

The methylation frequency of P73 gene was detected in 136/208 (65.4%), 72/108 (66.7%), 32/100 (32%) and 4/100 (4%) among HCC, liver cirrhosis, chronic hepatitis and control groups respectively. Out of them 88/208 (42.3%), 56/108 (51.9%), 24/100 (24%) and 4/100 (4%) were intermediately methylated among HCC, liver cirrhosis, chronic hepatitis C and control groups respectively with a statistically significant difference between the studied groups (p value<0.001) as shown in Table 2, Figure (1D, 2D).

In addition, HCC group was significantly different from chronic hepatitis C group (p value=0.037) and from control group (p value=0.001). Cirrhosis group was significantly different from chronic hepatitis C group (p value=0.058) and from control group (p value<0.001). Chronic hepatitis C group was significantly different from control group (p value=0.026) as shown in Table 3.

Clinicopathological features in relation to methylation: there was a statistically significant difference between patients with P14 positive and negative methylation regarding white blood cells (WBC) (p value=0.032), urea (p value=0.015), creatinine (p value=0.042) and liver tenderness (p value=0.011) of liver cirrhosis group; platelets of chronic hepatitis C group (p value=0.004) and there was no significant difference between patients with P14 positive and negative methylation regarding age, gender, smoking, DM and liver size.

In addition, there was a statistically significant difference between patients with P15 positive and negative methylation regarding platelets of liver cirrhosis group and chronic hepatitis C group; p value=0.027 and 0.008 respectively. On the other hand there was no significant difference between patients with P15 positive and negative methylation regarding white blood cells (WBC) and platelets of liver cirrhosis group.

### Table 3. Pairwise Comparison between the Studied Groups

<table>
<thead>
<tr>
<th></th>
<th>HCC</th>
<th>Cirrhosis</th>
<th>Chronic C</th>
<th>Control</th>
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<tbody>
<tr>
<td>P14</td>
<td>0.954a</td>
<td>0.035a</td>
<td>0.004a</td>
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<tr>
<td></td>
<td>0.050a</td>
<td></td>
<td>0.004a</td>
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<tr>
<td></td>
<td>0.546a</td>
<td></td>
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<tr>
<td>P15</td>
<td>0.409a</td>
<td>0.090a</td>
<td>&lt;0.001a</td>
<td></td>
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<tr>
<td></td>
<td>0.554a</td>
<td></td>
<td>0.024a</td>
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<tr>
<td></td>
<td>0.223a</td>
<td></td>
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<tr>
<td>MGMT</td>
<td>0.328a</td>
<td>0.016a</td>
<td>0.003a</td>
<td></td>
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<tr>
<td></td>
<td>0.002a</td>
<td></td>
<td>&lt;0.001a</td>
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<tr>
<td></td>
<td>0.189a</td>
<td></td>
<td></td>
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<tr>
<td>TP73</td>
<td>0.858a</td>
<td>0.037a</td>
<td>&lt;0.001a</td>
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<td></td>
<td>0.058a</td>
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<td></td>
<td>0.026a</td>
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</tbody>
</table>

- Correlation between HCC and Cirrhosis; *Correlation between HCC and Chronic Hepatitis C; **Correlation between HCC and control; +Correlation between Chronic Hepatitis C and control; p value is significant if less than 0.05
methylation regarding age, gender, smoking, DM, liver size, liver tenderness, splenomegaly and ascites.

On the other hand, there was a statistically significant difference between patients with O6 MGMT positive and negative methylation regarding age of HCC group; platelets and alanine aminotransferase (ALT) of chronic hepatitis C group; p value=0.040, 0.006 and 0.029 respectively. However, there was no significant difference between patients with O6 MGMT positive and negative methylation regarding gender, smoking, DM, liver size, liver tenderness, splenomegaly and ascites.

Moreover, there was a statistically significant difference between patients with P73 positive and negative methylation regarding liver tenderness of HCC group; liver size, WBC and prothrombin time (PT) of liver cirrhosis group; p value=0.007, 0.008, 0.017 respectively. However, there was no significant difference between patients with p73 positive and negative methylation regarding age, gender, smoking, DM, splenomegaly and ascites.

