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Title page

Utility of glomerular Gd-IgA1 staining for indistinguishable cases of IgA nephropathy or Alport syndrome

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Abstract

Background

Pathological findings in Alport syndrome frequently show mesangial proliferation and sometimes incidental IgA deposition, in addition to unique glomerular basement membrane (GBM) changes including thin basement membrane and/or lamellation. However, similar GBM abnormalities are also often observed in IgA nephropathy. Both diseases are also known to show hematuria, proteinuria, and sometimes macrohematuria when associated with viral infection. Therefore, it can be difficult to make a differential diagnosis, even based on clinical and pathological findings. Some recent articles demonstrated that galactose-deficient IgA1 (Gd-IgA1)-specific monoclonal antibody (KM55) could potentially enable incidental IgA deposition to be distinguished from IgA nephropathy.

Methods

We performed comprehensive gene screening and glomerular Gd-IgA1 and type IV collagen α5 chain immunostaining for five cases with both IgA deposition and GBM changes to confirm that Gd-IgA1 can help to distinguish these two diseases.

Results

Four of the cases were genetically diagnosed with Alport syndrome (Cases 1–4) and one was IgA nephropathy with massive GBM changes, which had a negative gene test result (Case 5). In Cases 1–4, glomerular Gd-IgA1 deposition was not detected, although there was positivity for IgA in the mesangial area. In Case 5, glomerular Gd-IgA1 deposition was observed.

Conclusion

Gd-IgA1 expression analysis could clearly differentiate these two disorders. This approach can be applied to identify these two diseases showing identical clinical and pathological findings.

Keywords

IgA nephropathy, Alport syndrome, Galactose-deficient IgA1, glomerular basement membrane

Introduction

Alport syndrome (AS) is a hereditary renal disorder caused by pathogenic variants in *COL4A3*, *COL4A4*, and *COL4A5*, resulting in abnormalities of the type IV collagen α3, α4, and α5 network of the basement membranes [1]. There are no specific light microscopy findings in AS, and it usually shows mesangial proliferation [2]. Incidental IgA deposition on immunofluorescence is occasionally observed in AS [3]. Electron microscopy reveals irregular thickening and thinning of the glomerular basement membrane (GBM), and lamellation and splitting in lamina densa. However, young patients or autosomal dominant AS (ADAS) cases show only thinning of the GBM (TBM). Conversely, IgA nephropathy (IgAN) is a common primary glomerulonephritis, defined by mesangial proliferative nephritis with mesangial IgA-dominant or codominant deposition. Ultrastructural abnormalities of GBM including diffuse thinning and/or lamellation are relatively common in IgAN [4-8]. Clinically, both of these diseases show hematuria and proteinuria from a young age, and sometimes macrohematuria associated with viral infection. They can even cooccur in some patients. Therefore, it can be difficult to differentiate them using only clinical and conventional pathological findings.

Significant advances in understanding the pathogenesis of IgAN have been made over the past two decades, in which galactose-deficient IgA1 (Gd-IgA1) has been reported to be essential [9]. A novel lectin-independent ELISA using a specific monoclonal antibody (KM55) recognizing a hinge region in human Gd-IgA1 has also recently been established [10, 11]. KM55 can detect glomerular Gd-IgA1 in tissues using immunohistochemistry [11], and Suzuki et al. revealed that KM55 can identify glomerular Gd-IgA1 deposition as a disease-specific marker of IgAN and IgA vasculitis with nephritis (IgAV-N) in adult patients [12]. However, some recent papers demonstrated that Gd-IgA1 immunostaining by

KM55 is not specific for IgAN; glomerular Gd-IgA1 deposition was also observed in patients with secondary IgAN including rheumatoid arthritis, Sjögren's syndrome and ulcerative colitis, lupus nephritis, primary membranous nephritis, membranoproliferative glomerulonephritis, and infection-related glomerulonephritis [13-16]. In our previous analysis, glomerular Gd-IgA1 staining was negative in all patients with incidental IgA deposition including AS; therefore, we reported that KM55 could have potential to distinguish incidental IgA deposition [15]. In addition, other recent articles demonstrated that weak or negative staining for Gd-IgA1 may be suggestive of incidental IgA deposits [14, 16].

We experienced five cases showing both GBM changes and IgA deposition on the glomerulus. Thus, we subjected them to Gd-IgA1 immunostaining by KM55 to clarify whether Gd-IgA1 staining can distinguish IgAN and AS.

