

## Pathological features of proteinuric nephropathy resembling Alport syndrome in a young Pyrenean Mountain dog

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**ABSTRACT.** The renal biopsy tissue from a 9-month-old, male Pyrenean Mountain dog with renal disorder and severe proteinuria was examined. Ultrastructural examination revealed multilaminar splitting and fragmentation of the glomerular basement membrane (GBM) and diffuse podocyte foot process effacement. Immunofluorescent staining for  $\alpha$ (IV) chains revealed presence of  $\alpha$ 5(IV) and complete absence of  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains in the GBM. Immunohistochemistry also revealed decreased and altered expression of nephrin and podocin in the glomeruli compared with normal canine glomeruli. These results suggested that the glomerular disease of the present case might be consistent with canine hereditary nephropathy resembling human Alport syndrome caused by genetic defect of type IV collagen, and indicated possible contribution of podocyte injury to severe proteinuria in this case.

**KEY WORDS:** Alport syndrome, canine, hereditary nephropathy, type IV collagen

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Hereditary nephropathy (HN) resulting from a defect of type IV collagen (collagen IV), a main component of the glomerular basement membrane (GBM), has been identified in several domestic dog breeds, and the symptom profile is analogous to human Alport syndrome (AS), except for the hearing loss and blindness [11, 14]. Human AS is a genetic disorder caused by an abnormality of collagen IV and characterized by progressive renal disease, typically involving hematuria, proteinuria, hearing loss and blindness [3, 9]. In human and dogs, the disease is diagnosed by presence of characteristic ultrastructural features of the GBM, immunofluorescence staining (IF) results using antibodies specific for the  $\alpha$ (IV) chain and genetic testing results. In particular, IF for several  $\alpha$ (IV) chains is a most reliable and useful diagnostic method and also can distinguish the autosomal type from the X-linked form due to the following characteristics of collagen IV [14]. Collagen IV has six distinct  $\alpha$ (IV) chains [designated as  $\alpha$ 1(IV) –  $\alpha$ 6(IV)] encoded by six genes (designated as *COL4A1* – *COL4A6*). Three different heterotrimeric units (designated as  $\alpha$ 1/ $\alpha$ 1/ $\alpha$ 2,  $\alpha$ 3/ $\alpha$ 4/ $\alpha$ 5 and  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6) are formed from the six  $\alpha$ (IV) chains [9], and these heterotrimers have different distributional features:  $\alpha$ 1/ $\alpha$ 1/ $\alpha$ 2 trimers widely distribute in the basement membrane (BM);  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6 trimers distribute mainly in Bowman's capsule

BM and tubular BM; and  $\alpha$ 3/ $\alpha$ 4/ $\alpha$ 5 trimers are limited to the GBM in the kidney [9, 14]. Mutations in *COL4A3*, *COL4A4* or *COL4A5* result in loss of  $\alpha$ 3/ $\alpha$ 4/ $\alpha$ 5 trimers from the GBM, leading to dysfunction of glomerular filtration. Therefore, it is useful for the diagnosis of human AS and canine HN to confirm the distribution of several  $\alpha$ (IV) chains within renal tissue using immunostaining.

The glomerular slit diaphragm (SD) is located between foot processes (FPs) of the podocytes and plays a critical role in selective filtration together with the GBM. SD-associated proteins, such as nephrin and podocin, are expressed in FPs and SDs, and altered expression of these proteins has been described in pathological situations involving podocyte injuries related to proteinuria in humans and in animal models [12, 21], including an AS mouse model [6, 23]. Expression of these proteins in normal dogs was demonstrated in a previously reported study [13]; another report described altered expression of these proteins in several glomerular diseases of dogs [10]; but it has not, to our knowledge, been described in canine HN. Here, we describe pathological features of proteinuric nephropathy resembling AS in a young Pyrenean Mountain dog and demonstrate altered expression of nephrin and podocin in the glomeruli.

