ORIGINAL ARTICLE

# Membranoproliferative pattern of glomerular injury associated with complement component 9 deficiency due to Arg95Stop mutation

Takayoshi Miura · Shin Goto · Seitaro Iguchi ·
Hisaki Shimada · Mitsuhiro Ueno · Shin-ichi Nishi ·
Ichiei Narita

## Abstract

*Background* Arg95Stop mutation of exon 4 in complement component 9 (C9) gene is common in individuals in Japan with C9 deficiency (C9D); however, understanding of the influences of C9D on human glomerulonephritis remains elusive.

*Methods* A total of 1288 patients with chronic kidney disease (CKD) were recruited from the hospitals in Niigata prefecture. They were screened for the Arg95Stop mutation of C9 gene by allele-specific PCR.

*Results* We identified two individuals with C9D among 1,288 CKD patients, a frequency comparable to that of the general Japanese population (0.16%). Case 1 involved a 44-year-old man presenting with nephrotic proteinuria. The hemolytic activity of CH50 was low, and the concentration of C9 was not detected. Sequencing of exon 4 of the C9 gene showed the Arg95Stop mutation. Renal biopsy revealed diffuse global mesangial proliferation with extensive duplication of glomerular capillary walls. Mesangial, subendothelial and subepithelial deposits were noticed with light and electron microscopy. Immunofluorescent study showed predominant mesangial IgA deposition. Case 2 involved a 62-year-old man presenting with proteinuria and hematuria. His CH50 level was decreased. Renal biopsy revealed diffuse global mesangial proliferation with extensive duplication of glomerular capillary walls. Immune deposits were also confirmed. The percentage of C9D among patients with mesangial proliferation and duplication of GBM in this study was 5.1%.

*Conclusion* These results suggested that the lack of membrane attack complex because of an Arg95Stop mutation of the C9 gene predisposed patients to pathognomonic glomerulonephritis.

**Keywords** C9 · MAC · MPGN · PCR

T. Miura · S. Goto (✉) · S. Iguchi · H. Shimada · M. Ueno ·
I. Narita
Division of Clinical Nephrology and Rheumatology, Niigata
University Graduate School of Medical and Dental Sciences,
Asahimachi-dori, Niigata 951-8510, Japan
e-mail: gotos@med.niigata-u.ac.jp

S. Goto
Department of Clinical Nephroscience, Niigata University
Graduate School of Medical and Dental Sciences, Niigata, Japan

S. Nishi
Blood Purification Center, Niigata University Hospital,
1-757 Asahimachi-dori, Niigata 951-8510, Japan

## Introduction

In the Japanese population the incidence of complement 9 deficiency (C9D) is approximately 1 in 1000 [1]. Previous reports for C9D showed that C9D individuals are usually found among healthy subjects, although C9D was described as being significantly associated with the development of meningococcal meningitis [2]. Genetic analyses have shown that a C-to-T transition leading to the TGA stop codon for Arg95 in exon 4 of the C9 gene (Arg95Stop) is common in Japanese C9D individuals [3]. From these studies, the prevalence of heterozygous carriers of the Arg95Stop mutation was determined to be 6.7%, consistent with the estimated frequencies of the homozygous Arg95Stop mutation (0.12%) [3].

There have been several reports of C9D in patients with glomerulonephritis [4–7]. These cases of glomerulonephritis include IgA nephropathy (IgAN), poststreptococcal glomerulonephritis and immune complex glomerulonephritis. However, no epidemiological findings of

glomerulonephritis with C9D in Japan have been available. Furthermore, lack of membrane attack complex, which is important in the progression of glomerular injury, might influence the morphology and clinical picture of glomerulonephritis. These issues prompted us to screen patients using allele-specific PCR to detect Arg95Stop mutation, and we examined the patients revealed to have C9D. Here we report two patients with C9D and glomerulonephritis showing mesangial proliferation and duplication of capillary walls.

