Detection of Aberrant p16INK4A Methylation in Sera of Patients With HCV Related Liver Diseases: An Egyptian Study

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ABSTRACT

Egypt reports the highest prevalence of HCV worldwide, the molecular mechanisms of acute hepatitis C virus (HCV) infection, end-stage hepatitis (cirrhosis), and hepatocellular carcinoma have been extensively studied, but little is known of the changes in liver gene expression during the early stages of liver fibrosis associated with chronic HCV infection. The p16INK4A tumor suppressor gene frequently displays genetic alteration in HCC tissues. The present study was performed to estimate the frequency of methylated p16INK4A in the sera of patients with Hepatitis C Virus (HCV) related chronic active hepatitis (CAH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC), and to evaluate p16INK4A role as a tumor marker of HCC. The sera of 17 CAH, 20 LC patients and 25 HCC patients were examined in this study. The methylation status of p16INK4A was evaluated by methylation-specific PCR of serum samples. Methylated p16INK4A was detected in 47.1% (8/17) of CAH patients, 5% (4/20) of LC patients and in 92% (23/25) of HCC patients. HBV markers were detected in (4/25) all had methylated p16INK4A. No association was demonstrated between p16INK4A methylation and serum AFP level in HCC group.

Conclusion: The results of this study indicate that aberrant DNA methylation contribute to hepatocarcinogenesis, it may be an early event during hepatocarcinogenesis. As the status of p16INK4A methylation was not associated with serum AFP level, it may have a complementary role with AFP as a tumor marker of HCC.

Key Words: p16INK4A Methylation; PCR, Methylation-Specific; Carcinoma, Hepatocellular; Liver Cirrhosis

INTRODUCTION

Hepatitis C virus (HCV) infection is gaining an increasing attention as a global health crisis. Egypt reports the highest prevalence of HCV worldwide, ranging from 6% to more than 40% among regions and demographic groups.¹ The molecular mechanisms of acute hepatitis C virus (HCV) infection, end-stage hepatitis (cirrhosis), and hepatocellular carcinoma have been extensively studied, but little is known of the changes in liver gene expression during the early stages of liver fibrosis associated with chronic HCV infection, that is the transition from normal liver of uninfected patients to the first stage of liver fibrosis.² The burden of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. This has been attributed to several biological (e.g. hepatitis B and C virus infection) and environmental factors (e.g. aflatoxin, AF). Previously, there was strong evidence that hepatitis B virus (HBV) was the major cause of HCC in Egypt, but more recently HCV has become the predominant factor associated with the more recent epidemic of HCC.³ Although alpha-fetoprotein (AFP) measurement and ultrasonography are useful surveillance tests for detecting HCCs at a stage at which they may be treated, both tests have limitations. Inactivation of tumor suppressor genes is important in the development of cancers, and leads to abnormal proliferation, transformation, invasion, and metastasis.⁴ Inactivation of tumor suppressor genes can occur due to silencing as a result of methylation of tumor suppressor gene promoters, as well as genetic mutation, loss of heterozygosity (LOH), or deletion of homozygosity.⁵,⁶,⁷ The genes most frequently involved are those encoding tumor suppressors. The p16INK4A tumor suppressor gene frequently displays genetic alteration in HCC tissues. Inactivation of this gene, which normally inhibits progression to the G1 phase of the cell cycle, is involved in the initiation of tumors. The methylation of p16INK4A is known to silence transcription of the gene⁸. The degree of p16INK4A methylation shows a wide range of variation (from 0 to 94%) in tumor tissues of
HCC patients\textsuperscript{(3,9-15)}, and this change has been detected in the sera of such patients\textsuperscript{(16)}. In a previous study done to estimate methylation status of CpG islands in HCCs from countries with various HCC risks including Egypt p16 methylation had statistically significant geographic variation (34.4\% in tumors from China and Egypt versus 12.2\% in tumors from the United States and Europe, difference = 22.2\%; 95\% confidence interval [CI] = 11.2\% to 33.2\%; P<.001)\textsuperscript{(17)}. The present study was performed to estimate the frequency of methylated \textit{p16\textsuperscript{INK4A}} in the sera of patients with Hepatitis C Virus (HCV) related chronic active hepatitis (CAH), liver cirrhosis (LC) and HCC patients, and also to evaluate \textit{p16\textsuperscript{INK4A}} role as a tumor marker of HCC.