The case was a 9-month-old, male Pyrenean Mountain dog with azotemia (BUN: 59.1 mg/dl, creatinine: 2.66 mg/dl), hypoalbuminaemia (albumin: 1.8 g/dl), proteinuria (urinary protein-to-creatinine ratio: 7.4 when first seen and 5.4 when biopsy was performed) and occult hematuria. The familial history of the case was unknown. A renal biopsy specimen was obtained by using a 14-gauge Tru-cut-type needle. Renal tissue from a healthy 12-month-old, male beagle dog was used for comparison.

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Two-thirds of the biopsy tissue was fixed in 10% neutral-buffered formalin and then embedded in paraffin wax. Sections (3  $\mu$ m) then were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) reagents for histological examination. For ultrastructural examination, the other one-third of the biopsy tissue was fixed in 2.5% glutaraldehyde and post-fixed in 1% OsO<sub>4</sub>. Fixed specimens were then dehydrated through ascending grades of alcohol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a JEOL 1210 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

For IF, rat monoclonal antibodies established in previous studies [7, 20, 24] were used as following specificity; H11, H22, H31 and H43 recognize, respectively, the human NC1 domain of the  $\alpha$ 1(IV),  $\alpha$ 2(IV),  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains, and H53, H65 and B66 recognize, respectively, the human helix of the  $\alpha$ 5(IV),  $\alpha$ 6(IV) chains and the bovine helix of the  $\alpha$ 6(IV) chain. The sections were pretreated by Target Retrieval Solution, pH 6.1 (Nichirei, Tokyo, Japan) at 100°C for 10 min in an autoclave and by collagenase, pH 7.6, 100 U/ml (Sigma, St. Louis, MO, U.S.A.; C2674) at 37°C for 30 min. As described in our previous report [13], nephrin and podocin were detected by using rabbit anti-human podocin (Sigma) and rabbit anti-canine nephrin established in our laboratory. The sections were pretreated by Target Retrieval Solution, pH 9.0 (Nichirei) at 121°C for 5 min in an autoclave (nephrin) or by trypsin (Sigma; T7168) at 37°C for 30 min (podocin). The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-rat IgG (Cappel, Aurora, OH, U.S.A.) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Tokyo, Japan).

Four exons (exons 3 and 30 of *COL4A4* and exons 9 and 35 of *COL4A5*) were previously reported as causative gene mutation sites in HN dogs [14]. Therefore, these exons were amplified by polymerase chain reaction (PCR) using genomic DNA from whole blood of the current case. Sequencing of the amplicons was performed by Dragon GenomicsCenter, TAKARA BIO INC. (Otsu, Japan).

Microscopically, only mild irregular thickening of the capillary walls and cystic dilation with hemorrhage of the Bowman's capsule were observed (Fig. 1). Mild interstitial inflammation, fibrosis and interstitial foam cells were observed.

The ultrastructural appearance of the GBM included distinctive morphological features of HN, namely, irregular and global thickening of the GBM with multilaminar splitting and fragmentation of the lamina densa (Fig. 2). Severe FP effacement of the podocytes was also observed.

By IF, the pattern of labeling for each  $\alpha$ (IV) chain in the case was different from that in the normal dog. The  $\alpha$ 1(IV) and  $\alpha$ 2(IV) chains were distributed in all basement membranes (BM) and mesangial regions in both animals; however, an increased intensity of these chains was observed in the case (Fig. 3A and 3B). Labeling of the  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains in the GBM was completely absent in the case (Fig. 3C and 3D). Although the intensity was reduced compared with the normal dog, labeling of the  $\alpha$ 5(IV) chain

was observed in the GBM and Bowman's capsule BM in the case (Fig. 3E). Additionally, labeling of the  $\alpha$ 6(IV) chain was observed in the GBM of the case, but not in the normal dog (Fig. 3F).

Expression of nephrin and podocin was in a sharp linear pattern along the GBM in the normal glomerulus. On the other hand, the nephrin staining pattern was shifted to a cytoplasmic and granular pattern in the case (Fig. 4A). Expression of podocin was decreased and its staining pattern was changed to a granular pattern, although remaining along the GBM (Fig. 4B).

The 4 previously-reported gene mutations of the  $\alpha$ (IV) chains causative of canine HN, mentioned above, were not detected (data not shown).