## Methods

### Patients

A total of 1,288 patients clinically diagnosed with chronic kidney disease (CKD) were recruited from the hospitals in Niigata prefecture, 1998–2008. Among them, 696 patients underwent renal biopsy, and their diagnoses were as follows: IgAN (433), membranous nephropathy (81), non-IgAN (67), lupus nephritis (31), minimal-change disease (26), diabetic nephropathy (22), membranoproliferative glomerulonephritis (MPGN; 20), purpura nephritis (12) and nephrosclerosis (4). The ethics committee of each institute gave approval for the study. Informed consent was obtained from all participants in the genetic studies and having renal biopsies.

### Mutation analysis

Genomic DNA was prepared from peripheral whole blood of 1288 patients using an automatic DNA isolation system (NA-100, Kurabo, Osaka, Japan). A 148-bp fragment spanning the exon 4 region of C9 was amplified directly from genomic DNA by PCR. Primers for amplification were 5′-GATACCTCACCTCCAGGGTTA-3′ (sense) and 5′-CACCTATGTCCCTCGCACAAA-3′ (anti-sense), as previously described [8]. PCR fragments were sequenced using an ABI PRISM 310 Automated Sequencer (Applied Biosystems Japan, Tokyo, Japan). Sequencing analysis detected an Arg95Stop mutation in exon 4 of the C9 gene in this patient (Fig. 1).

To elucidate the prevalence of C9D in CKD patients, we investigated the frequencies of the Arg95Stop mutation in the CKD patients using allele-specific PCR. Briefly, normal primer (5′-GTCACCATTACACCGAAGTAG), mutant primer (5′-GTCACCATTACACCGAAGTAA) and common primer (5′-CCAACTTGATATTGTGCAGTTTCG) were designed. The common primer and normal primer were used for the amplification of the normal C9 gene (N primer set), and the common primer and mutant primer were used for the amplification of the C9 deficiency mutant
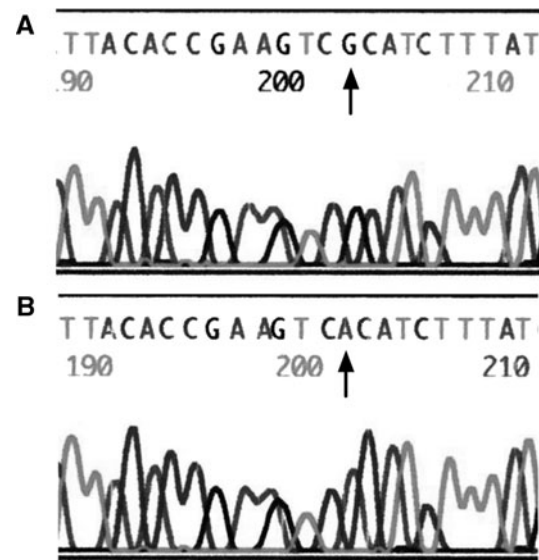


**Fig. 1** Arg95Stop mutation in exon 4 of the C9 gene of case 1. **a** Wild type of exon 4 of the C9 gene; **b** Arg95Stop mutation in exon 4 of the C9 gene of this case. *Arrow* represents G to A substitution of Arg95Stop mutation

gene (M primer set). Amplified PCR products were visualized on 1.5% agarose gel by ethidium bromide staining.

### Immunofluorescent study of the membrane attack complex

To detect membrane attack complex in renal biopsy specimens, cryostat sections were stained with a monoclonal mouse antibody against human C5b-9 (DAKO Japan, Tokyo, Japan) and followed with a FITC-labeled goat antibody against mouse IgG (Abcam, Cambridge, MA) as a secondary antibody. As a positive control, renal biopsy specimens of patients with SLE and membranous nephropathy were used.

