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Development of a Minimally Invasive Serum Test for Liver Cirrhosis

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Abstract: Phosphorylation is one of the most important post-translational modifications of proteins. Although cellular phosphorylation has been studied extensively, research on phosphoproteomes in patients' serum with hepatitis is limited. Serum phosphoproteins and phosphopeptides could be considered important disease biomarkers, as they play roles in cellular function. We previously reported higher serum levels of the phosphoZinc finger (pZNF) protein in patients with liver disease, including chronic hepatitis B (CHB), CHB-induced liver cirrhosis (CHB-LC), and CHB-induced hepatocellular carcinoma (CHB-HCC). To develop a minimally invasive pZNF protein-based diagnostic test for liver diseases (CHB, CHB-LC, HCC) from serum, we first synthesized a pZNF peptide based on our MALDI-ToF-MS peptide analysis data and developed polyclonal antibodies specific to pZNF. Using the pZNF-specific antibodies, an optimized Enzyme-Linked Immunosorbent Assay (ELISA) was then developed to screen sera from CHB (n=20), CHB-LC (n=17), CHB-HCC (n=20), and healthy volunteers (n=20). Results from the optimized ELISA test showed that sera from patients with liver diseases could be reliably detected, whereas those from healthy individuals could not (87.7% sensitivity and 76.9% specificity at a 300 cut-off; 82.6% sensitivity and 90.9% specificity at a 400 cut-off). Interestingly, the CHB-LC cohort stood out from the rest cohorts as it showed the highest expression of pZNF protein. At an 800-cut-off value, 17 of 17 CHB-LC samples were detected (above the cut-off), yielding a sensitivity of 100%. At the same cut-off value (800), only 2 of 60 samples were false positives, yielding a specificity of 96.8%. The two positive samples were from the CHB cohort, none from the HV cohort. Overall, we successfully developed a serum test (minimally invasive) that could detect CHB and CHB-induced liver diseases, particularly CHB-LC, with 100% sensitivity and nearly 100% specificity.

Keywords: Phosphorylation, Phospho-Zinc-finger protein, Liver cirrhosis, Biomarker, ELISA

INTRODUCTION

Phosphorylation is considered one of the most important post-translational modifications (PTM) of proteins. More than one-third of the protein is phosphorylated on serine (Ser or S, 86.4%), threonine (Thr or T, 11.8%), and tyrosine residues (Tyr or Y, 1.8%) [1].

Phosphorylation and dephosphorylation of proteins play a key role in regulating a wide spectrum of biological and cellular functions, including signal transduction, gene

expression, cell proliferation, and apoptosis [2]. The specific phosphorylation state of a polypeptide may also facilitate a better understanding of disease mechanisms and the discovery of biomarkers. The plasma proteome is typically used to identify the source of biomarkers using proteomics approaches [3-8]. Although cellular phosphorylation has been extensively studied, knowledge of the phosphoproteomes in the serum of patients with hepatitis is scarce. Whole sera are particularly attractive for biomarker studies due to their diagnostic potential and because of the ease and minimally invasive nature of sample collection [9-11]. Circulating phosphoproteins and phosphopeptides could serve as important disease biomarkers because of their well-established roles in cellular function. Body fluids are currently the choice of sample materials for health monitoring and diagnosis due to their relative availability. The analysis of phosphoproteins or free phosphopeptides in biological fluids, such as saliva, cerebrospinal fluid (CSF), and human serum, has recently been reported [12-14]. One of the main problems in studying samples using proteomic approaches is that targeted protein markers are present at very low concentrations, alongside other highly abundant protein families. One method is to use an antibody to study a phosphoprotein that selectively recognizes phosphorylated amino acid residues, thereby enabling a broader search for phosphoproteins [15]. A variety of experimental techniques were adopted for enrichment and detection of phosphorylated proteins. Currently, metal ion affinity chromatography of phosphopeptides using TiO2, followed by mass spectrometry (MALDI-ToF-MS), is in practice [16-17]. A fluorescent phosphorylation sensor dye is another sensitive reagent used to quantitatively analyze phosphoproteins, including protein kinase activity.

We previously observed higher expression of phosphoproteins in the sera of liver disease patients, namely chronic hepatitis B (CHB), CHB-induced liver cirrhosis (CHB-LC), and CHB-induced hepatocellular carcinoma (CHB-HCC), by Pro-Q diamond dye and phospho-specific monoclonal antibodies. MALDI-ToF-MS analysis of these phosphoproteins resulted in several peptides that matched with Zinc finger protein (ZNF) [18]. ZNFs, a large family of metalloproteins, are important regulators of cellular processes, including those related to liver cirrhosis. ZNFs play various roles in liver cirrhosis, both in the disease's development and its complications. Non-alcoholic fatty liver disease (NAFLD), now called Metabolic Dysfunction-Associated Steatohepatitis (MASH), which is a common chronic disease characterized by excessive fat accumulation in the liver without consumption of alcohol, is one of the most frequent causes of liver cirrhosis [19]. Increased expression of Zinc finger protein 267 in non-alcoholic fatty liver disease was observed [20]. In this study, we synthesized a phosphorylated ZNF peptide, CLKGFRNSpSALTKHQ, and developed a specific antibody against that peptide. Using this antibody, we then developed an Enzyme-Linked Immunosorbent Assay (ELISA) to detect liver diseases, particularly CHB-LC, with 100% sensitivity and nearly 100% specificity.

