# Detection of Anti-PLA2R Autoantibodies and IgG Subclasses in Post-allogeneic Hematopoietic Stem Cell Transplantation Membranous Nephropathy

*Xianghua Huang, MD, Weisong Qin, MD, Mingchao Zhang, MD, Chunxia Zheng, MD, Caihong Zeng, MD and Zhihong Liu, MD*

**Abstract:** *Background:* Membranous nephropathy (MN) is the most common glomerular disease of post–allogeneic hematopoietic stem cell transplantation (HSCT). Although this condition is now considered a renal complication of chronic graft-versus-host disease (cGVHD), the pathogenesis of this disease is not well established. *Methods:* Five patients with post-HSCT MN diagnosed by renal biopsy were selected for this study. The clinical and renal pathological data of these patients were analyzed, and anti-PLA2R (M-type phospholipase A2 receptor) autoantibodies and IgG subclasses were detected in the serum samples from the patients. *Results:* None of the 5 patients had a history of kidney disease. All the patients had a combination of cGVHD and proteinuria, which was in remission after an effective anti–graft-versus-host disease treatment. The immunofluorescent detection showed that IgG4 was the predominant IgG subclass, and the distribution of IgG4 was the same as that of nephrin. The anti-PLA2R autoantibodies were negative in 4 patients and positive in 1 patient. The levels of IgG2, IgG3 and IgG4 increased in the majority of the patients. *Conclusions:* Our data showed that the clinical course of post-HSCT MN patients was closely related to that of cGVHD. Although the renal pathology was similar to idiopathic MN, the negative result for the anti-PLA2R autoantibodies in the majority of the patients suggested that the formation of an immune complex occurs differently between these 2 diseases.

**Key Indexing Terms:** Membranous nephropathy; Graft-versus-host disease; Anti-PLA2R autoantibodie. **[Am J Med Sci 2013;346(1):32–37.]**

Membranous nephropathy (MN) in post–hematopoietic stem cell transplantation (HSCT) generally has been attributed to chronic graft-versus-host disease (cGVHD).[1] cGVHD may precipitate glomerular disease after HSCT via a complex interaction between donor T cells and host antigen-presenting cells. B cells also have received attention as possible effectors in cGVHD, although their role is not completely understood.[2] Animal models of cGVHD describe the kidney as a target organ with histopathologic features of MN, but the pathogenesis of post-HSCT MN and the role of cGVHD are not well established. Some specific autoantibodies, such as H-Y antibody, have been found to be correlated with cGVHD but only in male hematopoietic cell transplant recipients with female donors.[3]

MN is the most common cause of idiopathic nephrotic syndrome (NS) in adults[4] and is characterized by an accumulation of immune complexes on the outer glomerular basement membrane that causes a membrane-like thickening.[5] It has become clear that the binding of circulating autoantibodies to target antigens on the podocyte initiates the disease process. More recently, the M-type phospholipase A2 receptor (PLA2R) was identified as the major target podocyte antigen involved in adult autoimmune idiopathic MN.[6] The anti-PLA2R autoantibody can explain the pathogenesis of most idiopathic MN, but the pathogenesis of MN secondary to HSCT is largely unknown. It has been known for several years that the IgG deposits in the glomeruli of patients with idiopathic MN are predominantly IgG4,[7] whereas the IgG subclass in MN secondary to HSCT is unknown. In this study, we measured the anti-PLA2R autoantibodies and IgG subclass in serum samples from 5 patients and investigated possible mechanisms for the pathogenesis of the post-HSCT MN.

## MATERIALS AND METHODS

### Patient Selection

Five patients diagnosed with post–allo-HSCT MN by renal biopsy between January 2006 and July 2009 were included in this study. All the patients had undergone allo-HSCT with the primary disease classified as a hematological malignancy, had no history of renal disease before allo-HSCT and had MN confirmed by a renal biopsy; for all patients, the common causes of secondary MN, such as tumor-associated MN, hepatitis B virus–associated MN or lupus-associated MN, had been excluded. Patients' medical records were reviewed for demographic information, presenting clinical and laboratory findings, treatments and outcomes. The study was approved by the Institutional Review Board of Jinling hospital.

