Quantitative analysis of type IV collagen subchains in the glomerular basement membrane of patients with Alport syndrome with confocal microscopy

Jian Su, Zhi-Hong Liu, Cai-Hong Zeng, Wei-Gong, Hui-Ping Chen and Lei-Shi Li

Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, 210002, China

Abstract

Background. Alport syndrome (AS) is an inherited nephropathy characterized by glomerular basement membrane (GBM) abnormalities due to mutations in the type IV collagen genes. Through immunofluorescence analysis, the absence of \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains within the GBM has been shown in the majority of AS cases. In some atypical AS cases, however, staining of the GBM with antibodies against the \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains appeared normal. In this study, we studied these atypical AS cases by quantitative analysis of the expression of type IV collagen subchains in GBM.

Methods. Twelve patients diagnosed with AS, yet having normal staining for \( \alpha_3(IV) \) and \( \alpha_5(IV) \) chains in the GBM, were recruited. Quantitative analysis of type IV collagen subchains in the GBM was performed using confocal microscopy and immunofluorescence double label techniques.

Results. The absolute amounts of \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) were significantly lower in AS patients than that in normal subjects, associated with up-regulated expression of type IV collagen in GBM. It was found that eight cases had decreased ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \) in the GBM simultaneously; one had reduced levels of \( \alpha_3(IV)/IV \) and \( \alpha_5(IV)/IV \) but had a normal level of \( \alpha_4(IV)/IV \), and one had reduced \( \alpha_3(IV)/IV \) with normal \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \) levels. The remaining two patients had normal ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \).

Conclusions. Confocal analysis demonstrated for the first time that the ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \) in the GBM decreased in patients with AS, even though routine immunofluorescence staining for \( \alpha(IV) \) chains appeared normal. This result not only sheds light on the pathogenesis of AS, but also provides an alternative approach to diagnose atypical AS cases.

Keywords: alport syndrome; confocal microscopy; type IV collagen

Introduction

Alport syndrome (AS) is an inherited disorder of the glomerular basement membrane (GBM) characterized by haematuria, progressive renal failure and sensorineural hearing loss, frequently associated with ocular abnormalities [1]. The disease is caused by mutations in any one of the genes encoding the \( \alpha_3 \), \( \alpha_4 \) and \( \alpha_5 \) chains of type IV collagen (\( \text{COL4A3, COL4A4} \) and \( \text{COL4A5} \), respectively) [2,3]. \( \text{COL4A5} \) mutations lead to the most common form of AS which is X-linked, whereas \( \text{COL4A3} \) and \( \text{COL4A4} \) mutations are responsible for the autosomal recessive or dominant forms [4–6].

The availability of antibodies against \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains has made it possible to detect the changes in type IV collagen expression that occur as a result of mutations in the \( \text{COL4A3, COL4A4} \) or \( \text{COL4A5} \) genes. Generally, the GBM of male patients with X-linked AS (XLAS) is non-reactive to antibodies against the \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains. Women who are heterozygous for XLAS mutations frequently exhibit a mosaic expression for the \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains. In patients with autosomal recessive AS (ARAS), the GBM typically shows an absence of the \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) chains. Expression patterns of the type IV collagen chains in basement membranes of patients with autosomal dominant AS (ADAS) have rarely been studied [7].

In clinical practice, we found that some patients that fulfilled the diagnostic criteria of AS [8], showed normal staining for the \( \alpha_3(IV) \) and \( \alpha_5(IV) \) chains in the GBM.
It had been reported that in some genetically proven XLAS patients, staining of the GBM with antibodies against the $\alpha_3$(IV), $\alpha_4$(IV) and $\alpha_5$(IV) chains, appeared normal in immunohistochemical studies [9–11]. Most of these studies suggested that the types of underlying gene mutations were associated with the preservation of GBM expression of the $\alpha$(IV) chains. Thus, it is rational and helpful to diagnose these patients with genetic analysis. However, by now, genetic analysis is still not applicable to perform clinically. As confocal analysis is a very attractive and sensitive technique in the field of AS, in the present study, we used confocal laser scanning microscopy to quantitatively analyse the amounts of $\alpha_3$(IV), $\alpha_4$(IV) and $\alpha_5$(IV) chains in the GBM of patients with atypical AS. Our aim was to reveal potential quantitative abnormalities of the type IV collagen subchains in the GBM of patients with AS and to provide a new approach for the diagnosis of AS.

