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Proteomic Analysis for Cryofibrinogen: Associated Glomerulonephritis and Cryoglobulinemic Glomerulonephritis

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Abstract

Cryofibrinogen-associated glomerulonephritis (CF-GN) and cryoglobulinemic glomerulonephritis (CG-GN) are rare diseases that are both characterized by membranoproliferative glomerulonephritis (MPGN) with organized deposition of tubular or cylinder structures; however, they are distinguished by the absence or presence of immunoglobulins in CF-GN and CG-GN respectively. The diagnoses of the two disorders are based upon reversible cryoprecipitation of plasma or serum, clinical manifestations in the skin and the pathological characteristics seen during renal biopsy. Recently, a novel powerful laser microdissection (LMD) and liquid chromatography-tandem mass spectrometry (LCMS/MS) tool has been introduced to identify the components of electron dense deposits. In this review, we described the clinical characteristics and pathological features of CF-GN and CG-GN, and critically assessed the application of LCMS/MS to the diagnosis of kidney diseases among patients previously described. Notably, the practical information provided by proteomic analysis using LCMS/MS has an immense impact on establishing the specific diagnosis of these rare glomerulonephritis (GN) that have identical morphologies but different etiological molecules.

Keywords: Cryofibrinogen-associated glomerulonephritis; Cryoglobulinemic glomerulonephritis; Proteomic analysis

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Introduction

Molecular Features And Clinical Manifestations of CF and CG

Cryofibrinogen (CG) was identified in 1955 by Korst and Kratochvil as an abnormal protein in the plasma of a patient with migrating thrombophlebitis and lung cancer [1]. The protein is characteristically precipitated in plasma treated with an anti-coagulant, such as oxalate, citrate or edetic acid at low temperatures (4°C) for a period of 72 hours. Additionally, the precipitates are dissolved when the plasma is warmed to 37°C. Components of cryofibrinogen that are involved in the cryoprecipitation of cryofibrinogen include insoluble fibrin, fibrinogen, fibronectin, fibrin degradation products with albumin, cold insoluble globulins, factor VIII, and other plasma proteins [2]. On the other hand, cryoglobulin is mainly composed of immunoglobulins and complements. Therefore, a distinguishing characteristic between cryofibrinogen and cryoglobulin is that the precipitation of cryofibrinogen only occurs in cold plasma and not in cold serum, whereas the precipitation of cryoglobulin occurs in both cold serum and cold plasma.

Cryofibrinogenemia (CF) is classified as essential (primary) or secondary to other disorders. Diagnostic criteria for CF include, a sudden onset of skin manifestations that represents a constitutional symptom of cryoprecipitation in plasma, angiographic evidences of the abrupt occlusion of small to medium-sized arteries, typical skin biopsy findings, and elevated serum α1-antitrypsin and α2-macroglobulin [3]. The causes of secondary CF in published literature can be classified into four groups: 1) bacterial and viral infections, 2) autoimmune diseases including vasculitis, 3) lymphoproliferative disorders and 4) solid tumors [4-6]. Although not all patients with CF will have high plasma fibrinogen levels on blood examination, a temporal association between exposure to cold and the onset of skin symptoms has been reported in the literature [6]. The most specific finding of CF is demonstrated in the skin biopsy which shows cutaneous ischemia due to thrombi pluggin superficial and deep blood vessels. The skin is then characterized by cold sensitivity, livedo reticularis, Raynaud phenomenon, purpura ulcer, and gangrene.

On the other hand, cryoglobulin, first named by Lerner and Watson in 1947 is an abnormal circulating protein is composed of immunoglobulins [7]. CG precipitates in the serum of patients

