รูปแบบการแสดงออกของโปรตีนในซีรัมของผู้ป่วยโรคไวรัสตับอักเสบบีชนิดเรื้อรัง ก่อนและหลังรับการรักษาด้วยยา PEG-Interferon alfa-2b

นางสาวสุนิดา เกื้อกาญจน์

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SERUM PROTEOMIC PROFILE, PRE- AND POST-TREATEMNT, IN CHRONIC HEPATITIS B PATIENTS TREATED BY PEG-INTERFERON ALFA-2b

Miss Sunida Kuakarn

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สุนิดา เกื้อกาญจน์ : รูปแบบการแสดงออกของโปรตีนในซีรัมของผู้ป่วยโรคไวรัสตับอักเสบบีชนิดเรื้อรัง ก่อนและหลังรับการรักษาด้วยยา PEG-Interferon alfa-2b. (SERUM PROTEOMIC PROFILE, PRE- AND POST-TREATEMNT, IN CHRONIC HEPATITIS B PATIENTS TREATED BY PEG-INTERFERON ALFA-2b) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.พญ.ดร.ณัฏฐิยา หิรัญกาญจน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.นพ.พิสิฐ ตั้งกิจวานิชย์, 90 หน้า.

การติดเชื้อไวรัสตับอักเสบบีเรื้อรังอาจจะพัฒนาไปเป็นผังผืดในตับโรคตับแข็งและมะเร็งตับ และเป็นปัญหาทางด้านสาธารณสุขของประชาชนทั่วโลก วิธีการรักษาผู้ป่วยติดเชื้อไวรัสตับอักเสบบีเรื้อรังคือการรักษาด้วยภูมิคุ้มกันเช่น ยา PEG-Interferon alfa-2b แต่มีเพียง 30 %ของผู้ป่วยที่ตอบสนองต่อการรักษา คณะผู้วิจัยจึงใช้วิธีทางโปรติโอมิกมาศึกษาโปรตีนในซีรัมของผู้ป่วยติดเชื้อไวรัสตับอักเสบบีเรื้อรัง 19 คนที่ได้รับการรักษาด้วยยา PEG-Interferon alfa-2b เพื่อทำนายการตอบสนองต่อการรักษา ผู้ป่วยกลุ่มนี้ได้รับการติดตามผลการรักษาเป็นเวลา 3 ปีเพื่อจัดกลุ่มว่าเป็นกลุ่มที่ตอบสนองต่อการรักษา(n=9) หรือกลุ่มที่ไม่ตอบสนองต่อการรักษา (n=10) วิธีการศึกษาได้ทำโดยการใช้วิธีทูดีเจลและแมสสเปกโตรเมตรีมาศึกษาโปรติโอมิกในผู้ป่วยทั้งสองกลุ่มก่อนรักษา หลังการรักษาด้วยยาเพคอินเตอฟีรอนเป็นเวลา 24 สัปดาห์และ 48 สัปดาห์ จากการวิเคราะห์ความเข้มของจุดโปรตีนของคนไข้ทั้งสองกลุ่มพบว่า มีการแสดงออกของ 7 จุดโปรตีนที่แตกต่างก่อนได้รับการรักษา เมื่อติดตามการรักษาไปถึง 24 สัปดาห์พบว่ามีการเปลี่ยนแปลงอย่างมีนัยสำคัญของ 13 จุดโปรตีนในกลุ่มที่ตอบสนองต่อการรักษา และมีการเปลี่ยนแปลงอย่างมีนัยสำคัญของ 6 จุดโปรตีนในกลุ่มที่ไม่ตอบสนองต่อการรักษา โปรตีนที่สำคัญเหล่านี้ได้แก่ CD5 antigen-like precursor, α-2-HS-glycoprotein และ Apolipoprotein A-I หน้าที่ของโปรตีนเหล่านี้ได้แก่ การป้องกันทางภูมิคุ้มกัน เมตาบอลิซึมของไขมัน และโปรตีนระยะเฉียบพลัน

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SUNIDA KUAKARN : SERUM PROTEOMIC PROFILE, PRE- AND POST-TREATEMNT, IN CHRONIC HEPATITIS B PATIENTS TREATED BY PEG-INTERFERON ALFA-2b. ADVISOR : ASSOC.PROF.NATTIYA HIRANKARN, MD,Ph.D., CO-ADVISOR : ASSOC.PROF.PISIT TANGKIJVANICH, MD,Ph.D., 90 pp.

Chronic hepatitis B (CHB) virus infection may develop to liver fibrosis, cirrhosis, and hepatocellular carcinoma, and the disease remains a public health problem worldwide. Immunomodulatory therapies such as pegylated interferon is a major approach for the treatment of CHB patients but only 30% of them response to IFN type I treatment. Proteomic approach was employed to analyze the serum proteome in CHB patients receiving PEG-Interferon alfa-2b to predict treatment response. Nineteen patients with HBeAg + CHB were included. These patients were followed up for more than 3 years after treatment and were defined as sustained responders (n=9) and non responders (n=10). The 2-DE and MS/MS analysis were performed to compare the serum proteome before, after initial PEG-Interferon alfa-2b treatment for 24 weeks and 48 weeks. Quantitative intensity of 2-D gel from patients of the two groups before treatment showed 7 differentially expressed proteins. In addition, 13 protein spots were found to significantly changed in sustained responders, and 6 protein spots significantly changed at the end of the 24-week in nonresponders. These proteins are including CD5 antigen-like precursor, α-2-HS-glycoprotein and Apolipoprotein A-I. Functional characterizations of all proteins with respect to immunity protection, lipid metabolism, and acute phase protein.

Department : Microbiology Student's Signature .....
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ABBREVIATIONS

HBV	=	Hepatitis B Virus
HCV	=	Hepatitis C Virus
HBsAg	=	Hepatitis B surface antigen
HBeAg	=	Hepatitis e antigen
DNA	=	Deoxynucleic acid
RNA	=	Ribonucleic acid
cccDNA	=	Covalently closed circular Deoxynucleic acid
mRNA	=	messenger Ribonucleic acid
HCC	=	Hepatocellular carcinoma
PEG-IFN	=	pegylated interferon
NAs	=	Nucleoside or Nucleotide analogs
IRF	=	Interferon response factor
ISGF	=	Interferon stimulated gene factors
MHC	=	Major Histocompatibility
IL-12	=	Interleukin -12
DC	=	Dendritic cell
ALT	=	Alanine aminotransferase
HAI	=	Histological activity index
PCR	=	Polymerase Chain Reaction
HLA	=	Human leukocyte antigen
2-DE	=	Two dimension gel electrophoresis
HSP 27	=	Heat shock protein 27
SELDI-TOF	=	Surface-enhanced laser desorption/ionization time of flight
SELDI-MS	=	Surface-enhanced laser desorption/ionization mass

		spectrometry
LC/MS/MS	=	Liquid chromatography/temdem mass spectrometry
DIGE	=	Differential in-gel electrophoresis
ESI	=	electrospray ionization
ApoA1	=	Apo lipoprotein A1
nm	=	Nanometer
kb	=	Kilobase pair
IU	=	International unit
kDA	=	kilo Dalton
mM	=	Millimolar
cm	=	Centimeter
µg	=	Microgram
µL	=	Microliter
DTT	=	Dithiothreitol
IAA	=	iodoacetamide
SDS	=	sodium dodecyl sulfate
NR	=	Non responder
SVR	=	Sustained responder
ER	=	Endoplasmic Reticulum
CHB	=	Chronic hepatitis B
ORFs	=	open reading frames
IgG	=	immunoglobulin G
IgM	=	immunoglobulin M
MW	=	molecular weight

CHAPTER I

INTRODUCTION

One of the main community health troubles worldwide is hepatitis B virus (HBV) infection and it leads to liver fibrosis, liver cirrhosis, and liver cancer as we know hepatocellular carcinoma. Two major approaches for chronic hepatitis B treatment exist which are 1) immunomodulatory therapies for example pegylated interferon (PEG-interferon) and interferon-α 2) and antiviral drugs such as nucleoside or nucleotide analogs (NAs). NAs are inhibiting of viral replication process by inhibiting DNA polymerase enzyme(1) Immune-modulating therapy includes mainly pegylated Interferon-α and Interferon-α. The advantages of type 1 interferons include a fixed time of therapy, permanent off- treatment response, and no viral drug resistant(2) However, only 30% of the chronic HBV patients respond to IFN type I treatment(2, 3) Both viral and host factors are likely 2 key factors in determining treatment responses. For example, viral factors such as HBV DNA level, HBV genotype, HBsAg and HBeAg level, and HBV viral mutants(4, 5) Host factors for predicting a sustained virologic response in CHB patients with HBeAg-positive such as lower level of viral HBV DNA, higher level of alanine aminotransferase (ALT), older age, womankind, and lack of previous IFN therapy(6) Furthermore, host factors for predicting a sustained virologic response in HBeAg-negative patients such as youthfulness, womankind, higher level of alanine aminotransferase, lower level of viral HBV DNA(7) Host factors including HLA class II (HLA-DRB1*14 allele), polymorphisms of A(MxA)-88, level of interleukin-10, and the level of interleukin-12 have been published to help predicting treatment response(8-10) The biomarker that can predict treatment responses will be very useful and cost-effectiveness for the chronic hepatitis B (CHB) patients treatment.

Many researchers have been used proteomic technologies to study specific biomarkers in liver diseases e.g., for hepatocellular carcinoma (HCC)(11-13), HBV virus inflammation, HBV liver cirrhosis(14, 15), and hepatitis C virus (HCV) treatment responses(16) However, there is limited report of proteomic analyses in predicting

treatment responses in chronic hepatitis B patients. In this study, we used 2 dimension gel electrophoresis and mass spectrometry to study and observe the serum proteome changes in chronic hepatitis B patients who are sustained responders and non responders after PEG-Interferon alfa-2b treatment to predict treatment response.

Research objectives

1. To compare the profile of serum proteome in who are sustained

responders and non responders after PEG-Interferon alfa-2b treatment.

2. To assess the serum proteome in patients before treatment, after first PEG-Interferon alfa-2b treatment for 24 weeks and 48 weeks.

Hypothesis

Differential serum proteome profile in serum of CHB patients according to treatment by PEG-Interferon alfa-2b can predict treatment response.

Benefit

1. The discovery of biomarker from this study would help identify the patients who will benefit from the PEG-Interferon alfa-2b treatment in CHB patients.
2. The identification of different proteins after treatment between responder and non-responder group might help identify important protein as new target for treatment.

CHAPTER II

LITERATURE REVIEW

HEPATITIS B VIRUS

VIROLOGY OF HEPATITIS B VIRUS

One of the important virus in the family Hepadnaviridae is Hepatitis B virus. It is DNA virus and it is the member of genus orthohepadnavirus. Hepadnaviruses are noncytopathic hepatotropic virus, which have a strong favorite target cells in the liver cells. Their viral DNA can be found in the liver cells, but small numbers of their DNA can be detected in extrahepatic organs such as pancreas, kidney, and mononuclear cells(17)

Hepatitis B virus (HBV) virions are two-layered particles, their diameter is measured 40 to 42 nm. The HBV envelope is lipoprotein. It contains three related envelope surface antigens (small, middle and large HBV surface antigens). In the envelope, it contains of viral nucleocapsid or core. In the viral nucleocapsid is containing of the viral genomic DNA, which is relaxed-circular DNA length 3200 base pairs, and in the viral nucleocapsid also found DNA polymerase enzyme that is used for the viral DNA production(17)

The genome of HBV virus has four ORFs (open reading frames), which are the genetic codes of HBV. The preS–S (presurface–surface) region of the HBV genome has genetic codes for producing three HBV surface antigens (HBsAg) (small, middle, and large HBV surface antigens). The major protein is small HBsAg, which has 24-kD. Amino acid 121-149 of small HBsAg is major antigenic determinant or 'a' determinant, which is the antigen that stimulates host immune response to produce protective antibody against HBV. The middle protein is M (or preS2) protein, which is produced from pre-S2 and S gene. M protein may function as viral attachment on hepatocytes surface. The pre-S1, pre-S2 and S gene region have genetic codes for producing the large HBV surface protein or L protein or preS1 protein. The function of L protein is the

penetration to host-cells. The preC–C (precore–core) has genetic codes for producing hepatitis B core antigen (core protein-HBcAg) and hepatitis B e antigen (HBeAg). The core protein is the structural protein which is a part of the viral capsid. The core protein protects the HBV DNA from exogenous nuclease. It is the immune target of cellular immune response. HBeAg (16-kD fragment) is not the structural-protein of HBV, it is secreted into the blood and its function is not clear. The P coding region has the codons that encodes polymerase enzymes (DNA polymerase/reverse transcriptase enzyme), which is a multifunctional enzyme that important in the process of RNA encapsidation and DNA synthesis. The viral X protein (which is also known as HBx) is encoded by the X open reading frame. HBx is a multifunctional protein which is able to affect viral and host gene expression, modulation of host-cell signal transduction, DNA repair and also inhibition of protein degradation(Figure 1)(17)

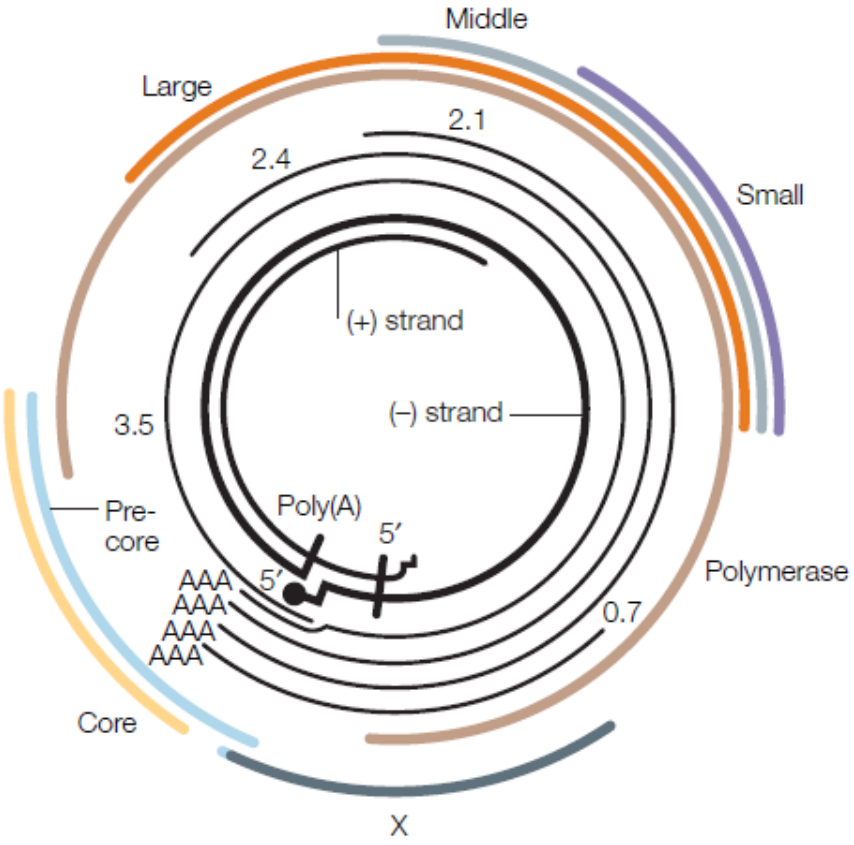


Figure 1. HBV genomic structure and translated proteins(17)

HBV has been divided into eight genotypes, depend on the geographic distribution. It has A to H genotypes and multiple subgenotypes. HBV genotypes show characteristic distribution: genotype A is pandemic but most prevalence in USA, Canada, Northern Europe, and South Africa. Genotype B and C can be found the most in Asia, especially South East Asia. Genotype D can be found in the Eastern Europe, Mediterranean area, and India.

Hepatitis B produces several antigens such as HBsAg, HBcAg, and HBeAg. HBsAg and HBeAg are secreted into the blood. These antigens can be identified in the blood by immunological assay such as enzyme immunoassay. These antigens will disappear when the body produces antibodies against them. HBsAg is the surface protein of HBV, can be detected in blood sample, body fluid, and liver cells. HBsAg is frequently detected as the first marker protein of acute hepatitis B viral infection. HBsAg can be found at the first week of infection but it is usually detected at 6-10 weeks after infection. By definition, the detection of HBsAg in blood sample is indicated that ongoing viral infection, it will be early or chronic infection. HBV chronic infection is defined as HBsAg persists longer than 6 months. The viral core component consists of endogenous viral DNA and hepatitis core antigen (HBcAg). HBcAg or core protein can be found in infected liver cells, but it cannot be found in the serum, but the antibody to HBcAg can be detected in the serum, the immunoglobulin M (IgM) will be detected first, following by immunoglobulin G (IgG). The antibodies to HBsAg (Anti HBs) and antibody to HBcAg were used to classified patients as having acute or chronic infection. IgM class antibodies to HBcAg (Anti HBc-IgM) is related to early infection. HBeAg is originated from precore protein which is cut by peptidase enzyme in endoplasmic reticulum (ER). It is synthesized while the viral replication process in the liver cells. HBeAg is an indicator that HBV has high rate replication at this time and also it is high rate of infection. Hepatitis e antigen can be detected 6-12 weeks after infection and it can persist for years in chronic HBV infection.

More than 400 million individuals worldwide have chronic infection with HBV. Every year, it causes 1 million people die of HCC or liver cirrhosis related to HBV(1) High prevalent areas for chronic HBV infection are East Asia, Southeast Asia and sub-

Saharan Africa (8.0–20.0%), intermediate prevalent areas for chronic HBV infection are Mediterranean countries, Eastern Europe, North Africa and Japan (2.0–8.0%), and low prevalent areas for chronic HBV infection (0.1–2.0%) are Western Europe and the USA(18) Figure 2 shows the Hepatitis B prevalence(19)

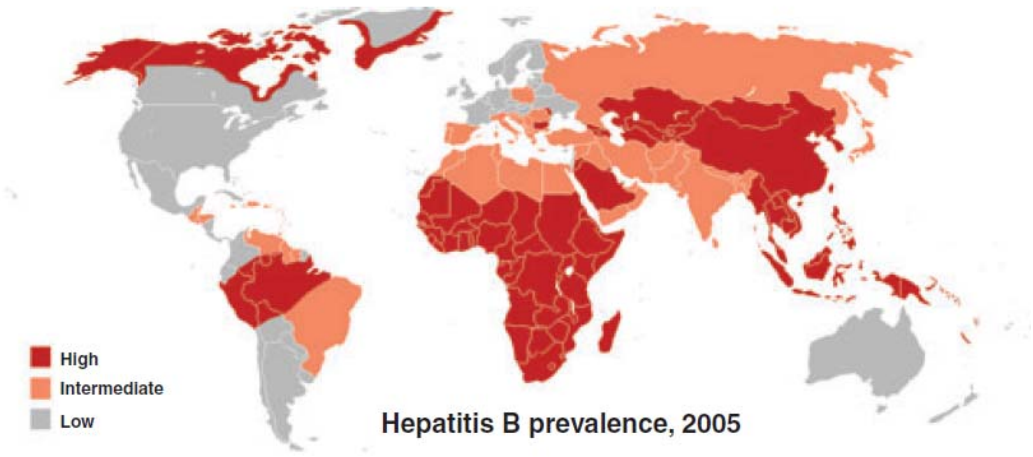


Figure 2. Hepatitis B prevalence(19)

Figure 3 shows the replication cycle of HBV. HBV virions bind to host cell-surface receptors and the viral core particles are internalized to host cytoplasm. After that HBV core particles move from cytoplasm to the nucleus of hepatocyte. In the hepatocyte nucleus, viral DNA genomes, which is relaxed circular DNA, in the core particles, are repaired to make a covalently closed circular DNA (cccDNA). The cccDNA is transcriped and translated to produce the HBV proteins such as viral surface protein, core protein, polymerase protein, and X proteins. After that, the capsids are assembled and corporate with genomic viral RNA in the cytosol, this process is called RNA packaging. This genomic viral RNA in the capsid is the template for producing viral DNA by reverse transcription. The resulting core particles with viral DNA can also bud into the endoplasmic reticulum to form enveloped, then they will be released from the cell, otherwise their genomes can be recycling into the nucleus for alteration to cccDNA(19)

