

# Differential Expression of Proteins Associated with Chronic Hepatitis B Virus Infection on HeLa cell lines

Arjunan S<sup>1</sup>, Abdulmuhsen AM<sup>2</sup>, Sudhakar M<sup>3</sup> and Sibi G<sup>\*3</sup>

<sup>1</sup>Department of Life Sciences, Kristu Jayanti College (Autonomous), Bangalore, India

<sup>2</sup>Department of Microbiology, Indian Academy Degree College-Autonomous, Bangalore, India

<sup>3</sup>Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore, India

\*Corresponding author: Sibi G, Ph.D, Head, Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore, India, Tel: +91-9986452875, E-mail: gsibii@gmail.com

**Citation:** Arjunan S, Abdulmuhsen AM, Sudhakar M, Sibi G (2018) Differential Expression of Proteins Associated with Chronic Hepatitis B Virus Infection on HeLa cell lines. *J Proteo Genomics* 2(1): 101

**Received Date:** July 27, 2018 **Accepted Date:** December 12, 2018 **Published Date:** December 14, 2018

## Abstract

Treatment of Hepatitis B infection is available with the help of existing drugs but the eradication of HBV infection is still under pipeline. The application of novel drugs and gene based therapies for HBV infection is need of the hour however the lack of efficient cell culture system, animal models for viral infection and replication acts as a major obstacle for novel therapies. This not only hampers the progress of HBV research but also stress the need for effective forthcoming therapeutics for Hepatitis B infection. Proteomic studies to understand the protein ubiquitination and to find molecular markers are the effective tools to study the HBV infection, pathogenesis and its control. This study involved the two dimensional gel electrophoresis of proteins followed by their analysis by mass spectrometry for identification and comparative protein expression profiles associated with Hepatitis B virus (HBV) infection. The Mascot analyses were applied which has identified the alteration of protein expression after viral infection using HeLa cell lines. Identification of highly unregulated proteins due to viral infection would help to understand the molecular mechanisms of HBV infection thus helps to discover novel diagnostic markers and therapeutic targets for eradication of HBV infection.

**Keywords:** Hepatitis B Virus; HeLa cells; HBS Ag; Proteomics; Non-Structural Proteins

## Introduction

Hepatitis B virus infection that leads to serious health conditions such as liver cirrhosis and hepatocellular carcinoma is a potentially life-threatening global health problem [1]. Significant human populations (~400 million) are chronically infected worldwide with Hepatitis B virus (HBV). Major factors liable to HBV infection are age of a person at the time of infection, virus (genome, HBV mutants, replication stage of HBV), and host nature (age, sex, and immunotolerance) [2-4]. The exogenous factors responsible for HBV infection are concurrent infection with other strains of hepatotropic viruses and alcohol consumption. The clinical spectrum of chronic Hepatitis B infection involves three phases namely subclinical, acute and chronic that has been identified as immune tolerant phase, active phase and inactive carrier phase. Chronic Hepatitis with elevated alanine aminotransferase accompanied by liver cirrhosis/fibrosis and liver inflammation is a major illness of HBV infection [2,3,5]. People (15-40%) with chronic Hepatitis B infection would progress to liver cirrhosis and other major liver diseases at later stages [6].

Defining the progress level that lead to chronic hepatitis remains challenging and this was owing to the factors including the inactive course of the disease, asymptomatic during the early stages, and heterogeneousness of the disease [7,8]. Recently, it was reported that some HBsAg negative individuals may develop chronic hepatitis B infection that can be detectable using molecular tests [9]. This could be due to the mutation that has occurred in the pre core structure of the Hepatitis B Virus through which the C gene is unable to produce HBsAg however producing HBcAg as an alternative. It was reported that this mutation occurs mostly in Asia and East European countries [10]. As a result, analyzing the diagnosis of HBV DNA in chronic carriers is required with new molecular techniques when compared to the ELISA which qualitatively and quantitatively detects HBsAg negative sera. Thus, the major aim of the present study was to evaluate the viral DNA in biological fluid of patients with chronic Hepatitis compared to a control group using the quantitative PCR and protein profiling by 2D gel electrophoresis and mass spectrometry.