MATERIALS AND METHODS

Serum Samples: Collection and Preparation

Serum samples from patients, viz., chronic hepatitis B (CHB, n=20), CHB-related liver cirrhosis (CHB-LC, n=17), CHB-related hepatocellular carcinoma (CHB-HCC, n=20), were collected from the Liver Clinic of Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. Sera from age and sex-matched healthy volunteers (HV, n=20) were

collected from the staff of PGIMER served as controls. The diagnosis of chronic hepatitis B patients was done based on clinical examination and biochemical tests (measurement of serum ALT, AST, ALP, γ-GT, albumin, bilirubin, prothrombin time, and virological investigations (HBsAg, HBeAg, AntiHBe, IgM, AntiHBc, HBV DNA >104 copies /ml in patients who are HBeAg negative). Hepatitis B-induced liver cirrhosis patients were diagnosed on the basis of clinical, biochemical, and radiological evidence, along with the presence of esophageal varices on endoscopy. HCC was diagnosed on the basis of EASL (European Association for the study of the liver) criteria, i.e., the presence of a hypervascular liver lesion confirmed by at least two imaging modalities (CT, MRI, USG) and an AFP value of more than 400 ng/ ml. All the serum samples were stored at -20°C till use. Written informed consent was obtained from each individual before sample collection. The ethical Committee of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, and the Chittaranjan National Cancer Institute, Kolkata, India, approved this study.

For the preparation of each serum sample, 10 µl of phosphatase inhibitor cocktail was mixed with 1 ml of the collected blood sample and incubated for a few minutes at room temperature. Afterward, the blood sample was centrifuged at 2500 x g for 15 min at 4°C, and the serum layer was collected and stored at -20°C till use.

Protein Estimation

Protein estimation of serum samples was done by using BioRad protein assay dye reagent in a 96-well microtiter plate. In brief, a standard BSA working solution (100 µg/ml) was prepared from a BSA stock solution (1 mg/ml) in PBS. Several dilutions of BSA were made, ranging from 2.5 to 40 µg/ml.

Serum samples for protein estimation were diluted 4000-fold and 8000-fold with PBS. To the samples in each well (160 µl) of the microtiter plate, BioRad protein assay reagent (40 µl) was added, and the plate was incubated at room temperature for 20 min; absorbance was measured at 595 nm. A standard curve was prepared using a BSA solution, and the protein concentration of the patients' samples was determined from it.

Preparation of Phospho-Zinc Finger (pZNF) Peptide Antibody

Peptide synthesis. To detect changes in the expression of the above-selected phosphoproteins by ELISA using a specific antibody against the phosphorylated proteins was considered a biomarker for liver diseases, including liver cancer. The complete peptide sequence of Zinc finger protein (135) in UniProt was searched using the selected peptide of Phospho Zinc finger protein obtained from MALDI-MS/MS in the study. The alignment of four ZNF (135) isomers shows that the isoforms share three common phosphopeptide sequences: peptide1: TRPKVKLSVLKQGIS; peptide2: CLKGFRNSSALTKHQ; peptide3: FTHSSLTKHQRTHT. The peptides were chosen on the basis of the phosphorylated site on the full-length protein. A Cys residue was added to the N-terminal of the peptide to facilitate the KLH conjugation.

KLH conjugation: Since the peptide is a small molecule and does not elicit an immune response on its own, it is important to conjugate it to a carrier molecule. Keyhole limpet hemocyanin (KLH) is the most popular and immunogenic carrier protein for the preparation of peptide antigens used for immunization and antibody production. KLH was

conjugated with the peptide by using Sulfo-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC). The peptide and the KLH were bound with a disulfide bond (covalent binding) between them. The peptide was selected based on the phosphorylation site in the full-length protein. A Cys residue was added to the N-terminal of the peptide to facilitate the KLH conjugation. The peptide has been synthesized by the peptide synthesizer. A control (non-phosphorylated) peptide has also been synthesized. Peptides are 1. CLKGFRNSpSALTKHQ, 2. CLKGFRNSSALTKHQ (Control).

Immunization of a rabbit: Two New Zealand white rabbits were immunized subcutaneously with KLH-conjugated phosphorylated peptides, 200 µg per rabbit, with Complete Freund's Adjuvant. Further immunizations were performed using 100 µg of conjugated peptide per immunization in Incomplete Freund's Adjuvant. Five immunizations were done on a weekly basis, and the rabbits were bled one week after the fifth immunization, and in the following weeks, immunization and bleeding were done on alternate weeks.