Methods

Ethical approval

All procedures performed in studies involving human participants were carried out in accordance with the ethical standards of the Institutional Review Board of Kobe University Graduate School of Medicine (IRB approval numbers B190137 and 301), and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants.

Study design and participants

This is a retrospective study. We enrolled five patients, all of whom were referred to our institute for gene testing. The pathological findings in all patients revealed both IgA deposition and GBM changes. Four of them (Cases 1–4) were genetically diagnosed with AS, and pathological findings revealed mesangial proliferation and glomerular IgA deposition. One patient (Case 5) was diagnosed with IgAN because pathological findings showed mesangial proliferative glomerulonephritis and IgA codominant deposition. This patient also showed diffuse TBM and lamellation, and genetic analysis did not detect any pathogenic variants in *COL4A3*, *COL4A4*, or *COL4A5*.

Clinical data at the time of renal biopsy in each patient are shown in Table 1. Light microscopic and electron microscopic images are shown in Fig. 1. The results of conventional immunofluorescence staining are shown in Table 2. The images and findings of immunofluorescence staining for IV collagen α2 and α5 are shown in Fig. 2 and Table 2. The results of Sanger sequencing in Cases 1–4 are shown in Table 3 and Online Resource 1.

The procedure of gDNA analysis by targeted next-generation sequencing (NGS) was performed as described previously using a custom panel that included *COL4A3*, *COL4A4*, and *COL4A5* [17, 18], and is presented in Online Resource 2. The protocol of double-immunofluorescence staining of IgA and Gd-IgA1 using frozen sections of renal biopsy specimens was conducted as described in our previous report [15], and the procedure is given in Online Resource 2. Data and images for A502 (Case 5) was published elsewhere [15].

Case 1 (A607)

The patient was a 45-year-old woman. Proteinuria and microscopic hematuria were detected when she caught a cold at age 40. After this episode, proteinuria and microscopic

hematuria were sustained, so angiotensin II receptor blocker was started. She underwent a renal biopsy at 45 years of age.

The specimen contained eight glomeruli. The cortex-to-medulla ratio was 1:1. On periodic acid–Schiff staining, slight mesangial proliferation was observed (Fig. 1-A). Sclerosis was detected in one glomerulus. No crescent, endocapillary proliferation, or adhesion was observed. Tubular atrophy was observed in 10% of interstitial tissue. On immunofluorescence, IgA deposition (1+) was detected in the mesangial area (Fig. 3-A, Table 2). Electron microscopic findings revealed TBM and electron-dense deposits in the intra-basement membrane (Fig. 1-A). A normal expression pattern of α2 and α5(IV) was observed in the GBM and Bowman's capsule (Fig. 2-A, Table 2).

The patient's father and uncle had received hemodialysis from the ages of 62 and 55, respectively. Her paternal grandmother had died of kidney disease, but the details were unknown. Her sister had been diagnosed with focal glomerular sclerosis. Targeted NGS and Sanger sequencing detected a novel heterozygous variant in exon 27 (c.1936G>A, p.Gly646Arg) in the *COL4A3* gene (Table 3), so she was diagnosed with ADAS.

Case 2 (A735)

The patient was a 37-year-old man. Microscopic hematuria had continued from the age of 3. A first renal biopsy at the age of 5 revealed TBM disease. Proteinuria appeared from the age of 16. He had been diagnosed with IgAN based on the findings of a second renal biopsy at 22 years of age. Therefore, he received angiotensin II receptor blocker and antiplatelet drug, which were not effective. He underwent a third renal biopsy at the age of 30, which indicated weak disease activity of IgAN. Tonsillectomy and prednisolone therapy were performed, but he did not achieve complete remission. Proteinuria was

maintained at around 1 g/day after discontinuing the prednisolone. Thus, a fourth renal biopsy was conducted at age 37.

The specimen contained 13 glomeruli. The cortex-to-medulla ratio was 4:1. On periodic acid–Schiff staining, mesangial proliferation was observed segmentally in half of the glomeruli (Fig. 1-B). Global sclerosis was observed in one glomerulus. No crescent, endocapillary proliferation, or adhesion was observed. Tubular fibrosis was observed in 10% of interstitial tissue. There was no tubular atrophy. On immunofluorescence, IgA deposition (1+) was detected in the mesangial area (Fig. 3-B, Table 2). Electron microscopic findings showed TBM with a wide range. Electron-dense deposit was detected in mesangial, endothelial, and intramembrane areas (Fig. 1-B). Immunostaining of α5(IV) expression was decreased in the GBM (Fig. 2-B, Table 2).