### Quantitative analysis of mesangial proliferation and duplication of GBM

To estimate the frequencies of C9D in patients who had membranoproliferative lesions, independent pathologists evaluated the mesangial proliferation and duplication of GBM in each renal biopsy of patients enrolled in this study. The severity of lesions was graded as follows: none, <25, 25–50, 50–75% and more than 75% observed glomeruli.

## Results

Among 1288 patients with kidney disease, 66 were heterozygous (5.1%) for the Arg95Stop mutation and 2 homozygous (0.16%) for the mutation. The frequency of

heterozygotes or homozygotes of the Arg95Stop mutation of the C9 gene in the patients in this study is compatible with that of the general Japanese population described in a previous study [3]. The profile of patients heterozygous for the mutation were 21 IgAN, 3 membranous nephropathy, 3 non-IgAN, and so on. Clinical data at enrollment were as follows: mean urinary protein excretion, $1.8 \pm 1.9$ g/day; mean serum creatinine, $1.1 \pm 1.1$ mg/dl; mean creatinine clearance, $79.3 \pm 34.4$ ml/min. Complement activities were within normal limits. Because complement components are acute reactant proteins, C9 proteins produced from a heterozygous genome are sufficient to maintain the total hemolytic complement activity. Clinical data of two patients homozygous for the mutation were as follows.
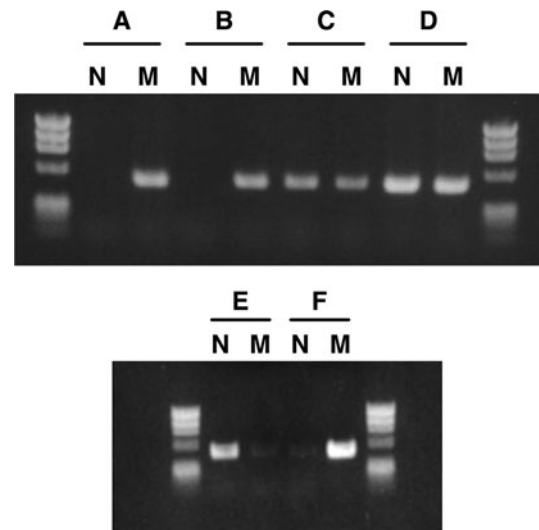
### Case 1

A 44-year-old man was admitted to the hospital because of nephrotic proteinuria and edema in his legs. Three years earlier, he had been diagnosed with proteinuria. One month earlier, he noted edema in his legs, which gradually became exacerbated.

On examination, the patient's blood pressure was 132/82 mmHg, and his pulse was 64 beats/min. His status was almost within normal limits except that the edema in his legs was noted to be slight. Neurological examination revealed impaired hearing and mild truncal ataxia.

Laboratory examinations showed a white cell count of 6030/ml, hemoglobin of 14.3 g/dl, hematocrit of 42.9% and platelet count of $29.5 \times 10^4$/ml. Other values included urea nitrogen 19 mg/dl, creatinine 1.1 mg/dl, sodium 141 mEq/l, potassium 3.7 mEq/l, chloride 106 mEq/l, calcium 8.1 mg/dl, phosphate 2.3 mg/dl, albumin 2.8 g/dl, total cholesterol 297 mg/dl, IgG 546 mg/dl, IgA 277 mg/dl and IgM 95 mg/dl. Anti-nuclear antibody, hepatitis B antigen and C antibody, as well as cryoglobulin, were all negative. Tumor markers were within normal limits. The urine was positive (3+) for protein at 3.2 g/day; the sediment contained many red blood cells and several white blood cells per high-power field. GFR decreased to 69.7 ml/min. The hemolytic activity of CH50 was low (14 U/ml), but the values of C3 and C4 were within normal limits. Although the concentrations of C1q, C2, C3, C4, C5, C6, C7 and C8 were all within normal limits, that of C9 was <0.5 mg/dl. To search for the cause of C9 deficiency, exon 4 of the C9 gene was sequenced, and a C-to-T transition leading to the TGA stop codon for Arg95 in exon 4 (Arg95Stop) was detected (Fig. 1). Allele-specific PCR disclosed that family members consenting to the genetics study had the Arg95Stop mutation (Fig. 2).

