Identification of C3GN Presenting in a Proband with Family History of Alport Syndrome

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Research

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Abstract

Background

Alport syndrome and C3GN are all rare kidney diseases and frequently responsible for familial hematuria, proteinuria and/or coexistent renal impairment. With the rapid development of molecular genetic testing, Alport syndrome have been restricted mostly to causal variants in the COL4A5 or COL4A3/COL4A4 genes. And a broad range of genetic contributors in the complement and complement-regulating proteins definitely implicate in the pathogenesis of C3GN.

Methods

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

Results

We describe a three-generation family with Alport syndrome showing a dominant maternally transmitted inheritance. Notably, renal biopsy showed the concurrent histological evidence of C3GN in the proband and further classified as CFHR5-related nephropathy due to a rare heterozygous variation in gene-encoding 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, the variation was predicted to deregulate complement activation by altering the structural property and enhancing C3b binding capacity to compete with CFH, which was in a good agreement with experimental data previously published.

Conclusions

The comorbidity findings between Alport Syndrome and C3GN indicate an underlying overlap, and await further study.

Introduction

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

Genetics were first used in nephrology with the first identification of a causal mutation for Alport syndrome in 1990 [3]. Alport syndrome is a rare hereditary disorder of basement membranes with an abnormal collagen IV composition characterized by hematuria, progressive renal failure, sensorineural deafness, anterior lenticonus and retinal flecks [4]. To date, the causes of familial hematuria nephropathies have been restricted mostly to variants in the COL4A5 or COL4A3/COL4A4 genes, responsible for the X-linked or autosomal inheritance [5–7]. Besides, analysis of data suggests that

familial microscopic hematuria attributable to the complement factor H-related protein 5 (CFHR5) gene is also associated with a rare renal disease termed C3 glomerulonephritis (C3GN), and further classified as CFHR5 nephropathy or CFHR5-related nephropathy [8–10].

Herein, we report a family with dominant maternally transmitted inheritance, in which 5 out of 8 members (I:1, II:1, II:4, III:1, and III:2) exhibit microscopic hematuria and proteinuria; 2 out of the 5 patients (II:1 and III:1) developed ESRD (end stage renal disease). The diagnosis of Alport syndrome is confirmed in the proband (III:1), his mother (II:1) and his maternal aunt's daughter (III:2) based on all available data. Besides, renal biopsy showed the comorbidity of C3GN and Alport syndrome in the proband based on a rare heterozygous c.508G > A (p.Val170Met) variant in the CFHR5 gene. And we provided several lines of evidence to support the contention that the variant CFHR5 c.508G > A was classified as pathogenic and may contribute to the genetic susceptibility to C3GN.

Methods

Family pedigree and clinical investigations

In this study, a family with persistent microscopic hematuria 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 3-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 UCSC (University of California, Santa Cruz) version hg19 (Genome Reference Consortium 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) [11]. This report recommends the use of specific standard terminology: 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign', and 'benign' to describe variants.

In brief, common variants with minor allele frequency (MAF) $\geq 1\%$ in any population are either 'likely benign' or 'benign'. Novel or rare variants that change protein sequence but have an unknown impact on protein function were classified as either 'likely pathogenic' or 'uncertain significance'. Ultra-rare variants with well-established in vitro or in vivo functional studies that indicate a damaging effect, coupled with multiple lines of computational evidence, were classified as 'pathogenic'.

Homology modeling of human CFHR5/C3b complex

Docking procedure has been described in more detail previously [12]. Starting from residues 23–569, the 3D homology models of CFHR5 were first generated by iterative threading assembly refinement (I-TASSER) server [13], where the one with the highest C-score was selected to be further refined by Fragment-Guided MD simulation (FG-MD) [14]. 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

Herein we investigated a three-generation Han Chinese family characterized by persistent microscopic hematuria associated with renal failure (Fig. 1a). The proband (III:1), a 11-year-old boy, was admitted to the hospital for hematuria and proteinuria during the physical examination. On admission, 24-h urine protein was 6.36 g/d, serum albumin is 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 umol/l, eGFR by CKD-EPI equation was 30.3 ml/min. Bilateral renal ultrasound supported chronic nephrology with stronger 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.

