Haptoglobin, an acute phase reactant as marker for chronic pancreatitis – evidence from serum proteome analysis

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ABSTRACT

Background and Aim: Serum proteome analysis is a novel tool to detect the protein markers for any pathological disease. Diagnosis of chronic pancreatitis is mainly based on the level of serum marker enzymes amylase and lipase. The aim of the present study is to evaluate other new serum protein markers for chronic pancreatitis in human subjects by proteome analysis. **Subjects and Methods:** Serum proteome analysis was carried out in groups of pooled serum samples from chronic pancreatitis patients (n-25) registered in the Department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital in the age group of 30 – 50. Normal serum samples (n-20) were pooled in groups from age and sex matched normal healthy volunteers. Serum proteome analysis was carried out by conducting 2D-electrophoresis, identifying differentially expressed proteins, isolating and excising the bands of interest, digesting the proteins by trypsin, separating of digested peptides by 2D Liquid Chromatography Mass spectroscopy (2D LC-MS) and identifying peptides by Mass Finger Printing Technique. The proteins were then confirmed by protein search engine programme MASCOT. Quantitative analysis of such protein(s) was carried out individually in all the samples for confirmation. **Results:** Among the 45 differentially expressed proteins, we have considered Haptoglobin-2 (Hp-2) as one of the marker, since Hp was quantitatively elevated significantly in all the samples considered for the study. Hp-2 isoform was also confirmed by native PAGE by using specific Benzidine-H_2O_2 stain. Being a natural acute phase reactant, Hp-2 level might have been elevated as a compensatory mechanism to counteract the tissue damage and to overcome the oxidative stress as an antioxidant because pancreatic tissue damage and inflammation is associated with the harmful effects of free radicals. **Conclusion:** Hp-2 isoform, a positive acute phase reactant with antioxidant property is identified as a marker for chronic pancreatitis by serum proteome analysis.

Keywords: Chronic pancreatitis, haptoglobin-2 (Hp-2), acute phase reactant, antioxidant, serum proteome.

INTRODUCTION

Pancreatitis is associated with painful inflammation of the pancreas and is one of the major gastrointestinal disorders with the gradual increase in the mortality rate of 2% per year. It occurs when the enzymes that digest food are activated in the pancreas itself that leads to auto digestion of the pancreatic cells.[1,2] Pancreatitis may be acute or chronic. Acute pancreatitis occurs suddenly, causes severe pain in the abdomen and lasts for a few days and often, resolves completely with treatment. It can be a life-threatening illness with severe complications. Chronic pancreatitis is inflammation of the pancreas that does not heal or improve but gets worse over time and leads to permanent damage. Chronic pancreatitis, like acute pancreatitis, occurs when digestive enzymes attack the pancreas and nearby tissues through cytokines, causing episodes of pain.[3–5] The incidence of acute pancreatitis

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is 19.5 per 100,000 populations and chronic pancreatitis is 8.3 per 100,000 individuals per year. Chronic alcohol intake and gallstone formation are two major causes for chronic pancreatitis.[6–9]

Most of the people with chronic pancreatitis experience upper abdominal pain, although some people have no pain at all.[10] Other symptoms include swollen and tender abdomen, nausea, vomiting, fever, a rapid pulse weight loss, diarrhea and fatty stools (steatorrhea). People with chronic pancreatitis often lose weight, even when their appetite and eating habits are normal. Less enzyme secretion cause poor digestion and leads to malnutrition.

Alcohol abuse is found to be the major cause of chronic pancreatitis. Alcohol is not only metabolized in liver but also in pancreas and produce reactive oxygen species (ROS) which initiate the pancreatic cell damage. The tissue injury stimulates the production of cytokines, which aggravates the cell damage to affect exocrine and endocrine function of pancreas.[11,12] Gall stone when exist on or near the head of pancreas, block the flow of pancreatic secretion to intestine and hence the secretions flow back to the gland and the enzymes digest the cells. This results in exocrine cell damage and release of lipase and amylase to the blood circulation. Till now these enzyme acts as markers for diagnosis of chronic pancreatitis.[13]

The diagnostic criteria for pancreatitis are "two of the following three features: 1) abdominal pain characteristic of acute pancreatitis, 2) serum amylase and/or lipase ≥3 times the upper limit of normal, and 3) characteristic findings of acute pancreatitis on CT scan".[14] Changes may also occur in other body chemicals such as glucose, calcium, magnesium, sodium, potassium, and bicarbonate.