with CG at a temperature of ≤ 37°C. CG has been classified into three subtypes based on the immunoglobulin components; Type I, Type II, and Type III by Brouet, et al. in [8]. Type I cryoglobulinemia contains monoclonal immunoglobulins [mainly immunoglobulin M (IgM), and rarely immunoglobulin G (IgG) or immunoglobulin A (IgA)]. Type II and Type III cryoglobulinemia are referred to mixed CG due to the detection of two types of immunoglobulins (i.e., IgG and IgM) in the cryoprecipitation. The cryoglobulin of type II mixed cryoglobulinemia is composed of a combination of monoclonal (usually IgM) and polyclonal immunoglobulins (usually IgG), whereas type III mixed CG is comprised of polyclonal IgM and polyclonal IgG. The type I CG is usually associated with B cell lymphoproliferative disorder, whereas type II and III CG are often linked to systemic autoimmune diseases, lymphoproliferative disorders and chronic infections such as hepatitis C virus (HCV) which comprise 80%-90% of the cases with mixed CG [9,10]. Cryoglobulin should be tested for in all patients presenting with typical manifestations of cryoglobulinemic vasculitis for instance orthostatic purpura, arthralgias and/or arthritis, weakness, peripheral neuropathy, and sicca syndrome, regardless of the presence or the absence of HCV or HBV infection. In such patients, other laboratory findings like urinary abnormalities, rheumatoid factor, and low C4 level in the serum are also suggestive of CG. The detection of circulating serum cryoglobulin, a common consequence of cryoglobulinemic vasculitis, is the most important diagnostic finding in patients with CG.

One of the most serious complications of vasculitis associated with CF and CG is the involvement of the kidneys. Cryofibrinogen-associated glomerulonephritis (CF-GN) has been recently identified as a renal manifestation of CF [11]. Terrier et al. demonstrated the presence of cryofibrinogen in 11 (11%) of 101 patients treated for the kidney disorders in s single university hospital, they also demonstrated the relationship between CF and thrombotic events in these patients [6]. Renal involvement has also been reported in 8 (13%) of 60 patients with CF by Saadoun, et al. and in 3 (38%) of 8 pediatric patients with CF by Chou HF, et al. [12,13]. Moreover, cryoglobulinemic glomerulonephritis (CG-GN) has been identified as a renal manifestation in patients with CG that is also a major risk factor for mortality and morbidity in these patients [14,15]. Renal involvement is more frequent encountered in mixed cryoglobulinemia than in type I cryoglobulinemia. In the previous research studies, many patients with HCV infection presented with mixed cryoglobulinemic vasculitis as a predominant extrahepatic chronic HCV infection [16]. Patients with chronic HCV infection and CG more frequently presented with arthralgia, hypertension, purpura, incidences of systemic vasculitis, and cardiovascular disease when compared to patients with chronic HCV without CG [16-18]. A typical clinical manifestation of cryoglobulinaemic vasculitis is the appearance of orthostatic purpura, whereas its typical pathological feature is leukocytoclastic vasculitis. The serological alterations consist of increasing mixed cryoglobulins with rheumatoid factor activity and low serum complement C4 (C4) [19,20]. Meanwhile, another report suggested that patients with mixed CG-GN without concurrent infection had poorer long-term outcomes with severe infection which ultimately contributed to majority of the deaths among this group of patients, when compared to patients with HCV-related CG-GN [21].

Technical Considerations for the Cryoprecipitation in CF and CG

The diagnosis of cryofibrinogenemic and cryoglobulinemic vasculitis principally depends on the laboratory detection of plasma cryofibrinogen or serum cryoglobuline respectively. Plasma cryofibrinogen can usually be detected in samples collected in whole blood collection tubes that contain a citrate anti-coagulant. On the other hand cryoglobulin precipitates are harvested from serum after complete elimination of blood clots is done in the early phase of sampling procedures [22,23]. The following steps that should be performed strictly, are very important in detecting cryofibrinogen and cryoglobuline in samples. For cryofibrinogen, the blood cells should be separated from the plasma by centrifugation immediately after the blood collection, whereas for cryoglobuline, a complete coagulation must to be performed before centrifugation for the blood cells separation. In both cases, these steps must always be performed at 37°C as much as possible. Additionally, at least 3 days of storage at 4°C are required to assess for the presence of cryoprecipitation in cold plasma or serum. After cryoprecipitation, the cryofibrinogens and cryoglobulins should be tested for their reversibility in the plasma and serum by rewarming the cryoprecipitates to a temperature of 37°C for 24 hours. False-positive results caused by heparin-precipitable proteins could be eliminated by this step, but the heparin had better be avoided to use as anti-coagulant for cryofibrinogen detection [19]. To identify components of cryoprecipitation, sensitive methodologies such as immunoelectrophoresis or immunofixation at 37°C ought to be used. It is important to note that false-negative results could easily be caused by leaving the samples at a lower temperature (i.e., below 37°C) before assessing for cryoprecipitation.

