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

M. Abdalla, Ph.D[1], M. Simkin[2], and Y. Haj-Ahmad, Ph.D[1,2]

[1]Brock University, St. Catharines, ON, Canada, [2]Norgen Biotek Corporation, Thorold, ON, Canada

Abstract

MicroRNAs (miRNA) are small endogenously expressed non-coding RNAs that negatively regulate expression of protein-coding genes at the translational level. Accumulating evidence, such as 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 miRNA signatures specific for Hepatitis C virus (HCV)-associated Hepatocellular carcinoma (HCC), miRNA expression profiling of 32 HCC post-HCV infected, 74 HCV-positive and 12 control individuals was carried out using whole genome expression profiling. Differential 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. The sensitivity and specificity of miR-618 for detecting HCC among HCV-positive individuals was found to be 64% and 68%, respectively. Whereas, the sensitivity and specificity of miR-650 were 72% and 58%, respectively. Additionally, the sensitivity and specificity for miR-618/650 in tandem were 58% and 75%, respectively. These predictive values are greatly improved compared to the traditional α-feto protein (AFP) level-based detection method. The proposed HCC miRNA signatures may therefore be of great value for the 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 persistently over many years (2). Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are the major risk factors for the development of HCC.
- The major potentially curative form of therapy is still surgical resection, however only 10% of patients are at operable stages upon disease discovery. This is mainly due to the absence of reliable tools for early diagnosis (3). Most chronically infected patients remain asymptomatic for many years and the 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 efficient (4). However, the widely used serological tumor markers for HCC, α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), have very low specificities and sensitivities.
- Therefore, the development of non-invasive biomarkers with high sensitivity and specificity 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-postitive group, HCV-postitive 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 for Liver Diseases at Menoufia University (Shebeen El-Kome, 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 12 normal individuals (Table 1). Confirmation of HCV genotype 4 presence in infected individuals was confirmed using RT-PCR.

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

	Control group	HCC post-HCV group	HCV-positive group
Number of patients	12	32	74
Age (mean ± SD)	28 ±4	50 ±8	36 ±18
HCV RNA*	-Ve	+Ve	+Ve
Sex	Male (6) Female (6)	Male (26) Female (6)	Male (50) Female (24)

Isolation and Quality Assessment of Total RNA from Urine. Total RNA was isolated from 3mL of urine using the Urine (Exfoliated cell) RNA Purification Kit (Norgen Biotek, Thorold, ON.). The isolation was performed using the manufacturer's protocol, with slight modifications. The purified miRNAs were loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to the manufacturer's instruction (Agilent Technologies).

MicroRNA Whole-Genome Expression Profiling. The differential miRNA expression profiling was performed using the miRNA expression profiling assay panel (Illumina, San Diego, CA.) at the SickKids genomic core facility (Toronto, ON, Canada). This assay is an adaptation of the proven DASL® (cDNA-mediated Annealing, Selection, Extension and Ligation) assay. Hybridization and scanning of miRNA expression bead array was performed according to the manufacturer's instructions (Illumina).

Relative Expression Profiling for Candidate miRNAs Using RT-qPCR. Relative miRNA expression levels for the 5 candidate miRNAs (miR-625, miR-532, miR-618, miR-516 and miR-650) were analyzed among the HCC post HCV-positive group, HCV-positive group and the control group. Candidate miRNAs were reversibly-transcribed using 3µl of RNA template and 0.5µl gene-specific stem-loop RT primer. For controls, minus RT reactions were also set up. The relative expression level (fold change) for each candidate miRNA within each group was calculated using the equation 2-ΔCt test / 2-ΔCt control.

