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(54) **Title**: SERUM BIOMARKERS OF HEPATITIS B VIRUS INFECTED LIVER AND METHODS FOR DETECTION THEREOF

(57) **Abstract**: The invention provides a method for detecting the presence of altered serum proteins in an Hepatitis B Virus (HBV)-infected patient with liver inflammation, comprising: obtaining a sample of serum from the patient; subjecting the sample to protein gel electrophoresis to separate proteins contained therein; staining proteins separated on the electrophoresis gel with silver nitrate solution; scanning the images of stained proteins into an image analysis scanner to obtain gel images; comparing the gel images to control samples of electrophoresis gels prepared from serum of normal patient and serum of HBV-infected patient with liver inflammation to determine whether the sample of serum from the patient contains specific serum proteins. This invention also provides serum protein biomarkers for the diagnosis of patients with HBV infection and liver inflammation.



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SERUM BIOMARKERS OF HEPATITIS B VIRUS INFECTED LIVER AND METHODS FOR DETECTION THEREOF

5 Background of the Invention

Hepatitis B virus (HBV), a serious infectious and widespread human pathogen, represents a major health problem worldwide. Chronic HBV infection has a very high risk of evolving into hepatocellular carcinoma. Although considerable progress
10 was made during the past several years, the pathogenesis of HBV infection is still elusive and a definite diagnosis of HBV-infected liver information still relies on biopsy histological test.

Hepatitis B virus (HBV) infection is one of the most common viral infections in
15 humans with approximately 2 billion people infected [1]. Among them, 350 million became chronically infected. This is of particular concern in Asia-Pacific areas such as Southern China [2]. Around 25-40% will eventually die of liver disease (viz. cirrhosis with or without hepatocellular carcinoma); the death rate being 50% for males and 15% for females. Epidemiological studies reveal that HBV infection is a
20 complicated condition and the pathogenesis of the infection is still not fully defined [2, 3]. Although many HBV markers such as hepatitis B surface antigen (HBsAg), hepatitis B surface antibody, hepatitis Be antigen, hepatitis Be antibody, hepatitis B core antigen, hepatitis B core antibody, IgM and IgG have been identified and used in diagnosing and monitoring the progress of disease, no single serological test can
25 unequivocally diagnose the infection [4]. For example, positive HBsAg is a hallmark for HBV, but negative HBsAg cannot exclude HBV infection [4]. Up to now, a definite diagnosis of HBV-infected liver inflammation still relies on a combination of serological, biochemical and histological examination.

30 Presently two classes of drugs are used for the treatment of chronic HBV [5, 6]. The first is a class of immunomodulators that act by modulating the immune response of the host to the HBV antigens. The second is a class of viral suppressors. The current best immune modulating drug, interferon α 2b, only has limited effectiveness, especially with Asian patients. The viral suppressing agents need to take a long time

to effectively decrease the level of HBV. Obviously, more specific and effective diagnosis and treatment methods are needed.

Summary of the Invention

This invention provides a method for detecting the presence of Hepatitis B Virus (HBV) infection in a patient, comprising: obtaining a sample of serum from the patient; subjecting the sample to protein gel electrophoresis to separate proteins contained therein; staining proteins separated on the electrophoresis gel with silver nitrate solution; scanning the images of stained proteins into an image analysis scanner to obtain gel images; comparing the gel images to control samples of electrophoresis gels prepared from HBV negative serum and HBV positive serum to determine whether the sample of serum from the HBV-infected patient contains altered serum proteins.

The invention also provides a serum biomarker for diagnosis of HBV infection and liver inflammation in a patient, wherein the serum biomarker comprises one or more of the following proteins: apolipoprotein A-I (apoA-I), apolipoprotein A-I fragments, haptoglobin β chain, haptoglobin, cleaved β chain, haptoglobin α 2 chain, apolipoprotein A-IV (apoA-IV), transthyretin, α 1-antitrypsin, α 1-antitrypsin fragments/isoforms, or DNA topoisomerases II (topo-II).

Brief Description of the Drawings

Figure 1 shows three representative 2D-gel images for normal, LNS and HNS serum samples respectively (A), and an enlarged master LNS gel displaying the common features of human serum proteins (B). (Norm: normal, LNS & HNS: low and high necroinflammatory score).

Figure 2 includes patterns of haptoglobin showing the alterations of the protein in Areas 1 & 2 (Norm: normal, LNS & HNS: low and high necroinflammatory score).

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Figure 3 shows protein alterations in Areas 3 & 4 concerning apoA-I, apoA-IV and transthyretin (TTR) (Norm: normal, LNS & HNS: low and high necroinflammatory score).

Figure 4 shows protein alterations in Areas 5 & 6 concerning α 1-antitrypsin (Norm: normal, LNS & HNS: low and high necroinflammatory score).

Figure 5 shows patterns of DNA topoisomerase II α showing the alterations of the protein in Area 7 (Norm: normal, LNS & HNS: low and high necroinflammatory score).

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Detailed Description of the Preferred Embodiments

The invention provides a method for detecting the presence of Hepatitis B Virus (HBV) infection in a patient having liver inflammation, comprising: (a) obtaining a serum sample from the patient; (b) separating the proteins present in the serum sample of the patient in order to determine the presence of biomarkers in the sample; and (c) comparing the proteins present in step (b) with proteins present in control serum samples of normal patients to determine whether the sample of serum from the HBV-infected patients contains altered serum proteins indicative of HBV infection of an inflamed liver.

The invention provides a method for detecting the presence of Hepatitis B Virus (HBV) infection in a patient, comprising: obtaining a sample of serum from the patient; subjecting the sample to protein gel electrophoresis to separate proteins contained therein; staining proteins separated on electrophoresis gel with silver nitrate solution; scanning the images of stained proteins into an image analysis scanner to obtain gel images; and comparing the gel images to control samples of electrophoresis gels prepared from serum of normal patients and serum of patients with HBV-infected and liver inflammation to determine whether the sample of serum from the patient contains altered serum proteins indicative of chronic HBV infection of inflamed liver. Preferably, the sample of patients with HBV infection and liver inflammation contains apolipoprotein A-I (apoA-I), apolipoprotein A-I fragments, haptoglobin β chain, haptoglobin cleaved β -chain, haptoglobin 2 chain, apolipoprotein A-IV (apoA-IV), transthyretin, α 1-antitrypsin, α 1-antitrypsin fragments/isoforms, or DNA topoisomerases II (topo-II). In another embodiment, this same serum for use in the method contains apolipoprotein A-I or a fragment thereof.