**PATIENTS & METHODS**

**Patients:**
This study included 17 patients with HCV chronic active hepatitis, 20 patients with liver cirrhosis and 25 patients with HCC selected from tropical medicine department, Kasr Al Aini hospital, Cairo University.

All patients were subjected to full clinical history and examination abdominal ultrasonography imaging, hepatic transaminasis, Viral markers : HCV testing using third generation ELISA, HBV surface antigen and HBc antigen, Alpha feto protein (AFP) and detection of aberrant hypermethylation of \textit{p16\textsuperscript{INK4A}}. Liver biopsy was done to all patients with CAH as well as quantitative HCV by PCR.

Patients with chronic active hepatitis were enrolled from patients candidates for interferon therapy before receiving their treatment. They had no clinical evidence of cirrhosis nor by abdominal ultrasonography or by histopathological examination of their liver biopsy. Liver cirrhosis was diagnosed by clinical examination confirmed by detection of ultrasonographic evidence of cirrhosis and laboratory conformation by AST/ALT ratio.

HCC was diagnosed by detection of focal hepatic lesion by abdominal ultrasonography the nature of which was proved to be HCC by finding an AFP >400 ng/dl, if AFP was below this cut off level patients had spiral CT if non conclusive patients had biopsy from the focal lesion. Patients with CAH an LC were followed up for 6 months to exclude undetected cancer.

**Methods**
Sampling:10 ml blood was collected from all patients and divided into two evacuated tubes. One on Na citrate for PT assay and another on dry tube where serum is separated for routine biochemical tests (total bilirubin, AST, ALT, ALP, Albumin), hepatitis viral markers (HBsAg and HCV abs), AFP and measurement of hypermethylated \textit{p16\textsuperscript{NK4A}}. Additional sample on EDTA was taken from those with CAH for quantitation of their HCV RNA by PCR.

**Analytical methods:**
Routine biochemical assays were done on Hitachi 917 chemistry autoanalyzer using kits supplied by Roche diagnostics.

Prothrombin time was done using STAGO ST4 coagulation analyzer using kits supplied by STAGO diagnostics.

AFP, HBsAg and HCV Abs were done by MEIA technique using Axsym autoanalyzer and kits were purchased by Abott diagnostics.

Quantitation of HCV RNA was done using the Roche Taqman real time quantitative PCR method.

Detection of Abnormal methylation of the \textit{p16\textsuperscript{NK4A}} gene; \textit{Isolation of DNA}:DNA was extracted from serum using kit supplied by Bio Basic (Bio Basic Inc. 160 Torbay Road, Mrkham Ontario L3R 1G6 Canada).

**Modification Reaction**
Conversion of unmethylated cytosine into uracil and leaving methylated cytosine as it is by Na-bisulfite using EzWay\textsuperscript{TM} DNA modification kit, supplied by Koma Biotech (1487 Gayang 3 dong, Gangseo-gu seoul 157-793, Korea). The modified DNA was eluted into 20\,\mu\text{L} of TE (10 mM Tris-\text{HCl}, 1 mM EDTA), to be used immediately as a template for MSP.

**Primer design for MSP**
Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment. To accomplish this, primer sequences were chosen for regions containing frequent cytosines (Cs) and CpG islands near the 3' end of the primers. This design was described by Herman et al\textsuperscript{(7)}. Two primer pairs for \textit{p16} gene provided from Biotec are described in table 1. Primers will be designed to amplify methylated specific primer pairs (p16-M) and unmethylated specific primer pairs (p16-U).
Bisulfite-modified DNA will be amplified. The PCR mixture contains 1× universal PCR buffer, dNTPs (each at 0.25 mM) (Fermentas: Fermentas GMBH Opelstrasse 9, D-68789 St.Leon-Rot), methylated or unmethylated primer, 1 unit of Taq polymerase (Fermentas), and modified DNA (100 ng) in a final volume of 12.5µL. Amplification was carried out in a Hybaid thermal cycler (Promega: Promega Corporation 2800 Woods Hollow Road-Madison, WI 53711-5399, USA) as follows: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min; with a final extension of 10 min at 72°C (Chu et al, 2004). PCR products (12.5µL) were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV transillumination.

Statistical Analysis: was performed using the SPSSSTM, version 11.0 for Windows software (SPSS Inc., Chicago, IL, U.S.A.). Comparison between clinical and laboratory data was done using ANOVA test. The frequency of P16 methylation was done using chi-square. The association of P16 methylation with other laboratory parameters was analyzed using MannWhitney test. P<0.05 is considered statistically significant.