Sera testing by ELISA: Immune sera were tested by ELISA. In brief, the ELISA plates were coated with free phosphorylated peptide in carbonate buffer, pH 9.6 (100 ng/100 µl/well) and incubated at 4°C overnight. After washing, the wells were blocked with 200 µl of blocking solution (0.25% non-fat skimmed milk in PBS-T). Anti-sera were added to each well (100 µl, diluted by 1:5000) and were incubated for 1 hour at room temperature on a rocking platform. After washing the wells, HRP-conjugated goat anti-rabbit secondary antibody was added at a 1:10,000 dilution and incubated for 1 hour at room temperature on a rocking platform. Pre-immunized sera were added at an equivalent dilution as negative controls. The wells were washed three times with PBS-T, and TMB/H_2O_2 substrate (Sigma) was added for color development. The reaction was stopped after 10 min by adding 50 µl of 1N H_2SO_4. The plates were read at 450 nm in an ELISA reader.

Purification of Phopho-specific antibody: Purification of the Phospho-Specific antibody was performed using a double peptide affinity chromatography method. For this purpose, two separate affinity chromatography columns were prepared by immobilizing the phospho- and non-phospho peptides on agarose beads, respectively.

First, the anti-sera were diluted at 1:2 and were passed through the phospho-specific column at 10 ml/hour. The flow-through was passed again through the same column to ensure antibody binding to the column. The column was then washed thoroughly with PBS to remove unbound or loosely bound proteins. The antibodies were eluted from the column by using glycine buffer (pH 3.0). The fractions were collected on Eppendorf tubes containing Tris buffer, pH 6.0. The fractions were screened for proteins at 280 nm. The active fractions were pooled and dialyzed against the PBS, pH 7.5.

Antibodies, thus generated, will have a mixture of antibodies against both phospho- and non-phospho peptides. The antibody solution is again passed through a chromatography column in which the non- phospho (control) peptide is immobilized. The purification process is repeated on this column using the protocol followed for the phospho-specific column. The non-phospho-specific antibodies will be bound to the column, and the flow-through will contain the phospho-specific antibodies.

The ELISA was performed against both purified peptides and free peptides, and the presence of specific antibodies was demonstrated.

Development of an Optimized ELISA

Serum of one single patient of each type and one single HV subject was taken for optimization of ELISA. For this purpose, varying concentrations of serum protein (0.005 to 1.25 µg/well/100 µl in PBS) were coated in duplicate onto individual wells of a microtiter plate (NUNC) and left overnight at 4 °C. The following day, the coating solution was removed completely, followed by the addition of 100 µl of blocking buffer (3% BSA in PBS per well), and the plate was incubated for 1 hour at room temperature. The wells were washed with 300 µl PBST (0.05% PBS, Tween 20) three times. Then 100 µl of diluted (1X PBST) rabbit [anti-phospho zinc finger protein] antibody (1:1000) was added to each well and incubated for 1 hour at room temperature. After washing the wells as before, enzyme-conjugated secondary antibody (anti-rabbit IgG-HRP, 1:10000 (1X PBST)/ 100 µl/well) was added to each well immediately and kept for 45 min at room temperature. The negative controls include secondary antibody only on one blank well and both primary and secondary antibody on another blank well (100µl each). After the usual washing, 100 µl of the substrate solution (TMB) was added per well, and the plate was read at 605 nm at 15, 30, 45, and 60 min. After subtracting the value of PBS (as blank) from the respective value of the patient's sample, the graph was plotted for each sample.

Determination of Phospho-Zinc Finger Protein in Serum Samples from Healthy Individuals and Patients with Liver Disease by the Optimized ELISA

The microtiter plate well was coated with each serum sample (0.1 µg/well/100 µl) from HV, CHB, LC, and HCC in quadruplicate and left at 4 °C overnight. The following day, after discarding the solution from the wells, 100 µl of blocking buffer (3% BSA in PBS) was added to each well, and the wells were incubated for 1 hour at room temperature. After washing each well 3 times in PBST, 100 µl of anti-phospho zinc-finger antibody (1:1000 in PBST) was added and incubated for 1 hour at 37 °C. After extensive washing with PBST 3 times, 100 µl of HRP-conjugated secondary antibody (1:10000 in PBST) was added and incubated at 37 °C for 45 min. The absorbance at 605 nm for each plate was recorded after 20 mins of TMB incubation. To normalize plate-to-plate variation, one internal positive control was also coated in quadruplicate on each plate. For other controls, PBS (substrate blank), secondary antibody only (negative control), and primary and secondary antibody (negative control) in quadruplicate were also added on the blank (not protein coated) wells.

Determination of Specificity and Sensitivity of the Assay

The developed ELISA was evaluated based on the sensitivity and specificity scores. The sensitivity and specificity were defined follows:

- ***Sensitivity*:** The ability of a test to correctly identify patients with a disease.
- ***Specificity*:** The ability of a test to correctly identify people without the disease.
- ***True positive***: The person has the disease and the test is positive.
- ***True negative*:** The person does not have the disease and the test is negative.
- ***False positive***: The person does not have the disease and the test is positive.

