Genetic Analysis of Focal Segmental Glomerulosclerosis in Thailand

จุฬาลงกรณ์มหาวิทยาลัย
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ที่มาของปัญหาการวิจัย: ความผิดปกติทางพันธุกรรมผู้ป่วยโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสมีความแตกต่างกันในแต่ละกลุ่มประชากร การศึกษานี้บรรยายลักษณะทางคลินิกและความผิดปกติทางพันธุกรรมของผู้ป่วยโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสในประเทศไทย และได้ศึกษาการแสดงออกของโปรตีนคอลลาเจนในชิ้นเนื้อไตสัมพันธ์กับความผิดปกติทางพันธุกรรมที่ตรวจพบด้วยการย้อมทางพยาธิวิทยา นอกจากนั้นได้ศึกษาการทำงานในระดับเซลล์เพื่อหาหลักฐานว่าการกลายพันธุ์ที่ตรวจพบเป็นสาเหตุของการเกิดโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสในผู้ป่วย

ระเบียบวิธีการวิจัย: ผู้ป่วยโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสที่ไม่พบสาเหตุจำนวน 53 รายที่ไม่มีความเกี่ยวข้องกันทางสายเลือดถูกรวบรวมเข้ามาในการศึกษา ผู้ป่วยจะถูกตรวจทางพันธุศาสตร์ด้วยเอกโซมซีเควนซิ่ง และใช้เทคนิคอิมมูโนฮิสโตเคมีเพื่อตรวจดูลักษณะของชิ้นเนื้อไตเพื่อหาความสัมพันธ์กับลักษณะทางคลินิกและผลตรวจทางพันธุศาสตร์ นอกจากนี้เรายังทำการตรวจในระดับเซลล์ด้วยการเทคนิคการสร้างโปรตีนคอลลาเจนและวัดแสงลูซิเฟอเรสเพื่อหาความสัมพันธ์ของการกลายพันธุ์ที่ตรวจพบด้วยเอกโซมซีเควนซิ่ง กับลักษณะทางคลินิก ลักษณะทางพยาธิวิทยาและผลตรวจทางพันธุศาสตร์

ผลการศึกษา: ผู้ป่วย 35 รายจาก 53 ราย (ร้อยละ 66) เป็นผู้ป่วยผู้ใหญ่ ผู้ป่วย 51 ราย (ร้อยละ 96.2) ไม่มีประวัติครอบครัวเป็นโรคไต การวินิจฉัยทางคลินิกก่อนเจาะไตเป็นกลุ่มอาการเนโฟรติกที่ไม่ตอบสนองต่อสเตียรอยด์ร้อยละ 58.5 และเป็นโรคไตที่มีโปรตีนรั่วในปัสสาวะร้อยละ 32.1 จากการตรวจด้วยเทคนิคเอกโซมซีเควนซิ่ง พบการกลายพันธุ์ก่อโรคในผู้ป่วย 6 ราย คิดเป็นร้อยละ 11.3 (6/53) ผู้ป่วยสองรายจาก 6 รายนี้มีประวัติครอบครัวเป็นโรคไต จากผู้ป่วย 6 รายที่พบการกลายพันธุ์ก่อโรค ผู้ป่วยสองรายมีการกลายพันธุ์ที่ไม่เคยตรวจพบมาก่อน โดยผู้ป่วยหนึ่งรายพบการกลายพันธุ์ในยีน *COL4A4* และผู้ป่วยอีกหนึ่งรายพบการกลายพันธุ์ในยีน *MAFB* ผู้ป่วยอีกสี่รายพบการกลายพันธุ์ที่เคยรายงานมาแล้วในยีน *CLCN5* ยีน *LMX1B* และยีน *COL4A4* การย้อมอิมมูโนฮิสโตเคมิสทรี่ของคอลลาเจนชนิดที่สี่อัลฟาห้าในชิ้นเนื้อไตพบว่าผู้ป่วยที่มีการกลายพันธุ์ที่ก่อโรคและไม่ก่อโรค มีการแสดงออกของโปรตีนคอลลาเจนชนิดที่สี่แอลฟาห้าทั้งสิ้น แสดงว่าการย้อมอิมมูโนฮิสโตเคมีของคอลลาเจนชนิดที่สี่ในชิ้นเนื้อไตไม่มีความสัมพันธ์กับการกลายพันธุ์ที่จำแนกตามเกณฑ์ อย่างไรก็ตามการตรวจในระดับเซลล์ด้วยการเทคนิคการสร้างโปรตีนคอลลาเจนและวัดแสงลูซิเฟอเรสแสดงให้เห็นว่าเซลล์สร้างโปรตีนคอลลาเจนชนิดที่สี่ได้ลดลงในเซลล์ที่มีการกลายพันธุ์ที่ก่อโรค แสดงว่าการกลายพันธุ์ที่ก่อโรคนี้ น่าจะก่อให้เกิดโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสในผู้ป่วยจริง

สรุป: การกลายพันธุ์ที่ก่อให้เกิดโรคพบได้ร้อยละ 11.3 ในผู้ป่วยโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิส ความผิดปกติที่พบมากที่สุดอยู่ในยีน *COL4A4* การย้อมอิมมูโนฮิสโตเคมีของคอลลาเจนชนิดที่สี่ในชิ้นเนื้อไตไม่มีความสัมพันธ์กับการกลายพันธุ์ที่จำแนกตามเกณฑ์ แต่การตรวจในระดับเซลล์พบว่าการกลายพันธุ์ที่ก่อโรคก่อให้เกิดโรคโฟคอลเซกเมนทอลโกลเมอรูโลสเคลอโรซิสในผู้ป่วย

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Suramath Isaranuwatchai : Genetic Analysis of Focal Segmental Glomerulosclerosis in Thailand. Advisor: Prof. KEARKIAT PRADITPORNSILPA, M.D. Co-advisor: Prof. KANYA SUPHAPEETIPORN, M.D.,Asst. Prof. ANKANEE CHANAKUL, M.D.

Background: The genetic variants spectra of focal segmental glomerulosclerosis (FSGS) vary among different populations. Here we described the clinical and genetic characteristics of biopsy-proven FSGS patients in Thailand. We also used special staining in renal biopsy tissue to describe protein expression related to the variants found by whole-exome sequencing (WES). Also, a functional study in cells was studied to investigate the etiologic evidence of the variants found by WES.

Methods: Fifty-three unrelated FSGS patients without secondary causes were included in our study. Whole-exome sequencing (WES) was subsequently performed. Immunohistochemistry (IHC) staining method was used to characterize the morphology of renal pathology for clinical and genomic correlation. Cell-based split-luciferase-based trimer formation assay was used to investigate whether the variances found by WES related to clinical, pathology, and genomic findings.

Results: Of 53 FSGS patients, 35 patients were adults (66.0%), and 51 patients were sporadic cases (96.2%). Clinical diagnosis before kidney biopsy was steroid-resistant nephrotic syndrome (SRNS) in 58.5%, and proteinuric chronic kidney disease in 32.1%. Using WES, disease-associated pathogenic/likely pathogenic (P/LP) variants could be identified in six patients including the two familial cases, making the P/LP detection rate of 11.3% (6/53). Of these six patients, two patients harbored novel variants with one in the *COL4A4* gene and one in the *MAFB* gene. Four other patients carried previously reported variants in the *CLCN5*, *LMX1B* and *COL4A4* genes. Protein expression study with IHC staining of α5(IV) collagen in kidney tissues were positive in kidney tissues of both P/LP variants and benign variants; therefore, IHC staining did not correlated with pathogenicity of variants classified. However, cell-based split-luciferase-based trimer formation assay of α345(IV) collagen showed decreased in protein expression of α345(IV) collagen in the cells with P/LP variants; hence, predicted that these P/LP variants were the cause of FSGS in the respective patients.

Conclusions: The overall P/LP variant detection rate by WES in biopsy-proven FSGS patients was 11.3%. The most identified variants were in *COL4A4*. IHC staining of α5(IV) collagen was not associated with pathogenicity of variants, but cell-based study can successfully demonstrated the etiologic evidence of *COL4A4* variants found by WES.

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Student's Signature ....

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CHAPTER I

BACKGROUND AND RATIONALE

Background

Focal segmental glomerulosclerosis (FSGS) is one of the most common glomerular diseases in Thailand and worldwide[1, 2]. Traditionally, FSGS was classified into primary FSGS and secondary FSGS[3]. In practice, nephrologists will investigate the secondary causes of FSGS, namely: obesity, reduced kidney mass, obstructive nephropathy, human immunodeficiency virus (HIV) infection, or other viral infections and medications. If no secondary causes are identified, the patients will be diagnosed with primary FSGS. They will be treated with immunosuppressive medications with high-dose corticosteroids as first-line treatment and calcineurin inhibitors as second-line treatment[4].

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In recent years, the monogenic mutation was identified as the cause of FSGS, and genetic FSGS was established as a new entity of FSGS. Single gene mutation was reported to cause FSGS in 10-44% of FSGS patients depending on the population ethnicity and age group studied[5-7]. The most common gene reported causing FSGS were genes in podocyte slit diaphragms such as *NPHS1*, *NPHS2*, *WT1*, *ACTN4*, *TRPC6*, *INF2*, *CD2AP* and *SCARB2*[8]. Some recent studies reported genes in the glomerular basement membrane (GBM), including *COL4A3*, *COL4A4*, and *COL4A5*, as the most common causes of FSGS[5]. Due to high costs and scarcity of genetic testing, not every FSGS

patient could be tested for genetic mutation. The selection of FSGS patients for genetic testing is challenging, and recommendations for genetic testing in FSGS patients are immature. Therefore, this study will be Thailand's first genetic study of FSGS patients.

The American College of Medical Genetics and Genomics and the Association of Molecular Pathology recommend genetic testing by next-generation sequencing (NGS) and analysis according to joint consensus(9). This guideline will classify variants found by NGS according to pathogenicity into five categories: pathogenic variants, likely pathogenic variants, variants of uncertain significance (VUS), likely benign variants, and benign variants. This classification shows the pathogenicity of a variant but does not show that the variant is the cause of disease in a patient. Since the most common gene mutations were in COL4A3/4/5 genes, we planned to further clarify the phenotype of our FSGS cases by immunohistochemistry (IHC) staining of α3(IV), α4(IV), and α5(IV) collagen in GBM of their kidney tissues. Moreover, a functional study in cells will be conducted to confirm the etiologic evidence of the newly found mutation in *COL4A3/4/5* genes.

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Research question

Primary research question

What is the genotypic spectrum and the most common genetic defect of FSGS in the Thai population?

Secondary research question

- Can immunohistochemistry (IHC) staining of α3(IV), α4(IV) and α5(IV) collagen in GBM of FSGS patients' kidney tissues further clarify the phenotypes of FSGS patients?
- Can functional analysis help elucidate the pathogenicity of the identified variants?

Objectives

- To characterize the genetic defects associated with FSGS in Thai patients
- To elucidate the functional consequence of the identified candidate variants associated with FSGS

Hypothesis

- There are genes responsible for FSGS in the Thai population and alterations in these genes could have a functional impact.
- There are indications for genetic testing in FSGS patients. Some subgroups of FSGS could benefit from genetic testing.

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- Immunohistochemistry (IHC) staining of α3(IV), α4(IV) and α5(IV) collagen in GBM of FSGS patients' kidney tissues might help clarify the phenotypes of FSGS patients.
- Functional analysis will help elucidate the pathogenicity of the identified variants.

Research design

This is a single-center study of Thai FSGS patients in King Chulalongkorn Memorial Hospital (KCMH).

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Conceptual framework

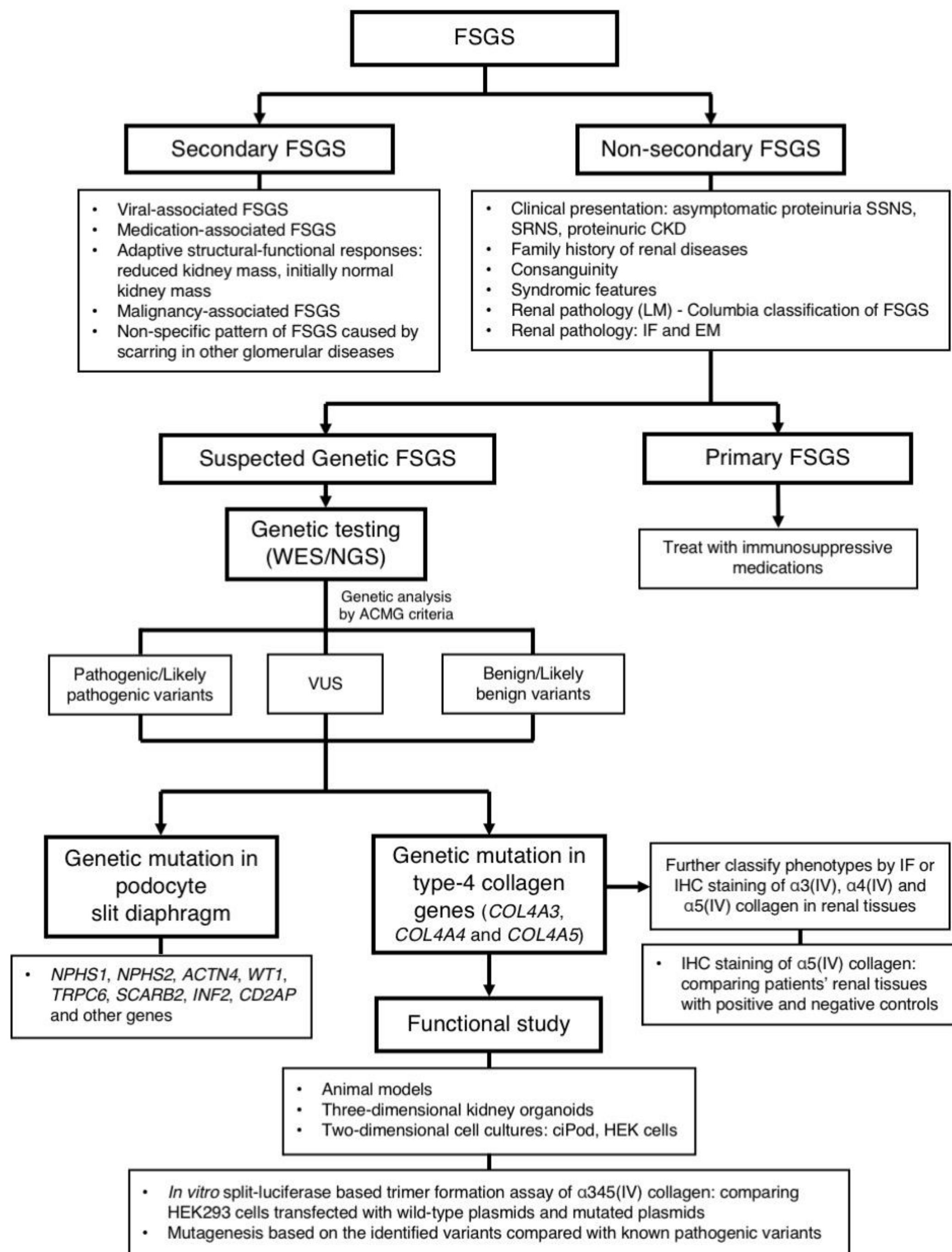

Figure 1: Conceptual framework of the study

ACMG = American College of Medical Genetics and Genomics, ciPod = conditionally immortalized podocytes, CKD = chronic kidney disease, EM = electron microscopy, FSGS = focal segmental glomerulosclerosis, HEK = human embryonic kidney, IF = immunofluorescent, IHC = immunohistochemistry, LM = light microscopy, NGS = next-generation sequencing, SRNS = steroid-resistant nephrotic syndrome, SSNS = steroid sensitive nephrotic syndrome, VUS = variants of uncertain significance, WES = whole-exome sequencing

CHAPTER II

REVIEW OF LITERATURE

Focal segmental glomerulosclerosis and genetic testing

Focal segmental glomerulosclerosis (FSGS) is a glomerular disease, and a pattern of glomerular injury, classified by the presence of segmental sclerosis of the glomerulus in renal biopsy pathology. FSGS is an important cause of steroid-resistant nephrotic syndrome in the pediatric population (about 7-10% of pediatric nephrotic syndrome) and the adult population (about 20-30% of adult nephrotic syndrome)[10]. FSGS is also one of the leading causes of early-onset chronic kidney disease (CKD)[11]. Moreover, FSGS is one of the most recurrent diseases after kidney transplantation leading to kidney allograft failure[12]. Currently, diagnosis of FSGS can be made only by kidney biopsy.

