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The Urinary Genomic and Proteomic Profiling of Hepatocellular Carcinoma Patients Infected with Hepatitis C Virus

M. Abdalla[1], and Y. Haj-Ahmad[1,2]. [1]Brock University, St. Catharines, ON, CANADA, [2]Norgen Biotek Corp., Thorold, ON, CANADA

Contact Information:
3430 Schmon Parkway
Thorold, ON, CANADA
Ph: (905) 227-8848
Fax: (905) 227-1061

Abstract

One of the problems which leads to the poor prognosis of cancer treatment is the absence of a biomarker which can detect the disease in an early stage. Cancer is usually detected in a late stage after being metastasized, which makes finding the primary tumor a hassle. Hepatitis C virus (HCV), as well as Hepatitis B virus (HBV), is considered one of the major causes for the development of Hepatocellular Carcinoma (HCC). The availability of an HBV vaccine has lowered the incidence of HCC caused by HBV infection, whereas the incidence of HCC post HCV infection is in a continuous increase. The objective of this study was to investigate the protein expression patterns and apoptotic DNA levels in HCC patients which occur as a result of HCV infection.

In this study, total proteins and total DNA were isolated from 1 mL of urine isolated from a HCC-post HCV positive group and a healthy control group. Total urinary proteins showed significant differences when resolved on 1D SDS-PAGE gel. Some of the differentially over-expressed proteins among HCC-post HCV positive group compared to healthy control group were excised from the gel and identified using MS/MS analysis. The identified proteins were related to the development of HCC in response to HCV infection. Urinary DNA showed the presence of high amounts of apoptotic DNA which was positively correlated to the levels of α-feto protein (AFP). Eight tumor suppressor genes (TSGs) were studied for their aberrant methylation among the HCC post HCV positive group. The number of the aberrant hyper-methylated TSGs was found to be correlated with both AFP and the urinary apoptotic DNA. This correlation shows a direct relation between the progression of disease and the shedding of degraded DNA into the circulation caused by apoptotsis. There was no correlation between the proteins and the apoptotic DNA isolated from those patients. A multidisciplinary systems biology approach was employed in order to use urine as a biological sample for this study, since urine is acquired in a non-invasive manner. This required developing novel techniques in order to overcome certain problems inherent the isolation of protein and DNA from urine.

Introduction

- Hepatocellular carcinoma (HCC) is one of the leading causes of deaths worldwide. HCC leads to 500,000 deaths per year, worldwide (1).
- Individuals chronically infected with Hepatitis B or C virus (HBV, HCV) are at high risk for developing HCC, with disease progression occurring persistently over many years (2).
- Approximately 370 million individuals are HBV positive and 130 million HCV positive, worldwide (3).
- Currently, the radiographic imaging techniques are not practical for mass-population screening diagnosis due to its invasiveness (4).
- The widely used serological tumor markers for HCC, α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), lack specificity and sensitivity (5).
- Urine offers a great chance for the development of novel, non-invasive assays for the diagnosis, monitoring and the early detection of diseases.
- Many advantages favor using urine over blood and tissues samples for cancer biomarker discovery; urine-based tests are absolutely noninvasive; urine is noninfectious for HIV and less infectious for many other pathogens (6).
- Urine is considered a specific filtrate of blood; the protein components of urine are qualitatively similar to those of blood but quantitatively more diluted (7).
- Circulating nucleic acids in the plasma are cleared from the circulation through the kidneys, and therefore, they can be detected in urine (8).
- These nucleic acids are extremely stable because they are normally wrapped within apoptotic bodies (9), or associated with protein to form DNA-protein complexes (10) to travel through the kidneys without being degraded.
- We hypothesize that comparing the urinary proteomic and genomic profiles of patients with Hepatocellular Carcinoma-associated hepatitis C virus infection versus non-diseased individuals should yield valuable data that will help in better understanding both the mechanism of HCV infection as well as the progression of this infection to the cancerous state.