Subjects and methods

Patients

Twelve patients were recruited for this study. AS was diagnosed according to clinical features, light and electron microscopic examination of renal biopsies [12]. Immunostaining for $\alpha$(IV) was performed in the biopsies under study prior to confocal evaluation. All the patients showed normal expression for $\alpha$(IV) and $\alpha$(V) chains in the GBM (Table 1).

Clinical features are presented in Table 2. Briefly, all the patients had microscopic haematuria and 64.3% of the patients had proteinuria. Neurosensory deafness was present in three patients. None of the patients had renal failure. Of the 12 patients, only nine had family pedigrees. Among them, five patients demonstrated X-linked transmission (cases A–E) and four patients were inconclusive, but suggestive of X-linked transmission (cases F–I) (Figure 1).

Controls consisted of the following:

(i) Normal kidney tissues from six donor kidneys (age from 20 to 40 years).

(ii) Ten patients with mild mesangial proliferative glomerulonephritis (MsPGN) (age from 20 to 40 years); there was no family history of renal diseases in these patients. Most of the patients with MsPGN were negative for IgG, IgA, IgM, C3, C4 and Clq in renal tissues. The alterations of GBM under electron microscopy (EM) were mild.

(iii) Four patients with thin basement membranous nephropathy (TBMN). The diagnosis of TBMN was applied when there was diffuse thinning of the GBM (at least 50% of the GBM was thinned) with an average width <250 nm and no thickening or splitting of the GBM was observed [13]. Clinical features and pathological findings are presented in Tables 1 and 2 (cases M–P).

These studies were approved by the Ethical Committee of Nanjing University. Informed consents were obtained from patients to use their tissue specimens for research purposes.

<table>
<thead>
<tr>
<th>Patients with X-linked AS</th>
<th>Histological pattern</th>
<th>IF(GBM)</th>
<th>IF (EBM)</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>FSGS</td>
<td>$\alpha_3$</td>
<td>$\alpha_4$</td>
<td>$\alpha_5$</td>
</tr>
<tr>
<td>B</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Patients suggestive of X-linked AS</td>
<td>IF</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>FSGS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Unknown mode of inheritance</td>
<td>IF</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Patients with TBMN</td>
<td>IF</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>MsPGN</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

MsPGN, mesangial proliferative glomerulonephritis; FSGS, focal segmental glomerulosclerosis; EM, electromicroscopic classification; IF, immunofluorescence.

$^a$Her son and brother showed absent $\alpha_3$, $\alpha_5$(IV) chain expression in GBM.

$^b$The respective mothers showed mosaic staining pattern of $\alpha_5$(IV) chain in EBM.

Histological examination

Renal specimens were obtained by percutaneous needle biopsy, fixed with 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin and cut into 2 $\mu$m sections. Sections were stained with haematoxylin and eosin, periodic acid-schiff, silver methenamine and Masson trichrome, and subsequently examined by light microscopy.

The GBM structure was evaluated using electron microscopic material. For each case, ultra-thin sections were observed using EM, and microphotographs were obtained. The characteristic lesions of GBM included thinning (GBM < 250 nm), thickening (GBM > 373 nm in adult men and GBM > 326 nm in adult women) and splitting and basket weaving of the lamina densa [14].

Immunofluorescence analysis of the skin

Skin biopsies were performed on all patients. The expression of the epidermal basement membrane (EBM) $\alpha_5$(IV) chain was detected using an indirect immunofluorescence method performed on frozen sections. Briefly, 5$\mu$m-thick cryostat sections were air-dried, fixed in acetone for 7 min and denatured by the exposure to 6 M urea in 0.1 M glycine/HCl buffer (pH 3.5) for 10 min to unmask the hidden epitope of the $\alpha_5$(IV) chain. After incubating with 10% FCS (fetal calf serum) for 4 min, sections were incubated with MAB5 (1:50, Wieslab, Sweden) overnight. FITC-conjugated rabbit antibodies against mouse immunoglobulins were used as secondary antibodies.
Table 2. Clinical manifestation of the patients