Pathological Features Of CF-GN and CG-GN

Membranoproliferative glomerulonephritis (MPGN) is a common pathological feature of CF-GN and CG-GN that could be identified on light microscopy. The histological features of MPGN include the characteristic endocapillary hypercellularity, lobular accentuation of the glomerular capillary tufts, “tram track” appearance and mesangial interposition upon silver staining. During the clinical course of the disease, this pattern may evolve into several phenotypic categories that include; minimally proliferative, mesangial proliferative, typical membranoproliferative (lobular) and sclerosing glomerulonephritis which are detected upon the examination of renal biopsies. Thrombi can often be found in the capillary lumina and arterioles of the glomeruli. In some cases, medium-sized renal arteries have fibrinoid necrosis in their vessel walls with intramural and perivascular leukocyte infiltration. In the glomeruli of patients with CF-GN, fibro-cellular crescent formation occasionally appears and the immunofluorescent examination usually reveals dominantly positive complement 3 (C3) along the capillary walls and in the segmental mesangial area in but no immunoglobulins are present [24]. In contrast, in the glomeruli of patients with CG-GN, the glomerular capillary walls stain positive for IgG, IgM, C3 and complement 1q (C1q) and have a granular pattern with focal and segmental accentuation in the mesangial area. In addition, the cryoglobulin thrombi composed

of IgG, IgM, C3, and C1q have been occasionally demonstrated in afferent arterioles in serial sections on immunofluorescence microscopy [25]. The characteristic organized deposits with randomly arranged tubular, cylinder, and fibrillary structures can be detected on electron microscopy in the capillary lumina, subendothelial, and mesangial regions in patients with CF-GN and CG-GN [11,24]. On the contrary, routine immunohistochemical methods used to examine renal biopsy specimens do demonstrate any specific and sensitive antigens related to CF and CG. Therefore, there is limited information regarding the diagnosis of CF-GN and CG-GN when you have the characteristic clinical symptoms and signs derived from vasculitic manifestations but not information regarding cryoprecipitation.

LMD and LCMS-MS in the Diagnosis of Various Kidney Diseases

Recently, laser microdissection (LMD) and liquid chromatography-tandem mass spectrometry (LCMS/MS) analysis have been introduced as a novel diagnostic technique in renal biopsy pathology. Proteomics can better identify and characterize the composition the deposits and factors involved in the pathogenesis of renal disorders even in the presence of small amounts of proteins. Using these techniques, PLA2R, THSD7A, and exotosin 1/2 have been established as etiological molecules and useful markers for membranous nephropathy [26-28]. DNAJB9 was also demonstrated as a novel etiological protein for fibrillary GN [29-31]. Recently, many investigators have described the proteomic analysis of renal amyloidosis in detail and proposed several novel entities of amyloidosis such as leukocyte cell derived chemotaxin 2 (LECT-2), fibrinogen-α, gelsolin, apolipoprotein A-I, II and IV, and transthyretin amyloidosis [32-43]. This method, that was applied for only small amount of samples containing 0.001 mm3 of laser microdissected glomeruli with amyloid deposition and 0.003 mm3 with immunoglobulin-associated GN from formalin fixed paraffin embedded sections digested with 0.3 μg trypsin, accurately demonstrated the causal factors in renal biopsy specimens [44-46]. Using this procedure, a list of proteins identified by MS signals is generated based on tryptic peptide fragments in one sample. The MS data shown by spectra that match particular peptides of proteins, and peptide identifications is then established at more than 95% probability based on the criteria by the Peptide Prophet algorithm. All proteins of in sample are then identified by the amino acid sequences of the identified peptides that have been listed in the database (UnipotKB, UniProt Consortium, https://www.uniprot.org/).

LCMS-MS Analysis in CF-GN and CG-GN

LMD and LCMS/MS can be used to confirm the presence of etiological molecules, fibrinogen, or immunoglobulin in CF-GN and CG-GN. Prior to assessing the glomerular proteomic data of CF-GN and CG-GN, it is vital to comprehend the proteomic data of normal glomeruli. To identify the major proteins of normal glomerular tissues and to elucidate the influence of the blood components in the glomerular capillaries Kawata, et al. compared the 0 hour-RBx and 1 hour-RBx samples from kidney transplant donors (Table 1) [45]. Compared to the 0 hour-Rbx in which the blood-derived proteins were eliminated by perfusion with preservation solution, the 1 hour-Rbx samples showed slight elevations of IgG1, IgA, light chain kappa, and albumin due to the presence of blood in glomerular capillaries. Although small amounts of plasma proteins are usually present in glomerular proteomic contents, considerable high total spectrum count (TSC) numbers of immunoglobulins and complements in the glomeruli of CF-GN and CG-CG imply that these molecules play a significant role in the glomerular deposition of cryofibrinogen and cryoglobulin.