The invention also provides a serum biomarker for diagnosis of an HBV infected liver inflammation in a patient, wherein the serum biomarker comprises one or more of the following identified proteins: apolipoprotein A-I (apoA-I), apolipoprotein A-I fragments, haptoglobin β chain, haptoglobin, cleaved β -chain, haptoglobin 2 chain, apolipoprotein A-IV (apoA-IV), transthyretin, α 1-antitrypsin, α 1-antitrypsin

fragments/isoforms, or DNA topoisomerases II (topo-II). In another embodiment, the serum biomarker contains purified apolipoprotein A-I or a fragment thereof.

5 The invention will be better understood by reference to the following experimental details, but those skilled in the art will readily appreciate that the specific examples detailed herein are illustrative and are not meant to limit the invention as described herein, which follow thereafter.

Experimental Details

10 I. Introduction

Proteomic analysis is a powerful technology recently developed to enhance our study on the diagnosis, treatment and prevention of human diseases [7, 8]. By comprehensively examining the different protein expression profiles (expression level, post-translational modification, interaction, etc.) between normal and diseased or drug-treated samples through 2-dimensional electrophoresis or protein chips, proteomics may provide information on new biomarkers, disease-associated targets and the process of pathogenesis. This technique has been extensively employed to investigate cancers and other diseases [9-11] but there is currently no report concerning the proteomic study on HBV infected liver inflammation. In this study, we used proteomics to globally analyze HBV-infected serum samples. By comparing to the normal serum samples, many significantly different protein expressions were identified. Detailed analysis of these proteins may reveal valuable information for the diagnosis and therapy of HBV-infected liver disease.

25 In this report, we used proteomics technology to globally exam HBV-infected serum samples aiming at searching for liver disease-associated proteins that can be used as serological biomarkers for diagnosis and/or target proteins for pathogenetic study. By comparing with normal and HBV negative serum samples, we found that at least seven proteins were significantly changed in sera of patients with HBV-infection and liver inflammation. These greatly altered proteins were identified to be haptoglobin β and α 2 chain, apolipoprotein A-I and A-IV, α 1-antitrypsin, transthyretin and DNA topoisomerase II α . The alteration of these proteins presents not only in their quantities but also in their patterns (or specificity), which can be correlated with the

necroinflammatory scores. In particular, apolipoprotein A-I displays heterogenous change in expression level with different isoforms and α 1-antitrypsin produces evidently different fragments implying diverse cleavage pathways. These unique phenomena appear specific to HBV infection. A combination simultaneously
5 considering the quantities and isoforms of these proteins could be a useful serum biomarker (or index) for HBV diagnosis and therapy.

II. Materials and Methods

A. *Human Subjects*

10 We studied 18 chronic hepatitis Be antigen positive Chinese subjects follow-up at the Hepatology Research Clinic (by GKK Lau), Queen Mary Hospital, Hong Kong SAR, China. Nine were in the immune-tolerant phase (Group 1) and 9 were in the immune-clearance phase (Group 2) (Table 1). They all had a liver biopsy for assessment or for pre-treatment assessment in accordance to the clinical trial protocol [12]. The
15 liver biopsies were assessed by 2 pathologists who were unaware of the patient's clinical findings. Both the histology and the modified histological activity index were assessed [13]. Serum samples collected at the time of liver biopsy were evaluated for serum ALT level and the serum HBV DNA was quantitated by the bDNA signal amplification assay (bDNA Quantiplex™ HBV DNA, Chiron, Emeryville, CA, USA)
20 [14]. Group 1 patients had high serum HBV-DNA ($6554 \pm 1731 \times 10^6/\text{ml}$), low ALT level ($33 \pm 14 \text{ IU/L}$) and low necroinflammatory scores (LNS) (≤ 2) and group 2 patients had low serum HBV DNA ($922 \pm 1388 \times 10^6/\text{ml}$), high ALT level ($427 \pm 253 \text{ IU/L}$) and high necroinflammatory scores (HNS) (≥ 7) (Table 1). In addition, 5 subjects with hepatitis B negative and ten normal subjects were used as control. All serum
25 samples were stored at -80°C until use. The protein concentration of all samples was determined by the method of Bradford.

B. *2D-Gel Electrophoresis*

The 2D electrophoresis was performed with Amersham Pharmacia IPGphor IEF and
30 Ettan Dalt six electrophoresis units using the protocol suggested by the company of Amersham Pharmacia. Briefly, $250 \mu\text{g}$ ($\sim 3 \mu\text{l}$) of serum sample was mixed into $340 \mu\text{l}$ rehydration solution containing 8 M urea, 4% CHAPS, 1mM PMSF, 20 mM DTT and 0.5% IPG buffer. Rehydration step was carried out with precast 18 cm IPG strips for

more than 10 hours under a low voltage of 30 V. IEF was run following a "step-wise" voltage increase procedure: 500 V and 1000 V for 1 hour each and 5000-8000 V for about 10 hours with a total of 64K Vh. After IEF, the strips were subjected to two step equilibration in the equilibration buffers containing 6 M urea, 30% glycerol, 2% SDS and 50 mM Tris-HCl (pH 6.8) with 1% DTT (w/v) for the first step and 2.5% IAA (w/v) for the second step. The strips were then transferred onto the second-dimensional SDS-PAGE which was run on 1.5 mm thick 12.5% polyacrylamide gels at 10°C.

10 **C. Silver Staining**

The gels were fixed in 40% ethanol and 10% acetic acid in water overnight, and then incubated in a buffer solution containing 30% ethanol, 41% sodium acetate and 0.2% sodium thiosulfate for 30 minutes. After washing three times in water for 5 minutes each, the gels were stained in 0.1% silver nitrate solution containing 0.02% formaldehyde for 40 minutes. Development was performed for 15 minutes in a solution consisting of 2.5% sodium carbonate and 0.01% formaldehyde. EDTA solution (1.46%) was used to stop the development and the stained gels were then washed three times in water for 5 minutes each.

20 **D. Image Acquisition and Analysis**

The stained gels were scanned in an ImageScanner (Amersham) operated by a software, LabScan 3.00, from Amersham Pharmacia Biotech. Intensity calibration was done with an intensity step wedge prior to gel image capture. Image analysis was carried out using the ImageMaster 2D Elite software 4.01 from Amersham Pharmacia. Image spots were initially detected, matched and then manually edited. Ten gel images of normal serum samples were averaged and set as the reference for comparison. Each spot intensity volume was processed by background subtraction and total spot volume normalization, the resulting spot volume percentage was used for comparison. Only those significant different spots (2-fold increase or decrease) were selected for analysis with mass spectrometry.