Proteomics is the large-scale study of proteins differentially expressed in diseases, and act as markers of diagnostic importance.[15,16] There is a great interest in proteomics because it gives a much better understanding of an organism than genomics. The level of transcription of a gene gives only a rough estimate of its level of expression into a protein. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Many proteins experience post-translational modifications that profoundly affect their activities. Many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules. Finally, protein degradation rate plays an important role in protein content.[17]

Serum proteome analysis is a novel tool to detect the protein markers for any pathological disease. Since chronic pancreatitis is mainly diagnosed based on the level of serum marker enzymes amylase and lipase, the current study was designed to evaluate whether any other proteins, which are released in to the blood stream from pancreas and others during pancreatitis, can be used as a biological marker to diagnose chronic pancreatitis by conducting serum proteome analysis.

MATERIALS AND METHODS

Reagents and chemicals

Immobilized pH gradient strips (IPG) of pH 3-10, (Immobiline Dry Strip 0.5 × 3 × 180 mm) was purchased from Genei, Bangalore, India and Sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, tetramethylethylenediamine (TEMED), tris, glycine, urea dithiothreitol (DTT), formaldehyde and other reagents for 2-DE and MS were purchased from Sigma Aldrich Company, Bangalore, India. All the other reagents and chemicals used were of analytical grade.

Subjects

The patients registered in the department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital, Chennai were enrolled in the study. The study included 25 chronic pancreatitis patients and 20 age and sex matched normal healthy individuals. They were at the age group of 30–50 years of both sexes. The patients were diagnosed for pancreatitis by endoscopy, abdominal X-ray and by exocrine and endocrine functional assay. The case history was obtained from each patient. All the patients and the control subjects did not have any major diseases affecting thyroid and bone and were not under any medical treatment including supplementation of antioxidants. The clinical history of patients is presented in Table 1.

Sample collection and processing

Blood sample was collected at sterile condition from each patient and normal subject by venous arm puncture, allowed to clot and immediately centrifuged at 3000 rpm for 20 min for serum separation. Serum separated were pooled in groups, stored in liquid N_2 at –20°C and processed for proteome analysis. Total protein concentration

Table 1 Clinical history of normal subjects and pancreatitis patients

Characteristics		Normal Subjects	Chronic Pancreatitis
Total number		20	25
Male / Female ratio		16/4	20/5
Age		30–50	30–50
Alcohol associated		–	10
Gall stone associated		–	7
Metabolic and other causes		–	3
Abnormal Liver function		–	5
Pancreatic function	Normal	20	–
	Abnormal	–	25

was estimated by the method of Bradford.[18] Major proteins like albumin, IgG, IgA, transferrin present in serum were removed by using suitable serum immunoaffinity columns.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE or 2-DE)

2-DE was carried out according to the procedures adopted by Westereier, Liu *et al.*, and Jianjun Shen *et al.*[19–21] Briefly, 1–3 µl (350 µg) of abundant protein depleted serum was rehydrated and subjected to the first-dimension isoelectric focusing in immobilized pH gradient strips (IPG) of pH 3-10 on Isoelectric focusing (IEF) cell for 46,000 Vhr at 20°C. Then, the gels were equilibrated for 30 minutes each in buffer I (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.1% DTT) and buffer II (50 nM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.25% iodoacetamide). Proteins get separated in first-dimension electrophoresis based on their isoelectric point (pI). The second-dimension separation by SDS-PAGE was carried out by using 12% SDS-polyacrylamide gel in Criterion Cell apparatus according to the protocol of the Biotech Laboratories, Yercaud, India. The IPG strips were placed on the surface of the second dimension gel, and the strips were sealed with 0.5% agarose in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) and electrophoresed for 4 hrs at 110 V. Then the gel was stained with Ezee-Blue and spots were visualized and photographed. Among the differentially expressed proteins, the spot of our interest was selected and subjected to tryptic digestion.[22]

Protein profiling and identification

Analysis of the tryptic digests was done with minor modifiction by Liquid Chromatography Mass spectrometry method (LC-MS)[23] and mass caliberation was carried out with 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifuloroacetic acid diluted 1:1 with solvent. Protein peptides were identified by Mass finger printing technique and then confirmed by protein search engine programme MASCOT. Among the various differentially expressed proteins haptoglobin was selected because many peptides were related with haptoglobin. Haptoglobin was further confirmed by quantitative analysis in all the individual samples.

Analysis of acute phase reactants

Determination of haptoglobin in serum

Quantitative analysis of Haptoglobin (Hp)[24] in serum was done based on the peroxidase activity of Hp-Hb complex by mixing the serum with excess of free haemoglobin.