### Renal Biopsy

Percutaneous renal biopsies were taken from all patients before the study. Renal tissues were stained with hematoxylin-eosin, periodic acid-Schiff, periodic acid-silver methenamine and Masson for light microscopy examination. Frozen sections were tested for immunoglobulins and complement deposition by direct immunofluorescence (IF). The IgG subclasses were evaluated by indirect IF with monoclonal antibodies directed to IgG1, IgG2, IgG3 and IgG4 in 5 patients.

### Double-Staining Indirect Immunofluorescent Microscopy

Cryosections measuring 3 μm in thickness were placed on silan-coated slides and dried at room temperature. The sections were then fixed in cold acetone for 5 minutes at 4°C and washed in phosphate-buffered saline (PBS). Mouse on mouse blocking reagent solution was added, and the slides were incubated for 60 minutes; next, the sections were incubated with mouse anti-human nephrin antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes, then incubated with biotin-labeled anti-mouse

*From the Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China.*

*Submitted April 04, 2012; accepted in revised form June 13, 2012.*

*Presented as a poster (SA-PO2305) at the annual meeting of the American Society of Nephrology, Denver, Colorado, November 16 through 21, 2010.*

*All authors declare no conflicts of interest.*

*Correspondence: Zhihong Liu, MD, Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, 305 East Zhongshan Road, Nanjing 21002, China (E-mail: zhihong–liu@hotmail.com).*

IgG (1:400; Sigma, St. Louis, MO) for 10 minutes, washed in PBS, incubated with dioxyfluoran Avidin DCS (1:200; Vector, Burlingame, CA) for 5 minutes, followed by another wash with PBS. Mouse on mouse blocking reagent solution was added a second time and incubated for 60 minutes, followed by a 60-minute incubation with mouse anti-human IgG4 antibody (1:400; Sigma) and a 10-minute incubation with biotin-labeled anti-mouse IgG. Next, the slides were washed in PBS and incubated with dioxyfluoran Avidin DCS for 5 minutes, washed again in PBS, incubated with Texas Red Avidin DCS (Vector) for 5 minutes and a final wash of PBS. The distribution of nephrin and IgG4 deposits was observed by confocal microscopy.

### Human Glomerular Extract

Kidneys from deceased donors, which were unsuitable for transplantation, were obtained from the Jinling hospital. Human kidneys were stripped of their capsules, and the cortex was obtained and minced. Glomeruli were collected using a series of graded sieves (Fisher Scientific, Waltham, MA) and washed 3 times with cold PBS at a pH of 7.4; the resultant preparation consisted of approximately 80% to 85% glomeruli. The glomerular pellet was initially resuspended in an equal volume of 100 mM Tris (pH 8) and 1 mM $MgCl_2$ and frozen at −80°C. An equal volume of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) was added with 1× Protease Inhibitor Cocktail Set I (Calbiochem/EMD Chemicals, Inc, Philadelphia, PA), and the glomeruli were homogenized on ice. RIPA-insoluble debris was removed by a 10-minute centrifugation at 14,000 rpm at 4°C. The human glomerular extract was incubated for 4 hours at 4°C with Protein G Plus (Thermo Fisher, Waltham, MA), and the beads were discarded to remove the contaminating human IgG.

### Extracts of HEK Cells Expressing Recombinant PLA2R

A plasmid vector containing full-length human PLA2R under the control of the cytomegalovirus promoter was transiently transfected into HEK293T cells. Cells were collected after culture and lysed with Tris buffer plus protease inhibitors. Insoluble cell debris was discarded after centrifugation.

### Detecting of PLA2R by Western blotting

Human glomerular extract or recombinant PLA2R was electrophoresed under nonreducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and then incubated with human serum at a dilution of 1:25. Sheep antibodies against human IgG4 (1:3000; Binding Site, San Diego, CA) were used as a secondary antibody. Donkey anti-sheep peroxidase–conjugated antibody was used as the detecting antibody at 1:5000 (Jackson ImmunoResearch, West Grove, PA). A commercial antibody to human PLA2R (Sigma-Aldrich, St. Louis, MO) was used at 1:400 to confirm the position of the PLA2R bands. Blots were incubated in chemiluminescent substrate (100 mM Tris, pH 8.5, 250 mM luminol, 90 mM p-coumaric acid and hydrogen peroxide) for 3 minutes and exposed to HyBlot CL autoradiography film (Denville Scientific, Inc, South Plainfield, NJ). The exposure times were typically 10 to 30 seconds for positive bands and up to 10 minutes for weak or negative bands. The film was developed using a Kodak X-OMAT 2000A processor.