E. Tryptic in-Gel Digestion

Protein spots were excised and transferred into siliconized 1.5 ml Eppendorf tubes. Gel chips were de-stained in a 1:1 mixture solution of 30 mM potassium ferricyanide

and 100 mM sodium thiosulfate and then equilibrated in 50 mM ammonium bicarbonate to get pH 8.0. After hydrated with acetonitrile and dried in a SpeedVac, the gels were rehydrated in a minimal volume of trypsin solution (10 µg/ml in 25 mM NH₄HCO₃) and incubated at 37°C overnight. The supernatant was directly applied
5 onto the sample plate with equal amount of matrix. If necessary, the in-gel digests were extracted subsequently with 50% and 80% acetonitrile, and then concentrated and de-salted by Zip tips prior to applying on the sample plate.

F. MALDI-TOF Mass Analysis and Protein Identification

10 Tryptic peptide mass spectra were obtained using a Voyage-DE STR MALDI-TOF mass spectrometer (Applied Biosystems). The instrument setting was reflector mode with 175 ns delay extraction time, 60-65% grid voltage, and 20k accelerating voltage. 250 Laser shots per spectrum were used to acquire the spectra with mass range from 600 to 2500 Daltons. The trypsin autolytic fragment peaks (906.5049,
15 1153.5741 and 2163.0570) serve as internal standards for mass calibration. Protein identification was performed by searching in NCBI nr protein database using MS-Fit (<http://prospector.ucsf.edu/>). The criteria for searching were set with 50 ppm or better mass accuracy, at least 4 matching peptide masses and molecular weight and pI matching estimated values from gels. Post-source decay MS/MS measurement and
20 MS-Tag (<http://prospector.ucsf.edu/>) searching were also performed to confirm the results from the MS-Fit. Species search was limited in Homo sapiens.

III. Results

A. Protein Separation

25 Two groups of HBV-infected serum samples together with control samples were applied to 2D-PAGE and proteins visualized by silver staining. 2D gels were run three times for each sample to minimize gel-to-gel variation. Figure 1A shows three representative gel images for normal, HBV-infected low and high necroinflammatory score serum (LNS and HNS), respectively. Figure 1B is an enlarged master LNS gel
30 displaying the common features of human serum proteins. Overall, the gel has a very similar pattern to the plasma map in the SWISS-2D database (<http://www.expasy.ch/ch2d/>) except lacking fibrinogen. More than 1000 spots were detected in a gel ranging from 6k to 20k Da of molecular mass with pI/s between 4 and 10. Many trains of spots represent those proteins that have primary structure

with different degrees of glycosylation and/or phosphorylation (isoforms), resulting in a progressive change in the *pI* and molecular weight. Spot volume comparison was made between three types of samples with assistance of the ImageMaster program. Significant and constant differences were found in at least seven areas shown in Fig. 5 1B.

B. Protein Identification

The protein spots that have significant differences were cut off and subjected to trypsin digestion, MALDI-TOF mass spectra measurement and database searching. 10 Where appropriate, protein identifications were confirmed by comparing spot locations and patterns to those in the SWISS database plasma map. Table 2 summarizes the identified proteins in the seven areas and their alterations among normal, LNS and HNS serum samples. Overall, the expressions of three proteins (groups) are suppressed and five proteins (groups) are enhanced in the sera of 15 patients with chronic HBV-infection and liver inflammation.

One of remarkable changes is shown in Figure 2 (Areas 1 & 2 in Figure 1B) concerning haptoglobin. Haptoglobin $\alpha 2$ chain, β chain and cleaved β chain present their own characteristic train patterns in 2D gels, featuring with three, seven and six 20 detectable isoforms respectively. Compared to that in normal samples, haptoglobin overall slightly increased or had no change in the LNS serum samples but was significantly suppressed in the HNS patients (Table 2). In some cases (30%) the protein was diminished to undetectable level. In contrast, a protein cluster highlighted in circle in the Area 1 of Figure 2 gradually increases its expression level 25 from undetectable in the control to partially visible in LNS and then to fully appearance in HNS.

Figure 3 displays another dramatic change occurring in apolipoprotein A-I (apoA-I) region (Area 3 in Figure 1B). Normal serum sample has three main apoA-I protein spots (isoforms 2, 1 and 0 from left to right)[15] with one to three very weak cleaved 30 fragment spots immediately below the main spots. In the chronic HBV-infected serum samples, the entire pattern of apoA-I profile was altered. Isoform 2 was significantly up-regulated, isoform 0 was evidently down-regulated, while isoform 1 remained unchanged (Table 2). In addition to the three major spots, the weak

fragment spots were significantly enhanced as well as a number of new spots appeared in the low molecular weight area (Fig. 3). These new spots were identified to have the primary sequence of apoA-I and they probably belong to the cleaved fragments of apoA-I or its other isoforms. It is also worth noting that overall the main apoA-I protein spots decrease their volumes in both LNS and HNS samples and the cleaved fragments of apoA-I or other isoforms appear in higher volumes in LNS than those in HNS samples (Table 2).

One more apoA-I fragment was observed in the even low molecular weight Area 4 (Fig.3). This spot appeared in both LNS and HNS samples but not in the normal serum samples. Similarly in the same Area 4, a spot of apoA-IV was identified in both HBV-infected samples but was undetectable in the control. The only one detectable spot in the Area 4 of normal samples was identified to be transthyretin. The expression level of this transthyretin isoform was apparently decreased in the serum of HBV carriers (Table 2).

Figure 4 shows the protein alterations in Areas 5 and 6 in Figure 1B. Three spots in Area 5 and two spots in Area 6 were markedly intensified in both LNS and HNS samples (Table 2). These spots are identified to have the same primary sequence with α 1-antitrypsin. This protein normally appears in a group of spots representing various phenotypes in the 2D gel ranging from pI 5.0-5.2, MW 55kDa (<http://www.expasy.ch/ch2d/>) (Fig. 1B). No significant difference in the level of the normal α 1-antitrypsin was found between control and HBV serum samples. However, α 1-antitrypsin level in the lower molecular weight areas (MW 38-39kDa) is greatly enhanced in HBV samples (Table 2). The increased expression level of the protein in the Area 6 is 2-fold more in HNS than that in LNS samples.

Spots in Area 7 were identified as DNA topoisomerase II (Topo II) (Fig. 5). Only one spot of this protein was detected in the normal serum whereas a group of five spots developed in HBV samples. As a result, the protein level was elevated about 20 fold in total in the HBV serum (Table 2). Given the fact that intact TopoII has a molecular weight of 180kDa, these spots in Area 7 are probably the low-molecule fragments.