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (Years)</th>
<th>Proteinuria (g/24 h)</th>
<th>Haematuria (10^4/ml)</th>
<th>Hearing loss</th>
<th>Ocular lesion</th>
<th>Scr (µmol/l)</th>
<th>BUN (mmol/l)</th>
<th>Family history</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients with X-linked AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>F</td>
<td>40</td>
<td>1.2</td>
<td>132</td>
<td>–</td>
<td>102</td>
<td>7.4</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>11</td>
<td>1.7</td>
<td>172</td>
<td>–</td>
<td>70</td>
<td>4.8</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>2</td>
<td>0.1</td>
<td>32</td>
<td>ND</td>
<td>44</td>
<td>5.6</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>19</td>
<td>0.7</td>
<td>92</td>
<td>–</td>
<td>87</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>23</td>
<td>0.2</td>
<td>162</td>
<td>–</td>
<td>53</td>
<td>6.2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Patients suggestive of X-linked AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>F</td>
<td>36</td>
<td>0.2</td>
<td>34</td>
<td>ND</td>
<td>52</td>
<td>5.3</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>M</td>
<td>41</td>
<td>4.5</td>
<td>32</td>
<td>+^a</td>
<td>58</td>
<td>4.8</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>F</td>
<td>35</td>
<td>0.58</td>
<td>195</td>
<td>–</td>
<td>68</td>
<td>13.4</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>M</td>
<td>17</td>
<td>1.0</td>
<td>4</td>
<td>+^a</td>
<td>87</td>
<td>4.4</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td>F</td>
<td>15</td>
<td>2</td>
<td>39</td>
<td>–</td>
<td>68</td>
<td>5.2</td>
<td>–</td>
</tr>
<tr>
<td>K</td>
<td>F</td>
<td>23</td>
<td>0.5</td>
<td>143</td>
<td>–</td>
<td>59</td>
<td>8.1</td>
<td>–</td>
</tr>
<tr>
<td>L</td>
<td>F</td>
<td>29</td>
<td>0.4</td>
<td>204</td>
<td>+^a</td>
<td>33</td>
<td>8.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Patients with TBMN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>F</td>
<td>30</td>
<td>0.2</td>
<td>165</td>
<td>–</td>
<td>62</td>
<td>5.8</td>
<td>+</td>
</tr>
<tr>
<td>N</td>
<td>M</td>
<td>25</td>
<td>0.4</td>
<td>1050</td>
<td>–</td>
<td>68</td>
<td>6.2</td>
<td>–</td>
</tr>
<tr>
<td>O</td>
<td>M</td>
<td>43</td>
<td>0.4</td>
<td>253</td>
<td>–</td>
<td>45</td>
<td>4.8</td>
<td>–</td>
</tr>
<tr>
<td>P</td>
<td>M</td>
<td>32</td>
<td>0.4</td>
<td>150</td>
<td>–</td>
<td>64</td>
<td>7.6</td>
<td>+</td>
</tr>
</tbody>
</table>

Scr, serum creatine; BUN, blood urea nitrogen; ND, not detected; M, male; F, female.

^aNeurosensory deafness.

Ratios of α3(IV), α4(IV) and α5(IV) to type IV collagen in the GBM

To quantitatively analyse the amounts of α3(IV), α4(IV) and α5(IV) chains in the GBM of patients with atypical AS, an immunohistochemical double label experiment was performed on formalin-fixed, paraffin-embedded sections as follows: following dewaxing, sections were treated with trypsin (0.25 mg/ml) for 4 min. After a wash with PBS (0.01 mol/l, pH 7.2), sections were incubated with 10% FCS (fetal calf serum) for 4 min. Then the sections were incubated with mouse anti-human type IV collagen monoclonal antibody (1:100, DAKO, Denmark; recognize a conformational epitope on a helical part of native type IV collagen) overnight at room temperature. After three washes with PBS, sections were incubated with TRITC-conjugated rabbit against mouse immunoglobulins (1:50, DAKO, Denmark) for 40 min. Then the sections were microwaved on low power for 10 min in a target retrieval solution (DAKO, Denmark) and cooled. Sections were treated with trypsin a second time and incubated with MAB3 (1:50, Wieslab, Sweden), rabbit polyclonal antibody to the α4(IV) chain (1:50) (a generous gift from Dr J. H. Miner) [15] and MAB5 (1:50) overnight. Sections were incubated with FITC-conjugated swine against rabbit immunoglobulins (1:50) for 40 min.