## Materials and Methods

### Cell Line

HeLa cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). The pH of the culture medium was maintained at  $7.2 \pm 0.2$  and incubated in a humidified 5% CO<sub>2</sub> incubator at 37 °C until the cell become confluent in the culture flask and subsequently sub cultured as required.

### Infection and Harvesting

After 24 hours of incubation, the cells were observed for 80% confluence. The media was discarded from the flask and added 2 ml of fresh media followed by 200 µl of HBV infected serum sample. Without disturbing the cells, the media was centrifuged at 2500 rpm for 30 minutes at 25 °C under sterile conditions. This was followed by incubating the flask immediately in the incubator for 2 hours. After incubation, the media was discarded and added 5 ml of fresh DMEM media followed by incubation for a period of 24 hours. The cells were observed for infection under a phase contrast microscope (cells with granulation). The floating cells were carefully suspended in the media, centrifuged at 1000 rpm and the pellet was used for further studies. In the case of attached cells, the media was discarded, rinsed with phosphate buffered saline and the cells were collected by using a cell scraper.

### Protein Extraction

The total proteins were extracted by following the conventional sample preparation method the infected cells were homogenized with liquid nitrogen using mortar and pestle. 20µl of cell lysis buffer (50 mM Tris-HCl, 8 M urea, 65 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1% Triton X-100) was added per mg of sample. The mixture was centrifuged at 10,000 rpm for 10 mins at 4 °C and the proteins were precipitated with ice-cold protein precipitation solution (ethanol/acetone/acetic acid = 50:50:0.1). The precipitate was suspended in ice cold acetone and 75% ethanol and again centrifuged at 12,000 rpm at 4 °C for 10 minutes. Acetone and ethanol were removed from the precipitate by evaporation and the proteins were redissolved in 50mM Tris HCl containing 8M urea and stored at -20 °C [11]. The total proteins were estimated by Bradford assay.

### Two-dimensional Electrophoresis

A total of 100 µg of precipitated protein was dissolved 80 µl of rehydration buffer (0.5 M Tris Cl, 8 M urea, 0.5% Triton X-100, 2mM dithiothreitol, 0.2% Ampholine). The first dimensional electrophoresis was performed in a Protein Isoelectric Focusing Unit according to the manufacturer's instructions (Invitrogen). The second dimensional electrophoresis was conducted on 12% polyacrylamide gel. The second electrophoresis running conditions involved constant 16mA for 30 min at 6 °C followed by constant 30mA per gel until the BPB dye reached the bottom of the gel. The 2-D gels were stained with Coomassie Brilliant Blue dye and then destained in a destaining solution until the protein bands were visualized. The molecular weight of separated protein bands were determined by comparing them with the molecular weight markers.

### Extraction of Protein and MALDI-TOF-MS Analysis

The protein spots were excised from the gel (sample) along with a control piece of gel from a blank region of the gel and processed in parallel with the sample. The gel pieces were washed with 500 µL of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and incubated at room temperature for 15 min and the procedure was repeated twice. This was followed by dehydrating the gel pieces in 100% acetonitrile for 5 mins and drying at room temperature for 10-20 mins. The gel pieces were then rehydrated in 150 µl reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 56 °C. This is followed by adding 100 µL of alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate), incubated for 30 min in the absence of light at room temperature and washed with 500 µL of wash solution. The gel pieces were dehydrated in 100 µL 100% acetonitrile for 5 min and completely dried.

This was followed by rehydrating the gel with 20 µL of protease digestion solution (20µg/mL) and digested overnight at 37°C. In the next step, the solution was centrifuged and to the supernatant, 50 µL of extraction solution (60% acetonitrile, 0.1% TFA) was added and sonicated for 10 min. The extracted peptides were dried by centrifugal evaporation to near dryness and added 5 µL of resuspension solution (50% acetonitrile, 0.1% TFA) and sonicated for 10 min. Centrifugation was carried out 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). The spots were allowed to dry completely and plate was loaded into Voyager.