C. Discussion

Haptoglobin, apoA-I and α 1-antitrypsin are among most abundant serum glycoproteins secreted by the liver. It is therefore reasonable that liver damage results in alterations of these proteins, which in turn can be used as biomarkers for monitoring the liver diseases. Recent efforts have been made to combine these proteins with other basic serum markers as an index for the diagnosis of HCV disease thus reducing the number of liver biopsies performed in patients with chronic HCV infection [16, 17]. Almost all these studies are based on the comparison of the amount of the marker proteins between normal and diseased samples. The current report examined the alterations of these biomarkers globally (their expression levels and patterns) through proteomic analysis and thus may provide more specific assessments for HBV infection.

Haptoglobin has been long used to study the serum of various liver diseases including HBV infection. However, contradictory results were reported. Earlier studies showed that haptoglobin level were decreased in chronic [18, 19] and acute viral hepatitis [20], other reports claimed that haptoglobin level changed in acute hepatitis [21] but no significant difference were observed in chronic hepatitis [22]. A more recent study revealed that haptoglobin increased in all acute viral hepatitis and in chronic HBV infection but decreased in patients with other chronic hepatitis [23]. These conflicting results may be due to the different measurements used, samples sources and stages of disease. Here we specifically examined the HBV serum at low (LNS) and high (HNS) inflammatory stages. Our results revealed that both haptoglobin α 2 and β chains slightly increased in the LNS serum but significantly decreased in the HNS samples (Table 2). This implies that HBV infection without inflammation may not cause serious liver damage, corresponding to the low ALT and high HBV-DNA immune-tolerant phase in the chronic HBV infection [6]. Meanwhile, advance stage of inflammation causes severe liver function impairment, resulting in the substantial decrease of both haptoglobin α and β chains secreted from the injured liver.

Many studies have shown that the level of apoA-I correlates with changes in the hepatocellular function in chronic liver disease [24-27]. A low level of the protein

implies the severity of liver cell injury. It is believed that apoA-I is controlled at posttranslational level [28] and liver damage decreases the apoA-I conversion from other isoforms [29]. Our results indicate that in chronic HBV infection apoA-I alteration occurs not only in its level but its whole pattern in the 2D gel (Figure 3). In particular, three main apoA-I isoforms change their expression level independently. This is the first time such phenomenon was observed in HBV infection. This heterogeneous alteration reflects different posttranslational control occurring to the different isoforms of apoA-I, which may be correlated with specific features or functions of the isoforms. A further identification of the specific modifications underlying these specific protein isoforms may provide the useful diagnostic information of HBV infection. The appearance of the new cluster of apoA-I spots also appears specific to HBV infection because no similar observation was revealed in other inflammations such as alcoholic, cirrhosis and other liver injury [30] (unpublished results). These new spots may contain the different isoforms of apoA-I that may not be detected by the common assay of nephelometry. This suggests that the observed decrease of apoA-I level in chronic hepatitis only corresponds to the alteration of the three main apoA-I isoforms. It is therefore possible to develop monoclonal antibodies specifically to the apoA-I isoforms for the serological assay of HBV infection. This possibility is under investigation.

20

Closely linked to apoA-I in a multigene cluster, apoA-IV is a glycoprotein synthesized by the human intestine [31, 32]. ApoA-IV has been suggested to have protective function against lesions and atherosclerosis [33-35] and have physiological role in modulating gastric function [36, 37]. Suppressed level of apoA-IV was found in the cases of inflammation [38], acute hepatitis [39] and cirrhosis [40]. The apoA-IV spot appears in the low molecular weight area of current HBV sera probably is a fragment of the entire protein (~45kDa). This implies that the observed decrease of apoA-IV is due to the protein cleavage during inflammation, another indicator for the assessment of HBV infection.

30

Transthyretin, a known negative acute-phase protein, was found to have significantly reduced concentration in various acute liver diseases [23, 41, 42]. We confirmed in the present report that at least one transthyretin isoform displayed a half of its

expression level in both LNS and HNS serum samples (Table 2), suggesting liver dysfunction occurs with the virus infection even before serious inflammation.

As one of the powerful inhibitors of apoptosis and caspase activation, α 1-antitrypsin
5 can inhibit many of the proteases released from dying cells and thus protects normal
tissues during periods of stress such as inflammation [43]. The observed increase of
 α 1-antitrypsin level in both acute and chronic hepatitis [19] may be the response of
self-protection of the liver. On the other hand, the deficiency of α 1-antitrypsin activity
has been shown to closely associate with liver diseases [44, 45]. This α 1-antitrypsin
10 deficiency can be inherited from mutant genes in some phenotype variants [46-48].
In the present study we found that α 1-antitrypsin increases in the lower molecular
weight (~40kDa) region. Considering the fact that α 1-antitrypsin is a single-chain
protein of 394 amino acids, 52kDa, we believe that the lower MW proteins probably
are the fragments rather than other phenotypes. Interestingly, these large fragments
15 spread out in two different *pI* areas, present in totally different patterns, and display
different increasing extents between LNS and HNS cases, indicating that these are
the products of different cleavage pathways. Further characterization of these
peptides may reveal underlying information helpful for understanding HBV infection
and for its diagnosis. Moreover, no such particular phenomenon was observed in
20 other liver inflammation, suggesting that the change could be specific to HBV
infection.

DNA topoisomerase II is an ATP-dependent enzyme that catalyses topological
genomic change by passing one DNA duplex through a transient break in a second
25 duplex [49]. The enzyme has two separately encoded isoforms, topoll α and topoll β .
Both forms are molecular targets for several widely used anticancer agents which
interrupt enzymatic DNA breakage-reunion, leading to a stop of DNA replication
processes and ultimately cell death [50]. The over-expression of topoll α was
suggested to have potential linkage with several aggressive tumors including HCC
30 [51], lung cancer [52, 53] and ovarian cancer [54]. The elevated mRNA level of
topoll β was also found in human ovarian cancer [54] and K562 cells [55]. The
greatly enhanced topoll β fragments currently found in HBV sera may reflect the
significant increase of topoll β expression related to HBV infection. Although whether

this alteration represents an initial sign of malignant cell transformation induced by HBV requires more detailed investigation, it may indicate that a DNA metabolic process is involved in HBV infection.

5 HBV disease is a complicated condition. Many serum biomarkers have been identified and used in clinical diagnosis based on their quantity change. However, different serological tests have various limitations in the diagnosis and management of chronic HBV infection [4]. This study demonstrated that 2D-PAGE electrophoresis can generate a comprehensive serological profile in which the HBV protein
10 biomarkers change patterns not only in their quantities but their qualities (or specificity). In particular, we have observed various alterations occurring with the different isoforms of these biomarkers, which are especially informative and useful for assessing HBV infection. Proteomic analysis provides a specific and suitable alternative to conventional measurements of HBV diagnosis and progression in the
15 clinical research setting. A global examination combining these basic serum markers, together with their special isoform alteration, could be useful in HBV therapy and thus substantially reduces the number of liver biopsies performed in patients with chronic HBV infection.

