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Research Article

Cirrhosis of liver: Comparative cross reactivity for quantification of SERPINA4/Kallistatin

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Abstract

Cirrhosis of liver is an end stage of chronic liver insults from varied etiologies which leads to impaired liver functions. Proteins expressed from liver and enters into circulation reflects degree of liver dysfunction. Serpins (Serine protease inhibitors) are class of plasma proteins expressed from liver; SERPINA4/Kallistatin is a multifunctional serpin clade A protein expressed from liver and concentration in serum is the reflection of extent of liver dysfunction. The present study aimed to compare cross reactivity of serpins for polyclonal and monospecific antibodies in both cirrhotic liver and healthy subjects. Blood samples were collected from 20 subjects (10 cirrhotic liver, 10 healthy) from R. L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. Separation of proteins was carried out by SDS-PAGE. Cross reactivity study was analyzed using western blot. Proteins present in cirrhotic liver and healthy subject's serum were separated by SDS PAGE. There was no band detection on both (cirrhotic liver and healthy) PVDF (polyvinylidene diflouride) membranes with polyclonal antibodies. However, a significant band was observed with protein of interest in healthy PVDF membrane with monospecific antibodies. There was no band in cirrhotic liver PVDF membrane even with monospecific antibodies. Comparative cross reactivity analysis of serpins in quantification of SERPINA4/Kallistatin in the present study demonstrated that there will not be any immunological cross reactivity between serpins and SERPINA4/Kallistatin due to the absence of identical epitope in cirrhotic liver and healthy subjects.

Keywords: Cirrhosis of liver; SERPINA4/Kallistatin; olyclonal; monospecific antibodies

Introduction

Cirrhosis of liver is reversible natural wound healing response which results in the formation of abnormal continuation of connective tissue production and deposition and regenerative nodular formation in response to chronic liver injury [1, 2]. It is an end result of chronic liver insults from varied etiologies with similar pathological characteristics *viz.*, degeneration, necrosis of hepatocytes, replacement of parenchyma by fibrotic tissue, regenerative nodules which leads to portal hypertension and finally liver failure [3]. Proteins which are expressed from liver and enters into circulation reflects degree of liver dysfunction and give potential insights for diagnosis of the disease [4].

Serpins (Serine Protease Inhibitors); plasma proteins mainly expressed from liver have similar structure and diverse functions [5]. With poor sequence homology; serpins share highly conserved core structure which is crucial for their inhibitory function [6]. Serpins exist as monomeric proteins in their native state. Reactive centre loop (RCL) of these proteins in secondary structure interacts with active site of proteases and inhibits their action. They undergo conformational changes in their structure which is crucial for their activity. Apart from inhibitory action, they acts as transporters for hormones, plays an important role in coagulation and blood pressure regulation [5].

Sequence similarity of serpins divided them into clades. Clades A to I in humans have 36 serpin coding genes and 5 pseudogenes. Clade A molecules are localized on chromosomes 1, 14 and X which are extracellular. Clade B serpins which are intracellular localized on chromosome 6 and 18. Clades A serpins are localized on chromosome 14 which are mainly expressed from liver (Table 1) [7, 8].

S. No	Name	Symbols	Synonyms	Chromosome	Biological function
1	SERPINA1	PI	α-1-antitrypsin, AAT	14q32.1	Inhibitor of neutrophil elastase
2	SERPINA2	PIL	ATR, ARGS	14q32.1	Indicates an ongoing process of pseudogenization
3	SERPINA3	AACT	ACT	14q32.1	Inhibitor of chymotrypsin and cathepsin G
4	**SERPINA4**	**PI4**	**KST, KAL, KLST, Kallistatin**	**14q32.1**	**Anti-inflammatory, anti angiogenic, anti-oxidant, anti-apoptotic**
5	SERPINA5	PLANH3	PA13, PROCI	14q32.1	Inhibits active C protein
6	SERPINA6	CBG	-	14q32.1	Non inhibitory; cortisol transporter
7	SERPINA7	TBG	-	Xq22.3	Thyroid hormone transport protein
8	SERPINA8	AGT	-	1q42.2	Angiotensinogen
9	SERPINA9	-	CENTERIN	14q32.1	Role in maintaining native B cell
10	SERPINA10	-	PZI	14q32.1	Inhibitory protein of activated coagulation factors Z and XI
11	SERPINA11	-	-	14q32.1	Pseudogene and uncharacterized
12	SERPINA12	-	Vaspin, OL-64	14q32.1	Inhibitory protein of kallikrein