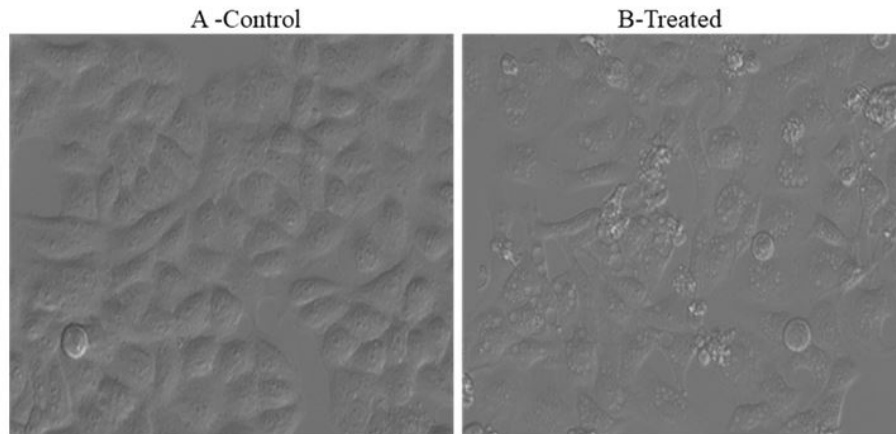
### Protein Database Analysis

The proteins were identified with peptide mass fingerprinting data using Mascot (<http://www.matrixscience.com>). Mascot Distiller was used to detect peaks to fit an ideal isotopic distribution to the experimental data

### Results

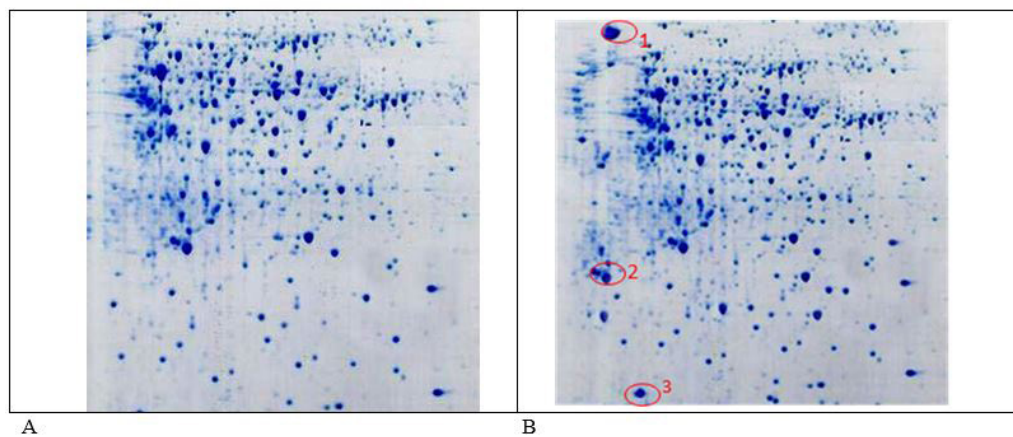
Proteomic analysis of virus requires a highly purified preparation of its virions. The lack of permissive cell lines supporting the

replication of Hepatitis B virus urged this study to use HeLa cell lines instead of Hepatic cell lines (HepaRG) to study the interaction and proteomic changes during the infection. After incubation with the serum, the cell lines showed some morphological changes as shown in (Figure 1).