V. References

The following references, referred to in the specification by numeral, are incorporated by reference herein as background and to provide the state of the art.

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Table 1. Summary of HBV-infected serum samples used. NIS refers to necroinflammatory score.

<i>Patients</i>	<i>Age</i>	<i>Sex</i>	<i>HBeAg</i>	<i>HBV-DNA</i>	<i>ALT</i>	<i>NIS</i>
Group 1						
1	41	M	+	5874	46	2
2	24	M	+	4032	21	1
3	22	F	+	10536	24	2
4	22	M	+	6876	52	2
5	44	F	+	6949	16	2
6	25	M	+	7443	36	1
7	34	F	+	6690	17	2
8	42	M	+	5408	45	2
9	50	F	+	5175	19	1
Group 2						
10	21	F	+	104	425	8
11	49	F	+	13	706	9
12	29	F	+	16	461	7
13	18	M	+	750	1050	8
14	29	M	+	476	277	9
15	31	F	+	454	307	7
16	54	M	+	4695	190	8
17	36	M	+	1320	200	7
18	49	M	+	471	253	7

5

Table 2. Summary of protein alterations in HBV infection serum samples (N/D: non-detectable)

<i>Protein</i>	<i>Area</i>	<i>Experimental MW(kDa)/pI</i>	<i>Normal (% vol)</i>	<i>LNS (% vol)</i>	<i>HNS (% vol)</i>
Haptoglobin α chain (7 spots)	1	37.2/5.0-5.6	3.242 \pm 1.021	3.196 \pm 1.243	0.873 \pm 0.661
Haptoglobin cleaved α chain (6 spots)	1	35.7/5.1-5.8	0.329 \pm 0.185	0.410 \pm 0.262	0.066 \pm 0.072
Haptoglobin α 2 chain (3 spots)	2	19.8/5.3-5.9	1.225 \pm 0.582	1.638 \pm 0.631	0.421 \pm 0.310
Apo A-I (isoform 1, 2 and 3)	3	23.8/5.1-5.2	0.136 \pm 0.082	0.416 \pm 0.179	0.359 \pm 0.220
			0.708 \pm 0.189	0.745 \pm 0.256	0.720 \pm 0.358
			2.893 \pm 0.424	1.210 \pm 0.401	1.326 \pm 0.718
Apo A-I fragments (10 spots)	3	22.3/5.0-5.1	0.037 \pm 0.031	1.043 \pm 0.622	0.474 \pm 0.284
	4	15.8/5.0	N/D	0.264 \pm 0.125	0.163 \pm 0.187
Apo A-IV	4	15.7/5.0	N/D	0.552 \pm 0.335	0.403 \pm 0.277

Transthyretin	4	15.9/5.1	0.493 ± 0.198	0.234 ± 0.216	0.237 ± 0.240
□1-Antitrypsin (normal)		54.4/5.0-5.2	2.935 ± 0.592	2.352 ± 0.825	2.384 ± 1.182
□1-Antitrypsin (fragments or isoforms)	5	38.9/4.6-4.7	0.012 ± 0.008	0.524 ± 0.352	0.522 ± 0.484
	6	38.2/5.4	N/D	0.063 ± 0.075	0.170 ± 0.152
	6	38.2/5.6	N/D	0.059 ± 0.041	0.183 ± 0.131
DNA Topoisomerase II□ (5 spots)	7	21.2/6.6-7.7	0.053 ± 0.027	0.937 ± 0.721	1.106 ± 0.664

WE CLAIM:

1. A method for detecting the presence of Hepatitis B Virus (HBV) infection in a patient having liver inflammation, comprising:
 - 5 (a) obtaining a serum sample from the patient;
 - (b) separating the proteins present in the serum sample of the patient in order to determine the presence of biomarkers in the sample; and
 - 10 (c) comparing the proteins present in step (b) with proteins present in control serum samples of normal patients to determine whether the sample of serum from the HBV-infected patients contains altered serum proteins indicative of HBV infection.

- 15 2. A method in accordance with claim 1, wherein the serum sample of HBV-infected patient contains altered expressions of apolipoprotein A-I (apoA-I), apolipoprotein A-I fragments, haptoglobin β chain, haptoglobin cleaved β -chain, haptoglobin 2 chain, apolipoprotein A-IV (apoA-IV), transthyretin, α 1-antitrypsin, α 1-antitrypsin fragments/isoforms, or DNA topoisomerases II (topo-II).

- 20 3. A method in accordance with claim 1, wherein the serum sample of HBV-infected patients contains apolipoprotein A-I or a fragment thereof.

- 25 4. A serum biomarker for diagnosis of an HBV infection in a patient having liver inflammation, wherein the serum biomarker comprises one or more of the following purified proteins: apolipoprotein A-I (apoA-I), apolipoprotein A-I fragments, haptoglobin β chain, haptoglobin, cleaved β -chain, haptoglobin 2 chain, apolipoprotein A-IV (apoA-IV), transthyretin, α 1-antitrypsin, α 1-antitrypsin fragments/isoforms, or DNA topoisomerases II (topo-II).

- 30 5. A serum biomarker for diagnosis of HBV-infection in a patient having liver inflammation comprising apolipoprotein A-I or a fragment thereof.

