

Identification of co-existence of Alport syndrome and C3 glomerulonephritis in a proband with family history

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Research

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Abstract

Background

Alport syndrome and C3 glomerulonephritis (C3GN) are rare kidney diseases, frequently responsible for familial haematuria, proteinuria, and renal impairment. With the rapid development of molecular genetic testing, Alport syndrome causes have been restricted mostly to variants in the *COL4A5* or *COL4A3/COL4A4* genes. Moreover, a broad range of genetic contributors in the complement and complement-regulating proteins are definitely implicated in the pathogenesis of C3GN.

Methods

We sought a family with persistent microscopic haematuria associated with renal failure. Clinicopathologic and follow-up data were obtained, and molecular genetic testing was used to screen for pathogenic variants.

Results

We describe a three-generation family with Alport syndrome showing a dominant maternal inheritance. Notably, renal biopsy showed the concurrent histological evidence of C3GN in the proband harbouring an uncommon heterozygous variation in *CFHR5*, c.508G>A. The alteration leads to replacement of a highly conserved residue at position 170 of the β -strand subunit of *CFHR5* (p.Val170Met). In silico analysis showed that the variation was predicted to deregulate complement activation by altering the structural properties and enhancing C3b binding capacity to compete with Complement Factor H (CFH), which was in line with experimental data previously published.

Conclusions

The comorbidity findings between Alport syndrome and C3GN indicate an underlying overlap and require further study.

Background

In China, a rare disease is defined as a condition that affects fewer than 500,000 people [1]. Approximately 80% of rare diseases have an identified genetic background with substantial geographic or ethnic variations in incidence [2].

Genetic analysis was first used in nephrology for the identification of a causal mutation for Alport syndrome in 1990 [3]. Alport syndrome is a rare hereditary disorder in which the glomerular basement membrane (GBM) have an abnormal collagen IV composition. It is characterized by haematuria, progressive renal failure, sensorineural deafness, anterior lenticonus and retinal flecks [4]. To date, the causes of familial haematuria nephropathies have been restricted mostly to variants in the *COL4A5* or *COL4A3/COL4A4* genes, responsible for X-linked or autosomal inheritance [5-7]. Moreover, analysis of

data suggests that familial microscopic haematuria attributable to the complement factor H-related protein 5 (*CFHR5*) gene is also associated with a rare renal disease termed C3 glomerulonephritis (C3GN) [8-12], defined as glomerular C3 position in the absence of significant amounts of immunoglobulin, C1q, and C4, and without intramembranous glomerular basement membrane dense deposits.

Herein, we report a family with dominant maternal transmitted inheritance, in which five out of eight members (I:1, II:1, II:4, III:1, and III:2) exhibited microscopic haematuria and proteinuria and two out of the five patients (II:1 and III:1) developed end-stage renal disease (ESRD). The diagnosis of Alport syndrome was confirmed in the proband (III:1), his mother (II:1), and his maternal aunt's daughter (III:2) based on all available data. Furthermore, renal biopsy showed the comorbidity of C3GN and Alport syndrome in the proband based on an uncommon heterozygous c.508G>A (p.Val170Met) variant in the *CFHR5* gene. Moreover, we presented several lines of evidence to support the contention that this variant is pathogenic and may contribute to the genetic susceptibility to C3GN.

Methods

Family pedigree and clinical investigations

In this study, a family with persistent microscopic haematuria associated with renal failure was recruited from Hangzhou, Zhejiang Province, China. All affected members were regularly followed up at the out-clinic until May 12, 2020. A brief history and physical examination were performed and blood, urine, skin, kidney samples were collected for determination of serum creatinine concentration, estimated glomerular filtration rate (eGFR), urinalysis, C3/C4 levels and histological manifestation.

Renal and skin biopsies processing

Biopsy specimens for light microscopy were fixed in alcohol formaldehyde acetic solution, embedded in paraffin, and cut into 2µm-thick sections. Sections were stained with Masson trichrome, hematoxylin and eosin, periodic acid–Schiff, and silver methenamine. Samples for immunofluorescence study were stained with fluorescein isothiocyanate-conjugated polyclonal antibodies to human IgA, IgG, IgM; C3; light chains (κ and λ); C4, C1q; and α5 chains of type IV collagen. Antigen was retrieved by EDTA solution as well as gastric enzyme, and elivision system was applied in IHC detection. Specimens for electron microscopy were fixed in glutaraldehyde and embedded in epon. All reports were reviewed by two pathologists.

