ANALYSIS OF THE PLASMA PROTEOME OF PATIENTS WITH CHRONIC HEPATITIS C INFECTION UNDERGOING TREATMENT WITH INTERFERON ALPHA AND RIBAVIRIN: A PILOT STUDY

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Abstract

Background - Chronic hepatitis C virus infection is a massive worldwide healthcare burden with estimated costs in the USA alone of over $5 billon per annum. The only current effective treatment is combination therapy with interferon alpha and ribavirin (IFNα + RBV) which is expensive and has significant side-effects. Unfortunately, it is only effective in up to

50% of those infected with HCV genotype 1. In this study the examination of the patterns for the protein expression followed the peg-IFNα + RBV treatment in patients whom were selected to demonstrate different durations of the disease depending upon their response. The current treatment usually gives an SVR rate of around 55 %. Through the virological and host-related factors, the predictive value of HCV kinetics during treatment seems to exceed that of all virus and host-related baseline factors for predicting the virologic response. The identification of easily measurable biomarkers which predict response to treatment either before or during the early stages would enable better selection of those who would benefit from starting or continuing treatment. The search for novel biomarkers in disease has been the focus of increased attention in recent years due to the advances made in proteomic technology. The plasma proteome is an attractive target for such studies because it is readily available from patients on a regular basis. It is also in contact with all tissues in the body and so may reveal differences in the abundance of proteins expressed in these tissues. Current proteomic technology allows the characterisation of plasma proteins across a wide dynamic range to include those secreted from cells at relatively high concentration to those there due to tissue leakage present at extremely low levels. In this pilot study the plasma from a small number of selected patients was subjected to proteomic analysis to investigate whether there were variations in plasma protein profile which could predict treatment efficacy.

AIMS - The article shows the first stages of an ongoing analysis of the plasma proteome. As the aim is to identifying the potential biomarkers which indicate either responsiveness or resistance to treatment of chronic HCV with interferon alpha and ribavirin.

Methods - Three patients chronically infected with Hepatitis C virus genotype 1b who were to be treated with pegylated Interferon alpha and Ribavirin were selected for the pilot study. The total length of treatment was to be 48 weeks and patients were followed up as out-patients where biochemical and virologic parameters were measured and used as indicators of response.

Peripheral blood samples were taken into EDTA at the start of treatment and again at 14 and 28 days. Blood was immediately cooled on ice and within 15 minutes centrifuged at 3000 rpm for 10 minutes to separate the plasma which was aliquoted and stored at -80°C.

The plasma was depleted of albumin and IgG using the Bio-Rad Aurum Serum protein mini kit. This method removed over 90% of the total albumin and immunoglobin, effectively enriching the other plasma proteins and maximising their resolution by electrophoresis. Protein concentration of the depleted plasma was estimated using the Bradford assay.

2D gel electrophoresis was performed on selected samples using GE Healthcare IPGphor IEF and Ettan units. Briefly, 70 µg depleted plasma protein was mixed with Sample Buffer (8M urea; 4% CHAPS; 2% Ampholytes; 50 mM DTT; 0.001% Pyronin Y) and rehydrated into immobilized pH Gradient (IPG) strips overnight (5-8 pH range).

Isoelectric focusing was carried out. The IPG strips were then equilibrated into Loading Buffer (8M Urea; 50 mM Tris, 2% Lauryl SDS, 0.001% Pyronin Y, pH 6.8) and reduced with 50 mM DTT, prior to alkylation with 20 mM iodoacetamide. The IPG strips were then run onto a 12% Polyacrylamide gel for SDS-PAGE. The gels were then washed briefly and stained with Colloidal Coomassie Blue. The gels were destained in water and imaged using a BioRad GS-800 Scanning Densitometer and PDQuest software.