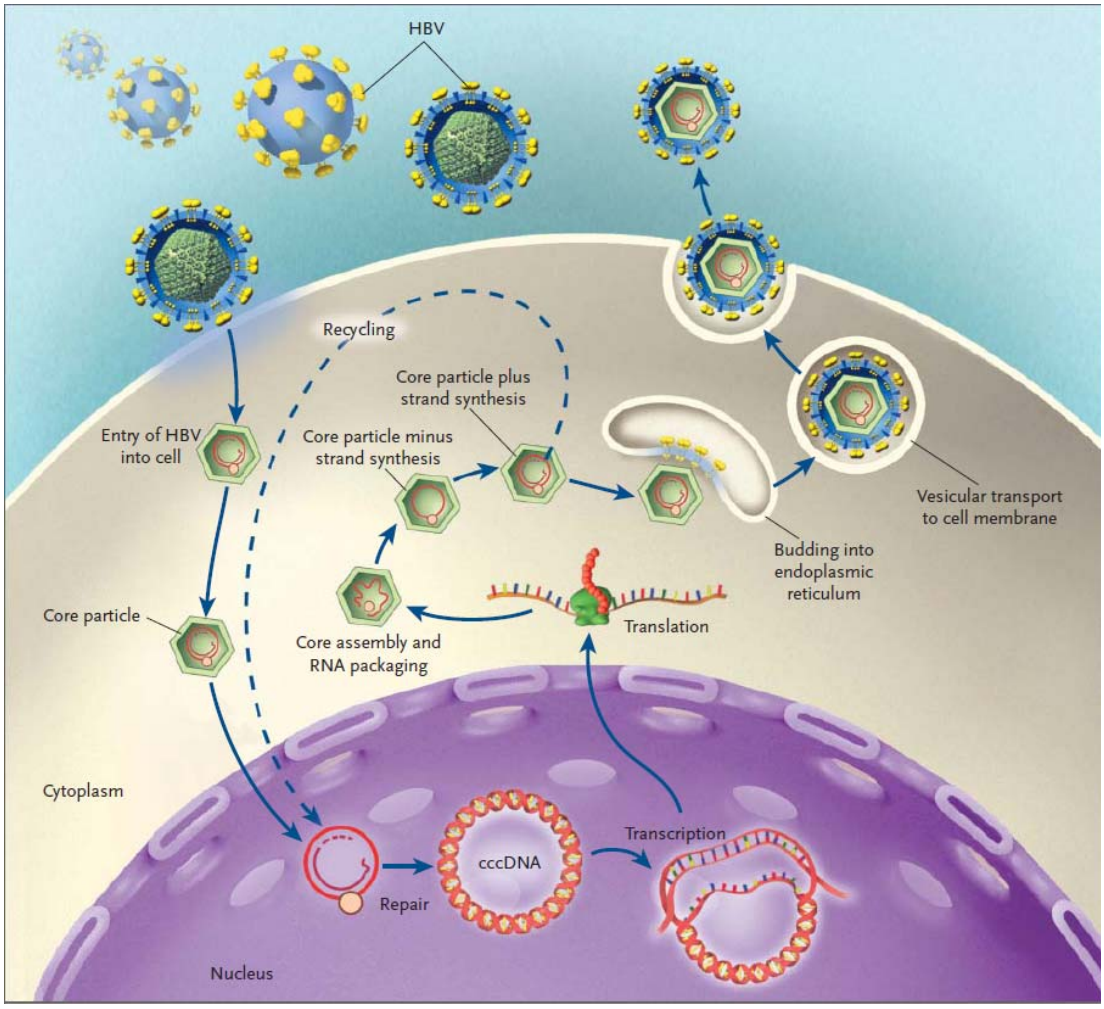


Figure 3. The replication cycle of HBV(17)

TRANSMISSION OF HEPATITIS B VIRUS

HBV is transmitted by percutaneous contact, sexual transmission, and perinatal exposure to infectious body fluid (blood, semen, breast milk, saliva, nasopharyngeal fluid, urine, and cervical secretion). Vertical or perinatal transmission is the major cause of HBV transmission especially in high prevalent areas, but unprotected sexual contact and needle sharing between intravenous drug users are the main cause of infection in low prevalent area such as the Western Europe and USA. For the intermediate prevalent areas, the major mode of HBV transmission are horizontal transmission of HBV occurs during childhood, most probably by contacting with non intact skin and sexual transmission.

People are at risk of HBV infection including: people who have many sexual partners; IV drug users who share needles and syringes; infants born to mothers with the virus; contact with infected family member and infected sexual partners; anyone in intimate contact with the infected person; recipients who receive infected plasma-derived products(20)

TREATMENT OF HEPATITIS B VIRUS

Two major approaches for chronic hepatitis B treatment exist which are 1) immunomodulatory therapies for example pegylated interferon (PEG-interferon) and interferon-α 2) and antiviral drugs such as nucleoside or nucleotide analogs (NAs). NAs are inhibiting of HBV DNA replication process by inhibiting DNA polymerase enzyme(1) Immune-modulating therapy includes mainly pegylated Interferon-α and Interferon-α. IFN-α acts by inducing the interferon-stimulated gene factors (ISGF). IFN-α triggers a non viral specific antiviral state in cells by activate natural killer cell (NK) cytotoxicity, stimulate MHC class I expression and regulate cell growth. Additionally, IFN-α induces cell mediated immune response by reduce IL-12 expression, stimulate CD4 and CD8 T cells to produce IFN-γ, stimulate other IFN- α / β cytokines, and increase macrophages phagocytic activity(19) However, studies using expression microarray in hepatoma cell lines transfected with HBV genome after treating with IFN type I reveal a number of up and down-regulated host genes. Therefore, unknown mechanisms related to IFN type I mode of action remain to be further characterized. Although the cost is quite expensive, immunomodulatory therapy has the advantage of fixed time of therapy, durable off- treatment response, and no viral drug resistant(2)

Clinical practice guidelines recommended that CHB patients should be monitored serum ALT levels and complete blood counts (CBC) every month when treated with PEG-Interferon alfa, and it is necessary to monitor the level of serum HBV DNA at week 12 and week 24 after therapy to validate primary response(21) HBV DNA decrease to low levels by Interferon alfa or NA therapy is related to disease reduction. The undetected HBV DNA level by real-time PCR assay is necessary to diminish the

possibility of drug resistance to NA, and confirm that virological repression level that will lead to histological improvement, biochemical reduction and prevention of complications. Undetectable HBV DNA levels also increases the opportunity of HBeAg loss, the presence of antibody to HBeAg and also increase the opportunity of HBsAg loss, and the presence of Anti HBs in patients(21)

However, only 30% of the chronic HBV patients respond to IFN type I treatment (2, 3) Both viral and host factors are likely 2 key factors in determining treatment responses. For example, viral factors such as HBV DNA level, HBV genotype, HBsAg and HBeAg level, and HBV viral mutants(4, 5) Host factors for predicting a sustained virologic response in CHB patients with HBeAg-positive such as lower level of viral HBV DNA, higher level of alanine aminotransferase (ALT), older age, female sex, and lack of previous IFN therapy(6) Furthermore, host factors for predicting a sustained virologic response in HBeAg-negative patients such as youthfulness, womankind, higher level of alanine aminotransferase, and lower levels of viral HBV DNA(7) Also host factors including HLA class II (HLA-DRB1*14 allele), polymorphisms of A(MxA)-88, level of interleukin-10, and interleukin-12 have been published that these host factors can predict treatment response(8-10) The biomarker that can predict treatment responses will be very useful and cost-effectiveness for the chronic hepatitis B (CHB) patients treatment.

INTRODUCTION OF PROTEOMICS

The finding that there are no more than 30,000 to 40,000 protein-encoding genes of human chromosomal(22) However, because of the occurrence of mRNA splicing in the process of transcription and the post-translational modification, the protein is much larger than protein-encoding genes, and possibly more than 100,000 different proteins(23) One gene can encode more than one protein but we found each protein can have many isoforms, which is the reason of post-translational modifications (Figure 4).

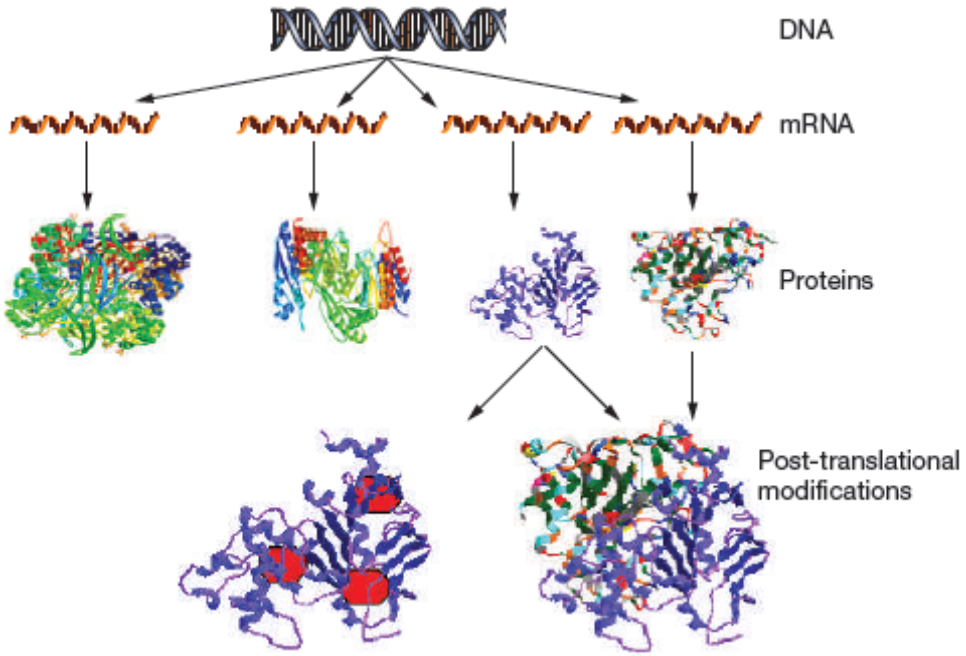


Figure 4. Protein synthesis(24)

Proteomic analysis is more interested studying than the analysis of genome. It is very useful for the reason that proteomic captures the all processes of gene expression in the cell including transcriptional, translational, and post-translational(25) The term proteome was established in 1994 by Marc Williams(24) The objective of proteomics study is the finding of new protein biomarkers, screening and diagnosis the disease, response treatment prediction, establishment of prognosis, and monitoring the progression of disease(24, 26)

Proteomic analysis can be applied to tissue samples, serum, plasma, urine, and body fluids (such as cerebrospinal fluid, plural fluid, gastric juice, peritoneal fluid, bronchoalveolar larvage, or bile). Serum or plasma is very useful for the studying of proteomic because of the collection of serum or plasma is also minimally invasive. There are various technologies used in proteomics. One of the most effective and commonly used methods is two-dimensional gel electrophoresis (2-DE) for proteins separation following by mass spectrometry for proteins identification and characterization. Other new method for protein profiles detection is protein microarrays that are in used for the expression analysis of set of proteins. Proteomic analysis begins

with protein extraction, next protein separation using either a gel-based (2-DE) or gel-free method (capillary electrophoresis; CE, liquid chromatography; LC). After that, separated proteins are identified by different types of mass spectrometry.

Figure 5 shows an overview of proteomic approaches in many various types of samples.

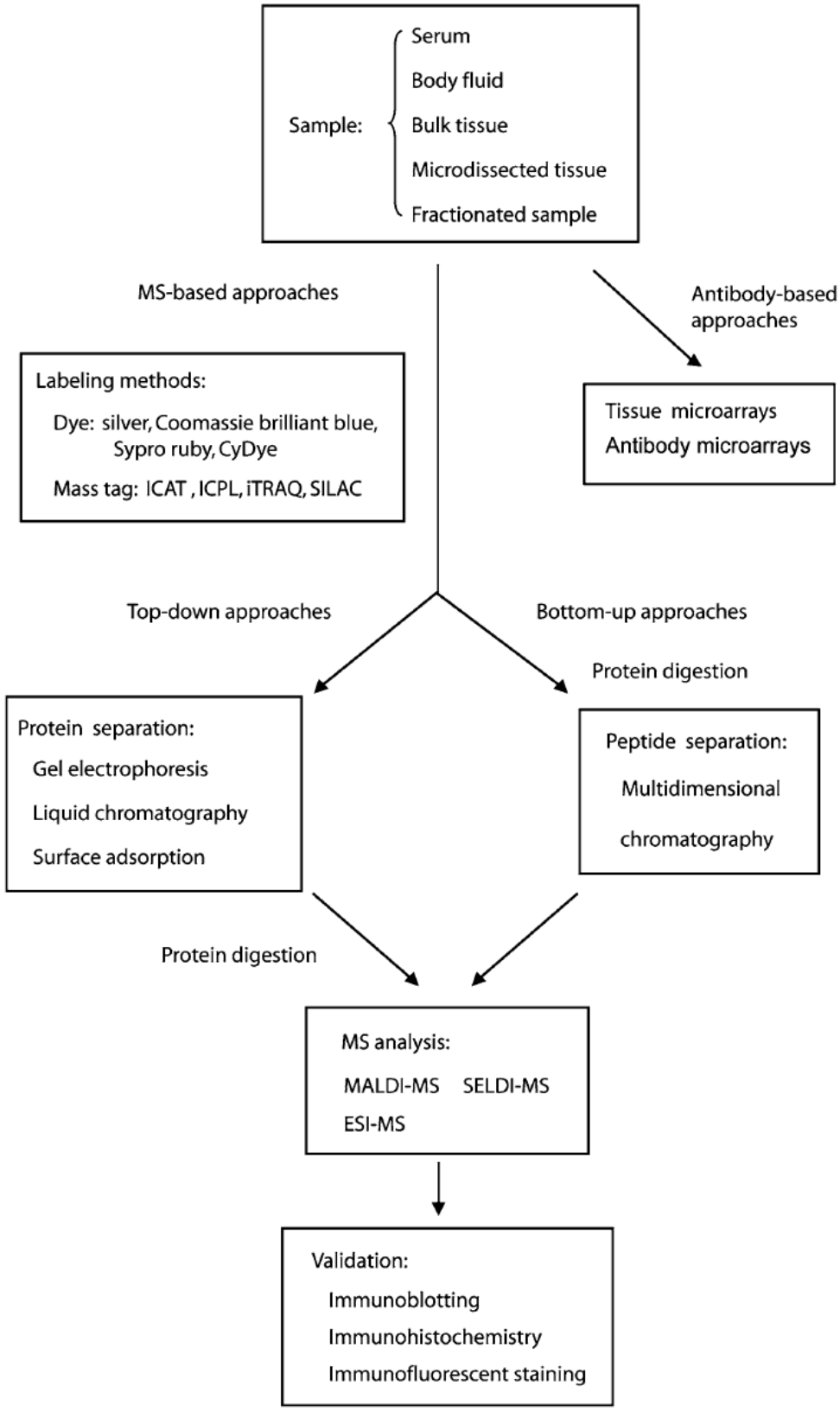


Figure 5. Proteomic approaches(25)

METHODS OF PROTEIN SEPARATION

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) is the method of separating and identify proteins on the basis of different molecular weights and charges of each protein(27) Isoelectric focusing or IEF is the first dimension of 2-DE, proteins are separated on the basis of the different isoelectric point (pI) of protein in this process. The isoelectric point (pI) is defined as pH for all protein, at this pH a protein will not be moving when apply the electric field and its net charge is zero. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the second-dimension, proteins are separated on the basis of the different molecular weight (MW) of protein in the present of sodium dodecyl sulphate (SDS). In the IEF process, proteins are applied to an immobilized pH gradient strip (IPG strips) under the controlled electric field. Protein will travel to the location of its pI (the net charge is zero) in the IPG strips. At its pI, the protein will be stopped moving. The second-dimension SDS-PAGE can be performed after IEF. SDS-PAGE is started with the equilibrating the IPG strips in SDS equilibration buffer. SDS detergent in the equilibration buffer will denature proteins and let the protein to form protein-SDS complexes which has negatively charge. Furthermore, the DTT (dithiothreitol) will reduce proteins in the IPG strips and iodoacetamide (IAA) will alkylate proteins in the IPG strips. DTT reduces every disulfide bonds found in the proteins by disrupt disulfide bonds between cysteine residues. The free sulfhydryl groups of the proteins are then blocked with an alkylating agent, iodoacetamide, to prevent disulfide bonds reformation. After treated the proteins with the equilibration buffer, the equilibrated IPG strips are then set on the SDS-PAGE gel. Under an electric field, the proteins in the IPG strips will be separated on the SDS-PAGE by size (depending on molecular weight). The pore size of the SDS-PAGE is depending on the acrylamide concentration, and by the ratio of acrylamide to bisacrylamide. TEMED is a free radical stabilizer, which is usually included to support polymerization. The separated proteins on the gels are stained by many methods such as comassie blue, silver stain or Sypro ruby staining. Protein spot quantification can be measured by its stain intensity. Protein spots are cut out of the stained gel. The excised spot must be digested by trypsin (this process called tryptic in

gel digestion), and the interesting spot will be identified in a mass spectrometer(28) The advantage of 2D gel electrophoresis is reproducibility, high-resolution, resolving capacity and simple to perform. Although these advantages, 2D gel electrophoresis still has some limitations. It cannot determine proteins which have their molecular weight smaller than 10 kDa, those at the hydrophobic membrane bound proteins or the extremes of pH. 2D gel electrophoresis has limitation to find out hydrophobic proteins. It also has limitation to find out complex protein–protein interactions, time consuming, no automation, labor task intensive, needs large volume of clinical samples, and low-throughput in clinical applications(24)

Differential in-gel electrophoresis

Differential in-gel electrophoresis (DIGE) method is the new technology for reproducibility and sensitivity improvement of results of 2D gel electrophoresis. DIGE is higher sensitivity than 2D gel electrophoresis by detecting the low molecular weight protein to 0.5 kDa. DIGE is less laborious task than 2D gel electrophoresis because it can be used pooling multiple samples (up to three samples) in to one single 2D gel and co-separated and visualized on the one 2D gel. DIGE labels the proteins by using the fluorescent dyes called cyanine dyes (for example Cy3, Cy5, and Cy7) on the basis of cyanine dyes are linked to lysine residues of the proteins. Although these advantages, DIGE still have some limitations, this method cannot identify protein without lysine residues. DIGE is expensive and low-throughput(24)

METHODS OF PROTEIN IDENTIFICATION

Mass spectrometry

Mass spectrometry is an analytical system which is very useful to classify the chemical compound of separated proteins on the strength of the mass to charge (m/z) ratio(29) The advantages of mass spectrometry is high sensitivity, high throughput and small sample volume used, but the disadvantages of mass spectrometry is need expert scientists who have specifically training, expensive and time consuming(24)

MS-based proteomic is started with labeling methods to label proteins of interest. The labeling methods including dye (such as silver, comassie brilliant blue,

Sypro ruby or CyDye) and mass tag (such as ICAT, ICPL, iTRAQ or SILAC) (Figure 5). After that, the labeled protein will be identified by Mass spectrometry in two approaches. First, the bottom-up approaches. In this approach, proteins must be digested by protease enzymes, then the digested proteins are separated by multidimensional chromatography. After that in the last step, the products are again analyzed by mass spectrometry. The resultant spectrum is evaluated by peptide databases. The top-down approaches start with separation steps (for example liquid chromatography or two-dimensional gel electrophoresis) for sample proteins separation. After that digest the protein of interest and in the last step, the peptide products are analyzed by mass spectrometry(25)

Mass spectrometer is composed of 3 parts 1) the ion generating source, 2) the mass analyzer and 3) the detector. The ion source is the technique applied to generate ion from the sample for mass spectrometry. The mass analyzer is used for evaluate the mass-to-charge (m/z) ratio of the ion that generated from the ion source. The detection system is used for recording the number of each mass-to-charge value. The ion generating source consists of Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). They are able to ionize large molecules for example proteins (Figure 6)(25)

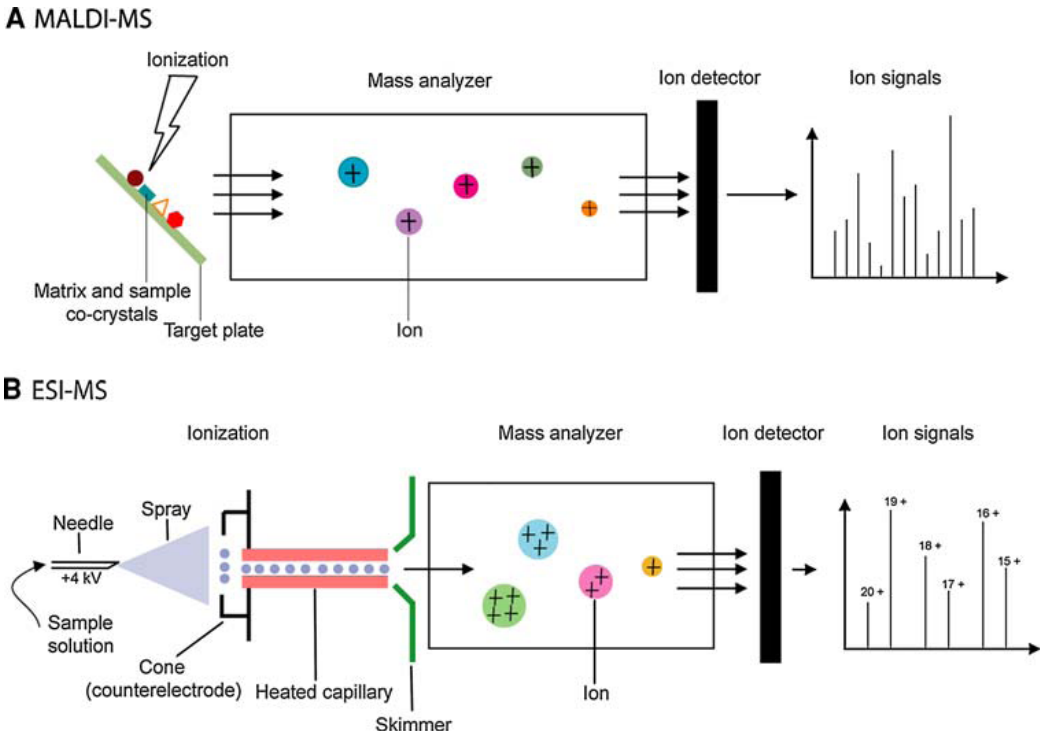


Figure 6. MALDI-MS and ESI-MS systems(25)

MALDI-MS is the ion source that ionizes protein samples in a solid phase. The protein samples are mixed to the dry crystalline matrix, and put the co-crystallized matrix on the target plate. Then, a pulsed lazer irradiated the co-crystallized matrix on the target plate, which causes desorption of ions to the gas phase. The resulting gaseous ionized particles pass through the high vacuum tube of the mass analyzer. The length of time that the peptide uses to pass through the vacuum tube is inversely relative to the weight of peptide. It means that in the curtain total of time, small peptides molecules will travel further than large peptides molecules. A mass map of peptide is created from MALDI is able to be evaluated with established protein/peptide databases. With electrospray ionization (ESI), liquid sample of peptide solution goes through the heated capillary at high voltages, the capillary is injecting the peptide liquid to a high vacuum compartment, and then the droplets evaporate and ionize. The charged ions are passing through a mass analyzer. The ionized molecules will be analyzed by the mass analyzer based on mass-to-charge ratio and sends all information to the detector.