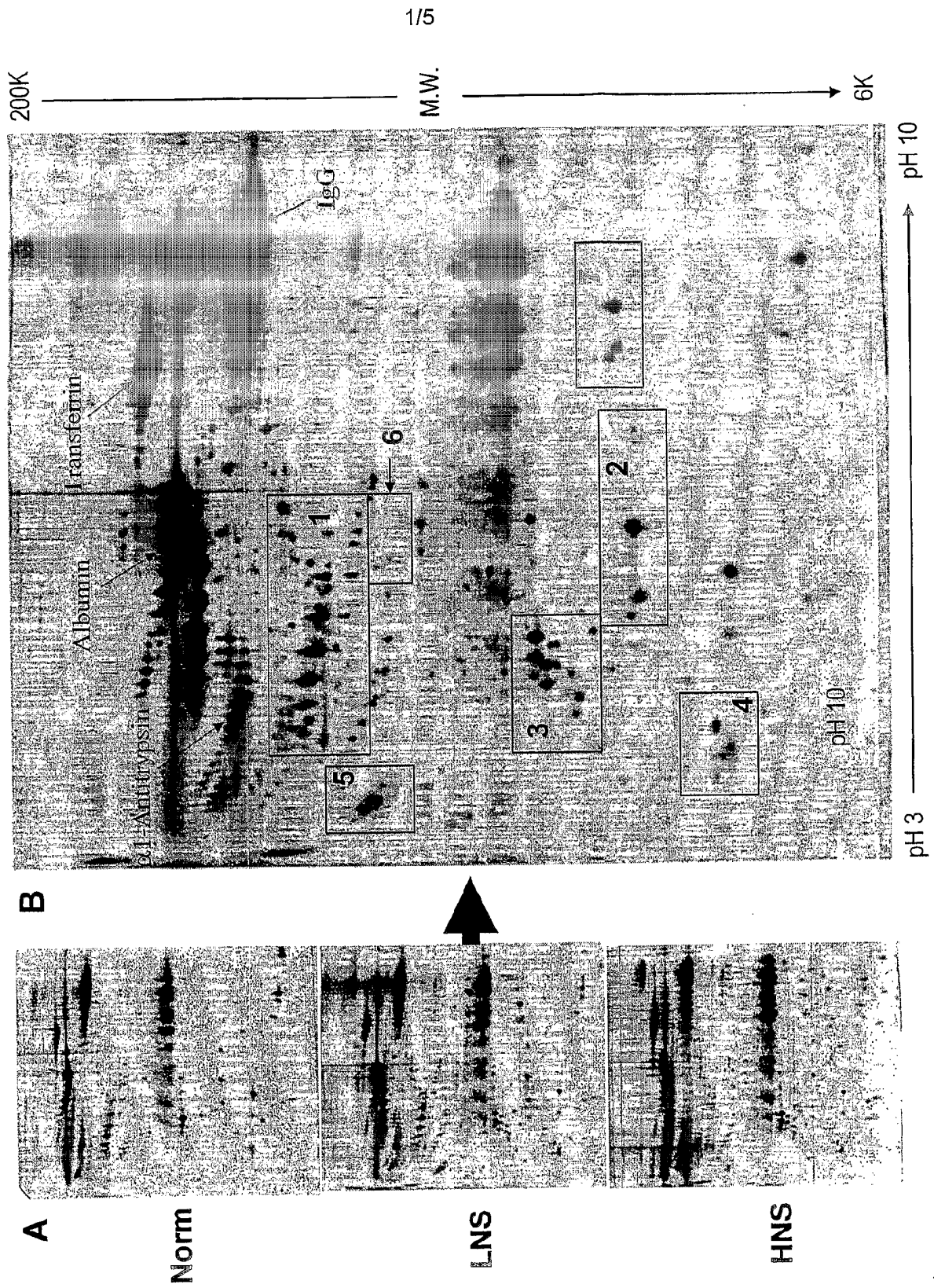


Fig. 1

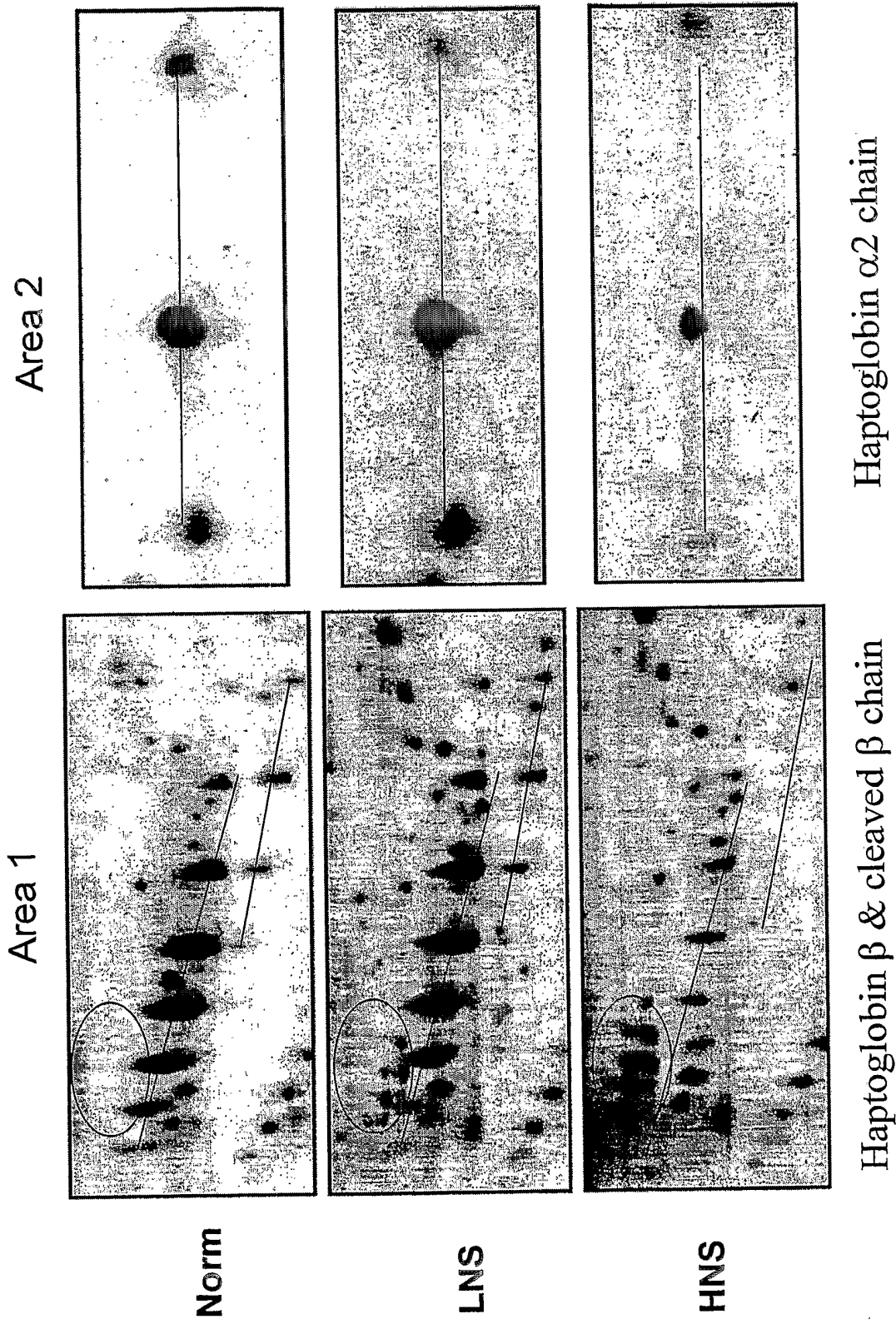


Fig. 2