FSGS present to the renal clinic with various clinical presentations, including asymptomatic proteinuria, nephrotic syndrome, or proteinuric CKD. After FSGS diagnosis by kidney biopsy, FSGS will be classified into primary FSGS and secondary FSGS[4]. There are many causes of secondary FSGS, namely, HIV infection, reduced kidney mass, and medications, as shown in Table 1[3]. If the secondary cause is identified, treatment will aim directly at the secondary cause without giving the immunosuppressive medication. Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) might be used to alleviate proteinuria in case of heavy proteinuria from secondary

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FSGS. Otherwise, FSGS will be classified as primary FSGS and will be treated with immunosuppressive treatment as in Table 2[4].

*Table 1: **Classification of FSGS***

Primary (idiopathic) FSGS
Secondary FSGS • Virus-associated FSGS: HIV-associated nephropathy (HIVAN), parvovirus B19 • Medication-associated FSGS: heroin-nephropathy, interferon-α, lithium, pamidronate/alendronate, anabolic steroids • Adaptive structural-functional responses are likely mediated by glomerular hypertrophy or hyperfiltration Reduced kidney mass: oligomeganephronia, unilateral kidney agenesis, kidney dysplasia, cortical necrosis, reflux nephropathy, surgical kidney ablation, chronic allograft nephropathy, any advanced kidney disease with the reduction in functioning nephrons Initially normal kidney mass: DM, hypertension, obesity, cyanotic congenital heart diseases, sickle cell anemia • Malignancy-associated FSGS: lymphoma

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• Nonspecific pattern of FSGS caused by kidney scarring in glomerular disease: focal proliferative glomerulonephritis (IgA nephropathy, lupus nephritis, pauci-immune glomerulonephritis), hereditary nephritis (Alport's syndrome), membranous nephropathy, Thrombotic microangiopathy
Genetic FSGS

DM = diabetes mellitus, FSGS = focal segmental glomerulosclerosis

Table 2: Treatment of primary FSGS

<table>
<tr><th>Treatment</th><th colspan="2">Medications and dosage</th></tr>
<tr><td>First-line regimen</td><td colspan="2">Prednisolone 1 mg/kg/day (max 80 mg/day)
Or alternate-day prednisolone 2 mg/kg (max 120 mg)</td></tr>
<tr><td rowspan="2">Second-line regimen</td><td>Cyclosporine 3-5 mg/kg/day</td><td rowspan="2">plus
Low-dose prednisolone (0.15 mg/kg/day) for 4-6 months, then taper over 4-8 weeks</td></tr>
<tr><td>Tacrolimus 0.1-0.2 mg/kg/day</td></tr>
<tr><td>Third-line regimen</td><td colspan="2">Combination of mycophenolate mofetil and high-dose dexamethasone</td></tr>
</table>

FSGS = focal segmental glomerulosclerosis

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Recently, genetic mutation, previously described as familial FSGS, was also found as one of the most common causes of sporadic FSGS[2] and was defined as a new

category of genetic FSGS. Genetic FSGS was defined as FSGS caused by monogenic mutation or single-gene mutation. Clinical characteristics of genetic FSGS vary widely due to different genetic mutations ranging from asymptomatic proteinuria to nephrotic syndrome to proteinuric CKD. Another noticeable clinical feature of genetic FSGS is that it rarely respond to immunosuppressive medications, including corticosteroids. This data led to the avoidance of immunosuppressive medication in patients with a confirmed diagnosis of genetic FSGS. Another important clinical feature is that patients with genetic FSGS rarely had a recurrence in allograft after kidney transplantation[13]. In contrast with primary FSGS, which commonly recurred in kidney allografts[12].

With the clinical benefit of correct diagnosis of genetic FSGS, genetic testing was crucial for managing FSGS patients. However, only some FSGS patients could undergo genetic testing since genetic testing was expensive and not available in every hospital. There is no consensus on genetic testing recommendation for FSGS patients. Current Kidney Disease: Improving Global Outcomes (KDIGO) guideline state that genetic testing is not recommended in all FSGS patients[4]. However, this guideline suggested that genetic testing could be considered in FSGS patients with a strong family history or syndromic features. Genetic testing might help determine the risk of FSGS recurrence after kidney transplantation. Other studies suggested genetic testing might be appropriate in different settings, such as in patients with steroid-resistant nephrotic

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syndrome (SRNS)[10, 14]. However, an acceptable recommendation for genetic diagnosis in FSGS patients remains incomplete.

Early articles in genetic testing of FSGS usually found that the most common gene mutations causing FSGS were genes in podocyte slit diaphragms such as *NPHS1*, *NPHS2*, *WT1*, *ACTN4*, *TRPC6*, *INF2*, *CD2AP*, and *SCARB2*[8]. However, some recent studies reported *COL4A3*, *COL4A4*, and *COL4A5* as the most common causes of FSGS[5]. Patients with *COL4A3/4/5* gene mutation were identified as Alport syndrome (AS). AS is a multi-system disorder involving kidneys, eyes, and hearing systems because α3(IV), α4(IV), and α5(IV) collagen are the common component of the basement membrane in these three organs. However, in FSGS patients with *COL4A3/4/5* gene mutations, no extra-renal manifestation was presented. The absence of extra-renal manifestation makes the genotype-phenotype correlation challenging for geneticists to establish the correlation between the *COL4A3/4/5* gene mutations and FSGS. There was also some confusion about the naming and classification of patients with *COL4A3/4/5* gene mutations with or without extra-renal manifestation, prompting nephrologists and geneticists to develop a new classification system of AS.

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Alport syndrome and its classifications

Alport syndrome (AS) is a disease presenting with renal failure, ocular and auditory abnormalities caused by genetic mutation of *COL4A3*, *COL4A4*, or *COL4A5*

genes. AS could be classified based on genetic mutation into two groups: X-linked AS (XLAS), which has a genetic mutation in the *COL4A5* gene on X chromosome, and autosomal AS, which has a genetic mutation in *COL4A3* and/or *COL4A4* genes on chromosome 2[15]. However, in clinical practice, due to high cost and low availability of genetic testing, patients with AS usually did not undergo genetic testing. Diagnosis of AS will usually depend on clinical characteristics and pathognomonic renal biopsy tissue, which includes basket weaving appearance of GBM in electron microscopy (EM).

The inherited disorders affecting GBM collagen include AS and thin basement membrane nephropathy (TBMN) or familial benign hematuria. Historically, clinicians classified these diseases based on a patient's clinical characteristics. TBMN, as the name represented, is diagnosed by EM showing thin GBM. Nephrologists used to believe that TBMN was a benign disease which means it will not progress to CKD or end-stage renal disease (ESRD). Hence, it was also known as familial benign hematuria. On the other hand, AS is a disease that will progress to CKD and eventually ESRD. AS is characterized by clinical syndrome involving kidneys, eyes and ears with compatible renal biopsy. However, from a molecular basis, TBMN and AS both have the mutation in type-4 collagen in the GBM. In adult GBM, type-4 collagen is a heterotrimeric molecule with the specific association of α3(IV), α4(IV), and α5(IV) collagen chain in 1:1:1 ratio. These chains are encoded by the genes *COL4A3*, *COL4A4*, or *COL4A5*. Since AS and TBMN result from mutations in the same genes, the term "collagen-IV related renal disease" has been

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suggested but has not been generally adopted by nephrologists(16). The Alport Syndrome Classification Working Group of International Society of Nephrology suggested using the term “AS” instead of “collagen-IV related renal disease” due to the familiarity of many clinicians(17, 18). Thus, the current recommendation for AS and related disorders classification are shown in Table 3.

Table 3: Classification system for AS and related disorders

from The Alport Syndrome Classification Working Group

Inheritance	Affected gene(s)	Genetic state	Estimated risk of ESRD
X-linked	*COL4A5*	Hemizygous (male)	100%
		Hemizygous (female)	Up to 25%
Autosomal	*COL4A3* or *COL4A4*	Recessive (homozygous or compound heterozygous)	100%
		Dominant	20% or more among those with risk factors for progression, <1% in the absence of risk factors*
Digenic	*COL4A3*, *COL4A4*, and *COL4A5*	*COL4A3* and *COL4A4* mutation in trans	Up to 100%

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Inheritance	Affected gene(s)	Genetic state	Estimated risk of ESRD
		COL4A3 and *COL4A4* mutation in cis	Up to 20%
		Mutation in *COL4A5* and *COL4A3* or *COL4A4*	Up to 100% (in the affected male)

AS = Alport syndrome, ESRD = end-stage renal disease, FSGS = focal segmental glomerulosclerosis, GBM = glomerular basement membrane, SNHL = sensorineural hearing loss

*Risk factors for progression: proteinuria, FSGS, GBM thickening and lamellation, SNHL, or evidence of progression in patient or family, genetic modifiers

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As shown in Table 3, genetic testing can predict the estimated risk of ESRD. Therefore, whenever possible, every AS case, diagnosed from clinicopathological data, should go through a genetic study. In patients with typical AS, the mutation is detected in almost 100% of cases[18]. It also should be noted that autosomal dominant AS, previously known as TBMN, could progress to ESRD in more than 20% of cases depending on risk factors, including proteinuria, FSGS, GBM thickening, GBM lamellation, SNHL, evidence of progression in patient or family and other genetic modifiers. Thus, genetic testing can also provide valuable knowledge of inheritance patterns for further genetic counselling.

As genetic testing was not readily available for most AS patients, clinicians used other tools to diagnose AS and predict disease progression for many years. Since renal

biopsy is a routine investigation in kidney patients, renal pathology, including immunofluorescent study (IF), immunohistochemistry (IHC), and EM, have been the mainstay for diagnosis and prognosis of AS. For EM, even though there are many pathognomonic characteristics of AS in EM, many EM characteristics did not present in early AS patients. Moreover, EM was not available in many hospitals due to high costs. Also, paraffin block tissues or frozen tissues might produce distorted EM morphology. Therefore, IF and IHC of α5(IV) collagen offer a better advantage if clinicians suspect AS after a renal biopsy.

IF and IHC staining of α3(IV), α4(IV), and α5(IV) collagen

Type-4 collagen staining is mainly studied in α5(IV) collagen. IF and IHC staining of α5(IV) collagen in the patient's GBM were used for diagnosis as an absence of α5(IV) collagen means XLAS diagnosis. Negative staining of α5(IV) collagen correlated with worse prognosis and more severe pathological changes in male XLAS patients[19]. However, patients with clinically milder XLAS were reported to have positive staining of α5(IV) collagen[20]. A study by Becknell et al. demonstrated that a novel missense mutation in the *COL4A5* gene in non-collagenous domain strongly correlates with clinical XLAS in a large family with 117 individuals across 7 generations, associated with positive staining α5(IV) collagen[21]. The authors suspected that severe kidney diseases in this family were not due to impaired deposition of α3α4α5(IV) collagen but may reflect a

specific functional impairment of the collagen network caused by the mutation. In short, XLAS patients with truncating mutations usually lead to loss of GBM staining of α5(IV) collagen. In contrast, a missense mutation or a mutation in non-collagenous domain can be associated with loss or intact α5(IV) collagen staining.

For autosomal AS, the absence of α5(IV) collagen can also be used to diagnose autosomal AS. Due to specific heterotrimerization of α3(IV), α4(IV), and α5(IV) collagens, the absence of α5(IV) collagen implies the absence of α3α4α5(IV) collagen, which can also be used for diagnosis and prognosis of autosomal AS. There was a study demonstrate weaker staining of α5(IV) collagen in a patient with a novel *COL4A4* nonsense mutation[(22)]. Another IF study of α5(IV) collagen in patients with autosomal recessive AS revealed normal positive staining of α5(IV) collagen[(23)]. This patient had SNHL and had *COL4A3* mutations in different alleles. The authors suspected that positive collagen staining in this patient might be associated with his preserved renal function. Normal or partial staining of α5(IV) collagen has also been observed in patients with autosomal recessive AS[(24, 25)]. Currently, there is no study about IF and IHC staining of α3(IV) and α4(IV) collagen in the diagnosis and prognosis of autosomal AS. Currently, there is no study to determine whether there are any changes in α3α4α5(IV) collagen in the GBM of FSGS patients with *COL4A3*, *COL4A4*, and *COL4A5* mutations, which do not have typical AS manifestation and typical AS lesions in kidney biopsy.

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Functional tests for *COL4A3*, *COL4A4*, and *COL4A5* genes

Many variants in *COL4A3*, *COL4A4*, and *COL4A5* genes were reported in AS and FSGS patients. There were no hot spots for mutations in these genes, and most of the variants reported were missense mutations. Hence, it was very challenging to classify these variants with the American College of Medical Genetics and Genomics (ACMG) guideline for variant interpretation because most of them will be classified as VUS. Currently, there was a consensus for variant interpretation for *COL4A3*, *COL4A4*, and *COL4A5* genes, which helped clinicians easier to interpret variants found in these type-4 collagen genes[17, 26]. The main difference in variant interpretation is that missense mutations in the collagenous domain of *COL4A3*, *COL4A4*, and *COL4A5* genes in glycine residues will usually be classified as likely pathogenic variants. However, classification with new recommendations leaves the same question unanswered, whether these variants were indeed the cause of FSGS or AS in the patient. The staining of α3α4α5(IV) collagen only classified the patients' phenotypes but did not identify whether or not the patients' COL4A3/4/5 variants caused kidney diseases. This question leads to an attempt to find a functional study of *COL4A3*, *COL4A4*, and *COL4A5* genes to determine the etiologic evidence of the variants in these three genes.

Currently, there are three main categories for functional tests of genetic mutation: animal models, three-dimensional organoids, and two-dimensional cell cultures[27]. There

were different advantages and disadvantages of these three methods, as shown in Table 4.