Discussion

Hepatocellular carcinoma is a major health problem worldwide. Epigenetic silencing of tumor-related genes due to the methylation of gene promoter regions plays an important role in carcinogenesis in HCC (Lee et al., 2003). In HCCs, a growing number of genes have been recognized as undergoing aberrant CpG island hypermethylation, which is associated with the transcriptional inactivation and loss of gene function, suggesting that CpG island hypermethylation is an important molecular mechanism for the development of HCC (Iwata et al., 2000). There was a characteristic male predominance in all our HCV related chronic liver disease groups and this was reported worldwide for HCC to be more prevalent in males that could be due to gender-based differences in carcinogen-metabolizing liver enzymes (Parkin et al., 2005). In this study, a significant difference in the hypermethylation status of all studied genes was noted within the different stages of chronic liver disease and ultimately HCC. In our analysis for the results, we observed an increase in methylation frequency of P14 gene between the studied groups being 48.1% in HCC cases with a statistically significant difference between the studied groups. The presence of p14 methylation could be attributed to that P14 was reported to be preferentially methylated in HCV related HCC (Yang et al., 2003). Matching with our results, Anzola et al. reported the methylation frequency of p14 in 41.9% out of 117 HCC cases (Anzola et al., 2004). Also, Liu et al. (2006) reported the frequency of p14 methylation in 36% out of 50 HCC cases (Liu et al., 2006) but they did not include normal controls in their comparison as we did. In contrast to our results, Yang et al. (2003) reported p14 methylation in only 6% out of 51 HCC cases and reported no methylation of p14 either in non-tumor or cirrhotic livers but 18% of their patients were HBV positive and 46% were negative for both HBV and HCV while all our patients were HCV positive. Also 29% of their patients were not on top of liver cirrhosis while all our patients were on top of liver cirrhosis. Moreover, Peng et al. (2002) reported p14 methylation frequency in 20% (8 out of 40) of HCC which is not matched with our results. Methylation of P15 is present not only in HCC, but was also reported at low frequencies in chronic hepatitis and liver cirrhosis samples (Fukai et al., 2005; Shih et al., 2006). Silencing of P15 through promoter methylation may be involved in virus-induced hepatocarcinogenesis (Yang et al., 2003). On the other hand, analysis of epigenetic changes on p15 tumor suppressor gene in serum DNA may be a valuable biomarker for early detection in populations at high risk of HCC (Zhang et al., 2007). In our study, there was an increase in the methylation frequency of P15 gene between the studied groups being 44.2% out of 208 HCC cases with a statistically significant difference between the studied groups. Matching with our results, Yang et al. reported p15 methylation in 47% out of 51 HCC cases but with no methylation of p15 in either non tumor or cirrhotic livers which is not matched with our results as we have 33.3% methylation frequency of p15 gene in cirrhotic patients. Moreover, Liu et al. (2006) reported the frequency of p15 methylation in (58%) out of 50 HCC cases versus (50%) out of 50 pericancer tissues but they did not include normal controls in their comparison. In contrast to our results, Zhang et al. (2007) reported that aberrant methylation was found in serum DNA 1 to 5 years before clinical HCC diagnosis; they reported P15 methylation in 22% out of 50 HCC cases. However, they reported that out of 50 control cases, none had methylation of p15 which is matched with our results (Zhang et al., 2007). On the other hand, Iyer et al. (2009) reported P15 methylation frequency in 14.2% HCC Egyptian patients in tissues and 10.7% for plasma. Most of their patients were HCV positive and only 4 patients had combined HBV and HCV infection similar to our patients but they only compared the methylation profile of the tumor suppressor genes in tumor tissues and plasma from the same HCC patient without any controls (Iyer et al., 2009). The p73 gene encodes a protein structurally and functionally homologous to P53. In our study; the methylation frequency of P73 gene was 65.4% out of 208 HCC cases with a statistically significant difference between the studied groups. Liu et al. (2006) reported the frequency of p73 methylation in (16%) out of 50 HCC cases, which is not matched with our results, versus (0%) out of 50 pericancer tissues, which is nearly similar to our results, but they did not include normal controls in their comparison (Liu et al., 2006). In contrast to our results, Yang et al. (2003) reported p73 gene methylation in only 6% out of 51 HCC cases but with no methylation of p73 either in neighboring non-tumor or cirrhotic livers which is not matched with our results as the methylation frequency of P73 gene in our series was 66.7% in cirrhotic patients (Yang et al., 2003). Also, Zhao et al. (2005) reported the frequency of promoter hypermethylation of p73 in (5.0%) out of 40 HCC. However, none of hypermethylation of the gene was detected in corresponding non-tumor liver tissues (Zhao et al., 2005). Long-term inflammation of the liver induced by HCV infection may generate reactive oxygen species (ROS) to promote DNA damage, and so the relationship between ROS and epigenetic alterations of O6MGMT in hepatocytes requires further clarifications. This might
suggest the critical role of the defects in DNA repair mechanisms in HCV induced HCC (Pal et al., 2010). In our analysis for data, the methylation frequency of O6MGMT gene was 40.4% out of 208 HCC cases with statistically significant difference between the studied groups. Comparing our results with Matsukura et al., they reported MGMT promoter methylation in 21.8% (10 out of 46 HCC cases) and they also reported significant difference between the studied groups of HCC, liver cirrhosis and normal controls (Matsukura et al., 2003). Moreover, Liu et al. reported the frequency of O6MGMT methylation in (16%) out of 50 HCC cases versus (10.4%) out of 50 pericancer tissues but they did not include normal controls in their comparison (Liu et al., 2006). In contrast to our results, Nomoto et al. (2007) reported no methylation at all of MGMT either in HCC, liver cirrhosis or normal controls of only 19 Japanese patients with 13 patients were HCV positive and the rest had chronic HBV. The variability of our results could be attributed to several factors including the sensitivity of the PCR and the differences in CpG sites, the etiological factors contributing to HCC and the geographical differences (Nomoto et al., 2007). However, these do not rule out the involvement of excision repair mechanism in hepatocarcinogenesis.

References