Renal biopsy was performed to reveal the cause of proteinuria and hematuria. Light microscopic examination showed diffuse global mesangial proliferation with



**Fig. 2** Allele-specific PCR detected the Arg95Stop mutation in case 1 as well as the family members and in case 2. *N* normal primer set, *M* mutation primer set. *A* Case 1, *B* brother of case 1, *C* sister of case 1, *D* mother of case 1, *E* normal control, *F* case 2

lobulation of tufts and segmental duplications of glomerular capillary walls in PAS and PAM stains. Paramesangial and subendothelial deposits were observed in Masson-trichrome stain (Fig. 3a). The tubulointerstitium had focal cell infiltration and fibrosis. In immunofluorescent study, IgA was predominantly detected in the mesangium and along the capillary walls (Fig. 3b). IgG and C3c (Fig. 3c) were also detected, but weakly, in the same pattern. C5b-9 was not detected in both glomeruli and tubulointerstitium (Fig. 4a, c). Electron microscopy confirmed the existence of paramesangial and subendothelial and subepithelial deposits (Fig. 3d, e).

With the diagnosis of IgAN, prednisolone and the angiotensin II receptor antagonist, losartan, were administered. At first, the patient's urine protein decreased in response to the treatment; however, in an outpatient department, proteinuria gradually increased to 2+ or 3+ by dipstick testing. Nine years later, approximately 1 g/day of urinary protein was excreted, and serum creatinine was 1.7 mg/dl.

### Case 2

A 62-year-old man admitted to the hospital because of urine abnormalities and hypocomplementemia found by medical check-up. Physical examination revealed that his status was almost within normal limits on admission. Laboratory data showed a white cell count of 5,500/ml, hemoglobin of 14.4 g/dl, hematocrit of 41.3% and platelet count of $22.2 \times 10^4$/ml, serum creatinine 0.8 mg/dl, urea nitrogen 21.0 mg/dl, albumin 4.1 g/dl, sodium 139 mEq/l, potassium 4.2 mEq/l, chloride 104 mEq/l, calcium 9.1 mg/dl,
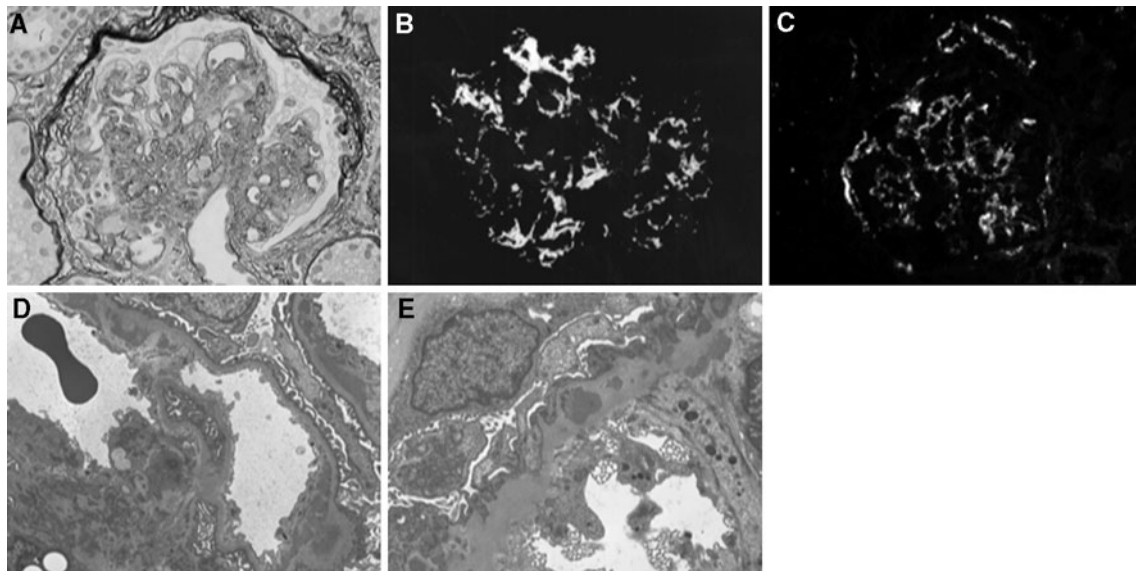
**Fig. 3** Case 1. **a** Light microscopic examination showed lobulation of tufts in focal glomeruli, and subendothelial and paramesangial deposits were extensively observed. **b** IgA was predominantly detected in the mesangium and along the capillary wall. **c** C3c was also detected in the mesangium and along the capillary wall. **d**, **e** Electron microscopy showed the existence of paramesangial and subendothelial deposits

