Promising Candidate Urinary MicroRNA Biomarkers for the Early Detection of Hepatocellular Carcinoma Among High-Risk Hepatitis C Virus Egyptian Patients

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Abstract
MicroRNAs (miRNAs) are small endogenously expressed non-coding RNAs that negatively regulate expression of protein-coding genes at the translational level. Accumulating evidence, including aberrant expression of miRNAs, suggests that they play a role in the development of cancer. They have been identified in various tumor types, demonstrating that different sets of miRNAs are usually deregulated in different cancers. To identify the miRNAs signature specific for Hepatitis C virus (HCV)-associated Hepatocellular carcinoma (HCC). We first list a list of 42 HCC post-HCV infected, 74 HCV-positive and 12 control individuals was carried out for whole genome expression profiling. Different expression of two individual miRNAs between control and high risk HCV patients was detected and found to possibly target genes related to HCC development and progression. There was a significant difference in expression HCC among HCV-positive individuals was found to be 64% and 68%, respectively. Whereas, the sensitivity of 62% and 56%, respectively. Additionally, the sensitivity and specificity for miR-618,690 in tandem were 59% and 84%, respectively. These predictive values are greatly improved compared to the traditional a-fetoprotein (AFP) level-based detection method. The proposed HCC miRNA signatures may therefore be of great value for early diagnosis of HCC, before the onset of disease in HCV-positive patients. The significance of this approach is amplified by the use of urine as a sample source as it offers a non-invasive approach for developing screening methods that can reduce mortality rates.

Introduction
• Hepatocellular carcinoma (HCC) is one of the leading causes of deaths worldwide and is responsible for 500,000 deaths per year (1). Individuals chronically infected with hepatitis B or C virus (HBV, HCV) are at high risk for the development of HCC, with disease progression occurring paradoxically over a long latency between infection and development of HCC provides an important window of time during which individuals can be monitored for disease progression and intervention could be effective (4). However, the widely used serological tumor markers for HCC, α-fetoprotein (AFP) and des-y-carboxy prothrombin (DCP), have very low specificities and sensitivities.
• Therefore, the development of non-invasive biomarkers with high specificity and sensitivity that can be used for large-scale clinical investigations would be highly beneficial.

Objectives of the Study
1) To conduct a miRNA expression profile in urine samples collected from HCC post-HCV positive group, HCV positive group as well as from healthy control groups to seek out deregulated miRNAs in HCV- positive individuals.
2) To evaluate the use of deregulated miRNAs as putative non-invasive urinary biomarkers for screening high-risk patients for the early detection of HCC.

Materials and Methods

Urine Sample Collection. Urine samples were collected from the general hospital at Alexandria University (Alexandria, Egypt) and the National Institute of Endemic Diseases (Alexandria, Egypt). The 3 groups participating in this study were divided as follows: 32 patients with HCC post-HCV infection, 74 patients with chronic HCV infection and 15 normal individuals as control group. The urine samples from all patients were obtained in 3 ml urine sample tubes. The samples were kept at -20°C until use.

Table 1. Clinical Pathological Parameters of the Subjects in this Study.

<table>
<thead>
<tr>
<th>Group</th>
<th>HCC post-HCV</th>
<th>HCV-positive</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Patients</td>
<td>12</td>
<td>32</td>
<td>74</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>58 ± 5</td>
<td>50 ± 8</td>
<td>60 ± 8</td>
</tr>
<tr>
<td>Sex</td>
<td>Male (6)</td>
<td>Male (26)</td>
<td>Male (50)</td>
</tr>
<tr>
<td></td>
<td>Female (5)</td>
<td>Female (44)</td>
<td>Female (50)</td>
</tr>
</tbody>
</table>

Isolation and Quality Assessment of Total RNA from Urine. Total RNA was isolated from 3 ml of urine using the Urilute (Euritol) RNA purification kit (Norgen Biotech, Thorold, ON, Canada). The isolation of RNA was performed using the manufacturer’s protocol. A spectrophotometric assay was performed to determine the concentration of the isolated RNA and its purity. The quality of the isolated RNA was assessed by gel electrophoresis, and the integrity of the RNA was confirmed by the ratio of the 18S and 28S bands.

