Plasma gelsolin protein: a candidate biomarker for hepatitis B-associated liver cirrhosis identified by proteomic approach

Cristina Marrocco[1], Sara Rinalducci[1], Ashraf Mohamadkhani[2], Gian Maria D'Amici[1], Lello Zolla[1]

[1]Department of Environmental Sciences, University of Tuscia, Viterbo, Italy; [2]Digestive Disease Research Centre, Shartati Hospital, Tehran University of Medical Science, Tehran, Iran.

Background. Despite the significant improvement in internal medicine and supportive therapy in recent years, liver fibrosis/cirrhosis remains a serious health issue in hepatitis B virus (HBV) infected patients. Invasive liver biopsy is presently the best means of diagnosing cirrhosis, but it carries a significant risk and has well recognised limitations such as sampling error, hence the importance in developing early diagnosis biomarkers. With this aim, we performed a pilot proteomic study to assess this as a strategy for plasma marker detection in patients suffering from HBV-associated liver cirrhosis.

Methods. Plasma from eight chronic HBV-infection patients and from eight HBV-related cirrhotic patients were selected and proteome profiles were created by two-dimensional electrophoresis. The strategy included the use of ProteoMiner enrichment kit for the reduction of highly abundance proteins (e.g. albumin and IgG) prior to proteomic analyses with the goal to improve detection of novel candidate markers.

Results. One reproducible spot was found to be completely repressed in plasma samples from cirrhotic patients and mass spectrometry analysis identified this a specific variant of the gelsolin actin-depolymerizing factor. Though further investigations are needed, especially in term of clinical validation, to our knowledge this is the first time that gelsolin is proposed as potential biomarker in HBV-related liver pathologies.

Conclusions. Our findings confirm the potential utility of gelsolin either as a prognostic marker or a replacement therapeutic agent to alleviate liver injury.

Keywords: hepatitis B virus (HBV)*;* inactive chronic HBV-infection; HBV-associated liver cirrhosis; human plasma; biomarker discovery.

Introduction

Hepatitis B virus (HBV) is the prototype member of the family Hepadnaviridae that also includes viruses that can infect higher primates such as chimpanzees, and lower primates such as tupaia[1]. Approximately 350 million individuals has been infected with HBV and each year, an estimated 1 million persons die from chronic complications of the disease. Although chronic hepatitis B infection has a worldwide distribution, the vast majority of infected persons reside in Asia, the Middle East or Africa[2], where there is a concomitant high incidence of hepatocellular carcinoma (HCC)[3]. HBV is a non-cytopathic virus and chronic hepatitis B is developed when the immune response that normally clears the infection fails to have a function or is too weak to be effective. Thus, infections are almost always chronic following exposure of children younger than 1 year or of immunocompromised individuals[4-6]. HBV infection may or may not be symptomatic and the outcome of

infection to a large extent is determined by the immune status of the individual[7]. Successful clearance and resolution of infection also depends on the age and immune status of the individual. The complications of chronic HBV infection are well known and include liver cirrhosis, liver cancer as well as liver failure[8,9]. Cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by fibrous scar tissue as well as regenerative nodules, leading to progressive loss of liver function. Liver cirrhosis could be reversible, and accurate diagnosis is crucial to the management of patients. Pathologic diagnosis with liver biopsy has long been the gold standard for assessing the degree of fibrosis, but it is an invasive procedure with inherent risk and sampling variability. Plasma-based tests of liver cirrhosis have attracted more attention in recent years because plasma sample can be easily obtained from blood collection of patients[10]. Human blood plasma is one of the most important proteome from a clinical and medical point of view and the discovery of new biomarkers is a very challenging process which has become the basis for preventive medicine. However, plasma is also the most complex human-derived sample for proteomic analysis because it contains the widest dynamic range of cellular protein species in the body. In fact, several plasmatic proteins are synthesized in the liver and the majority of these change their structures and abundance in response to liver disease[11]. Tens of thousands of proteins, with their cleaved or modified forms, have been estimated to be present in the plasma. A small number of proteins such as albumin, immunoglobulins, α-1-antitrypsin, transferrin, and haptoglobins are present in concentrations in the milligram to tens of milligrams per milliliter range and together account for as much as 90% of the total plasma protein called "highly-abundant proteins" (HAP)[12]. On the other hand, a large number of proteins, including many that are, or could be, diagnostically significant are comprised into "low-abundant proteins" (LAP) because when such proteins are released into around 6 L of blood, their final concentration becomes extremely low. The presence of "highly-abundant proteins" and "low-abundant proteins" represents the major problem in proteome studies which use plasma and serum samples because the first protein content masks the second one. Depletion of abundant plasma proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers[13].To overcome the above-described difficulties, prefractionation methods have been recently developed[14] and a novel sample preparation tool is now commercially available under the trade name of ProteoMiner[15]. This protein enrichment technology is based on the interaction of complex protein sample with large, highly diverse library of hexapeptides bound to a chromatographic support where each unique hexapeptide binds to a unique protein sequence. High-abundance proteins quickly saturate their ligands because of the bead capacity limits binding capacity and excess protein is washed out during the procedure. In contrast, low-abundance proteins are concentrated on their specific ligands, thereby decreasing the dynamic range of sample proteins. When analyzed in downstream applications (e.g., electrophoresis) the number of proteins detected is dramatically increased[16]. This peculiar property of revealing novel low-abundance species is of extremely interest within biomarker discovery investigations.