### Determination of IgG Subclass Levels

Standard nephelometry was used to determine the absolute amounts of the IgG subclasses in patients' sera at diagnosis.

## RESULTS

### Clinical Characteristics of the Patients

Five patients were included in this study; the clinical characteristics of the patients at the time of MN diagnosis are summarized in Table 1. All patients had a malignant hematological disease and underwent allo-HSCT, with a sibling donor in 4 cases and an unrelated donor in 1 case. The HLAs were all matched; the major complication of the transplantation was acute graft-versus-host disease (GVHD), and 4 of 5 patients had a history of acute GVHD. None of the 5 patients had a history of kidney disease, and the renal disease onset was 10 to 22 months post-HSCT, with a clinical manifestation of NS at disease onset. In our study, one patient had hematuria, the urine protein was between 3.27 and 6.54 g per 24 hours and albumin was 21.2 to 34.1 g/L; all patients had normal renal function, the anti-nuclear antibody test of 3 cases was positive, and the complement and of all cases was normal. In all the cases, cGVHD occurred before renal disease, and the main organs involved in cGVHD were the oral cavity and the skin. The renal pathologic diagnosis of all patients was MN. After treatment with a combination of immunosuppressive agents (Table 2), 1 patient achieved complete remission and the other 4 patients achieved partial remission. All patients survived and are currently in remission from GVHD.

### Renal Pathology

The light microscopy of periodic acid-Schiff–stained slides showed that the basement membranes were thin and delicate, the capillaries were all patent, the glomeruli had no apparent abnormalities, and the tubules were normal with an absence of interstitial nephritis (Figure 1A). In periodic acid-silver methenamine-stained slides, the spikes were barely visible, whereas the MASSON staining showed diffuse red discrete granular deposits on the epithelial side of the glomerular basement membrane (Figures 1B and 1C). Electron microscopy showed dense subepithelial immune deposits and diffuse effacement of the foot processes of epithelial cells (Figure 1D). The IF confirmed the granular immune deposition of IgG along the capillary loops (Figure 1E) and granular deposition of C3 along the glomerular capillary wall (Figure 1F) and demonstrated that C4 and C1q deposition was positive in some patients (Table 1).

### Staining of IgG Subclasses

The IgG subclasses that were deposited are summarized in Table 1. The IgG4 was the predominant IgG subclass in these patients (presenting in all analyzed patients) and was positively associated with IgG1 deposits and negatively associated with IgG3 deposits (Figures 2A–C).

### Double Immunofluorescence Staining for IgG4 and Nephrin

Confocal microscopic analysis suggested that there were several foci of colocalization of IgG4 and nephrin along the glomerular capillary walls in all patients (Figures 2D–F). These results indicated that nephrin and IgG4 were colocalized.

### The Results of Anti-PLA2R Antibody

The anti-PLA2R autoantibodies were negative in cases 1, 2, 3 and 5. A serum sample from case 4 was reactive with PLA2R, which was visualized as a 185-kDa protein band. Serum from case 4 also recognized recombinant human PLA2R, which appears smaller, likely because of incomplete glycosylation *in vitro* (Figure 3).

TABLE 1. Clinical and pathological characteristics of patients with post–allogeneic hematopoietic stem cell transplantation membranous nephropathy

| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 |
|---|---|---|---|---|---|
| Sex/age (yr)/hemopathy | M/52/CML | F/46/AML-M1 | F/20/ALL-L1 | M/22/CML | M/49/AML-M0 |
| Conditioning regimen | BU/CY | TBI/CY | BU/CY | BU/CY | BU/CY |
| Donor/HLA-match | Sib/identical | Sib/identical | Unrelated/identical | Sib/identical | Sib/identical |
| GVHD prophylaxis | CsA/MTX | CsA/MTX | CsA/MTX/ATG | CsA/MTX | CsA/MTX |
| History of aGVHD/cGVHD | Y/Y | N/Y | Y/Y | Y/Y | Y/Y |
| Treatment for cGVHD | CsA + P | N | P | P | FK506 |
| Time between HSCT and MN (mo) | 19 | 10 | 23 | 14 | 22 |
| Time between cGVHD diagnosis and MN (mo) | 12 | 5 | 4 | 9 | 2 |
| Time between immunodepressant withdrawal and MN (mo) | 8 | 2 | 1 | 4 | 16 |
| cGVHD signs at the time of MN diagnosis | Y | Y | Y | Y | Y |
| Laboratory examination at the time of MN diagnosis | | | | | |
| Proteinuria (g/24 h)/SCr (mg/dL)/Alb (g/L) | 4.95/0.81/28.8 | 4.56/0.69/29.7 | 3.27/0.60/21.2 | 6.54/0.65/34.1 | 4.59/0.8/25.6 |
| ANA/C3/C4/HV test/cryoglobulin | −/−/−/− | 1:16/−/−/− | −/−/−/− | 1:16/−/−/− | 1:128/−/−/− |
| TC (mmol/L)/TBIL (mmol/L)/ALT (U/L) | 6.91/8.4/26 | 5.76/9.3/11 | 14.05/8.2/74 | 6.95/11.2/32 | 7.98/15.2/20 |
| Immunofluorescence for renal biopsy | | | | | |
| IgG/C3/C4/C1q | 2+/1+/1+/1+ | 2+/−/−/− | 2+/2+/1+/1+ | 2+/2+/1+/1+ | 2+/1+/−/− |
| IgG subclasses | | | | | |
| IgG1/IgG3/IgG4 | 2+/−/3+ | 1+/−/2+ | 2+/−/2+ | 2+/−/3+ | 2+/−/2+ |

aGVHD, acute graft-versus-host disease; Alb, albumin; ALL, acute lymphocytic leukemia; ALT, alanine aminotransferase; AML, acute myeloid leukemia; ANA, anti-nuclear antibody; BU, busulfan; cGVHD, chronic graft-versus-host disease; CML, chronic myeloid leukemia; CsA, cyclosporine A; CY, cyclophosphamide; F, female; FK506, tacrolimus; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; HV test, hepatic virus test (polymerase chain reaction test for hepatitis B virus and hepatitis C virus DNA); M, male; MN, membranous nephropathy; MTX, methotrexate; N, no; P, prednisone; Scr, serum creatinine; Sib, sibling; TC, total cholesterol (normal range, 3.6–6.0 mmol/L); TBIL, total bilirubin; Y, yes; "−"means negative; "+" means positive.

### IgG Subclasses

The concentrations of the IgG subclasses are described in Table 3. The level of IgG1 was within the normal range for all the patients except one, whereas the levels of IgG2, IgG3 and IgG4 increased in the majority of the patients.

## DISCUSSION

Renal injury is a common complication of HSCT,[8] and 15% to 66% of patients eventually develop chronic kidney disease.[9] However, NS is a rare renal complication, with an overall incidence of only 1% to 2.4%, as reported by 2 large cohort studies.[10,11] Renal biopsy findings have demonstrated that MN is the most common pathological change, occurring in approximately two-thirds of post-HSCT NS patients. A review of the literature revealed that up to 61 cases of MN had been reported in English.[12]

MN is the most common form of immune complex–mediated glomerulonephritis that has been described in the setting of HSCT. Since the first report by Hisse et al,[13] the

TABLE 2. Treatment and outcome of patients with membranous nephropathy after hematopoietic stem cell transplantation

| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 |
|---|---|---|---|---|---|
| Initial treatment for MN | FK506 (0.1 mg/kg/d), MMF (1.5 g/d), P (30 mg/d) | TW (60 mg/d), P (30 mg/d) | TW (60 mg/d), MMF (1.5 g/d), P (30 mg/d) | CsA (5 mg/kg/d), P (30 mg/d) | FK506 (0.1 mg/kg/d), P (30 mg/d) |
| Proteinuria (g/24 h) before treatment/2 mo after treatment/6 mo after treatment | 4.95/2.47/NA | 4.56/0.4/0.1 | 3.27/1.08/0.5 | 6.54/2.36/0.98 | 4.59/1.28/NA |
| The signs for cGVHD after treatment | Acute erythroderma remission | Anorexia remission, weight increased | Mouth ulcers and acute erythroderma remission | Dyspigmentation remission | The symptom of dry eyes remission |
| Medical adjustment | NA | Drug withdrawal after 9 mo | Dose reduced for MMF and P | Convert CsA to FK506 | Maintenance initial therapy |
| Outcome/survival | PR/Y | CR/Y | PR/Y | PR/Y | PR/Y |

cGVHD, chronic graft-versus-host disease; CsA, cyclosporine A; FK506, tacrolimus; MMF, mycophenolate mofetil; MN, membranous nephropathy; NA, not available; P, prednisone; TW, *Tripterygium wilfordii*.