The amino acid sequences of the proteins or peptides information alone or both can be generated by ESI, depending on the coupled mass spectrometry(24) Aebersold, R. reported that “Mass analyzers can be divided in 4 types; 1) time-of-flight (TOF), 2) ion-trap(IT), 3) quadrupole(Q), and 4) Fourier transform ion cyclotron (FTICR)”(28) Mass analyzers can be stand alone or put together in tandem mass spectrometer to take benefit of the powers of each analysis.

Tandem mass spectrometer platforms are the combination of more than one mass analyzer joined together, which perform MS/MS. The first mass analyzer selects an ion of interest then the ion of interest is sent to the collision cell. The inert gas the collision cell will make partial fragmentation at peptide bonds, and then pass the fragmented products through a second mass analyzer. The second mass analyzer provides amino acid sequence pattern of the resulting fragmentation(25)

Surface enhanced laser desorption ionization (SELDI) is used to separate and identify proteins on the principle of surface adsorption. There have many kinds of SELDI chips depending on the protein properties for example hydrophobic, hydrophilic, charged (anionic, cationic), or other surface (metallic, antibody, enzymes, receptors). This SELDI chips can be put into mass spectrometer, and the mass spectrometry can read them. The mass spectrometry will generate complex mass spectrum. The advantage of SELDI is high throughput, simplicity, use less volume of samples, and it is automation. Table 1 explains the advantages and disadvantages of proteomic technologies.

Table 1. Advantages and disadvantages of proteomic technologies(24)

Proteomic technology	Advantages	Disadvantages
2-DE	Mass range 10–200 kDa High-resolution Post-translational modifications detected quantifiable	Basic and hydrophobic proteins not resolved Labor intensive Time-consuming Cannot detect low abundance proteins Large sample volume Low throughput
DIGE	Direct comparison of up to three samples	Expensive Low throughput Excludes lysine-free residues
Mass spectrometry	Small sample volume (µl) High throughput High sensitivity	Specialized technology Expertise needed Time-consuming
SELDI	Simplicity Additional purification/enrichment Automated Rapid/high throughput Minimal sample preparation	Pre-fractionation Nonspecific binding to ProteinChips® Lack of standardized statistical analysis Semi-quantitative Not able to identify protein without further investigation

Plasma/serum proteomics in Liver diseases

Many researchers have demonstrated that serum or plasma proteins are classified as potential biomarkers in cancers for example ovarian cancer(30, 31), prostate cancer(32), breast cancer(33, 34) and hepatocellular cancer(11, 35) In the earlier state of neoplastic diseases, they do not demonstrate clinical symptoms of this disease until the disease is progressing in advanced stages. According to this reason, investigators tried to find tumor-associated biomarkers that can be very helpful for early on finding of the pathological state. Such as Rui Z *et al*. has observed that “14-3-3 sigma protein and HSP 27 protein in serum are the potential tumor-associated markers for breast cancer. They have found these difference biomarkers between normal

healthy and breast cancer patients by using 2-DE"(33) Petricoin *et al.* has observed that "Surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) technique could profile sera samples of different stages of ovarian cancer patients together with normal controls and this profile had 100% sensitivity of and 95% specificity to differentiate ovarian cancer from non-cancer"(30) In addition, Ye B *et al* has observed that "haptoglobin alpha are serum potential tumor-associated biomarkers in patients with ovarian cancer by Surface-enhanced laser desorption/ionization mass spectrometry and liquid chromatography/tandem mass spectrometry technologies"(31)

Proteomic technologies have been reported in specific biomarkers study for hepatocellular carcinoma (HCC). Steel LF *et al.* has identified that "two proteins: C3 component and ApoA1: their levels are low in patients with diagnosis of HCC and they might be potential biomarkers for HCC"(11) Yang MH *et al.* have observed that "six proteins (Annexin VI, Complement component 9, ceruloplasmin, serum amyloid A4, serum amyloid A2, and serum amyloid A1 isoform 2) have found in HCC patients serum compared with normal serum by using a combination of 2-DE and nano-high-performance liquid chromatography electrospray ionization tandem mass spectrometry (nano-HPLC–ESI-MS/MS)"(13) Feng JT *et al*. have observed that "there have eight different proteins expression between healthycontrol, HBV patients, and HCC groups, including AFP, Transferrin, Transthyretin, α-1-antitrypsin, Clusterin, Haptoglobin-α-2-chain, Ceruloplasmin, and Heat shock protein 27(HSP27) by using a combination of 2-DE and MALDI-TOF-MS/MS. And they have validated HSP27 and have found that this protein was a potential tumor-associated biomarkers for HCC"(12)

Proteomic technologies have been used to study hepatitis B virus (HBV) inflammation. He QY *et al*. has observed that "seven proteins such as haptoglobin β and α2 chain, α 1-antitrypsin, apolipoprotein A-I (isoform 1, 2 and 3), apolipoprotein A-IV, transthyretin, and DNA topoisomerase II β might be considered as HBV biomarkers. They have used 2-DE to compare these proteins in normal serum samples and HBV serum samples"(14) Marrocco C *et al*. has reported that "gelsolin protein is repressed in HBV-associated liver cirrhosis compared with CHB patients who were asymptomatic by using 2-DE and nanoLC-MS/MS"(15) MA Hui *et al.* has reported that "serum α-2-HS

glycoprotein levels in CHB patients treated by PEG-Interferon alfa-2b were significantly up-regulated at 24 weeks after treatment in the HBeAg seroconversion group"(36)

Proteomic technologies have also been used to monitor the serum proteome change in CHC patients treated PEG-Interferon alfa-2b in plus ribavirin to predict treatment response. Paradis V. *et al.* has reported that "they used SELDI-TOF/MS to study serum profiles in CHC patients treated PEG-Interferon alfa-2b combination of ribavirin, the results have shown that 37 m/z peaks were significantly variations during treatment in SVR, but only 1 m/z peak was different in NR"(16) In 2011, Fujita N *et al.* has reported that "ApoAI, albumin, and ApoCI are over-expressed in SVR, whereas hemopexin, transferrin, CTAP-III, and platelet factor 4 are down-regulated in NR, in the pretreatment serum of CHC patients treated with PEG- Interferon combination with ribavirin"(37) The summary of proteomic studies about CHB and CHC patients have shown on table 2.

Here, we want to study and monitor the serum proteome change in patients with chronic hepatitis B virus infection treated with PEG-Interferon alfa-2b to predict treatment response. Additionally, we want to compare the different of serum proteome in patients who are sustained virologic response and non response after PEG-Interferon alfa-2b treatment.

Table 2 Proteomic survey relevent to HBV and HCV

Disease	Source of sample	Analytical techniques	Study size	Principal finding	Citation
HBV	Serum	2-DE MALDI-TOF MS	15 normal 9 LNS , 9 HNL (pooled samples)	HSP27 was significantly up-regulated in HCC 7 proteins with different expression (Haptoglobin β&cleved chain, Apo A1, ApoAIV, Transthyrin, α1-antitrypsin, DNA topoisomerase Haptoglobin α2 chain)	He Q.Y. *et al.* (2003)
HCC(HBV-related)	Serum	2-DE MALDI-TOF MSMS	20HCC, 20 HBV, 20 normal (pooled samples)	8 proteins with different expression (Transferrin, Transthyrin, α1-antitrypsin, Clusterin, Haptoglobin α2 chain, Ceruloplasmin, HSP27, Alpha fetoprotein) HSP27 up-regulated in HCC	Feng J.T. *et al.* (2005)
HBV	HepG2 HepG2.2.15 treated with IFN 5000 IU/ml	2-DE MALDI-TOF MS/MS	Triplicate 2-DE	11 proteins up-regulated in HepG2.2.15 6 proteins down-regulated in HepG2.2.15 Validation by WB: Peroxiredoxin and Grp94 up-regulated in HepG2.2.15 in HepG2.2.15, GST-pi down-regulated Prohibitin up-regulated	Wang JH *et al.* (2009)
HBV associated liver cirrhosis	Plasma	2-DE nanoLC-MS/MS	8 inactive CHB 8 HBV related liver cirrhosis	Gelsolin down-regulated in HBV related liver cirrhosis	Marrocco C *et al.* (2010)

LNS low necroinflammatory score, HNS high necroinflammatory score

Table 2 Proteomic survey relevent to HBV and HCV continued

Disease	Source of sample	Analytical techniques	Study size	Principal finding	Citation
HBV related liver fibrosis	Plasma	DIGE MALDI-TOF MS	Screening; 7 normal 27 HBV related liver fibrosis (pooled samples) Validation; 42 normal 24 Mild fibrosis 32 large fibrosis 12 Early cirrhosis	Clusterin downregulated along fibrosis development Perixiredoxin II up-regulated along fibrosis development	Lu Y *et al*.(2010)
HBV treated PEG-IFN α2b	Serum	2-DE LC-ESI-MS/MS	4 HBe seroconversion 24 non HBe seroconversion (pooled samples)	Up-regulated proteins in SVR (α2-HS-glycoprotein, Haptoglobin, Leucine-rich-α2-glycoprotein) Down-regulated proteins in NR (Leucine-rich-α2-glycoprotein, Apo CIII precusor, α-albumin) Validation: α2-HS-glycoprotein significantly up-regulated at 4 week after treatment	MA Hui *et al*. (2011)
HCV treated PEG-IFN plus ribavirin	Serum	SELDI-TOF/MS	96 chronic hepatitis C (68 SVR and 28 NR)	50 m/z peaks with different expression SVR 37 m/z peaks significantly variation during treatment NR 1 m/z peak different during treatment	Paradis V. *et al*. (2006)
HCV treated PEG-IFN plus ribavirin	Serum	SELDI-TOF/MS Chromatography SDS-PAGE TOF/MS(MS/MS)	10 SVR 12 TR 10 NR	Up-regulated proteins in SVR (ApoAI, albumin, ApoCI) Down-regulated proteins in SVR (Hemopexin, Transferrin, CTAP-III Platelet factor 4)	Fujita N. et al. (2011)

SVR Sustained responder, NR non responder, TR Transient responder

CHAPTER III

MATERIALS AND METHODS

Methodology Scheme

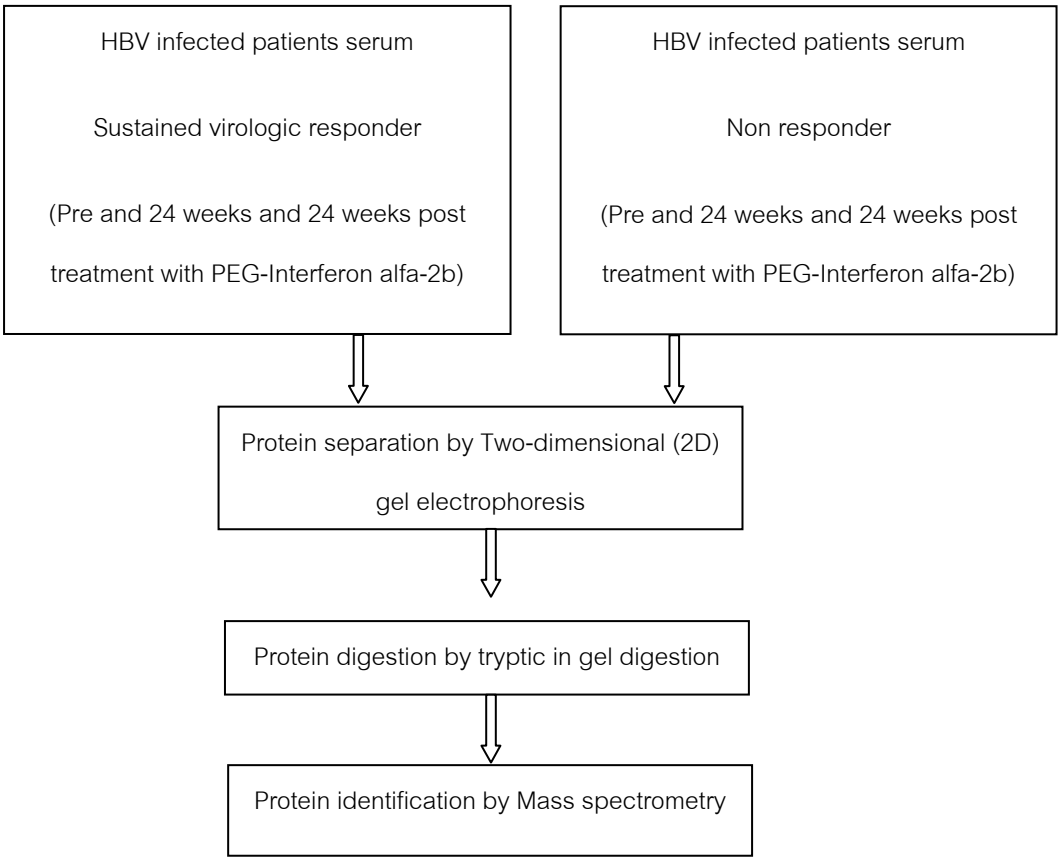


Figure 7. Methodology Scheme.

PART I: Collection of samples

Human subjects

Chronic hepatitis B Patients followed up at the King Chulalongkorn Memorial Hospital. All patients received Peg-IFN-α2b (1.5 mg/kg per week) subcutaneously for 48 weeks and assessed therapy response.

Assessment of efficacy

In HBeAg-positive patients, sustained virologic response was characterized by undetectable HBeAg and found Anti-HBe in blood (HBeAg seroconversion) and HBV viral load < 2000 IU/ml at week 48 after treatment. In HBeAg-negative patients, sustained virologic response was characterized by HBV viral load < 200 IU/ml at week 48 after treatment(38) Patients without sustained virologic response were defined as non responders.

The patients were fulfilled the following criteria: (1) patients who could be detected HBsAg and for HBeAg in blood samples for at least 6 months (2) Absence of chronic liver disease from other reasons such as, haemochromatosis, autoimmune chronic hepatitis, or chronic hepatitis from other viruses (Cytomegalovirus or Epstein Barr Virus).

Sample size

3 sustained responders (SVR) and 3 non responders (NR) were used to study and monitor the kinetics of serum proteome before starting treatment, and after initial PEG-Interferon alfa-2b treatment at week 24 and week 48.

9 sustained responders and 10 non responders were used to compare the expressed proteins before treatment.

Serum collection

For each patient, serum was collected before start treatment, after initial PEG-Interferon alfa-2b treatment at week 24 and week 48. Serum samples were kept at -80°C until start of 2-DE and LC-MS/MS analysis. The serum were collected from the project "Virological and Histological Efficacy of PEG-Interferon alfa-2b Therapy in Thai Patients with Chronic Hepatitis B" (IRB approval No. 368/2005) and stored at -80°C at Division of Gastroenterology Chulalongkorn University Hospital. The procedure of this study was agreed by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University.

PART II: Optimizing serum pretreatment before 2D gel electrophoresis

Before proteomic analysis, the high abundant proteins (albumin and IgG) in the serum were removed using ProteoPrep Blue Albumin Depletion Kit (from Sigma-Aldrich, Inc, Missuuri, USA) according to the company's protocol. ProteoPrep Blue Albumin Depletion Kit medium contains Cibacron Blue and Protein A/G. Cibacron Blue has been reported that it binds to human serum albumin(39) Protein A has been reported that it binds to the Fc portion of IgG, while Protein G has been reported that it binds to both the Fc and Fab portion of IgG(40) 50 µl of each serum sample was added to the top of medium in the column, incubate at room temperature for 10 minutes for the adsorption of sample into the medium. After that, centrifuge the column and collection tube at 10,000-12,000 rpm for 60 second at 4°C. The eluate was reapplied from the collection tube to the top of the medium again. Incubate 10 minutes following by centrifuge the column in the same collection tube at 12,000 rpm for 60 second at 4°C. Add 100 ml of Equilibration Buffer to the top of the medium bed for washing the remaining unbound proteins from the spin column by centrifuge for 1 minute. The protein concentration were measured by Bio-Rad Bradford total protein assay kit (Biorad Laboratories, Inc., Redmond, WA, USA)(41), using bovine serum albumin (BSA) as a standard curve.

PART III: Serum proteins separation by 2D gel electrophoresis

The Immobiline Dry strip (pH 4-7, length 7 cm, Amersham Biosciences, Uppsala, Sweden) was rehydrated with 150 µg protein in 125 µl rehydration buffer containing 9M urea, 2% CHAPS, 0.002 % w/v bromophenol blue, 0.8% (w/v) DTT, 1% IPG buffer for 14 h at room temperature. IEF was performed using IPGphor IEF apparatus (Amersham Biosciences, Uppsala, Sweden) with a total of 8000 Vhrs. Then the strip was equilibrated in equilibration buffer containing 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 50 mM Tris-HCL (pH 8.8) with 1 % DTT w/v for the first and 2.5 %

w/v iodoacetamide for the second step. Equilibrated strips were then placed on SDS-polyacrylamide gels, 12.5% acrylamide, and sealed with 0.5% (w/v) agarose. SDS-PAGE was performed using SE 260(right) Mini-Vertical Units (GE healthcare, Uppsala, Sweden) at constant electric field (15 mA per gel) until the bromophenol blue tracking dye was arrived to the bottom of the gel. Proteins spot were stained by Coomassie Brilliant Blue G-250 stain(42)

Image analysis

1. For studying and monitoring the kinetics of serum proteome before starting treatment , after initial PEG-Interferon alfa-2b treatment for 24 weeks and 48 weeks.

Thirty-one two-dimension stained gels were scanned using an ImageScanner (Amersham Biosciences, Uppsala, Sweden). The stained 2-D gel were analyzed by the software, ImageMaster 2D Platinum (GE Healthcare, Uppsala, Sweden) following this spot detection parameters; (1) minimal area = 5 pixels, (2) smooth factor = 2.0, and (3) saliency = 50. The gel image which has the maximum number of spots was selected to be a reference gel. The spots on reference gel were matched to the spots on other gels. Thirty-one gels were separated into 2 groups, 15 for the sustained responders and 16 for the non responders. In each group was divided into 3 sub-groups, 9 for SVR and 10 for NR for pre-treatment with PEG-Interferon alfa-2b, 3 for 24 weeks post treatment with PEG-Interferon alfa-2b and 3 for 48 weeks post treatment with PEG-Interferon alfa-2b. The spots will be compared between pre-treatment sample and post treatment samples in the same groups (Non response or sustained virologic response), and the spots will be compared between non responder and sustained virologic responder group.

2. For comparison the expressed proteins before PEG-Interferon alfa-2b treatment.

Nineteen two-dimension stained gels were scanned using an ImageScanner

(Amersham Biosciences). The stained 2-D gel were analyzed by the software, ImageMaster 2D Platinum (GE Healthcare), using the parameters the same as topic 1. Nineteen gels were separated into 2 groups, 9 for the sustained virologic response and 10 for the non response.

Statistic analyses for image analysis

SPSS version 17.0 was used for statistical analyses. Value of spots intensities are mean±SEM. Independent sample *t*-tests were used to evaluate baseline characteristics of patients (The *P*-value cut off for Independent sample *t*-tests was 0.05). Mann-Whitney *U* test were used to evaluate different protein expression among two groups. The significantly differentially expressed protein with the *P*-value for Mann-Whitney *U* test was < 0.05 were selected and subjected to further mass spectrometry analysis.