Targeted exon sequencing and Sanger sequencing

Genomic DNA was isolated from peripheral blood of a three-generation pedigree of 8 members using the Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. Coding exons reference sequences of the individuals (II:1, II:2, III:1 and III:2) were targeted for the Illumina high-through sequencing, and reads were aligned to the human genome reference sequence (hg19/GRCh37). The approximately 95% of reads were mapped to the target regions at an average of 20×. We examined

copy number, rare, and common variants. All the disease-related sites were selected and Sanger sequencing was further performed in all 8 members. Primers were indigenously designed using the primer premier 5.0 program (Lalitha, 2000) and shown in Supplementary Table S1. The purified PCR products were directly sequenced using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit. The analyses were completed on an ABI-3500Dx Genetic Analyzer (Applied Biosystems).

In silico evaluation of pathogenicity

The ProtParam tool was used to compute various physical and chemical parameters including the molecular weight and theoretical pI. The complete amino acid sequence data of the human protein *CFHR5* (GenBank accession: AAI11774.1) were obtained from the NCBI (National Center for Biotechnology Information) and alignments done by the EMBL-EBI (European Bioinformatics Institute). The SIFT (Sorting Intolerant From Tolerant), SNAP (Screening for Non-acceptable Polymorphisms), PolyPhen-2 (Polymorphism Phenotyping v2), and Mutation Taster were utilized to evaluate possible biologic effects of genetic aberration impact on protein structure.

Variant interpretation

In 2015, standards and guidelines for the interpretation of sequence variants were updated by the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP) and the College of American Pathologists (CAP) [13]. This report recommends the use of specific standard terminology: 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign', and 'benign' to describe variants. This report recommends the use of specific standard terminology: 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign', and 'benign' to describe variants, based on criteria using typical types of variant evidence (e.g., population data, computational and predictive data, functional data, segregation data and de novo data).

In brief, an allele frequency in a control population that is greater than expected for disorder or with lack of segregation among affected individuals was considered strong support for a benign interpretation or, if over 5%, it is considered as 'benign'. Novel or rare variants that lead to splicing defect or amino acid change coupled with multiple lines of computational evidence, or cosegregate with disease, were classified as 'pathogenic' or 'likely pathogenic', and variants with well-established in vitro or in vivo functional studies that indicate a damaging effect were labeled 'pathogenic'. Variants of 'uncertain significance' are more common: other criteria shown above are not met or the criteria for benign and pathogenic are contradictory.'

Homology modeling of human *CFHR5*/C3b complex

Docking procedure has been described in more detail previously [14]. Starting from residues 23-569, the 3D homology models of *CFHR5* were first generated by iterative threading assembly refinement (I-TASSER) server [15], where the one with the highest C-score was selected to be further refined by Fragment-Guided MD simulation (FG-MD) [16]. Potential energy of refined protein was calculated by

“Calculate Energy” protocol of Discovery Studio (DS) 3.0. The crystal structure of ligand C3b (PDB ID: 2WII) was taken from protein data bank (PDB).

By applying “Prepare Protein” protocol of DS, energy minimization was performed to clean the protein molecule by adding missing atoms, inserting missing loops, assigning charges and fixing CHARMM force fields. A total of 2000 docking poses were generated by ZDOCK (CHARMM-based DOCKER) protocol and incorporated. The top1 model was collected as the probable complex structure. Hydrogen-bonding networks can be displayed by Molecular Operating Environment (MOE). Binding free energy of protein-protein interaction was estimated by the Calculate Mutation Energy (Binding) protocol within DS.

Results

Clinical presentation and follow-up

We investigated a three-generation Han Chinese family characterized by persistent microscopic haematuria associated with renal failure (Fig. 1a). The proband (III:1), a 11-year-old boy, was admitted to the hospital for haematuria and proteinuria. On admission, 24-h urine protein was 6.36 g/d and serum albumin was 24.4 g/L. Microscopic urinary analysis showed urine sediment containing ++~+++ red cells per high-power field. His blood urea nitrogen level was 15.73 mmol/L, serum creatinine level was 266 μ mol/L and eGFR by CKD-EPI equation was 30.3 mL/min. Bilateral renal ultrasound revealed abnormal changes with strong and unevenly distributed echoes, and unclear demarcation between the renal cortex and parenchyma. Sensorineural hearing impairment and vision abnormalities, as well as other clinical signs, were not detected.