In our work, comparison between plasma proteomes from patients with liver cirrhosis associated to hepatitis B infection and chronic HBV-infection patients who were asymptomatic, has been performed to show a possible approach for plasma biomarker discovery and validation. Before proteomic analysis, the sample has been treated by ProteoMiner™ Protein Enrichment Technology and then analysed by 2D-IEF-SDS-PAGE and MS/MS to search for candidate markers of this liver disease.

Materials and methods

Plasma samples

With patients' consent, we collected plasma samples from a total of 16 HBsAg positive and HBeAg negative subjects attending the Hepatitis Clinic of Shariati Hospital, Tehran University of Medical Sciences. 8 were patients with inactive chronic HBV-infection and 8 with HVB-related liver cirrhosis. Only patients with HBV DNA detected using a non-commercial hemi-nested PCR (sensitivity of approximately 100 copies/mL)[17] were recruited for this study, whereas patients with undetectable HBV DNA, or those with evidence of concomitant hepatitis C or D virus infection, HIV infection, autoimmune, drug-induced or other causes of chronic liver disease

were excluded. Inactive chronic HBV-infection defined as chronic hepatitis B with persistently normal ALT levels for 6 months prior to enrolment. Cirrhosis was defined as the presence of fibrosis stage 4 or more than 4 in HAI score[18]. Demographic data and plasma samples were collected at the initial assessment before liver biopsy and stored at -70°C prior to analysis. None of patients received antiviral treatment prior to liver biopsy. HBV DNA was quantified in the Light-Cycler (Roche) using the RealART™ HBV LC PCR (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The protocol for this study was approved by the ethics committee of Shariati Hospital. Detailed information on subjects enrolled in proteomics analyses are shown in Table I.

ProteoMiner enrichment

Before proteomic analysis, each plasma sample was pre-fractioned using the ProteoMiner™ kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The protein concentration of sample was 50mg/mL.

The equalized plasma samples were stored at –80 °C until use.

Quantification of plasma protein concentration

The protein concentration of each sample was determined according to Bradford[19] using BSA as a standard curve. The protein concentration was estimed on whole plasma sample before and after ProteoMiner treatment.

Two-dimensional electrophoresis

To remove lipids, proteins were precipitated from a desired volume (containing 400 µg of proteins) of each sample with cold (4 °C) acetone (80% v/v) over-night, then centrifuged at 18000 g for 20 min. The supernatant was removed and the pellet was air-dried and then solubilized in the focusing solution 8 M urea, 4% (w/v) CHAPS, 0.5% (w/v) pH 4-7 carrier ampholyte (Bio-lyte; Bio-Rad, Hercules, CA, USA) and 40 mM Tris base with continuous stirring. Proteins were subsequently reduced (10 mM tributylphosphine, 1 h) and alkylated (40 mM IAA, 1 h). To prevent over-alkylation, iodoacetamide (IAA) excess was destroyed by adding 10 mM DTE. IEF was performed using Biorad Multiphore II and Dry Strip Kit (Bio-Rad-Protean-IEF-Cell-System). Seventeen centimeter IPG strips (Bio-Rad, Hercules, CA, USA) pH 4-7 were rehydrated overnight with 345 µL of rehydratation solution containing 8 M urea, 4% (w/v) CHAPS, 0.5% (w/v) pH 4-7 carrier ampholyte (Bio-lyte; Bio-Rad, Hercules, CA, USA), 10 mM DTE and 100µL of sample was loaded using the cup-loading method. The total product time × voltage applied was 80 000 V h for each strip at 20 °C. For the second dimension, IPG strips were incubated in the equilibration solution [6 M urea, 50 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 3% (w/v) SDS, 0.002% (w/v) bromophenol blue] for 30 min with gentle agitation. Equilibrated strips were then placed on SDS-polyacrylamide gels, 16 cm × 20 cm, 11% acrylamide, and sealed with 0.5% (w/v) agarose.