PART IV: Protein identification by LC-MS/MS analysis

In gel tryptic digestion

Protein spots were cut from the Commassie Brilliant Blue stained gels and subjected to in-gel digestion using an in-house method developed by Jaresitthikunchai *et al*. (2009)(43) Protein spots were destained with 50% methanol and 50 mM ammonium bicarbonate, dehydrated with 100% acetonitrile (ACN). The gel plugs were subjected to reduction and alkylation in 10mM of DTT and 100mM iodoacetamide at room temperature for 1 h. The gel pieces were dehydrated twice with 100% ACN for 5 min after alkylation. Subsequently, the gel pieces were digested in 10 µl trypsin (modified porcine trypsin, sequencing grade, Promega, Medison, WI) solution (20 ng in 10 mM ammonium bicarbonate in 50% acetonitrile, ACN) and incubated at room

temperature for overnight. The peptides were extracted twice by adding 30 µl of solution containing 50% ACN and 0.1% formic acid. The extracted solutions were dried in a heat box at 40°C and kept at -80°C for further mass spectrometry analysis. Prior to mass spectrometry analysis, the peptide mixtures were re-dissolved in 10 µl of 0.1 % formic acid.

Protein identification by LC/MS/MS analysis

Peptide mixtures were injected into Ultimate 3000 LC System (Dionex, USA) combined to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany)) with electrospray at flow rate of 300 nl/min to a nanocolumn. A solvent gradient (solvent A: 0.1% formic acid in HPLC grade water; solvent B: 0.1% formic acid in 80% acetonitrile) was run for 65 min.

Database analysis

Proteins were identified with peptide mass mapping data by searching software Mascot (MatrixScience, London, UK) http://matrixscience.com. A database search (NCBInr) was performed using the following set up: species was selected as homo sapiens/human, trypsin digest, the number of missed clevages sites was allowed up to 1, cysteines modified by carbamidomethylation, variable modification was oxidation (Methionines), mass values was monoisotopic, peptide Mass Tolerance±1.2 Da, Fragment Mass Tolerance ± 0.6 Da(15) Probability based Mowse score greater than 43 were considered significant ($p<0.05$).

CHAPTER IV

RESULTS

PART I: Patient characteristics

Baseline characteristics of patients with sustained virologic responders and non responders are shown in Table 3-4.

Table 3. Baseline characteristics of patients with sustained virologic responders and non responders [a)].

	Sustained virologic responders (n=9)	Non responders (n=10)	*P* value
Age (years)	29.67±8.29	36.90±6.40	0.047
Gender (male:female)	7:2	8:2	0.912
ALT level (U/l	103.88±105.04	130.50±75.77	0.609
HBV DNA (copies/ml)	(11.07± 8.52)$\times 10^6$	(14.35± 8.44)$\times 10^6$	0.411
HBeAg	Positive	Positive	Not significant

a) For comparison the expressed proteins before PEG-Interferon alfa-2b treatment.

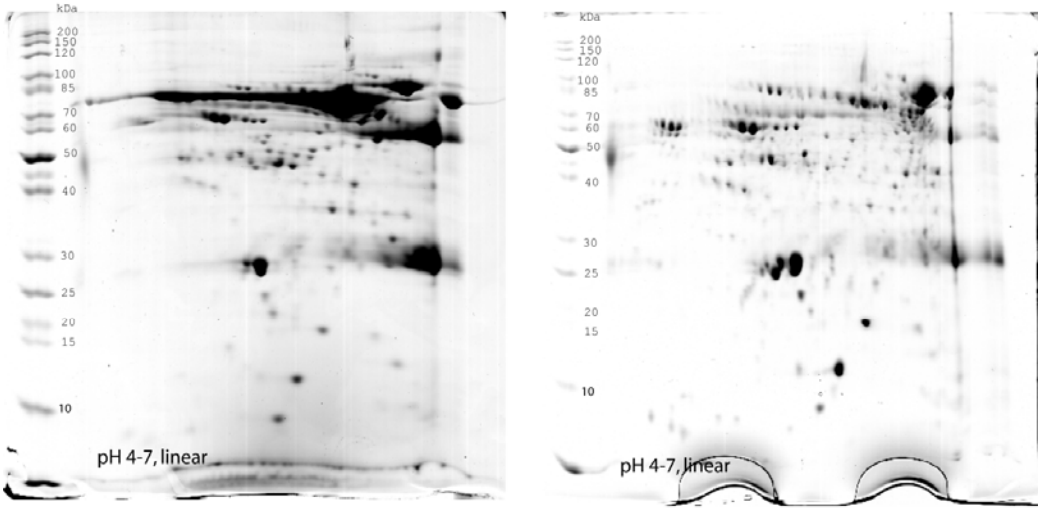
Table 4. Baseline characteristics of patients with sustained virologic responders and non responders [b)].

	Sustained virologic responders (n=3)	Non responders (n=3)	*P* value
Age (years)	29.3 ± 11.9	35.7 ± 10.5	0.528
Gender	Male	Male	Not significant
ALT level (U/l)	250.0 ± 121.6	155 ± 66.5	0.435
HBV DNA (copies/ml)	(13.46± 8.56)✕10^6	(18.77± 2.14)✕10^6	0.356
HBeAg	Positive	Positive	Not significant

b) For studying and monitoring the kinetics of serum proteome after initial PEG-Interferon alfa-2b treatment for 24 weeks and 48 weeks.

PART II: Optimizing serum pretreatment before 2D gel electrophoresis

Before performing electrophoresis on serum collected from patients, the efficiency of ProteoPrep Blue Albumin Depletion Kit was tested. Figure 8 shows two representative maps of serum samples (chronic HBV infection) before and after treatment with ProteoPrep Blue Albumin Depletion Kit. As expected, in the untreated sample, albumin predominated the gel, obscuring signals from less abundance proteins. When an equal quantity of protein is pre-treated with ProteoPrep Blue Albumin Depletion Kit, generated 2D gels showed a dramatically improved resolution and a greater number of spots were detected.



(A) Un-depleted alb and IgG (B) Depleted alb and IgG

Figure 8. 2D gel (linear IPGs pH 4-7, 7 cm length) of serum samples of chronic HBV infection patients before (A) and after (B) treatment with ProteoPrep Blue Albumin Depletion Kit (Sigma-Aldrich, Inc, Missuri, USA). Total protein load is 150 µg.

PART III: Serum proteins separation by 2D gel electrophoresis

1. Identification of protein spots on the gel.

We identified 88 protein spots in the sustained responder gel as the reference for matching, cut, in-gel digestion and analyzed by LC/MS/MS. The data of all identified proteins including protein scores, accession number from NCBI, percent of sequence coverage, theoretical isoelectric points (pI), and molecular weights (MW) were summarized in Table 5.

Table 5. List of identified proteins.

Spot	protein identity	NCBI ID	score	%cov	MS/MS pI	MW(kDa)
1	haptoglobin Hp2	gi|223976	530	27	6.23	42.34
3	haptoglobin Hp2	gi|223976	511	27	6.23	42.34
138	haptoglobin precursor	gi|306882	373	35	6.24	45.86
141	haptoglobin precursor	gi|306882	66	24	6.24	45.86
57	haptoglobin [Homo sapiens]	gi|1212947	394	42	6.27	38.941
4	albumin, isoform CRA_c	gi|119626066	660	42	6.39	27.67
33	albumin, isoform CRA_b	gi|119626065	539	38	6.96	61.12
193	albumin, isoform CRA_j	gi|119626073	650	73	6.40	26.91
199	serum albumin [Homo sapiens]	gi|28592	153	27	6.05	71.316
264	serum albumin [Homo sapiens]	gi|62113341	115	31	5.85	71.092
301	serum albumin precursor	gi|6013427	563	35	5.91	71.176
257	serum albumin precursor	gi|6013427	78	17	5.91	71.176
312	serum albumin precursor	gi|6013427	151	37	5.91	71.176
207	albumin [Homo sapiens]	gi|332356380	781	49	5.73	68.484
280	albumin [Homo sapiens]	gi|332356380	201	32	5.73	68.484
9	proapolipoprotein [Homo sapiens]	gi|178775	448	39	5.45	28.94
20	proapolipoprotein [Homo sapiens]	gi|178775	798	95	5.45	28.94
11	chain A, crystal structure of lipid-free human apolipoprotein A-I	gi|90108664	1347	78	5.27	28.061
12	chain A, crystal structure of lipid-free human apolipoprotein A-I	gi|90108664	200	76	5.27	28.061
16	chain A, crystal structure of lipid-free human apolipoprotein A-I	gi|90108664	923	95	5.27	28.061

Table 5. List of identified proteins continued.

Spot	protein identity	NCBI ID	MS/MS score	%cov	pI	MW(kDa)
17	chain A, crystal structure of lipid-free human apolipoprotein A-I	gi|90108664	1121	95	5.27	28.061
26	chain A, the structure of pentameric human serum amyloid P component	gi|576259	174	43	6.12	23.358
32	Vitamin D-binding protein	gi|139641	56	17	5.40	54.526
89	Vitamin D-binding protein	gi|139641	530	62	5.40	54.526
37	apolipoprotein J precursor	gi|178855	270	24	6.27	49.342
38	apolipoprotein J precursor	gi|178855	230	41	6.27	49.342
39	apolipoprotein J precursor	gi|178855	132	41	6.27	49.342
43	apolipoprotein J precursor	gi|178855	72	33	6.27	49.342
44	apolipoprotein J precursor	gi|178855	255	45	6.27	49.342
311	apolipoprotein J precursor	gi|178855	216	39	6.27	49.342
49	Chain A, Apo-human serum transferrin (non-glycosylated)	gi|110590597	690	51	6.58	76.81
55	Zn-alpha2-glycoprotein	gi|38026	257	52	5.71	34.942
58	apolipoprotein A-IV precursor [Homo sapiens]	gi|178779	652	76	5.22	43.358
59	chain C, human complement component C3c	gi|78101271	513	70	4.79	40.204
61	PRO0684 [Homo sapiens]	gi|6855601	93	43	5.37	46.862
69	Ig alpha-1 chain C region	gi|113584	61	32	6.08	38.486
86	Alpha-2-HS-glycoprotein	gi|112910	494	40	5.43	40.098
87	Alpha-2-HS-glycoprotein	gi|112910	422	29	5.43	40.098
93	Chain A, Alpha1-Antitrypsin	gi|157831596	675	69	5.37	44.280

Table 5. List of identified proteins continued.

Spot	protein identity	NCBI ID	score	%cov	MS/MS pI	MW(kDa)
99	Chain A, Alpha1-Antitrypsin	gi|157831596	571	73	5.37	44.280
102	Chain A, Alpha1-Antitrypsin	gi|157831596	601	67	5.37	44.280
178	Chain A, Alpha1-Antitrypsin	gi|157831596	84	56	5.37	44.280
96	Alpha1-Antitrypsin	gi|151302818	424	53	5.51	46.978
97	Chain A, Alpha1-Antitrypsin	gi|157831596	399	64	5.37	44.280
121	hemopexin precursor	gi|386789	269	46	6.57	52.254
124	hemopexin precursor	gi|386789	180	41	6.57	52.254
128	hemopexin precursor	gi|386789	209	42	6.57	52.254
167	hemopexin precursor	gi|386789	375	55	6.57	52.254
123	alpha-1-B-glycoprotein – human	gi|69990	247	45	5.65	52.479
125	alpha-1-B-glycoprotein – human	gi|69990	329	50	5.65	52.479
127	alpha-1-B-glycoprotein – human	gi|69990	334	55	5.65	52.479
149	serotransferrin precursor	gi|4557871	650	44	6.81	79.280
145	CD5 antigen-like precursor	gi|5174411	443	67	5.28	39.603
202	CD5 antigen-like precursor	gi|5174411	70	33	5.28	39.603
146	albumin [Homo sapiens]	gi|332356380	243	41	5.73	68.484
150	serotransferrin precursor	gi|4557871	986	48	6.81	79.280
153	serotransferrin precursor	gi|4557871	734	57	6.81	79.280
171	chain A, the intact and cleaved human antithrombin Iii complex as a model for serpin-proteinase interactions	gi|999513	396	58	5.95	49.350
173	chain A, the intact and cleaved human antithrombin Iii complex as a model for serpin-proteinase interactions	gi|999513	210	49	5.95	49.350
198	transferrin, isoform CRA_c	gi|119599572	568	58	6.67	48.669
201	PRO2619 [Homo sapiens]	gi|11493459	278	42	5.96	58.513
206	transferrin, isoform CRA_c	gi|119599572	196	62	6.67	48.669

Table 5. List of identified proteins continued.

Spot	protein identity	NCBI ID	score	%cov	MS/MS pI	MW(kDa)
229	Ig J-chain [Homo sapiens]	gi|532598	42	27	4.62	16.040
294	serum vitamin D-binding protein precursor [Homo sapiens]	gi|181482	413	63	5.40	54.612
296	Vitamin D binding protein isoform 1 precursor [Homo sapiens]	gi|32483410	499	63	5.32	54.480
106	unnamed protein product	gi|34527233	370	40	6.08	54.127
109	unnamed protein product	gi|34527233	354	45	6.08	54.127
297	unnamed protein product	gi|34527290	332	42	6.55	54.372
298	unnamed protein product	gi|34527290	328	33	6.55	54.372
23	immunoglobulin light chain	gi|218783338	252	65	5.95	24.162
299	immunoglobulin light chain	gi|218783338	747	68	5.95	24.162
309	Alpha-2-HS-glycoprotein	gi|112910	507	40	5.43	40.098
310	Alpha-2-HS-glycoprotein	gi|112910	475	26	5.43	40.098
500	chain A, crystal structure of cys10 sulfonated transthyretin	gi|126030594	645	89	5.35	13.761
501	haptoglobin [Homo sapiens]	gi|3337390	319	10	6.14	38.722
502	prealbumin [Homo sapiens]	gi|219978	134	17	5.52	16.023
503	haptoglobin [Homo sapiens]	gi|3337390	183	11	6.14	38.722
504	unnamed protein product	gi|28590	172	5	5.92	71.246
505	chain A, Nmr structure of human of cys10 sulfonated transthyretin	gi|14277770	241	21	4.57	10.177
506	apolipoprotein C-III	gi|521205	106	16	5.23	10.815
507	chain A, structure and dynamics of Human apolipoprotein C-Iii	gi|186972736	77	21	4.72	8.759
508	unnamed protein product	gi|34069	59	6	5.13	39.309
509	haptoglobin-like protein	gi|292158	164	10	6.42	39.496

Table 5. List of identified proteins continued.

Spot	protein identity	NCBI ID	MS/MS score	%cov	pI	MW(kDa)
510	proapolipoprotein	gi|178775	174	16	5.45	28.944
511	chain A, crystal ctructure of cys10 sulfonated transthyretin	gi|126030594	110	37	5.35	13.761
512	hypothetical protein	gi|51476390	77	1	5.88	71.353
513	cytokeratin 9 [Homo sapiens]	gi|435476	133	5	5.19	62.320
514	transferrin precursor (AA at 8) [Homo sapiens]	gi|339485	91	30	8.63	8.041

2. Comparison the expressed proteins between sustained responders and nonresponders before PEG-Interferon alfa-2b treatment.

Nineteen specimens were tested for protein separation by 2D gel electrophoresis, in gel digestion and protein identification by LC/MS/MS. They were divided into 2 groups, 9 for the sustained responders and 10 for the nonresponders. There were 7 protein spots found to be different with respect to protein intensity between sustained responders and nonresponders (Table 6). There were 4 proteins were significantly higher expressed in sustained responders: 1) Chain A, Alpha-1- Antitrypsin, 2) albumin, isoform CRA_b [Homo sapiens], 3) CD5 antigen-like precursor [Homo sapiens], 4) albumin [Homo sapiens]. Three proteins were significantly higher expressed in non responders: 1) Chain A, crystal structure of lipid-free human apolipoprotein A-I, 2) Chain C, human complement component C3c, 3) alpha-2-HS-glycoprotein (Figure 9, 10). Functional characterization of these proteins can be summarized into 5 groups: 1) protease inhibitor: alpha-1-antitrypsin, 2) transport protein and protein binding: serum albumin, 3) acute phase response: alpha-2-HS-glycoprotein 4) immunity protection: CD5 antigen precursor, and complement component C3c, 5) cholesterol metabolism: human apolipoprotein A-I.

Table 6. Proteins whose serum levels were significantly differed among sustained responders (SVR) and nonresponders (NR) before PEG-Interferon alfa-2b treatment.

Spot	protein identity	NCBI ID	score	%cov	pI	MW (kDa)	Relative intensity (mean ± SEM) NR	SVR	Ratio SVR/NR	T-test *P* values
Protease inhibitor										
97	Chain A, Alpha-1- Antitrypsin	gi|157831596	399	64	5.37	44.280	0.2764±0.0327	0.4275±0.0609	2.34	0.038
Transport protein and protein binding										
33	albumin, isoform CRA_b[Homo sapiens]	gi|119626065	539	38	6.96	61.12	0.0330±0.0121	0.0764±0.0144	2.31	0.033
146	albumin [Homo sapiens]	gi|332356380	243	41	5.73	68.484	0.0602±0.0182	0.1516±0.0333	2.52	0.024
Acute phase protien										
309	Alpha-2-HS-glycoprotein	gi|112910	507	40	5.43	40.098	0.7165±0.0238	0.4782±0.0851	0.67	0.012
Immunity protection										
59	Chain C, Human Complement Component C3c	gi|78101271	513	70	4.79	40.204	0.3969±0.0391	0.2675±0.0403	0.67	0.034
145	CD5 antigen-like precursor [Homo sapiens]	gi|5174411	443	67	5.28	39.603	0.1086±0.0192	0.1903±0.0197	1.75	0.009

Table 6. Proteins whose serum levels were significantly differed among sustained responders (SVR) and nonresponders (NR) before PEG-Interferon alfa-2b treatment. Continued.