**Fig. 4** **a** Immunofluorescent study revealed that no stain for C5b-9 was observed in glomeruli of case 1. **b** In a SLE patient used as a positive control, obvious staining for C5b-9 was observed in inflamed glomeruli. **c** C5b-9 staining was not observed in tubulointerstitium of patient 1. **d** Significant tubular C5b-9 deposition in patients with membranous nephropathy as a positive control
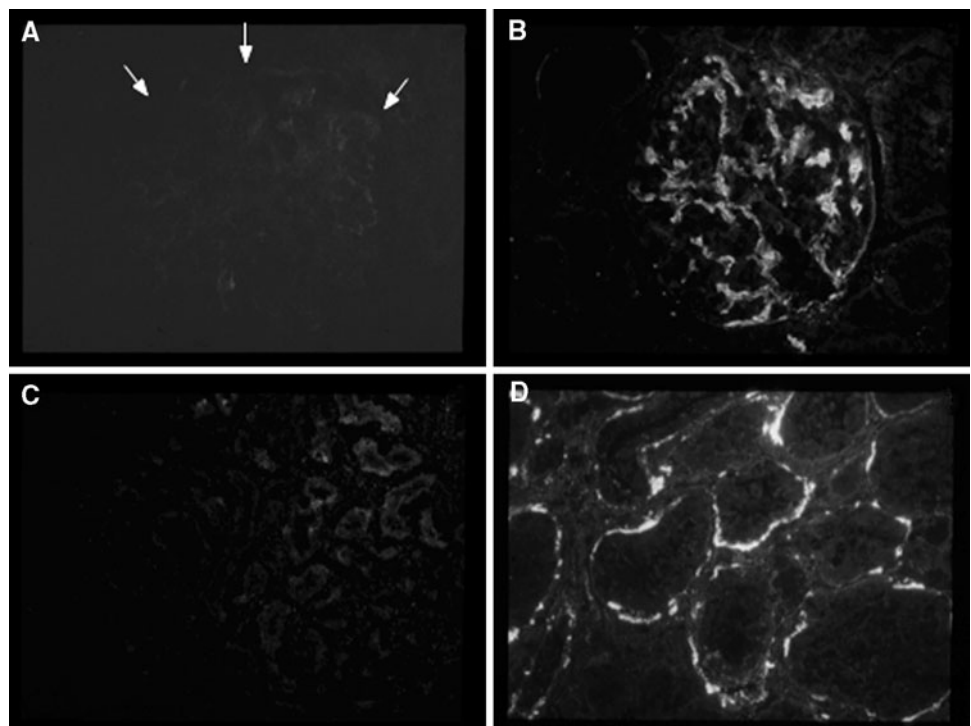


phosphate 3.9 mg/dl, albumin 4.5 g/dl, total cholesterol 179 mg/dl, IgG 1004 mg/dl, IgA 271 mg/dl, IgM 130 mg/dl, C3 66 mg/dl, C4 36 mg/dl and CH50 16.2 U/ml. Antinuclear antibody, hepatitis B antigen and C antibody, as well as cryoglobulin were all negative. Tumor markers were within normal limits. Urinary protein excretion was 0.68 g/day, and creatinine clearance was 102 ml/min.