CR defined as proteinuria <0.4 g/24 hours; PR defined as proteinuria deceased more than 50% of baseline and <3.5 g/24 hours.

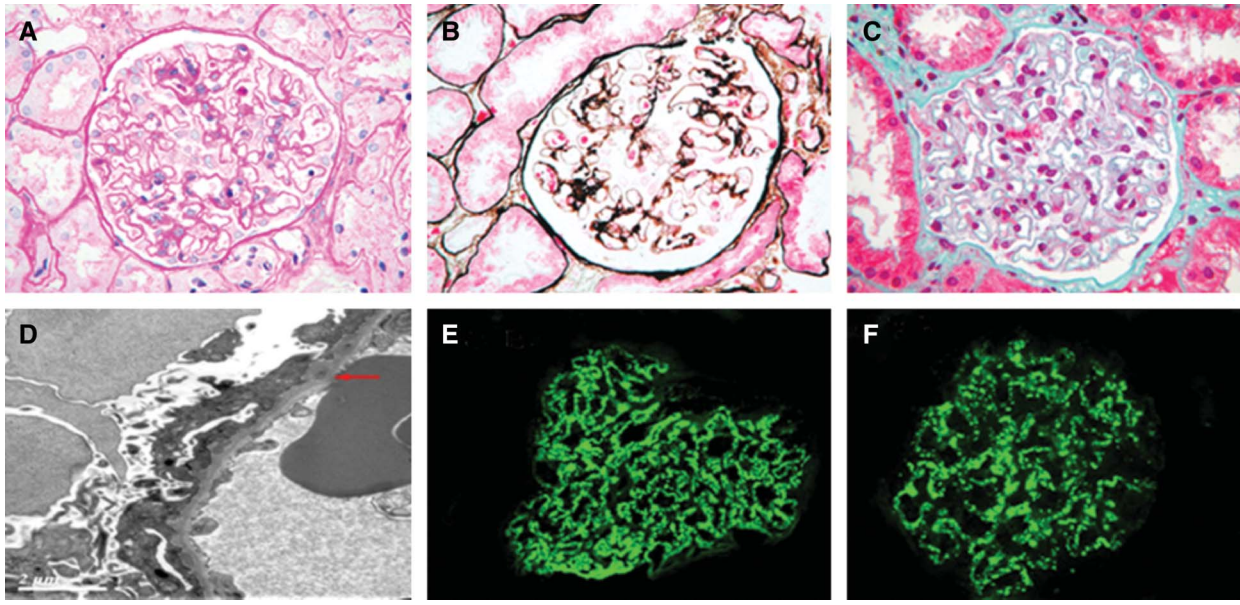


FIGURE 1. The renal histology of post–allo-HSCT membranous nephropathy patients. (A) Case 3, Light micrograph (×400, periodic acid-Schiff stain): The basement membranes are thin and delicate, the capillaries are all patent, the glomerulus has no apparent abnormalities as determined by light microscopy, the tubules are normal and there is an absence of interstitial nephritis. (B) Case 2, Light micrograph (×400, periodic acid-silver methenamine stain) shows barely visible spikes. (C) Case 4, Light micrograph (×400, MASSON stain) shows diffuse red discrete granular deposits on the epithelial side of glomerular basement membrane. (D) Case 3, electron micrograph shows dense subepithelial immune deposits (red arrow) and diffuse effacement of the foot processes of epithelial cells consistent with membranous nephropathy. (E) The immunofluorescence confirmed granular immune deposition for IgG along the capillary loops (×400). (F) The immunofluorescence confirmed granular deposition of C3 along glomerular capillary wall (×400).

total number of reported cases is 61, which accounts for 64% of HSCT-related glomerular diseases.[12] Reddy et al[10] and Terrier et al[14] presented the 2 largest series of cases with 5 cases of MN each. Most cases of MN have been associated with cGVHD; the clinical course of MN was also correlated with the evolution of cGVHD in our study. Furthermore, none of the 5 patients had a history of kidney disease, which excludes the recurrence of MN. All our patients had cGVHD concurrent with proteinuria, and the onset of MN was related to the withdrawal of immunosuppression; after an effective