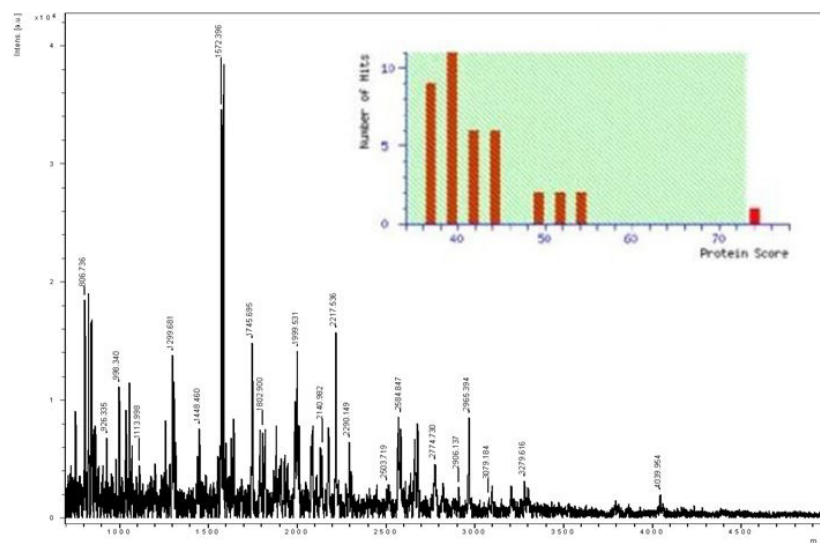
**Figure 1:** Changes in the protein spots under 2-D Electrophoresis (a) Control cell lines; (b) treated cell lines

The cells were multiplied in normal manner during the first week of incubation however large number of cells were lysed or detached later. To investigate the alteration of the protein expression in HeLa cells infected with HBV *in vitro*, protein lysates from the control and serum treated HeLa cells were subjected to 2-D electrophoresis (Figure 2a and b).



**Figure 2:** Changes in the protein spots under 2-D Electrophoresis (a) Control cell lines; (b) treated cell lines

Following staining, the gels were digitized using digital camera and the spots were analyzed. It was found that the intensity of around 7-9 protein spots were altered after HBV serum treatment. Amongst them, three brighter bands were cut and eluted from



**Figure 3:** MALDI-TOF-MS analysis of spot 1 and its Mascot score

the gel for further analysis and identification by MALDI-TOF-MS analysis. The peptide mass fingerprinting (PMF) maps were shown in figure 3, 4 and 5 for spot 1, 2 and 3 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 shown in (Figure 3,4 and 5). The Mascot analysis had identified the proteins as envelope glycoprotein, Non-structural protein 3 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 mechanisms involved in the up regulation of these envelope glycoprotein and Non-Structural Proteins.