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Methods

- Urine samples were collected from the Alexandria University General Hospital and the Institute of Liver Diseases at El Monofya University (Egypt) upon the approval of the Brock University Research Ethics Board. There were 44 samples collected; 32 from patients in the HCC post-HCV group and 12 from patients in the control group.
- Total proteins were isolated from 1mL urine samples using the ProteoSpin™ Urine Protein Concentration Micro Kit (Norgen Biotek).
- Protein samples were quantified using the Bio-Rad Protein Assay. Protein samples (20µL out of each 100µL elution) were run on 15% SDS-PAGE gels for qualitative analysis.
- Total urinary cell-free DNA was isolated from 2mL urine sample using the Urine DNA Isolation Kit (Norgen Biotek).
- The urinary apoptotic nucleic acids (~150-200bp) were quantified using gel densitometry and the quantitative FastRunner DNA Ladder (Norgen Biotek). DNA samples (20µL out of each 100µL elution) were run on 2% agarose gels for qualitative and quantitative analysis.
- Methylation-sensitive restriction enzyme digestion PCR was done using two methylation sensitive and resistant restriction enzymes (DpnI and AciI, respectively) to study the aberrant methylation profile of 8 tumor-suppressor genes (Table 2). GAPDH was used as an internal control for digestion.
- MALDI MS/MS analysis of seven protein bands was analyzed on a Q-STAR XL (York University, Proteomic Facility) with an o-MALDI source (MDS Sciex/ Applied Biosystems Inc.). MS and MS/MS analyses were performed manually.

Results

A. Urine Proteins from Healthy Control Group **B. Urine Proteins from Patients with HCC Post-HCV**

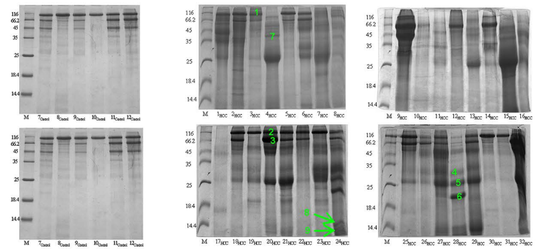

Figure 1. SDS-PAGE gels (15%) of total urinary proteins isolated from 1mL urine samples (ProteoSpin™ Urine Protein Concentration Micro Kit, Norgen Biotek) of control healthy individuals (Panel A) and individuals with HCC post HCV-positive infection (Panel B). A total of 20µL out of each 100µL elution was loaded onto a 15% SDS-PAGE gel and run at 200 V for 75 minutes. The bands which were excised for MALDI MS/MS analysis are labelled with green numbers on the gels (Panel B). Lane M is the Unstained Protein Molecular Weight Marker (Fermentas, #SM0431).

Table 1. Proteins Identified from the Excised Protein Bands Analzyed by Tandem Mass Spectrometry

Band Number	Protein Identified
1	Tamm-Horsfall protein (uromodulin) Serum Albumin
2,3	Serum Albumin
4	Complex forming glycoprotein HC
5	Immunoglobulin lambda light chain Immunoglobulin Kappa light
6	Retinol binding protein
7	Alpha-1-acid glycoprotein Serum Albumin
8	Immunoglobulin light chain
9	Ig G1 H Nie

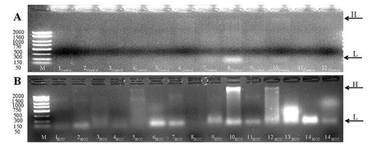

Figure 2. Agarose gels of DNA isolated using the Urine DNA Isolation Kit (Norgen Biotek) from 2mL urine samples of control healthy individuals (A) and individuals with HCC post HCV-positive infection (B). A total of 20 µL out of each 100 µL elution was loaded onto 2% agarose gels and run at 170V for 25 minutes. M is the FastRunner DNA Ladder (Norgen Biotek). Pictures were taken using AlphaImager™ 2200 from AlphaInnotech. H refers to high molecular weight DNA, while L refers to low molecular weight DNA.