- ***False negative***: The person has the disease and the test is negative.

Sensitivity and specificity scores were then calculated using the following formula

$$\%\text{Sensitivity} = \frac{\text{True Positive}}{\text{True positive + False negative}} \times 100$$

$$\%\text{Specificity} = \frac{\text{True Negative}}{\text{True negative + False positive}} \times 100$$

For the calculation of the sensitivity and specificity of the assay, various cut-off values were set and any value above the cut-off was considered positive and the value below the cut-off was considered negative. Specificity of the assay was calculated from the HV samples as the assay was expected to be negative for HV samples. Sensitivity was calculated from the disease samples (CHB, LC, and HCC) as the assay is expected to be positive for these samples.

RESULTS

Characteristics of Procured Serum Samples

The detailed clinicopathological parameters of the samples are shown in Table 1.

Table 1: Clinicopathological parameters of samples.

		CHB (n=20)	CHB-LC (n=17)	CHB-HCC (n=20)	Healthy Volunteer (n=20)
Age	Min-Max	22-60	35-65	28-77	22-40
Sex	Male	15	15	17	12
	Female	5	2	3	8
HBV DNA	Min-Max	2000-8000	315-2500000	Undetectable-1160000	-
HBsAg	Positive	20	20	17	-
	Negative	-	-	-	-
HBeAg	Positive	5	3	20	-
	Negative	15	14	-	-
Bilirubin	Min-Max	0.2-3.6	0.29-3	0.1-3.8	0.1-2
Albumin	Min-Max	2.1-4.6	2-4.18	2.2-4.26	3.4-4.8
AST	Min-Max	18-193	22.7-260	40.7-116	2-40
ALT	Min-Max	15-148.9	21-190	40.5-156	2-41
SAP	Min-Max	27-257	25-309	37-262	42-128

Protein Concentration of the Serum Samples

The standard curve of BSA was almost linear (R^2 = 0.9978) through the protein concentration range (2.5 to 20 µg/ml) (Fig. 1). The two different dilutions (4000 and 8000) of each unknown sample fell in the linear zone of the standard curve. The protein concentration of each unknown sample was calculated by multiplying by 4000 or 8000 and taking the average of these two values. The calculated protein concentrations of the serum samples were in the range of 82 to 120 mg/ml.

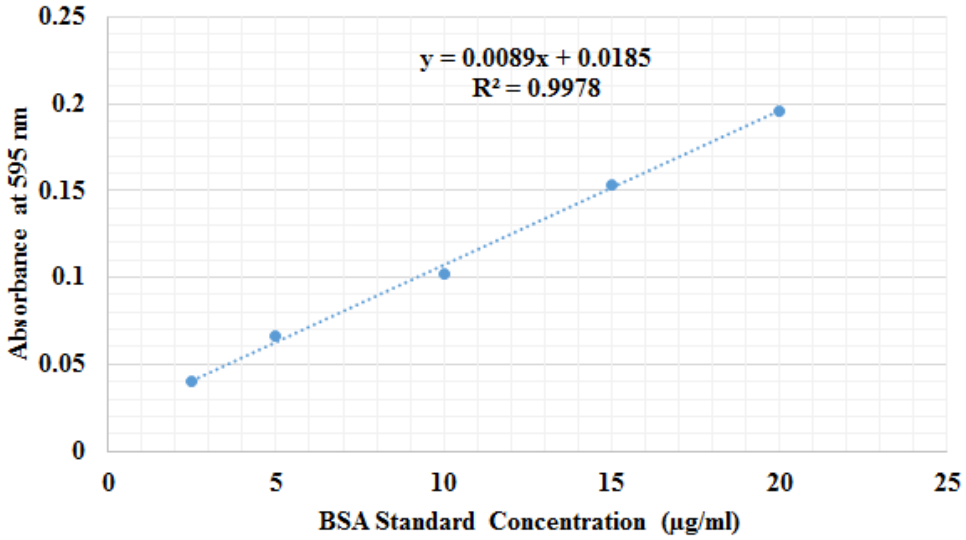

Fig. 1: BSA standard graph.

The synthesized peptide, after purification, was found to be homogeneous, as demonstrated by mass spectrometry (Fig. 2).

