ABSTRACT

A major obstacle to the research on the development of drug and gene-based therapies for HBV infections has been the lack of an efficient cell culture system or a readily available small-animal model, permissive for viral infection and replication. Lack of a robust in vitro cell culture system has seriously hampered the progress of HBV research. Development of high throughput proteomics approach provides a new tool to study the pathogenesis of HBV and identification by molecular markers. Proteomics has been used to characterize the molecular events occurring in various disease processes. In this study the two dimensional electrophoresis (2-DE) followed by mass spectrometry (MS) analysis approach was used for identification and comparative expression profiles of protein level that may be associated with HBV infection. The Mascot analysis identified the proteins as envelope glycoprotein and Non structural protein 5 which were highly unregulated after viral infection. Such approaches are expected to establish the molecular definition of the HBV infection and contribute to the discovery of diagnostic markers and therapeutic targets.

Keywords: HBV, Hela, 2D electrophoresis, MALDI-TOF-MS.

INTRODUCTION

Hepatitis B virus (HBV) is the most common infection with approximately one third of the world population has been infected with this infection.\(^1\) Chronic HBV infection is common by an estimate in 350 million persons globally, and carriers of HBV are at increasing risk of developing cirrhosis, hepatic decomposition and hepatocellular carcinoma.\(^2\) The evolution of HBV has led to the present existence of various genotypes, sub genotypes, mutants, recombinants, and even quasispecies of HBV.\(^3\) At present HBV can be classified into 9 genotypes from A to I.\(^4, 5\) based on a nucleotide divergence in the entire genome of at least
8%, with specific and characteristic geographical distributions. Genotype B predominates in Asia (China, Indonesia and Vietnam).\[6\]

The treatment of HBV can promote the appearance of quasispecies.\[7\] For example, the use of nucleoside-analogs can cause drug resistant polymerase mutations that can also affect the overlapping HBsAg reading frame.\[8\] Another problem is the appearance of HBV escape mutants that are resistant to nucleotide-analogs after long term treatment. Furthermore, it is described that selective pressure due to HBV vaccination can cause HBsAg mutations that escape the neutralization by vaccine-induced antibodies.\[9\] Currently, chronic HBV infections are treated with pegylated interferon-alpha in combination with nucleoside/nucleotide analogs.\[10\] In the majority of cases this treatment suppresses the viral replication successfully, but there is a very low cure rate for chronic HBV infection; however, HBV remains a major health problem with an estimated 400 million chronic infections worldwide.\[11\] This emphasizes the need for additional therapy strategies to increase the success rate of the current chronic HBV treatments.

Only a few infection models are available to study HBV infection because HBV is very tissue and species specific.\[12\] Infection of primary human hepatocytes would be the most appropriate model but human liver tissue is only seldom available and the preparation of a sufficient amount of susceptible hepatocytes from a tissue sample is difficult. For reasons that are not clear, infection of primary hepatocytes and established cell lines with hepatitis viruses has produced poor viral replication and low viral yields and has suffered from poor reproducibility.\[13\] There is already some previous proteomic analysis of HBV-related hepatocarcinogenesis still needs to be further clarified. The aim of the present study was to carry out a differential profiling of proteins after HBV infection using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The results presented here are expected to obtain some clues to further study the carcinogenic mechanisms, or identify some possible molecular markers for HBV.

**MATERIALS AND METHODS**

**Cell line**
HeLa is cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% of Fetal Bovine Serum (FBS). The pH of culture media is maintained at 7.2 ± 0.2 and incubated in 5% CO\(_2\) at 37°C until the cell become confluent in the culture flask and subsequently sub-cultured as required.
Infection & Harvesting

After 24hrs of incubation the cells were observed for 80% confluence. Discard the media from the flask and add 2ml of fresh media, add 200µl of the infected Serum sample to the flask, keeping one as the control. Mix the media gently without disturbing the cells. Centrifuge the flask in a swing rotor at 2500 rpm for 30 minutes at 25°C (in sterile condition). After centrifugation immediately transfer the flask to the incubator for two hours. After discard the media from the flask and add 5ml of fresh media incubate for 24hrs. Observe the cells for infection under a microscope (infected cells shows granulation). If cells are floating, carefully suspend them in the media, centrifuge them at 1000 rpm and use the pellet for further studies. If the cells are adhered, discard the media, rinse with PBS and use a rubber policeman (cell scraper) to collect the cells.