Table 1: Classification of SERPIN Clade A and chromosomal location

Kallistatin (SERPINA4, serpin family A member 4, tissue kallikrein inhibitor), belongs to clade A serpins; an acidic glycoprotein with a molecular weight of 58kD and isoelectric pH ranges from 4.6 to 5.2. It is mainly expressed from liver cell (Hep G2 and Hep 3B) and encoded by the *SERPINA4* gene with 5 exons and 4 introns mapped to chromosome 14q31-32.1 [9, 10]. Multifunctional protein, SERPINA4/Kallistatin shows inhibitory action on human tissue kallikrein [11]. SERPINA4/Kallistatin expressed from liver enters into circulation and concentrations vary in different chronic liver diseases (fibrosis, cirrhosis and hepatocellular carcinoma) [12].

Interference of serpins in quantification of SERPINA4/Kallistatin with antibodies may mislead the diagnosis of extent of the chronic liver disease. Hence, in the present study, an attempt has been made to identify immunological cross reactivity between SERPINA4/Kallistatin and other serpins for polyclonal and monospecific (monoclonal alternative) antibodies in cirrhotic liver and compared with healthy subjects.

Materials and Methods

Samples

Blood samples were collected from 20 subjects: 10 clinically and diagnostically proven cirrhotic liver subjects with varying degree and varied etiology; age and gender matched 10 healthy subjects **(Table 2)** from R. L. Jalappa Hospital and Research Centre, attached to Sri Devaraj Urs Medical College, A constituent institute of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. Individuals diagnosed with cirrhosis of liver caused by alcoholic liver disease (ALD) and non-alcoholic fatty liver diseases (NAFLD) based on clinical history and symptoms *viz.*, ascites, encephalopathy, jaundice and altered biochemical parameters were included in the study. Individuals with diabetes and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia and cancer were excluded from the study. Collection of blood samples from cirrhotic liver and healthy subjects was carried out after obtaining informed consent and study is approved by Institutional Ethical Committee (**DMC/KLR/IEC/61/2016-17**).

Controls				Cases			
Sample ID	**Gender**	**Age**	**Etiology**	**Sample ID**	**Gender**	**Age**	**Etiology**
C1	M	36	NA	D1	M	36	ALD
C2	M	28	NA	D2	M	28	ALD
C3	M	60	NA	D3	M	60	ALD
C4	F	26	NA	D4	F	26	NAFLD
C5	M	35	NA	D5	M	35	ALD
C6	F	26	NA	D6	F	26	NAFLD
C7	M	58	NA	D7	M	58	ALD
C8	M	30	NA	D8	M	30	ALD
C9	M	55	NA	D9	M	55	ALD
C10	M	30	NA	D10	M	30	ALD

Table 2: Details of 20 subjects (10 liver cirrhotic cases, 10 healthy age and gender matched controls) used for cross reactivity analysis

Abbreviations: C: Control; D: Diseased; M: Male; F: Female; NA: Not Applicable; ALD: Alcoholic Liver Disease; NAFLD: Non Alcoholic Fatty Liver Disease

Serum separation

Serum was separated from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 minutes. Serum was stored at – 20ºC for further analysis. All the samples were used to find out cross reactivity of other serpins with SERPINA4/Kallistatin for polyclonal and monospecific (monoclonal alternative) antibodies by western blot after protein segregation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Reagents

Primary polyclonal and monospecific (monoclonal alternative) antibodies specific for SERPINA/Kallistatin along with secondary antibodies conjugated with HRP specific for primary antibodies were procured from R&D systems, USA. Other chemicals of analytical grade were procured from Bio-Rad and Sigma Aldrich, USA.

SDS-PAGE

SDS gels were prepared as per standard protocol. Cirrhotic liver and healthy subject's serum samples were loaded in different gels (two sets; for polyclonal and monospecific antibodies) and SDS-PAGE was carried with duplication at 25 mA per gel in 1x SDS running buffer. After electrophoresis, gels were incubated in fixing solution (7% acetic acid and 10% methanol) at room temperature for 20 minutes. At this point, the gels were transferred onto a polyvinylidene diflouride (PVDF) membrane for western blot and duplicate gels were subjected for staining with colloidal Coomassie brilliant blue in a shaker at room temperature for 2 hours. Excess staining solution was removed and the gels were washed with 10% acetic acid and placed in deionized water for destaining till the appearance of bands [13, 14].