Numerous mutation sites are confirmed in *COL4A3*, *COL4A4* or *COL4A5* gene in human AS [1, 18]. They are scattered throughout many exons, and there are no hotspots, making AS difficult to diagnose using genetic testing. In the current case, mutation was not detected at any previously-reported mutation sites in HN dogs. However, we diagnosed this case as HN resembling AS based on the typical ultrastructural findings of GBM and disappearance of  $\alpha$ 3(IV) and  $\alpha$ 4(IV) chains from the renal tissue. Additionally, appearance of  $\alpha$ 5(IV) chains in the GBM and Bowman's capsule indicated autosomal type HN, because *COL4A3* and *COL4A4* are on chromosome 25, and *COL4A5* and *COL4A6* are on the X chromosome in dogs [17]. Therefore, in the X-linked type, because a defect of  $\alpha$ 5(IV) chains causes loss of  $\alpha$ 3/ $\alpha$ 4/ $\alpha$ 5 and  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6 trimers, all  $\alpha$ 3(IV),  $\alpha$ 4(IV),  $\alpha$ 5(IV) and  $\alpha$ 6(IV) chains should disappear from both the GBM and Bowman's capsule BM. Although increased expression of  $\alpha$ 1/ $\alpha$ 1/ $\alpha$ 2 trimers in the glomeruli is commonly observed in human AS and canine HN, increased expression of  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6 trimers was only reported in the autosomal type of canine HN [16, 20]. In a previous study, expression of  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6 trimers in the GBM was normally observed in normal dogs more than about 45 months old, but not in those younger than 30 months [15], whereas it was not observed in humans regardless of age [19, 22]. This characteristic property of normal dogs may relate to the expression of  $\alpha$ 5/ $\alpha$ 5/ $\alpha$ 6 trimers in young dogs with the autosomal type canine HN.

In human AS and canine HN, mutations of collagen IV cause GBM abnormalities and subsequent podocyte injuries lead to massive proteinuria, and finally cause end-stage renal disease. In general, it is thought that podocyte injury with loss of the SDs is a major cause of marked albuminuria and nephrotic syndrome [2, 6]. However, proteinuria in AS, especially in the early stages of the disease, is thought to be associated with damage to the GBM rather than to primary loss of the SDs and FP effacement, and it is considered to be a secondary response to proteinuria and the abnormal GBM [5, 8]. But, at the same time, it has been indicated that multiple glomerular cell types participate in the pathogenic process underlying AS progression and podocyte damage may be a precipitating factor in disease progression [4, 6, 23]. In the previous reports of canine HN, FP effacement was noted, but expression of SD-associated proteins was not examined. In this case, massive proteinuria and also altered expression