10 µg samples of plasma for 1D SDS-PAGE were added to four-times the volume of Loading Buffer. Samples were kept cool and then electrophoresed through a 12% SDS-PAGE gel. The gels were then either stained with Colloidal Blue or imaged, or electroblotted onto PVDF membrane. The membrane was blocked with 5% milk powder and then probed with mouse monoclonal antibody to human haptoglobin (Sigma H6395). The membrane was then washed thoroughly with PBS/Tween, incubated with antibody to mouse immunoglobulins-HRP (Dako P0260) and washed again with PBS/Tween. The membrane was developed using ECL plus (GE Healthcare) and the image acquired on a BioRad Fluor-S multi-imager.

Spots and bands were excised from the polyacrylamide gels and dried under vacuum. Proteins were digested using trypsin in 30 mM Ammonium Bicarbonate. The samples were concentrated under vacuum and extracted using a mixture of acetonitrile and 0.1% Formic Acid. Peptides were separated and identified using electrospray mass spectrometry (LC-MS/MS). The peptides of the mixture were separated using reverse-phase chromatography over a C18 column (Dionex) onto a Waters Q-ToF Micro. Tandem mass spectrometry data were analysed using the Mascot programme (Matrix Science).

Results - A number of differences were seen in patients compared with controls when the plasma was analyzed using 2D electrophoresis. Perhaps the most striking being the absence of haptoglobin alpha chain forms which were identified by electrospray mass spectrometry (Fig 1).

Further analysis of albumin-depleted plasma by 1D SDS-PAGE found that two of the patients showed a considerable increase in an approximately 80 kDa protein band in samples taken at days 14 and 28 on treatment when compared with a pre-treatment sample (Fig 2).

This protein was identified as deriving from the haptoglobin precursor using LC-MS/MS, the patients who exhibited the increase in the ~80 kDa protein were a responder and a transient

responder to treatment. The patient who did not respond to treatment showed no temporal increase in this band. The increase in the 80 kDa form of haptoglobin in responders was confirmed using immunoblotting probed with an antibody raised against haptoglobin (Fig 3).

The molecular weight of this band is anomalous. Haptoglobin usually appears as a band of around 20 kDa when strongly reduced and denatured. There have been reports of processed haptoglobin forming dimeric and tetrameric complexes when not fully denatured. The buffer used was not the classic Laemmli buffer and the samples were not boiled, so there may have been interaction between the sub-units. Alternatively, these may be interacting with another protein or ligand or these could be splice-variants of the haptoglobin precursor. Further work is under way to determine whether this is a robust finding that has broader implications for a wider cohort of patients.

Discussion and Conclusions - Haptoglobin has been implicated in liver disease for some time although some contradictory results have been seen. Recently it has been included in a panel of tests, the so-called 'Fibrotest', which it has been suggested can estimate the degree of hepatic fibrosis where serum haptoglobin levels inversely correlate with fibrosis.

In this study we have shown that in a small number of untreated HCV patients there is a reduction of haptoglobin compared to normal plasma. However, during treatment some individuals show a dramatic increase in a high molecular weight form of haptoglobin. It is unlikely that this is due to any hemolytic events associated with Ribavirin treatment, as this is usually associated with reductions in serum haptoglobin. Also, most patients had reasonably stable serum hemoglobin during the period of study. In this limited pilot study the patients which exhibited an increase in plasma haptoglobin soon after the commencement of IFNα+R therapy were those that responded to treatment by reduction of viral load to undetectable levels; one albeit transiently. The mechanism of the increase in haptoglobin in response to treatment is unknown, and it remains to be seen whether changes in plasma haptoglobin are consistently associated with responses to treatment in chronic hepatitis C virus infection.

Haptoglobin is a positive acute phase reactant and as such plasma levels may be expected to be disrupted during periods of inflammation in the liver, although no association with the degree of hepatic inflammation has been shown. This of course may have been confounded by the negative correlation seen between serum haptoglobin and liver fibrosis.

