Methylated Gene p15 and APC Gene as Biomarkers in Detection of HCC Versus MRI Diffusion in Cirrhotic Patients

MONA M. SOLIMAN, M.D. 1; HANAN Sh. MOHAMMED, M.D. 2; MOHAMED Z. ABD ELRAHMAN, M.D. 3; DALIA A. NIGM, M.D. 4; AMAL A. MAHMOUD, M.D. 5; MOUSTAFA E.M. RADWAN, M.D. 6 and REEM M. ELKADY, M.D. 7

The Departments of Internal Medicine 1, 2, Clinical Pathology 3, 4, 5 and Diagnostic Radiology 6, 7, Faculty of Medicine, Assuit University

Abstract

Background/Objectives: MRI of the liver is an important tool for detection and characterization of focal liver lesions. Epigenetic biomarkers is a noninvasive approach for early detection of HCC in high-risk patients. We work to determine the sensitivity and specificity of methylated gene p15 and Adenomatous polyposis coli gene in detection of HCC aiming to replace liver biopsy especially in patients with bleeding diathesis and to make a comparison between laboratory biomarkers and MRI in their abilities in detection of HCC.

Methods: Sixty cirrhotic patients with hepatic focal lesions subjected to the following investigations: Hepatitis C virus anti-bodies, Hepatitis B surface antigen, Methylated gene p15 and Adenomatous polyposis coli gene in serum by Polymerase chain reaction, Abdominal Ultrasound, Liver biopsy and MRI diffusion abdomen.

Results: Methylated gene p15 is more sensitive and specific than MRI in detection of HCC. The combination of methylated gene p15 and MRI raise the sensitivity and specificity in detection of HCC. Adenomatous polyposis coli gene is less sensitive and less specific than methylated gene p15 and MRI in detection of HCC.

Conclusion: Methylated gene p15 is more sensitive and specific than MRI and Adenomatous polyposis coli gene in detection of HCC. The combination of methylated gene p15 and MRI diffusion together will increase sensitivity of HCC detection up to 100% and this will make diagnosis more accurate and decreases the need for liver biopsy in diagnosis.


Introduction

LIVER cirrhosis and its complications are major clinical problems that carry a considerable risk of disability and death [1]. Magnetic resonance imaging (MRI) has more advantages than ultrasound, computed tomography (CT), positron emission tomography (PET), or any other imaging modality in diagnosing focal hepatic masses [2]. The use of epigenetic biomarkers is a noninvasive approach for early detection of hepatocellular carcinoma in a population at high-risk. Identification of methylated DNA sequences in serum that correspond to cancer-related epigenetic events in occult tumors has been suggested as a potentially valuable biomarker for preclinical detection of malignant lesions. In fact, several studies of hepatocellular carcinoma have shown that methylated p 15 is present in serum at the time of cancer diagnosis. Thus, aberrantly methylated promoter sequences corresponding to these genes represent potentially valuable epigenetic biomarkers for early (preclinical) diagnosis of hepatocellular carcinoma [3]. Up to our knowledge no studies correlate MRI and methylated genes in detection of HCC.

Aim of the study:

- To determine the sensitivity and specificity of methylated gene p 15 and APC genes in detection of HCC aiming to replace liver biopsy especially in patients with bleeding diathesis.
- To determine sensitivity and specificity of MRI in detection of HCC.
- To make a comparison between laboratory biomarkers (methylated gene p15, APC gene) and MRI in their abilities in detection of HCC.

Patients and Methods

Randomized double-blind pilot study was done on 60 cirrhotic patients with hepatic focal lesions in out patient clinic in Assuit University Hospital in the period of July 2013 to December 2014, all patients were subjected to the following:
Laboratory and imaging investigations: Blood urea, Serum creatinine, Liver function tests by Hitachi 902, Complete blood picture by celldyne, prothrombin time and concentration by symex, HCV anti-bodies by Architect system in a two step immunoassay, using chemiluminescent microparticle immunoassay (CMIA) technology, for the qualitative detection of anti- HCV in human serum and plasma, HBV s antigen by Architect system in one step immunoassay for the qualitative detection of HBV s antigen in human serum and plasma using CMIA technology, with flexible assay protocols, referred as Chemiflex, Methylated gene p15 and APC gene in serum by (real time PCR).