Results

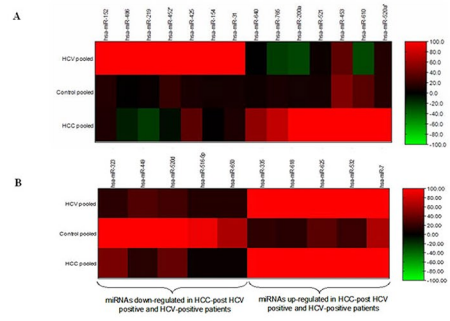

Figure 1. Heat Map of miRNA Expression in HCC-post HCV Positive, HCV Positive and Control Group. A) MiRNA up-regulated in HCC-post HCV positive and HCV positive group. B) MiRNA de-regulated in both HCC post HCV-positive and HCV-postitive group relative to the control group. The signal intensity from each miRNA tested in either the HCC or the HCV groups was normalized against its equivalent in the control group. Scanning the TargetScan and MiRanda databases (26) revealed that the commonly up-regulated miRNAs (miR-625, miR-532 and miR-618) were likely playing a significant role in the development of HCC among high risk HCV positive patients. The methyl-CpG binding domain protein 2 gene (MBD2) is likely to be targeted by miR-625. MiR-532 likely targets ligase I, DNA, and the ATP-dependent gene (LIG 1), whereas, the putative target of miR-618 was found to be the low density lipoprotein-related protein 12 (LRP12).

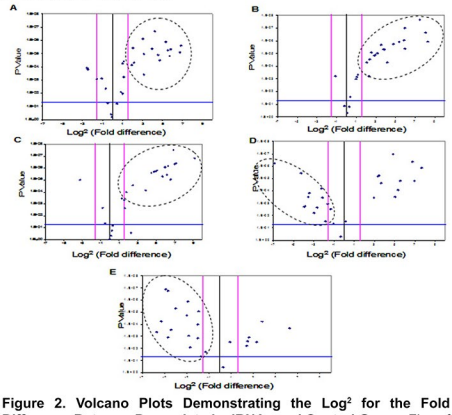

Figure 2. Volcano Plots Demonstrating the Log² for the Fold Difference Between Deregulated miRNAs and Control Group. Five of the miRNAs, three up-regulated (miR-625, miR-532 and miR-618) and two down-regulated (miR-516-5P and miR-650), whose levels were significantly different in the urine pooled from the HCC post HCV-positive group relative to the control group, were studied as potential HCC biomarker(s) since their putative targets have a role in cancer development. A Volcano Plot graphs the log² for the fold difference in A) miR-625, B) miR-532, C) miR-618 D) miR-516-5p and E) miR-650 expression versus its p value from the t-test among HCC-post HCV positive group. The black line indicates a fold-change in gene expression of 1. The pink lines indicate the desired fold change in miRNA expression threshold (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 difference ≥ 3 and $p < 0.05$.

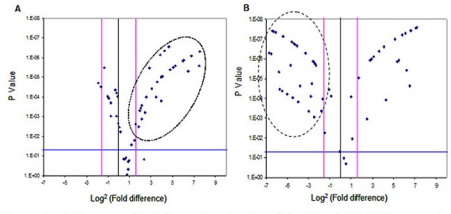

Figure 3. A Volcano Plot Graphing the Log² for the Fold Difference in A) miR-618 and B) miR-650 Expression Versus its p Value from the t-Test Among HCV Positive Group. The black line indicates a fold-change in gene expression of 1. The pink lines indicate the desired fold-change in gene expression threshold (3). 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 ≥ 3 and $p < 0.05$. Since the deregulation of expression of miR-618 and miR-650 appeared to be a characteristic event among HCC patients, with 72% of HCC-post HCV positive patients exhibiting this deregulated pattern of expression, the expression levels of miR-618 and miR-650 were examined among the 74 HCV positive patients using RT-qPCR in order to evaluate the potential of using these aberrant miRNA expression signatures as an HCC biomarker. MiR-618 was significantly up-regulated in 35 of the 74 HCV positive patients (A), while, miR-650 was significantly down-regulated in 42 of the 74 HCV positive patients (B).

Table 2. Criteria Used to Determine Sensitivity and Specificity of miRNA Biomarkers miR-618 and miR-650. To evaluate the potential of using aberrant expression of either miR-618, miR-650 or both for diagnostic purposes, the AFP levels and CT scan results of all HCV-positive patients were updated two years after initial sample collection. Using the criteria in this table, the results from each patient could be classified as true positive, true negative, false positive or false negative.