On renal biopsy, light microscopy showed diffuse global mesangial proliferation with diffuse segmental duplication and membranous transformation of glomerular capillary walls (Fig. 5a). Mesangial, subendothelial and subepithelial deposits were found in almost all glomeruli in Masson-trichrome stain. Immunofluorescence study showed granular deposition of IgG and C3 in the mesangium and capillary

walls. Electron microscopy showed mesangial and subendothelial deposits (Fig. 5b). The histological diagnosis was comparable to MPGN. Then, the patient was treated with ACE inhibitor, dipyridamole and aspirin. After 16 years, his renal function was well preserved (creatinine clearance 122 ml/min), although he had moderate proteinuria (1.33 g/day). Persistence of a low value of CH50 (13 U/ml) despite a normal range of C3 and C4 suggested that he had C9D. Allele-specific PCR revealed that he was homozygous for the Arg95Stop mutation of the C9 gene (Fig. 2e).

## Frequencies of C9D in patients with mesangial proliferation and duplication of GBM

Both of the two cases reported here showed extensive glomerular injuries with mesangial proliferation and duplication of GBM. The number of patients who have both mesangial proliferation and duplication of GBM with a severity of at least 25% in this study was 39. Therefore, the percentage of C9D among patients with these membranoproliferative lesions in this study was 5.1%.

## Discussion

In this study, we analyzed the frequencies of Arg95Stop mutations in CKD patients. Of about 1200 patients, 2 were homozygous for this mutation. The frequencies of the mutation (0.16%) did not differ from that of a general population in Japan reported previously.

In experimental glomerulonephritis models, C5b-9 generation in the glomerulus is necessary to express the full lesions of anti-Thy-1 glomerulonephritis [9]. In contrast, several case reports have described patients with C9D and glomerulonephritis including IgAN [4–7]. Alexopoulos et al. [10] showed no correlation among glomerular T cells, monocytes/macrophages and the deposition of C5b-9. Furthermore, MAC was not detected in renal biopsy specimens in the previous cases nor in our case, whereas glomerular injury of IgAN obviously developed [5, 7]. A case of C9D associated with dermatomyositis, which has been implicated with MAC in the pathogenesis, has also been reported [11]. Furthermore, an association study with the Arg95Stop mutation revealed that there was no difference in the frequency of the mutation between SLE patients and controls, indicating that C9D is not implicated in SLE susceptibility [12]. Therefore, MAC is unlikely to be essential for the development of human glomerulonephritis.

Lack of MAC due to C9D might affect histopathological lesions in the kidney. Both cases in this study exhibited extensive duplication of the glomerular basement membrane in their renal biopsy. Inherited complement component deficiencies are described in patients with MPGN. Serum levels of complement were measured in various glomerulonephritis patients and normal subjects, and the deficiencies were found with significantly higher frequency among MPGN patients [13]. Involvement of C2, C3, factor B, C6, C7 and C8 was reported. Recently, lack of some other complement regulator, such as factor H and factor I, was also documented to play an important role in the development of MPGN [14, 15]. Indeed, the percentage of C9D among patients with mesangial proliferation and duplication of GBM in this study was 5.1%, which is higher than the estimated frequencies of C9D in the general population in Japan (0.12%). In addition to the two cases in this study, previously reported adult C9D cases with IgAN also exhibited partial membranoproliferative lesions [4]. Some investigators suggested that complement deficiency predisposes the individual to chronic infections and subsequent immune complex formation, and impairs the solubilization, disaggregation and clearance of deposited immune complexes [16]. Indeed, the distribution of glomerular deposits in patient 1 (IgAN) was distinctive. The sites of electron-dense deposits in patient 1 were the mesangial, subendothelial and subepithelial spaces. Yoshioka et al. [7] reported three pediatric IgAN patients who showed a similar distribution of deposits to that of our patients. Although it is hard to verify the hypothesis from