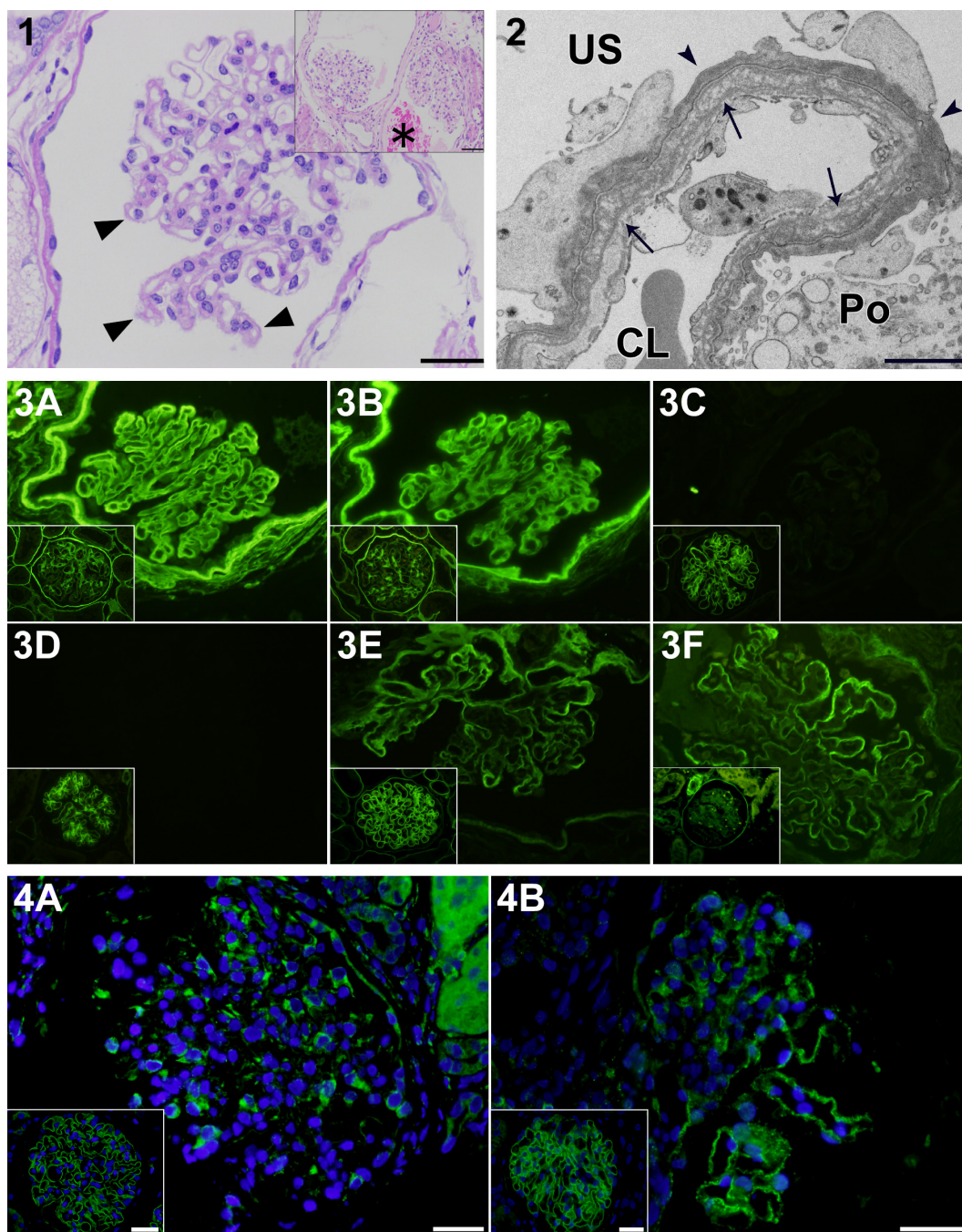


Fig. 1. Light microscopic features of the biopsy specimen. Mild and irregular thickening of the capillary walls is observed (arrowheads). PAS stain. Bar, 30  $\mu\text{m}$ . Inset: cystic dilation with hemorrhage of the Bowman's capsule is observed (asterisk). H.E. stain. Bar, 50  $\mu\text{m}$ .

Fig. 2. Transmission electron microscopic features of the biopsy specimen. The lamina densa of the GBM is irregularly and globally thickened with multilaminar splitting and fragmentation (arrows). Severe foot process effacement is also observed (arrowheads). CL, capillary lumen; US, urinary space; Po, podocyte. TEM. Bar, 2  $\mu\text{m}$ .

Fig. 3. Immunofluorescence for several  $\alpha(\text{IV})$  chains in the glomeruli of the case and a normal dog (respective insets).  $\alpha 1(\text{IV})$  and  $\alpha 2(\text{IV})$  chains (A and B, respectively) were increased in the GBM;  $\alpha 3(\text{IV})$  and  $\alpha 4(\text{IV})$  chains (C and D, respectively) were completely absent in the GBM;  $\alpha 5(\text{IV})$  and  $\alpha 6(\text{IV})$  chains (E and F, respectively) were observed in both the GBM and Bowman's capsule BM. IF.  $\times 400$ . Inset: expression pattern of respective  $\alpha(\text{IV})$  chains in the normal glomeruli.

Fig. 4. Immunofluorescence staining for nephrin (A) and podocin (B) in the glomeruli of the case and a normal dog (respective insets). Expression of both molecules in the case was decreased, and the staining patterns were shifted from the sharp linear pattern observed along the glomerular capillary walls of normal glomeruli to a coarse granular pattern. IF. Bar, 3  $\mu\text{m}$ .

of these SD-associated proteins were observed, accompanied by severe FP effacement and GBM abnormality. As in the AS mouse models [4, 6, 23], the GBM abnormality might be followed by severe podocyte injury in this case.