To confirm the clinical diagnosis, renal biopsy of the proband was performed. The changes on light microscopy are nonspecific and include diffuse hyperplasia in the glomerular mesangial cells and matrix, with segmental sclerosis and crescent formation. Immunohistochemical and immunofluorescence studies all showed patchy GBM expression of collagen a5 (IV) and complete absence in Bowman's capsule and distal tubular BM (basement membrane) (Fig. 2a & 2b). Electron microscopic analysis identified pathological characteristics accompanying with noticeably segmental uneven thickness with a laminated appearance of the dense layer within the basement membrane (100–400 nm) (Fig. 2c). Besides, interstitial fibrosis/tubular atrophy containing lipid-laden foam cells were obvious (Fig. 2d).

The proband (III:1) has a family history of hematuria, as his mother (II:1) presented with hematuria, proteinuria, and develop ESRD before age 33 years. And a skin biopsy was performed in his mother, which reveals that there was segmental absence of the collagen IV a5 chain within epidermal basement membrane (Fig. 2e). His maternal grandma (I:1), maternal aunt (II:4) and aunt's daughter (III:2) also had urinary abnormalities characteristic by asymptomatic hematuria and proteinuria with normal eGFR. His aunt's daughter (III:2) had a renal biopsy by age 13, showing segmental uneven thickness with a laminated appearance of the dense layer in GBM (160–400 nm) by electron microscopic (Fig. 2f) and scanty C3 staining along the capillary wall. His father (II:2), his maternal grandpa and aunt's husband is phenotypically normal.

Notably, a marked C3 staining was detected to locate along the mesangium by immunofluorescence analysis (Fig. 2g) in the proband (III:1) with the corresponding electron-dense deposits under the electron microscopy (Fig. 2i). During follow-up, the proband was commenced on hemodialysis 3 months later and received a kidney transplant after one year.

Findings on targeted exome-based next-generation sequencing 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 using Sanger sequencing. Genetic analysis identified the same heterozygous c.508G > A coding variant (p.Val170Met) in exon 6 of the CFHR5 gene derived from 3 affected individuals: the proband (III:1), his mother (II:1) and his maternal grandma (I:1) (Fig. 1b). The variant is ultra-low frequent in the general population (1000 Genomes: minor allele frequency [MAF] = 0.1%; ExAC Browser: MAF = 0.06%), and the mutated A allele carriers are all heterozygous. The nonsynonymous alteration leads to the replacement of a valine, strictly conserved among organisms (Fig. 3a), by a methionine residue. The Screening for four publicly available programs (SIFT, SNAP, PolyPhen-2 and Mutation Taster) independently predicted the replaced amino acid was “damaging” to 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 gene COL4A3, COL4A4 or COL4A5 that encode the $\alpha 3$, $\alpha 4$ or $\alpha 5$ chains of type IV collagen.

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

CFHR5 is a single-chain polypeptide composed of 9 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 neighboring residues (Leu171 and Val187) changes (Fig. 3b). DS 3.0 analysis predicted the calculated potential energy of the mutant type protein is -15180.97 kcal/mol compared to -15133.86 kcal/mol for mutant one, which implied that p.Val170Met could lead an increase in conformational stability of the CFHR5 protein.

As with CFH and other 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 can be seen in Fig. 3c, the three-dimensional (3D) model demonstrated that Val170 is not to be 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 hematuria, 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 the damage to the base membrane of several organs. Approximately 85% of families have X-linked inheritance with mutations in COL4A5 gene [3, 15–17], and most of the others have autosomal

recessive disease with alterations in both copies of COL4A3 or COL4A4 [18–20]. Autosomal dominant inheritance is very rare and results from heterozygous COL4A3 or COL4A4 variants.