**Fig. 5** Case 2. **a** Light microscopic examination showed diffuse mesangiocapillary glomerulonephritis with extensive duplication of GBM. **b** Electron microscopy revealed duplication of GBM and paramesangial, subendothelial and subepithelial deposits in observed glomeruli
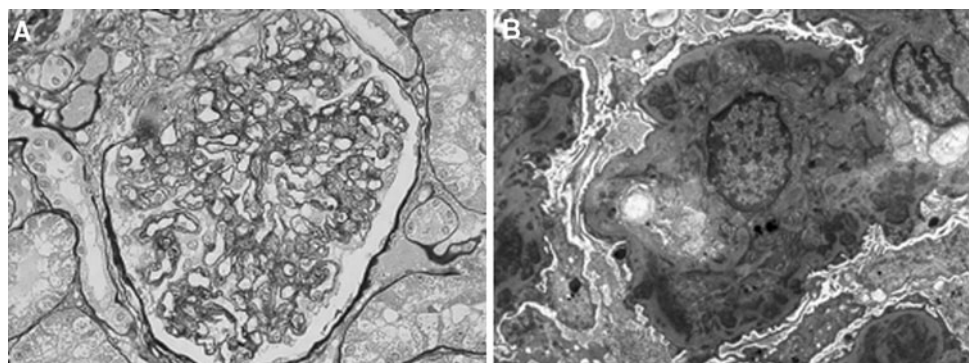
the findings in a few cases, underlying links might exist between the lack of MAC due to C9D and these distinctive pathological lesions.

It is well known that the degree of tubulointerstitial injury, rather than glomerular damage, is correlated with impairment of renal function [17–19]. In several animal models, MAC plays a functional role in mediating chronic tubulointerstitial disease [20–22]. Alexopoulos et al. [10] revealed that, in patients with IgAN, tubular deposition of MAC was related to the number of interstitial T cells and monocytes/macrophages, implicating MAC in the tubulo-interstitial injury. Therefore, it is possible that IgAN patients with C9D have milder tubulointerstitial injury and preserved renal function. In fact, in previously reported IgAN patients with C9D [5, 7], the histology regarded as indicative of the progressive course of human IgAN was not found in any of these patients. In this study, the renal function in patient 2 was well preserved despite moderate proteinuria. However, patient 1, who showed extensive glomerular injury at the time of diagnosis, had persistent proteinuria for 9 years, and his renal function gradually declined. Although MAC plays important roles in tubulo-interstitial injury, C5b-8 was reported to have non-lytic biological effects [23]. Sublytic doses of C5b-8 can stimulate nucleated cells to release several mediators [23], and formation of the C5b-8 complex also induces a number of proinflammatory activities [24], which could result in progression of glomerular and tubulointerstitial injury. It is possible that other precipitating factors such as hemodynamic changes and/or activation of the intrarenal renin-angiotensin system could have exerted their effects on the inflammation of the kidneys in patient 1. Further investigation with follow-up of more C9D patients is necessary to determine the possible role of C9D in progressive renal injury.