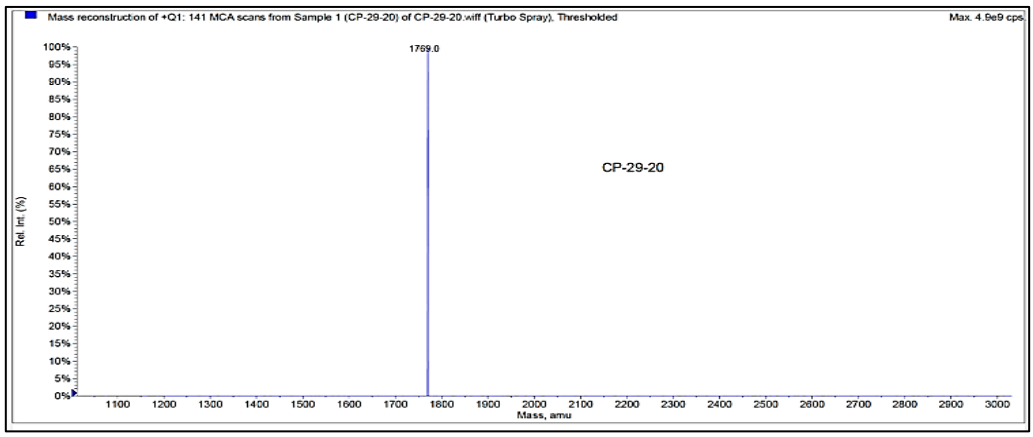

Fig. 2: Graph represents mass spectra of purified peptide (CP-29-20).

The immune response against the p-ZNF peptide-KLH conjugate was evident as the first bleed from both rabbits reacted with the p-ZNF peptide on plastic wells (Fig. 3). The interaction of the pre-immune serum (used as a negative control) was only 5.9% (for rabbit A) and 12% (for rabbit B). The antibodies were somewhat specific to the phospho-peptide,

as the non-phospho-peptide showed less absorbance at 405 nm, 73% (1.28 vs 1.74) for rabbit A and 53% (0.45 vs 0.86) for rabbit B. However, the antibodies were affinity-purified (see above) and used for the downstream applications.

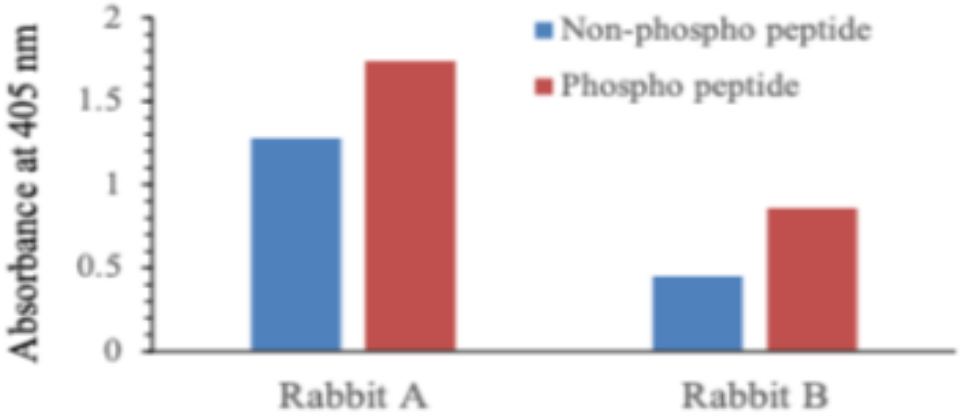

Fig. 3: ELISA showing interaction of the first bleed with the phospho-peptide and non-phospho-peptide coated at 200 ng/well. Five thousand dilutions of either the antibody or the pre-immune serum were used, and after incubating with secondary antibody-HRP, the plate was developed with ABTS substrate solution and read at 405 nm after 15 min.

Optimization of ELISA

The absorbance value of negative control was more or less same as PBS blank. After subtracting the PBS blank, the graph was plotted for each sample (Fig. 4). For each sample, antibody interaction was steadily increased with the increased amount of antigen approximately up to 0.08-0.1 µg/well before reaching to a plateau. The TMB substrate interaction was increased over time as observed by the absorbance at 605 nm, but it was not linear (*e.g.* not doubling after 30 min reaction compared to the 15 min reaction).