Table 4: Comparison between animal models, three-dimensional organoids and two-dimensional cell cultures

	Two-dimensional cell cultures	Three-dimensional organoids	Animal models
Physiologic representation	Limited	Semiphysiologic	Physiologic
Vascularization and immune system	No	No	Yes
High-throughput screening	Yes	Yes	No
Manipulability	Excellent	Good, but may have experimental variability	Limited
Biobanking	Yes	Yes	Yes, but only at cellular level
Genome editing	Yes	Yes	Yes, but it may require generation of embryonic stem cells
Modeling for organogenesis	Poor	Suitable for cell-cell communication, morphogenesis	Yes, but often confounded by complex tissue environment

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Different functional tests are suitable for different circumstances, depending on the objectives of the studies. Animal models offered physiologic representation including vascularization and immune system; however, the complicated system and limited manipulability made animal models difficult for studies in high-throughput screening of many patients with variants in *COL4A3*, *COL4A4*, and *COL4A5* genes. Functional studies in kidney organoids are currently developed to understand the extracellular matrixes and GBM of the kidneys[28]. Nonetheless, kidney organoids are still in early stage of development; thus, currently premature for studying *COL4A3*, *COL4A4*, and *COL4A5* genes. For two-dimensional cell cultures, there were two primary types of cells for the functional studies of *COL4A3*, *COL4A4*, and *COL4A5* genes: conditionally immortalized podocytes (ciPod) and human embryonic kidney (HEK) cells. Functional studies of *COL4A3*, *COL4A4*, and *COL4A5* gene mutations in conditionally immortalized podocytes (ciPod) have been done in past[29, 30]. However, due to the long cell-dividing time, rarity, and vulnerability of the ciPod, functional studies in ciPod were not popular. Also, functional studies in ciPod might not be suitable for high-throughput tests to verify the variants found in *COL4A3*, *COL4A4*, and *COL4A5* genes by genetic testing. In contrast, HEK cells are easy to study, easy to manipulate with short cell-dividing time. With these advantages, functional study in HEK cells can further be developed into a high-throughput screening for many patients who underwent NGS.

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Cell-based split-luciferase-based trimer formation assay of α345(IV) collagen

In 2018, Omachi et al. used split-luciferase-based trimer formation assay to determine the effects of variants in a cell model[31]. The concept is that when cells express proteins from *COL4A3*, *COL4A4*, and *COL4A5* genes, three strands of type-4 collagen will form heterotrimer of α345(IV) collagen. The researcher will used split nanoluciferase binary technology (NanoLuc BiT) system in which a subunit (large Bit [LgBiT], and small BiT [SmBiT]) was fused to an α(IV) monomer. When the heterotrimer of α345(IV) collagen was normal, LgBiT and SmBiT will fused normally, and luminescence could be measured, as shown in Figure 2. If missense variants lead to malformation of heterotrimer, the LgBiT and SmBiT might not fused, and measured luminescence will be decreased.

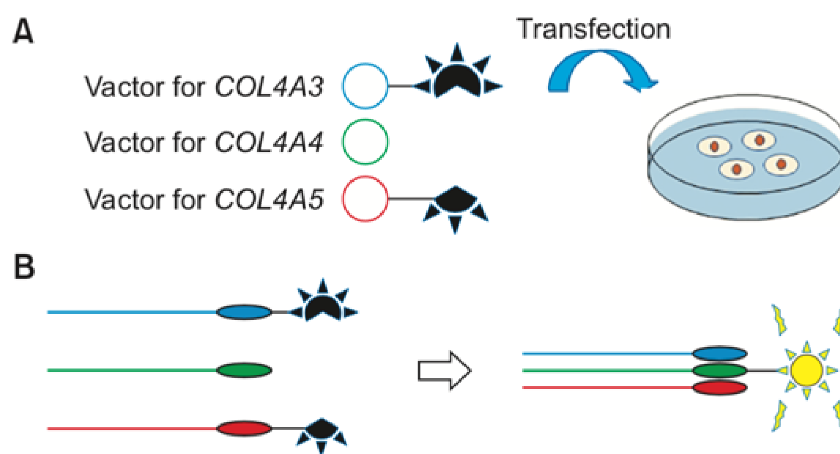

*Figure 2: **Cell-based Split-luciferase-based trimer formation assay of α345(IV) collagen***

A: Vectors for COL4A3-SmBiT, COL4A4 and COL4A5-LgBiT were transfected into HEK293 cells; B: HEK293cells will express proteins from three vectors. If the structures of three protein were complete and normal, SmBiT and LgBiT will fused properly. Luminescence can be measured and compared between cells with wild-type vectors and cells with mutated vectors

This cell-based technique can determine the etiologic evidence of *COL4A5* variants found by NGS or Sanger sequencing. By comparing luminescence to the wild-type, the luminescence of cells with a pathogenic variant was less than the luminescence of wild-type cells[32]. This technique could also be used for high-throughput screening of *COL4A5* variants. However, with the method developed by Omachi et al., the same method could be used for variants in *COL4A3* and *COL4A4* genes.

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CHAPTER III

MATERIALS AND METHODS

Population and sample

- **Target population** Thai FSGS patients
- **Study population** Thai FSGS patients in KCMH
- **Sample size**

Having data in hand, and very limited knowledge about the variability in clinical characteristics within groups and most importantly we have no single outcome variable: formal sample size calculation is unnecessary. However, according to previous data, about 10-40% of FSGS patients have positive genetic testing. Therefore, at least 10 FSGS patients should be included to guarantee one positive case in our study.

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Inclusion criteria

Patients must meet the inclusion criteria as follows:

1. Patients with the age of one year and above
2. Patients who underwent kidney biopsy and had segmental sclerosis in either LM or IF

Exclusion criteria

1. Patients who had testing positive for anti-HIV antibody, hepatitis B antigen and anti-HCV antibody.
2. Patients with secondary causes of FSGS which include:
   a. obstructive nephropathy
   b. systemic lupus erythematosus (SLE)
   c. obesity with the body mass index above 40 kg/m^2
   d. use of medication known to cause FSGS, such as heroin, interferon-α, lithium, pamidronate, alendronate
   e. conditions with reduced kidney mass including, oligometanephronia, unilateral kidney agenesis, kidney dysplasia, surgical kidney ablation

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Operational definition

1. Pediatric patients: aged 1-18 years
2. Nephrotic syndrome

   A clinical syndrome defined by generalized edema with nephrotic-range proteinuria (Pediatric patients: urine protein creatinine ratio [UPCR] $\geq$ 2.0 or $\geq$3+ dipstick. Adult patients: urine protein more than 3.5 g/day or by UPCR more than 3.5) and hypoalbuminemia (serum albumin less than 3.0 g/dL) with or without hypercholesterolemia (serum cholesterol more than 200 mg/dL) and lipiduria

3. Steroid-resistant nephrotic syndrome (SRNS)

   Pediatric patients: nephrotic syndrome, lack of remission at 4 weeks of therapy with oral corticosteroid at 2 mg/kg/day.

   Adult patients: nephrotic syndrome, does not respond to steroid treatment, a dose equivalent to prednisolone 1 mg/kg/day for 16 weeks.

4. Steroid-sensitive nephrotic syndrome (SSNS)

   Nephrotic syndrome patients who respond well to corticosteroid treatment and did not meet the criteria as SRNS

5. Asymptomatic proteinuria

   Patients who presented with any amount of proteinuria, did not have generalized edema and estimated glomerular filtration rate [eGFR] more than 60 ml/min/1.73 m^2.

6. Chronic kidney disease (CKD)

   Patients with eGFR of less than 60 ml/min/1.73 m^2 for more than 3 months.

7. Early-onset CKD

   CKD inpatients with aged less than 25 years.

8. End-stage renal disease (ESRD)

   CKD patients who undergo maintenance dialysis, either hemodialysis (HD) or peritoneal dialysis (PD), for more than 28 days.

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Study methodology

Screening and patient recruitment

We reviewed the renal pathology reports of patients who underwent kidney biopsy at King Chulalongkorn Memorial Hospital from January 2000 to December 2020. Patients who met the inclusion criteria and exclusion criteria listed above were then contacted by telephone and made an appointment for counseling and recruitment at the renal clinic. At the renal clinic, patients were informed about genetic testing for research purpose. The participant had freedom in deciding whether to participate in the study or not. The participants who decided to participate in genetic testing had to give written informed consent for this study before starting the research protocol. The Ethic Committee of Faculty of Medicine, Chulalongkorn University, approved the protocol of this study (Med Chula IRB no.1516/2562) in compliance with the International guidelines for human research protection as Declaration of Helsinki and International Conference on Harmonization in Good Clinical Practice.

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Genetic Counseling

All patients will be informed about the diagnosis of FSGS and how genetic testing might facilitate the treatment of their FSGS. They will further be interviewed about the clinical history of their illness and their family history of any renal diseases. They will be informed about the purpose of this research. The patients will also be informed that if the

result of genetic study might change the treatment, the results will be discussed with the responsible clinicians for further appropriate treatment of the patients.

Blood sampling

3 ml of whole blood in an EDTA tube (about 2 teaspoons) would be drawn from each patient for genetic testing with WES.

Data collection

Clinical characteristics and renal pathology were retrieved and reviewed. The patient's clinical presentations were classified into four main categories: asymptomatic proteinuria, SSNS, SRNS, and proteinuric CKD.

Genetic testing

Genomic DNA was extracted from peripheral blood leukocytes. The DNA sample was prepared as an Illumina sequencing library. The sequencing libraries were enriched by TruSeq® Exome Kit (Illumina Inc., Illumina, San Diego, CA) and were sequenced onto NextSeq 500 System (Illumina, San Diego, CA). Sequence reads were mapped against UCSC hg19 using Burrows-Wheeler Alignment (BWA) software. Variant calling was performed using GATK with *HaplotypeCaller*.

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Variant Interpretation

Golden Helix Genome Browser (Nagoya, Aichi-pref., Japan) and BaseSpace Variant Interpreter (Illumina, San Diego, CA) were used for genetic data analysis. We used the gene list to screen variants identified by WES. The gene list was shown in Table 5. Eighty-seven genes were previously reported as causative genes or genes associated with FSGS. Phenocopy disease genes, responsible for diseases that resembled FSGS shown by renal histopathology such as AS or Fabry's disease, were included in this gene list.

Table 5: ***Gene list***

No	Gene	Location	Inh.	Protein
1	*ACSL4*	Xq23	XL	acyl-CoA synthetase long-chain family member 4
2	*ACTN4*	19q13.2	AD	Alpha actinin 4
3	*ADCK4*	19q13.2	AR	Aarf domain containing kinase 4 (Coenzyme Q8B)
4	*AGXT*	2q37.3	AR	Alanine-glyoxylate aminotransferase
5	*ALG1*	16p13.3	AR	Asparagine-linked glycosylation 1
6	*ALG13*	Xq23	XL	UDP-N-acetylglucosaminyltranstease subunit
7	*ALMS1*	2p13.1	AR	Centrosome and basal body associated protein ALMS1
8	*ANLN*	7p14.2	AD	Anillin actin-binding protein
9	*APOE*	19q13.32	AR	apolipoprotein E
10	*APOL1*	22q12.3	AR	Apolipoprotein L-1
11	*ARHGAP24*	4q21.23	AD	Rho-GTPase activating protein

No	Gene	Location	Inh.	Protein
12	*ARHGDIA*	17q25.3	AR	Rho GDP-dissociation inhibitor alpha
13	*AVIL*	12q14.1	AR	Advillin
14	*CD2AP*	6p12.3	AD	CD2-associated protein
15	*CD151*	11p15.5	AR	CD151 antigen
16	*CFH*	1q31.3	AR	Complement factor H
17	*CLCN5*	Xp11.23	XL	Chloride voltage-gated channel 5
18	*COL4A1*	13q34	AD	Collagen type 4 alpha 1
19	*COL4A3*	2q36.3	AD	Collagen type 4 alpha 3
20	*COL4A4*	2q36.3	AD	Collagen type 4 alpha 4
21	*COL4A5*	Xq22.3	XL	Collagen type 4 alpha 5
22	*COQ2*	4q21.22	AR	Cerevisiae homolog of Q2 (Coenzyme Q10)
23	*COQ6*	14q24.3	AR	Cerevisiae homolog of Q6 (Coenzyme Q6)
24	*COQ8B*	19q13.2	AR	Coenzyme Q8B
25	*CRB2*	9q33.4	AR	Crumbs Drosophila homolog of 2, cell polarity complex component
26	*CTNS*	17p13.2	AR	Cystinosin, lysosomal cysteine transporter
27	*CUBN*	10p13	AR	Cubilin
28	*DAAM2*	6p21.2	AR	dishevelled associated activator of morphogenesis 2
29	*DGKE*	17q22	AR	Diacylglycerol kinase epsilon 64-KD
30	*E2F3*	6p22.3	AD	E2F transcription factor 3
31	*EMP2*	16p13.13	AR	Epithelial membrane protein 2
32	*EYA1*	8q13.3	AD	EYA transcriptional coactivator and phosphatase 1

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No	Gene	Location	Inh.	Protein
33	*FAT1*	4q35.2	AR	Fat tumor suppressor Drosophila, homolog of 1
34	*FN1*	2q35	AD	Fibronectin 1
35	*FRAS1*	4q21.21	AR	Fraser extracellular matrix complex subunit 1
36	*GLA*	Xq22.1	XL	Galactosidase alpha
37	*GPC5*	13q31.3	AR	Glypican 5
38	*INF2*	14q32.33	AD	Inverted formin 2
39	*ITGA3*	17q21.33	AR	Integrin alpha-3
40	*ITGB4*	17q25.1	AR	Integrin beta-4
41	*KANK1*	9p24.3	AR	KN motif and Ankyrin repeat domain containing protein 1
42	*KANK2*	19p13.2	AR	KN motif and Ankyrin repeat domain containing protein 2
43	*KANK4*	1p31.3	AR	KN motif and Ankyrin repeat domain containing protein 4
44	*LAGE3*	Xq28	XL	L antigen family member 3
45	*LAMA5*	20q13.33	AR?	Laminin subunit alpha 5
46	*LAMB2*	3p21.31	AR	Laminin beta-2
47	*LAMB3*	1q32.2	AR	Laminin beta-3
48	*LMNA*	1q22	AD	Lamin A, Lamin C
49	*LMX1B*	9q33.3	AD	LIM hoemobox transcription factor 1 beta
50	*MAFB*	20q12	AD	MAF bZIP transcription factor B
51	*MAGI2*	7q21.11	AR	Membrane-associated Guanylate kinase, WW and PDZ domains-containing 2
52	*MTTL1*	M	M	Transfer RNA. Mitochondrial, leucine 1

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No	Gene	Location	Inh.	Protein
53	*MTTL2*	M	M	Transfer RNA. Mitochondrial, leucine 2
54	*MUC1*	1q22	AD	Mucin 1, cell surface associated
55	*MYH9*	22q12.3	AD	Myosin heavy chain 9
56	*MYO1E*	15q22.2	AR	non-muscle Myosin 1E
57	*NEIL1*	15q24.2	AR	Nei like DNA glycosylase 1
58	*NPHS1*	19q13.12	AR	Nephrin
59	*NPHS2*	1q25.2	AR	Podocin
60	*NUP93*	16q13	AR	Nucleoporin 93-KD
61	*NUP107*	12q15	AR	Nucleoporin 107-KD
62	*NUP160*	11p11.2	AR	Nucleoporin 160-KD
63	*NUP205*	7q33	AR	Nucleoporin 205-KD
64	*NXF5*	Xq22.1	XL	Nuclear RNA export factor 5
65	*OCRL*	Xq26.1	XL	Phosphatidylinositol bisphophate-5-phosphatase
66	*OSGEP*	14q11.2	AR	O-sialoglycoprotein endopeptidase
67	*PAX2*	10q24.31	AD	Paired box 2
68	*PDSS2*	6q21	AR	Prenyl diphosphate synthase, subunit 2
69	*PLCE1*	10p23.33	AR	Phospholipase C epsilon-1
70	*PMM2*	16p13.2	AR	Phosphomannomutase 2
71	*PODXL*	7q32.3	AD	Podocalyxin like protein 1
72	*PTPRO (GLEPP1)*	12p12.3	AR	Protein-tyrosine phosphate receptor-type O