## References

1. Fukumori Y, Yoshimura K, Ohnoki S, et al. A high incidence of C9 deficiency among healthy blood donors in Osaka, Japan. Int Immunol. 1989;1:85–9.
2. Nagata M, Hara T, Aoki T, et al. Inherited deficiency of ninth component of complement: an increased risk of meningococcal meningitis. J Pediatr. 1989;114:260–4.
3. Kira R, Ihara K, Takada H, et al. Nonsense mutation in exon 4 of human complement C9 gene is the major cause of Japanese complement C9 deficiency. Hum Genet. 1998;102:605–10.
4. Hironaka K, Makino H, Amano T, et al. Immune complex glomerulonephritis in a pregnant woman with congenital C9 deficiency. Intern Med. 1993;32:806–9.
5. Kanda E, Shimamura H, Tamura H, et al. IgA nephropathy with complement deficiency. Intern Med. 2001;40:52–5.
6. Maruyama K, Arai H, Ogawa T, et al. C9 deficiency in a patient with poststreptococcal glomerulonephritis. Pediatr Nephrol. 1995;9:746–8.
7. Yoshioka K, Takemura T, Akano N, et al. IgA nephropathy in patients with congenital C9 deficiency. Kidney Int. 1992;42:1253–8.
8. Witzel-Schlomp K, Spath PJ, Hobart MJ, et al. The human complement C9 gene: identification of two mutations causing deficiency and revision of the gene structure. J Immunol. 1997;158:5043–9.
9. Brandt J, Pippin J, Schulze M, et al. Role of the complement membrane attack complex (C5b-9) in mediating experimental mesangioproliferative glomerulonephritis. Kidney Int. 1996;49:335–43.
10. Alexopoulos E, Papaghianni A, Papadimitriou M. The pathogenetic significance of C5b-9 in IgA nephropathy. Nephrol Dial Transplant. 1995;10:1166–72.
11. Ichikawa E, Furuta J, Kawachi Y, et al. Hereditary complement (C9) deficiency associated with dermatomyositis. Br J Dermatol. 2001;144:1080–3.
12. Kanemitsu S, Ihara K, Kira R, et al. Complement component 9 deficiency is not a susceptibility factor for SLE. Lupus. 2000;9:456–7.
13. Coleman TH, Forristal J, Kosaka T, et al. Inherited complement component deficiencies in membranoproliferative glomerulonephritis. Kidney Int. 1983;24:681–90.
14. Ault BH. Factor H and the pathogenesis of renal diseases. Pediatr Nephrol. 2000;14:1045–53.
15. Rose KL, Paixao-Cavalcante D, Fish J, et al. Factor I is required for the development of membranoproliferative glomerulonephritis in factor H-deficient mice. J Clin Invest. 2008;118:608–18.
16. Zhou XJ, Silva FG. Membranoproliferative glomerulonephritis. In: Jennette JC, Olson JL, Schwartz MM, Silva FG, editors. Heptinstall's pathology of the kidney. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 253–319.
17. Alexopoulos E, Seron D, Hartley RB, et al. The role of interstitial infiltrates in IgA nephropathy: a study with monoclonal antibodies. Nephrol Dial Transplant. 1989;4:187–95.
18. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. Am J Kidney Dis. 1992;20:1–17.
19. Striker GE, Schainuck LI, Cutler RE, et al. Structural-functional correlations in renal disease. I. A method for assaying and classifying histopathologic changes in renal disease. Hum Pathol. 1970;1:615–30.
20. Nangaku M, Pippin J, Couser WG. Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephrotic syndrome. J Am Soc Nephrol. 1999;10:2323–31.
21. Nangaku M, Pippin J, Couser WG. C6 mediates chronic progression of tubulointerstitial damage in rats with remnant kidneys. J Am Soc Nephrol. 2002;13:928–36.
22. Rangan GK, Pippin JW, Couser WG. C5b-9 regulates peritubular myofibroblast accumulation in experimental focal segmental glomerulosclerosis. Kidney Int. 2004;66:1838–48.
23. Torbohm I, Schonermark M, Wingen AM, et al. C5b-8 and C5b-9 modulate the collagen release of human glomerular epithelial cells. Kidney Int. 1990;37:1098–104.
24. Kilgore KS, Schmid E, Shanley TP, et al. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. Am J Pathol. 1997;150:2019–31.