During follow-up, the proband was started on haemodialysis three months later and received a kidney transplant after one year. The proband (III:1) had a family history of haematuria, as his mother (II:1) presented with haematuria, proteinuria, and developed ESRD before the age of 33 years old. His maternal grandma (I:1), maternal aunt (II:4), and aunt’s daughter (III:2) also had urinary abnormalities characterized by asymptomatic haematuria and proteinuria with normal eGFR. His father (II:2), his maternal grandpa, and aunt’s husband were phenotypically normal.

To confirm the clinical diagnosis, renal biopsy of the proband was performed. The biopsy sample contained fifteen glomeruli on light microscopy: global glomerular sclerosis in four glomeruli, segmental sclerosis in three other glomeruli (Fig. 2a), and four crescents were found (Fig. 2b). Diffuse and global mild-moderate proliferation of mesangial cells and mesangial matrix were shown in the remaining glomeruli (Fig. 2c). Besides, interstitial fibrosis/tubular atrophy containing lipid-laden foam cells were obvious (Fig. 2d). Immunofluorescence studies showed patchy GBM expression of α 5 (IV) collagen and complete absence in Bowman’s capsule and distal tubular basement membrane (BM) (Fig. 2e), compared with control (Fig. 2f). A skin biopsy was performed on his mother (II:1), which revealed that there was

segmental absence of the collagen IV $\alpha 5$ chain within the epidermal basement membrane (Fig. 2g), compared with control (Fig. 2h).

Electron microscopy identified pathological characteristics accompanied with noticeably segmental uneven thickness with unsmooth spiculation and suspected lamination of the dense layer within the basement membrane in the proband (Fig. 2i). His affected aunt's daughter (III:2) had a renal biopsy at age 13, showing segmental uneven thickness with a laminated appearance of the dense layer in GBM (Fig. 2j).

Notably, a marked C3 deposition (++ to +++) in the mesangial region was detected in the proband under immunofluorescence microscopy (Fig. 2k), with the corresponding lump electron-dense deposits and mild-moderate mesangial proliferation observed with electron microscopy (Fig. 2l). Immunofluorescence examination revealed none or trace positivity of IgA, IgG, IgM, C4, C1q, Fib, κ and λ fragments.

Findings on targeted exome-based next-generation sequencing (NGS) and Sanger sequencing

To make a precise diagnosis for the true pathogenic mechanism affecting the family, we performed whole exome-based NGS and further confirmed the results with Sanger sequencing. Genetic analysis identified the same heterozygous c.508G>A coding variant (p.Val170Met) in exon 6 of the *CFHR5* gene in three affected individuals: the proband (III:1), his mother (II:1) and his maternal grandma (I:1) (Fig. 1b). The variant presents low frequent in the general population (1000 Genomes: minor allele frequency [MAF] = 0.1%), and the mutated A allele carriers are all heterozygous. Compared across ethnicities, this variant is more frequent in East Asians (1000 Genomes: 0.6%), especially in the Chinese Han population (1000 Genomes: 1%). The nonsynonymous alteration leads to the replacement of a valine, strictly conserved among organisms (Fig. 3a), by a methionine residue. The screening with four publicly available programs (SIFT, SNAP, PolyPhen-2, and Mutation Taster) independently predicted that the replaced amino acid was "damaging" to the protein structure/function (SIFT score 0.004; SNAP score 20; PolyPhen2 score 0.999; Mutation Taster: might be affected).

No suspicious disease-causing variants were detected in the genes *COL4A3*, *COL4A4*, or *COL4A5* that encode the $\alpha 3$, $\alpha 4$, or $\alpha 5$ chains of type IV collagen, respectively.

In silico functional prediction for *CFHR5* c.508G>A, p.Val170Met

CFHR5 is a single-chain polypeptide composed of nine complement control protein domains (also known as CCPs). The residue p.Val170Met in *CFHR5* occurs in the β -strand region of the CCP3. The isoelectric point (pI) is found to be the same for both wild type and mutant proteins (pI 6.8). Molecular weight of the mutant (64.45 kDa) protein is similar to that of wild-type protein (64.42 kDa). In the native structure, the Val residue, located in the buried surface, is not involved in any intramolecular interactions. As substituted by Met170, the original intramolecular hydrogen-bonding distance between the side chain of its neighbouring residues (Leu171 and Val187) changes (Fig. 3b). DS 3.0 analysis predicted the potential energy of the mutant type protein is -15180.97 kcal/mol compared to -15133.86 kcal/mol for the wild

type one, which implied that p.Val170Met could lead to an increase in conformational stability of the *CFHR5* protein.