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No	Gene	Location	Inh.	Protein
73	*RCAN1*	21q22.12	AD	regulator of calcineurin 1
74	*SCARB2*	4q21.1	AR	Lysosomal integral membrane protein 2
75	*SGPL1*	10q22.1	AR	Sphingosine-1-phosphate lyase 1
76	*SMARCAL1*	2q35	AR	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily A like protein 1
77	*SYNPO*	5q33.1	AD	Synaptopodin
78	*TP53RK*	20q13.12	AR	TP53-regulating kinase
79	*TPRKB*	2p13.1	AR	TP53RK- binding protein
80	*TRPC6*	11q22.1	AD	Transient receptor potential cation channel subfamily C member 6
81	*TTC21B*	2q24.3	AR	Tetratricopeptide repeat domain-containing protein 21B
82	*UMOD*	16p12.3	AD	Uromodulin
83	*WDR19*	4p14	AR	WD (tryptophan-aspartic acid) repeat domain 19
84	*WDR73*	15p25.2	AR	WD (tryptophan-aspartic acid) repeat domain 73
85	*WT1*	11p13	AD	Wilms tumor 1
86	*XPO5*	6p21.1	AR	Exportin 5
87	*ZEB1*	10p11.2	AR	Zinc finger E-box binding homeobox 1
88	*ZMPSTE24*	1p34.2	AR	Zinc metalloprotease STE 24

AD = autosomal dominant, AR = autosomal recessive, Inh. = inheritance, M = mitochondrial, XL = X-linked

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The algorithm used for variant interpretation was as follows. Five criteria (I-V) were first applied, including (I.) coding consequences, (II.) read depth more than 20, (III.) within the gene list of 87 genes associated with FSGS, (IV.) with allele frequency of less than 1:1,000 in genes with autosomal recessive (AR) inheritance and less than 1:10,000 in genes with autosomal dominant (AD) or X-linked (XL) inheritance, and (V.) one variant in genes with AD or XL inheritance but two variants in genes with AR inheritance. All candidate variants were evaluated by clinical geneticists and nephrologists and were then classified according to joint consensus recommendations by the American College of Medical Genetics and Genomics and the Association of Molecular Pathology[9]. For *COL4A3*, *COL4A4*, and *COL4A5* genes, we used recommendations for variant interpretation as previously suggested by expert consensus[17, 26].

Immunohistochemistry (IHC) protocol

We used anti-collagen IV α3 (antibody produced in rabbit, product number SAB4500376, Lot number 3112197, Sigma-Aldrich, Saint Louis, MO), anti-collagen IV α4 (antibody produced in rabbit, product number SAB4500380, Lot number 3112198, Sigma-Aldrich, Saint Louis, MO) and anti-collagen IV α5 (antibody produced in rabbit, product number SAB4500381, Lot number 3112199, Sigma-Aldrich, Saint Louis, MO) as primary antibodies. At first, used normal renal tissue from nephrectomy cases and normal surveillance kidney allograft. We calibrated our protocol until we got the most distinct

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protocol. Negative control was then applied for confirmation of negative IHC staining. Negative control was selected from patients with clinical, pathological, and genetic confirmation of male AS. For patients with variants in COL4A3/4/5, their renal pathology tissues will undergo IHC protocol. The protocol was as follows:

1. Paraffin-embedded kidney biopsy samples sectioned to make 2 μm paraffin section
2. Deparaffinization as following
    a. Xylene: dip slide rack in xylene for 10 minutes
    b. 95% alcohol: 10 dips in 95% alcohol for 3 times
    c. Running tap water for 1 minute
3. Protease-induced epitope retrieval with proteinase K at room temperature then wash with buffer, the timing for antigen retrieval with proteinase K is varied as in Table 6
4. Block endogenous peroxidase activity by EnVision FLEX peroxidase-blocking reagent for 10 minutes, then wash with buffer
5. Block non-specific background by EnVision FLEX Antibody diluent for 10 minutes, then wash with buffer
6. Primary antibody staining with anti-collagen IV α3, anti-collagen IV α4 or anti-collagen IV α5 overnight (> 16 hours), then wash with buffer
7. EnVision FLEX+ Rabbit Linker for 15 minutes, then wash with buffer

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8. Secondary antibody for 20 minutes, then wash with buffer

9. DAB (diaminobenzidine) for 10 minutes, then wash with running tap water

10. Hematoxylin 5 dips, then wash with running tap water

11. Dehydration and mounting

The timing and concentration of primary antibodies differ for each collagen protein, as shown in Table 6.

Table 6: Comparison between IHC staining protocol for α3(IV) collagen, α4(IV) collagen and α5(IV) collagen

	α3(IV) collagen	α4(IV) collagen	α5(IV) collagen
Antigen retrieval	Proteinase K 5 minutes	Proteinase K 10 minutes	Proteinase K 10 minutes
Primary antibody	Conc. 1:50 Overnight > 16 hours	Conc. 1:50 Overnight > 16 hours	Conc. 1:50 Overnight > 16 hours
Linker	15 minutes	15 minutes	15 minutes
Secondary antibody	20 minutes	20 minutes	20 minutes
DAB	10 minutes	10 minutes	10 minutes

Conc. = concentration, DAB = diaminobenzidine, IHC = immunohistochemistry

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After IHC staining was done, two certified renal pathologists will separately and blindly examine the IHC staining of α3(IV) collagen, α4(IV) collagen, and α5(IV) collagen of these patients without knowing the details of the cases. Due to the lack of previous classification, we classified IHC staining of α3(IV) collagen, α4(IV) collagen, and α5(IV) collagen as positive, equivocal, and negative. The positive result was defined as the same staining intensity as the positive control, and the negative result as the same staining intensity as the negative control and if the case was stained somewhere between, we classified it as equivocal staining. If there was any incongruence in the results between two renal pathologists, they would re-examine the IHC together to find agreeable results without prior knowledge of previous results. The renal pathology results from the renal pathologists were then correlated with patients' genetic data.

Cell-based Split-luciferase-based trimer formation assay of α345(IV) collagen

Part 1: Normal *COL4A3*, *COL4A4*, and *COL4A5* plasmids

The cell-based study was done in a human embryonal kidney (HEK)293 cells. We used Collagen IV (*COL4A3*) (NM_000091) Human Tagged ORF Clone (Code: ORGRC223010), Collagen IV (*COL4A4*) (NM_000092) Human Tagged ORF Clone (Code: ORGRC211841), Collagen IV (*COL4A5*) (NM_000495) Human Tagged ORF Clone (Code: ORGRC217680) as plasmids for producing *COL4A3*, *COL4A4*, and *COL4A5* genes, respectively. We also used NanoBiT® Protein:Protein Interaction (PPI) System for

providing LgBiT and SmBiT for protein fusion. Collagen plasmids and vectors are shown in figure X. In our study, *COL4A3* plasmid must be cloned to fused with pBiT2.1-C [TK/SmBiT] Vector and *COL4A4* plasmid with pBiT1.1-C [TK/LgBiT] Vector. We designed a primer for PCR amplification of *COL4A3*-SmBiT and *COL4A4*-LgBiT, as shown in Table 7.

*Table 7: **Primers for PCR amplification of COL4A3-SmBiT and COL4A4-LgBiT***

Primer for		Primer sequence	GC(%)	Tm
***COL4A3*-SmBiT**	Forward primer	GCTCAGGGGAATTCGCCACCATGAGCGCCCGGACC	66.7	72.7
	Reverse primer	CACCACCGCTCGAGGTGTCTTTTCTTCAT	57.1	69.2
***COL4A4*-LgBiT**	Forward primer	GCTCAGGGGAATTCGCCACCATGTGGTCTCTGCAC	60.6	68.8
	Reverse primer	CACCACCGCTCGAGGCTATACTTCACGCA	60.6	67.9

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After PCR cloning, we checked the sequence of *COL4A3*-SmBiT and *COL4A4*-LgBiT by using primers as in Table 8.

*Table 8: **Primers for sequence checking of COL4A3-SmBiT and COL4A4-LgBiT***

Primer for	Primer sequence	GC(%)	Tm
COL4A3-SmBiT part 1 (A3Sm1)	TGTCCCCGGAAGAAATATATTT	36.4	51.5
COL4A3-SmBiT part 2 (A3Sm2)	TTCTCCTGGACTTCCAGGCACC	59.1	61.1
COL4A3-SmBiT part 3 (A3Sm3)	GGAAGTGAGGGAGTCAAGGGCA	59.1	61.1
COL4A3-SmBiT part 4 (A3Sm4)	GGTCCCCAGGAAATACAGGTCT	54.5	58.3
COL4A3-SmBiT part 5 (A3Sm5)	ACTGGGTTGTCCTGGAAAAATG	45.5	55.6
COL4A3-SmBiT part 6 (A3Sm6)	GGCCAGAGAGGAACCCCAGGAG	68.2	63.6
COL4A3-SmBiT part 7 (A3Sm7)	TGGGCCCTCCAGGAATCAGAGG	63.6	63.0
COL4A3-SmBiT part 8 (A3Sm8)	AATTGGGCCAAAAGGACCACCT	50.0	59.6
COL4A3-SmBiT part 9 (A3Sm9)	CTTGAGCCTTATATAAGCAGAT	36.4	49.7
COL4A4-LgBiT part 1 (A4Lg1)	TGTCCCCGGAAGAAATATATTT	36.4	51.5
COL4A4-LgBiT part 2 (A4Lg2)	AGGGGACAAAGGAGATAAGGGT	50.0	57.5
COL4A4-LgBiT part 3 (A4Lg3)	CCTCGGGGGGATCCTGGTTCCT	68.2	65.0
COL4A4-LgBiT part 4 (A4Lg4)	AGGGAGACTTGGGGCTCCCTGG	68.2	65.3
COL4A4-LgBiT part 5 (A4Lg5)	TCCACCTGGTTTTCGTGGTGAC	54.5	59.6
COL4A4-LgBiT part 6 (A4Lg6)	GCAGAGGGATGTCCTGGCGCAA	63.6	64.3
COL4A4-LgBiT part 7 (A4Lg7)	GGTGCCCAGGTGATCACGGGAT	63.6	63.8

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Primer for	Primer sequence	GC(%)	Tm
COL4A4-LgBiT part 8 (A4Lg8)	ACATGGATTTCCTGGGCCACCT	54.5	61.2
COL4A4-LgBiT part 9 (A4Lg9)	TACTGGCTGGCCAGCGCTGCGC	72.7	69.4
COL4A4-LgBiT part 10 (A4Lg10)	CCTTAAAAGAAAGCCAGGCCCA	50.0	57.9

Transfection and luminescence measurement

These two proteins (PRKACA and PRKAR2A) will combine after being expressed by HEK293 cells forming a functional enzyme with a bright luminescence signal, which can be detected. We used SmBiT-PRKACA Control Vector and LgBiT-PRKAR2A Vector as positive controls. For negative control, HEK293 cells without transfection were used.

Transfection was done via Amaxa™ 4D-nucleofector™ transfection protocol for HEK293 [ATCC®] as follows:

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1. Prepared HEK293 cells in T75 flask, for using 1 million HEK293 cells per transfection
2. Prepared SF 4D-Nucleofector X solution with Nucleofector™ solution 82 uL and supplement 18 uL per transfection
3. Mixed 4D-Nucleofector™ solution with plasmids (plasmid concentration 1.0-1.5 mcg/plasmid)

4. Mixed 1 million cells of HEK293 cells with solution from number 3 then pipette into Nucleocuvette™ Vessels
5. Put Nucleocuvette™ Vessels into Nucleofector™ X-unit machine using CM-130 program
6. Rinse the Nucleocuvette™ Vessels with cultured medium using an amaxa certified pipette. Then transfer the cells into 6-well plate

After transfection, incubate 6-well plate in incubator for 24 hours, then change media 3-4 hours before sub-cultured into 96-well plate for the experiment. Cultured for another 24 hours, then measured luminescence at 48 hours after transfection. Luminescence measurement was done using NanoBiT® Protein:Protein Interaction (PPI) System as following protocol.

1. Replace medium in 96-well plate with Optimem 100 uL
2. Incubate 96-well plate for 20 minutes
3. Prepared Nano-Glo live cell reagent by mixing Nano-Glo live cell substrate and Nano-Glo LCS dilution buffer in 1:19 ratio (20X dilution)
4. Add 25 uL of Nano-Glo live cell reagent to each well in 96-well plate
5. Incubate 96-well plate for another 20 minutes
6. Measure luminescence by Microplate machine

Results of luminescence should be reported as relative light unit (RLU).

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Part 2: Mutagenesis

For the mutagenesis part, we designed primers for mutagenesis in *COL4A4*-LgBiT plasmid, as in Table 9. We also select two known pathogenic mutations in *COL4A4* gene, as negative controls. Unfortunately, no pathogenic mutation in *COL4A4* gene underwent confirmed functional test for etiologic evidence of the mutation. Therefore, we select the pathogenic mutations reported in multiple patients by multiple institutes. We selected c.2906C>G, p.Ser969Ter as a nonsense mutation control and c.1396G>A, p.Gly466Arg as missense mutation control.

*Table 9: **Primers for mutagenesis in COL4A4-LgBiT plasmid***

Primer for		Primer sequence	GC(%)	Tm
905delG	Forward primer	TATTCCTGGATTTCCAGG	44.0	58.0
905delG	Reverse primer	CTTTTTCTCCTTTTGCCC	44.0	58.0
1805G>A	Forward primer	GGACCTCCAGaGGATCATGAAG	55.0	61.0
1805G>A	Reverse primer	TGGATCCCCTTTTTCTCC	50.0	61.0
2752G>A	Forward primer	AGGTTTTCCCaGAGAAAGAGGAAAGCCTG	48.0	68.0
2752G>A	Reverse primer	GGGAAACCAGGCAGCCCC	72.0	72.0

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Primer for		Primer sequence	GC(%)	Tm
Non-sense mutation control	Forward primer	GCTATCATTTgACAAAAGGGAAC	39.0	58.0
	Reverse primer	CATTTCTCCTTCATCTCC	44.0	56.0
Missense mutation control	Forward primer	TGGGAACCCCaGACCACAAGG	62.0	66.0
	Reverse primer	ACACTACAGTATATCACACTTGATC	36.0	62.0

After mutagenesis was done, we selected five clones from each mutagenesis for sequencing. Then we pick the correct sequence for the experiment as in part 1. Results of luminescence should be reported as relative light unit (RLU).

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CHAPTER IV

RESULTS

Patients' characteristics

53 patients met the inclusion and exclusion criteria. The clinical, laboratory and pathological characteristics of FSGS patients are shown in Table 10. 52.8% of patients (28/53) were male. The most common age group at the time of kidney biopsy was 19-45 years (19/53 = 35.9%), followed by 45-50 years (13/53 = 24.5%), 6-18 years (12/53 = 22.6%), 1-5 years (6/53 = 11.3%) and age more than 60 years (3/53 = 5.7%). Two patients had a family history of kidney diseases (2/53 = 3.8%). Only one patient had extra-renal manifestation compatible with Noonan syndrome. Two-thirds (35/53 = 66.0%) were adult patients older than 18. The most common clinical diagnosis/syndrome was SRNS (31/53 = 58.5%), followed by proteinuric CKD (12/53 = 22.6%), SSNS (5/53 = 9.4%) and asymptomatic proteinuria (5/53 = 9.4%). Most of our patients received immunosuppressive medications at some point during treatment. All FSGS patients who received immunosuppressive medications received prednisolone as the first line of treatment (40/53 = 75.5%). Calcineurin inhibitors, both cyclosporin and tacrolimus, were the most common second line treatment, followed by cyclophosphamide and mycophenolate mofetil. Some patients received many immunosuppressive medications because they failed to respond to second line treatment. The mean follow-up time in our

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study was 9.5 years. Currently, most of our patients were identified as asymptomatic proteinuria (20/53 = 37.7%) and proteinuria CKD (16/53 = 30.2%). 22.7% of our patients (12/53) progressed to ESRD with a median time from FSGS diagnosis of 8 years.