RESULTS

The clinical and laboratory features of each group are shown in Table 1. The average age of patients in the three groups was 45.6±11.6 years in CAH, 51.2±8.8 years in LC and in HCC were 50±9.2 years old with no statistical significance between them. The three groups were also sex matched. There was a highly significant difference in AFP level in HCC group compared to the other two groups. HBV markers were found in 10%(2/20) who had methylated p16INK4.

<table>
<thead>
<tr>
<th>Table 1: Clinical and laboratory features of Chronic active hepatitis, Liver cirrhosis and HCC patients</th>
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<tbody>
<tr>
<td>**Chronic active</td>
</tr>
<tr>
<td>**hepatitis, n=17</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Clinical presentation</td>
</tr>
<tr>
<td>Right hypochondrial pain</td>
</tr>
<tr>
<td>Jaundice</td>
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<tr>
<td>Ascites</td>
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<tr>
<td>Haematemesis</td>
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<tr>
<td>Lower limb oedema</td>
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<tr>
<td>Hepatic encephalopathy</td>
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<tr>
<td>Child Pugh scoring</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>Viral markers</td>
</tr>
<tr>
<td>Positive HBV</td>
</tr>
<tr>
<td>Positive HCV</td>
</tr>
<tr>
<td>Alpha-fetoprotein ng/ml</td>
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*Highly significant difference among groups with no significant difference between LC, CAH.
On comparing the frequency of p16 methylation among groups, there was a significant difference among them with the highest frequency in HCC group, table 2.

Table 2: Frequency of P16 methylation in the three studied groups

<table>
<thead>
<tr>
<th>P16 methylation</th>
<th>CAH</th>
<th>HCC</th>
<th>LC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>8(47.1%)</td>
<td>23(92%)</td>
<td>4(20%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>9(52.9%)</td>
<td>2(8%)</td>
<td>16(80%)</td>
<td></td>
</tr>
</tbody>
</table>

After subdividing the groups according their p16 methylation, there was no associations between the status of p16 methylation and serum AFP, or tumor size in HCC patients and between p16 methylation status and age, sex, or Child score in HCC and LC (P>0.05). In CAH group, there was no association between p16/INK4A methylation and PCR or Metavir score of fibrosis (P = 0.539) or activity (P = 0.858).

Table 3: Association between p16 methylation and AFP levels in patients with HCC

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>25&lt;sup&gt;th&lt;/sup&gt;-75&lt;sup&gt;th&lt;/sup&gt; percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated, n= 23</td>
<td>664</td>
<td>173-22400</td>
<td>0.097</td>
</tr>
<tr>
<td>Unmethylated, n= 2</td>
<td>6</td>
<td>5.7-6.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Association between p16 methylation and quantity of HCV RNA by PCR in CAH group

<table>
<thead>
<tr>
<th>P16 methylation</th>
<th>HCV RNA</th>
<th>Median</th>
<th>25&lt;sup&gt;th&lt;/sup&gt;-75&lt;sup&gt;th&lt;/sup&gt; percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated, n=8</td>
<td>5236.5</td>
<td>1009.2-39794.7</td>
<td>0.248</td>
<td></td>
</tr>
<tr>
<td>Unmethylated, n=9</td>
<td>56443.0</td>
<td>10675.5-887727.5</td>
<td></td>
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</tr>
</tbody>
</table>

P16 methylation discriminated HCC from cirrhosis and CAH with sensitivity of 92% and specificity of 80% in cirrhosis and 53% in CAH with positive predictive value of 85% and 74% versus LC and CAH, respectively.

Figure 1: Agarose gel electrophoresis is showing PCR products (234bp) of p16 gene in liver cirrhosis patients
Lane M: Ladder DNA marker (100,200,300,…etc).
Lane 1: No PCR product for methylated p16 gene
Lane 2-4: PCR products for unmethylated p16 gene.
Lane 5: Faint PCR product for methylated p16 gene
Figure 2: Agarose gel electrophoresis is showing PCR products (234bp) of p16 gene in HCC patients
Lane M: Ladder DNA marker (100, 200, 300, … etc).
Lane 1-3: No PCR product for unmethylated p16 gene
Lane 4-6: PCR products for methylated p16 gene.