Further proteomic analysis of this patient group is ongoing and represents a powerful tool in the search for potential biological markers which may help in the management of therapy.

Keywords : Chronic hepatitis C infection, interferon alpha, ribavirin

Introduction

HCV remains an important cause of liver disease globally and affects over 170 million of people worldwide. It has been estimated that about 3–4 million individuals are newly infected each year [1].

HCV primary infection leads to persistent viremia in 80-85% of cases, and around 60% can develop chronicity [2]. Meanwhile, 20% of those reach the end stage of liver disease, cirrhosis and /or hepato-cellular carcinoma (HCC) – depending on the variation between virus genotypes and subtypes [3-6].

Currently, treatment with pegylated interferon and ribavirin is the standard of care although a number of new drugs are undergoing clinical trials. Non-responsiveness to IFNs is fairly common and rates vary in HCV infection [7]. Numerous studies showed that less than 50% of patients with hepatitis C virus infection respond to IFN-alpha. This ratio is expected to increase based on identification of factors that the response to therapy and the likelihood of a patient achieving a sustained virological response. These include HCV genotype, genetic variation in the HCV NS5A gene, age, sex, geographic and ethnic group, and host genetics [8-15].

The variation in patient's response to viral therapy has emerged the need to identify other markers which allow patients to be stratified as potential non-responders [16, 17].

Chronic hepatitis C is associated with a largely unexplained iron overload and higher oxidative stress. Furthermore, ribavirin treatment leads to higher serum and liver iron levels which may due to the haemolytic anaemia induced by ribavirin treatment [18].

Proteomic studies of HCV infection have been particularly limited, for several reasons, including the lack of a good cell culture model and the need for large amounts of protein for conventional proteomic analysis [19, 20].

With development in both proteomic methodologies [21, 22], and cell culture models of HCV infection [15, 23], it has been made possible to characterize the whole host cell protein response within the context of the complete set of HCV genes in vitro.

Refined multidimensional liquid chromatographic (LC) separations, coupled with mass spectrometry (MS) for proteome analysis, have also allowed global experiments to be performed, using less protein and obtaining more sensitivity, throughput and dynamic range than previous proteomic techniques required [24-26].

Liver fibrosis or cirrhosis are considered to be the deleterious consequences of CHC [27-29] and can be characterized by striking medications of proteins, including synthesis of the

extracellular matrix proteins. This in turn suggests that the proteomics may provide a new insight in diagnosing fibrosis and cirrhosis [20, 30].

Several studies showed by using 2D gel electrophoresis that the expression of more than forty proteins could be altered when the cells were activated, either in vivo or in vitro [31, 32].

Surface-enhanced laser desorption/ionization time-of-light mass spectrometry (SELDI-TOF MS) technology can be applied to obtain the protein quickly and can also perform comparative analysis according to the patient's phenotype. This approach shows the advantage of making the first step the fractionation of the proteome according to its adaption on different affinity surfaces, followed by adsorption and time-of-light analysis for the proteins [33].

According to a study conducted by Su (2002) on the determination of protein pathways and the genomic analyses of liver biopsies from infected chimpanzees, using a sub genomic replicon model, the accumulation of the free fatty acids associated with transcriptional changes in the host genes involved in lipid metabolism can be determined [34].

In another study, Jacob (2005) demonstrated that transfection of Huh-7.5 cells with a full-length HCV replicon induces several protein abundance changes with disturbances in the lipid metabolism [31].

Proteomics and the genome-wide study of protein expression in human beings represent an expanding area, and the proteome technology is based on a subtractive procedure, when the protein patterns can be compared with the identified markers [35].

Proteomic characterization that has been used in human HCC cell lines [27] lead to establishing an expanded reference map for human liver tissue and carrying out a differential profiling of proteins for normal and tumour tissues of human HCC. Sixteen protein spots have been identified and exhibited significant abundance alterations in matched sets of normal and HCC tissues and proposed these proteins as candidates for HCC-associated proteins.