In conclusion, the GBM abnormality caused by genetic defect of collagen IV in canine HN might associate podocyte injury leading to proteinuria and progress of the disease.

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## REFERENCES

- Artuso, R., Fallerini, C., Dosa, L., Scionti, F., Clementi, M., Garosi, G., Massella, L., Epistolato, M. C., Mancini, R., Mari, F., Longo, I., Ariani, F., Renieri, A. and Bruttini, M. 2012. Advances in Alport syndrome diagnosis using next-generation sequencing. *Eur. J. Hum. Genet.* **20**: 50–57. [Medline] [CrossRef]
- Brinkkoetter, P. T., Ising, C. and Benzing, T. 2013. The role of the podocyte in albumin filtration. *Nat. Rev. Nephrol.* **9**: 328–336. [Medline] [CrossRef]
- Cosgrove, D. 2012. Glomerular pathology in Alport syndrome: a molecular perspective. *Pediatr. Nephrol.* **27**: 885–890. [Medline] [CrossRef]
- Cosgrove, D., Rodgers, K., Meehan, D., Miller, C., Bovard, K., Gilroy, A., Gardner, H., Kotelianski, V., Gotwals, P., Amatucci, A. and Kalluri, R. 2000. Integrin  $\alpha$ 1 $\beta$ 1 and transforming growth factor- $\beta$ 1 play distinct roles in alport glomerular pathogenesis and serve as dual targets for metabolic therapy. *Am. J. Pathol.* **157**: 1649–1659. [Medline] [CrossRef]
- Gubler, M. C. 2008. Inherited diseases of the glomerular basement membrane. *Nat. Clin. Pract. Nephrol.* **4**: 24–37. [Medline] [CrossRef]
- Hamano, Y., Grunkemeyer, J. A., Sudhakar, A., Zeisberg, M., Cosgrove, D., Morello, R., Lee, B., Sugimoto, H. and Kalluri, R. 2002. Determinants of vascular permeability in the kidney glomerulus. *J. Biol. Chem.* **277**: 31154–31162. [Medline] [CrossRef]
- Harvey, S. J., Zheng, K., Jefferson, B., Moak, P., Sado, Y., Naito, I., Ninomiya, Y., Jacobs, R. and Thorner, P. S. 2003. Transfer of the  $\alpha$ 5(IV) Collagen Chain Gene to Smooth Muscle Restores in Vivo Expression of the  $\alpha$ 6(IV) Collagen Chain in a Canine Model of Alport Syndrome. *Am. J. Pathol.* **162**: 873–885. [Medline] [CrossRef]
- Heidet, L., Cai, Y., Guicharnaud, L., Antignac, C. and Gubler, M. C. 2000. Glomerular expression of type IV collagen chains in normal and X-linked Alport syndrome kidneys. *Am. J. Pathol.* **156**: 1901–1910. [Medline] [CrossRef]
- Hudson, B. G., Tryggvason, K., Sundaramoorthy, M. and Neilson, E. G. 2003. Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N. Engl. J. Med.* **348**: 2543–2556. [Medline] [CrossRef]
- Ichii, O., Yabuki, A., Sasaki, N., Otsuka, S., Ohta, H., Yamasaki, M., Takiguchi, M., Namiki, Y., Hashimoto, Y., Endoh, D. and Kon, Y. 2011. Pathological correlations between podocyte injuries and renal functions in canine and feline chronic kidney diseases. *Histol. Histopathol.* **26**: 1243–1255. [Medline]
- Kashtan, C. E. 2002. Animal models of Alport syndrome. *Nephrol. Dial. Transplant.* **17**: 1359–1362. [Medline] [CrossRef]
- Kawachi, H., Miyauchi, N., Suzuki, K., Han, G. D., Orikasa, M. and Shimizu, F. 2006. Role of podocyte slit diaphragm as a filtration barrier (Review Article). *Nephrology (Carlton)* **11**: 274–281. [Medline] [CrossRef]