Assay of CRP in serum

The method of Wadsworth C & Wadsworth E[25] was adopted for the assay of C-reactive protein in the serum samples. The CRP-latex test is a rapid agglutination procedure for the direct determination of C-reactive protein. The reagent, latex particles suspension coated with specific anti human C-reactive protein antibodies, agglutinates in the presence of CRP in the serum sample.

Assay of Alpha-2 macroglobulin in serum

The method of Jesperson *et al.*,[26] was used for the determination of alpha-2 macroglobulin in serum samples of chronic pancreatitis patients and normal subjects.

Assay of C3a in serum

C3a in serum was also done based on the method of Dati *et al.*[27] Anti-human C3a antibody is mixed with samples containing C3a to form insoluble complex. This complex cause an absorbance change, depending upon the C3a concentration that can be quantified by comparing with the calibration curve obtained with known concentration of C3a.

Polyacrylamide gel electrophoresis (PAGE) of serum Hp isoform separation

Polyacrylamide gel electrophoresis method for Hp-isoform separation was done by the method of Davis.[28] Equal volume of serum and Hb solution (3 g/100 ml) were mixed and loaded in the well containing 10% polyacrylamide gel pH 8.8 and electrophoresed at 4°C with a starting current of 50 V for 10 minutes and thereafter the current was

maintained at 100 V per well for 2 hours. The separated bands were detected using Benzidine - H_2O_2 stain.

Statistical analysis

The results were presented as mean ± SD and the statistical significance of mean values between different groups was determined by applying student's t-test.

RESULTS AND DISCUSSION

Serum proteome analysis is a recently developed technique and finds application in diagnosis of many metabolic and inflammation related disorders. It is very interesting to find out that 45–50 abnormal proteins were differentially expressed in the sera of chronic pancreatitis patients than the normal subjects. Among the proteins observed, haptoglobin 2 (Hp 2), pre pro haptoglobin and haptoglobin related protein precursors were significantly elevated in chronic pancreatitis patients [Figure 1a]. All these proteins spot have shown greater than 60% matching score and confirmed their significant elevation. Interestingly the other proteins expressed were monoclonal Ig M antibody, Ig kappa light chain, collagen type VII, zinc finger FYVE domain-containing protein 19 and N-acetyl glucosaminase-1- phosphate transferase etc., [Figure 1, 1b, c].

2D nano LC-MS results of spot 1

Database: NCBInr 20070713 (5222402 sequences; 1811015345 residues)

Taxonomy: Homo sapiens (human) (181549 sequences)

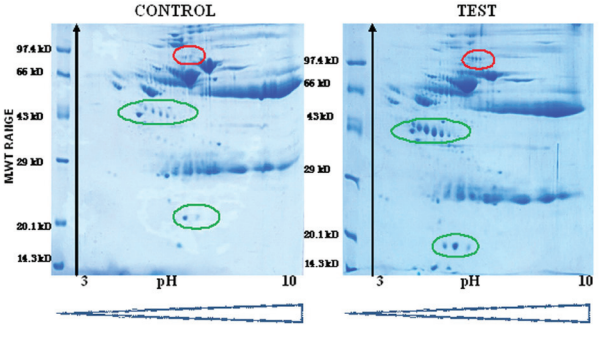

Figure 1. Comparison of the 2D gel electrophoresis of serum proteins of normal subjects (Control) and chronic pancreatitis patients (Test) in 12% SDS-PAGE (second dimension) in which 350 µg of protein was loaded into IEF. The figure is the representative of 5 individual experiments conducted by using pooled serum samples.

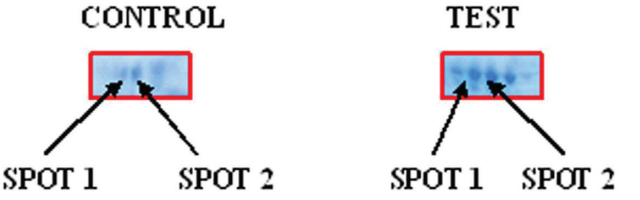

Figure 1a. Cropped 2D gel images of selected proteins in normal subjects (Control) and chronic pancreatitis patients (Test) in which Spot 1 and Spot 2 are selected for the study.