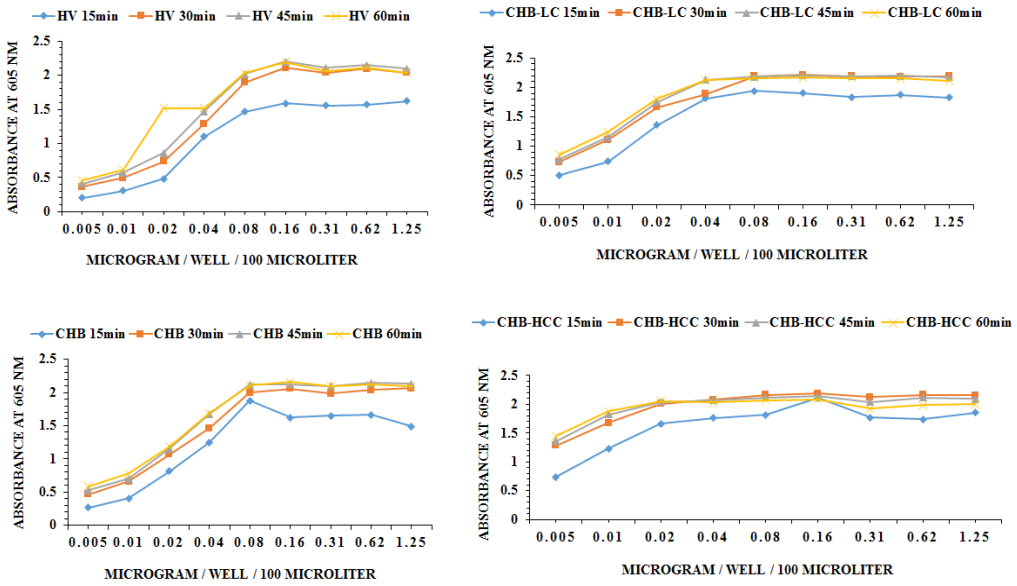

Fig. 4: ELISA showing the binding of the anti-p-ZNF antibody to the varying concentrations of serum protein (0.005 to 1.25 µg/well/100µl). The plate was read at

605 nm after TMB substrate addition at four different time points (a, 15 min; b, 30 min; c, 45 min, and d, 60 min).

To confirm the above protein concentration range (0.08-0.1 µg/well/100 µl) to be coated for the follow-up ELISA experiments, four different samples (one from each category i.e., HV, CHB, LC, and HCC) were applied to the wells in varying concentrations (0.0125 to 3.2 µg/well/100 µl) and performed ELISA as described above. Figure 5 shows the absorbance (605 nm) values for all four samples after 20 min substrate incubation. For most samples, antibody interaction increased steadily with increasing antigen amount, up to approximately 0.2 µg/well, before reaching a plateau.

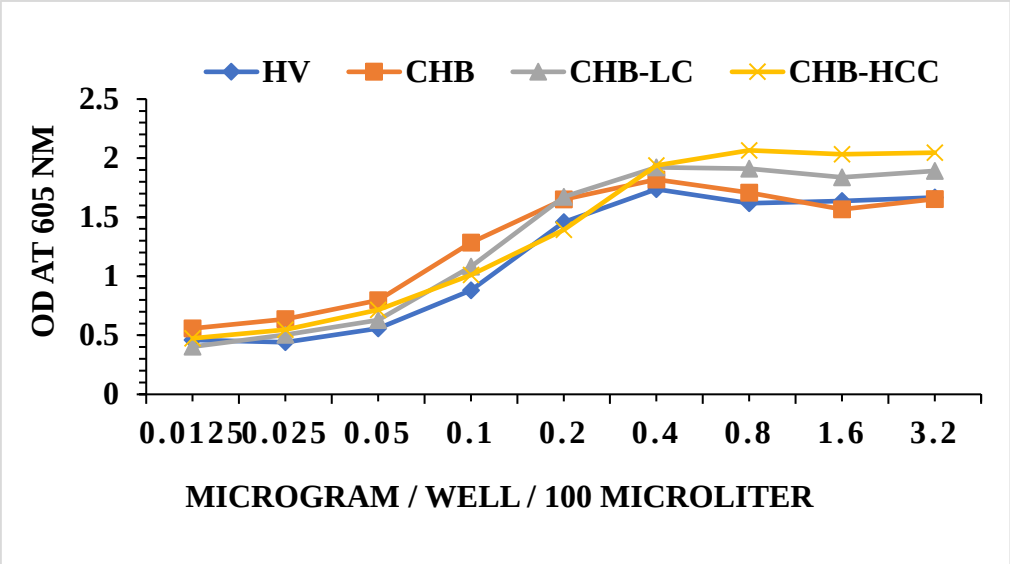

Fig. 5: Absorbance at 605 nm for varying concentrations (0.0125 to 3.2 µg/well/100µl) of each serum sample following the above ELISA protocol and TMB substrate incubation for 20 min.

Based on the experiments above (Fig. 4 and 5), the coating concentration of 0.1 µg/well/100 µl and the TMB substrate reaction time of 20 min were chosen for the follow-up ELISA experiments.

Optimized ELISA to Detect p-ZNF in Various Serum Samples

The optimized ELISA was then performed to detect p-ZNF in serum samples from HV, CHB, LC, and HCC. The negative control absorbance was essentially the same as that of the PBS blank. The absorbance value of the internal positive control across all plates was consistent (variation of only 8-10%). The obtained absorbance was normalized after subtracting the PBS blank. The normalized data were then converted based on the volume (from 0.1 µg/well/100 µl to per µl serum sample). The final result was obtained after multiplying the protein concentration for each sample, as shown in Fig. 6. The highest value was observed in LC samples (~800-1700), followed by CHB (~250-900) and HCC (~200-650). The HV samples had the minimum values (~150-550).