The patient's mother and maternal grandmother had a history of hematuria. Targeted NGS and Sanger sequencing detected a novel heterozygous variant in exon 42 (c.3809G>A, p.Gly1270Asp) in the *COL4A5* gene (Table 3), so the patient was diagnosed with X-linked AS (XLAS).

Case 3 (A818)

The patient was a 25-year-old man. Proteinuria and microscopic hematuria were identified from the age of 7. He underwent kidney biopsy and pathological findings demonstrated TBM disease. He experienced episodes of macrohematuria when suffering from tonsilitis. The proteinuria was maintained, so a second kidney biopsy was performed at 25 years of age. His sister had been diagnosed with IgAN from pathological findings and underwent tonsillectomy and methylprednisolone pulse therapy.

The specimen contained 22 glomeruli. The cortex-to-medulla ratio was 9:1. On periodic acid–Schiff staining, slight mesangial proliferation was observed (Fig. 1-C). Global sclerosis was observed in one glomerulus. No crescent, endocapillary proliferation, or adhesion was observed. Very slight interstitial fibrosis, tubular atrophy, and invasion of inflammatory cells were observed. There was foamy degeneration along the tubular epithelium. On immunofluorescence, IgA deposition (2+) was detected in the mesangial area (Fig. 3, Table 2). Electron microscopic findings showed TBM segmentally. Foam cell formation in epithelial and endothelial cells was widely noted. Electron-dense deposit was detected in the intramembrane area (Fig. 1-C). Immunostaining of α5(IV) expression was decreased in the GBM (Fig. 2-C, Table 2).

The patient's mother had a history of urinary abnormality including proteinuria. Targeted NGS and Sanger sequencing detected a novel heterozygous variant in exon 31 (c.2519G>A, p.Gly840Glu) in the *COL4A5* gene (Table 3), so the patient was diagnosed with XLAS.

Case 4 (A876)

The patient was a 50-year-old woman. Microscopic hematuria was identified at age 6. At 50 years of age, proteinuria, microscopic hematuria, and kidney dysfunction were detected by medical check-up, so she underwent a kidney biopsy.

The specimen contained 13 glomeruli. On periodic acid–Schiff staining, mesangial proliferation was observed in four glomeruli. Sclerosis was observed in one glomerulus (Fig. 1-D). No crescent, endocapillary proliferation, or adhesion was observed. Slight interstitial fibrosis and invasion of inflammatory cells were observed. On immunofluorescence, IgA deposition (1+) was detected segmentally in the mesangial area

(Fig. 3-D, Table 2). Electron microscopic findings showed TBM and lamellation of basement membrane. Slight electron-dense deposit was detected in the paramesangial area (Fig. 1-D). A normal expression pattern of α5(IV) was observed in the GBM and Bowman's capsule (Fig. 2-D, Table 2).

The patient's father had received hemodialysis from the age of 37 and suffered from hearing loss from his 60s. Her sister had a history of hematuria. Targeted NGS and Sanger sequencing detected a novel heterozygous variant in IVS 12 (c.687+5G>C) in the *COL4A5* gene (Table 3), so the patient was diagnosed with XLAS.

Case 5 (A502)

The patient was a 13-year-old woman. Microscopic hematuria and proteinuria were identified upon school urinary screening for the first time at age 12. She underwent a renal biopsy because of continuing microscopic hematuria and low-grade proteinuria for 1.5 years.

A total of 28 glomeruli were counted with focal mesangial proliferation. Fibrocellular crescent was observed in five glomeruli (Fig. 1-E). No endocapillary proliferation nor sclerosis was detected. The interstitium and blood vessels were unremarkable. On immunofluorescence, IgA deposition (3+) was detected in the mesangial area (Fig. 3-E, Table 2). Electron microscopic findings showed diffuse TBM and apparent lamellation (Fig. 1-E). No electron-dense deposit was noted. A normal expression pattern of α5(IV) was observed in the GBM and Bowman's capsule (Fig. 2-E, Table 2).

The patient had no particular family history including chronic kidney disease, dialysis, or hearing loss. Targeted NGS did not detect any pathogenic variants in *COL4A3*, *COL4A4*, and *COL4A5*.

Results

In cases genetically diagnosed with AS, glomerular Gd-IgA1 deposition was not detected, although IgA deposition was observed in the mesangial area globally (Cases 1–3) or segmentally (Case 4). In Case 5, glomerular Gd-IgA1 deposition (3+) was noted, localized in the mesangial area with IgA. The results of double-immunofluorescence staining for IgA and Gd-IgA1 are shown in Fig. 3 and Table 2.