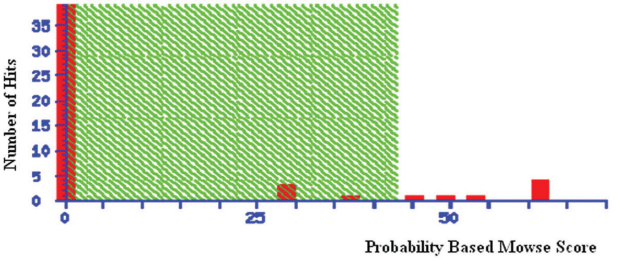

Figure 1b. Probability based mowse score of proteins in spot 1. Ions score is –10*Log (p), where p is the probability that the observed match is a random event. Individual ions score >43 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

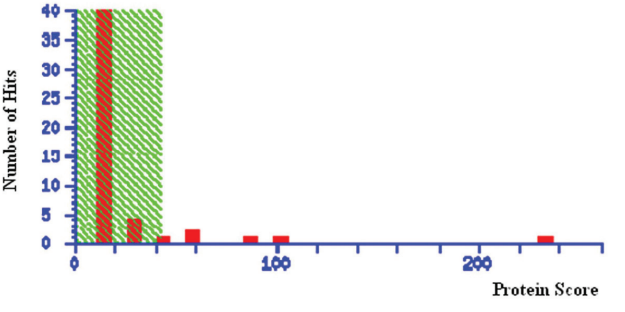

Figure 1c. Mascot score histogram of proteins in spot 2. Ions score is –10*Log (p), where p is the probability that the observed match is a random event. Individual ions score >42 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Significant hits

gi|223976 - haptoglobin Hp2, Mass: 42344, Score: 62, Queries matched: 18.

gi|1495458 - haptoglobin-related protein [Homo sapiens], Mass: 44076, Score: 62, Queries matched: 9.

gi|306880 - preprohaptoglobin, Mass: 38941, Score: 62, Queries matched: 19.

gi|123510 - haptoglobin-related protein precursor, Mass: 39496, Score: 62, Queries matched:10.

gi|68052314 - zinc finger CCCH domain-containing protein 13, Score: 54, Queries matched:7.

gi|34367817 - hypothetical protein [Homo sapiens], Score: 49, Queries matched: 1.

gi|4884413 - hypothetical protein [Homo sapiens], Mass: 29103, Score: 44, Queries matched:1.

2D nano LC-MS results of spot 2

Database: NCBInr 20100813 (11662491 sequences; 3984258230 residues)

Taxonomy: Homo sapiens (human) (232854 sequences)

Protein hits

gi|223976 - haptoglobin Hp2, Mass: 42344, Score: 233, Matches: 12 (11).

gi|306882 - haptoglobin precursor [Homo sapiens], Mass: 45860, Score: 103, Matches: 10 (7).

gi|189054178 - unnamed protein product [Homo sapiens], Mass: 66151, Score: 83, Matches: 1 (1).

gi|41388186 - monoclonal IgM antibody light chain [Homo sapiens], Mass: 26008, Score: 65, Matches: 2 (1).

gi|229271 - haptoglobin alpha1S, Mass: 9367, Score: 60, Matches: 3 (2).

gi|3169770 - immunoglobulin kappa light chain [Homo sapiens], Mass: 23320, Score: 50, Matches: 1 (1).

gi|71891729 - KIAA1503 protein [Homo sapiens], Mass: 514649, Score: 28, Matches: 3.

gi|257097163 - Chain A, Crystal Structure of Human Eif2b Alpha, Mass: 35000, Score: 27, Matches: 2.

gi|27526777 - steerin2 protein [Homo sapiens], Score: 23, Matches: 3.

gi|74729457 - RecName: Full = Putative uncharacterized protein C8orf61, Score: 22, Matches:4.

In the present investigation, haptoglobin-2 has been taken into consideration. Haptoglobin is an acute phase reactant synthesized mostly in liver and act to reduce the harmful effects of inflammation. Inflammation is often associated with extracellular hemolysis due to which hemoglobin is released from erythrocyte. RBC when lysed release free iron to the circulation. This may induce Fenton's reaction to produce excess of reactive oxygen species. Haptoglobin produced at this situation binds with hemoglobin and these Hp-Hb complexes are large enough to prevent or reduce iron mediated free radical formation and renal loss of hemoglobin.[29] The complexes are removed very rapidly by hepatic kupffer cells where the proteins are degraded and the iron and amino acid reutilized safely.