Spot	protein identity	NCBI ID	score	%cov	pI	MW (kDa)	Relative intensity (mean ± SEM) NR	SVR	Ratio SVR/NR	T-test *P* values
Cholesterol metabolism										
17	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I	gi|90108664	1347	78	5.27	28.06	9.4993±0.5044	6.0364±1.0047	0.64	0.005

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

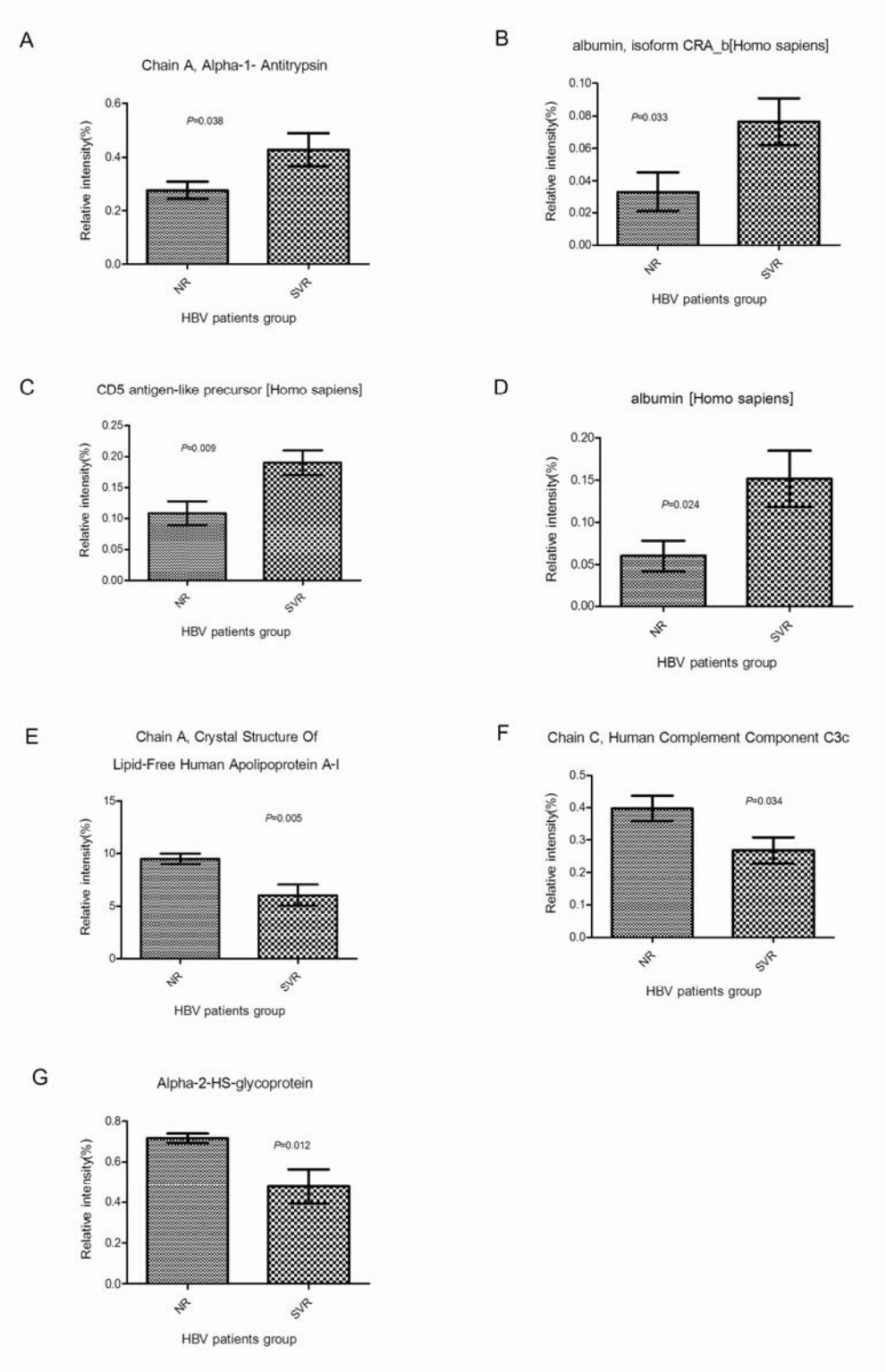
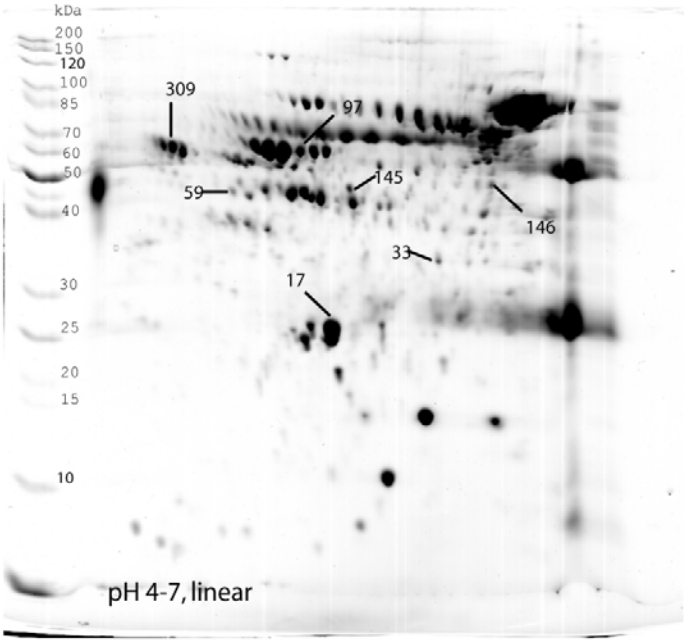
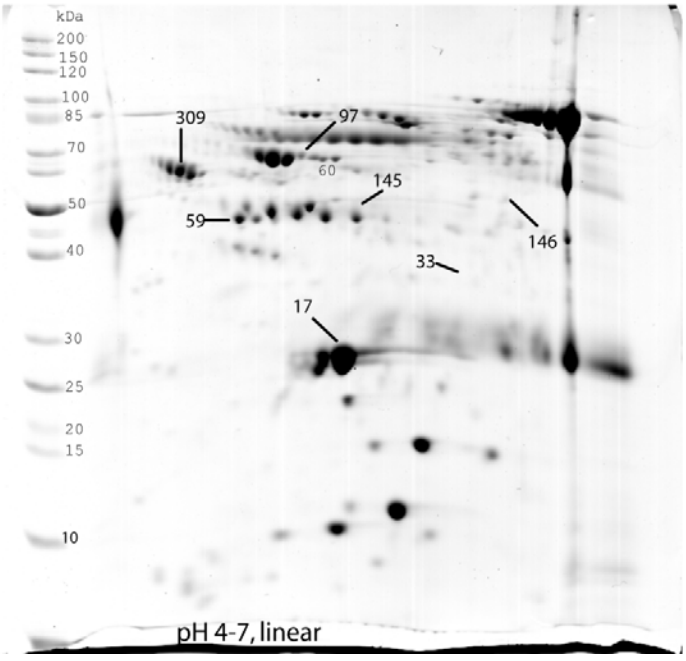


Figure 9. Proteins whose serum levels were significantly differed among sustained responders and nonresponders before treatment. A, B, C, D up-regulated in sustained response. E, F, G up-regulated in non responder. Value of plotted are mean +/- SEM.



(A) sustained responder



(B) nonresponder

Figure 10. 2-DE gels of serum proteins spots were significantly differed among (A) sustained responders and (B) nonresponders before treatment. Total protein load is 150 µg.

3. Comparison different protein expression at 24 weeks and 48 weeks.

There were 14 protein spots found to be different with respect to protein intensity at 24 weeks between sustained responders and nonresponders (Table 7) (Figure 11,12), and there were 2 protein spots found to be different with respect to protein intensity at 48 weeks between sustained responders and non responders (Table 8) (Figure 13,14). Functional characterization of these proteins can be summarized into 4 groups: 1) protease inhibitor: alpha-1-antitrypsin, 2) transport protein and protein binding : serum albumin, serotransferrin precursor, transferrin, albumin,isoform CRA_c, haptoglobin, vitamin D-binding protein 3) acute phase response: serum amyloid P Component 4) immunity protection such as CD5 antigen like protein, Ig alpha-1 chain C region and immunoglobulin light chain. All this proteins, except vitamin D-binding protein, are high expressed in sustained responders compared with non responders.

Table 7. Proteins whose serum levels were significantly differed among Sustained responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 24 weeks.

Spot	protein identity	NCBI ID	score	%cov	pI	MW (kDa)	Relative intensity (mean ± SEM) NR	SVR	Ratio SVR/NR	Mann-Whitney *P* values
Transport protein and protein binding										
4	albumin, isoform CRA_c [Homo sapiens]	gi|119626066	660	42	6.39	27.67	0.0385±0.0000	0.0971±0.0270	3.60	*p*=0.046
141	haptoglobin precursor [Homo sapiens]	gi|306882	66	24	6.24	45.86	0.0180±0.0000	0.0436±0.0074	2.42	*p*=0.046
150	serotransferrin precursor [Homo sapiens]	gi|4557871	986	48	6.81	79.28	0.0908±0.0000	0.1954±0.0236	2.15	*p*=0.046
199	serum albumin [Homo sapiens]	gi|28592	153	27	6.05	71.316	0.0000±0.0000	0.0476±0.0094	absent in NR	*p*=0.037
201	PRO2619 [Homo sapiens]	gi|11493459	278	42	5.96	58.513	0.0000±0.0000	0.0744±0.0139	absent in NR	*p*=0.037
206	transferrin, isoform CRA_c [Homo sapiens]	gi|119599572	196	62	6.67	48.669	0.0000±0.0000	0.0766±0.0117	absent in NR	*p*=0.037
207	albumin [Homo sapiens]	gi|332356380	781	49	5.73	68.484	0.0000±0.0000	0.1930±0.0499	absent in NR	*p*=0.037
264	serum albumin [Homo sapiens]	gi|62113341	115	31	5.85	71.092	0.0000±0.0000	0.0514±0.0096	absent in NR	*p*=0.037
280	albumin [Homo sapiens]	gi|332356380	201	32	5.73	68.484	0.0000±0.0000	0.1398±0.0462	absent in NR	*p*=0.037
312	serum albumin precursor [Homo sapiens]	gi|6013427	151	37	5.91	71.176	0.0000±0.0000	0.0561±0.0179	absent in NR	*p*=0.037
Acute phase protein										
26	chain A, the structure of pentameric human serum amyloid P component	gi|576259	174	43	6.12	23.358	0.0698±0.0000	0.0922±0.0061	1.32	*p*=0.046

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

Table 7. Proteins whose serum levels were significantly differed among Sustained responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 24 weeks. Continued

Spot	protein identity	NCBI ID	score	%cov	pI	MW (kDa)	Relative intensity (mean ± SEM) NR	SVR	Ratio SVR/NR	Mann-Whitney *P* values
Immunity protection										
69	Ig alpha-1 chain C region	gi|113584	61	32	6.08	38.486	0.0233±0.0000	0.0547±0.0045	2.35	*p*=0.046
202	CD5 antigen-like precursor [Homo sapiens]	gi|5174411	70	33	5.28	39.603	0.0000±0.0000	0.0348±0.0037	absent in NR	*p*=0.037
299	immunoglobulin light chain [Homo sapiens]	gi|218783338	747	68	5.95	24.16	0.0000±0.0000	0.1709±0.0238	absent in NR	*p*=0.037

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

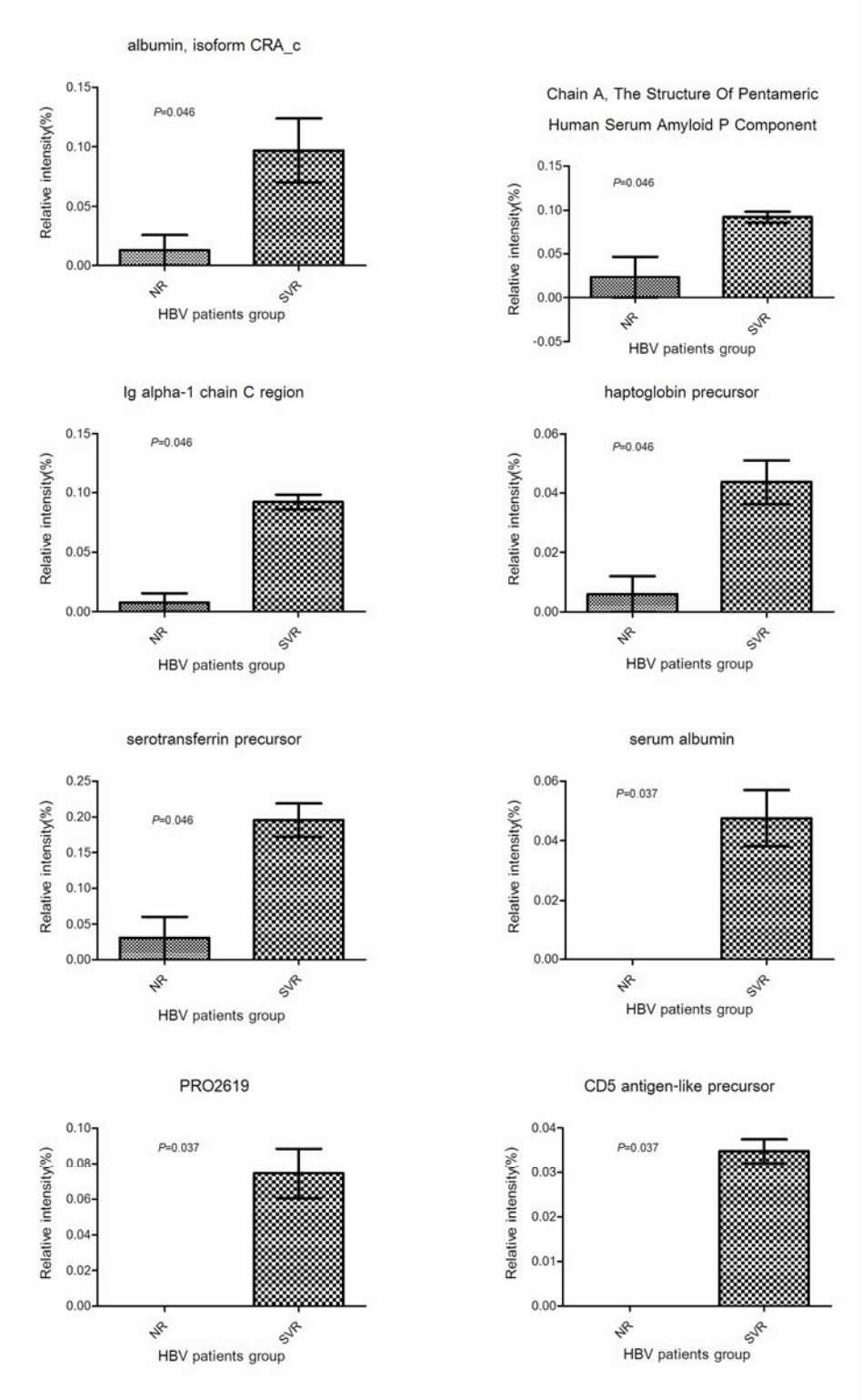


Figure 11. Proteins whose serum levels were significantly differed among sustained responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 24 weeks. Value of plotted are mean +/- SEM.

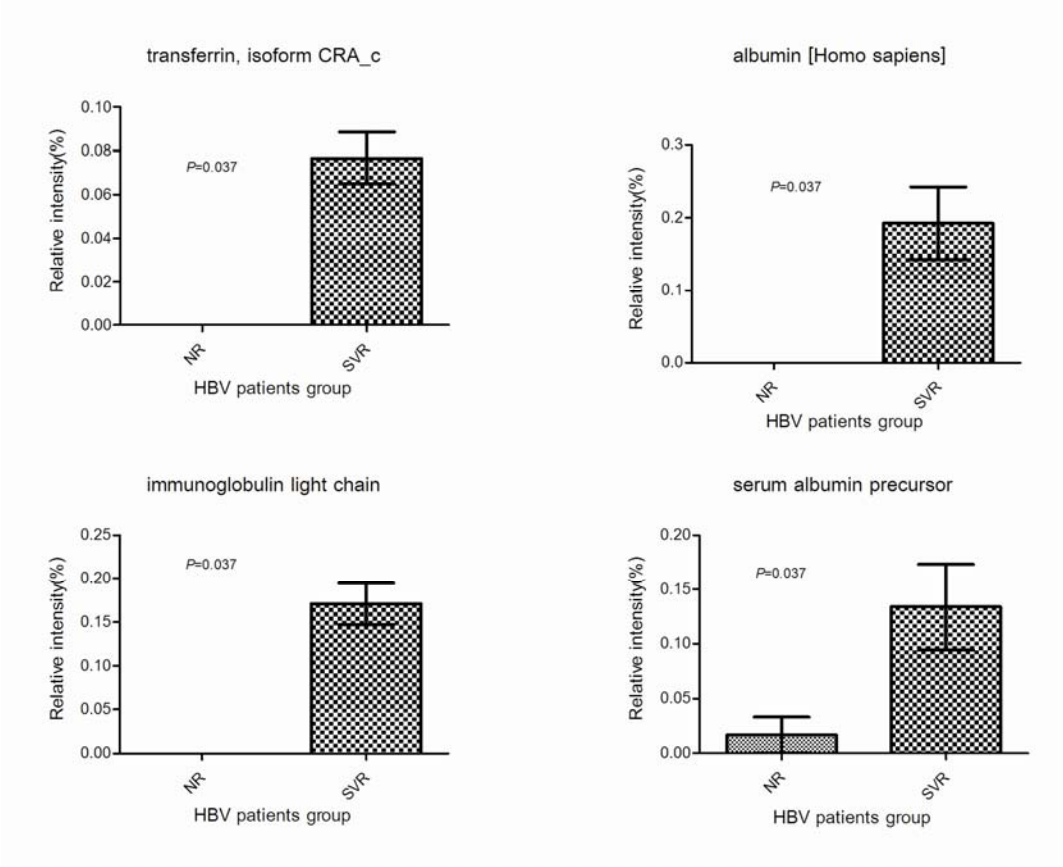
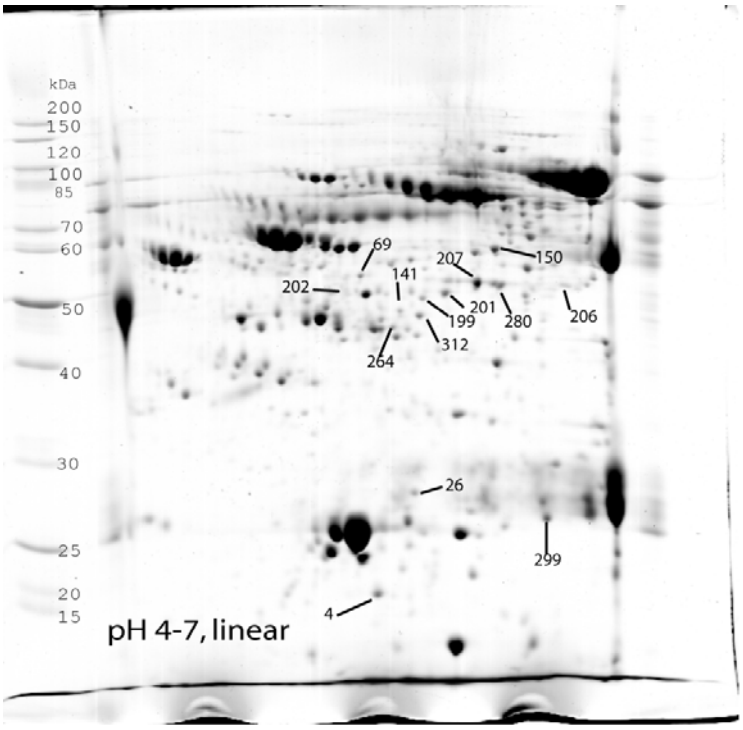
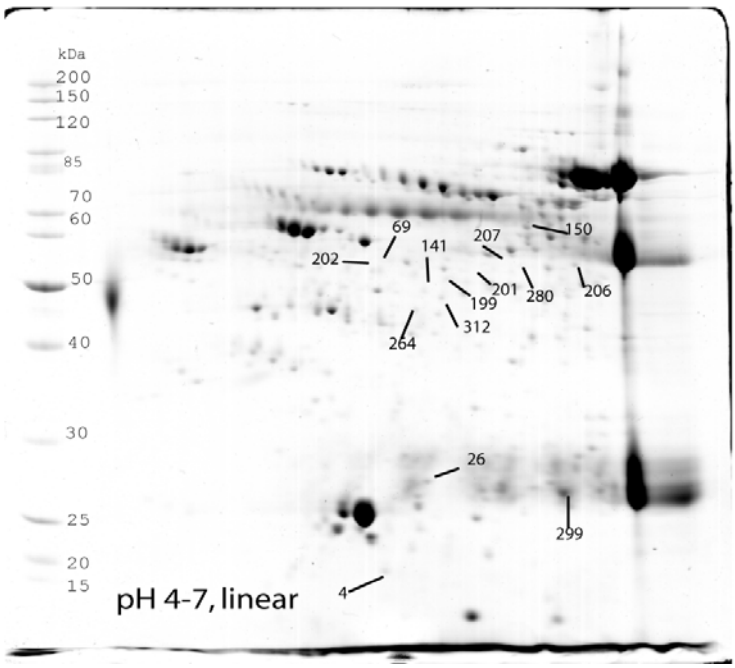


Figure 11. Proteins whose serum levels were significantly differed among sustained responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 24 weeks continued. Value of plotted are mean +/- SEM.



(A) sustained responder



(B) nonresponder

Figure 12. 2-DE gels of serum proteins spots were significantly differed among (A) sustained responders and (B) nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 24 weeks.

Table 8. Proteins whose serum levels were significantly differed among sustained virologic responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 48 weeks.