Table 10: Clinical, laboratory and pathological characteristics of FSGS patients in our study

Categories	Number of Patients (%)
Clinical characteristics	
Sex	
• Male	28/53 (52.8%)
Age at kidney biopsy	
• Age 1-5 years	6/53 (11.3%)
• Age 6-18 years	12/53 (22.6%)
• Age 19-45 years	19/53 (35.9%)
• Age 45-60 years	13/53 (24.5%)
• Age > 60 years	3/53 (5.7%)
Family history of kidney diseases	
• Positive	2/53 (3.8%)
Extra-renal syndromic manifestation	
• Positive	1/53 (1.9%)
Clinical diagnosis/syndrome	
• Asymptomatic proteinuria	5/53 (9.4%)
• SSNS	5/53 (9.4%)
• SRNS	31/53 (58.5%)
• Proteinuric CKD	12/53 (22.7%)
Immunosuppressive medications received	40/53 (75.5%)

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Categories	Number of Patients (%)
• Prednisolone	40/53 (75.5%)
• Cyclosporin	17/53 (32.1%)
• Tacrolimus	6/53 (11.3%)
• Cyclophosphamide	8/53 (15.1%)
• Mycophenolate mofetil	4/53 (7.5%)
• Others	2/53 (3.8%)
Mean time of follow-up	9.5 years
Current status	
• No proteinuria and normal creatinine	4/53 (7.5%)
• Asymptomatic proteinuria	20/53 (37.7%)
• Proteinuric CKD	16/53 (30.2%)
• ESRD	12/53 (22.7%)
• Dead	1/53 (1.9%)
Median time from FSGS diagnosis to ESRD	8 years
Pathological characteristics by light microscopy (N = 53)	
Columbia classification of FSGS	
• Tip lesion	11/53 (20.7%)
• Hilar/Perihilar lesion	4/53 (7.5%)
• Cellular variant	2/53 (3.8%)
• Collapsing variant	2/53 (3.8%)
• Not otherwise specified (NOS)	34/53 (64.2%)
Pathological characteristics by immunofluorescent study (N = 42)	
• Negative or non-specific IF staining	17/42 (40.5%)
• Only segmental IgM and/or C3 staining	11/42 (26.2%)
• Mesangial IgM and/or C3 staining	13/42 (30.9%)
• Others*	1/42 (2.4%)
Pathological characteristics by electron microscopy (N = 18)	

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Categories	Number of Patients (%)
Podocyte foot process effacement	
• No	0/18 (0%)
• Focal	12/18 (66.7%)
• Diffused	6/18 (33.3%)
Microvillous transformation	
• No	2/18 (11.1%)
• Focal	11/18 (61.1 %)
• Diffused	5/18 (27.8%)
Irregular GBM	10/18 (55.6%)
Multilayering of GBM	0/18 (0%)

CKD = chronic kidney disease, ESRD = end-stage renal disease, FSGS = focal segmental glomerulosclerosis, GBM = glomerular basement membrane, IF = immunofluorescent study, IgM = immunoglobulin M, SRNS = steroid-resistant nephrotic syndrome, SSNS = steroid-sensitive nephrotic syndrome

*One other case had trace coarse granular staining of IgM, kappa, and lambda along the capillary loop with focal linear C3 staining along Bowman's capsule

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The most common FSGS subtype according to Columbia classification was not-otherwise specified (FSGS, NOS) (34/53 = 64.2%), followed by tip lesion (11/53 = 20.7%), hilar or perihilar lesion (4/53 = 7.5%), cellular variant (2/53 = 3.8%) and collapsing variant (2/53 = 3.8%). IF was obtained in 42 patients. The majority had negative IF staining (17/42 = 40.5%). Mesangial IF staining and segmental IF staining of IgM and/or C3 were present in 12 cases (12/42 = 28.5%) and 11 cases (11/42 = 26.2%), respectively. EM was obtained in 18 patients. All patients with EM had podocyte foot process effacement (FPE). There were 12 patients (12/18 = 66.7%) with focal FPE and six patients (6/18 = 33.3%)

with diffused FPE. Microvillous transformation was identified in 13 patients (13/18 = 72.2%). Irregular GBM was found in ten patients (10/18 = 55.6%). None of the patients in our study had multilayering of GBM.

Genetic results

Of the 53 unrelated cases, 52 and one family underwent WES using only one member (the proband; singleton), and three members (the proband and parents; trio), respectively. Overall, six of 53 patients had pathogenic/likely pathogenic (P/LP) variants (6/53 = 11.3%) as defined by ACMG criteria. Of the six variants identified, two were novel. These variants included three missense, two nonsense, and one frameshift. Of these six P/LP variants, three were found in the *COL4A4* gene, one in the *CLCN5* gene, one in the *LMX1B* gene, and one in the *MAFB* gene. Detailed information on the clinical and pathological of patients with P/LP variants is shown in Table 11. Of the six patients with P/LP variants, two were pediatric patients, and four were adult patients. Four received immunosuppressive medications during the course of their treatment; however, none of them response to immunosuppressive medications. This data confirmed that patients with genetic FSGS did not respond to immunosuppressive medications. Hence, genetic testing is crucial for the management of non-secondary FSGS. Two had family history of renal diseases. Three had nephrotic-range proteinuria and three had sub nephrotic-range proteinuria. This data also confirmed that proteinuria in patients with genetic FSGS can

be varied from sub nephrotic-range to nephrotic-range proteinuria. Three patients progressed to ESRD and two patients progressed to proteinuric CKD. Columbia classification of FSGS was hilar type in three patients (50%) and not-otherwise specified (NOS) in three patients (50%). All of them had negative or segmental IF staining. EM revealed focal podocyte FPE. Sequence alignment and conservation of the novel variants are shown in Figure 3. Sequence alignment of both patients showed high read depth, which confirmed the variants in all patients. Both amino acids; glycine in position 302 in the *COL4A4* gene for patient E and and cysteine in position 46 in the *MAFB* gene for patient F; showed genetic conservation across many animal species including humans. Genetic conservation across many animal species in these amino acids is supporting data that an alteration of these amino acids might cause structural or functional changes in the protein. Fifteen patients harbored variants of uncertain significance (VUS), as shown in Table 12. Ten of patients with VUS presented with SRNS. Three patients progressed to ESRD and four patients progressed to proteinuric CKD. VUS were found in many genes including *ACSL4*, *ALMS1*, *ANLN*, *ARHGAP24*, *DAAM2*, *INF2*, *FN1*, *LMNA*, *LMX1B*, *MYH9*, *PAX2*, *SYNPO* and *TRPC6*. Further evaluation with genetic testing in patients' families or functional tests might be required to identify whether these VUS truly cause FSGS in these patients or not.

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Table 11: Clinical, laboratory, pathologic characteristics, and genetic variants of patients with pathogenic or likely pathogenic mutation (N = 6)

	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Clinical characteristics						
Age at kidney biopsy (years)	4	9	52	52	38	47
Sex	Male	Male	Female	Female	Female	Male
Clinical diagnosis/syndrome	Asymptomatic proteinuria	SRNS	Proteinuric CKD	SRNS	SRNS	Proteinuric CKD
Family history of kidney diseases	No	Yes	No	No	Yes	No
Extra-renal syndromic manifestation	None	None	None	None	None	None
Immunosuppressive medication received	Prednisolone	Prednisolone, CNIs	None	Prednisolone	Prednisolone	None
Current status	Asymptomatic proteinuria	ESRD on PD	Proteinuric CKD	Proteinuric CKD	ESRD on HD	ESRD on PD
Age at ESRD (years)	N/A	15	N/A	N/A	48	61

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	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Laboratory characteristics						
Serum creatinine at biopsy (mg/dL)	0.19	0.40	1.20	1.70	2.09	3.20
Proteinuria at biopsy (gm/day)	4.50 (on ACEI)	2.40 (on ACEI)	2.15 (on ACEI)	2.60 (on ARB)	4.07 (on ARB)	5.04 (No ACEI/ARB)
Current serum creatinine (mg/dL)	0.30	N/A	1.58	3.87	N/A	N/A
Current proteinuria (gm/day)	5.42 (on ACEI)	N/A	1.38 (on ARB)	1.71 (on ARB)	N/A	N/A
Pathological characteristics						
Columbia classification of FSGS (LM)	NOS	NOS	Hilar	Hilar	NOS	NOS
Immunofluorescent staining	No data	Negative	Segmental IgM staining	Negative	Segmental IgM and C3 staining	No data
Podocyte foot process effacement (EM)	Focal	No data	No data	Focal	No data	Focal
Microvillous transformation (EM)	Yes	No data	No data	Yes	No data	No

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	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Irregular GBM (EM)	No	No data	No data	No	No data	No
Genetic variants						
Gene	*CLCN5*	*LMX1B*	*COL4A4*	*COL4A4*	*COL4A4*	*MAFB*
Inheritance	AD	AD	AD	AD	AD	AD
Chromosome (HG19)	chrX:49855147	chr9:129455598	chr2:227942792	chr2:227919418	chr2:227967530	chr20:39317353
NM	NM_001127898.3	NM_001174146.1	NM_000092.4	NM_000092.4	NM_000092.4	NM_005461.4
Type of mutation	Nonsense	Missense	Missense	Missense	Frameshift indels	Nonsense
Known/Novel	Known[33]	Known[34]	Known[35, 36]	Known[37, 38]	Novel	Novel
Variant	c.2119C>T (p.Arg707Ter)	c.737G>A (p.Arg246Gln)	c.1805G>A (p.Gly602Glu)	c.2752G>A (p.Gly918Arg)	c.905delG (p.Gly302ValfsTer23)	c.138C>A (p.Cys46Ter)
Original AA group	Positively charged	Positively charged	Non-polar aliphatic	Non-polar aliphatic	Non-polar aliphatic	Polar, uncharged
Changed AA group	Stop codon	Polar uncharged	Negatively charged	Positively charged	N/A	Stop codon
AF (Thailand database)	0	0	0	0	0	0
AF (gnomAD, whole population)	0	0	0	9/280892 = 0.00003185	0	0
M-CAP prediction	N/A	0.813	0.893	0.897	N/A	N/A

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	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
		(Pathogenic)	(Pathogenic)	(Pathogenic)		
Polyphen-2 prediction	N/A	1 (Damaging)	1 (Damaging)	1 (Damaging)	N/A	N/A
PROVEAN prediction	N/A	-3.91 (Deleterious)	-7.64 (Deleterious)	-7.17 (Deleterious)	N/A	N/A
Sift prediction	N/A	0 (Damaging)	0 (Damaging)	0 (Damaging)	N/A	N/A
Variance conclusion	Pathogenic	Likely Pathogenic	Likely pathogenic	Likely pathogenic	Pathogenic	Pathogenic
ACMG criteria	PVS1+PM2+PM4	PS3+PM2+PP3	PM1+PM2 +PP3+PP5	PM1+PM2 +PP3+PP5	PVS1+PM2+PM4	PVS1+PM2+PM4

ACEI = angiotensin-converting enzyme inhibitors, ACMG = American College of Medical Genetics and Genomics, ARB = angiotensin receptor blockers, CKD = chronic kidney disease, EM = electron microscopy, ESRD = end-stage renal disease, IgM = immunoglobulin M, FSGS = focal segmental glomerulosclerosis, GBM = glomerular basement membrane, HD = hemodialysis, M-CAP = Mendelian clinically applicable pathogenicity score NOS = not otherwise specified, PD = peritoneal dialysis, Polyphen-2 = polymorphism phenotyping v.2, PROVEAN = protein variation effect analyzer, SRNS = steroid-resistant nephrotic syndrome, Sift = sorting intolerance from tolerance

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Table 12: ***Patients with variants with uncertain significance (VUS) (N = 15)***

Patients	Sex	Age at FSGS diagnosis (years)	Clinical presentation	Current status	Gene	Inheritance	Variance	Protein	Allele frequency (Thai database)	Allele frequency (gnomAD, East Asian)	Computational model*
G1800276	F	5	SRNS	Proteinuric CKD	*ARHGAP24*	AD	c.1190G>A	p.(Gly397Asp)	0.0078	0.0012	Conflicting results
G2100488	F	91	SRNS	Proteinuric CKD	*ARHGAP24*	AD	c.1875T>G	p.(Ser625Arg)	0	0	Pathogenic/Damaging
G6458	M	5	SRNS	Asymptomatic proteinuria	*ANLN*	AD	c.1760T>A	p.(Met587Lys)	0.0018	0	Conflicting results
G6787	F	8	SRNS	Asymptomatic proteinuria	*DAAM2*	AR	c.602T>C	p.(Ile201Thr)	0.0009	0.00006	Pathogenic/Damaging
					DAAM2	AR	c.803G>A	p.(Arg268Gln)	0	0.0003	Pathogenic/Damaging
					INF2	AD	c.2255G>A	p.(Arg752His)	0.0009	0.0002	Benign/Tolerated
G6914	M	56	SRNS	ESRD on HD	*FN1*	AD	c.3187A>G	p.(Thr1063Ala)	0	0.002	Conflicting results
G7294	M	13	SRNS	Asymptomatic proteinuria	*ANLN*	AD	c.1462G>A	p.(Val488Ile)	0	0.0003	Benign/Tolerated
G7384	F	35	SSNS	No proteinuria and normal creatinine	*LMNA*	AD	c.1279C>T	p.(Arg427Cys)	0	0.0001	Conflicting results
					SYNPO	AD	c.2206G>C	p.(Glu736Gln)	0	0	Conflicting results

Patients	Sex	Age at FSGS diagnosis (years)	Clinical presentation	Current status	Gene	Inheritance	Variance	Protein	Allele frequency (Thai database)	Allele frequency (gnomAD, East Asian)	Computational model*
G7392	F	42	Proteinuric CKD	ESRD on PD	*FN1*	AD	c.5577C>G	p.(Ile1859Met)	0	0	Benign/Tolerated
					ACSL4	AD	c.1007A>G	p.(Tyr336Cys)	0	0.0002	Conflicting results
G7393	F	54	SSNS	Asymptomatic proteinuria	*MYH9*	AD	c.4391G>A	p.(Arg1464His)	0	0.0006	Pathogenic/Damaging
G7420	F	41	SRNS	Proteinuric CKD	*LMX1B*	AD	c.1073C>T	p.(Ser358Phe)	0	0.0003	Conflicting results
G7535	F	50	Asymptomatic proteinuria with normal renal function	Asymptomatic proteinuria	*ANLN*	AD	c.659T>A	p.(Phe220Tyr)	0	0.0001	Benign/Tolerated
					MYH9	AD	c.1108+7C>T	N/A	0	0.0003	N/A
G7571	M	50	SRNS	Proteinuric CKD	*TRPC6*	AD	c.2084A>G	p.(Tyr695Cys)	0	0.0001	Conflicting results
G7614	M	33	SSNS	No proteinuria and normal creatinine	*PAX2*	AD	c.541C>T	p.(Pro181Ser)	0	0	Conflicting results
G7619	F	10	SRNS		*ALMS1*	AR	c.249C>A	p.(His84Gln)	0	0.001	Conflicting results