Discussion

It has been reported that Gd-IgA1 staining could distinguish incidental IgA deposition from IgAN [14-16]. Here, Gd-IgA1 negativity in Cases 1–4 indicated that IgA deposition occurred incidentally and they were diagnosed only with AS. Conversely, Gd-IgA1 positivity in Case 5 suggested that this patient suffered from IgAN, despite the pathological findings showing massive GBM changes, which are typical of AS.

Immunofluorescence staining for α5(IV) is one approach for diagnosing AS. However, we previously identified normal expression of α5(IV) in 29% of patients with male XLAS, 20% of patients with autosomal recessive AS, and all patients with ADAS [17, 19, 20]. Thus, it should be noted that normal α5(IV) staining pattern is observed in AS, especially ADAS [17]. Actually, in Case 1 with ADAS and in Case 4 with XLAS, normal expression of α5(IV) was observed. In addition, α5(IV) expression on the GBM can change in glomerular diseases including IgAN [21, 22]. Kamimura et al. demonstrated that partial reduction of α5(IV) expression in normally appearing GBM was observed in 42% of patients with IgAN [21]. Masuda et al. also detected holes, fractures, and rupture of α5(IV)

in the GBM among IgAN patients [22]. Therefore, the α5(IV) expression pattern could not ultimately contribute to differentiating AS from IgAN.

Pathological findings of AS showed unique GBM changes including TBM and/or lamellation. However, ultrastructural abnormalities of the GBM are relatively common in IgAN, and it shows various changes including thinning, lamellation, irregular thickening, disruption, and lysis [4-8]. TBM is the most common feature, which is often noted focally [4, 5], but Case 5 revealed diffuse thinning and lamellation, resembling the findings of AS. Some authors indicated a significant association between TBM in IgAN and severe hematuria [6], but others did not [7]. A correlation between the severity of GBM changes and the amount of proteinuria was also reported [5, 7]. Yoshikawa et al. demonstrated that subepithelial dense deposits and polymorphonuclear leukocytes may play roles in the lytic process [8]. Notably, the GBM changes in IgAN may occur even in patients with a milder clinical course, like Case 5.

Recently, genetic analysis using NGS has enabled comprehensive screening of various genes including *COL4A3*, *COL4A4*, and *COL4A5* [18]. Especially in patients with ADAS, genetic analysis is the only approach for diagnosis because of a lack of extra-renal symptoms, nonspecific light microscopy findings such as mesangial proliferation or focal segmental glomerulosclerosis, and a normal expression pattern of α5(IV). Although the frequency of ADAS has been estimated to be 5% in patients with AS [17], we speculate that some cases might have been misdiagnosed as IgAN. Stapleton et al. identified two families with variants in *COL4A3* and *COL4A5* by whole-exome sequencing among 10 families who were previously diagnosed with familial IgAN [23]. Some cases of XLAS and ARAS, initially diagnosed with IgAN by biopsy pathological findings, have also been reported [3]. Therefore, it is strongly recommended to conduct gene testing for the

definitive diagnosis of AS. Conversely, in Case 5, NGS did not detect any pathogenic variants in *COL4A3*, *COL4A4*, or *COL4A5*. It has been reported that NGS can detect nearly 95% of all missense and nonsense variants, insertions and deletions, and most splicing variants near intron–exon boundaries [24]. In other words, a few variants in AS cannot be detected by NGS, so it cannot be confirmed that Case 5 does not suffer from AS.

Gd-IgA1-specific monoclonal antibody has recently been established [11], but its specificity remains controversial [14, 13, 15, 16]. In recent reports, all cases of IgAN showed Gd-IgA1 positivity, although the immunostaining intensity differed among patients [12, 14, 13, 15, 16]. Therefore, Gd-IgA1 negativity could rule out the possibility that patients suffer from IgAN. In this study, glomerular Gd-IgA1 deposition was not observed in Cases 1–4, which suggests that they suffered only from AS, unaccompanied by IgAN. However, in Case 3, we evaluated Gd-IgA1 staining with the fourth biopsy specimen, so it may be too early to conclude that this patient had not suffered from IgAN since childhood because disease activity might have been suppressed under various treatments, and low disease activity of IgAN might have affected the negativity for Gd-IgA1.