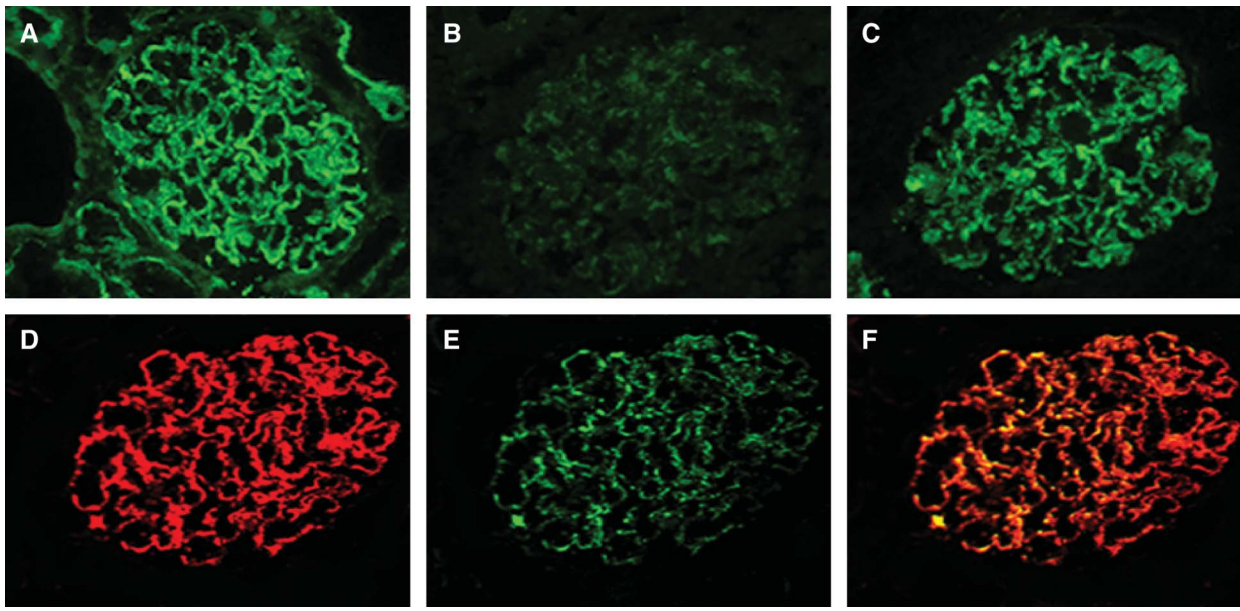


FIGURE 2. The IgG subclasses and confocal microscopic analysis of IgG4 and nephrin. (A) IgG1 deposition along the capillary loops (×400). (B) IgG3 deposition is negative. (C) IgG4 deposition along the capillary loops (×400). (D) Red fluorescent signals indicate IgG4 deposition (×400). (E) Green fluorescent signals indicate nephrin deposition (×400). (F) Confocal microscopic analysis of IgG4 and nephrin deposition in glomeruli showing apparent colocalization along the glomerular capillary walls.

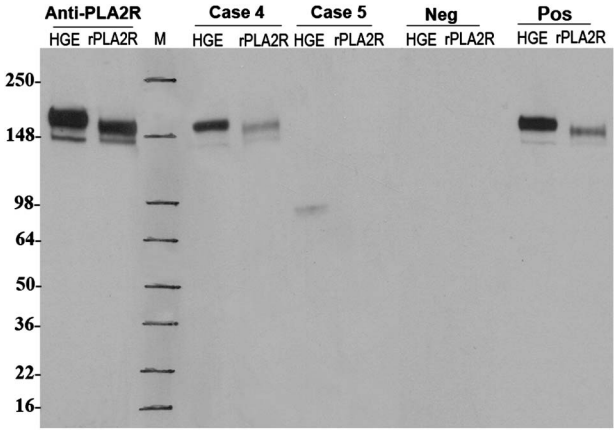


FIGURE 3. Detection of anti-PLA2R in patients with graft-versus-host disease Western blot of serum samples from 5 patients with graft-versus-host disease: Extracts of human glomeruli (HGE) and recombinant human PLA2R (rPLA2R) were electrophoresed under nonreducing conditions and immunoblotted with patient serum (Case 1–Case 5) at 1:25 and detected with anti-human IgG4. Case 4 and positive control serum recognized PLA2R in HGE and the smaller cell expressed rPLA2R. Recognition was confirmed using a commercial polyclonal anti-PLA2R antibody. Case 5 showed a weak band with the size of approximately 90 kDa. Case 1, Case 2 and Case 3 were negative and lacking any reactive bands. M. Protein standard (in kilodaltons).