Fluorescence images were collected and analysed by laser scanning confocal microscopy (LSM510, Zeiss). Briefly, for double-labelled sections, the dual channel mode was employed and sections were scanned simultaneously at both the wavelengths (488/543 nm) with the laser intensity, confocal aperture, gain and black-level settings kept constant [16]. A vertical scan was performed to determine the plane of greatest intensity of fluorescent signal within the specimen; a single horizontal scan was subsequently performed at that plane [17]. In each patient, five glomeruli were viewed and 10 glomerular capillary walls per glomerulus were selected randomly (Figure 2A and B). The fluorescence intensity at the selected areas, linearly correlated with the number of pixels, was quantitatively analysed using the standard imaging analysis software of an LSM510 system. The level of background fluorescence was demonstrated by selecting a line across the image. The positive threshold for the FITC channel was represented by 300 pixel units, while 400 pixel units represented the threshold for the TRITC channel (Figure 3A and B). The ratios of each individual α3(IV), α4(IV) and α5(IV) chain to type IV collagen [α3(IV)/IV, α4(IV)/IV or α5(IV)/IV] was represented as the ratio of FITC to TRITC mean fluorescence intensity in the total area of the selected glomerular capillary walls in all the glomeruli examined [18] (Figure 2C–H).

\[
\frac{\text{Fluorescence intensity of } \alpha_3(\text{IV})}{\text{Fluorescence intensity of type IV collagen (FITC)}}
\]

Statistical analysis

Values were expressed as mean±SD. Data were processed using SPSS software Version 10.0. Values were considered statistically significant when \(P<0.05\).

Results

Light microscopy

In the patients with AS, 10 biopsies showed mild increases of extracellular matrix in the mesangial...
Fig. 1. Pedigrees of the patients. Cross-hatched symbols denote individuals with renal diseases but no renal dysfunction and solid symbols denote an individual with renal failure due to unknown glomerulonephritis.
regions (MsPGN); two biopsies showed focal segmental glomerulosclerosis (FSGS). Quite a few interstitial foam cells were present in three patients (cases B, F, J). Four patients with TBMN showed very mild mesangial proliferation (Table 1).

**Electron microscopy**

An EM image suggestive of AS was one of the main criteria used to diagnose AS for the present study. Thus, all the patients showed altered GBM; that is, variable (from light/focal to severe/diffuse) combinations of thinning, thickening, splitting and basket weaving of the lamina densa of the GBM (Table 1). Six patients (cases A, B, F, H, J, L) showed diffuse, moderate to severe basket weaving of the lamina densa, with variable thinning and thickening of the GBM (Figure 4A). Six patients (cases C, D, E, G, I, K) mainly demonstrated thinning of the GBM, with variable thickening of the GBM and focal splitting or basket weaving of the lamina densa. The thinning area ranged from 30–50% in individual capillaries (Figure 4B and C).

**Distribution of \( \alpha_5(IV) \) chain in the epidermal basement membrane of patients with AS**

All but one patient (case D) showed normal \( \alpha_5(IV) \) chain immunostaining in skin biopsies (Table 1). Expression of the \( \alpha_5(IV) \) chain in the skin biopsy of case D was absent and did not correlate to the expression of the \( \alpha_5(IV) \) chain in the kidney biopsy. Furthermore, the respective mothers of cases B and D demonstrated a mosaic staining pattern of the \( \alpha_5(IV) \) chain in their skin biopsies.

**Ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \) in the GBM of patients with AS**

The mean immunofluorescence intensity of \( \alpha_3–5(IV) \) chains including type IV collagen from normal subjects, MsPGN and AS patients was demonstrated in Table 3. Statistical analysis indicated that the amounts of \( \alpha_3(IV) \), \( \alpha_4(IV) \) and \( \alpha_5(IV) \) were significantly lower in AS patients than that in normal subjects, while the amount of type IV collagen was higher in AS. In patients with MsPGN, the amounts of \( \alpha_3(IV) \), \( \alpha_4(IV) \), \( \alpha_5(IV) \) and type IV collagen were similar to that in normal subjects.

As the absolute amounts of \( \alpha(IV) \) chains are influenced by age, sex and maybe varied individually, we further investigated the ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \). The results demonstrated that ratios of \( \alpha_3(IV)/IV \), \( \alpha_4(IV)/IV \) and \( \alpha_5(IV)/IV \) were significantly lower in AS patients than that in normal subjects (Table 4, Figure 5).