Table I - Demographic, biochemical and virologic characteristics of patients with chronic hepatitis B

	Patients with chronic hepatitis B (n = 8)	Patients with cirrhosis (n = 8)	P value
Age (Years)	37 ± 7	44 ± 7.5	0.07
Gender M/F	4/4	6/2	-
Viral load (copies/mL)	6.9 ± 10.3 E3	647 ± 169 E3	0.3
ALT (IU/L)	30 ± 7	72 ± 23	0.001
AST (IU/L)	32 ± 6	79 ± 28	0.002
Platelets × 103 mm^{-3}	174 ± 48	158 ± 56	0.5
Prothrombin time(s)	12.3 ± 0.4	13.9 ± 1.4	0.01
Albumin (g/L)	4.5 ± 0.4	4.2 ± 0.3	0.1
HAI score	4.5 ± 1.6	9.5 ± 1.4	0.001
Bilirubin Total (mg/dL)	1.0 ± 0.2	2.3 ± 0.9	0.05

SDS-PAGE was performed using the Protean II xi Cell, large gel format (Bio-Rad) at constant current (35 mA per gel) at 7 °C until the bromophenol blue tracking dye was approximately 2-3 mm from the bottom of the gel. Protein spots were stained by Coomassie Brilliant Blue G-250 stain[20].

Image analysis

Sixteen two-dimension stained gel were digitized and image analysis was performed with Progenesis SameSpots software vers. 2.0 (Nonlinear Dynamics), which allows novel spot detection. The gel image showing the highest number of spots and the best protein pattern was chosen as a reference template, and spots in a standard gel were then matched across all gels. Each gel was analysed for novel spot detection and background subtraction.

In-Gel Digestion

Protein spots were carefully excised from Coomassie stained gels and subjected to in-gel trypsin digestion according to Shevchenko and colleagues[21] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/μL of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min the supernatant was removed and discarded, 20 μL of 50 mM NH_4HCO_3 were added to the gel pieces and digestion allowed to proceed at 37 ° C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 μL of 5% FA (Formic Acid).

Protein identification by MS/MS

Peptide mixtures were separated using a nanoflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 10 μL was loaded by the autosampler onto a homemade 2 cm fused silica precolumn (75 μm I.D.; 375 μm O.D.; Reprosil C18-AQ, 3 μm (Ammerbuch-Entringen, DE)) at a flow rate of 2 μL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 minutes over the precolumn in-line with a homemade 10-15 cm resolving column (75 μm I.D.; 375 μm O.D.; Reprosil C18-AQ, 3 μm (Ammerbuch-Entringen, Germany)). Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). Capillary voltage was 1.5-2 kV and a dry gas flow rate of 10 L/min was used with a temperature of 230 °C. The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching the National Center for Biotechnology Information non-redundant database (NCBInr, version 20100129, www.ncbi.nlm.nih.gov) using the Mascot program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide Mass Tolerance ± 1.2 Da, Fragment Mass Tolerance ± 0.9 Da, missed cleavages 2. For positive identification, the score of the result of (–10 x Log(P)) had to be over the significance threshold level ($P < 0.05$).

Results

Application of ProteoMiner™ Technology

Inactive chronic HBV-infected plasma samples together with cirrhotic samples were analysed by 2D IEF-SDS-PAGE and proteins subsequently visualized by coomassie staining. Before to perform electrophoresis analysis on plasma collected from patients, efficiency of ProteoMiner™ Technology was tested. Figure 1 shows two representative maps of plasma control samples (inactive hepatitis B infection) before and after treatment with ProteoMiner enrichment kit. As expected, in the untreated sample albumin dominated the gel, obscuring signals from less abundance proteins. When an equal amount of protein is pre-treated with ProteoMiner, generated 2D gels showed a dramatically improved resolution and a greater number of spots was detected.