In addition, some articles evaluated glomerular Gd-IgA1 staining for patients with incidental IgA deposition [14-16]. Incidental glomerular IgA deposition is not generally related to disease onset or progression, and it may occur even in healthy individuals [25]. From these studies, weak or negative staining for Gd-IgA1 may be suggestive of IgA deposition [14-16]. In Case 5, glomerular Gd-IgA1 deposition was detected with high intensity, localized in the mesangial area with IgA. This suggested that IgA deposition might not occur incidentally, which means that the Gd-IgA1 staining result ultimately contributes to accurately diagnosing IgAN. Therefore, we concluded that glomerular Gd-IgA1 analysis could clearly differentiate AS and IgAN. This approach can be applied for

these two diseases, which are sometimes very difficult to accurately diagnose from the clinical and conventional pathological findings.

This study had some limitations. First, we could not analyze serum Gd-IgA1 levels or the relationship between serum levels of Gd-IgA1 and glomerular Gd-IgA1 deposition intensity because insufficient blood samples were obtained. Second, we used frozen sections of biopsy specimens, as in our previous study [15], while other studies used paraffin-embedded sections.

In conclusion, it is sometimes difficult to distinguish AS and IgAN using clinical and conventional pathological findings alone. The expression of α5(IV) could also not ultimately differentiate these two diseases. Therefore, we strongly recommend performing gene testing for a definitive diagnosis of AS. In addition, Gd-IgA1 staining could contribute to an accurate diagnosis in cases with glomerulonephritis accompanied by GBM abnormality and mesangial IgA deposition. More analyses are required to evaluate the utility of glomerular Gd-IgA1 staining.

Table 1. Characteristics of enrolled patients

No.	Patient ID	Age at renal biopsy	Sex	uPro/Cr (g/g・Cr)	uRBC (/HPF)	Serum Alb (g/dL)	Serum creatinine (mg/dl)	eGFR (ml/min/1.73 m^2)
1	A607	45	female	0.55	50-99	4.1	0.57	88.9
2	A735	37	male	2.57	50-99	3.6	0.78	90.3
3	A818	25	male	2.40	5-9	3.5	0.80	98.0
4	A876	50	female	0.46	20-30	4.5	0.71	68.0
5	A502	13	female	0.19	>100	3.9	0.68	93.7

The data obtained at the time of renal biopsy are shown. uPro/Cre ratio, urine protein creatinine ratio; uRBC, urine red blood cell; Alb, albumin; eGFR, estimated glomerular filtration rate; HPF, high-power field.

Table 2. Results of immunofluorescence staining

Case	Patient ID	Gd-IgA1	IgA	IgG	IgM	C3	C4	C1q	Fib	α2	α5
1	A607	−	+	+	+/−	+	−	−	−	normal	normal
2	A735	−	+	+/−	+	+/−	+/−	+ – +/−	+/−	normal	decreased
3	A818	−	2+	−	2+	+/−	n/d	−	+	normal	decreased
4	A876	−	+ (s)	+	+ (s)	+ (s)	−	+/−	+/−	normal	normal
5	A502	3+	3+	2+	1+	1+	3+	−	n/d	normal	normal

Immunofluorescence intensity was analyzed in frozen sections and defined as generally negative/trace (+/−), 1+, 1/2+, 2+, 2/3+, and 3+ in Gd-IgA1, IgA, and other conventional immunofluorescence. All deposits were observed in the mesangial region. “s” indicates segmental deposit; the rest of the deposits were observed as a diffuse pattern in glomerulus. The evaluation of IV collagen α2 and α5 was classified as normal or decreased expression.

Table 3. Results of gene test for Alport syndrome

Case	Patient ID	Gene	Exon/intron	cDNA	Amino acid	Mutation	Previous reports
1	A607	*COL4A3*	Exon 27	c.1936G>A	p.Gly646Arg	missense	novel
2	A735	*COL4A5*	Exon 42	c.3809G>A	p.Gly1270Asp	missense	novel
3	A818	*COL4A5*	Exon 31	c.2519G>A	p.Gly840Glu	missense	novel
4	A876	*COL4A5*	Intron 12	c.687+5G>C	-	splice site	novel

Figure Legends

Fig. 1

Pathological findings in renal biopsy of each patient. (a) Light microscopic findings. All images are magnified at 200×. (b, c) Electron microscopic findings. The pathological findings are described in the Method section.

Fig. 2

Results of double-immunofluorescence staining for IV collagen α2 and α5 using frozen biopsy sections. First column, type IV collagen α2 staining; second column, type IV collagen α5 staining; third column, merged images. Bar indicates 100 μm. In Cases 1, 2, and 5, normal expression patterns of α2(IV) and α5(IV) were observed in the GBM and Bowman's capsule. In Cases 3 and 4, α5(IV) expression was decreased while α2(IV) showed normal expression.