**Methylated p15 primer sequence:**
- Forward primer 5'-GCGTTCGTATTTTGCG-TTT- 3'
- Reverse primer 5'-CGTACAATAACCGAACGACCA- CCGA- 3'

**Methylated APC primer sequence:**
- Forward primer 5'-TTATATGTCGGTTACGT-GCGTTTATAT- 3'
- Reverse primer 5' – GAACCAAAACGCTCCCAT- CCGA- 3'

**Liver biopsy:** Liver biopsy was done after checking prothrombin time and concentration and platelet count by Guillotine biopsy needle (medical devices - GTA) 16G x 20 cm via Della vittoria, s Quistello (MN) –Italy.

**Statistical analysis:**
Data entry and statistical analysis were done by SPSS version (Statistical Package for Social Science) version 19. Data for continuous variables were expressed as mean, standard deviation and standard error. Categorical variables were expressed as absolute numbers and percentages. Comparison between two groups was analyzed by chi-square test, p-value of less than 0.05 was considered to be statistically significant. ROC curve analysis which was done using “medcalc 7”.

**Results**
Table (1) shows that the study includes sixty patients; 12 patients (20%) <50 years and 48 patients (80%) 50 years, fifty male patients (83.3%) and 10 females (16.7%). Patients from rural area are 42 (70%) and from urban area are 18 (30%).

Number of HCV +ve patients were 34 (56.7%) and HCV -ve patients were 26 (43.3%).

HBV S Ag +ve patients were 4 (6.7%) and HBV s Ag –ve patients were 56 (93.3%).

Methylated gene p15 +ve patients were 42 (70%) and methylated gene p 15 –ve patients were 18 (30%).

Table (2) displays APC gene +ve patients are 24 (40%) and APC gene –ve patients are 36 (60%).

There is significant positive methylated gene p15 in HCV Ab positive patients compared to HCV Ab –ve and there is more positive cases for APC gene in HCV +ve patients compared to HCV Ab –ve but the difference is not significant.

Table (3) shows MRI and liver biopsy in diagnosis of cirrhotic patients with focal lesion where there is 40 cirrhotic cases (66.7%) diagnosed as having HCC by MRI compared to 48 cases (80%) diagnosed as having HCC by liver biopsy.

There is insignificant difference between patients less than 50 years old and more than 50 years regarding the presence of methylated gene p 15 and APC gene with p-values = 0.765 and 0.305 respectively (Fig. 1).

There is insignificant difference between males and females regarding the presence of methylated gene p15 and APC gene with p-values = 0.593 and 0.617 respectively (Fig. 1).

There is insignificant difference between rural and urban patients regarding the presence of methylated gene p15 and APC gene with p-values = 0.862 and 0.745 respectively (Fig. 1).

Methylated gene p15 is more sensitive and specific than MRI and APC gene in detection of HCC (Fig. 2).

The combination of methylated gene p 15 and MRI diffusion together will increase the sensitivity of HCC detection up to 100% (Fig. 3).
Table (1): Baseline data of cirrhotic patients with focal lesion.

<table>
<thead>
<tr>
<th></th>
<th>No. (n=60)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 years</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>50 years</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td><strong>Sex:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>83.3</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td><strong>Residence:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>Urban</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td><strong>HCV anti-bodies:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>34</td>
<td>56.7</td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>43.3</td>
</tr>
<tr>
<td><strong>HBs antigen:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>6.7</td>
</tr>
<tr>
<td>Negative</td>
<td>56</td>
<td>93.3</td>
</tr>
<tr>
<td><strong>Methylated gene p15:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td><strong>Adenomatous polyposis coli gene (APC):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>60</td>
</tr>
</tbody>
</table>

Table (2): Methylated gene p15 and Adenomatous polyposis coli gene (APC) according to HCVAb.