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Patients	Sex	Age at FSGS diagnosis (years)	Clinical presentation	Current status	Gene	Inheritance	Variance	Protein	Allele frequency (Thai database)	Allele frequency (gnomAD, East Asian)	Computational model*
				Asymptomatic proteinuria	*ALMS1*	AR	c.4246C>T	p.(Arg1418Trp)	0	0.002	Conflicting results
G7702	M	3	SRNS	ESRD on HD	*FN1*	AD	c.989A>G	p.(Gln330Arg)	0	0	Conflicting results
					MYH9	AD	c.519-4G>A	N/A	0	0.00005	N/A

CKD = chronic kidney disease, ESRD = end-stage renal disease, HD = hemodialysis, N/A = not applicable, PD = peritoneal dialysis, SRNS = steroid-resistant nephrotic syndrome, SSNS = steroid-sensitive nephrotic syndrome

* We used four computational models: M-CAP, Polyphen-2, PROVEAN, and Sift. Conflicting results mean there are conflicting results between these four models. Pathogenic/Damaging and Benign/Tolerated means all four computational models provided the same result

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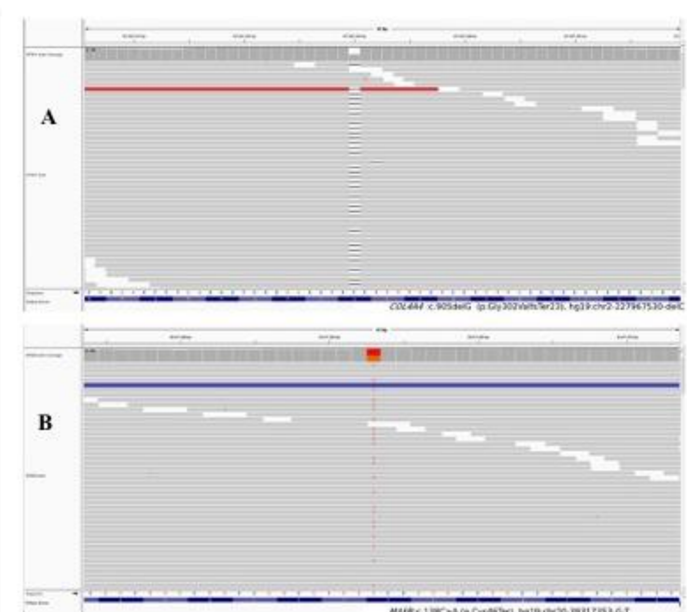
Figure 3: Screenshots of WES alignments of three novel variants and sequence alignment of partial amino acid sequences of *COL4A4* (A, B) and *MAFB* (C) from various species.

A: (Left) WES alignment of patient E's pathogenic variant (*COL4A4*, c.905delG, p.Gly302ValfsTer23), (Right) Glycine in position 302 in *COL4A4* gene had genetic conservation across many animal species. B: (Left) WES alignment of patient F's pathogenic variant (*MAFB*, c.138C>A, p.Cys46Ter), (Right) Cysteine in position 46 in *MAFB* gene had genetic conservation across many animal species.



COL4A4:p.Gly302ValfsTer23

Human	A	K	G	E	K	G	I	P	G	F	P
Rhesus	A	K	G	E	K	G	I	P	G	F	P
Mouse	I	K	G	E	K	G	I	P	G	F	P
Dog	P	K	G	E	K	G	I	P	G	F	S
Elephant	A	K	G	E	K	G	I	V	G	F	P
Chicken	-	-	-	-	-	-	-	-	-	-	-
X_tropicalis	-	-	-	-	-	-	-	-	-	-	-
Zebrafish	E	K	G	E	K	G	V	I	G	L	P
Fruit fly	L	T	G	E	K	G	D	P	G	L	P
C-Elegan	V	K	G	E	K	G	L	P	G	P	P

Patient E
COL4A4
c.905delG
p.Gly302ValfsTer23

MAFB:p.Cys46Ter

Human	P	-	G	R	P	C	T	R	L	Q	P
Rhesus	P	-	G	R	P	C	T	R	L	Q	P
Mouse	P	-	G	R	P	C	T	R	L	Q	P
Dog	P	-	D	A	S	C	A	V	G	S	P
Elephant	P	-	G	R	P	C	T	R	L	Q	P
Chicken	S	-	G	R	H	C	T	R	L	Q	P
X_tropicalis	A	-	I	R	P	C	N	R	L	Q	P
Zebrafish	A	N	I	R	Q	C	N	R	L	Q	P
Fruit fly	-	-	-	-	-	-	-	-	-	-	-
C-Elegan	-	-	-	-	-	-	-	-	-	-	-

Patient F
MAFB
c.138C>A
p.Cys46Ter

In subgroup analysis, the mutation detection rate in adult patients was 11.4% (4/35). The mutation detection rate in pediatric patients was 11.1% (2/18). All patients with a family history of renal diseases (2/2 = 100%) had P/LP variants, one with a novel variant, c.905delG (p.Gly302ValfsTer23) in *COL4A4*, and the other with a known variant, c.737G>A (p.Arg246Gln) in *LMX1B*. In 51 sporadic cases, the mutation detection rate was 3.9% (2/51). Among patients presenting with SRNS, the mutation detection rate was 6.5% (2/31). Among patients presenting with SSNS, no disease-associated variants were identified. There were some characteristic features in renal pathology associated with genetic FSFS. All patients with genetic FSGS had hilar or NOS lesions in LM. None of them had tip lesion or cellular or collapsing variant. All three genetic FSGS patients with EM results had focal FPE with microvillous transformation.

The median age of disease onset and end stage renal disease (ESRD) of these 53 cases was 33 and 47 years, respectively. Among FSGS patients with disease-associated variants, the median age of onset and ESRD was 42.5 and 48 years, respectively.

The clinical and pathological characteristics of patients with P/LP variants are shown in Table 11. Patient A presented with a febrile urinary tract infection at the age of one year and a history of passing stones at four years of age. Investigations revealed nephrotic-range proteinuria, hypercalciuria, and nephrocalcinosis. The urinary concentration of low-molecular-weight proteins and urinary β2MG were not measured.

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Given the male gender, laboratory data and the absence of signs of other diseases, Dent disease was primarily considered. He was briefly treated with high-dose prednisolone with an absence of response. He underwent a renal biopsy and was diagnosed with FSGS, NOS with focal FPE in EM. To establish a definitive molecular diagnosis, exome sequencing was performed and a known hemizygous nonsense variant (c.2119C>T, p.Arg707Ter) in the CLCN5 gene was identified, resulting in a diagnosis of Dent disease type 1[33]. Currently, he had good renal function and his proteinuria was controlled by an angiotensin converting enzyme inhibitor (ACEI).

Patient B presented with SRNS and did not respond to prednisolone, cyclosporin, and tacrolimus. His renal biopsy showed FSGS, NOS with negative IF staining. His renal function rapidly declined until he reached ESRD six years after his initial presentation. His father also had kidney transplantation (KT) at the age of 37. The pedigree is shown in Figure 4A. Both were found to have a known missense variant (c.737G>A, p.Arg246Gln) in the *LMX1B* gene[34]. Currently, patient B underwent peritoneal dialysis and is awaiting KT.

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Patient C presented with proteinuric CKD. Her urinalysis showed microscopic hematuria without history of gross hematuria. She received no immunosuppressive medication but was treated with an angiotensin receptor blocker (ARB). Her kidney biopsy showed hilar FSGS with segmental IgM staining in IF. WES revealed a known missense

variant in *COL4A4* (c.1805G>A, p.Gly602Glu)[35, 36]. After 17 years of follow-up, she was still at CKD stage 3b.

Patient D had SRNS, which did not respond to prednisolone. She did not have a history of episodic macroscopic hematuria, but her urinalysis showed microscopic hematuria. Her kidney biopsy showed hilar FSGS, negative IF staining and focal FPE in EM. WES revealed a known missense variant in *COL4A4* (c.2752G>A, p.Gly918Arg)[37, 38]. After eight years of follow-up, she was at CKD stage 4 and treated with ARB.

Patient E presented with SRNS and did not respond to prednisolone. Her urinalysis showed microscopic hematuria without history of gross hematuria. Her renal function gradually declined until she reached ESRD in ten years. She also had a family history of kidney disease, with her pedigree shown in Figure 4B. Her younger brother had a history of dialysis, but he died before patient E was offered genetic testing. WES revealed a novel deletion in *COL4A4*, leading to frameshift and premature stop codon (c.905delG, p.Gly302ValfsTer32). Now she underwent hemodialysis, waiting for KT. Genetic mutation in this family cannot be proven familial or de novo.

Patient F presented to our clinic with CKD and proteinuria of 5.04 g/day. He did not receive any immunosuppressive medication. His renal biopsy showed FSGS, NOS with focal FPE in EM. He was found to have a novel nonsense variant (c.138C>A, p.Cys46Ter) in the *MAFB* gene. His renal function gradually declined until he reached ESRD in 13 years. Currently, he underwent peritoneal dialysis, also waiting for KT.

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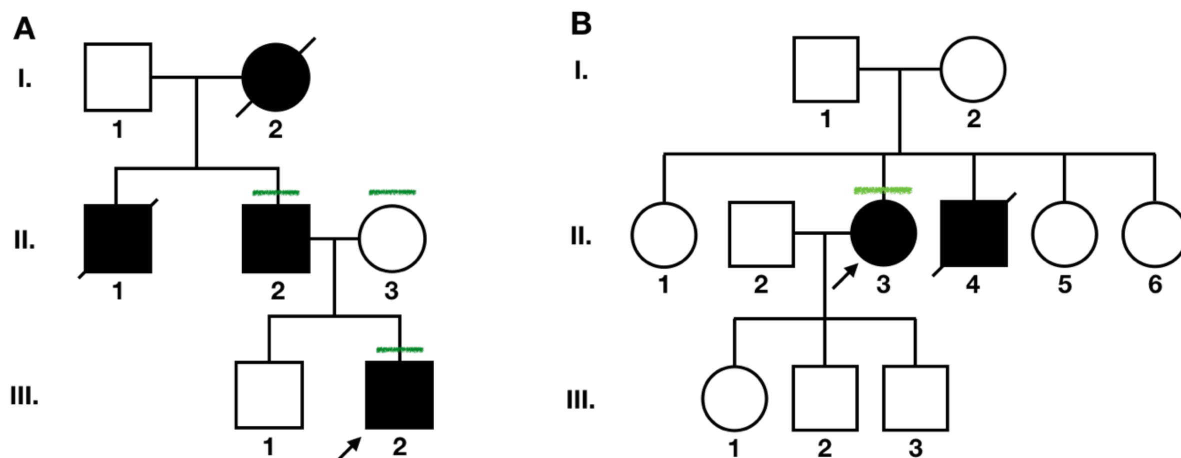

Figure 4: ***Pedigrees of two FSGS patients with positive family history***

Patient B's pedigree is shown in figure 4A. Patient E's pedigree is shown in figure 4B.

Mutation analysis was performed in patients with green bar.

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Genetic testing in a family of FSGS patients with positive family history

Patient B had a strong family history of renal diseases. Patient B himself presented with SRNS, but his father presented with advanced stage CKD. At first, we did not know that both patients, a father, and a son, had the same mutation because their clinical presentation was very different. In 1999, patient II-2 (Figure 4A), a 33-year-old man who presented to our renal clinic with dyspnea and orthopnea was diagnosed with CKD stage 5 and pulmonary edema. He received treatment to delay CKD progression without kidney biopsy as imaging showed bilateral small kidney size. Eventually, 8 months after being presented to the clinic, he received the initiation of hemodialysis and continued for three years before undergoing a deceased donor kidney transplantation. Six months post-

transplant, he developed nephrotic-range proteinuria. The allograft biopsy revealed thickening of glomerular capillary loops compatible with membranous nephropathy. He had been put on angiotensin converting enzyme inhibitors (ACEIs). Currently, he is 54 years of age and has a stable renal function with proteinuria of 0.6 gram per day. His latest serum creatinine level was 1.7 mg/dL and 24-hour urine creatinine clearance was 55 ml/min.

In 2007, a son of patient II-2 (in Figure 4A), patient B, an 8-year-old boy, was diagnosed with nephrotic syndrome. He had been treated with high-dose corticosteroid for 3 months but did not respond. The kidney biopsy revealed FSGS pattern without cellular proliferation (Figure 5). Immunofluorescence staining was negative for IgG, IgM, and IgA. Electron microscopy (EM) showed extensive effacement of the podocyte foot process without electron-dense deposition. He was diagnosed with steroid-resistant primary FSGS and was switched to cyclosporine for two years. The proteinuria remained in nephrotic range and a repeated kidney biopsy showed the progression of FSGS. His renal function gradually declined and peritoneal dialysis was initiated when he was 15. Currently, he is 21 years of age and on the waiting list for cadaveric kidney transplantation.

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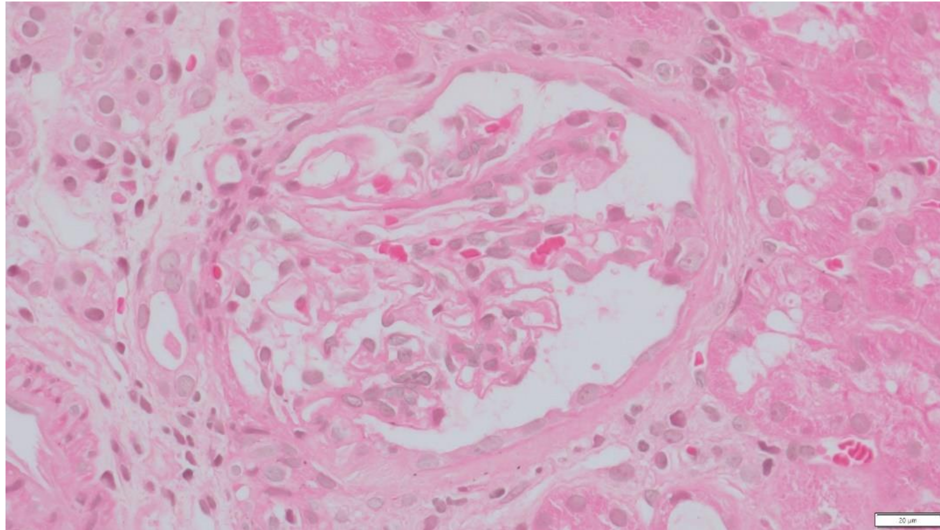

Figure 5: ***Renal pathology of LMX1B-associated nephropathy in patient 2***

The renal pathology (H&E staining) showed focal adhesion in the glomerulus, compatible with FSGS pattern. Columbia classification of FSGS is NOS. A scale bar in figure 1B equals 20 μm.

We identified the renal disease in three generations in this family, as shown in Figure 4A. Patient I-2 died at the age of 30 years with unidentified kidney disease as comorbidity. Patient II-1 had the end-stage renal disease (ESRD) at the age of 49 years and subsequently received a kidney transplant. However, he died from severe infection seven months after the transplantation.