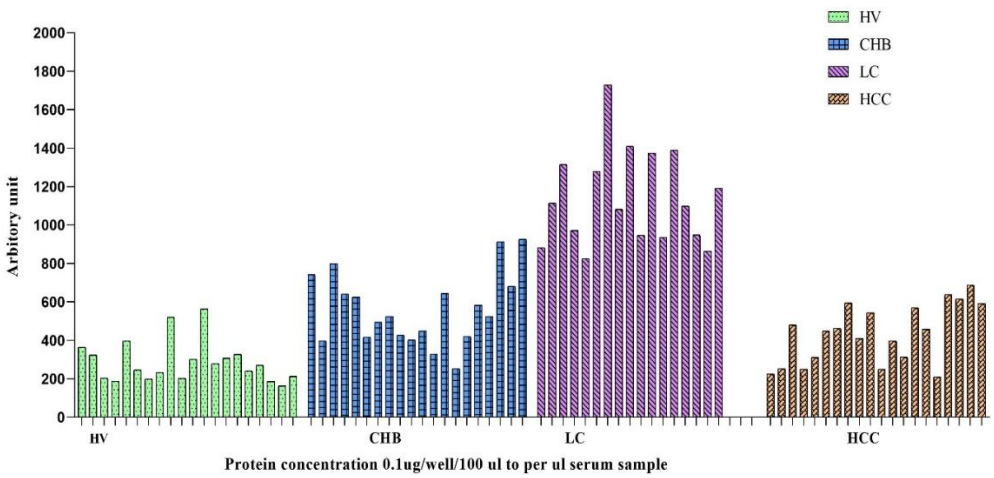

Fig. 6: The normalized data (in arbitrary units) per microliter of serum sample from healthy individuals and patients with liver disease.

Sensitivity and Specificity of the Test

The normalized data shown in Figure 6 above were used to determine the sensitivity and specificity of the test for detecting liver disease (CHB, LC, and HCC) relative to normal. The test was also evaluated to detect LC only over other cohorts. For this purpose, several thresholds were considered. At a 200-cut-off value, 56 out of 57 disease samples (CHB, LC, and HCC) were positively detected (above the cut-off value), so sensitivity was calculated as 98.3% (Table 2). At the same cut-off value, of 20 true-negative samples, only 6 were negative (below the cut-off), and 14 were false positives, so the specificity was 58.8%. At a 300-cut-off value, 49 of 57 disease samples were detected, and 14 of 20 HV samples were negative, yielding 87.7% sensitivity and 76.9% specificity. Similarly, at a 400-cut-off value, 45 of 57 disease samples were detected, and 18 of 20 HV samples were negative, yielding 82.6% sensitivity and 90.9% specificity.

Table 2: Sensitivity and specificity of the test to detect liver disease

Cut-off value	%Sensitivity	%Specificity
200	98.3	58.8
300	87.7	76.9
400	82.6	90.9

For the detection of LC only, we used a cut-off value of 800 as the LC samples had an arbitrary value of ~800-1700 (see Fig. 6). At an 800-cut-off value, 17 out of 17 LC samples were positively detected (above the cut-off value), so sensitivity was calculated as 100% (Table 3). As the focus was on detecting the LC sample only, other cohorts (HV, CHB, and HCC) were considered negative samples. At the same cut-off value (800), out of 60 true-negative samples, 58 were negative (below the cut-off value), and only 2 were false positives, yielding a specificity of 96.8%. The two positive samples were from the CHB cohort, none from the HV cohort.

Table 3: Sensitivity and specificity of the test to detect liver cirrhosis

Cut-off value	%Sensitivity	%Specificity
800	100	96.8

DISCUSSION

Phosphorylation is a dynamic post-translational modification of proteins. This process plays a critical role in regulating a wide spectrum of biological events and cellular functions, including signal transduction, gene expression, cell proliferation, and apoptosis. Cell signaling pathways are intimately dependent on phosphorylation and dephosphorylation of proteins. Phosphorylation of protein is strictly controlled by the interplay of two kinds of enzymes, viz., protein kinases and phosphatases, through sequential phosphorylation and dephosphorylation [21]. It has been known that aberrant phosphorylation is associated with deregulation of different pathophysiology of the system and was found to occur in tissue, serum, saliva, and urine of the patients of different diseases, including liver diseases and cancer [22]. Aberrant phosphorylation and glycosylation were observed in liver diseases, chronic hepatitis B (CHB), chronic hepatitis C (CHC), CHB/CHC-related liver cirrhosis, and CHB/CHC-related hepatocellular carcinoma (HCC) [23-32]. Aberrant glycosylation and phosphorylation were also reported in metabolic dysfunction-associated fatty liver disease (MAFLD), metabolic dysfunction-associated steatohepatitis (MASH), alcohol-associated liver disease (ALD), and alcoholic liver cirrhosis (ALC). Routine biochemical tests are a preliminary diagnostic process. For a more accurate diagnosis, imaging techniques are considered, though they are expensive and not readily available at all diagnostic centers. Liver biopsy is the gold standard for the diagnosis of liver cirrhosis and liver cancer; however, this technique is not suitable for many reasons, namely, the risk of complications and discomfort to patients. Thus, to minimize the need for biopsy, it is important to explore non- invasive biomarkers that can compensate for the deficiencies of current ones. The phosphorylated proteins are secreted from cells into the circulation and are readily available in serum. 2D gel electrophoresis of the sera of three patient groups, CHB, CHB-LC, and CHB-HCC, and control subjects, followed by MALDI-ToF-MS analysis, revealed high expression of phospho Zinc finger protein (ZNF 135) of 70 kDa. UniPort-based search revealed three peptides' sequences of pZNF protein (TRPKVKLSVLKQGIS, CLKGFRNSALTKHQ, and FTHSSSLTKHQRTHT) as obtained by MALDI-MS/MS analysis in our earlier study [18]. The peptide 2 (CLKGFRNSSALTKHQ) was selected for antibody production because its terminal cysteine residue is suitable for conjugation to maleimide-activated KLH. In addition, this peptide sequence matches the sequences of four isoforms of ZNF, so the antibody against this peptide will recognize all isoforms of ZNF.