<table>
<thead>
<tr>
<th></th>
<th>Positive No.</th>
<th>Positive %</th>
<th>Negative No.</th>
<th>Negative %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylated gene p15:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>94.1</td>
<td>10</td>
<td>38.5</td>
<td>0.004*</td>
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<tr>
<td>Negative</td>
<td>2</td>
<td>5.9</td>
<td>16</td>
<td>61.5</td>
<td></td>
</tr>
<tr>
<td><strong>Adenomatous polyposis coli gene (APC):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
<td>52.9</td>
<td>6</td>
<td>23.1</td>
<td>0.098</td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
<td>47.1</td>
<td>20</td>
<td>76.9</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): MRI and liver biopsy in diagnosis of cirrhotic patients with focal lesion.

<table>
<thead>
<tr>
<th>MRI diagnosis of cirrhotic patients with focal lesion</th>
<th>Liver biopsy in cirrhotic patients with hepatic focal lesion</th>
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<tbody>
<tr>
<td>MRI Diagnosis</td>
<td>No. (n=60)</td>
</tr>
<tr>
<td>Dysplastic nodules</td>
<td>8</td>
</tr>
<tr>
<td>Haemangioma</td>
<td>2</td>
</tr>
<tr>
<td>HCC</td>
<td>40</td>
</tr>
<tr>
<td>HCC versus metastasis</td>
<td>2</td>
</tr>
<tr>
<td>Liver cysts</td>
<td>2</td>
</tr>
<tr>
<td>Regenerating nodules</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. (1): Relation of methylated gene p15, APC gene to age, gender and residence.
Fig. (2): Sensitivity and specificity of APC gene, Methylated gene p15 and MRI in detection of HCC.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+PV</th>
<th>−PV</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC gene</td>
<td>41.67</td>
<td>66.67</td>
<td>83.3</td>
<td>22.2</td>
<td>0.542</td>
</tr>
<tr>
<td>Methylated gene p15</td>
<td>87.50</td>
<td>100.00</td>
<td>100.0</td>
<td>66.7</td>
<td>0.937</td>
</tr>
<tr>
<td>MRI</td>
<td>79.17</td>
<td>83.33</td>
<td>95.0</td>
<td>50.0</td>
<td>0.812</td>
</tr>
</tbody>
</table>

Fig. (3) Sensitivity and specificity of combined genes (APC gene and methylated gene p15) and combined (Methylated gene p15 and MRI) in detection of HCC.

**Discussion**

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and one of the main causes of cancer-related death worldwide. It is well documented that hepatitis B, hepatitis C virus infections, aflatoxin B exposure and heavy consumption of alcohol are important risk factors for HCC. In addition, numerous genetic abnormalities associated with the development of HCC have been described [4].

Hepatocellular carcinoma is the fifth most common cancer in men and the seventh in women worldwide. Most of cancer burden (85%) is borne in developing countries [5].
Hepatocellular carcinoma (HCC) is a common disorder worldwide and ranks 2nd and 6th most common cancer among men and women in Egypt. HCC has a rising incidence in Egypt mostly due to high prevalence of viral hepatitis and its complications [6]. Abnormal gene expression in cancerous cells often occurs as a result of genetic mutations, but recent studies suggest aberrant DNA methylation as an alternative mechanism of tumor pathogenesis [7].

Hepatocellular carcinoma (HCC) is caused by both genetic and epigenetic alterations of several tumor-associated genes. In particular, DNA methylation of tumor suppressor genes has been described as one of the major epigenetic alterations in HCC. Aberrant promoter hypermethylation of tumor suppressor genes (TSGs) involved in cell proliferation, apoptosis, cell adhesion, DNA repair and detoxification is frequently detected in HCC, resulting in loss of the corresponding gene function [8].

In addition, up-regulation of DNA–methyltransferase) DNMT 1, 3a and 3b has been detected in HCC compared to non-neoplastic or normal liver, indicating a close correlation between DNMT up-regulation and promoter hypermethylation-mediated inactivation of TSGs. However, the mechanism and its biological significance are largely unknown [9].

Our study was done on 60 cirrhotic patients; 50 males and 10 females. Twelve Patients <50 years and 48 patients 50 years as HCC is more common in older patients this contributes with Ding & Wang, [5] who concluded that HCC usually occurs after the age of 40 years and reaches a peak at approximately 70 years of age.

For biomarkers to be ideal they must not affected by age, gender and residency [8]. In this study there is insignificant relationship between methylated gene p 15, APC gene with both age and gender.