As with Complement Factor H (CFH) and other Complement Factor H Related Proteins (CFHRs), *CFHR5* regulates the complement cascade by binding and interacting with the macromolecular protein ligand C3b. The C3b fragment is a glycoprotein composed of the modified C3- α chain (C3 α') and the intact C3- β chain (C3 β). We then simulated the probable native *CFHR5*/C3 β complex structure. As it can be seen in Fig. 3c, the three-dimensional (3D) model demonstrated that Val170 is not the binding site for protein C3b. However, further binding free-energy calculation showed that p.V170M slightly increases the binding affinity of *CFHR5*/C3 β complex by -0.76 kcal/mol.

Discussion

Alport syndrome is characterized by haematuria, renal failure, and extra-renal alterations, such as: hearing loss, lenticonus, and retinal flecks [4-7]. The disease is caused by changes in the collagen type IV chains, resulting in damage to the basement membrane of several organs. Approximately 85% of families have X-linked inheritance with mutations in *COL4A5* gene [3, 17-19], and most of the other affected families have autosomal recessive disease with alterations in both copies of *COL4A3* or *COL4A4* [20-22]. Autosomal dominant inheritance is very rare and results from heterozygous *COL4A3* or *COL4A4* variants.

In our study, we comprehensively examined the history of a three-generation family. The proband (III:1) presented progressive deterioration of renal function with persistent haematuria and proteinuria, and underwent a kidney transplant at age 12. His family history was significant, as her mother progressed to ESRD, over a 10-year follow-up period of haematuria and proteinuria. Besides, his maternal grandma (I:1), maternal aunt (II:4), and aunt's daughter (III:2) had asymptomatic microscopic haematuria (<30/HP) and proteinuria (<0.5g/d) with normal renal function. Characteristic pathological changes were observed in renal biopsy specimens from the proband (III:1) and his maternal aunt's daughter (III:2), including segmental uneven thickness with lamellation and splitting of the dense layer in GBM under electron microscopy. In addition, immunostaining showed nearly complete loss for $\alpha 5$ (IV) collagen chain in GBM and complete absence in Bowman's capsule and distal tubule basement membrane in the proband. His mother (II:1) exhibited segmental distribution of $\alpha 5$ (IV) expression in the skin BM. According to the diagnostic algorithm published in 2013 [7], the diagnosis of Alport syndrome was established in the proband (III:1), and his mother (II:1) and his maternal aunt's daughter (III:2) based on pedigree study, clinical manifestation, and skin/renal biopsy.

The *COL4A3* and *COL4A4* genes are located on chromosome 2, while the *COL4A5* gene is located on the X chromosome, these genes encode the collagen IV $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains, respectively. In normal individuals, the collagen IV $\alpha 3\alpha 4\alpha 5$ chains are highly expressed and co-distributed within the mature kidney (GBM and distal tubular BM), cochlea, and eye, and the collagen IV $\alpha 5\alpha 5\alpha 6$ network occurs in the skin BM and kidney (Bowman's capsule) [5]. X-linked Alport syndrome accounts for the majority of Alport syndrome cases, arising from mutations in the *COL4A5* gene. In the proband and his mother, morphologic

phenotype demonstrated reduced and depleted expression of the collagen IV $\alpha 5$ chain in kidney and/or skin. Further clinical and genealogical study indicated that the affected proband (III:1) had much more severe disease than the females [his mother (II:1), maternal grandma (I:1), maternal aunt (II:4) and aunt's daughter (III:2)] in the family, and male-to-male transmission was absent. Hence, the mode of inheritance was suspected to be X-linked.

Molecular genetic testing is one of the criteria for the diagnosis of Alport syndrome with a high sensitivity and specificity (>90%) [7, 23, 24]. High-throughput next generation sequencing (NGS) technology can improve the diagnosis of Alport syndrome by providing molecular confirmation of *COL4A3*, *COL4A4*, or *COL4A5* mutations. We performed whole-exome sequencing for four individuals (II:1, II:2, III:1, and III:2); however, we did not identify any variant that could be possibly disease-causing in the three type IV collagen genes. It is possible that a small proportion of variants may have been missed owing to unfavourable insurance coverage or for other reasons. Alternatively, there could be a rare deep intronic variant affecting splicing and only detectable by RNA analysis. In addition, pathogenic genes in families with Alport syndrome may not be confined to a few regions. Known *COL4A3*, *COL4A4*, and *COL4A5* genes are scattered throughout many exons, making it difficult to develop predictive genetic tests.