Comparison between plasma proteome of both inactive HBV infected patients and patients suffering from HBV-associated liver cirrhosis

The experimental procedure involved large 2D gel electrophoresis (17 cm, pI 4-7) conducted on 8 plasma samples from inactive HBV-infected patients and 8 plasma samples from patients with hepatitis B virus-related liver cirrhosis. Eight biological and three technical replicates (for a total of 24 maps) per condition were performed and cross-compared by

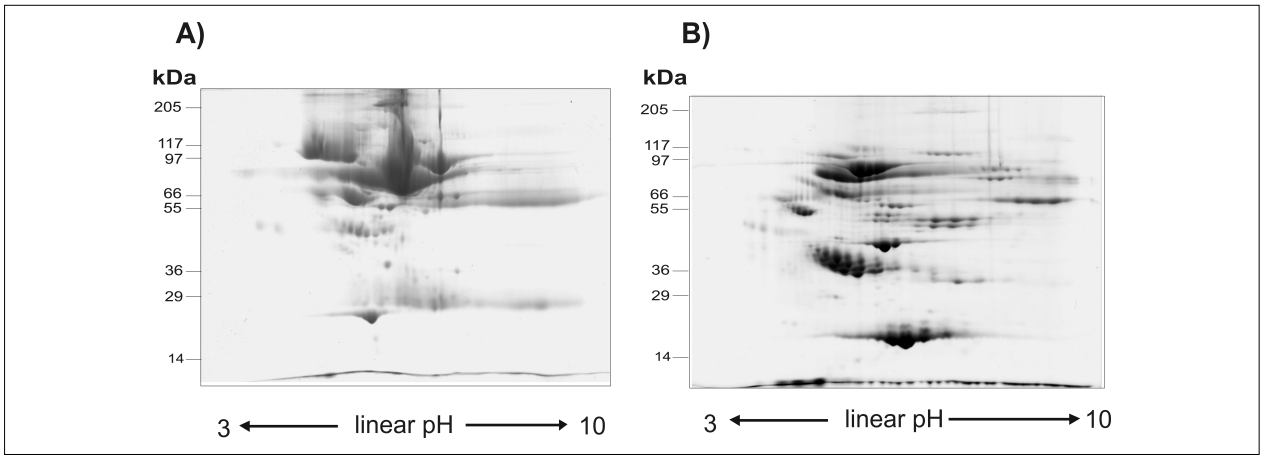

Figure 1 - Small size 2D gels (linear IPGs pH 3-10, 7 cm length) of plasma samples from inactive chronic HBV-infection patients before (A) and after (B) treatment with ProteoMiner enrichment kit (Bio-Rad Laboratories, Hercules, CA, USA). Total protein load: 200 µg.