In our study, we took a comprehensive three-generation family history. The proband (III:1) was presented a course of progressive deterioration of renal function with persistent hematuria and proteinuria, and underwent a kidney transplant at aged 12. His family history is significant for progressing to ESRD in her mother, over a 10-year follow-up period of hematuria and proteinuria. Besides, His maternal grandma (I:1), maternal aunt (II:4) and aunt's daughter (III:2) had asymptomatic microscopic hematuria (≥ 30 /HP) and proteinuria (≥ 0.5 g/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, complete absence in Bowman's capsule and distal tubule basement membrane in the proband, and 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 is established in the proband (III:1), 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 reside on chromosome 2 while the COL4A5 gene is located on the X chromosome, which encode the collagen IV $\alpha 3$, $\alpha 4$ and $\alpha 5$ chain, 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 of 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) are much more severe than females (his mother (II:1), maternal grandma (I:1), maternal aunt (II:4) and aunt's daughter (III:2)) in the pedigree, and male-to-male transmission is 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 ($\geq 90\%$) [7, 21, 22]. High-throughput next generation sequencing (NGS) technology can improve the diagnosis of Alport syndrome by providing molecular confirmation of COL4A3, COL4A4, or COL4A5 mutations. Then we performed whole-exome sequencing for 4 individuals (II:1, II:2, III:1, III:2), however, we did not identify any variation in thought to be possibly disease-causing in the three type IV collagen genes. It is possible that a small proportion of variants may have been missed. Alternatively, 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.

Noteworthy, 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), while normal in the proband and other affected family members. Excessive glomerular C3 fragment deposition, with scanty C1q and immunoglobulin, 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 is suspicious. The findings above prompted us to take a further investigation of the complement system in the index family. A broad range of genetic contributors definitely implicate in the pathogenesis of C3GN [23–25], then we screened a set of complement genes.

We identified a rare heterozygous missense variant c.508G > A (p.V170M; rs201073457) in the complement regulatory gene CFHR5 in 3 members in this family (I:1, II:1 and III:1). CFHR5 colocalizes with complement-containing glomerular immune deposits in a variety of glomerular pathologic states [26]. Based on genetic studies, copy number variations in CFHR5 is implicated in C3GN [8, 27–28], but significant enrichment of disease-associated rare variants is less [29]. 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. Then we simulated 3D homology-modeled structures, which indicated mutant-type CFHR5 increased conformational stability and induced an increase in the C3b-binding affinity. Additionally, the rare alteration has been previously reported in a patient with aHUS [30] and verified to exhibit significantly higher C3b binding capacity compared with wild-type CFHR5 in vitro functional assays [31]. The above computational and functional assessments indicated that CFHR5 c.508G > A may be responsible for the C3GN phenotype in the proband.

Conclusions

Our study reports a pedigree characterized by microscopic hematuria and variety in the rapidity 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. The cases of Alport Syndrome complicated with C3GN has never been reported, and underlying overlap awaits 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 denoted males and females respectively. Roman numbers indicate generations. Arrow indicated the proband (III:1). Family members carrying the heterozygous CFHR5 rare 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 a negative control.

Figure 2

The pathological features of kidney and skin samples in the proband and his mother. The proband (III:1) demonstrated partial loss for the collagen IV a5 chain in GBM and complete absence in Bowman's capsule and distal tubule basement membrane (a, IHC×400 & b, IF×400) by immunostaining, and a large amount of lipid-laden foam cells interstitial infiltration under the light microscope (d, MA×200). Electron microscopic analysis identified pathological characteristics accompanying with noticeably segmental uneven thickness with a laminated appearance of the dense layer in the GBM (c, ×6000). In addition, there was segmental absence of the collagen IV a5 chain within epidermal basement membrane (e, IF ×200) in the proband's mother (II:1). His aunt's daughter (III:2) showed segmental uneven thickness with a laminated appearance of the dense layer in GBM (160-400nm) by electron microscopic (f). Remarkably, a

marked C3 staining was detected in the proband to locate along the mesangium by immunofluorescence analysis (g, IF×200) with the corresponding electron-dense deposits under the electron microscopy (i, ×6000), while scanty IgA was detected (h, IF×200).

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 acid 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 (C3β) were generated with the DS 3.0. CFHR5 is denoted by pink, C3β (residues 1-642, PDB 2WII) is shown in turquoise. The binding regions of the CFHR5/ C3β complex are presented in yellow. The Val residue 170 of CFHR5 is highlighted with a CPK model.

Supplementary Files

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