**Ratios of \( \alpha_3(IV)/IV \) and \( \alpha_5(IV)/IV \) in AS**

In five patients with XLAS (Table 5), compared with normal controls, three had simultaneously significant decreases of \( \alpha_3(IV)/IV \) and \( \alpha_5(IV)/IV \) (<95%CI), one (case D) had slightly reduced \( \alpha_3(IV)/IV \) but \( \alpha_5(IV)/IV \) was normal and one (case E) had normal ratios of \( \alpha_3(IV)/IV \) and \( \alpha_5(IV)/IV \).

In patients suggestive of XLAS (Table 5), three out of four patients showed simultaneous decreases.
Fig. 3. Profiles of fluorescence intensity constructed by selecting a line across the images showed peaks and troughs (backgrounds). 
(A) The profile of fluorescence intensity of α3(IV) [or α4(IV), α5(IV)] in glomeruli; (B) the profile of fluorescence intensity of type IV collagen in glomeruli.

Fig. 4. Electron micrographs of GBM from patients with AS: (A) thickening and basket weaving of the lamina densa; (B) segmental splitting of the GBM; and (C) thinning of the GBM (×10000).
of α3(IV)/IV and α5(IV)/IV, and one (case I) patient had normal ratios of α3(IV)/IV and α5(IV)/IV. In patients with an unknown mode of inheritance (Table 5), all three patients showed decreases of α3(IV)/IV and α5(IV)/IV.

Cumulative results indicate that in 10 out of 12 unrelated atypical AS patients (most of them were X-linked), a decrease of α3(IV)/IV or α5(IV)/IV, or both, was detectable by confocal microscopy, though normal staining for α3(IV) and α5(IV) chains had previously been exhibited in kidney sections.

Ratio of α4(IV)/IV in AS
Quantitative results indicated that some patients with an evident diagnosis of XLAS had normal ratios of α3(IV)/IV and α5(IV)/IV. Expression of the α3(IV) chain was not always associated with the α5(IV) chain. As the α4(IV) chain is also involved in the incorporation of the type IV collagen network into the GBM and the corresponding gene COL4A4 was one of the main mutation genes in ARAS, we further investigated the expression of the α4(IV) chain in these patients. All 12 patients showed normal staining for the α4(IV) chain in the GBM when using standard immunofluorescence analysis. Quantitative results demonstrated that the level of α4(IV)/IV was identical to that of α3(IV)/IV and α5(IV)/IV with the exception of cases D and F. Case D showed slightly reduced α3(IV)/IV, normal α4(IV)/IV and α5(IV)/IV. Case F showed reduced α3(IV)/IV, α5(IV)/IV and normal α4(IV)/IV. These data indicate the discordance in the expression levels of the α3(IV), α4(IV) and α5(IV) chains in some patients with AS.

Relationship between ratios of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV and structure of GBM
Using electron microscopy, we divided the patients into two groups: patients with typical EM findings (six cases) and patients with thin and thick GBM (six cases). Statistical analysis demonstrated that the levels of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV were unrelated with the severity of the structure of GBM.

Ratios of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV in TBMN
Although the amount of α5(IV) chain was lower in patients with TBMN compared with normal subjects, the levels of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV were normal in all four patients with TBMN (Table 5).

Discussion
The AS is caused by mutations in any of the three COL4A3, COL4A4 or COL4A5 genes. Genetic mutation detection has been considered as sufficient diagnostic criterion for AS [19–21]. However, mutation analysis of AS genes remains a tedious and sometimes an unsuccessful task due to the large size of the genes and the high number of polymorphisms. In the meantime, the diagnosis of AS rests on an approach exemplified by careful collection and analysis of clinical features, histological examination, electron microscopic and immunohistochemical examination of basement membranes [8,22]. Unfortunately, however, in clinical practice, we often found that some patients with a family history of renal diseases and typical GBM

| Table 3. The mean immunofluorescence intensity of α3–α5(IV) and type IV collagen in normal subjects, MsPGN, TBMN and AS |
|----------------|----------------|----------------|----------------|----------------|
|                | MsPGN          | TBMN           | AS             | Normal subjects |
| α3(IV)         | 2064.6 ± 299.2 | 2002.3 ± 375.8 | 2086.2 ± 140.1 | 1459.8 ± 248.6 |
| α4(IV)         | 2055.1 ± 268.5 | 2303.6 ± 328.4 | 1787.2 ± 126.5 | 1354.7 ± 144.9 |
| α5(IV)         | 1779.1 ± 259.7 | 1702.9 ± 280.7 | 1722.8 ± 279.2 | 1786.4 ± 338.5 |
| Normal subjects| 2196.0 ± 367.0 | 2261.8 ± 158.3 | 2166.0 ± 113.7 | 1356.6 ± 237.1 |