Spot	protein identity	NCBI ID	score	%cov	pI	MW (kDa)	Relative intensity (mean ± SEM) NR	SVR	Ratio SVR/NR	Mann-Whitney *P* values
Transport protein and protein binding										
32	Vitamin D-binding protein	gi|139641	56	17	5.40	54.526	0.0651±0.0201	0.0000±0.0000	absent in SVR	*p*=0.037
257	serum albumin precursor [Homo sapiens]	gi|6013427	78	17	5.91	71.176	0.0000±0.0000	0.0512±0.0129	absent in NR	*p*=0.037

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

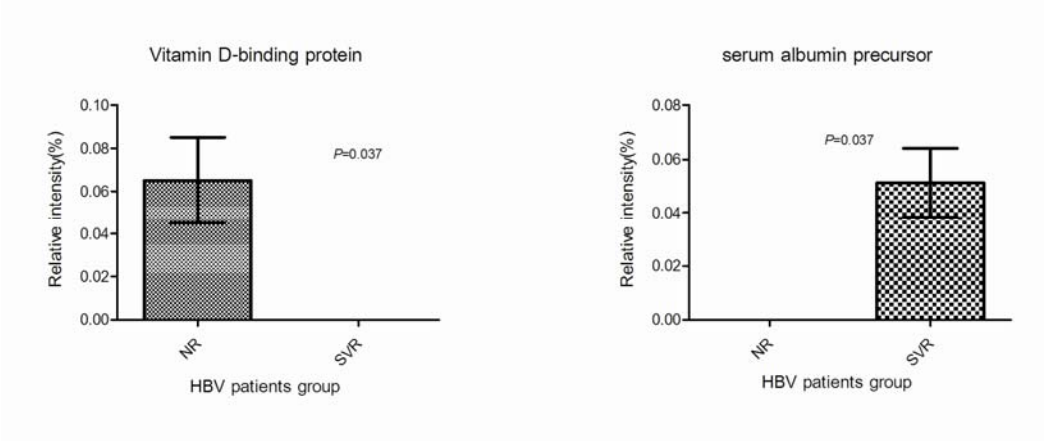
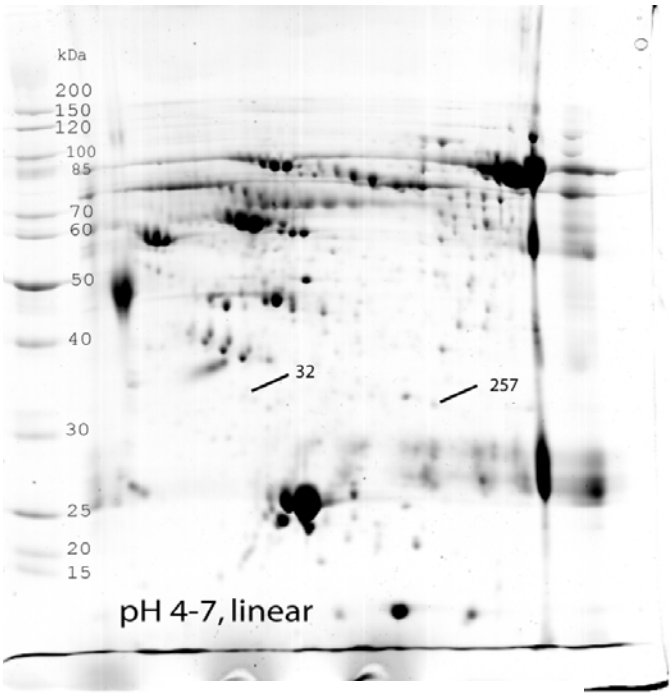
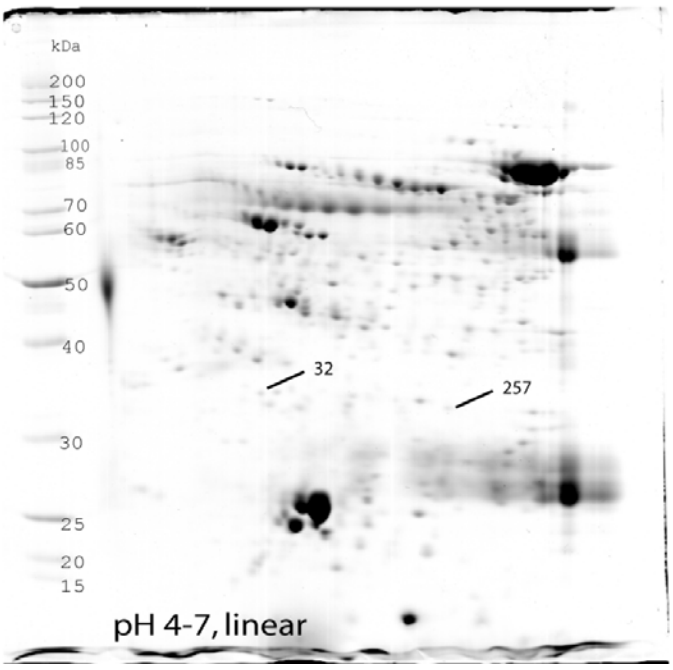


Figure 13. Proteins whose serum levels were significantly differed among sustained responders and nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 48 weeks. Value of plotted are mean +/- SEM.



(A) sustained responder



(B) nonresponder

Figure 14. 2-DE gels of serum protein spots were significantly differed among (A) sustained responders and (B) nonresponders at the timepoint after initial PEG-Interferon alfa-2b treatment for 48 weeks.

4. **Comparison and monitoring the kinetics of serum proteome before starting treatment, after initial PEG-Interferon alfa-2b treatment for 24 weeks and 48 weeks between group of patients with sustained responders and nonresponders.**

All specimens were tested for protein separation by 2D gel electrophoresis, in gel digestion and protein identification by LC/MS/MS. Compared with baseline, 13 protein spots were found to significantly change at the end of 24-week treatment in the sustained response group, and 6 protein spots were significantly changed at the end of the 24-week treatment in nonresponders (Table 9). Compared with the end of 24-week treatment, 6 protein spots were found to significantly change at the end of 48-week treatment in the sustained response group, and 5 protein spots were significantly changed at the end of the 48-week treatment in nonresponders (Table 10) (Figure 15,16,17). Functional characterization of these proteins can be summarized into 5 groups: 1) protease inhibitor: antithrombin Iii 2)transport protein and protein binding: serum albumin, serotransferrin precursor, transferrin, isoform CRA_c, haptoglobin, vitamin D-binding protein 3) acute phase response: serum amyloid P component, alpha-2-HS-glycoprotein 4) immunity protection such as CD5 antigen like protein, Ig alpha-1 chain C region, Ig J-chain and immunoglobulin light chain 5) cholesterol metabolism: proapolipoprotein,

Table 9. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline in sera of patients in the sustained responders and nonresponders.

Group	Spot No.	protein identity	Fold change	NCBI ID	Δ relative intensity	Mann-Whitney
	Cholesterol metabolism					
	20	proapolipoprotein [Homo sapiens]	↑ 5.17	gi|178775	0.1525	*p*=0.018
	Acute phase protein					
	26	Chain A, the structure of pentameric human serum amyloid P component	↑ 6.65	gi|576259	0.0783	*p*=0.021
	86	Alpha-2-HS-glycoprotein	↑ 2.39	gi|112910	0.3773	*p*=0.021
	309	Alpha-2-HS-glycoprotein	↑ 2.04	gi|112910	0.4987	*p*=0.021
	Protease inhibitor					
	171	Chain A, the intact and cleaved human antithrombin Iii complex as a model for serpin-proteinase interactions	↑ 9.66	gi|999513	0.1481	*p*=0.005
Sustained virologic responders	173	Chain A, the intact and cleaved human antithrombin Iii complex as a model for serpin-proteinase interactions	↑ 8.01	gi|999513	0.0694	*p*=0.005
	Transport protein and protein binding					
	149	serotransferrin precursor [Homo sapiens]	↑ 3.43	gi|4557871	0.1165	*p*=0.019
	201	PRO2619 [Homo sapiens]	↑ 4.95	gi|11493459	0.0594	*p*=0.023
	207	albumin [Homo sapiens]	↑ 6.97	gi|332356380	0.1653	*p*=0.010
	280	albumin [Homo sapiens]	↑ 7.33	gi|332356380	0.1206	*p*=0.018

Table 9. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline in sera of patients in the sustained responders and nonresponders. Continued

Group	Spot No.	protein identity	Fold change	NCBI ID	Δ relative intensity	Mann-Whitney
	Immunity protection					
Sustained	202	CD5 antigen-like precursor [Homo sapiens]	↑ absent at baseline	gi|5174411	0.0348	*p*=0.001
virologic	229	Ig J-chain [Homo sapiens]	↑ absent at baseline	gi|532598	0.0923	*p*=0.001
responders	299	immunoglobulin light chain [Homo sapiens]	↑ absent at baseline	gi|218783338	0.1709	*p*=0.001
	Cholesterol metabolism					
	20	proapolipoprotein [Homo sapiens]	↑absent at baseline	gi|178775	0.2383	*p*=0.001
	Protease inhibitor					
	171	Chain A, the intact and cleaved human antithrombin Iii complex as a model for serpin-proteinase interactions	↑2.39	gi|999513	0.0896	*p*=0.026
Nonresponders	**Transport protein and protein binding**					
	4	albumin, isoform CRA_c [Homo sapiens]	↓0.04	gi|119626066	-0.2832	*p*=0.011
	264	serum albumin [Homo sapiens]	↓absent at 24 wk	gi|62113341	-0.1196	*p*=0.011
	Serum protein					
	123	alpha-1-B-glycoprotein – human	↑1.38	gi|69990	0.1610	*p*=0.018
	Acute phase protein					
	310	Alpha-2-HS-glycoprotein	↓0.46	gi|112910	-0.4119	*p*=0.011

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

Table 10. Differentially changed serum levels of proteins at 48 weeks after treatment compared with 24 weeks after treatment in sera of patients in the sustained responders and nonresponders.

Group	Spot No.	Protein identity	Fold change	NCBI ID	Δ relative intensity	Mann-Whitney
	Transport protein and protein binding					
	201	PRO2619 [Homo sapiens]	↓0.09	gi|11493459	0.0676±0.01	*p*=0.046
Sustained	264	serum albumin [Homo sapiens]	↓absent at 48 wk	gi|62113341	0.0514±0.01	*p*=0.037
virologic	207	albumin [Homo sapiens]	↓0.03	gi|332356380	0.1879±0.08	*p*=0.046
responders	202	CD5 antigen-like precursor [Homo sapiens]	↓0.27	gi|5174411	0.0253±0.02	*p*=0.046
	Immunity protection					
	229	Ig J-chain [Homo sapiens]	↓absent at 48 wk	gi|532598	0.0923±0.06	*p*=0.037
	264	serum albumin [Homo sapiens]	↓absent at 48 wk	gi|62113341	0.0514±0.01	*p*=0.037
	299	immunoglobulin light chain [Homo sapiens]	↓absent at 48 wk	gi|218783338	0.1709±0.04	*p*=0.037
	Transport protein and protein binding					
	4	albumin, isoform CRA_c [Homo sapiens]	↑9.33	gi|119626066	0.1069±0.01	*p*=0.046
Nonresponders	150	serotransferrin precursor	↑5.34	gi|4557871	0.1312±0.12	*p*=0.046
	32	Vitamin D-binding protein	↑absent at 24 wk	gi|139641	0.0651±0.27	*p*=0.034
	Acute phase protein					
	26	Chain A, the structure of pentameric human serum amyloid P component	↑11.37	gi|576259	0.2415±0.27	*p*=0.046
	Immunity protection					
	69	Ig alpha-1 chain C region	↑9.53	gi|113584	0.0662±0.06	*p*=0.046

Relative intensity; [(volume of spot/volume over all the spots in the gel)x100], NCBI National Center for Biotechnology Information

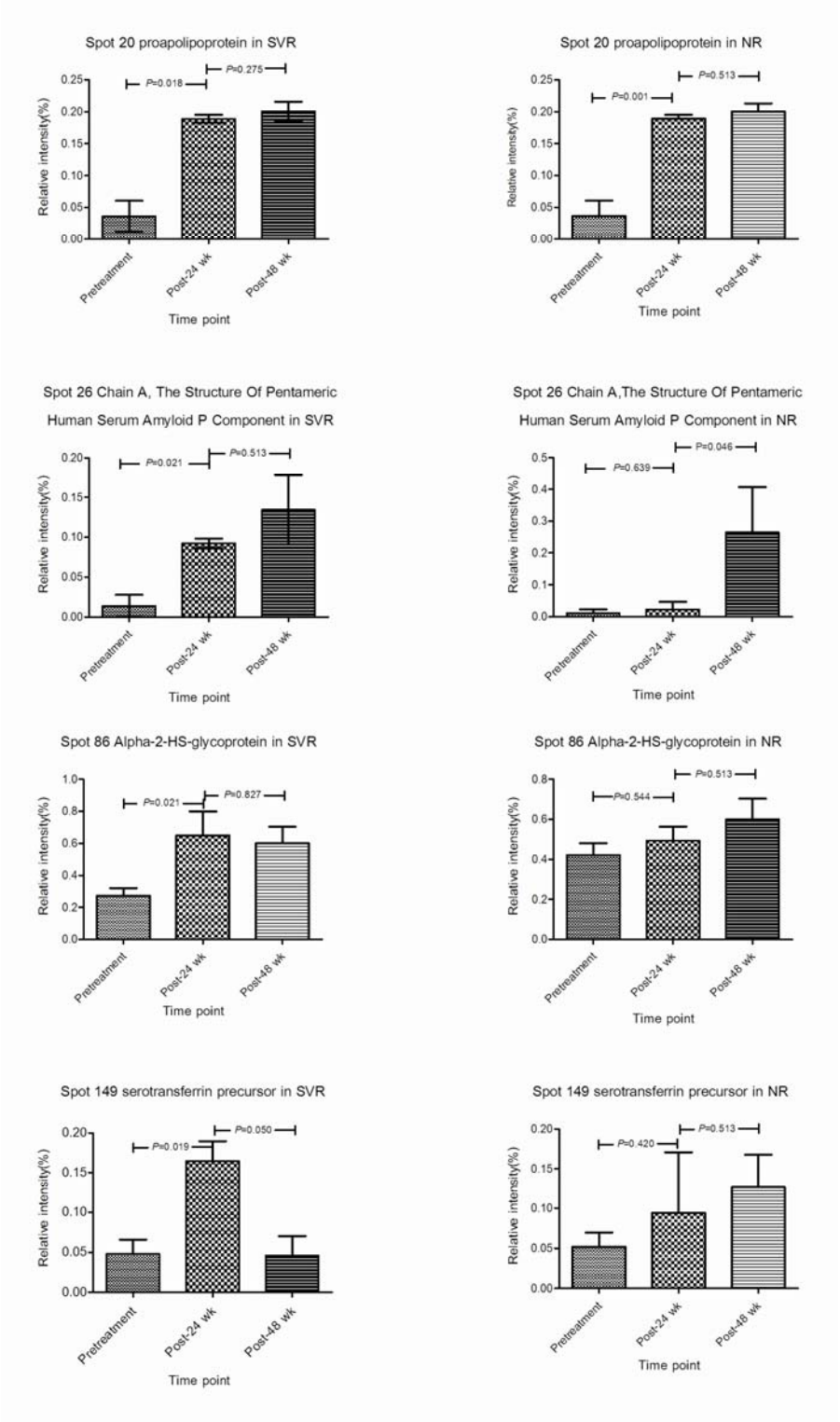


Figure 15. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline, serum levels of proteins at 48 weeks after treatment compared with 24 weeks after treatment in sera of patients in the SVR and NR. Value of plotted are mean +/- SEM.

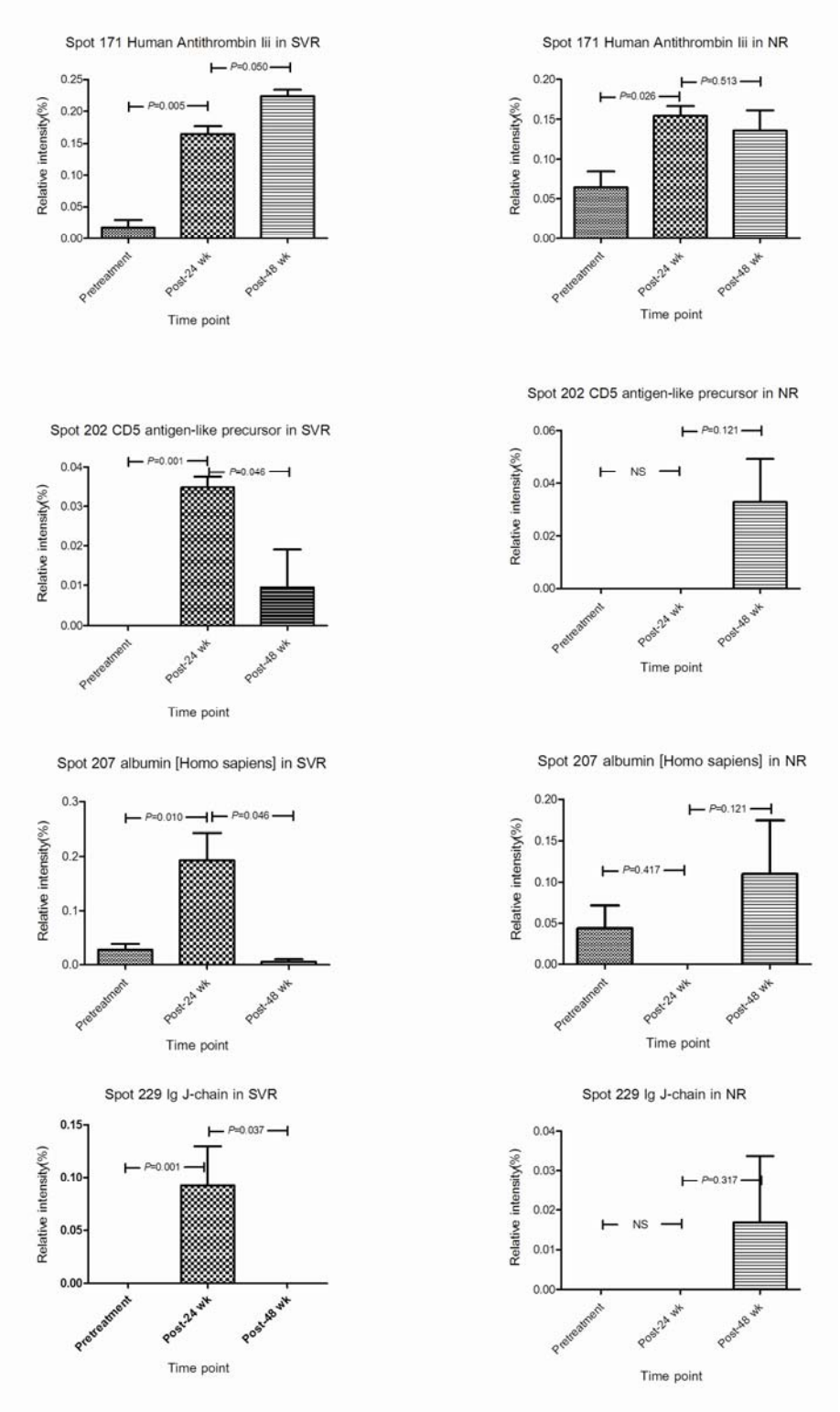


Figure 15. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline, serum levels of proteins at 48 weeks after treatment compared with 24 weeks after treatment in sera of patients in the SVR and NR continued. Value of plotted are mean +/- SEM.

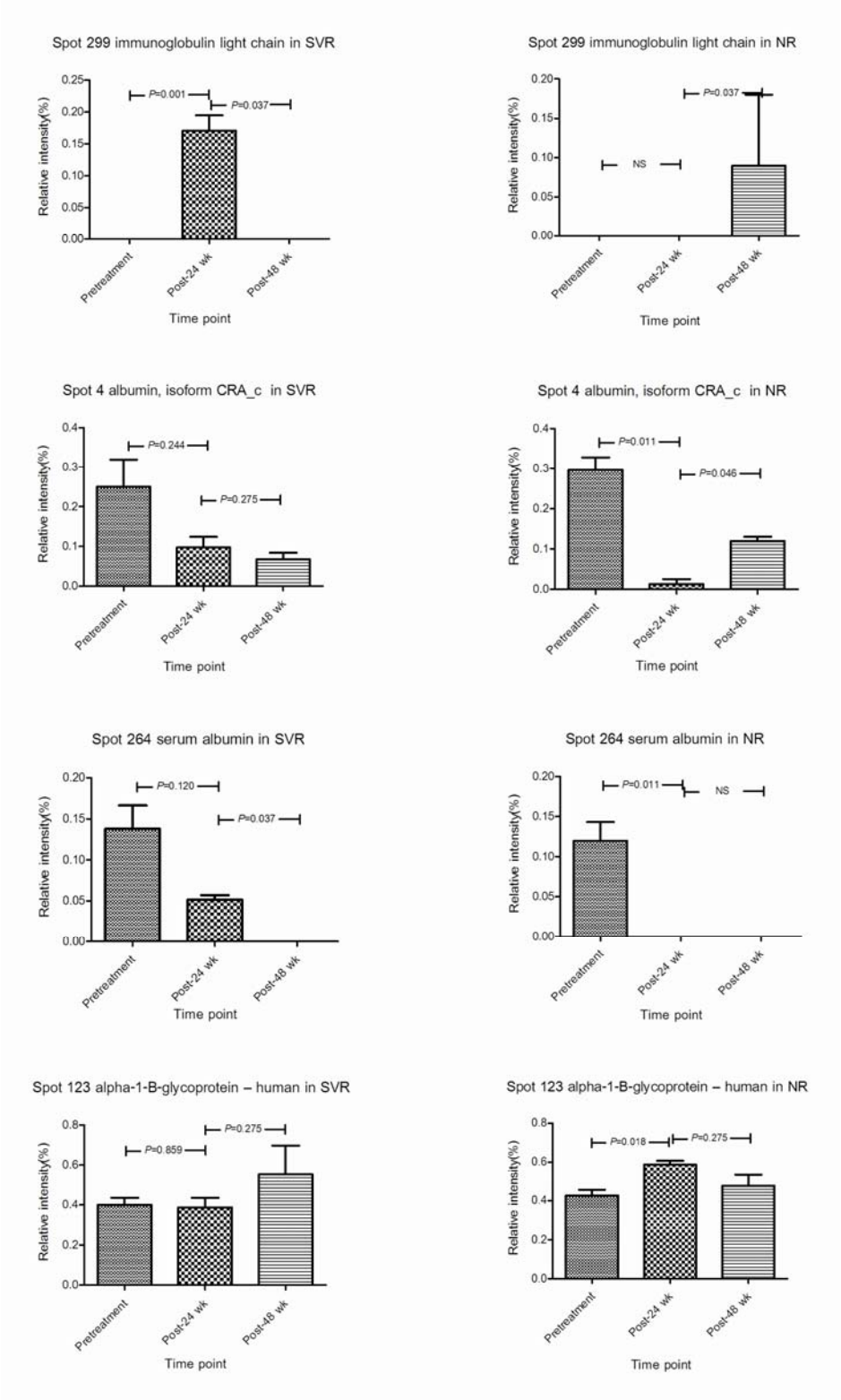


Figure 15. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline, serum levels of proteins at 48 weeks after treatment compared with 24 weeks after treatment in sera of patients in the SVR and NR continued. Value of plotted are mean +/- SEM.