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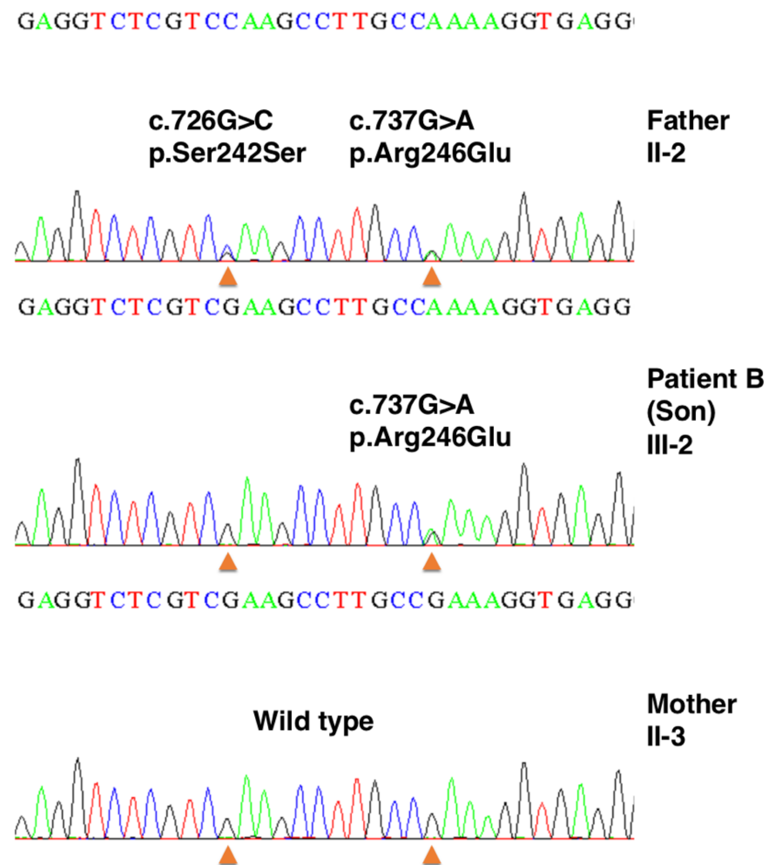

Figure 6: PCR-Sanger sequencing of patient B's family

PCR-Sanger sequencing revealed a missense mutation in the *LMX1B* gene [NM_002316.3, c.737G>A, p.Arg246Glu] in family members II-2 (proband, father), and III-2 (son). There was a synonymous variant in II-2 (father) [c.726G>C, p.Ser242Ser] which did not pass to his son and was absent in II-3 (mother).

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We further investigated the possibility of the same genetic defect in this family by WES of the father (II-2) and his wife (II-3). The analysis revealed that the father (II-2) had a heterozygous for a previously reported pathogenic missense mutation, c.737G>A

(p.Arg246Gln) in the *LMX1B* gene (NM_002316.3), same as patient B. PCR-Sanger sequencing confirmed the presence of this mutation in both patient B and his father as shown in Figure 6. Notably, patient II-2 also harbored a synonymous variant, c.726G>C, resulting in the same amino acid (serine), but he did not pass it to his son. Re-evaluation for extra-renal manifestations of both patients (patient B and his father) revealed normal nails and normal elbows, knees, and pelvis by X-rays.

Renal pathology results

For positive control, the IHC staining of α3(IV) collagen, α4(IV) collagen, and α5(IV) collagen were inarguably positive, as shown in Figure 7. A five-year-old male patient with clinical AS was a negative control. He had intermittent gross hematuria and proteinuria with normal renal function. He also had mild right sensorineural hearing loss. His eye examination was normal. He also had a family history of AS. Light microscopy (LM) finding was normal and immunofluorescent study (IF) finding was non-specific. Electron microscopy (EM) showed irregularly thin GBM with focal podocyte foot process effacement. No GBM splitting or multi-layering was observed in EM. WES of this patient revealed a novel pathogenic nonsense mutation (c.4599C>A, p.Cys1533Ter), which confirmed the diagnosis of X-linked AS. IHC staining of α3(IV) collagen, α4(IV) collagen and α5(IV) collagen of this patient is negative, as shown in Figure 7. This patient is 11 years old with normal renal function and minimal proteinuria.

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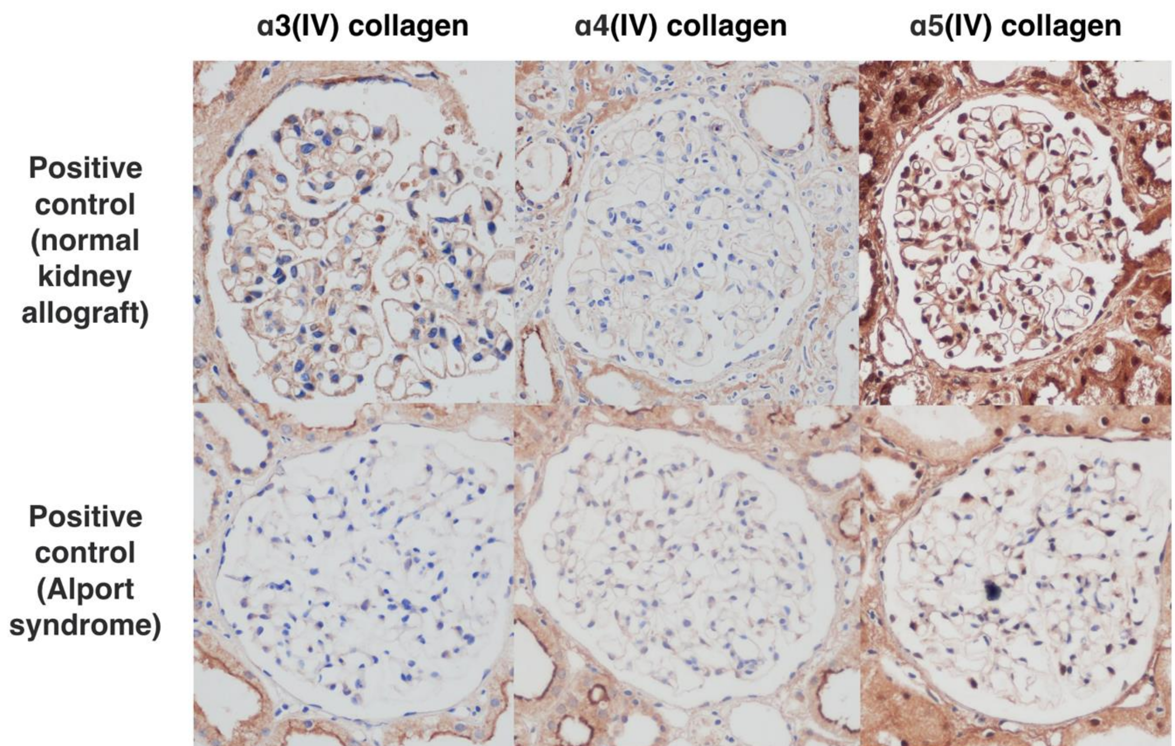

*Figure 7: **IHC staining of α3(IV) collagen, α4(IV) collagen and α5(IV) collagen***

Upper row: From left to right, IHC staining of α3(IV) collagen, α4(IV) collagen and α5(IV) collagen of positive control. Positive staining can be seen as brown line along glomerular basement membrane. Lower row showed significantly weaker IHC staining of α3(IV) collagen, α4(IV) collagen and α5(IV) collagen in negative control compared with positive control in upper row.

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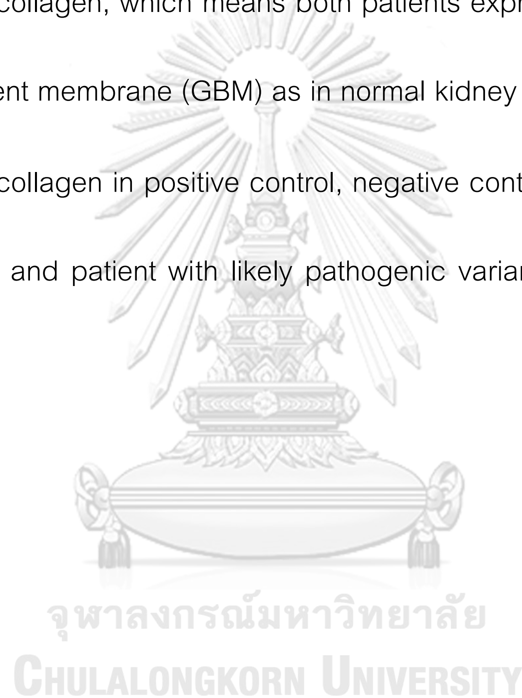
From WES results, we had three patients with P/LP variants in the COL4A4 genes, which are patient C (c.1805G>A, p.Gly602Glu), patient D (c.2752G>A, p.Gly918Arg), and patient E (c.905delG, p.Gly302ValfsTer32). Patient C's renal tissue was discarded from the pathology department because the biopsy was done more than 15 years ago.

Therefore, we did IHC staining of α3(IV) collagen, α4(IV) collagen and α5(IV) collagen in patient D, patient E, and the available renal tissues of the other FSGS patients in our study. The staining of α3(IV) collagen and α4(IV) collagen varied significantly among patients. Therefore, we used only α5(IV) collagen for interpretation. For FSGS patients with pathogenic and likely pathogenic variants in *COL4A4*, both of them had positive staining of α5(IV) collagen, which means both patients express α345(IV) trimer in their glomerular basement membrane (GBM) as in normal kidney of positive control. The IHC staining of α5(IV) collagen in positive control, negative control, patient with pathogenic variant (patient E), and patient with likely pathogenic variant (patient D) are shown in Figure 8.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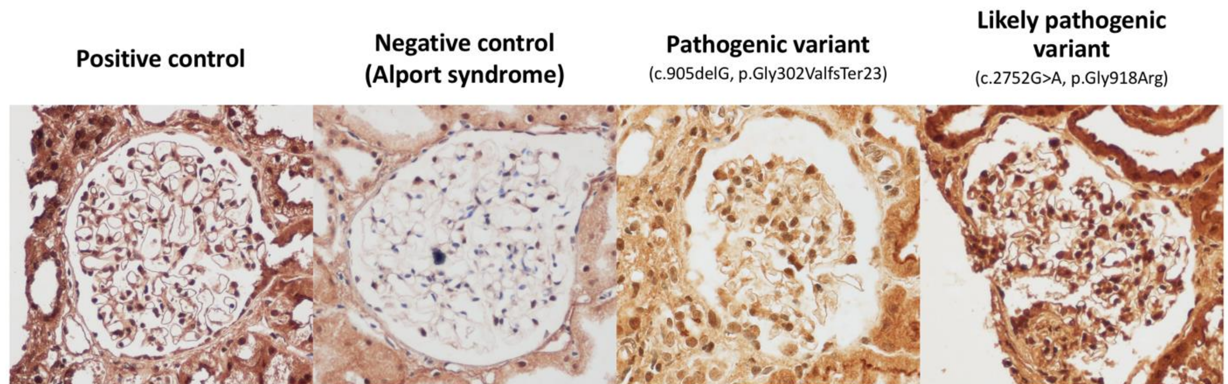

Figure 8: ***Immunohistochemistry staining of α5(IV) collagen***

From left to right: IHC staining of α5(IV) collagen in the positive control, negative control (a male Alport syndrome with confirmed clinical and genetic diagnosis), a patient with pathogenic variant in *COL4A4* (patient E, c.905delG, p.Gly302ValfsTer23) and a patient with likely pathogenic variant in *COL4A4* (patient D, c.2752G>A, p.Gly918Arg). The IHC staining showed that IHC staining of α5(IV) collagen of patient D and E was positive despite having P/LP variant in the *COL4A4* gene.

Cell study results

PCR amplifications for *COL4A3*-SmBiT vector and *COL4A4*-LgBiT vector were done. The sequence of *COL4A3*-SmBiT vector was checked by nine primers (A3Sm1-A3Sm9). The sequence of *COL4A4*-LgBiT vector was checked by ten primers (A4Lg1-A4Lg10). The sequencings were correct in both *COL4A3*-SmBiT and *COL4A4*-LgBiT. Figure 9 showed both vectors and their primers. Primers are shown with small colored segments under both vectors, labelled A3Sm1 to A3Sm9 and A4Lg1 to A4Lg10. Primers were designed to cover both targeted genes and nanoluciferases (SmBiT and LgBiT). The sequencings of each primers were overlapped as shown in Figure 9.

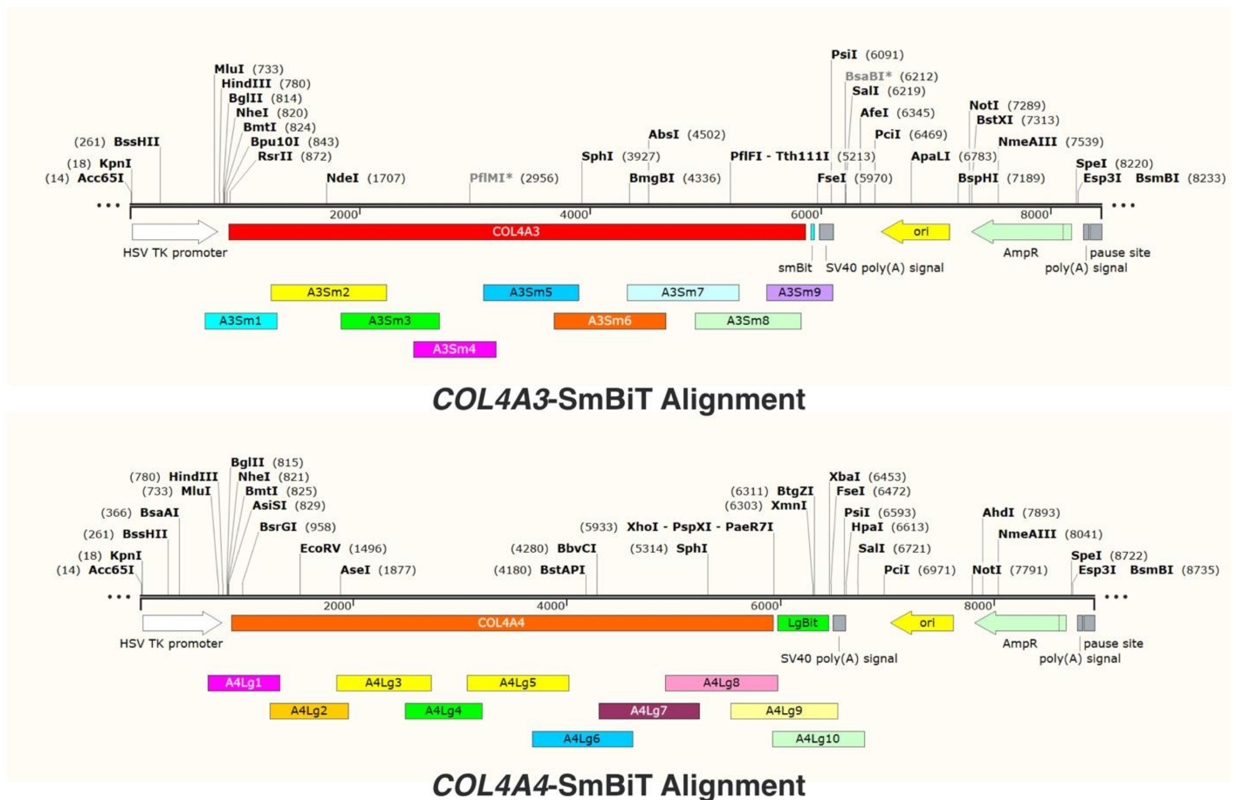

Figure 9: ***Sequence alignment of COL4A3-SmBiT and COL4A4-LgBiT Vectors***

COL4A3-SmBiT and *COL4A4*-LgBiT vectors are showed with primer below the vectors. Primers were designed to cover *COL4A3* and *COL4A4* genes and SmBiT and LgBiT.