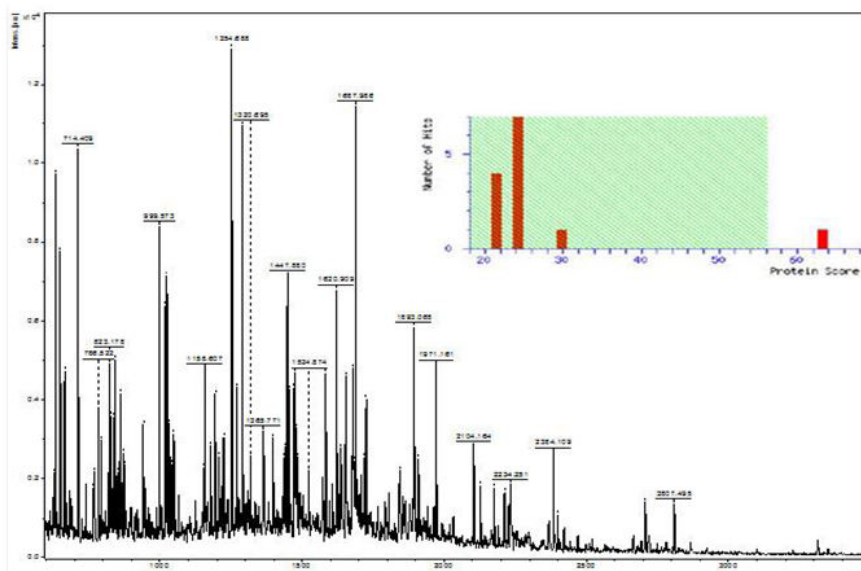


Figure 4: MALDI-TOF-MS analysis of spot 2 and its Mascot Score

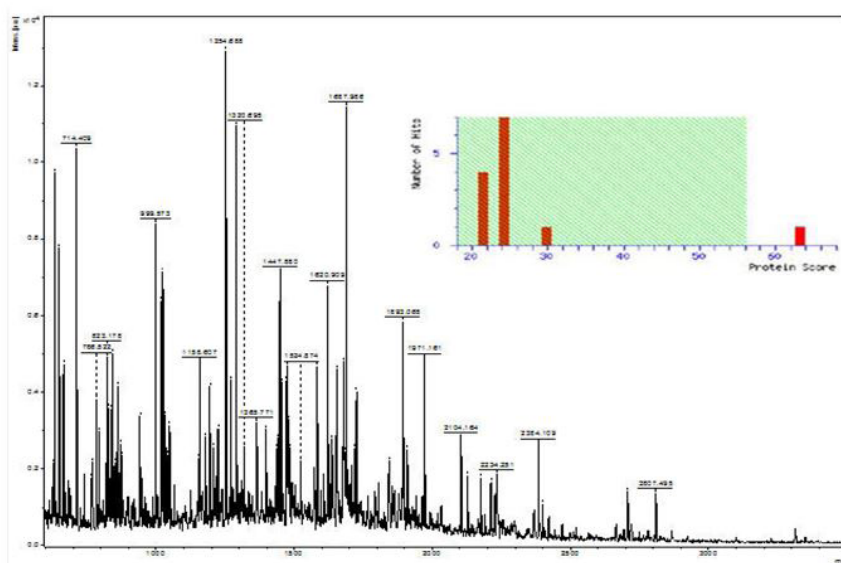


Figure 5: MALDI-TOF-MS analysis of spot 3 and its Mascot Score

## Discussion

Understanding the pathogenetic nature of HBV infections would help to discover novel drugs for viral infection but the lack of cell lines supporting the HBV replication slow down the studies. Identification of differential expression of proteins associated with HBV infection through proteomic analysis is a promising approach to investigate viral infections. Such findings will identify targets for new therapeutic drugs and vaccines for chronic viral infections. In this study, differential expression of proteins in HeLa cell lines infected with HBV serum was identified. The selection of HeLa cells was based on the previous report that HBV infected HeLa cells exhibited cytopathic effects similar to transfection in hepatocytes [12]. In another study, liposome mediated binding of HBS antigen to HeLa cell surface was confirmed by immunoelectron microscopy [13]. Infection of HeLa cells by HBV recombinant poliviruses is reported previously [14]. Similarly, successful propagation of HCV replicons was reported by Zhu *et al.* Hence, there are evidences that use of HeLa cells to study the upregulation of proteins by Hepatitis infection [15]. In this study, the phenomenon was supported by cytopathic changes in HeLa cells exposed to HBV by formation of enlarged and detached cells



when observed under microscope. Though immuno staining is one of the effective techniques to confirm the HBV infection of cell cultures, this study considered the upregulation of proteins in infected cell lines as strong evidence to support the investigation.

Comber et al., had studied the HBV protein expression in infected HepG2 and DE19 cell lines and identified that MHC-I restricted epitopes derived from HBV proteins were activating T cells. The findings revealed the use of these epitopes to develop therapeutic vaccine against HBV infection [16]. In another study, HepaRG cell lines were described as tool to understand the Hepatitis B viral replication thereby allowing researchers to develop antiviral drugs (Gripon *et al*) [17]. The findings of this study is correlating with the earlier reports in which in vitro infection of HepG2 cells with HBV resulted in the secretion of three envelope antigens (Mabit *et al.*) [18]. The identification of envelope glycoprotein through Mascot analysis strongly supports the earlier findings that HBV infection leads to expression/secretion of envelope antigens in cell lines. The expression of other proteins upon HBV infection is documented earlier where increased expression of ERp57 in HBV-related hepatocellular carcinoma was observed [19]. The differential expression of proteins in HBV transfected HepG2.2.15 was studied by She et al. It was found that 133 unique proteins were differentially expressed with Ectonucleotide pyrophosphatase ENPP2 as the most significantly up-regulated protein upon HBV replication [20].

## Conclusion

In conclusion, the findings not only yield evidence that the expression of specific proteins in HeLa cell lines during viral infection/replication but also proved the application of appropriate cell line supporting the HBV replication. The scope of the work involves the identification of specific proteins that are up-regulated during HBV replication thus would provide insights into finding of factors/pathways to develop new therapeutic drugs against chronic HBV infections.

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