

Original Article

Antigen retrieval with protease digestion applied in immunohistochemical diagnosis of Alport syndrome

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Abstract

Background. Immunofluorescence (IF) staining of type IV collagen α chains on fresh frozen renal tissue is a convenient and accurate diagnosis technique for Alport syndrome (AS), which is restricted in the application with a non-frozen section. An alternative method for a paraffin-embedded section is needed in order to extend the application in various specimens. In this study, immunohistochemical staining of type IV collagen α chains on paraffin-embedded renal sections was investigated.

Methods. Three antigen retrieval methods including autoclave heating, pepsin digestion and protease digestion were tried on paraffin-embedded renal sections from two X-linked male AS patients, two X-linked female AS patients and two autosomal recessive AS patients. Two patients with isolated haematuria were also involved. Normal portions of nephrectomized kidneys from two patients with renal tumours were used as controls. The immunohistochemical staining pattern of type IV collagen was compared with that of IF staining.

Results. The results showed that both the autoclave heating method and protease digestion can be used for antigen retrieval for type IV collagen α chains. Compared to autoclave heating, protease digestion had the advantage of being less time consuming, with less renal sections being lost from the slides; it was the most optimal antigen retrieval method in this study. After protease digestion for 40 min, type IV collagen $\alpha 3$ and $\alpha 5$ chains showed a clear and proper distribution pattern in AS patients, haematuria patients and controls, which is the same as that of IF staining.

Conclusions. Compared to the autoclave heating method, protease antigen retrieval is more convenient and effective and can be used to restore type IV collagen α chains on paraffin-embedded renal sections for the diagnosis of AS.

Keywords: Alport syndrome; antigen retrieval; diagnosis; paraffin-embedded renal sections; protease digestion

Introduction

Alport syndrome (AS) is a hereditary renal disease manifested by haematuria, sensorineural hearing loss and progressive renal failure; it is caused by mutations of *COL4A3*, *COL4A4* and *COL4A5* genes that encode type IV collagen $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains, respectively. Type IV collagen is the main component of the glomerular basement membrane (GBM); it is composed of six α chains, including $\alpha 1$ – $\alpha 6$ chains. The gene mutations result in the characteristic abnormal immunofluorescence (IF) staining pattern of type IV collagen α chains on renal tissues [1,2]. The X-linked form of AS is caused by *COL4A5* mutations, which results in negative staining of $\alpha 3$ (IV), $\alpha 4$ (IV) and $\alpha 5$ (IV) along the GBM and Bowman's capsule (BC) in male patients and mosaic linear staining of $\alpha 3$ (IV), $\alpha 4$ (IV) and $\alpha 5$ (IV) on GBM and BC in female patients. In autosomal recessive (AR) AS, which is caused by *COL4A3* or *COL4A4* mutation, the staining of $\alpha 3$ (IV), $\alpha 4$ (IV) and $\alpha 5$ (IV) is negative along the GBM, but the staining of $\alpha 5$ (IV) remains positive along the BC [3]. The type IV collagen $\alpha 1$ chain is widely distributed in the basement membrane irrespective of AS. IF staining of type IV collagen α chains on a renal frozen section has been established for the diagnosis of AS and is applied in practice in our laboratory [1] as well as some other laboratories routinely. However, the method is restricted in the application with non-frozen sections [4]. In addition, some archival paraffin-embedded renal tissues need to be reassessed for the patients who are suspected of having AS. An alternate technique for paraffin-embedded sections, which is more convenient to store and transport, needs to be investigated. To our knowledge, the immunostaining method for collagen IV α chains on paraffin-embedded renal sections is rare; only an autoclave heating antigen retrieval method has been developed in Japan [5]. For immunostaining on a paraffin-embedded section, antigen retrieval is the most crucial step. In this study, we investigated three different antigen retrieval methods including autoclave heating, pepsin digestion and protease digestion in order to find an optimal immunohistochemistry diagnosis technique for AS on paraffin-embedded sections.