Fig. 3

Results of double-immunofluorescence staining for IgA and Gd-IgA1 using frozen biopsy sections from patients with Alport syndrome accompanied by IgA deposition (Cases 1–4) and IgA nephropathy with diffuse basement membrane change (Case 5). First column, IgA staining; second column, Gd-IgA1 monoclonal antibody (KM55) staining; third column, merged images. Bar indicates 100 μm. Glomerular Gd-IgA1 deposition was not detected, but IgA was localized in the mesangial area in Cases 1–4. Glomerular Gd-IgA1 deposition was detected, localized in the mesangial area with IgA in Case 5.

Compliance with ethical standards

Conflicts of interest

Kazumoto Iijima reports grants from Daiichi Sankyo, Co., Ltd., personal fees from Kyowa Kirin Co., Ltd., and Integrated Development Associates, and has a patent on antisense nucleotides for exon skipping therapy in Alport syndrome issued. Kandai Nozu has received consulting fees from Kyowa Kirin Co., Ltd.; lecture fees from Kyowa Kirin Co., Ltd., Novartis Pharmaceuticals Corporation, and Chugai Pharmaceutical Co., Ltd.; and has filed a patent application on the development of antisense nucleotides for exon skipping therapy in Alport syndrome.

Ethical approval

All procedures performed in studies involving human participants were carried out in accordance with the ethical standards of the Institutional Review Board of Kobe University Graduate School of Medicine (IRB approval number B190137) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Comprehensive informed consent was obtained from all individual participants in the study and/or their guardians regarding the use of the patients' clinical data.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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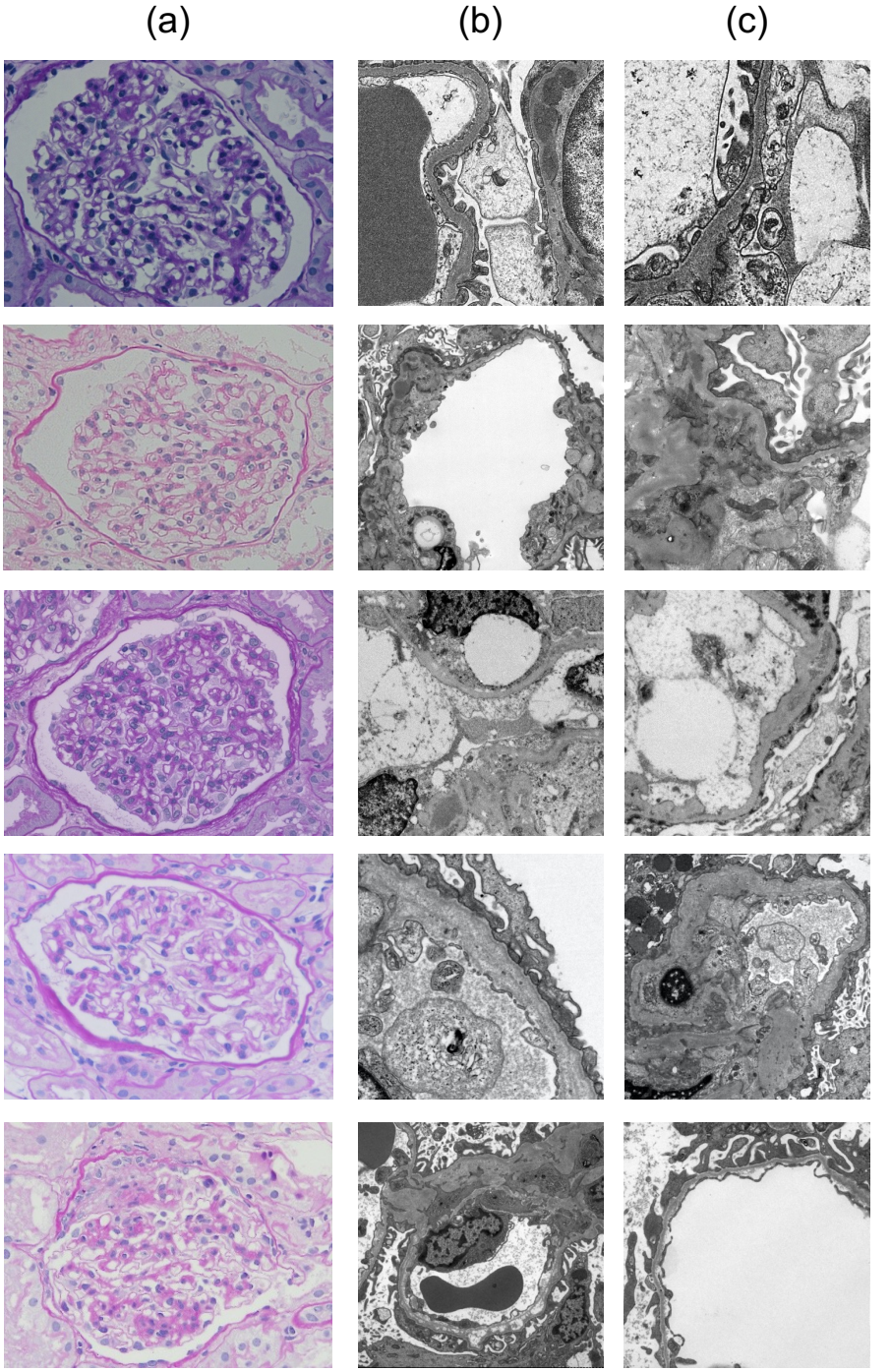
Fig. 1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(b)
(c)
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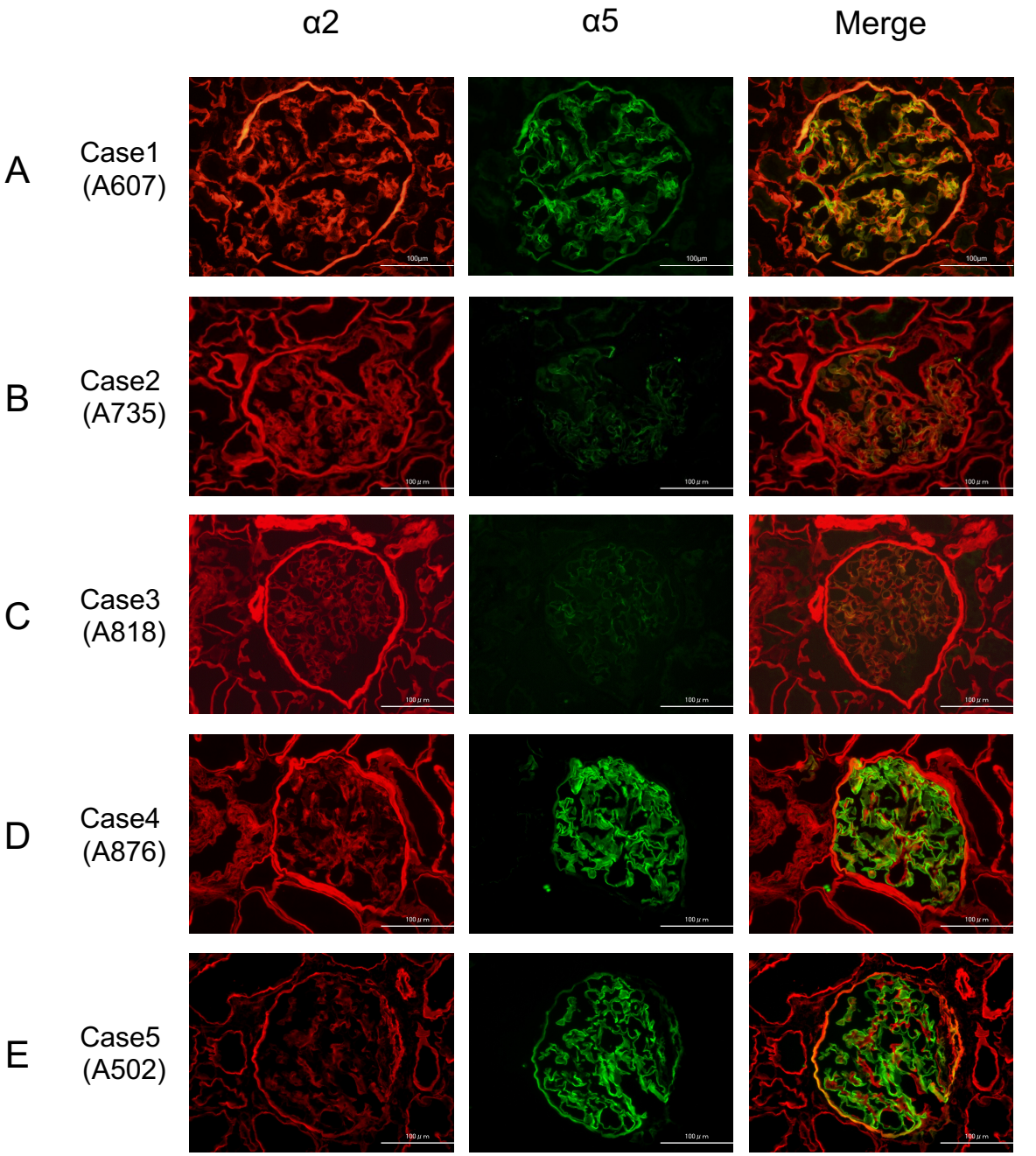
Fig. 2