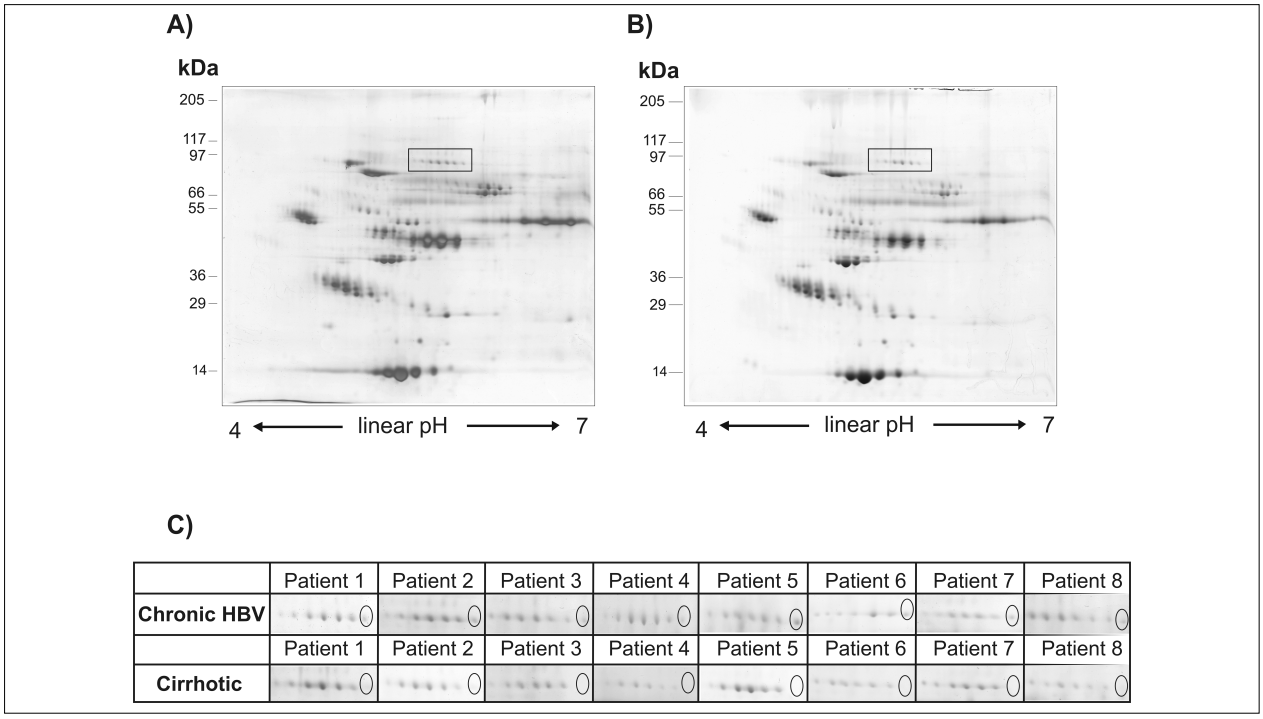

Figure 2 - Representative analytic 2D-gels (linear IPGs pH 4-7, 17 cm length) of ProteoMiner pre-fractioned plasma samples. A, inactive HBV-infected patients. B, HBV-related cirrhotic patients. Total protein load: 400 µg. Boxes indicate gel regions which are zoomed in the panel below. C, close-up views of the 2D gel area showing the reproducible difference of the candidate marker protein. Ellipse indicates gelsolin spot totally repressed in patients suffering from HBV-associated liver cirrhosis.

image software analysis Progenesis SameSpot (NonLinear Dynamics, Newcastle, UK) which generated the reference maps shown in Figure 2A-2B. As can be seen, the 2DE pattern is similar between samples. We found 624 spots that were common to all the gels and searching for qualitative changes resulted in only one spot of difference which was detected in 2-DE gels of inactive HBV infected plasma and was completely repressed in 2-DE map of plasma from patients with HBV-associated liver cirrhosis. Figure 2C displays sections of 2DE images showing reproducible detection of the candidate

protein in all the inactive HBV infected patients which were analyzed. Interestingly, this protein appeared as the more basic spot of a train pattern, typically due to post-translational modifications (PTMs). The spot of interest was excised from the gels, digested by trypsin and peptide mixtures were then analysed by LC-ESI-MS/MS for protein identification. Mass spectrometry analysis identified this completely repressed protein as gelsolin actin-depolymerizing factor (ADF). Results are summarized in Table II.

Discussion

Currently, lack of robust biomarkers still limits evaluation of hepatic fibrosis stages and progression in chronic diseases, especially in HBV infection[22,23]. Liver biopsy remains the gold standard for assessment of hepatic fibrosis, but it is an invasive procedure with inherent risk and sampling variability[24]. In addition, the diagnostic accuracy depends on the size of the biopsy specimens[25]. Furthermore, intra- and inter-observer variation for interpretation of biopsies is 10-20%, even among experienced pathologists[26]. Serum-based tests of liver fibrosis have attracted more attention in recent years. In particular, comparison of proteomes of disease and control serum samples has been shown to be a possible approach for discovering serum biomarkers of liver diseases associated with hepatitis B infection[27-30]. Surface enhanced laser desorption/ionization (SELDI) was used to develop fingerprinting models for discriminating different stages of fibrosis and predicting fibrosis, cirrhosis and hepatocellular carcinoma in chronic HBV infections[31-33]. Although serum/plasma became an important resource for proteome analysis, and several depletion and fractionation technologies have been developed to remove highly abundant proteins such as albumin and immunoglobulin G, little study has been reported on the use of enrichment pre-treatment in biomarker discovery for HBV-associated liver diseases[34]. Recently, the ProteoMiner technology has been proposed as a promising and powerful alternative to common immuno-subtraction tools and a flurry of applications emerged in the literature[15]. For the first time, with this study the use of ProteoMiner was tested in searching new potential candidate markers in plasma samples of (HBV)-related cirrhotic patients. Though many differential proteins or discriminatory patterns were demonstrated in previous studies, most of them were focused on end-stage liver diseases, especially on hepatocellular carcinoma[35-37]. Moreover, these studies looked at differentially expressed proteins and not to absolute protein expression