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Subjects and methods

Specimens

Renal biopsy specimens were obtained from two X-linked AS male patients, two X-linked AS female patients and two autosomal recessive AS patients. The age ranged from 4 to 13 years. The diagnosis of AS was made under the criteria proposed by Flintner [6]. Five patients were diagnosed with AS as well as their inheritance patterns with the IF staining of α (IV) chains on basement membranes and ultrastructural changes of the GBM revealed by electron microscopy. One patient was diagnosed with the IF staining of α (IV) chains on basement membranes and molecular study, and *COL4A5* 4183 del C was revealed. All patients with AS in this study presented with haematuria and proteinuria. Two patients presented with hypertension and two with mild sensorial hearing loss. Two patients with isolated haematuria (one male and one female) who have been excluded from the diagnosis of AS by gene analysis were also involved; they presented with microscopic haematuria: one glomerular haematuria and one non-glomerular haematuria assessed by urinary red blood cell morphology analysis. Normal portions of nephrectomized kidneys due to renal tumours from two adults were used as controls. Both of them did not have glomerulonephritis, AS and AS family history. The fresh frozen specimens from patients and controls were cut into sections with a thickness of 6 μ m. Formalin-fixed specimens were embedded by paraffin and cut into 4- μ m sections. This study was approved by the Ethic Committee of Peking University First Hospital.

Antibodies

The murine monoclonal antibodies (Wieslab, Lund, Sweden) against NC1 domain of α 1(IV), α 3(IV) and α 5(IV) were used.

Antigen retrieval

Three different antigen retrieval methods including autoclave heating, pepsin digestion and protease digestion were investigated on control sections. According to the reports from Natio [5], after removal of paraffin, the sections were put into a 3.65 g/L HCL solution and autoclave (SANYO MLS-3020, Japan) heated at 115°C for 6 min and 121°C for 6 min, respectively. In the pepsin digestion method group, after removal of paraffin, the sections were digested by pepsin (Invitrogen, USA) for 5, 10, 15 and 30 min, respectively, in chambers at 37°C. In the protease digestion group, the sections were incubated with protease (Lab Vision Corporation, USA) for 10, 20, 30 and 40 min, respectively, in chambers at 37°C. The effect of antigen retrieval was compared by the damage of renal sections after staining, staining intensity with different antibodies and the proper distribution pattern of type IV collagen α chains.

Immunoperoxidase staining

After antigen retrieval, the sections were incubated with monoclonal antibodies against type IV collagen α 3 and

α 5 chains, respectively, for 30 min at room temperature; the phosphate buffered saline instead of primary antibodies was used as a negative control and monoclonal antibody against type IV collagen α 1 chain as a positive control. After rinsing with phosphate buffered saline, the antigens were detected by a PV 9000 two-step Immuno-histological Staining kit (GBI Labs, Mukilteo, WA, USA) according to the procedure provided by the company.

Indirect IF staining

Indirect IF staining of type IV collagen α chains was performed on the frozen sections according to the procedure reported previously [1,2]. After incubation with primary antibodies and secondary antibodies, the sections were observed under IF microscopy.

Results

The staining pattern of type IV collagen α chains on fresh frozen sections

On renal sections from controls, the IF staining of the type IV collagen α 3 chain showed a continuous linear pattern along the GBM and tubular basement membrane (TBM) (Figure 1A), negative on BC; the type IV collagen α 5 chain showed a continuous linear pattern along the GBM, BC and partial TBM (Figure 1B). The same α 3(IV) and α 5(IV) staining patterns on renal sections from haematuria patients (Figure 1I and J) as that of controls were revealed. The IF staining of α 3(IV) and α 5(IV) was negative on the GBM, TBM and BC on sections from X-linked AS male patients (Figure 1C and D). On renal sections from X-linked AS females, the IF staining of α 3(IV) showed a mosaic linear pattern along the GBM, partial TBM and negative on BC; α 5(IV) showed a mosaic linear pattern along the GBM, partial TBM and BC (Figure 1E and F). On renal sections from autosomal recessive AS patients (Figure 1G and H), IF stainings of α 3(IV) and α 5(IV) were negative on the GBM, whereas α 5(IV) was still positive on the BC and partial TBM. The type IV collagen α 1 chain was widely distributed on renal basement membranes in all patients.

The distribution of type IV collagen α chains with different antigen retrieval methods (Table 1)

The effects of different antigen retrieval methods were compared by the staining intensity and pattern of type IV collagen α chains, as well as the degree of renal tissue damage after antigen retrieval. By autoclave heating, although both α 3(IV) and α 5(IV) could be restored like previous reports, two different temperatures were needed to restore two different antigens. Unexpectedly, partial tissue sections (three out of eight sections) were damaged and lost from the slides during the process. After pepsin digestion, only α 5(IV) could be restored whereas α 3(IV) could not. After protease digestion for 40 min, both α 3(IV) and α 5(IV) were clearly stained and properly distributed on paraffin-embedded renal sections (Figure 2A and B), which was the