Notably, a marked C3 staining with the corresponding electron-dense deposits along the mesangium was detected by immunofluorescence and electron microscope analysis of the kidney biopsy in the proband (III:1). Serum C3 was reduced in his mother (II:1), but it was normal in the proband and other affected family members. Excessive glomerular C3 fragment deposition without immunoglobulin, mesangial proliferation and crescent formation showed some morphological features of C3GN. In addition, the proband and his mother were affected more severely than other members. In the pedigree, familial C3GN was suspicious. The findings above prompted us to further investigate the complement system in the index family. A broad range of genetic contributors are definitely implicated in the pathogenesis of C3GN [25-27], so we screened a set of complement genes.

We identified an uncommon heterozygous missense variant c.508G>A (p.V170M; rs201073457) in the complement regulatory gene *CFHR5* in three members of this family (I:1, II:1 and III:1). Based on genetic studies, copy number variations in *CFHR5* are implicated in C3GN [8, 29-30], but significant enrichment of disease-associated rare variants is less [31]. The c.508G>A in the gene *CFHR5* generates a nonsynonymous alteration at amino acid position 170 (Val in wild-type and Met in mutant), which is strictly conserved among organisms. In silico programs (SIFT, SNAP, PolyPhen-2, and Mutation Taster) independently indicated the *CFHR5* c.508G>A to be potential functional variant. *CFHR5* colocalizes with complement-containing glomerular immune deposits in a variety of glomerular pathologic states [28]. The exact mechanism of complement regulation is unknown but is probably related to the C3b binding capacity. Then, we simulated 3D homology-modelled structures, which indicated mutant-type *CFHR5* increased conformational stability and induced an increase in the C3b-binding affinity. Additionally, this alteration has been previously reported in a patient with aHUS [32] and verified to exhibit significantly higher C3b binding capacity compared with wild-type *CFHR5* in *in vitro* functional assays [33]. The above

computational and functional assessments indicated that *CFHR5* c.508G>A may have contributed to the C3GN phenotype in the proband.

Conclusions

Our study reports a pedigree characterized by microscopic haematuria and variety in the speed of onset of renal failure. The diagnosis of Alport syndrome is confirmed in the proband (III:1), his mother (II:1) and his maternal aunt's daughter (III:2). Most notably, the proband revealed morphological features of C3GN based on genetic complement dysregulation background with concurrent histological evidence of Alport syndrome. Cases of Alport syndrome complicated with C3GN has never been reported, and underlying overlap requires further study.

Abbreviations

CFHR5: Complement Factor H-related Protein 5; C3GN: C3 glomerulonephritis; ESRD: end stage renal disease; eGFR: estimated glomerular filtration rate; MAF: minor allele frequency; BUN: blood urea nitrogen; sCr: serum creatinine; GBM: glomerular basement membrane; BM: basement membrane; NGS: next generation sequencing; CKD: chronic kidney disease; WT: wild type; MT: mutant type.

Declarations

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Author's contributions

Experiment performance, data analysis and interpretation, figure preparation and manuscript writing: Yin Ding. Histological and laboratory analysis and interpretation: Xuanli Tang, Yuanyuan Du. Study design, clinical analysis and interpretation: Hongyu Chen, Dongrong Yu, Bin Zhu. Patient recruitment, counseling and follow-up, study design: Bohan Yuan. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are contained within the manuscript and the appendix, and available from the corresponding author on reasonable request. The totality of the data cannot be shared based on patient confidentiality concerns.

Ethical approval and consent to participate

All of the procedures performed in studies involving human participants were carried out in accordance with the ethical standards of the Ethics Committee of the Hangzhou hospital of traditional Chinese medicine (institutional review board approval number: 2020KY055) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent forms for participation in the study were obtained, where participants are children (under 16 years old) from their parent.

Consent for publication

Not applicable.

Competing interests

The authors declare that there exist no conflicts of interest.