The sixteen protein spots corresponded to 11 proteins with a significant abundance of normal and tumour tissues, which were identified by using peptide mass fingerprinting. Kim et al found that these proteins potentially played a role in the tumour genesis and progression of human HCC and were therefore useful markers for diagnostics or targets for therapeutic intervention. As a basic tool for the proteome analysis of liver tissues, they expanded a large 2-DE reference map for human liver tissue [35].

In 2009, Mas et al provided important information for elucidating the different steps of HCC carcinogenesis, although their findings are limited due to their diagnostic values. Still, their identification of non-invasive HCC markers of antigenic proteins showed that these markers may be useful in evaluating the HCC patients after therapy, in particular in after liver transplantation [32].

The human plasma proteome is now promising a revolution in disease diagnosis and therapeutic monitoring, since it provides potentially the largest range of the human proteins. However, all types of plasma are readily available it is also one of the most difficult protein-containing samples to analyses, on account of the large proportion of albumin (55%), the wide dynamic range of abundance of other proteins and the tremendous heterogeneity of its predominant glycoproteins. In addition, plasma proteins contain all the tissue proteins, such as the leakage markers, plus very numerous distinct immunoglobulin sequences [37].

Patients and Methods:

Three patients out of ten chronically infected with Hepatitis C virus who were treatment naïve were selected for the pilot study. A written consent form and study information sheet were given to each patient. The study was approved by the local research ethics committee of Royal Free Hospital. Patients were treated with pegylated Interferon alpha dose and Ribavirin dose. Patients were treated for 48 weeks and patients were followed up as out-patients where biochemical and virologic parameters were measured and used as indicators of response.

Proteomic analysis

Peripheral blood samples were taken into EDTA tubes at the start of treatment and again at 14 and 28 days. Blood was immediately cooled on ice and within 15 minutes centrifuged at 5000 RPM for 10 minutes to separate the plasma which was aliquoted and stored at-80°C The plasma was then depleted of albumin and IgG using the Bio-Rad Aurum Serum protein mint kit which removes over 90% of the total albumin and immunoglobin, effectively enriching the other plasma proteins and maximising their resolution by electrophoresis. Protein concentration of the depleted plasma was then measured using the Bradford assay.

2D gel electrophoresis was performed on selected samples using GE Healthcare IPGphor IEF and Ettan units according to the manufacturer's instructions. Briefly, 70 ug depleted plasma protein was mixed with Sample Buffer (8M urea, 4% CHAPS; 2% Ampholytes; 50 mM DTT; 0.001% Pyronin Y) and rehydrated into immobilized pH Gradient (iPG) strips overnight (5-8 pH range). Iso-electric focusing was carried out and the IPG strips were then

equilibrated into Loading Buffer (BM Urea, 50 mM Tns, 2% Lauryl SDS, 0.001% Pyronin Y, pH 6.8) and reduced with 50 mM DTT, prior to alkylation with 20 mM iodoacetamide.

The IPG strips then run onto a 12% polyacrylamide gel for SDS-PAGE. The gels are then washed briefly and stained with Colloidal Coomassie Blue. The gels were destained in water and imaged using a BioRad GS- 800 Scanning Densitometer and PDQuest software.

Western blotting

10 ug samples of plasma for 1D SDS-PAGE were then added to four-times the volume of loading buffer. Samples were kept cool and then electrophoresed through a 12% SDS-PAGE gel. The gels then either stained with Colloidal Blue and imaged, or electro-blotted onto PVDF membranes. The membrane was blocked with 5% milk powder and then probed with mouse monoclonal antibody to human haptoglobin (Sigma H6395). The membrane was then washed thoroughly with PBS/Tween, incubated with antibody to mouse immunoglobulin's-HRP (Dako P0260) and washed again with PBS/Tween. The membrane was developed using ECL plus (OF Healthcare) and the image acquired on a BioRad Fluor-S multi-imager KOa O 14 21. Spots and bands were excised from the polyacrylamide gels and dried under vacuum. Proteins were digested using trypsin in 30 mM Ammonium Bicarbonate. The samples were concentrated under vacuum and extracted using a mixture of acetonitrile and 0.1% Formic Acid. Tandem mass spectrometry data were then analysed using the Mascot programme (Matrix Science).