Genetic variance of both Hp α1 and Hp α2 exists and differentially expressed in various pathological conditions. Among the phenotypes of haptoglobin Hp 1-1 (86 KD) 16%, Hp 2-1 (86-300 KD) 48% and Hp 2-2 (170–900 KD) 36%, variations are observed mostly in phenotype Hp 2-2.[19],[30–32] Clinically the phenotype Hp 2-2 is associated with the risk of cardiovascular diseases, myocardial infarction[33,34] and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.[35,36] The Hp 1-1 phenotype was reported to be protective in two critical vascular complications of diabetes mellitus: diabetic nephropathy and restenosis after percutaneous transluminal coronary angioplasty.[37] Haptoglobin is synthesized as a single polypeptide chain and proteolytically cleaved to form a short α chain and a long β chain which are connected through a disulfide bond. Haptoglobin is found in serum of all mammals, but this polymorphism exists only in humans.[38,39]

The results of serum proteome analysis were also confirmed by the spectrophotometric quantification of total haptoglobin. The total haptoglobin level was found elevated significantly in all the chronic pancreatitis patients when compared to that of normal subjects [Table 2]. Since pancreatitis is associated with inflammatory process, the other inflammatory markers CRP, alpha-2 macroglobulin, C3a were also quantified but significant elevation was seen only in Hp level.

From the above investigation, it has been observed that Haptoglobin-2 (Hp-2) level was enhanced in the sera of chronic pancreatitis patients. Native polyacrylamide gel electrophoretic (PAGE) analysis of Hp for its various isoforms was carried out for confirmation. The results show that there is a significant difference in the pattern of Hp isoform in the sera of chronic pancreatitis patients when compared to that of normal healthy volunteers [Figure 2]. The data shows that both Hp 2-1, Hp2-2 have expressed abnormally in the sera of chronic pancreatitis patients that is Hp 2-2 hypersecretion in 50% of the patients and isoform Hp2-1 in rest of the patients [Figure 2. L2 & L3]. L4 of the same picture show normal expression of Hp 1

Table 2 Levels of acute phase reactants in normal subjects and chronic pancreatitis patients

Groups	Haptoglobin (mg/dl)	C3a (mg/dl)	CRP (mg/dl)	Alpha-2 Macroglobulin (mg/dl)
Normal Subjects (n-20)	220.9 ± 40.0	21 ± 2.8	0.6 ± 0.06	120.5 ± 24.6
Chronic Pancreatitis Patients (n-25)	297.6 ± 43.0*	25 ± 3.37$	0.8 ± 0.09*	146.0 ± 21.5#

*Values are expressed as mean ±SD for 'n' subjects in each group. C3a = Complement component 3a, CRP = C-reactive protein. Statistically significant variations are expressed as *p < 0.001, #p < 0.01, $p < 0.05 when the values are compared between normal subjects and chronic pancreatitis patients.*

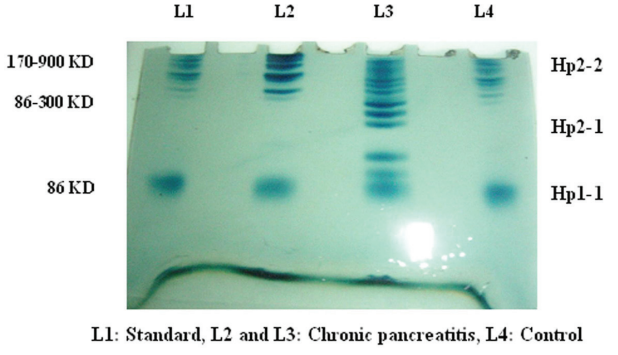

Figure 2. Native Polyacrylamide gel electrophoresis showing Haptoglobin isoforms. The figure is the representative of 5 individual experiments conducted by using pooled serum samples.

and Hp 2 and could be compared to that of standard in the rate of migration as well as in range of molecular weight. The native PAGE of Hp is a supportive evidence for the results obtained in serum proteome analysis because it is highly essential to prove the result of serum proteome analysis either by gene expression or quantification of proteins.

CONCLUSION

The present study from serum proteome analysis concludes that Hp-2 isoform, a positive acute phase reactant and functional antioxidant can be considered as a useful diagnostic protein marker for chronic pancreatitis. Since the pathogenesis of chronic pancreatitis is attributed to the formation of reactive oxygen species and the reactions mediated by them, Hp-2 might have been elevated to prevent free radical mediated lipid peroxidation reaction either in the circulation or in pancreas. The elevated level of total haptoglobin observed in the present investigation might be the compensatory mechanism to clear the free radicals as far as possible. The future study on the level of Hp-2 gene expression in pancreas and in liver can add more evidence for the role of Hp in the pathogenesis of chronic pancreatitis.

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