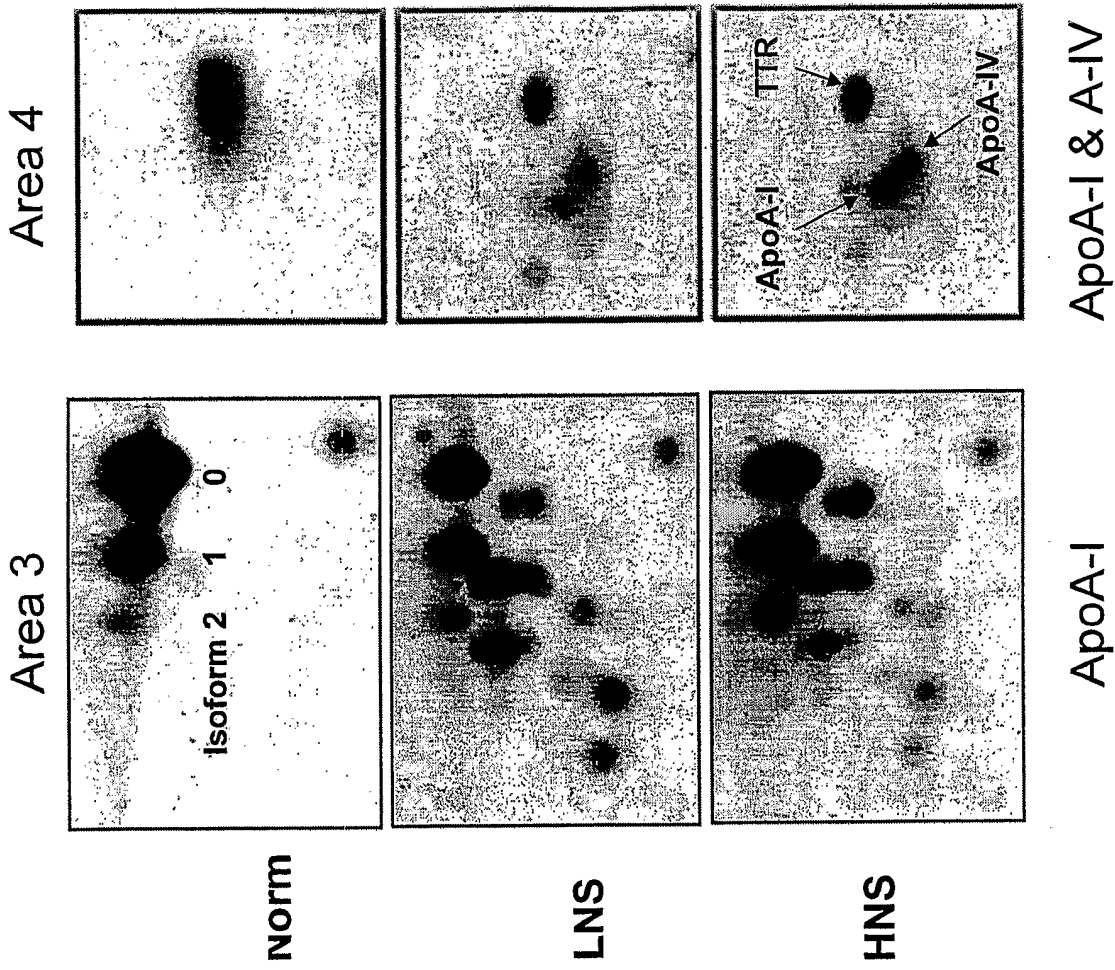


Fig. 3

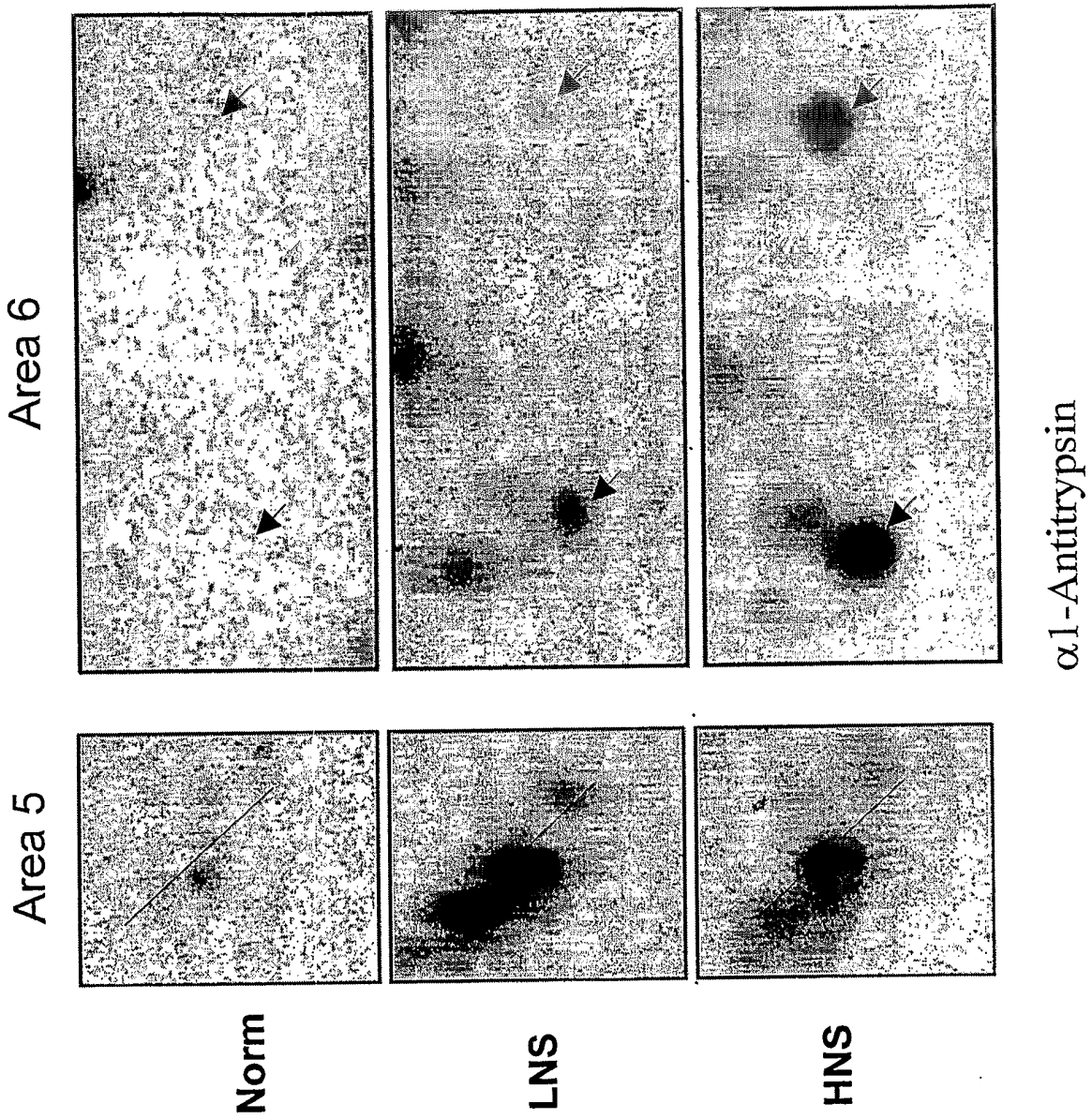
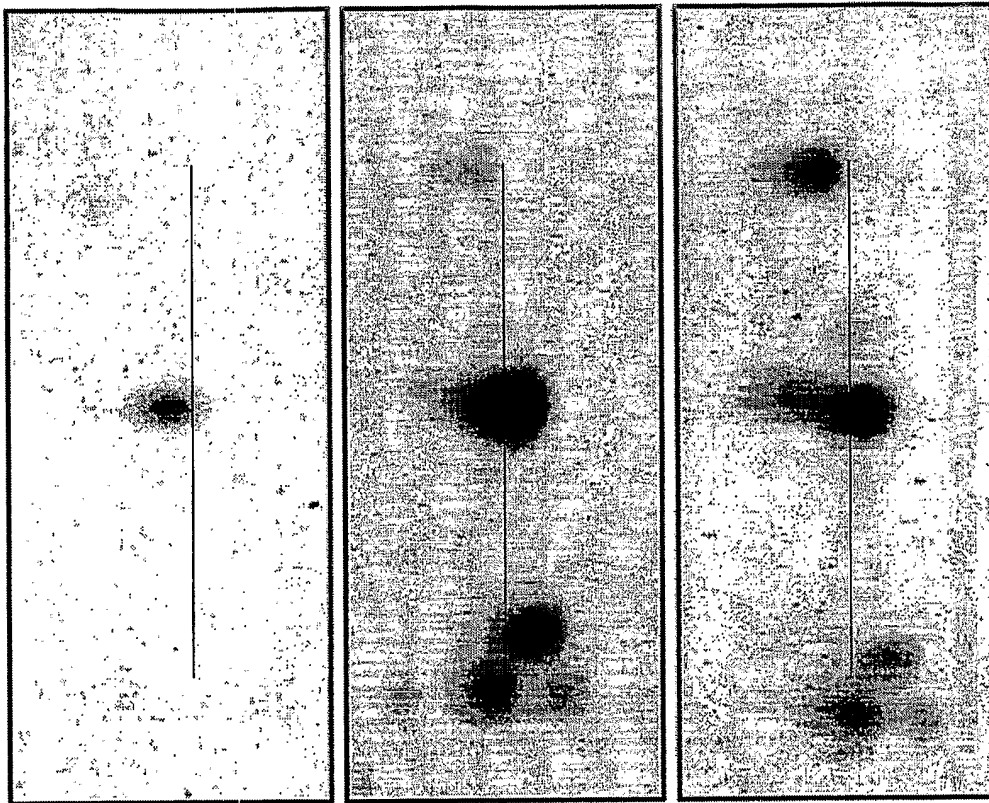