Results

Characteristics of patients

The study included 10 patients infected with HCV. There were 3 male and 7 female patients. Male patients were 1 TR, 1 NR and 1R. Female patients were 2 TR, 3 NR and 2 R (table 1).

Table1: Frequency by sex of responders and non-responders for HCV treatment

Name	Sex	Sample date	Date of treatment	Kind of Respond	Over all samples #
Pt1	M	15/10/2001	15/10/2001	TR	1
		05/11/2001			2
		01/12/2001			3

Pt2	M	17/01/2000	17/01/2000	NR	4
		22/08/2000			5
		14/12/2000			6
Pt3	F	28/11/2001	28/11/2001	TR	7
		13/03/2002			8
		27/03/2002			9
Pt4	F	08/08/2001	16/03/2001	R	10
		12/09/2001			11
		20/09/2001			12
Pt5	F	20/09/2001	20/09/2001	TR	13
		24/10/2001			14
		22/11/2001			15
Pt6	F	26/04/2000	02/10/2001	NR	16
		05/10/2001			17
		14/11/2001			18
Pt7	F	05/02/2001	05/02/2001	R	19
		07/03/2001			20
		04/04/2001			21
Pt8	F	17/01/2000	20/01/2000	NR	22
		23/08/2000			23
		17/11/2000			24
Pt9	M	20/09/2006	20/09/2006	R	25
		25/10/2006			26
		21/12/2006			27
Pt10	F	17/09/2006	17/09/2006	NR	28
		21/10/2006			29
		18/12/2006			30

Proteomic Results

The patients plasma were run on the SDS-PAGE of the time into treatment, and (figure 1) shows the transient responder and the non-responder all with the responder which framed in a red dotted boxes in order to show the Hp band which identified by the LC-MS/MS.

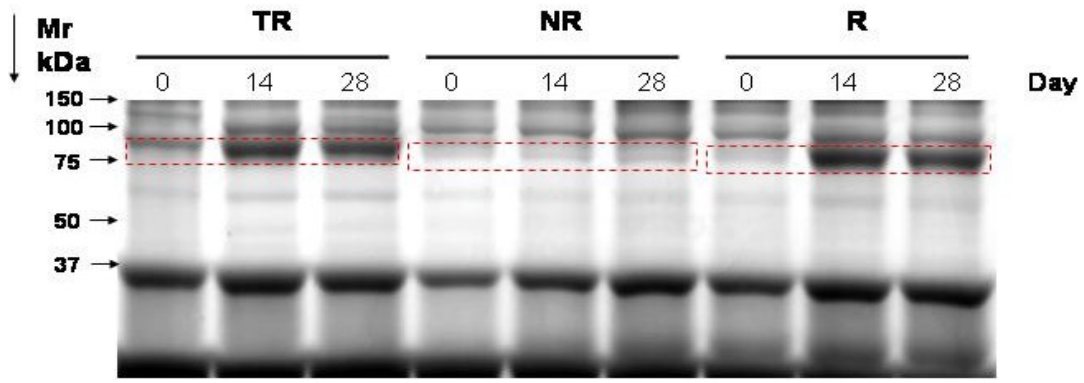

Figure 1. Patient plasma samples run on SDS-PAGE. Time into treatment at which sample take shown along the top in days. TR-transient responder, NR=non-responder, R=responder. Red dotted boxes show the haptoglobin bands identified by LC-MS/MS.