Protein extraction

The cells were grinded with Liquid Nitrogen using Mortar pestle. 20 µL of protein extraction buffer (50 mM Tris-Hcl, 2% SDS, 10mM DTT, 10% Glycerol) was added per mg of sample. Ensured the tubes are closed properly. Centrifuged for 1 min at 10,000 rpm and proteins was precipitated with ice-cold acetone. The Precipitate was centrifuged at 12,000rpm at 4°C for 10 minutes. Acetone was removed by evaporation and the protein was dissolved in 2% SDS solution and storied at -20°C. The Protein quantity was estimated by Bradford assay.

Two-dimensional Electrophoresis

A total of 100 µg of protein was used for 2D electrophoresis. The first dimensional electrophoresis (isoelectric focusing) was carried out with a Protein Isoelectric Focusing Unit according to the manufacturer’s instructions. The second dimensional electrophoresis was conducted on 12% polyacrylamide gel. The second electrophoresis running conditions were as follows: constant 16 mA for 30 min at 6°C followed by constant 30 mA per gel until the BPB dye reached the bottom of the gel. The 2- D gels were stained with Comassie Brilliant Blue dye. The gel was then destained in a destaining solution for a few times until protein bands were visualized. The molecular weight of protein bands were determined by comparing them with the molecular weight markers.

Extraction of protein and MALDI-TOF-MS analysis

Protein spots were excised from the gel and a control piece of gel was cut from a blank region of the gel and processed in parallel with the sample. The gel pieces washed with 500 µL of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and incubated at room
temperature for 15 min and repeated twice. Dehydrated the gel pieces in 100% acetonitrile for 5 min, removed the acetonitrile with a pipette and then completely dry gel pieces at room temperature for 10-20 min. Rehydrated gel pieces in 150 µL reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 56 ºC. Then 100 µL alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) and incubate for 30 min in the dark at room temperature and washed with 500 µL of wash solution. Dehydrated gel pieces in 100 µL 100% acetonitrile for 5 min and completely dried. Rehydrated the gel with 20 µL of protease digestion solution (20 µg/mL). Digested overnight at 37 ºC. Centrifuged and to the supernatant 50 µL of extraction solution (60% acetonitrile, 0.1% TFA) was added and sonicated for 10 min. Dried the extracted peptides by centrifugal evaporation to near dryness. Added 5 µL of resuspension solution (50% acetonitrile, 0.1% TFA) and sonicated for 10 min. Centrifuged and 0.5 µL of sample was placed on MALDI plate followed by 0.5 µL of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% acetonitrile, 0.1% TFA). Allowed the spots to dry completely and loaded the plate into Voyager.

Database analysis
Proteins were identified with peptide mass fingerprinting data using Mascot (http://www.matrix science.com). Mascot Distiller was used to detect peaks to fit an ideal isotopic distribution to the experimental data.

RESULT AND DISCUSSION
Viral proteomic analysis requires a highly purified preparation of virions. There was no available permissive cell line capable of supporting productive replication of HBV. For this study we used HeLa cell lines instead of Hepatic cell lines (HepaRG), and studied the interaction and proteomic changes during the infection. After incubation with the serum the cell lines shows some morphological changes as shown in the fig. 1.

![Figure 1](image_url)

Figure 1. The treatment of HBV positive serum to HeLa cell lines (a) control cell line,
(b) HBV serum treated cell lines

The cells had multiplied in normal manner during the first week, but later large number of cells were lysed or detached. To investigate the alteration of the protein expression in HeLa after treatment in vitro, protein lysates from the control and serum treated HeLa cells were subjected to 2-D electrophoresis (Fig. 2).

![Figure 2. The 2-D Electrophoresis map indicated protein spots changed in volume after serum treatment in HeLa cells (a) Control cell lines, (b) treated cell lines](image)

Following staining, the gels were digitized using digital camera and analyzed the spots. We found that the intensity of more than 7-9 protein spots was altered after serum treatment. Amongst them two most brighter bands were cut and eluted from the gel for further analysis and identification by MALDI-TOF-MS analysis. The peptide mass fingerprinting (PMF) maps were shown in figure 3 and 4 for spot 1 and 2 respectively. The PMF data were used to search the SWISS-PROT database with Mascot software. The corresponding Mr value was considered for identification of proteins and the Mascot score for the matched protein was showed in figure 3 and 4.

![Figure 3. MALDI-TOF-MS analysis of spot 1 and Mascot score.](image)
CONCLUSION
The Mascot analysis identified the proteins as envelope glycoprotein and Non structural protein 5 which were highly unregulated after viral infection. The envelope proteins are known to mediate the host antiviral response during viral infection. Additional experiments will be required in order to further understand signaling mechanism involved in up regulation of the envelope glycoproteins.

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REFERENCES