Descriptions of identified proteins	Total spectrum count	+
(average ± SD)	+	+
	0 hour-RBx	1 hour-RBx
Immunoglobulins	+	+
Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3	-	-
Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	2 ± 1	6 ± 2
Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2	<1	<1
Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2	1 ± 0	3 ± 1
Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	9 ± 3	19 ± 7
Complement proteins	+	+
Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	3 ± 1	2 ± 1
Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=2	1 ± 0	1 ± 0
Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4	-	-
Complement component C6 OS=Homo sapiens GN=C6 PE=1 SV=3	-	-
Complement component C9 OS=Homo sapiens GN=C9 PE=1 SV=2	3 ± 1	2 ± 1
Other plasma proteins	+	+
Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3	-	-
Apolipoprotein B 100 OS=Homo sapiens GN=APOB PE=1 SV=2	-	-
Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2	<1	<1
Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	-	-
Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3	<1	<1
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	47 ± 12	97 ± 33
Structural proteins	+	+
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=4	13 ± 3	12 ± 14
Collagen alpha-2(IV) chain OS=Homo sapiens GN=COL4A2 PE=1 SV=4	9 ± 5	7 ± 2
Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4	9 ± 4	7 ± 8
Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4	25 ± 6	14 ± 9
Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	-	-

Abbreviations: 0 hour-RBx: time-zero renal biopsy; 1 hour-RBx: time-1 hour renal biopsy; Ref.: reference; -: not detected	+	+

Table 1: LCMS/MS fingdings of normal glomerular tissue using 0 hour-RBx and 1 hour-RBx of patients [45].

Using this technique, Sethi et al illustrated the composition of the glomerular deposits in four patients with CF-GN [47]. Although no tubular substructure was detected in any of the four patients, all patients tested positive for cryofibrinogens in their plasma. The proteins that are usually detected by LCMS are divided into four groups: Amyloidogenic proteins in the serum, structural proteins, immunoglobulins, and complement proteins (Table 2). Glomeruli of patients with CG-GN did not contain amyloidogenic proteins such as apolipoprotein E, but contained large spectra numbers for IgM chain C region and IgG-1 chain C region (Table 2). Large spectra numbers of structural proteins (such as basement membrane heparan sulfate and fibronectin) and C3 were also detected in patients with CG-GN, nevertheless, these structural proteins can also be detected in normal glomeruli at relatively lower levels (Table 1).

Furthermore, the clinical features, kidney biopsy findings, and proteomic analysis of the cryoprecipitate extracted from plasma for two patients with CF-GN were described by Sethi, et al. [11]. In this study, kidney biopsy examination showed MPGN weak segmental capillary wall fibrinogen and no immunoglobulin deposition. Special organized deposits with large-bore multilayered tubular structures were observed in the glomeruli and cryoprecipitate was also observed on electron microscopy. When LCMS analysis was performed for the cryoprecipitates, this revealed that the major protein was fibrinogen that consisted of fibrinogen α, β and γ chains coexisting with smaller amounts of albumin, α2-macroglobulin, haptoglobin, C3, filamin, and apolipoprotein B-100 [11]. Ibuki, et al. documented a patient with CF-GN with similar clinical manifestations and laboratory findings as the one described by Sethi, et al. [24]. In this patient, kidney biopsy showed a membranoproliferative pattern with crescent formation and dominant C3c deposition in which subendothelial deposits were observed. Additional ultrastructural analysis of organized glomerular deposits of glomeruli and cryoprecipitates extracted from plasma revealed randomly arranged large fibrils with large central bores and double layer structures. Large TSC number of fibrinogen-α, β, and γ chains, fibronectin, filamin-A, and C3 were also identified by LMD-LCMS/MS. This is considerably different from the glomerular proteomic results observed in normal glomeruli observed from 1 hour-RBx patients (Table 1).