Western blot

Proteins separated by SDS-PAGE were transferred onto PVDF membranes separately for both polyclonal and monospecific antibodies using a Transblot-Blot SD semi dry transfer cell (Bio-Rad) at 15 V for 2 hours (1x transfer buffer: Tris/Glycine with 20% Methanol). After transfer, PVDF membranes were kept for blocking using blocking buffer (5% skimmed milk powder in 1x PBST) and incubated over night at 4ºC. After overnight blocking, PVDF membranes were washed with 1x PBST thrice for 3 minutes each. Primary antibodies (polyclonal and monospecific antibodies separately) were diluted (1:100) and PVDF membranes were incubated in diluted primary antibody solution at room temperature with slow shaking on rocker for 2 to 3 hours. PVDF membranes were washed with 1x PBST thrice for 3 minutes each [15, 16].

Secondary antibody (polyclonal and monospecific antibodies separately) was diluted (1:5000) and PVDF membranes were incubated in diluted secondary antibody solution at room temperature with slow shaking on rocker for 2 to 3 hours. After incubation, PVDF membranes were washed with 1x PBST thrice for 3 minutes each. 12.5 mL Tris buffer (pH 7.35), 30 µl of 30% H_2O_2, a pinch of DAB were added into detection tray, mixed well and PVDF membranes were kept into the tray. The tray was gently shaken for a period of 10 minutes until the colour developed in the control lane [15, 16].

Results

Immunological cross reactivity analysis by using polyclonal antibodies

Proteins were separated according to molecular weight in both diseased (D1 to D10) and healthy (C1 to C10) by using SDS-PAGE along with corresponding molecular weight marker. Western blot analysis allowed identification of cross reactivity of serpins with SERPINA4/Kallistatin in diseased and healthy samples by using polyclonal antibodies specific for SERPINA4/Kallistatin followed by secondary antibodies conjugated with HRP.

PVDF membranes of both cirrhotic liver and healthy did not show any band which revealed that there will not be any cross reactivity of other serpins with polyclonal antibodies specific for SERPINA4/Kallistastin (**Figure 1**). However, polyclonal antibodies with more sensitivity were failed to capture SERPINA4/Kallistatin in both cirrhotic and healthy subjects.

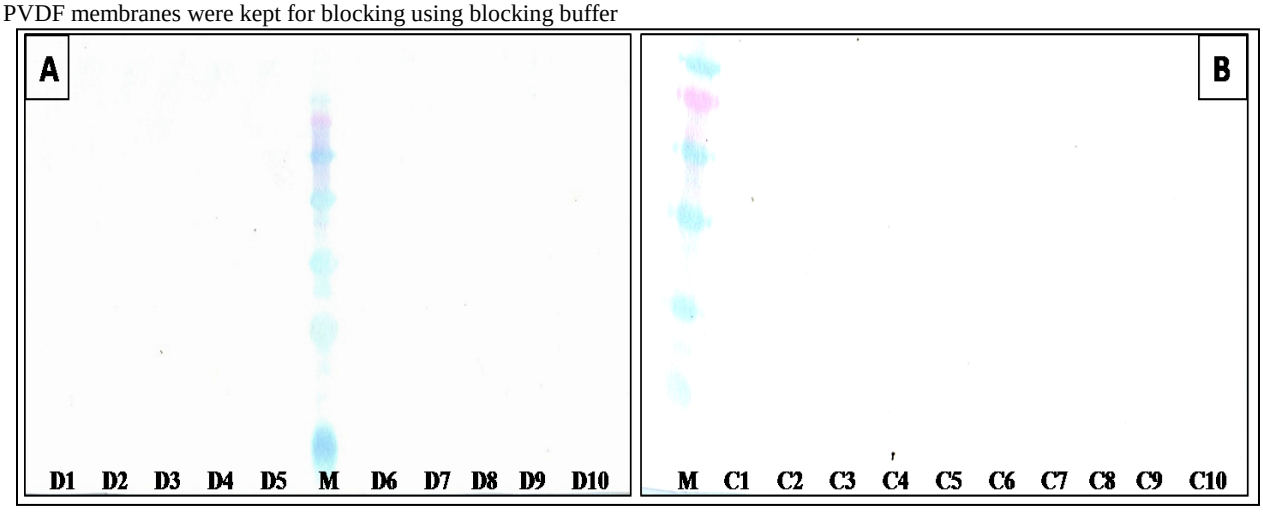

Figure 1: Western blot analysis for immunological cross reactivity with polyclonal antibodies