Here in this study, we selected pZNF as a target protein to explore its diagnostic potential in liver cirrhosis. Liver cirrhosis is the end-stage condition of chronic liver disease [33-37]. Cirrhosis of the liver is permanent scarring that damages the liver, deteriorates its function, and ultimately leads to liver failure. The ALD, MAFLD, and hepatitis B-and hepatitis C-induced diseases are the most common causes of cirrhosis. For diagnosis, the patient must undergo routine blood tests, including liver function tests (liver enzymes, proteins, and bilirubin) and prothrombin time measurement. Imaging tests like abdominal ultrasound, CT scan, or MRI can show the size, shape, and texture of the liver. Elastrography can be used

to measure liver stiffness or fibrosis. Finally, a liver biopsy can confirm cirrhosis. However, these tests are expensive and not available to all diagnostic centers. Our minimally invasive diagnostic approach was to determine the highest pZNF protein expression among sera from CHB, CHB-LC, and CHB-HCC; for this, we used a direct ELISA with an anti-pZNF peptide antibody. The antibody against phospho Zinc finger peptide was developed by immunization of rabbit, purified by affinity chromatography. ELISA was optimized, and the phosphor-Zinc finger protein was measured in serum samples from individuals with liver disease. The normalized data, expressed in arbitrary units per microliter of serum from healthy individuals and patients with liver disease, were used to determine the sensitivity and specificity of the test for detecting liver disease (CHB, LC, and HCC) over normal (Fig 6). The test was also evaluated to detect LC only over other cohorts. For this purpose, several thresholds were considered. At a 200-cut-off value, the sensitivity of CHB, CHB-LC, and HCC was calculated as 98.3% (Table 2) when 56 out of 57 patients' samples were found above the cut-off value and were considered positive. At the same cut-off value, out of 20 HV samples, 14 truly negative samples were false positives because they were above the cut-off, and 6 samples were below the cut-off and were regarded as negative. So, the specificity was 58.8%. A similar calculation shows 87.7% sensitivity and 76.9% specificity when the cut-off value is set to 300. Similarly, at a 400 cut-off value, 82.6% sensitivity and 90.9% specificity were determined.

For the detection of LC, only a cut-off value of 800 was used, since the LC samples had arbitrary values of ~800-1700 (Fig 6). At an 800-cut-off value, 17 out of 17 LC samples were marked positive, as shown above the cut-off value, so the sensitivity was calculated 100%. Since our focus was on detecting LC samples only, other cohorts, e.g., HV, CHB, and HCC, were considered negative samples. At the same cut-off value of 800, out of 60 true negative samples, 58 samples were detected below the cut-off value, so they are negative, and only 2 samples from the CHB cohort were found to be false positives. Therefore, the specificity was 96.8%.

CONCLUSION

Our previous study shows the higher expression of pZNF in liver diseases, namely CHB, CHB-LC, and CHB-HCC. Our effort was to identify phosphozinc finger proteins as non-invasive biomarkers of CHB, CHB-LC, and CHB-HCC. Direct ELISA was performed using an anti-pZNF peptide antibody on sera from the above liver disease patients. It revealed that CHB-LC patients showed the highest expression of pZNF protein. Sensitivity was found 100% and specificity 97% employing the cut-off value of 800. Thus, pZNF can be considered a non-invasive biomarker of chronic hepatitis B-induced liver cirrhosis (CHB-LC). Further study with larger patient cohorts will exemplify the merit of pZNF as a promising non-invasive diagnostic marker of CHB-LC.

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Author Contribution

BPC conceptualized the project plan. BPC and HA designed the experimental protocols. SP, RG, and MB performed the experiments. YB collected and processed the patients' samples. AD selected the patients based on clinical history and biochemical parameters and imaging results. BPC, CKP, SD guided the experiments. BPC, HA analyzed the experimental results and drafted the manuscript. CKP edited the manuscript. The work was done in two centers, CNCI, Kolkata and PGIMER, Chandigarh. The ethical committee of these two centers approved the study.

Conflict of Interest

All authors declare no potential conflicts of interest.

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