A western blotting (WB) analysis for the patients plasma were examined by the mouse monoclonal Ab to human Hp in the time during treatment. At (figure 2 A-C) the 80KDa of Hp band are framed in red dotted boxes and run on 1D gel.

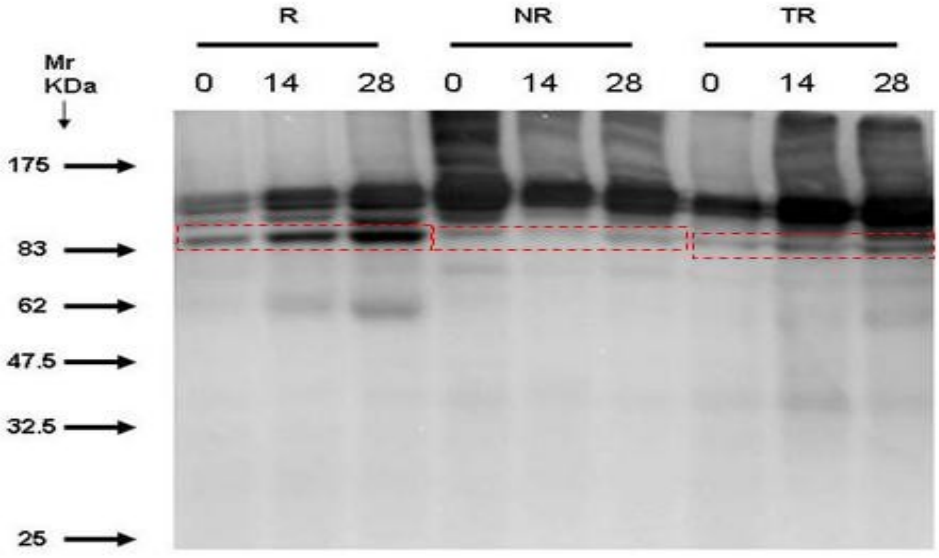

Figure 2. Western blot analysis of patient plasma samples stained with mouse monoclonal antibody to human haptoglobin. Time into treatment shown along the top in days. ~80kDa haptoglobin identified by red dotted boxes.

When the plasma was analyzed using 2D electrophoresis, a number of differences were seen in patients compared to the controls. Perhaps the most striking being the absence of haptoglobin alpha chain forms which were identified by electrospray mass spectrometry (figure 3).