DISCUSSION

Lehman and Wilson, 2009 claimed that there is high hepatitis C virus (HCV) incidence in Egypt and suggested that HCV may lead to substantial health consequence over the next 10-20 years (1). HCV infection is closely linked to development of hepatocellular carcinoma. But the molecular mechanisms involved in hepatocarcinogenesis of HCV remain poorly understood. Many authors believe that HCV can not directly change the structure of the host genes like hepatitis B virus by integration because HCV is an RNA virus. The precise mechanisms leading to aberrant methylation of CpG islands in neoplastic cells are not known. Moreover, little is known of the link between viral infections and methylation machinery. Human immunodeficiency virus has been shown to induce methylation of interferon through increased DNA methyltransferase activity (18).

The tumor suppressor gene p16INK4A exhibits variations, including methylation, that are involved in the process of carcinogenesis, so we aimed to estimate the frequency of methylated p16INK4A in the sera of patients with (HCV) related chronic active hepatitis (CAH), liver cirrhosis (LC) that represents stages of HCV infection, estimate frequency of methylated p16INK4A in HCC patients, and also to evaluate p16INK4A role as a tumor marker of HCC.

In our study, we found highly significant difference in the frequency of serum p16INK4A methylation among groups with higher percentage in HCC (92%). In CAH it was 47.1% while in LC it was only 20%. There have been few serum studies of p16INK4A methylation in HCC patients. Wong et al. (16) reported abnormal p16INK4A methylation in 60% of sera and in 73% of tissue in HCC patients. Chu et al. (19), detected abnormal p16INK4A methylation in 48% of the sera of HCC patients, Kaneto et al. (16) detected abnormal p16INK4A methylation in 23.5 % of the biopsies from chronic active hepatitis patients. This may be due to the lack of a standardized method of detection and to the diversity in the clinical courses of patient groups.

In our study, we had cirrhotic patients with abnormal p16INK4A methylation, who were followed for 6 months and showed no evidence of malignancy. This could be explained by Kaneto et al. (14) results as the analysis of serial samples from individual patients with methylation positive HCC revealed that loss of p16 expression with promoter methylation occurred in 18 of 20 patients at the stage of chronic hepatitis without clinically detectable
carcinoma, that suggest that methylation of the p16 promoter and the resulting loss of p16 protein expression are early events in a subset of hepatocarcinogenesis and that their detection is useful in the follow up of patients with a high risk of developing HCC, such as those with hepatitis B or C viral infections.

It has been inferred that abnormal p16INK4A methylation can also be detected in the sera of patients with cirrhosis because it is frequently found in nontumorous tissues. In our study groups the least frequency of methylation was detected in the liver cirrhosis group. In contrast with our results, several previous studies have failed to detect methylation of the p16 promoter in chronic non-tumour liver diseases, including those of viral origin. These conflicting observations may be due to differences in the methods for detection of methylation and/or in the ethnic and aetiological backgrounds of individuals. However, these factors in our study were similar to those in the study of Kurita and colleagues. We can explain our result by that more meticulous methods may be required to detect p16INK4A methylation in cirrhosis patients, as the amount of circulating DNA is lower in the sera of cirrhosis patients than in that of HCC patients.

Although further study is necessary to clarify the link between hepatitis virus infections and methylation of p16 in hepatocarcinogenesis, detection of p16 promoter methylation could be a useful molecular marker to follow up patients with a high risk of developing HCC, such as those with HBV or HCV infections. It would be of interest to prospectively examine whether patients with p16 methylation positive cirrhosis or chronic hepatitis have a higher risk of developing HCC compared with those without p16 methylation.

In our study, the incidence of methylated p16INK4A DNA was not correlated with AFP levels in the sera of HCC patients. Wong et al. reported a significant correlation between the methylation of circulating DNA and serum AFP levels in HCC patients. There is a danger in generalizing the negative association from this result, however, as the study only included 2 cases of HCC without methylation which could explain this non-correlation.

Our study suggested that methylated p16INK4A DNA may play an important role as a tumor marker in detection of HCC. The state of serum p16INK4A methylation discriminated HCC from cirrhosis and CAH with sensitivity of 92% and specificity of 80% in cirrhosis and 53% in CAH with positive predictive value of 85% and 74% versus LC and CAH, respectively.

Therefore, serum methylation may be a useful complementary tool to the AFP test. Finally, our data provide evidence that HCV infection may accelerate the methylation process and suggests a continuum of increasing methylation with persistent viral infection and carcinogenesis in the liver.

We recommend further studies on plasma or sera as large variations in incidence may be expected due to differences in sample materials, detection methods, and/or subjects selected. We recommend further analysis, including a number of non-virus associated cases, is required to clarify the possible intriguing relationship between methylation of the p16 promoter and chronic hepatitis virus infections.

REFERENCES