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Figures

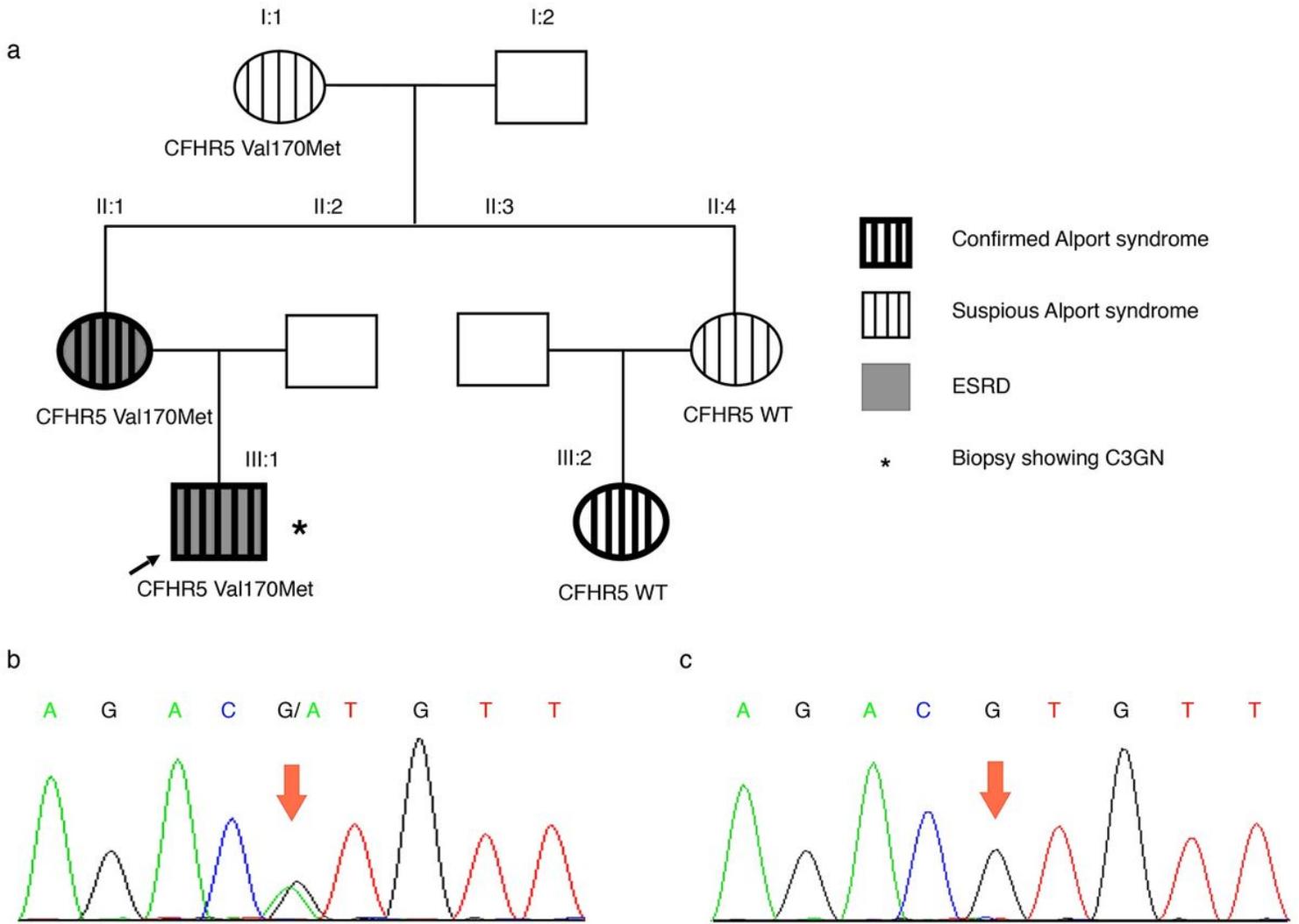


Figure 1

Family pedigree and Sanger sequencing chromatograms. a Pedigree structure of the family with Alport syndrome. Squares and circles denote males and females respectively. Roman numbers indicate generations. Arrow indicate the proband (III:1). Family members carrying the heterozygous CFHR5 genetic variant (c.508G>A; Val170Met) are highlighted. Individuals II:4 and III:2 do not carry the Val170Met variant. b Sequence electropherogram shows a heterozygous CFHR5 c.508G>A (p.Val170Met) variation (arrow) in individual I:1, II:1, and III:1. c Sequence electropherogram shows the negative control.