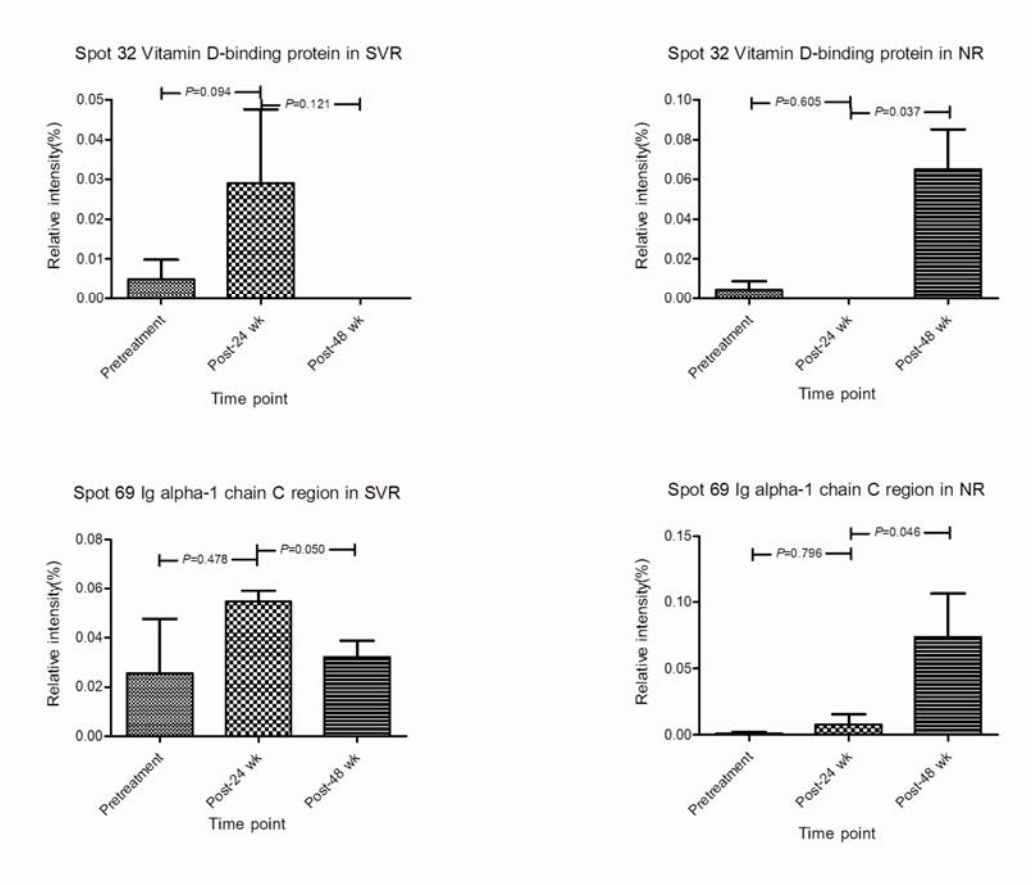
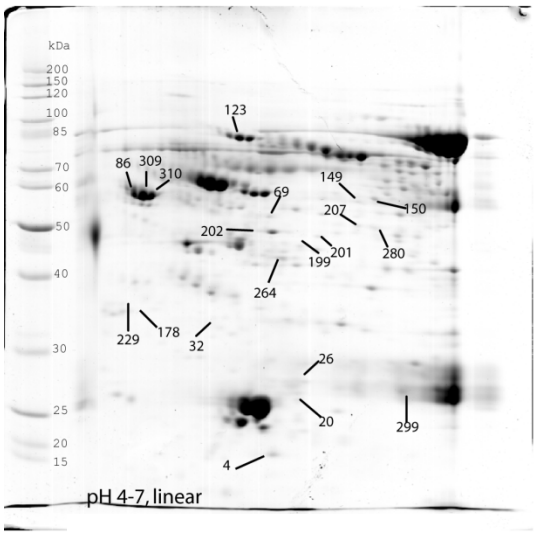
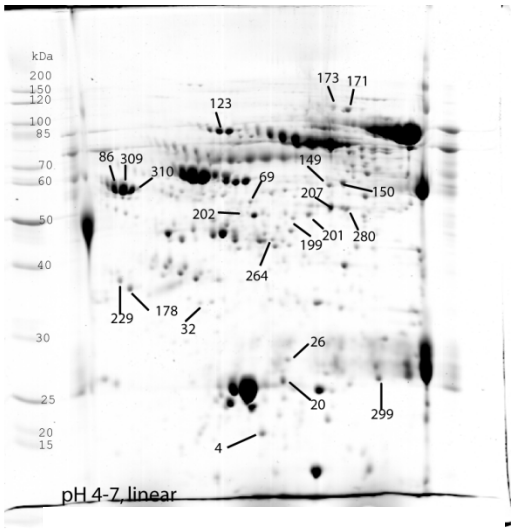


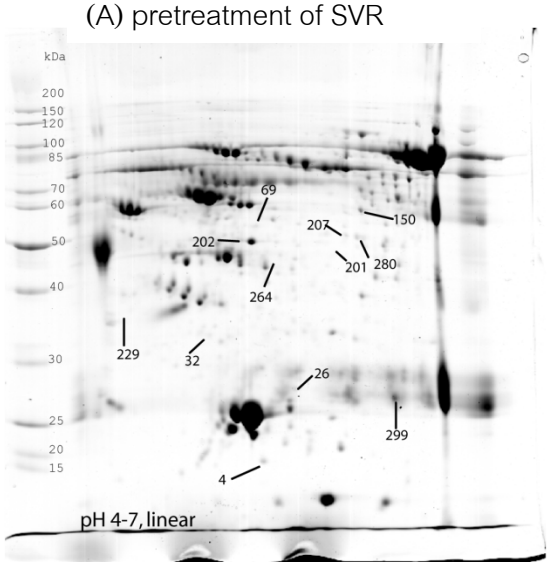
Figure 15. Differentially changed serum levels of proteins at 24 weeks after treatment compared with baseline, serum levels of proteins at 48 weeks after treatment compared with 24 weeks after treatment in sera of patients in the SVR and NR continued. Value of plotted are mean +/- SEM.



(A) pretreatment of SVR

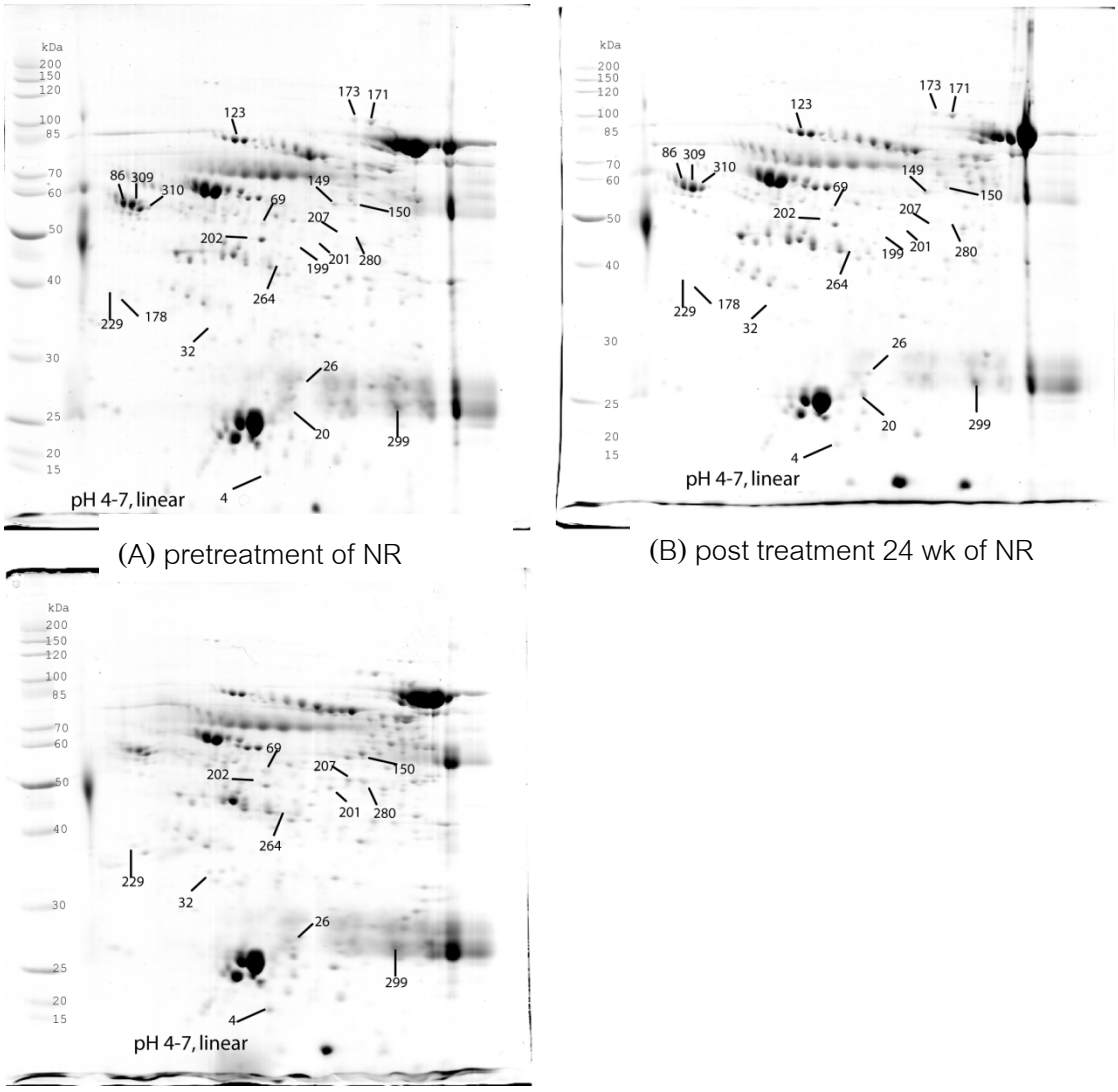


(B) post treatment 24 wk of SVR



(C) post treatment 48 wk of SVR

Figure 16. 2-DE gels of serum proteins spots were found to significantly change at the end of 24-week treatment and the end of the 48-week treatment in sustained responder. (A) pretreatment of SVR, (B) post treatment 24 wk of SVR, (C) post treatment 24 wk of SVR.



(A) pretreatment of NR

(B) post treatment 24 wk of NR

(C) post treatment 48 wk of NR

Figure 17. 2-DE gels of serum proteins spots were found to significantly change at the end of 24-week treatment and the end of the 48-week treatment in nonresponder.

(A) pretreatment of NR, (B) post treatment 24 wk of NR, (C) post treatment 24 wk of NR.

CHAPTER V

DISCUSSION

One of the main community health troubles worldwide is hepatitis B virus (HBV) infection and it leads to liver fibrosis, liver cirrhosis, and liver cancer as we know hepatocellular carcinoma. Two major approaches for chronic hepatitis B treatment exist which are 1) immunomodulatory therapies for example pegylated interferon (PEG-interferon) and interferon-α 2) and antiviral drugs such as nucleoside or nucleotide analogs (NAs). NAs are inhibiting of HBV DNA replication process by blocking DNA polymerase enzyme(1) Immune-modulating therapy includes mainly (Pegylated) Interferon-α and Interferon-α. The advantages of type 1 interferons include a fixed therapy time, durable off- treatment response, and no viral drug resistant(2) However, only 30% of the chronic HBV patients respond to IFN type I treatment(2, 3) Both viral and host factors likely 2 key factors in determining the treatment responses. Therefore, the biomarkers that can be used to predict treatment responses will be very useful and cost-effectiveness for CHB patients treatment.

Many researchers have tried to discover potential host biomarker to predict HBV treatment response by using: 1) single–nucleotide polymorphisms (SNP), 2) mRNA gene expression in PBMC and liver cells, 3) host immune response by detecting cytokines level, T cell response and Toll like receptor on monocyte, but little study has been reported about proteomic approach to predict response treatment. The proteomic approach is considered to be used for analysis protein expression. It can be used with various specimens such as tissue, serum, plasma, or body fluids. In this study we choose serum samples from patients to study proteomic biomarker because 1) serum collection is non-invasive, 2) new biomarkers especially proteins from the liver can secrete to the serum, 3) there has non hypothesis screening for new proteins, and 4) serum proteomic can detect post translational modification. In this case we have chosen 2-DE and mass spectrometry for protein separation and identification because

2-DE has high resolution, it can detect post-translational modifications and mass range 10–200 kDa proteins. Mass spectrometry is high sensitivity and high throughput. This approach has proved to identify new biomarker in many systems for example ovarian cancer, prostate cancer, breast cancers, and hepatocellular carcinoma. Anyway, in 2-DE also has limitation, it cannot detect low abundant proteins because the high abundant protein for example albumin and IgG can suppress low abundant proteins expression. To solve this solution, albumin and IgG were removed from all serum samples before electrophoresis, using ProteoPrep Blue Albumin Depletion Kit. Albumin (~45 mg/ml) and IgG (~10 mg/ml) are two main protein components of serum, representing 60–70% and 10–20 % of the total serum protein(44) When the high abundant proteins were depleted from serum, the low abundant proteins spots were more clearly expression. In contrast, the low abundant protein could bind to albumin and IgG. The high abundant proteins removing might be a reason of the important low abundant proteins that bound to albumin and IgG losing at the same time(45) If we deplete the more numbers of abundant proteins, the more numbers of important proteins which binding to albumin and IgG may possibly loose(46) For this reason, we choose to remove only albumin and IgG to decrease low abundant protein-bound albumin or IgG depletion. As expected, in the untreated sample albumin predominated the gel, obscuring signals from less abundance proteins. When an equal quantity of protein is pre-treated with ProteoPrep Blue Albumin Depletion Kit, generated 2D gels showed a significantly improved resolution and clearly protein spots were detected.

Recently, researchers have reported the novel serum biomarkers predicting interferon treatment response in patients. Gandhi, KS. *et al*. has reported that “alpha-2 macroglobulin, fibrinogen beta chain, and apolipoprotein A1 as the novel serum biomarkers in Multiple Sclerosis patients serum treated by interferon-β”(47) Fujita, N., *et al.* has reported that “Hemopexin, apolipoprotein A1 and transferrin as the novel serum biomarkers in the pretreatment serum of HCV patients treated with PEG-Interferon in combination with ribavirin”(37) Also, the report from MA Hui. *et al* has shown that “alpha-2-HS-glycoprotein level maybe a serum biomarker for prediction hepatitis e seroconversion in CHB patients treated by PEG-interferon alfa-2b”(36) The summary of

previous studies about biomarker of HCV, HBV, and multiple sclerosis predicting treatment response compared with our study has shown in table 11.

Table 11. Proteomic studies to predict treatment response in HBV, HCV, and multiple sclerosis treated with interferon.

Disease	Sample	Method	Study size	Principal finding
HBV treated PEG-IFN α2b MA Hui *et al*. [2011]	Serum	2-DE LC-ESI-MS/MS	4 HBe seroconversion 24 non HBe seroconversion (pooled samples)	Up-regulated proteins in SVR - Leucine-rich-α2-glycoprotein) - α2-HS-glycoprotein - Haptoglobin Down-regulated proteins in NR - Leucine-rich-α2-glycoprotein) - Apo CIII precursor - α-albumin
HCV treated PEG-IFN plus ribavirin Fujita N. *et al*. [2011]	Serum	SELDI-TOF/MS Chromatography SDS-PAGE	10 SVR 12 TR 10 NR	Up-regulated proteins in SVR - Apolipoprotein AI - albumin - Apolipoprotein CI Down-regulated proteins in SVR - Hemopexin - Transferrin - CTAP-III
Multiple sclerosis treated IFN-β Gandhi KS *et al*. [2010]	Serum	DIGE MALDI-TOF	3 clinical responders 3 non responders	Up-regulated proteins in responders - Alpha 2 macroglobulin - Fibrinogen beta chain - Apolipoprotein AI
This experiment	Serum	2-DE LC/MS/MS	9 SVR 10 NR	Up-regulated proteins in SVR - alpha-1- antitrypsin - albumin, isoform CRA_b - CD5 antigen-like precursor - serum albumin Down-regulated proteins in SVR - apolipoprotein A-I - chain C, human complement component C3c - alpha-2-HS-glycoprotein

The major objective of our study is to discovery serum biomarker from the patient before start the treatment. We compared the significant expressed proteins before PEG-Interferon alfa-2b treatment from 9 sustained responders compared to 10 non responders. Interestingly, there were 7 protein spots found to be different with respect to protein intensity between sustained responders and nonresponders. The 4 proteins that were significantly up-regulated in sustained responders are 1) chain A, alpha-1- antitrypsin, 2) albumin, isoform CRA_b, 3) CD5 antigen-like precursor and, 4) albumin [Homo sapiens]. Three proteins were significantly up-regulated in non responders: 1) chain A, crystal structure of lipid-free human apolipoprotein A-I, 2) chain C, human complement component C3c, 3) alpha-2-HS-glycoprotein. Compared our results with the results of MA Hui *et.al*, they did not find the significantly different proteins expression before treatment, but they found the significantly up-regulated of alpha-2-HS-glycoprotein at 24 week post treatment compared with baseline in sustained responders group same as our experiment. The different results of our experiment and MA Hui *et.al* report at the baseline maybe possible because of this reason: 1) their experiment did not deplete high abundant proteins, 2) they pooled the sera in the process on 2-DE, and 3) we have much more sample size in the experiment. Compared our results with the results of Fujita N. *et al*. in HCV, we found the serum albumin up-regulated at the baseline in sustained responders, but we have the different results from them as we have found apolipoprotein A-I down-regulated in sustained responders but they have found apolipoprotein A-I up-regulated in sustained responders. The different results of our experiment and Fujita N. *et al*. report at the baseline maybe possible because of this reason: 1) we used the different method for proteomic analysis, they used SELDI-TOF/MS but we used 2-DE with LC-MS/MS, and 2) we have studied in the different disease. Compared our results with the results of Gandhi KS *et al*. in multiple sclerosis, we have found the apolipoprotein A-I is low expression at the baseline in clinical responders before treatment, but they didn't find different expression protein. They have found apolipoprotein A-I up-regulated in clinical responders same as our result that we have found apolipoprotein A-I up-regulated in SVR. The different results of our experiment and Gandhi KS *et al*. report at the baseline maybe possible because of

this reason: 1) we used the different method for proteomic analysis, they used DIGE, which is more high sensitivity but we used 2-DE stained with comassie blue, which is less sensitivity, and 2) we have studied in the different disease. This proteins need to be validated by other methods. We are in process of validation level of some proteins by ELISA in CHB. Table 12 shows the comparison results of our study and other previous studies.

Table 12. Result comparison between our results with the previous reports.

	Similar report	Different report
Our report	Up regulated of alpha-2-HS-glycoprotein at 24 week after treatment	Found 7 protein spots significantly expressed before treatment
MA Hui *et.al*	Up regulated of alpha-2-HS-glycoprotein at 24 week after treatment	No found different expression protein before treatment
Our report	serum albumin up-regulated at the baseline in SVR	apolipoprotein A-I down-regulated in SVR
Fujita N. *et al*.	serum albumin up-regulated at the baseline in SVR	apolipoprotein A-I up-regulated in SVR
Our report	apolipoprotein A-I up-regulated in SVR	apolipoprotein A-I is low expression in SVR before treatment
Gandhi KS *et al*.	apolipoprotein A-I up-regulated in clinical responders	No found different expression protein before treatment

We also analyzed proteins with significant alterations related with PEG-Interferon alfa-2b treatment in CHB patients at 24 weeks to identify significant up-regulated or down-regulated proteins that associated with treatment responses. Compared with baseline, 13 protein spots were significantly changed at the end of 24 weeks treatment in the sustained responders, and 6 protein spots were significantly changed at the end of the 24 weeks treatment in nonresponders. Level of CD5 antigen like protein, proapolipoprotein, serum amyloid P component, alpha-2-HS-glycoprotein, serotransferrin precursor, antithrombin Iii, immunoglobulin light chain, Ig J-chain and serum albumin were up-regulate as compared with baseline in sustained responders.