anti-GVHD treatment, the patients' proteinuria was also in remission. All these clinical characteristics were similar to those of previous reports.[10,14]

It was clear that the formation of subepithelial immune complexes could occur by more than 1 pathogenetic mechanism, including *in situ* formation through the reaction of circulating autoantibodies with a native podocyte antigen, deposition of the immune complexes from the circulation or the planted antigen mechanism, in which antibodies in the circulation deposit by interacting with nonnative antigens artificially planted in the subepithelial space because of a biophysical or immunologic affinity for glomerular basement membrane structural elements.[15] The high prevalence of anti-PLA2R antibodies has been demonstrated in idiopathic MN worldwide.[6,16] The positive rate of anti-PLA2R antibodies in the United States, Europe and China was 70%,[6] 78%[17] and 82%,[16] respectively, so detection of anti-PLA2R antibodies is a sensitive test for idiopathic MN.

We tested the anti-PLA2R antibodies in serum samples taken when the patients had active NS, and the results of the anti-PLA2R autoantibodies tests were negative in 4 of 5 patients (with 1 positive result). The previous study[6] demonstrated that the anti-PLA2R autoantibody test had 100% specificity in idiopathic MN patients, so we considered that the coincidence of cGVHD and idiopathic MN post-HSCT maybe one possible explanation for the patient with positive anti-PLA2R autoantibody result. Such coincidence has been proved in other secondary causes of MN, including hepatitis B virus– and tumor-associated MN.[16] This result suggests that the anti-podocyte antibody of post-HSCT MN may be different from that of idiopathic MN. Podocyte-associated proteins might serve as targets for the circulating alloimmune antibodies that are directed against either a podocyte antigen expressed in the recipient but absent from the donor or against an allovariant,[18] perhaps reflecting alloimmunization to a minor histocompatibility antigen expressed in the glomerulus.[19] Although the negative finding of anti-PLA2R autoantibodies among the 4 of 5 patients is highly suggestive of a pathogenic mechanism other than idopathic MN, it does not exclude the possibility of idiopathic MN, particularly because 1 of 5 patients had a positive anti-PLA2R autoantibody findings highly specific for idiopathic MN.

Immunopathogenesis is important in MN. The IF findings showed that 3 patients had codeposits of C4 and/or C1q associated with Ig in glomerular deposits, indicating the activation of the classical complement pathway. Further investigation showed that the deposits of both IgG1 and IgG4 were observed, which is similar to the deposits of IgG4 in idiopathic MN.[20] IgG4 seems to play an important role in autoimmune idiopathic MN.[21] IgG4 is a subclass that does not activate complement, has a low affinity for Fc receptors and is the least abundant in the serum; however, the concentration of IgG4 in the serum is not increased in patients with idiopathic MN.[22] In contrast, all IgG subclasses are present in lupus-associated MN, but IgG1 and IgG2 predominate in malignancy-associated MN.[23] The IgG4 could be part of an *in situ* complex formed there from the binding of IgG4 to a podocyte antigen or an antigen planted there due to cGVHD. However, the exact mechanism responsible for MN as a manifestation of cGVHD is still unclear.

MN is the most common cause of post-HSCT NS, and our data showed that the clinical course of post-HSCT MN patients was closely related to that of cGVHD. Although there are several limitations of our study, including small patient numbers and no repeat test for anti-PLA2R autoantibodies after treatment, the negative result for the anti-PLA2R autoantibodies in most of the patients suggested that the formation of immune complexes is different. We speculate that the patients might have circulating pathogenic antibodies to a podocyte antigen other than PLA2R; however, additional studies are needed to resolve this question.

TABLE 3. The concentration of IgG subclasses for 5 patients

| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 |
|---|---|---|---|---|---|
| IgG1 (mg/dL) (reference: 416–1084 mg/dL) | 95.8 | 808.8 | 356.5 | 441.3 | 728.7 |
| IgG2 (mg/dL) (reference: 175–351 mg/dL) | 678.1 | 674.1 | 349.8 | 341.8 | 707.3 |
| IgG3 (mg/dL) (reference: 15–47 mg/dL) | 73.5 | 77.6 | 81.9 | 54.3 | 127.9 |
| IgG4 (mg/dL) (reference: 2–20 mg/dL) | 41.6 | 42.1 | 16.3 | 112.1 | 125.2 |

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