A: Western blot with diseased serum; **M**: Pre stained marker; **D**: Diseased subjects (D1 to D10; cirrhosis of liver); **B**: Western blot with control serum; **C**: Healthy subjects (C1 to C10)

Immunological cross reactivity analysis by using monospecific (monoclonal alternative) antibodies

Separation of proteins according to molecular weight was achieved by using SDS-PAGE along with pre-stained molecular marker in both healthy (C1 to C10) and diseased (D1 to D10) samples. Western blot analysis allowed identification of cross reactivity of serpins with SERPINA4/Kallistatin in diseased and healthy samples using monospecific (monoclonal alternative) antibodies specific for

SERPINA4/Kallistatin followed by secondary antibodies conjugated with HRP.

PVDF membrane of healthy (**Figure 2**) showed bands corresponding to SERPINA4/Kallistatin molecular weight revealed that monospecific antibodies have ability to capture protein of interest which is in pg/mL. However, no other bands were observed in healthy PVDF membranes which indicate that there will not be any cross reactivity of other serpins with monospecific (monoclonal alternative) antibodies.

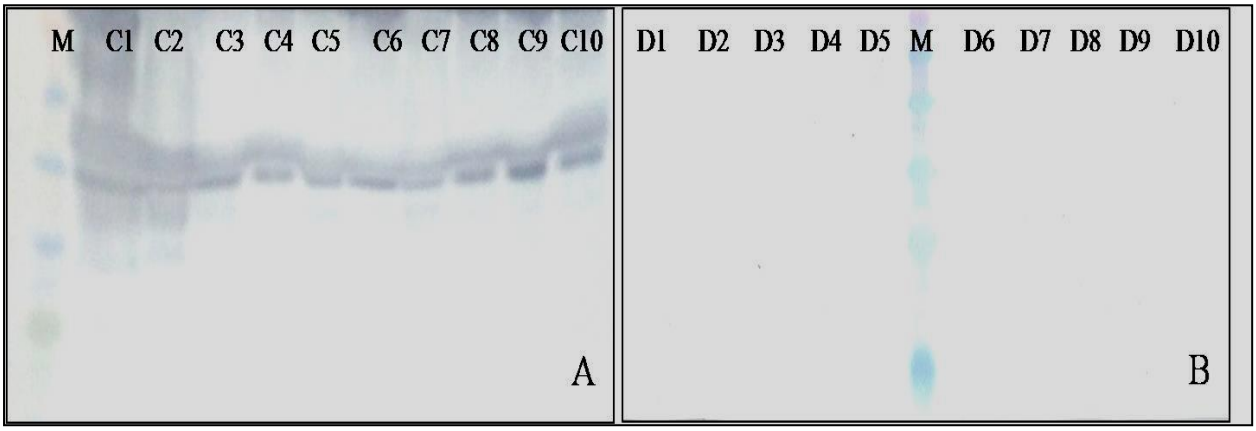

Figure 2: Western blot analysis for immunological cross reactivity with monospecific (monoclonal alternative) antibodies

A: Western blot with healthy serum; **M**: Pre stained marker; **C**: Healthy subjects (C1 to C10); **B**: Western blot with control serum; **D**: Diseased subjects (D1 to D10; cirrhosis of liver)

Cirrhotic liver PVDF membrane did not show any band corresponding to molecular weight of SERPINA4/Kallistatin which revealed that there will be decreased concentration of SERPINA4/Kallistatin in cirrhotic liver subjects when compared to healthy subjects. There were no other bands in cirrhotic liver PVDF membrane that showed no cross reactivity of other SERPINs with SERPINA4/Kallistatin.