- Kobayashi, R., Kamiie, J., Yasuno, K., Ogihara, K. and Shiota, K. 2011. Expression of nephrin, podocin, alpha-actinin-4 and alpha3-integrin in canine renal glomeruli. *J. Comp. Pathol.* **145**: 220–225. [Medline] [CrossRef]
- Lees, G. E. 2013. Kidney diseases caused by glomerular basement membrane type IV collagen defects in dogs. *J. Vet. Emerg. Crit. Care (San Antonio)* **23**: 184–193. [Medline] [CrossRef]
- Lees, G. E., Kashtan, C. E., Michael, A. F., Helman, R. G., Naito, I., Ninomiya, Y., Sado, Y. and Kim, Y. 2000. Expression of the alpha6 chain of type IV collagen in glomerular basement membranes of healthy adult dogs. *Am. J. Vet. Res.* **61**: 38–41. [Medline] [CrossRef]
- Lees, G. E., Helman, R. G., Kashtan, C. E., Michael, A. F., Homco, L. D., Millichamp, N. J., Ninomiya, Y., Sado, Y., Naito, I. and Kim, Y. 1998. A model of autosomal recessive Alport syndrome in English cocker spaniel dogs. *Kidney Int.* **54**: 706–719. [Medline] [CrossRef]
- Lowe, J. K., Guyon, R., Cox, M. L., Mitchell, D. C., Lonkar, A. L., Lingaas, F., Andre, C., Galibert, F., Ostrander, E. A. and Murphy, K. E. 2003. Radiation hybrid mapping of the canine type I and type IV collagen gene subfamilies. *Funct. Integr. Genomics* **3**: 112–116. [Medline] [CrossRef]
- Myers, J. C., Jones, T. A., Pohjolainen, E. R., Kadri, A. S., Goddard, A. D., Sheer, D., Solomon, E. and Pihlajaniemi, T. 1990. Molecular cloning of alpha 5(IV) collagen and assignment of the gene to the region of the X chromosome containing the Alport syndrome locus. *Am. J. Hum. Genet.* **46**: 1024–1033. [Medline]
- Ninomiya, Y., Kagawa, M., Iyama, K., Naito, I., Kishiro, Y., Seyer, J. M., Sugimoto, M., Oohashi, T. and Sado, Y. 1995. Differential expression of two basement membrane collagen genes, COL4A6 and COL4A5, demonstrated by immunofluorescence staining using peptide-specific monoclonal antibodies. *J. Cell Biol.* **130**: 1219–1229. [Medline] [CrossRef]
- Nowend, K. L., Starr-Moss, A. N., Lees, G. E., Berridge, B. R., Clubb, F. J., Kashtan, C. E., Nabity, M. B. and Murphy, K. E. 2012. Characterization of the genetic basis for autosomal recessive hereditary nephropathy in the English Springer Spaniel. *J. Vet. Intern. Med.* **26**: 294–301. [Medline] [CrossRef]
- Pavenstädt, H., Kriz, W. and Kretzler, M. 2003. Cell biology of the glomerular podocyte. *Physiol. Rev.* **83**: 253–307. [Medline] [CrossRef]
- Peissel, B., Geng, L., Kalluri, R., Kashtan, C., Rennke, H. G., Gallo, G. R., Yoshioka, K., Sun, M. J., Hudson, B. G. and Neilson, E. G. 1995. Comparative distribution of the alpha 1(IV), alpha 5(IV), and alpha 6(IV) collagen chains in normal human adult and fetal tissues and in kidneys from X-linked Alport syndrome patients. *J. Clin. Invest.* **96**: 1948–1957. [Medline] [CrossRef]
- Ryu, M., Mulay, S. R., Miosge, N., Gross, O. and Anders, H. J. 2012. Tumour necrosis factor-alpha drives Alport glomerulosclerosis in mice by promoting podocyte apoptosis. *J. Pathol.* **226**: 120–131. [Medline] [CrossRef]
- Sado, Y., Kagawa, M., Kishiro, Y., Sugihara, K., Naito, I., Seyer, J. M., Sugimoto, M., Oohashi, T. and Ninomiya, Y. 1995. Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different alpha chains of human type IV collagen. *Histochem. Cell Biol.* **104**: 267–275. [Medline] [CrossRef]