This coincides with Iyer et al., [10] who had used 5 methylated genes (APC, FHIT, p15, p16 and E-cadherin) in detection of HCC and for each gene, the mean age among patients with a methylated promoter was compared where they did not observe a significant association between promoter methylation and age for any of the genes.

Furthermore, Iyer et al., [10] investigated the association between gender and methylation status for each gene by comparing the proportion of methylation in males and females where insignificant relation of APC and p15 with gender was concluded.

In our study there is insignificant relationship between methylated gene p 15, APC gene and residence this coincides with Iyer et al., [10] as they found no significant relationship between methylated genes (APC, FHIT, p 15, p 16 and E-cadherin) and residency.

In this study positive methylated gene p 15 was present in more cases with HCV +ve patients versus HCV-ve patients (32 cases versus 10 cases respectively) with significant difference as hepatitis C virus infection might promote methylation of certain genes in HCC patients [11]. HCV infection may contribute to hepato carcinogenesis through enhancing methylation of multiple genes [12].

There is more positive cases for APC gene in HCV +ve patients patients compared to HCV Ab–ve but the difference was not significant this matched with Edamoto et al., [13] who found increase in promoter methylation of p 15 among HCC patients with HCV infection.

Methylation is significantly more abundant in HCV-positive livers compared with normal liver tissues including APC gene [14].

APC gene has low sensitivity (41.67%) and specificity (66.67%) in detection of HCC compared to methylated gene p 15 which has sensitivity of (87.50%) and specificity of (100%) in detection of HCC. Sensitivity and specificity of both genes together (APC gene and methylated gene p15) increase sensitivity to 91.67% but not affect specificity in detection of HCC in studied patients.

These findings support the notion that methylation specially for p 15 is important in HCC detection concordant with Hua, [15] who identified that the six methylated genes (APC, GSTP1, RASSF1A, CDKN2A, RUNX3 and SFRP 1) having the discriminatory power for HCC and concluded that 100% cases of HCC had at least one promoter of methylated genes. Also, Wong et al., [16] have found that circulating tumor DNA and HCC cells were detectable in the peripheral blood in patients when examining p 15 methylation.

Zhang et al., [17] prospectively examined epigenetic changes in tumor suppressor genes for predicting HCC development in a cohort of high-risk subjects as p15 where promoter hypermethylation were detected in DNA from serum samples collected up to 9 years before clinical diagnosis compared with controls. These molecular changes
may be a valuable biomarker for early detection, risk assessment in high-risk populations, and monitoring the clinical course of HCC.

Magnetic resonance imaging (MRI) has the highest accuracy in diagnosing cirrhotic HCC. However, the published efficacy in detecting early HCC has been less consistent, and the reported sensitivity of the described criteria for HCC <2cm is as low as 30%. This is due to several reasons: early HCC may retain significant portal blood supply and does not show the “diagnostic” delayed washout [18]. Approximately 17% of small HCC appear hypovascular at imaging. With improved MRI technology and field strength, small (<2cm) flash-enhancing foci are increasingly seen in the cirrhotic liver, and only a proportion of these lesions represent HCC. The distorted texture of the cirrhotic liver also causes poor detection of small HCC. In short, any endeavor to improve HCC diagnosis needs to interrogate other biological changes subsequent to hepatocarcinogenesis besides neovascularity [19].

Diffusion-weighted imaging (DWI) is a functional MRI technique that has increasingly been used a cancer-imaging tool in clinical practice. Advances in MRI technology, particularly in gradient strength and parallel imaging, have greatly expanded the use of DWI in extra-cranial oncological imaging. The utility of DWI in detecting, characterizing, histological grading, and assessing treatment response in various abdominal malignancies including HCC has been extensively studied [20].

Park et al., [21] have assessed the role of DWI for lesion detection and characterization, including HCC. For example, in a prior study from their group, they demonstrate higher sensitivity of DWI compared with standard breath-hold T2WI sequence for HCC detection (80.5% versus 53.9%, respectively; \(p<0.001\)). Only few studies have specifically focused on HCC detection in the cirrhotic liver, especially in comparison with contrast-enhanced imaging. Only one of these studies has correlated DWI with liver explant findings, and showed lower sensitivity of DWI for HCC detection, compared with contrast-enhanced T1-weighted imaging (CET1WI) (45%-55% sensitivity for DWI, compared with 92%-100% for CET1WI).