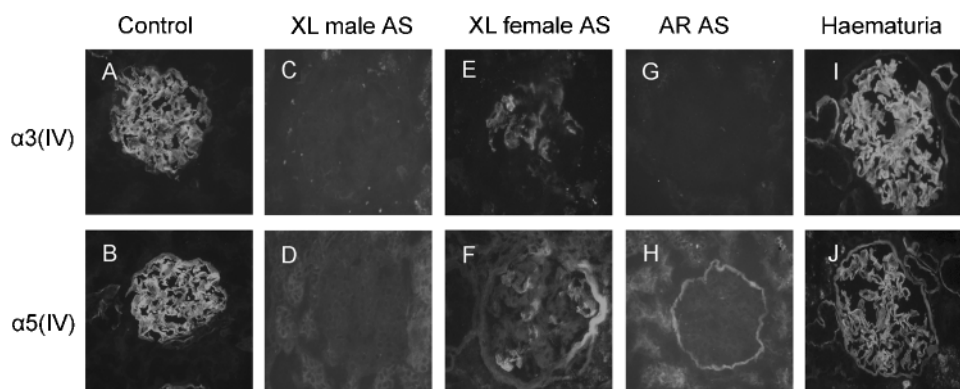


Fig. 1. Immunofluorescence staining of collagen IV $\alpha 3$, $\alpha 5$ chains on fresh frozen renal sections of controls, Alport syndrome patients and haematuria patients (IF, $\times 400$). **A, B:** The staining of type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections from controls. **C, D:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on sections from X-linked male Alport syndrome. **E, F:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections of X-linked female Alport syndrome. **G, H:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections of autosomal recessive Alport syndrome. **I, J:** The staining patterns of type IV collagen α chains on renal sections of haematuria patients.

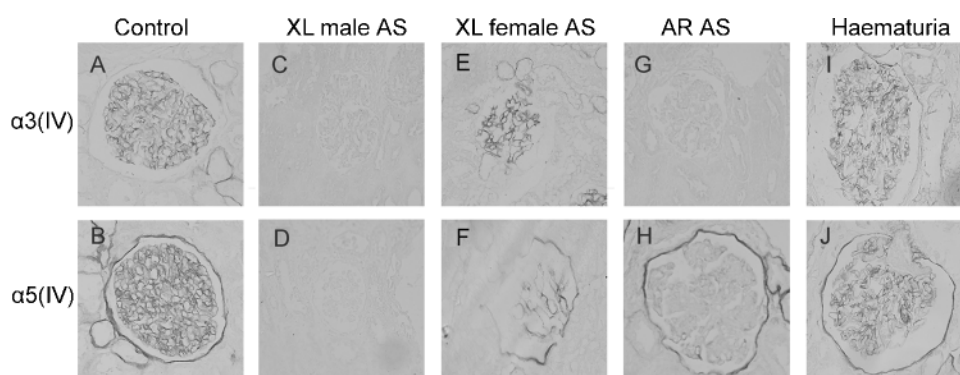


Fig. 2. Immunostaining of type IV collagen $\alpha 3$ and $\alpha 5$ chains on paraffin-embedded renal sections of controls, Alport syndrome patients and haematuria patients (LM, $\times 400$). **A, B:** The staining of type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections from controls. **C, D:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on sections from X-linked male Alport syndrome. **E, F:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections of X-linked female Alport syndrome. **G, H:** Type IV collagen $\alpha 3$ and $\alpha 5$ chains on renal sections of autosomal recessive Alport syndrome. **I, J:** The staining patterns of type IV collagen α chains on renal sections of haematuria patients.

same as the result of IF staining on frozen sections, and the tissue was preserved well.

IV collagen α chains on paraffin-embedded renal sections were comparable to that with IF staining.

The immunostaining of type IV collagen α chains on paraffin-embedded sections from patients

After antigen retrieval by protease digestion for 40 min, immunoperoxidase staining was performed on patients with AS and haematuria. On sections from the X-linked form of AS male patients (Figure 2C and D), both $\alpha 3$ (IV) and $\alpha 5$ (IV) were negative on the GBM and BC. On renal sections from the X-linked form of AS female patients (Figure 2E and F), the staining of $\alpha 3$ (IV) showed a mosaic linear pattern along the GBM and negative on the BC, and $\alpha 5$ (IV) showed a mosaic linear pattern along the GBM and BC. On sections from autosomal recessive AS patients (Figure 2G and H), the staining of $\alpha 3$ (IV) and $\alpha 5$ (IV) were negative on the GBM, but the staining of $\alpha 5$ (IV) was positive along the BC. The staining pattern of type IV collagen α chains on specimens from haematuria was the same as controls (Figure 2I and J). All the immunostaining patterns of type

Discussion

IF staining of type IV collagen α chains on renal sections has been used for diagnosis of AS for >10 years. Although valuable and believable [2], the method is restricted in the application with non-frozen sections. Unlike a paraffin-embedded section, frozen section preparation and microscopy for the IF method need special facilities that are not available in a lot of local hospitals as well as laboratories. Furthermore, the archival renal tissues of suspected patients need to be reassessed for definite diagnosis. Therefore, it is necessary to set up an alternative method of staining type IV collagen α chains on paraffin-embedded sections.