Fig. 4

Area 7



Norm

LNS

HNS

DNA Topoisomerase II β

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2004/000405

A. CLASSIFICATION OF SUBJECT MATTER

C12Q1/00, C12Q1/04, C12Q1/70, G01N33/50, G01N33/573, G01N33/576, G01N33/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)


WPI, PAJ, EPODOC, CNKI, CPRS, MEDLINE (HEPATITIS, HBV, HEPATITIS B VIRUS, ANTITRYPSIN, TRANSTHYRETIN, HAPOTOGLOBIN, APOLIPOPROTEIN, APO, TOPOISOMERASE, TOPO)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Comparative analysis of plasma lipoprotein components assessed by ultracentrifugation in primary biliary cirrhosis and chronic hepatitis. Tacikowski,-T; Milewski,-B; Dzieniszewski,-J; Nowicka,-G; Walewska-Zielecka,-B. Med-Sci-Monit. 2000 Mar-Apr; 6(2): 325-9.see abstract.	1-5
A	Serum total lipids, lipoprotein cholesterol, and apolipoprotein A in acute viral hepatitis and chronic liver disease. Vergani,-C; Trovato,-G; Delu,-A; Pietrogrande,-M; Dioguardi,-N. J-Clin-Pathol. 1978 Aug; 31(8): 772-8. see abstract.	1-5
A	Alpha 1-antitrypsin and other acute phase reactants in liver disease. Carlson,-J; Eriksson,-S. Acta-Med-Scand. 1980; 207(1-2): 79-83. see abstract.	1-5

Further documents are listed in the continuation of Box C. See patent family annex.

<p>* Special categories of cited documents:</p> <p>“A” document defining the general state of the art which is not considered to be of particular relevance</p> <p>“E” earlier application or patent but published on or after the international filing date</p> <p>“L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>“O” document referring to an oral disclosure, use, exhibition or other means</p> <p>“P” document published prior to the international filing date but later than the priority date claimed</p>	<p>“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>“&” document member of the same patent family</p>
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Date of the actual completion of the international search 17.Jul.2004	Date of mailing of the international search report 29 . JUL 2004 (29 . 07 . 2004)
Name and mailing address of the ISA/CN 6 Xitucheng Road, Jimen Bridge, Haidian District, Beijing, 100088. P. R. China. Facsimile No. (86-10)62019451	Authorized officer KANG Yuning Telephone No. (86-10)62085093 <div style="float: right; border: 1px solid black; padding: 2px; text-align: center;">  </div>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2004/000405

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The serum protein profile in chronic hepatitis, cirrhosis and liver cancer. Hiramatsu,-S; Kojima,-J; Okada,-T-T; Inai,-S; Ohmori,-K. Acta-Hepatogastroenterol-(Stuttg). 1976 Jun; 23(3): 177-82. see abstract.	1-5
A	Serum haptoglobin type and liver cirrhosis. Hirayama,-C; Nakamura,-M; Koga,-S. Humangenetik. 1975 Jun 19; 28(2): 139-46. see abstract.	1-5
A	Prealbumin rather than albumin is a more sensitive indicator of acute liver disease. Yasmin,-M-Y; Aziz,-B; Nazim,-M; Madhavan,-R-K. Malays-J-Pathol. 1993 Dec; 15(2): 147-50. see abstract.	1-5
A	Human DNA topoisomerase I-mediated cleavage and recombination of duck hepatitis B virus DNA in vitro. Pourquoi,-P; Jensen,-A-D; Gong,-S-S; Pommier,-Y; Rogler,-C-E. Nucleic-Acids-Res. 1999 Apr 15; 27(8): 1919-25. see abstract.	1-5