As expected, proteins related with immunity protection such as CD5 antigen-like protein, immunoglobulin light chain, Ig J-chain had the same alteration pattern in sustained responders (Figure 18). They were higher expression in the serum of sustained responders compared to non-responders at 24 weeks after initial PEG-Interferon alfa-2b treatment, which is in accordance with the role of interferon in modulating the immune systems, Furthermore the proteins related with immunity protection were significantly changed at the end of 24 weeks treatment in the sustained response compared with baseline. The most prominent data of our study is that the significant expression of CD5 antigen-like protein. Its protein level was higher in responder group even before the treatment compared with nonresponder group. Then it continued to up-regulated in SVR after treatment of PEG-Interferon alfa-2b for 24 weeks. This protein was not detected in nonresponders before treatment and 24 weeks after treatment. Therefore, we choose this protein to further validate by ELISA. However, note that the level of CD5L is decreased in responder group at the end of treatment (48 weeks) and start to increase in the non-responder group. It is maybe possible because of the delay immune response in nonresponders (Figure 19).

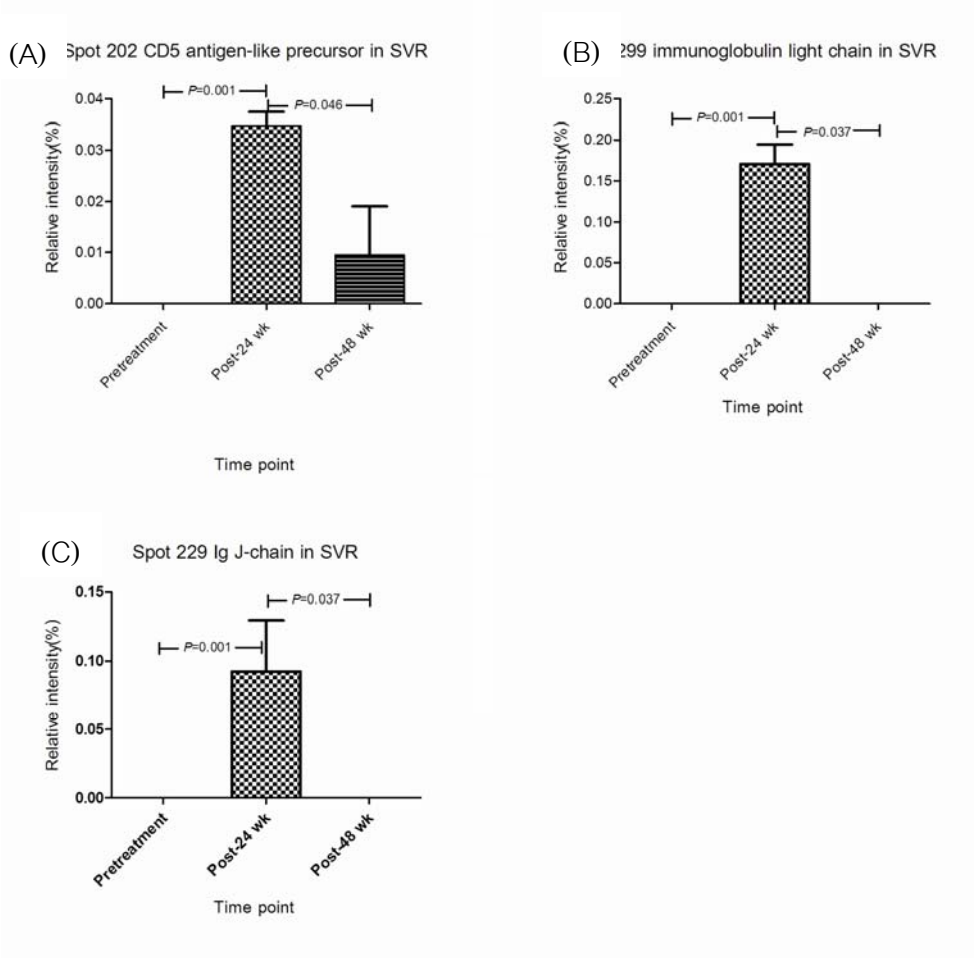


Figure 18. Proteins related with immunity protection (A) CD5 antigen-like protein, (B) immunoglobulin light chain, (C) Ig J-chain pattern in sustained responders.

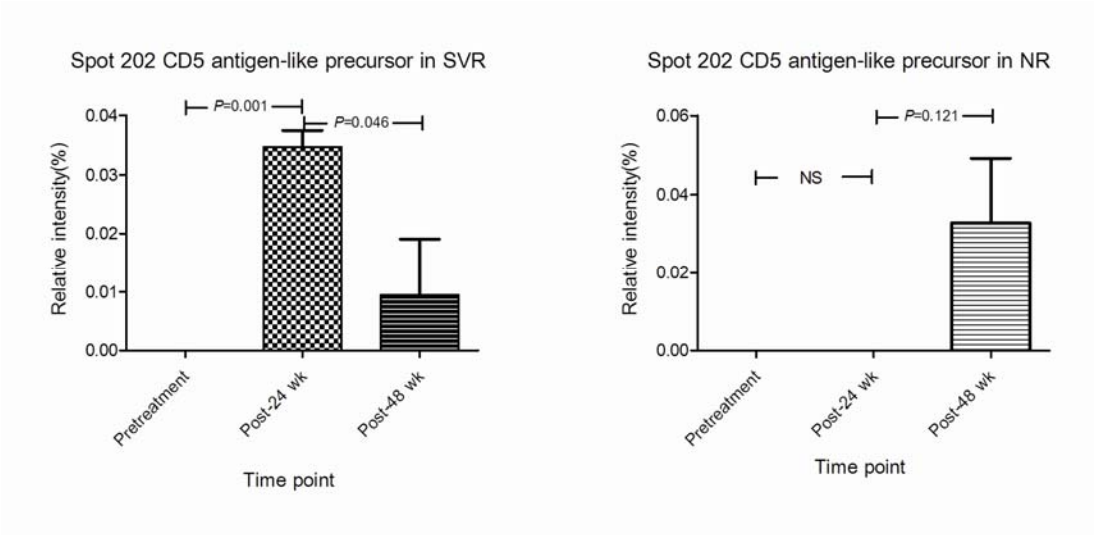


Figure 19. Pattern of CD5L alteration after treatment in sustained responders and nonresponders.

Tissot, J.D., *et al* has reported that “CD5 antigen-like molecule (CD5L; Spα) has reported that it is belonging to the scarvenger receptor cysteine-rich (SRCR) superfamily of proteins. Its molecular weight is 38-kDa. It is soluble glycoprotein, consist of 3 SRCR domains, and it could be expressed by macrophages which living in lymphoid tissues such as thymus, but not in non-lymphoid tissues”(48) Arai, S. *et al.* has reported that “CD5L plays a role in the innate and adaptive immune response regulation”(49) Tissot, J.D., *et al* has reported that “’CD5L also plays a role in the homeostasis of immunoglobulin M(IgM)”(48), and anti-apoptotic effects(50) CD5L was reported that it was up-regulated in bronchoalveolar lavage in asthma compared with healthy control(51), it was up-regulated in serum of liver cirrhosis compared with healthy control(52), and it also was up-regulated in serum in atopic dermatitis compared with healthy control(53) Recently report has suggested that CD5L plays a role in resistance to apoptosis resistant inducing in T cells and NKT cells and supports host defense mechanism(54) From our study, CD5L was significant up-regulated in sustained responders, suggesting that it could be potential marker for interferon treatment response.

In this study, α-2-HS-glycoprotein appeared to be a novel candidate as early marker for predicting sustained virologic response in CHB treated by PEG-Interferon alfa-2b. α-2-HS-glycoprotein was increased expression in sustained responders before treatment, whereas it was decreased expression in non responders. Compare with baseline, it was also up-regulated in SVR 24 weeks after treatment of PEG-Interferon alfa-2b, whereas this protein was down-regulated in nonresponders at 24 weeks after treatment.

α-2-HS-glycoprotein is an high abundant protein produced from liver and also in osteoblasts, and concentrated into mineralized tissues. It is belonging to a member of the cystatin super family(55) α-2-HS-glycoprotein is involved in different conditions for

example metabolic disease, tumor, and sepsis(56, 57) Variations of this protein have been reported in patients with liver diseases(58-60) Some observation found low serum levels of α-2-HS-glycoprotein in patients with acute drug-induced hepatitis, alcoholic hepatitis, and chronic autoimmune hepatitis, as well as in primary biliary cirrhosis, fatty liver, and HCC patients. In 2011, Xia-Hong Dai *et al.* has reported that "α-2-HS-glycoprotein is an independent marker of liver injury and a prognostic marker of hepatitis B virus chronicity, they suggested that it may perhaps decrease liver inflammation by partly inhibiting release of inflammatory factors from activated peripheral blood mononuclear cells"(61) MA Hui *et al.* has reported that "α-2-HS-glycoprotein levels in CHB patients serum were significantly up-regulated at the end of 24 week therapy in the HBeAg seroconversion patients"(36) Recently report of MA Hui *et al.* is correlated with our study that the level of α-2-HS-glycoprotein is significantly altered in patients treated by PEG-Interferon alfa-2b. Thus, α-2-HS-glycoprotein might be a possible biomarker for prediction PEG-Interferon alfa-2b treatment response in CHB.

In this experiment, protein with the function of transport such as apolipoprotein A1 and serotransferrin had the same pattern of increase at the 24 weeks after PEG-Interferon alfa-2b treatment compared with baseline (Figure 20), which implied the malfunction of the liver with the progress of liver cell injury. Apolipoprotein A1 (Apo A-1), as one composition of high-density lipoprotein (HDL), is secreted by hepatocytes and small intestine. Apo A-1 has important anti-inflammatory properties(62, 63) It is reported that inflammatory and viral infection are correlated with decreased Apo A-1 and serum HDL levels(64) Many previous studies have reported that the level of Apo A-I correlates with CHB and CHB -related HCC(65, 66) Some researchers reported that in patients with severe liver cell injury would have a low level of Apo A-I. It is considered that ApoA-I management is happened at the post-translational level(67), and liver cell injuries reduces the ApoA-I alteration(68) He QY *et al*. has reported that "ApoA-I level was significantly up-regulated in normal sample compared with its level in chronic HBV infection and it also changed in their pattern of isoforms, especially three main isoforms of apoA-I change their expression level separately"(14) Recently report of White, I.R., *et*

al has shown that “Apo A-I expression was reduced in chronic hepatitis C virus infection patients with advanced disease compared with mild fibrosis, it is indicated that downregulation of Apo A-I is correlated with disease progression”(69) Other report from Ye Lu *et al.* has shown that “apolipoprotein was down-regulated along fibrosis progress in CHB patients compared with normal samples”(45), also the report from Ren F. *et al.,* has shown that “Apo A-I was down-regulated in patients with chronic hepatitis B and patients with acute-on-chronic liver failure compared with normal healthy”(70)

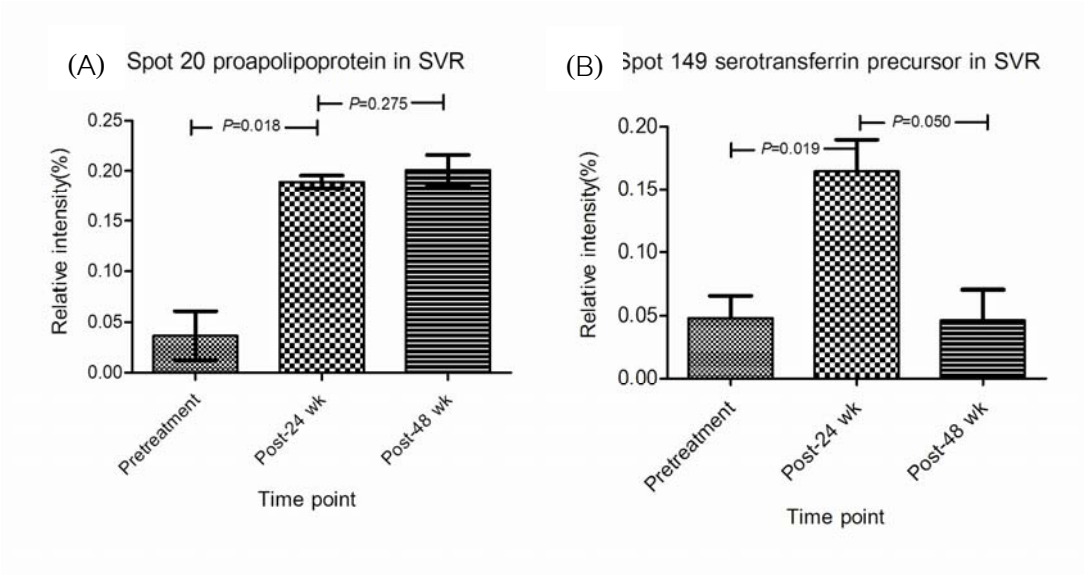


Figure 20. Proteins related with transport. (A) proapolipoprotein, (B) serotransferrin pattern in sustained responders.

In this study, Apo A-I was increased expression in non responders before treatment, compared with sustained responders. It may be related with the disease progression. Compare with baseline, Apo A-I was also up-regulated in SVR 24 weeks after treatment of PEG-Interferon alfa-2b, and the same as this protein was up-regulated in nonresponders at 24 weeks after treatment, suggesting that this alteration of this proteins is implied the regeneration of the liver cells.

Serotransferrins is transport proteins which carrying the iron from assimilation site to storage site or utility site. Serotransferrins is synthesized in the liver.

Furthermore, it may function as cell proliferation stimulation. In this study, serotransferrin was up-regulated in SVR at 24 weeks after treatment of PEG-Interferon alfa-2b compare with baseline, and it also was up-regulated in SVR 24 weeks at the timepoint 24 weeks compared with NR. Previous studies of Feng, J.T., *et al* have shown that "the level of serotransferrin was down-regulated in HBV infection patients and HCC compared with healthy normal"(12), and also report from Kim, W.K., *et al* have reported that "serotransferrin was down-regulated in atopic dermatitis patients compared with healthy normal"(53) Ye Lu *et al.* has shown that "serotransferrin was down-regulated along fibrosis progress in CHB patients compared with normal samples"(45) From our study, suggesting that the up-regulation of serotransferrin after treatment of PEG-Interferon alfa-2b may relate with the regeneration of the liver cells.

However, little report about these profile proteins using protein microarrays or mRNA expression to study about serum HBV proteomic treated by interferon, only one report from Joe Gray *et al.* has reported that "CD5L protein and mRNA expression reveals hepatic fibrosis severity in non alcoholic-fatty liver disease"(71)

There were some limitation in 2-DE we use in our study: 1) hydrophobic proteins and small proteins which have molecular weight less than 15 kDa could not be detected, 2) comassie blue staining has low sensitivity, the low abundance proteins might not be detected. To resolve this limitation, new technologies with more sensitivity should be used such as: 1) 2D-DIGE labeling method, 2) cleavage isotope-coded affinity tags (cICAT).

In the future studies, we plan to validate this proteins (CD5L precursor and α-2-HS-glycoprotein) with the CHB subjects to confirm this proteins as candidate markers for predicting PEG-Interferon alfa-2b treatment in CHB patients.

CHAPTER VI

CONCLUSION

In this study, we have shown the serum protein profiles in chronic hepatitis B patients (SVR and nonresponders) before and after treatment of PEG-Interferon alfa-2b. We have found several significant different proteins before treatment, and found several proteins that changed significantly during the time of treatment. These significant different proteins before treatment proteins were also correlated with those that changed significantly during the time of treatment. The significant different proteins are including CD5 antigen-like precursor and α-2-HS-glycoprotein. Comparison of serum proteomic in SVR and nonresponders before and after treatment of PEG-Interferon alfa-2b could be useful as prediction serum markers for treatment response in chronic HBV infection patients. Further validations of these markers for clinical practice are underway.

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APPENDIX

APPENDIX

Reagents for 2-DE gel electrophoresis

1. Bromophenol blue stock solution

Reagents	Final concentration	Amount
Bromophenol blue	1%	100 mg
Tris-base (FW 121.1)	50 mM	60.57 mg
Double distilled water		To 10 ml

Stored at 4°C.

2. Rehydration buffer

Reagents	Final concentration	Amount
Urea (FW= 60.06)	9 M	13.6 g.
CHAPS	2% (w/v)	0.5 g.
Bromophenol blue	0.002% (w/v)	50 µl of 1% solution
Double distilled water		To 25 ml

Stored in 1 ml aliquots at -20°C.

3. SDS equilibrium buffer

Reagents	Final concentration	Amount
Tris base, pH 8.8	50 mM	10 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% v/v	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophemol blue	0.002 % (w/v)	400 µl of 1% solution
Double distilled water		To 200 ml

This is a stock solution. Prior to us DTT or iodoacetamide is added. Aliquots in 10 ml tube and store at - 20°C.

4. 4X resolving gel buffer

Reagents	Final concentration	Amount
Tris-base (FW 121.1)	1.5 M	181.7 g
Double distilled water		To 750 ml
HCL (FW 36.46)		Adjust to pH 8.8
Double distilled water		To 1,000 ml

Filter solution through a 0.45 µm filter. Stored at 4°C.

5. SDS electrophoresis buffer (10X)

Reagents	Final concentration	Amount
Tris-base (FW 121.1)	25 m M	30.3 g
Glycine (FW 75.07)	192 mM	144.0 g
SDS (FW 288.38)	0.1 % (w/v)	10.0 g
Double distilled water		To 1000 ml

Stored at 25 °C.

6. Agarose sealing solution

Reagents	Final concentration	Amount
SDS electrophoresis buffer (1X)		25 ml
Agarose	0.5 %	0.125 g
Bromophenol blue	0.002 % (w/v)	50 µl of 1% solution

Add all ingredients into 100 ml Erlenmeyer flask. Heat in a microwave on low until the agarose is completely dissolved. Stored at room temperature.

7. Stock colloidal Comassie brilliant blue G-250

Reagents	Final concentration	Amount
Ammonium sulfate (FW 131.13)	10 % (w/v)	50 g
Comassie Brilliant blue G-250		10 ml
Phosphoric acid		6 ml
Double distilled water		To 500 ml

Store at room temperature.

Working Comassie Brilliant blue G-250 was performed by mixing 100 ml of stock colloidal Comassie brilliant blue G-250 with 25 ml of methanol.

8. 10 % Ammonium persulphate

Reagents	Final concentration	Amount
Ammonium persulphate (FW 228.20)	10 % (w/v)	0.1 g
Double distilled water		1 ml

Fresh preparation prior to use.

9. 10 % SDS

Reagents	Final concentration	Amount
SDS (FW 288.38)	10 % (w/v)	5 g
Double distilled water		To 50 ml

Filter solution through a 0.45 µm filter. Stored at room tempurature.

10. Fixing solution

Ethanol	200	ml
Acetic acid	50	ml
Double distilled water	250	ml

Store at room temperature.

11. 12.5 % acrylamide gel

40% acrylamide gel	3.125	ml
1.5 M Tris-HCl pH 8.8	2.5	ml
Double distilled water	4.23	ml
10 % SDS	0.1	ml
10 % Ammonium persulfate	0.05	ml
TEMED*	3.3	µl

TEMED = N,N,N',N'-tetramethylethane-1,2-diamine

Reagents for in gel tryptic digestion

1. 20 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
Ammonium bicarbonate (FW 79.06)	20 mM	79.6 mg
HPLC grade water		To 50 ml

2. 10 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
20 mM Ammonium bicarbonate	10 mM	25 ml
HPLC grade water		25 ml

3. 10 mM DTT/10 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
20 mM Ammonium bicarbonate	10 mM	2 ml
DTT (FW 154.25)	10 mM	3.085 mg
Fresh preparation prior to use.		

4. 100 mM IAA/10 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
20 mM Ammonium bicarbonate	10 mM	2 ml
Iodoacetamide (FW 184)	100 mM	36.8 mg
Fresh preparation prior to use.		

5. 50%ACN/10 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
20 mM Ammonium bicarbonate	10 mM	10 ml
Acetonitrile	50 % (v/v)	10 ml

6. 20 ng trypsin in 50%ACN/10 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
50%ACN/10 mM Ammonium bicarbonate		1 ml
Sequencing grade modified trypsin 20 ng		20 µg

7. 30% Acetonitrile

Reagents	Final concentration	Amount
Acetonitrile	30 % (v/v)	1.5 ml
HPLC grade water		3.5 ml

8. 0.2 % formic acid

Reagents	Final concentration	Amount
Formic acid	0.2 % (v/v)	20 µl
HPLC grade water		9.98 ml

9. 50%Acetonitrile /0.1% formic acid

Reagents	Final concentration	Amount
0.2 % formic acid	0.1 % (v/v)	5 ml
Acetonitrile	50 % (v/v)	5 ml

10. 50% methanol /50 mM Ammonium bicarbonate

Reagents	Final concentration	Amount
Ammonium bicarbonate (FW 79.06)	50 mM	ml
HPLC grade water		ml
Methanol	50 % (v/v)	ml

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