| Table 4. Ratios of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV in normal subjects, MsPGN, TBMN and AS |
|----------------|----------------|----------------|----------------|----------------|
|                | MsPGN          | TBMN           | AS             | Normal subjects |
| α3(IV)/IV      | 1.330 ± 0.179  | 1.483 ± 0.165  | 1.053 ± 0.270  | 1.462 ± 0.081  |
| α4(IV)/IV      | 1.554 ± 0.251  | 1.665 ± 0.093  | 1.038 ± 0.265  | 1.555 ± 0.145  |
| α5(IV)/IV      | 1.364 ± 0.171  | 1.395 ± 0.163  | 0.963 ± 0.379  | 1.510 ± 0.180  |

Values are means ± SD; MsPGN, mesangial proliferative glomerulonephritis; TBMN, thin basement membranous nephropathy; AS, Alport syndrome.

a
b
changes, showed normal staining for α3(IV) and α5(IV) chains in the GBM which often challenges clinicians to the diagnosis of AS. In this study, we used confocal microscopy and attempted to develop a method that might be applicable for the diagnosis of AS for regular clinical use.

Criteria for recruiting our patients were based on clinical features, histological examination and electron microscopic examination of basement membranes. As TBMN is also characterized by a family history of renal disease and persistent haematuria that resembles the clinical presentation of AS, we specifically investigated the ultrastructure of the GBM in order to exclude TBMN. All 12 patients showed an altered GBM under electron microscopy. Among them, six patients showed classical ultrastructural changes of AS and six patients showed variable thinning, thickening, splitting or basket weaving of the lamina densa. Combined with the clinical features and histological findings, all of these 12 patients were considered as AS patients.

As the absolute amounts of α3(IV) chains are influenced by age, sex and may be varied individually, in this study, we prefer to analyse the changes of α3(IV)/IV rather than merely evaluate the amount of any α(IV) chain. Our results demonstrated that 10 out of 12 unrelated atypical AS patients had decreased levels of α3(IV)/IV, α4(IV)/IV and α5(IV)/IV or all three, in the GBM. In addition, for cases A, B and D, the demonstration of abnormalities in α(IV) chains was known in the family either in GBM or EBM.

![Fig. 5. Comparison of the signal intensity for α3(IV)/IV (A), α4(IV)/IV (B) and α5(IV)/IV (C) in the GBM among atypical AS, MsPGN and normal kidney tissues.](image1)

![Fig. 6. Immunofluorescence staining for human type IV collagen in glomerulus from normal control subjects (A, C and E) and patients with AS (B, D and F). (A and B) Double-staining for α3(IV) and type IV collagen; (C and D) double-staining for α4(IV) and type IV collagen; (E and F) double-staining for α5(IV) and type IV collagen. Green corresponds to α3(IV), α4(IV) or α5(IV), respectively and red to type IV collagen (×400).](image2)
The confocal study was consistent with these findings and showed that \( \alpha 3(IV)/IV \), \( \alpha 4(IV)/IV \) or \( \alpha 5(IV)/IV \) significantly decreased in these patients. The results were exciting and indicated that confocal microscopy analysis was a very attractive technique in the field of AS. However, it must be pointed out that in our observed cases, although the pure population of AS with X-linked transmission was considered (cases A–E), the results were heterogeneous and one patient had normal ratio of \( \alpha 3(IV)/IV \), \( \alpha 4(IV)/IV \) and \( \alpha 5(IV)/IV \). That is, cases remain where \( \alpha (IV) \) chain distribution does not explain AS or the small abnormalities of \( \alpha (IV) \) chains in these patients cannot be detected even by confocal microscopy. For these patients, the definite diagnosis will depend on genetic analysis. Regardless, analysing the ratios of \( \alpha 3(IV) \), \( \alpha 3–5(IV)/IV \), 3–5(IV)/IV, \( \alpha 4(IV)/IV \) and \( \alpha 5(IV)/IV \) in the GBM will improve the detection rate of atypical AS, especially in regular clinical practice.