MicroRNA Whole-Genome Expression Profiling. The differential miRNA expression profiling was performed using the miRNA expression profiling associated with HCC. Using the Total RNA, the microRNA expression profiling was performed using the Illumina Small RNA Sequencing Kit (Toronto, ON, Canada). This assay is an adapter-based protocol that uses a modified Illumina Solexa Nextera extender (Toronto, ON, Canada). The Illumina Hiseq 2000 sequencer was used to sequence the captured miRNAs. The miRNA expression data analysis was performed using the Illumina’s miRNA expression analysis software.

Relative Expression Profiling for Candidate miRNAs Using RT-qPCR. Relative miRNA expression levels were measured using real-time RT-qPCR. The expression of the candidate miRNA was quantified using a standard curve method. The relative expression of each candidate miRNA was determined from the expression level of the housekeeping gene 18S rRNA.

Results

Figure 1. Heat Map of miRNA Expression in HCC-post HCV Positive, HCV Positive and Control Group. A) miRNA up-regulated in HCC-post HCV positive and HCV positive group. B) miRNA down-regulated in both HCC post HCV positive and HCV positive group relative to the control group. The signal intensity from each miRNA tested in either the HCC or the HCV groups was normalized against its equivalent in the control group. C) The expression profiling and functional enrichment analysis of the deregulated miRNAs in HCC patients with HCV infection showed that the majority of up-regulated miRNAs were involved in cancer-related pathways and processes.

Figure 2. Volcano Plots Demonstrating the Log2 Fold Difference Between Deregulated miRNAs and Control Group. Five of the miRNAs identified as deregulated in the HCV-infected patients (miR-592-32 and miR-618) and the two down-regulated (miR-565-1P and miR-650), whose levels were significantly different in the urine samples from the HCC post-HCV positive group relative to the control group, were studied as potential HCC biomarkers (6). Since their putative targets have a role in cancer development, their deregulated expression may be associated with HCC. The deregulated miRNAs identified in this study may be useful for the early detection of HCC.

Figure 3. A Volcano Plot Graphing the Log2 Fold Difference in Expression of A) miR-96 and B) miR-499 Expression versus its P value from the T-Test Among HCC Positive Group. The black line indicates a fold-change in gene expression threshold of 2. The blue line indicates the desired threshold for the p value of the t-test (P < 0.05). Samples with a significant miRNA deregulation (dotted oval) were chosen at a fold change > 2 and p < 0.05. The deregulation of miR-96 in the HCC positive patients and the expression levels of miR-618 and miR-650 were examined among the HCC post-HCV positive patients using RT-qPCR in order to evaluate the potential of these aberrant miRNA expression signatures as an HCC biomarker. miR-618 was significantly up-regulated in 35 of the 74 HCC positive patients (A), while miR-650 was significantly down-regulated in 42 of the 74 HCV positive patients (B).

Conclusions

The sensitivity and specificity of miR-618 and miR-650 for detecting HCC among HCV-positive patients, based on AFP levels and AFP on radiotherapy, is in no correlation between the relative expression of miR-618 and miR-650 and AFP levels. The positive predictive value (PPV) of miR-618 and miR-650 for correctly identifying true HCC positive patients was 66%, and the negative predictive value (NPV) for correctly identifying true negative HCC patients was 67%. Whereas, the sensitivity and specificity of miR-618 for detecting HCC among HCV- positive patients were 72% and 58%, respectively, with an overall diagnostic accuracy of 66%. The PPV of miR-618 for correctly identifying true HCC positive patients was 62% and the NPV for correctly identifying true negative HCC patients was 68%. Additionally, the sensitivity and specificity for miR-618 and miR-650 were 50% and 73%, respectively, with an overall diagnostic accuracy of 66%. The PPV of miR-618 and miR-650 for correctly identifying true HCC positive patients was 66% and the NPV for identifying true negative HCC patients was 70%.

References


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