Ct Scan Result	AFP Level	Fold Change miR-618 (HCV/ Control)		Fold Change miR-650 (HCV/ Control)	Classification
Positive	≥ 400ng/L	≥ 3		≤ -3	True Positive
Negative	< 400ng/L	≥ 3	OR	≤ -3	False Positive
Negative	< 400ng/L	≤ 3		≥ -3	True Negative
Positive	≥ 400ng/L	≤ 3		≥ -3	False Negative

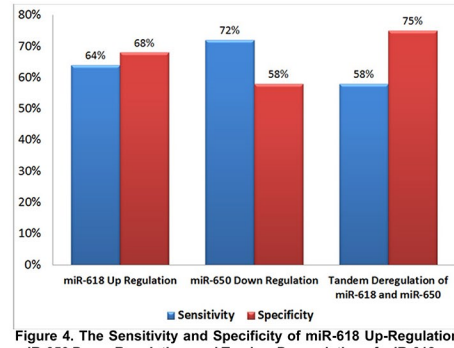

Figure 4. The Sensitivity and Specificity of miR-618 Up-Regulation, miR-650 Down-Regulation, and Tandem Deregulation of miR-618 and miR-650 for Detecting HCC Among HCV-Positive Patients, Based on AFP Levels and CT Scan Results. There was no correlation between the relative expression of miR-618 or miR-650 and AFP levels. The positive predictive value (PPV) of miR-618 for correctly identifying true HCC positive patients was 66% and the negative predicative value (NPV) for correctly identifying true negative HCC patients was 67%. Whereas, the sensitivity and specificity of miR-650 for detecting HCC among HCV-positive patients were 72% and 58%, respectively, with an overall diagnostic accuracy of 65%. The PPV of miR-650 for correctly identifying true HCC positive patients was 62% and the NPV for correctly identifying true negative HCC patients was 69%. Additionally, the sensitivity and specificity for miR-618/ miR-650 tandem were 58% and 75%, respectively, with an overall diagnostic accuracy of 69%. The PPV of miR-618/miR-650 for correctly identifying true HCC positive patients was 68% and the NPV for correctly identifying true negative HCC patients was 70%.

Conclusions

- 10 miRNAs commonly deregulated in both the HCC-post HCV group and the HCV positive group were discovered.
- miR-618 and miR-650 were chosen as potential biomarkers, based on their deregulation percentages among the HCC-post HCV and the HCV positive group. This is in accordance with their putative roles as regulators of genes involved in HCC progression and development. MiR-618 up-regulation suppresses the expression of the tumor-suppressor gene (LPR12) and miR-650 down-regulation leads to the over-expression of TARF4.
- The sensitivities and specificities of using miR-618, miR-650, or miR-618/650 in tandem were found to be greatly improved compared to the traditional α-feto protein (AFP) level-based detection method. The proposed HCC miRNA signatures may therefore be of great value for the early diagnosis of HCC, before the onset of disease in HCV-positive patients.
- This study also made use of urine as the sample source for biomarker detection, which is significant because urine is a non-invasive sample, and there is virtually no limit to sample volume when urine is used. Therefore the use of urine in screening methods is a very practical method to reduce HCC mortality rates worldwide.

References

1. Varnholt H, *et al.* MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology. 2008; 47: 1223 - 1232.
2. Steel LF, *et al.* A proteomic approach for the discovery of early detection markers of hepatocellular carcinoma. Dis Markers. 2001; 3: 179-189.
3. He QY, *et al*. Toward the proteomic identification of biomarkers for the prediction of HBV related hepatocellular carcinoma. J Cell Biochem. 2008; 3: 740-752.
4. Steel LF, *et al.* A proteomic approach for the discovery of early detection markers of hepatocellular carcinoma. Dis Markers. 2001; 3: 179-189.

NORGEN BIOTEK CORPORATION

Contact Information:
Norgen Biotek
3430 Schmon Parkway
Thorold, ON CANADA L2V 4Y6
Phone: (905) 227-8848
demerdashm@yahoo.com

Brock University