A decrease in the ratio of \( \alpha (IV) \) chains to type IV collagen can be consequent to (i) decrease in the absolute amount of \( \alpha (IV) \) chains, (ii) increase in the amount of type IV collagen (may be due to compensatory accumulation of \( \alpha 1\alpha 1\alpha 2 \) [23–25] or (iii) a combination of both factors. Our results are in keeping with the third possibility and demonstrated a significant decrease in the ratio of \( \alpha (IV)/IV \) in patients with AS.

From the above results, we could suggest that in a number of AS patients, apparently normal renal distribution of \( \alpha 3–5(IV) \) chains may be an artifact because of the limitation of routine immunofluorescence technique. The mutations of type IV collagen genes in these patients may be small or different from typical AS [9,10], resulting in partial preservation of the expression of \( \alpha 3–5(IV) \) chains in GBM. It was regrettable that genetic analysis was not performed in our studies, but the confocal study and the observation that the ratio of \( \alpha 3–5(IV)/IV \) was reduced in AS patients provided a practical approach to diagnose these atypical AS.

One interesting observation in our study is that in case D, the ratio of \( \alpha 5(IV)/IV \) was normal in GBM, but the expression of the \( \alpha 5(IV) \) chain in the skin biopsy was absent. The discordance of the \( \alpha 5(IV) \) chain distribution in the EBM and GBM has been reported before [26]; two possible explanations were provided for this particular result. The first explanation is that a mutation in COL4A5 can have different effects on the \( \alpha 3 \), \( \alpha 4 \), \( \alpha 5(IV) \) network of the GBM compared with the \( \alpha 5 \), \( \alpha 5 \), \( \alpha 6(IV) \) network of the EBM [27]. Despite the absence of the \( \alpha 5(IV) \) chain in the skin, the protein might sometimes be incorporated correctly into the renal network. The second explanation is that skin and kidney come from different developmental origins; the skin comes from the ectoderm and the kidney from the mesoderm [28]. The expression of the \( \alpha 5(IV) \) chain may vary widely in different tissues from the same individual. The other 11 patients had positive staining of the \( \alpha 5(IV) \) chain in the skin, which was not consistent with X-linked inheritance. One recent study using confocal microscopy demonstrated that the apparently normal skin distribution of the \( \alpha 5(IV) \) chain in a number of XLAS patients was the consequence of an optical artifact. A focal interruption and irregular shape of \( \alpha 5(IV) \) chain expression could be detected by three-dimensional reconstruction of the EBM [29]. Thus, confocal microscopy examination of skin biopsies may also be an alternative approach to the diagnosis of AS.

It is also interesting that in patients with TBMN, the amount of \( \alpha 5(IV) \) chain decreased while the amount of

---

### Table 5. Ratios of \( \alpha 3(IV)/IV \), \( \alpha 4(IV)/IV \) and \( \alpha 5(IV)/IV \) in the glomeruli of patients with AS and TBMN