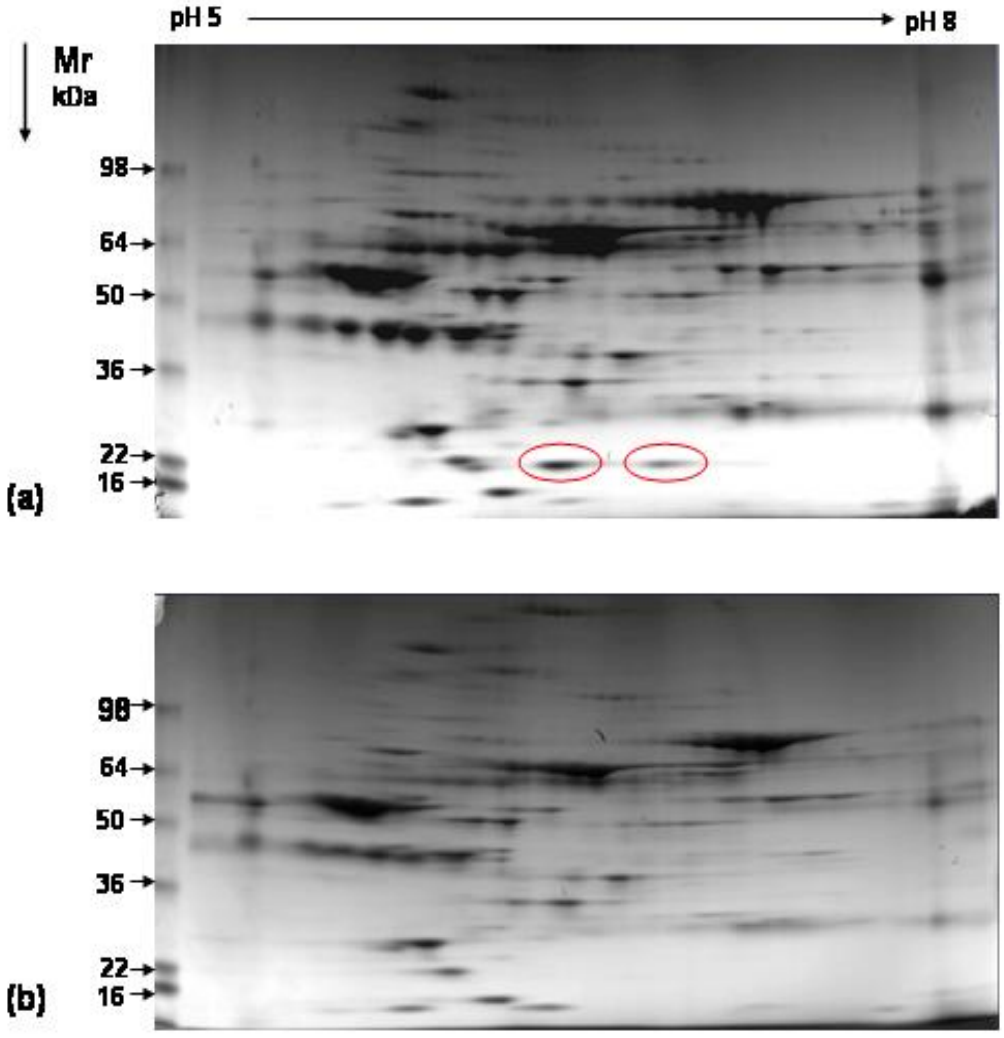

Figure 1. Representative 2D gel images of normal and hepatitis C virus infected plasma (a) normal (b) hepatitis C. Red ellipses identify Haptoglobin alpha chain

Further analysis of albumin-depleted plasma by 1D SDS-PAGE found that two of the patients showed a considerable increase in an approximately 80 kDa protein band in samples taken at days 14 and 28 on treatment when compared with a pre-treatment sample.

This protein was identified as deriving from the haptoglobin precursor using LC-MS/MS. The patients who exhibited the increase in the ~80 kDa protein were a responder and a transient responder to treatment.

The patient who did not respond to treatment showed no temporal increase in this protein in responders which was confirmed by using immunoblotting probed with an antibody raised against haptoglobin.

Discussion

In this study we have shown that in a small number of untreated HCV patients there is a reduction of haptoglobin compared to normal plasma. However, during treatment some individuals show a dramatic increase in a high molecular weight form of haptoglobin.

In this limited pilot study the patients which exhibited an increase in plasma haptoglobin soon after the commencement of IFNα+R therapy were those that responded to treatment by reduction of viral load to undetectable levels; one albeit transiently.

It is unlikely that this is due to any haemolytic events associated with Ribavirin treatment, as this is usually associated with reductions in serum haptoglobin. Also, most patients had reasonably stable serum hemoglobin during the period of study.

Haptoglobin is a positive acute phase reactant and as such plasma levels may be expected to be disrupted during periods of inflammation in the liver, although no association with the degree of hepatic inflammation has been shown. This of course may have been confounded by the negative correlation seen between serum haptoglobin and liver fibrosis.

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The mechanism of the increase in haptoglobin in response to treatment is unknown, and it remains to be seen whether changes in plasma haptoglobin are consistently associated with responses to treatment in chronic hepatitis C virus infection.

Further proteomic analysis of this patient group is ongoing and represents a powerful tool in the search for potential biological markers which may help in the management of therapy.

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