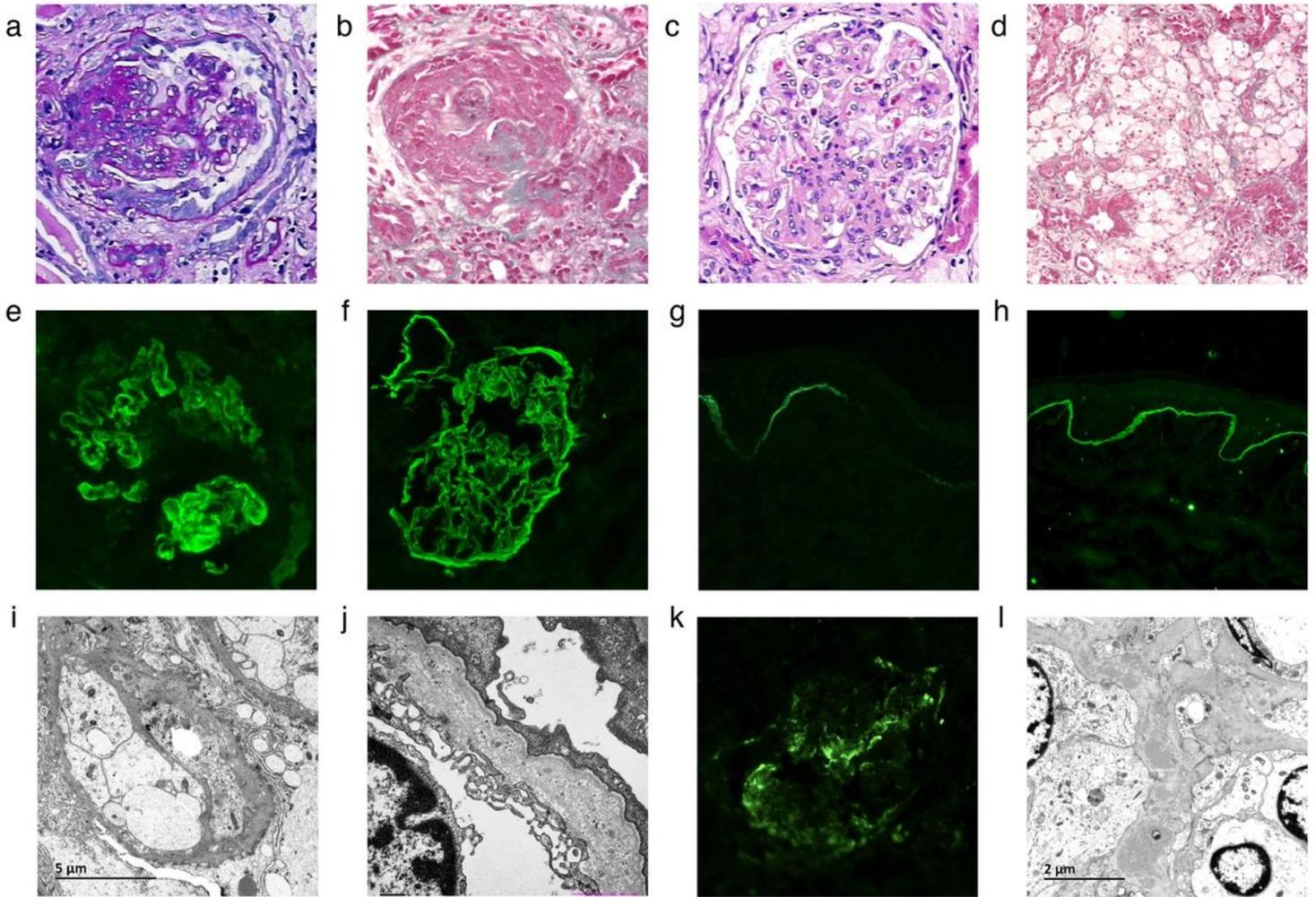


Figure 2

The pathological features of kidney and skin samples in the proband (III:1), his mother (II:1), and His affected aunt's daughter (III:2). Light micrograph: The proband shows segmental sclerosis (a, PAS×200), crescent formation (b, MA×200), diffuse and global mild-moderate mesangial proliferation (c, HE×200), and interstitial fibrosis/tubular atrophy containing lipid-laden foam cells (d, MA×200). Immunofluorescent stain: The proband reveals partial loss for the collagen IV a5 chain in GBM and complete absence in Bowman's capsule and distal tubule basement membrane (e, IF×400), compared with control (f, IF×400). A skin biopsy of his mother demonstrates segmental absence of the collagen IV a5 chain within the epidermal basement membrane (g, IF×200), compared with control (h, IF×200). Electron micrograph: Segmental uneven thickness with unsmooth spiculation of the dense layer in the GBM is found in the proband (i, ×6000), and uneven thickness with a laminated appearance is shown in his aunt's daughter (j, ×15000). Remarkably, a marked C3 staining is shown in the proband to locate along the mesangium by immunofluorescence analysis (k, IF×200) with the corresponding electron-dense deposits under the electron microscopy (l, ×10000).