Table II - Detailed MS/MS peptide sequence analysis of the identified candidate biomarker

Protein name	Mr, kDa theor/exper [a]	pI theor/exper [a]	NCBI Accession No.	Peptides identified by MS/MS m/z	charge state	start-end [b]	sequence	Mascot Ion Score
				500.76	2+	33-43	R.GASQAGAPQGR.V	42
				418.90	3+	62-72	K.AGKEPGLQIWR.V	36
				425.76	2+	169-177	K.KGGVASGFK.H	51
				361.61	2+	170-177	K.GGVASGFK.H	46
				638.36	2+	178-188	K.HVVPNEVVVQR.L	66
Gelsolin				441.73	2+	361-368	K.TASDFITK.M	36
[Homo	86.04/88.0	5.9/5.7	gi|4504165	915.46	2+	374-390	K.QTQVSVLPEGGETPLFK.Q	54
sapiens]				528.76	2+	554-564	R.EGGQTAPASTR.L + pyro-Glu (N-term E)	31
				378.22	2+	578-584	R.AVEVLPK.A	55
				660.39	2+	585-597	K.AGALNSNDAFVLK.T	66
				919.46	2+	598-615	K.TPSAAYLWVGTGASEAEK.T	93
				444.25	2+	616-623	K.TGAQELLR.V	60
				758.30	2+	627-648	R.AQPVQVAEGSEPDGFWEALGGK.A	55
				488.81	2+	721-729	K.TEALTSAKR.Y	44
				379.22	2+	742-748	R.TPITVVK.Q	46

[a] theoretical Mr/pI was calculated with Mr/pI tool on the Expasy web site (http://expasy.org/tools/pi_tool.html)
[b] start-end positions of identified peptides were calculated against complete amino acid sequence of the protein

changes (newly expressed or totally repressed spots). Furthermore, to our knowledge, our preliminary study is the first on plasma samples of patients suffering of HBV-associated liver cirrhosis. Interestingly, our findings demonstrated the repression of a gelsolin-containing spot. Gelsolin is a highly conserved, multifunctional actin-binding protein initially described in the cytosol of macrophages and subsequently identified in many vertebrate cells. A unique property of gelsolin is that in addition to its widely recognized function as a cytoplasmic regulator of actin organization, the same gene expresses a splice variant coding for a distinct isoform, plasma gelsolin, which is secreted into extracellular fluids[38]. The secreted form of gelsolin was implicated in a number of processes such as the extracellular actin scavenging system and the presentation of lysophosphatidic acid and other inflammatory mediators to their receptors, in addition to its function as a substrate for extracellular matrix-modulating enzymes[38]. Consistent with these proposed functions, blood gelsolin levels decrease markedly in a variety of clinical conditions such as acute respiratory distress syndrome, sepsis, major trauma, prolonged hyperoxia, malaria, and liver injury[39-44]. Therefore, gelsolin could not be considered as a specific marker of HBV-related cirrhosis. However, our identified spot corresponded to a specific isoform of gelsolin. In fact, gelsolin protein generated spot trains in 2D maps, typically due to post-translational modifications or to small differences in amino acid composition (usually a splice variant). This progressive change in the pI and Mr of protein spots on serum/plasma 2-DE maps was reported before[29,30] and detection of specific modified isoforms is a pathological hallmark[45]. As a rule of thumb, the correlation between blood gelsolin levels and several critical clinical conditions suggests the potential utility of gelsolin as a prognostic marker as well as the possibility for therapeutic replenishment of gelsolin to alleviate the injurious cascades in these settings[38]. On the other hand, the presence or absence of a particular isoform for this protein may be specific for a certain disease. To this regard, deeper investigations are being carried out in our lab with the intent to further characterize the detected gelsolin isoform which was found to be repressed in plasma samples of patients suffering from liver cirrhosis associated with hepatitis B infection. Additional experimentation is also needed to validate this protein as a real biomarker with approval of clinical efficiency. At any rate, the indication of new candidate markers may help in early diagnosis of HBV-related liver diseases.

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Correspondence: Lello Zolla
Dipartimento di Scienze Ambientali
Università della Tuscia
Largo dell'Università, snc
01100 Viterbo (VT), Italy
E-mail: zolla@unitus.it