The study by Piana et al., (2011) which assessed the role of DWI versus CET1WI (using extracellular gadolinium chelates) for the detection of HCCs >1cm in a large number of patients reported higher sensitivity for DWI compared with CET1WI for HCC detection. In their study, the sensitivity of conventional MRI criteria (wash-in/wash-out) for the diagnosis of HCC was 59.6% compared with 81.7%-72.5% for DWI alone [18].

Radiological diagnosis of (HCC) in cirrhosis is now so well-established that it obviates routine histological confirmation. Arterial enhancement followed by washout is considered a diagnostic feature of HCC > 1 cm by the American Association for the Study of Liver Diseases (AASLD) and European Association for Study of Liver Diseases (EASL) [22].

In our study MRI diffusion has sensitivity of (79.17) and specificity of (83.33) in detection of HCC.

The combination of methylated gene p15 and MRI diffusion together will increase sensitivity of HCC detection up to 100% and this will make diagnosis more accurate and no need of liver biopsy in diagnosis.

Conclusion:
1- Methylated gene p15 and APC gene have insignificant relationship to age, sex and residency making them good markers for detection of HCC.
2- Methylated gene p15 is more sensitive and specific than MRI and APC gene in detection of HCC.
3- The combination of methylated gene p15 and MRI diffusion together will increase the sensitivity of HCC detection up to 100% making the diagnosis more accurate and replacing liver biopsy especially in patients with bleeding tendency.

Recommendations:
- Multiple methylated genes should be done in serum to increase sensitivity in diagnosis of HCC
- Quantitative methylated gene p 15 and APC gene to detect the cut off values in detection of HCC is mandatory.

References
3- RIVENBARK A.G. and COLEMAN W.B.: The Use of Epigenetic Biomarkers for Preclinical Detection of Hepa-


الملخص العربي

التحليل/الأهداف: الريتين المغناطيسي للكبد هو آداة هامة للكشف وتنوع البؤر الكبدية. المؤشرات الحيوية الجينية هو نهج موسع للكشف المبكر عن سرطان الكبد في المرضى أصحاب الخطرة العالية. ونحن نعمل لتحقيق حساسية وخصوصية مثلى جين P15 وجين APC في الكشف عن سرطان الكبد هادئين إلى استبدال خزعة الكبد خاصة في المرضى الذين يعانون من نزيف وإجراء مقارنة بين المؤشرات الحيوية المخبرية والتصوير بالرنين المغناطيسي في قدرتهم على الكشف عن سرطان الكبد.

الأساليب:

ستكون مريض تليف كبد مع بؤر كبدية يخضعون للفحوصات التالية:
- الأجسام المضادة للالتهاب الكبدى (سي)، مصل الالتهاب الكبدى (بي) السطحي.
- ميثل جين P15، جين APC بواسطة تفاعل البلمرة المشتسل.
- فحص البطن بالصوتيات فوق الصوتية، رنين مغناطيسي.
- خزعة الكبد.

النتائج: ميثل جين P15 أكثر حساسية وخصوصية من التصور بالرنين المغناطيسي في الكشف عن سرطان الكبد. إضافة ميثل جين APC هو أقل حساسية وأقل خصوصية من ميثل P15 والرنين المغناطيسي تزيد الحساسية والخصوصية في الكشف عن سرطان الكبد. جين P15 والتصوير بالرنين المغناطيسي في الكشف عن سرطان الكبد.

الاستنتاج: ميثل جين P15 هو أكثر حساسية وخصوصية من التصور بالرنين المغناطيسي وجين APC في الكشف عن سرطان الكبد.

والجمع بين ميثل جين P15 والرنين المغناطيسي يؤدي إلى زيادة حساسية الكشف عن سرطان الكبد بنسبة تصل إلى 100% وهذا سيجعل التشخيص أكثر دقة وقليل من الحاجة إلى خزعة الكبد في التشخيص.