<table>
<thead>
<tr>
<th>Patients with X-linked AS</th>
<th>( \alpha 3/IV )</th>
<th>Col IV</th>
<th>( \alpha 3/IV )</th>
<th>( \alpha 5/IV )</th>
<th>Col IV</th>
<th>( \alpha 5/IV )</th>
<th>( \alpha 4/IV )</th>
<th>Col IV</th>
<th>( \alpha 4/IV )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1685.9</td>
<td>1666.7</td>
<td>1.01</td>
<td>1575.2</td>
<td>2087.9</td>
<td>0.75</td>
<td>1762.5</td>
<td>1798.5</td>
<td>0.98</td>
</tr>
<tr>
<td>B</td>
<td>1671.1</td>
<td>1836.4</td>
<td>0.91</td>
<td>1288.8</td>
<td>2406.5</td>
<td>0.54</td>
<td>1489.5</td>
<td>1752.3</td>
<td>0.85</td>
</tr>
<tr>
<td>C</td>
<td>1906.5</td>
<td>1777.7</td>
<td>1.07</td>
<td>1896.4</td>
<td>2222.1</td>
<td>0.85</td>
<td>1559.2</td>
<td>1658.7</td>
<td>0.94</td>
</tr>
<tr>
<td>D</td>
<td>1718.8</td>
<td>1431.2</td>
<td>1.20</td>
<td>1893.5</td>
<td>1179.6</td>
<td>1.61</td>
<td>1681.3</td>
<td>1235.5</td>
<td>1.36</td>
</tr>
<tr>
<td>E</td>
<td>2155.9</td>
<td>1351.7</td>
<td>1.59</td>
<td>2228.3</td>
<td>1360.0</td>
<td>1.64</td>
<td>2120.6</td>
<td>1442.6</td>
<td>1.47</td>
</tr>
<tr>
<td>Patients suggestive of X-linked AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1659.7</td>
<td>1784.6</td>
<td>0.93</td>
<td>1821.5</td>
<td>1917.3</td>
<td>0.95</td>
<td>2010.2</td>
<td>1526.0</td>
<td>1.32</td>
</tr>
<tr>
<td>G</td>
<td>1532.3</td>
<td>1824.7</td>
<td>0.74</td>
<td>1576.1</td>
<td>2101.5</td>
<td>0.75</td>
<td>1314.0</td>
<td>1932.4</td>
<td>0.68</td>
</tr>
<tr>
<td>H</td>
<td>2020.6</td>
<td>2432.2</td>
<td>0.83</td>
<td>1569.4</td>
<td>2350.6</td>
<td>0.67</td>
<td>1847.1</td>
<td>2040.2</td>
<td>0.91</td>
</tr>
<tr>
<td>I</td>
<td>2001.3</td>
<td>1380.2</td>
<td>1.45</td>
<td>1958.2</td>
<td>1386.4</td>
<td>1.41</td>
<td>2003.2</td>
<td>1464.2</td>
<td>1.37</td>
</tr>
<tr>
<td>Patients with TBMN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>2138.3</td>
<td>1469.9</td>
<td>1.45</td>
<td>1883.2</td>
<td>1451.5</td>
<td>1.30</td>
<td>2442.1</td>
<td>1554.1</td>
<td>1.57</td>
</tr>
<tr>
<td>N</td>
<td>2380.7</td>
<td>1383.1</td>
<td>1.72</td>
<td>1694.6</td>
<td>1394.2</td>
<td>1.22</td>
<td>2248.9</td>
<td>1377.0</td>
<td>1.63</td>
</tr>
<tr>
<td>O</td>
<td>1751.3</td>
<td>1233.3</td>
<td>1.42</td>
<td>1662.7</td>
<td>1057.5</td>
<td>1.57</td>
<td>1875.7</td>
<td>1123.2</td>
<td>1.67</td>
</tr>
<tr>
<td>P</td>
<td>1950.0</td>
<td>1455.2</td>
<td>1.34</td>
<td>1908.4</td>
<td>1281.2</td>
<td>1.49</td>
<td>2647.6</td>
<td>1475.4</td>
<td>1.79</td>
</tr>
</tbody>
</table>

IFI, immunofluorescence intensity; Col IV, type IV collagen.
type IV collagen and the ratio of $\alpha_5(IV)/\alpha_1$ was within the normal range compared with the normal subjects. As TBMN is an autosomal dominant disease caused by mutations in \textit{COL4A3} or \textit{COL4A4} [30,31], the reduced absolute amount of $\alpha_3(IV)$ chain with normal $\alpha_3(IV)$ and $\alpha_4(IV)$ chains is not clear. Confocal analysis will be an alternative technique to consider for differentiating AS and TBMN.

In conclusion, this is the first report to quantitatively analyse type IV collagen subchains in the GBM of patients with AS and the first to find that most of the patients appear to have decreased ratios of $\alpha_3(IV)/\alpha_4(IV)$ or $\alpha_5(IV)/\alpha_1$. This observation increases our knowledge of the molecular pathogenesis of AS and also provides an alternative approach for making a diagnosis on atypical AS patients.

Conflict of interest statement. None declared.

References

2. Mariyama M, Zheng KG, Yang FT, Reenders ST. Colocalization of the genes for the $\alpha_3(IV)$ and $\alpha_4(IV)$ chains of type-IV collagen to chromosome 2 bands 2-q35–q37. \textit{Genomics} 1992; 13: 809–813
15. Miner JH, Sanes JR. Collagen IV $\alpha_3$, $\alpha_4$, and $\alpha_5$ chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. \textit{J Cell Biol} 1994; 127: 879–891

Received for publication: 28.9.05
Accepted in revised form: 15.2.06