Descriptions of identified proteins	CF-GN			CG-GN [47] (Glo.)			
	(CP) 11]	(Glo.) [46]	(Glo.) [24]	Case 1	Case 2	Case 3	Case 4
Immunoglobulins							
Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3	NM	NM	-	22	20	40	37
Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	NM	2	14	16	6	14	17
Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2	NM	NM	5	9	-	-	-
Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	NM	NM	15	14	11	15	18
Ig lambda chain C regions OS=Homo sapiens GN=IGLC1 PE=1 SV=1	NM	NM	NM	6	1	4	3
Complement proteins							
Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	91	NM	84	14	6	8	9
Complement C4-B OS=Homo sapiens GN=C4A PE=1 SV=2	NM	NM	16	-	-	-	-
Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4	NM	NM	9	-	-	-	-
Complement component C6 OS=Homo sapiens GN=C6 PE=1 SV=3	NM	NM	3	-	-	-	-
Complement component C9 OS=Homo sapiens GN=C9 PE=1 SV=2	NM	NM	19	4	-	-	-
Other plasma proteins							
Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3	100	NM	-	NM	NM	NM	NM
Apolipoprotein B 100 OS=Homo sapiens GN=APOB PE=1 SV=2	170	NM	-	NM	NM	NM	NM
Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2	260	15	19	NM	NM	NM	NM
Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	212	5	15	NM	NM	NM	NM
Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3	187	7	13	NM	NM	NM	NM
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	39	NM	68	NM	NM	NM	NM
Structural proteins							
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=4	NM	NM	51	55	12	6	18
Collagen alpha-2(IV) chain OS=Homo sapiens GN=COL4A2 PE=1 SV=4	NM	NM	28	15	11	7	14
Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4	154	18	57	50	22	18	33
Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4	97	NM	55	NM	NM	NM	NM
Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	34	NM	-	NM	NM	NM	NM
Abbreviations: CF-GN:Cryofibrinogen-Associated Glomerulonephritis; CG-GN Cryoglobulinemic Glomerulonephritis; CP:Cryoprecioitation exatrated from the plasma; Glo:Glomeruli; Ref: reference; NM: not mentioned in the manuscript; -: not detected. All data are showed by total spectrum count.							

Table 2: LCMS/MS fingdings in CF-GN and CG-GN [11,24,46,47].

When comparing proteomic results of patients with CF-GN to those of patients with CG-GN, increasing immunoglobulins such as IgM are more prominent and the low TSC numbers of IgG, IgA, light chain C regions are present in the glomeruli of patients with CG-GN (Table 2). On the contrary, C3 and C9 as complement proteins are present in high TSC numbers in the plasma cryoprecipitate and glomeruli from CF-GN patients. The other blood components such as α2-macroglobulin, haptoglobin, apolipoprotein B-100, and albumin were also found in the cryoprecipitate and glomeruli from CF-GN patients as well as the etiologic proteins; fibrinogen α, β, and γ chains. Some structural proteins such as basement membrane-specific heparan sulfate proteoglycan core protein, fibronectin, filamin-A, and collagen alpha-2(IV) chain can be identified in the glomeruli of CF-GN and CG-GN at various degrees of TSC numbers (Table 2). We also analyzed the pathological features and LCMS/MS results of three patients with biopsy-proven MPGN compatible with CF-GN or CG-GN but no cryoprecipitate could be detected in the serum and plasma in the repetitive blood tests (unpublished data). However, the characteristic pathological findings suggested of CG-GN or CF-GN, and LCMS/MS identified IgM, fibronectin, and fibrinogens (α, β, γ) in the glomerular extracts were present, Notably, these features are distinguished from kidney diseases that have organized deposits such as renal amyloidosis, fibrillary nephropathy, immunotactoid nephropathy, and immune complex nephritis which are usually difficult to delineate from the diagnosis of CF-GN or CG-GN. These results indicated that various blood-derived proteins and glomerular structural proteins are concomitantly involved in the cryoprecipitates of CF and CG and the composition of these proteins may be different in each disease entity, or may vary on a case by case basis. Future investigations could aide in accumulating the results of LCMS/MS to support a definitive diagnosis and explore the novel markers of CF-GN and CG-GN.

Conclusion

In conclusion, proteomic analysis is a promising new diagnostic tool for renal biopsy specimens. In addition, results of LCMS/MS could provide information on the specific proteins that contribute to the formation of CF and CG in the glomeruli. Further investigations should be conducted to enhance our understanding of glomerular components in the patients with CF and CG and to accurately diagnose CF-GN and CG-GN by LCMS even among patients without evidence of cryoprecipitation. LCMS/MS analysis is a highly reproducible method that facilitates further studies of renal diseases in not only CF-GN and CG-GN, but also various glomerular diseases.

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