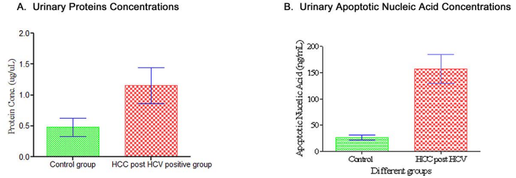

Figure 3. Histograms showing (A) the urinary protein concentrations and (B) the levels of urinary apoptotic nucleic acid (mean ± CI) isolated from the HCC-post HCV positive group and the healthy control group. For both the proteins and the nucleic acids the means were significantly different at $p < 0.05$.

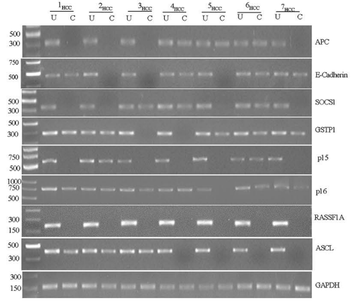

Figure 4. Representative 1.2% agarose gels showing the methylation profile of 8 tumor-suppressor genes among the HCC post HCV positive group. U=Uncut, C=Cut.

Urine Proteomic Results

- Some protein bands were consistently present in all patient and control samples
- Unique protein populations appeared or disappeared consistently within each pateint group, which could be a consequence of the viral infection or due to the tumor
- Quantitatively, total urinary protein concentrations purified from the HCC post HCV-positive group (mean of 1.153µg/µl ± 0.8) were found to be significantly higher from that purified from the control group (mean of 0.4775µg/µl ± 0.2) with a *p* value of 0.0068 (Figure 3, Panel A)
- Partial sequencing using tandem mass spectrometry identified 9 different proteins, which are listed in Table 1

Urine Genomic Results

- Two bands were identified, a high moleuclar weight gDNA (H) and a low molecular weight DNA (L) (Figure 2)
- The amount of urinary apoptotic nucleic acids present in the HCC post-HCV patients (mean of 157.26 ng per mL of urine) was found to be significantly higher than those of the control individuals (mean of 26.93 ng/mL) (Figure 3)
- Aberrant methylation profiles were found in all the 32 HCC patients with various numbers of TSGs methylated (Figure 4). The mean number of the methylated TSGs was 4.75. The majority of HCC post-HCV patients has ≥ 4 genes methylated (29/32, 90.60%)
- A minority of these HCC patients has only 3 genes (1/32, 3.13%) or 2 genes (2/32, 6.25%) methylated. The most frequently methylated TSGs were: RASSF1A (93.75%), p16 (71.87%), ASCL and GSTP1 (59.30%), APC and SOCS-1 (53.12%), which were present in more than half of the patients (data not shown)
- The rest of the TSGs, p15 and E-Cadherin, were aberrantly methylated in less than half of the patients (43.75% and 40.00%, respectively) (data not shown)

Conclusions and Significance

1. Valuable information can be obtained from analyzing the genomic and proteomic profile of urine in HCC post HCV-positive patients.
2. Albumin is the most abundant serum protein (11), and it was identified in 4 bands analyzed by MALDI MS/MS.
3. Tamm-Horsfall protein (uromodulin) is an abundant soluble urinary protein that is secreted by a nephron segment downstream from the proximal renal tubule (12), and was identified in the urine samples from the patients.
4. AGP is a member of the acute phase proteins and is considered as a natural anti-inflammatory and immuno-modulatory agent (13). Its presence in HCC post HCV-positive patients is expected as a response towards the viral infection.
5. Complex forming glycoprotein HC (protein HC) was also identified, and it has been reported that this protein is likely to be a normal constituent of human urine (14).
6. The rest of the proteins identified were immunoglobulins. Their abundance in the urine of HCC post HCV-positive patients is likely to be as an immune response against the HCV infection.
7. The high levels of urinary apoptotic nucleic acids is likely to be a characteristic feature among HCC post HCV-positive patients.
8. The isolated urinary DNA was of a very good quality and quantity, which enabled us to study the methylation profiles of 8 different TSGs using the sensitive Methylation-sensitive restriction enzyme digestion PCR.
9. Aberrant CpG island methylation is likely to be a characteristic feature among HCC post HCV-positive patients.
10. This work proved that urine can be efficiently used as a non invasive sample source in studying the genomic and proteomic profiles of HCC.

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