α2
α5
Merge
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B
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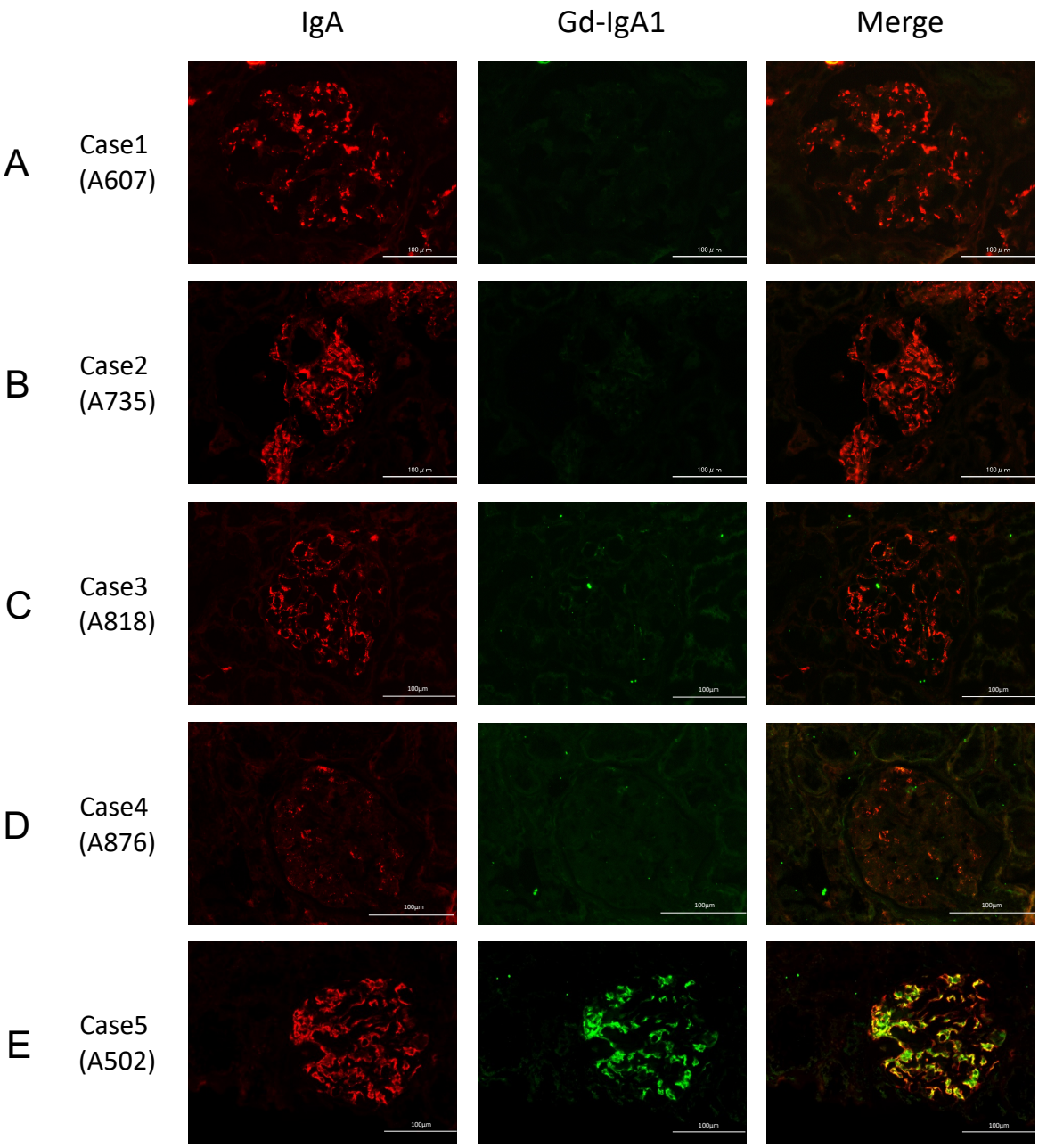
Fig. 3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