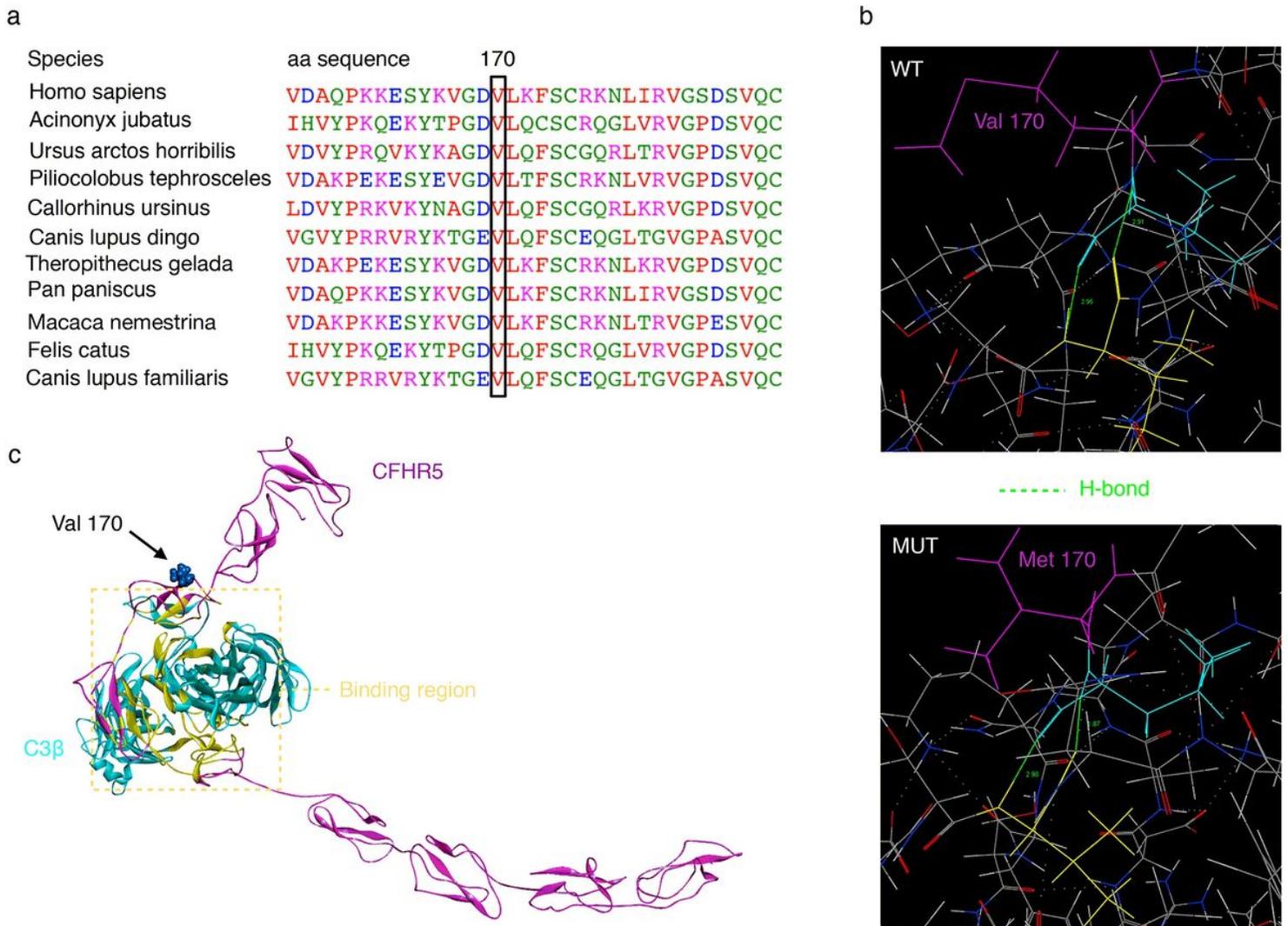


Figure 3

Functional characterization of the CFHR5 p.Val170Met variant. **a** Alignment of the CFHR5 protein in different species shows the conservation of the V170 residue. The concerned amino acids are boxed. **b** Generated models show the discrimination of local intramolecular hydrogen bonding interactions between Val170 and Met170 by MOE. Hydrogen bonds are shown with a fluorescent green dotted line representation. **c** Structure of CFHR5 in complex with the ligand C3b- β chain (C3 β) were generated with the DS 3.0. CFHR5 is denoted by pink, C3 β (residues 1-642, PDB 2WII) is shown in turquoise. The binding regions of the CFHR5/C3 β complex are presented in yellow. The Val residue 170 of CFHR5 is highlighted with a CPK model.

Supplementary Files

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