Discussion

Study of human biology and disease in respect to proteomic studies is a major practical challenge due to lack of well validated antibodies to many of the human proteins [17]. Protein affinity reagents are fundamental tools for basic and wide range of applications in biomedical research [18]. In human protein atlas, 80% of the antibodies are polyclonal antibodies which imply binding to multiple epitopes which increase the risk of cross specificity towards other proteins. Cross reactivity for antibodies is a major problem in diagnostic and therapeutic application; essential to measure cross reactivity of given antibody against full proteome by using western blot, immunohistochemistry, immunofluorescence or sandwich immunoassays [19, 20].

Proteins share stretches of their primary structure which is identical or differ only by few amino acid residues and some proteins with similar functions have domains with surface patches of high similarities. An antibody targeting an epitope in one of these regions shows cross reactivity to other proteins than the intended target, making the results from an assay with this antibody unreliable and hard to interpret. Availability of well characterized antibodies provides valuable resource for diagnostic studies of the corresponding protein [21]. Mapping of linear epitopes of a polyclonal antibody followed by sequential epitope specific capture using synthetic peptides generates single epitope specific antibodies i.e. monospecific (monoclonal alternative) antibodies [19].

In our previous study using monoclonal antibodies to rule out cross reactivity, serpins did not show any cross reactivity with antibodies specific for SERPINA4/Kallistatin due to absence of identical epitope despite similarity in chemical properties, minor amino acids sequence resemblance and mapped on same gene. But due to less sensitivity (5ng/lane), monoclonal antibodies failed to capture protein of interest both in cirrhotic and healthy PVDF membranes. Multifactorial liver cirrhosis may not induce polymerization which directs to share identical epitope of serpins. This might be the reason for no cross reactivity in cirrhotic liver subjects [8].

Polyclonal antibodies have ability to recognize multiple epitopes, in the present study; these antibodies specific for SERPINA4/Kallistatin did not cross react with other serpins. Even though they are more sensitive; failed to capture protein of interest in both cirrhotic liver and healthy PVDF membranes whose concentrations are in pictogram/mL. Sequential affinity purification of polyclonal antibodies will generate epitope specific antibodies (monospecific antibodies) based on epitope mapping by using synthetic peptides [19].

Monospecific (monoclonal alternative) antibodies specific for SERPINA4/Kallistatin also did not exhibit cross reactivity with other serpins in both cirrhotic and healthy PVDF membranes in present study. These antibodies were able to capture protein of interest in healthy PVDF membranes. But they failed to capture SERPINA4/Kallistatin in cirrhotic liver PVDF membranes. Due to decreased synthetic capacity of liver in cirrhosis, concentrations of SERPINA4/Kallistatin might be decreased in circulation. This could be the reason for no band formation in cirrhotic PVDF membranes even with monospecific (monoclonal alternative) antibodies. On the other hand, renaturation of target protein in western blot analysis might be the reason for no band formation. This limitation could be overcome in Enzyme Linked Immuno Sorbant Assay (ELISA) as native protein is going to bind with specific antibodies in quantification analysis [19].

Comparison of cross reactivity analysis with polyclonal and monospecific (monoclonal alternative) antibodies concluded that serpins do not cross reactive with antibodies specific for SERPINA4/Kallistatin. Monospecific (monoclonal alternative) antibodies are more sensitive and more specific with single linear specific epitope to form double sandwich quantitative ELISA than polyclonal antibodies. Monospecific (monoclonal alternative) antibodies are well characterized antibodies for protein studies especially in clinical diagnosis to capture protein of interest whose concentrations will be in pg/mL. During ELISA development, validation of newly developed methodology should be carried out to check interference of other factors (buffer components, sample matrix, compliment and rheumatoid factor). Further quantitative

studies of Kallistatin may provide potential insights for diagnosis of chronic liver diseases [8, 22].

Conclusion

Comparative cross reactivity analysis of serpins in quantification of SERPINA4/Kallistatin in the present study demonstrated that there will not be any immunological cross reactivity between serpins and SERPINA4/Kallistatin due to the absence of identical epitope in cirrhotic liver and healthy subjects. Monospecific (monoclonal alternative) antibodies are well characterized antibodies with single linear specific epitope in the development of quantitative ELISA for protein studies especially in clinical diagnosis which are capable to capture protein of interest whose concentrations are in pg/mL; high sensitivity and high specificity.

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Conflict of interest

Authors declare no conflict of interest

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