The antigen retrieval step was thought to be as the key process influencing the immunohistochemical staining on paraffin-embedded sections [7]. The common antigen retrieval methods available now include a heating method [8]

Table 1. The effect of different antigen retrieval methods on the staining of type IV collagen α chains in control renal specimens

		Autoclave heating		Pepsin digestion				Protease digestion				IF
		115°C	121°C	5'	10'	15'	30'	10'	15'	30'	40'	
		Partial lost	Partial lost	Well	Well	Well	Partial lost	Well	Well	Well	Well	
$\alpha 3(\text{IV})$	GBM	—	+	—	—	—		±	±	±	+	+
	BC	/	/	/	/	/	/	/	/	/	/	/
	TBM	±	+	—	—	—		±	±	±	+	+
$\alpha 5(\text{IV})$	GBM	—	+	±	±	+		±	±	±	+	+
	BC	+	—	±	±	+		±	±	±	+	+
	TBM	+	+	±	±	+		±	±	±	+	+

GBM: glomerular basement membrane; BC: Bowman's capsule; TBM: tubular basement membrane; IF: immunofluorescence.
 —: negative; ±: weak positive; +: positive; /: no expression in normal kidneys.

and enzyme digestion. Among heating methods, the autoclave heating method has been successfully used for diagnosis of AS, but the procedure is time consuming because it needs two different steps to restore different antigens on the GBM and BC separately [5]. Pepsin digestion is a common way of enzyme digestion used for antigen retrieval, which has been successfully used for collagen IV staining in mouse brain [9]. For formalin-fixed specimens, protease digestion was thought to be a better antigen retrieval method; it has been successfully used for other antigen retrieval in renal tissues but not used for type IV collagen [10,11]. The effects of different antigen retrieval methods for collagen IV alpha chains were compared by the tissue integrity, the staining intensity and pattern of the antigen restored. For control specimens, protease digestion of 40 min was the most optimal method for glomerular type IV collagen α chains. Although the results showed that the autoclave heating method can restore type IV collagen $\alpha 3$ and $\alpha 5$ chains and help diagnosis of AS as previously reported, during the antigen retrieval process, some tissues on the specimen (three out of eight sections) were destroyed and lost from the slides. The tissue loss might be due to the heating process [12]. Only alpha 5(IV) can be restored by pepsin digestion antigen retrieval method, the mechanism underlying it is not clear. After protease antigen retrieval at 37°C for 40 min, type IV collagen $\alpha 3$ and $\alpha 5$ chains were restored perfectly, and the staining was clear and properly distributed without tissue loss, which demonstrated the superiority of this method compared to other methods in this study.

To further confirm the effect of the protease antigen retrieval method, we applied it to the paraffin-embedded renal sections from AS patients and haematuria patients; the same results as those with the IF method were achieved. We also repeated the immunostaining on patients with different genotypes of AS, haematuria and control and confirmed the stability of the method for the diagnosis of AS. The antigen retrieval method of protease digestion has been used for other antigen retrievals on renal tissues and was thought to be a better antigen retrieval method for formalin-fixed paraffin-embedded renal sections, but it has not been used in the staining of type IV collagen. For the first time, our results showed that protease digestion could be used for the antigen retrieval of type IV collagen α chains and applied to the diagnosis of AS. Compared with autoclave heating, the protease digestion antigen retrieval method is easy to op-

erate, less time consuming and there is less tissue wastage. This technique can also be used in retrospective analysis on paraffin-embedded specimens and in specimen convenient transportation between different laboratories. Although the sensitivity and specificity of the technique need further confirmation, we conclude that the protease antigen retrieval method can assist in a more convenient diagnosis and even retrospective diagnoses. It provides a new alternative technique for diagnosing AS. Although the method worked well for the antibodies we used in this study, additional tests are needed for other anti-type IV collagen chains antibodies.

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Conflict of interest statement. None declared.

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