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We measured the concentration of plasmid DNAs for transfection as in Table 13. Because we needed 1.50 ng of plasmid DNA for transfection, we calculated the plasmid amount used for transfection as shown in Table 13. Then, we did the transfection according to our protocol.

Table 13: ***Plasmid DNA concentration***

Plasmid	DNA concentration	Amount used for transfection
COL4A3-SmBiT	983.5 ng/uL	1.52 uL
COL4A4-LgBiT	997.9 ng/uL	1.50 uL
COL4A5	1,044.0 ng/uL	1.44 uL

After transfection, we measured the luminescence results for negative control (no transfection) and wide type (*COL4A3*-SmBiT, *COL4A4*-LgBiT and *COL4A5*). The luminescence of wild type will be used as a reference which will be referred as 100% and can be used as positive control for split-luciferase-based trimer formation assay in HEK293 cells. The luminescence of negative control was at 17.45% of RLU from cells expressing wide type as shown in Figure 10. This result implied that untransfected HEK293 cells did not have the same luminescence as the wild type.

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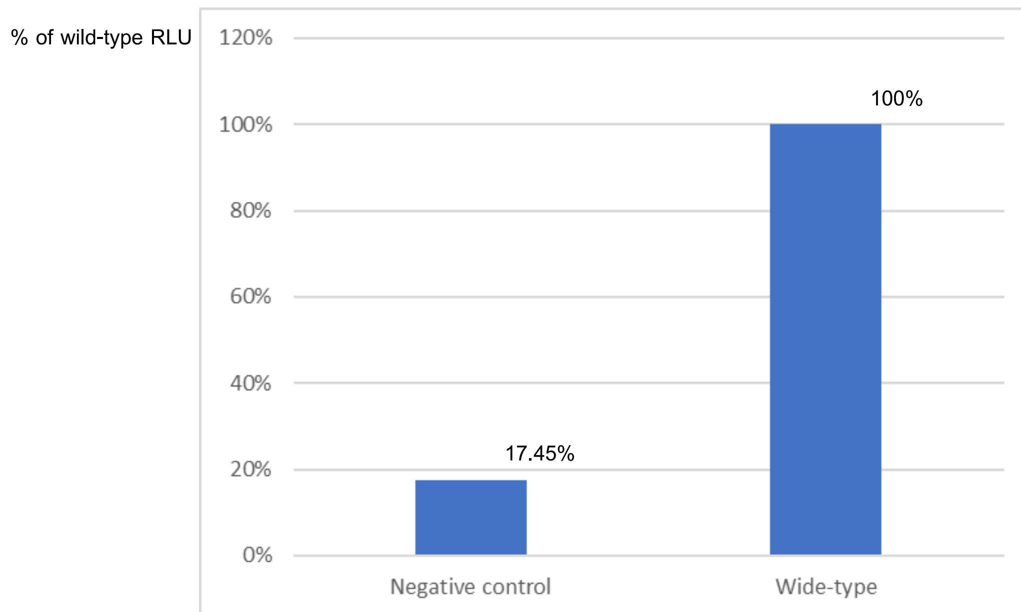

Figure 10: ***Results from the luminometric measurement of wide-type and negative control***

The luminescence of negative control was at 17.45% of RLU from cells expressing wide type (positive control).

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After we successfully demonstrated that the system for cell-based split-luciferase-based trimer formation assay in HEK293 cells can distinguished between wild-type and negative control, we proceeded to mutagenesis with P/LP variants we found in our patients. We selected the P/LP variants in patient C, who had a known missense variant in *COL4A4* (c.1805G>A, p.Gly602Glu), and patient D who had a known missense variant in *COL4A4* (c.2752G>A, p.Gly918Arg), and patient E who had a novel deletion in *COL4A4*, leading to frameshift and premature stop codon (c.905delG, p.Gly302ValfsTer32). We also used a known nonsense mutation (c.2906C>G, p.Ser969Ter) and a known missense mutation (c.2906C>G, p.Gly466Arg) as controls. We

did the mutagenesis and re-sequencing to confirm the mutation in plasmids, as shown in Figure 11. The pink arrows showed the site of desired mutations. The above row showed the sequences of wild-type plasmid. The below row showed the sequences of mutated plasmids, from left to right, c.905delG, c.1805G>A, c.2752G>A, c.2906C>G, and c.2906C>G. All five mutagenesis had the correct mutations as we desired.

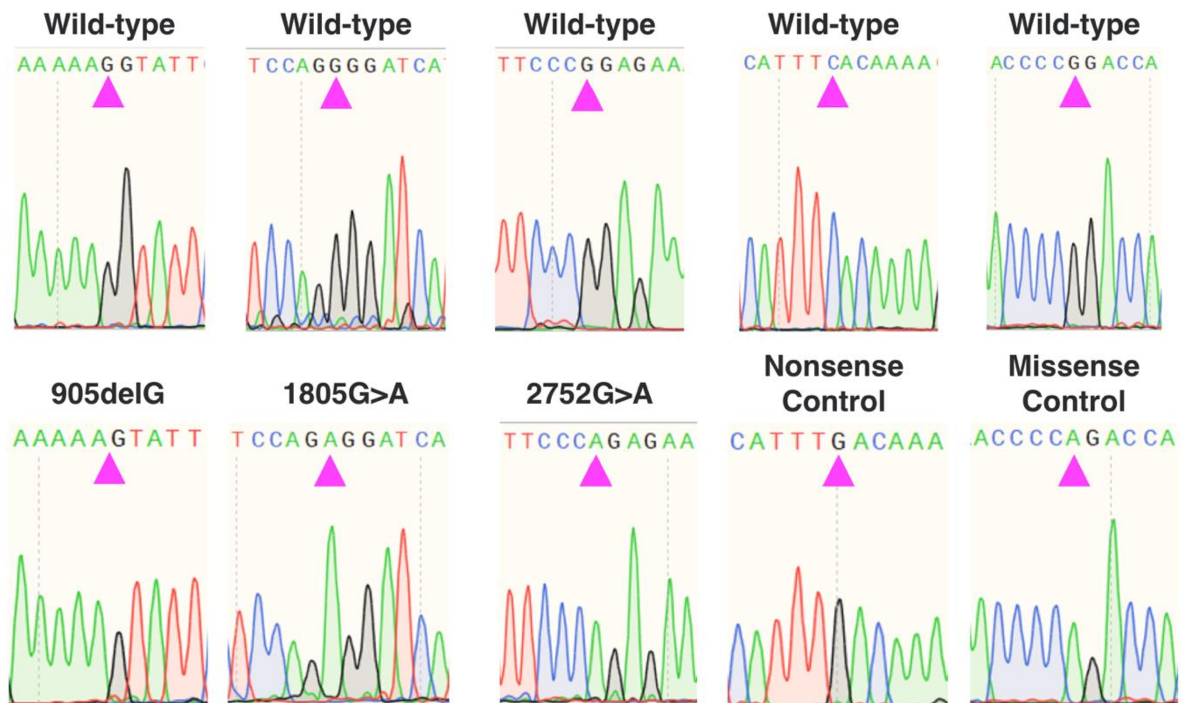

*Figure 11: **PCR-Sanger sequencing of COL4A4-LgBiT plasmid after mutagenesis compared with wild-type plasmid***

The above row showed chromatograms of wild-type plasmid. The below row showed chromatograms of mutated plasmids, from left to right, c.905delG, c.1805G>A, c.2752G>A, c.2906C>G, and c.2906C>G.

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After sequencing, we proceeded to transfection processes. We measured plasmid DNA concentration as shown in Table 14. Because we needed 1.50 ng of plasmid DNA for transfection, we calculated the plasmid amount used for transfection as shown in Table 14. We did the transfection again with the same protocol.

Table 14: ***Plasmid DNA concentration of COL4A4-LgBiT in mutagenesis***

Plasmid	DNA concentration	Amount used for transfection
COL4A4-LgBiT: 905delG (patient 5)	895.9 ng/uL	1.67 uL
COL4A4-LgBiT: 1805G>A (patient 3)	1,028.3 ng/uL	1.46 uL
COL4A4-LgBiT: 2752G>A (patient 4)	950.0 ng/uL	1.58 uL
COL4A4-LgBiT: nonsense control	992.1 ng/uL	1.51 uL
COL4A4-LgBiT: missense control	1,028.6 ng/uL	1.46 uL

After transfection, we measured luminescence results of the mutagenesis. The luminescence in HEK293 cells that transfected with wild-type plasmids will be calculated as 100% (Left in Figure 12). The results for mutagenesis are as follows (from left to right): 905delG (patient 5) was at 33.68% of RLU from cells expressing wide type, 1805G>A (patient 3) was at 30.94% of RLU from cells expressing wide type, 2752G>A (patient 4) was at 19.74% of RLU from cells expressing wide type, nonsense control was at 22.50% of RLU from cells expressing wide type, and missense control was at 23.58% of RLU from cells expressing wide type as shown in Figure 12. The luminescence of all five experiments were significantly lower than the wild-type which implied that the heterotrimers of α345 collagen was not created or not formed correctly. Thus, SmBiT and

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LgBiT cannot fused properly and luminescence was measured significantly lower than the positive control (wild-type).

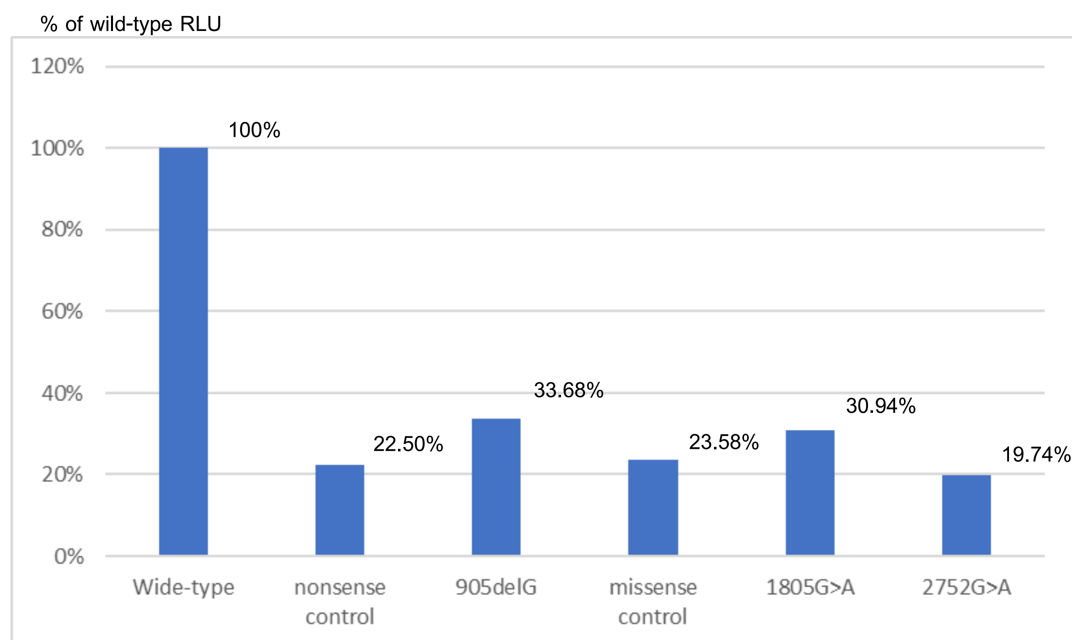

Figure 12: ***Results from the luminometric measurement of wide-type, three cases from out cohort nonsense control and missense control***

The luminescence of nonsense control and missense control were at 22.50% and 23.58% of RLU from cells expressing wide type (positive control), respectively. The luminescences of cells expressing mutated plasmid were at 33.68%, 30.94% and 19.74% of RLU from cells expressing wide type (positive control) for c.905delG, c.1805G>A and c.2752G>A, respectively.

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CHAPTER V

DISCUSSION

In this study, we analyzed the clinical and genetic data of 53 biopsy-proven FSGS patients. Using whole exome sequencing, P/LP variants were identified in six patients (11.3%). All patients with a family history of renal diseases (2/2 = 100%) had P/LP variants. In 51 sporadic cases, the mutation detection rate was 7.8% (4/51). The prevalence of genetic FSGS in our study appeared to be lower than the previous study, which had a mutation detection rate of around 11-26%, as shown in Table 15. This variation in the prevalence of mutation detection rate might be due to different population races, age, family history of renal diseases, and percent of consanguinity in the cohort.

Genetic testing is very important for management decisions, as 40 patients in our cohort (75.5%) received immunosuppressive medications to treat presumed primary FSGS. Only five patients (9.4%) responded to the treatment, and none of them were found to harbor causative variants. These findings also supported previous recommendations not to do genetic testing in SSNS patients[14] because genetic testing in patients presenting with SSNS yields a very low or negative results. 31 of them (58.5%) met the criteria of SRNS. However, causative variants were identified in only two (6.5%). Half of these patients with genetic FSGS (3/6 = 50%) did not receive any immunosuppressive medication. This could occur because nephrologists decided not to prescribe

immunosuppressive medications in 24.5% of our cohort, even though the patients had FSGS with no apparent secondary causes. The reasons for not treating FSGS patients with immunosuppressive drugs might include advanced glomerulosclerosis and IFTA in kidney biopsy or clinical presentation not compatible with nephrotic syndrome. The practice in KCMH was in line with the new 2021 KDIGO guideline for glomerular diseases(4). Nephrologists in KCMH usually applied clinicopathological parameters to select only some patients to be treated with corticosteroids, including the clinical presentation of nephrotic syndrome and diffused podocyte foot process effacement in EM.

The *COL4* genes (*COL4A3*, *COL4A4*, and *COL4A5*), encode collagen type 4, are essential for normal GBM. Mutations in these genes have been implicated as the cause of Alport syndrome. Three of our patients (3/6 = 50.0%) had a disease-associated variant in the *COL4A4* gene, which was in line with previous studies(4, 39). In addition, several studies have demonstrated that *COL4A3-5* mutations are associated with FSGS pathology. We identified three disease-associated variants in the *COL4A4* gene in unrelated patients. All were found at the conserved glycine residue (Table 2). Glycine residues in inter-collagenous domains were highly conserved from *H sapiens* (humans) to *X tropicalis* (frogs) and critical for collagens to be folded and function as normal GBM(26). The missense mutations at the glycine residue are increasingly reported to be likely pathogenic without a functional study. However, some of these missense mutations

were re-classified as benign after functional studies were performed to evaluate the molecular effect[32]. Therefore, further studies are required to confirm the disease-variant association and elucidate the underlying mechanism.

Our study also emphasized the importance of intrafamilial variability in genetic kidney diseases, as shown in patient B’s family. The patient B family who had a *LMX1B* causative variant suggested an interesting intrafamilial and interfamilial variability of the same mutation. We further reviewed the previous reports of patients with a known missense variant (c.737G>A, p.Arg246Gln) in the *LMX1B* gene, as shown in Table 16.

Table 15: ***Comparison between our FSGS cohort and previous studies***

	Our Cohort	Santin CJASN 2011	Sadowski JASN 2015	Gast NDT 2016	Bierzynska KI 2017	Sen JMG 2017	Warejko CJASN 2018	Gribouval KI 2018	Yao CJASN 2019	Ammar JHG 2021	Braunisch EJHG 2021	Miao Mayo 2021
References		(8)	(40)	(5)	(41)	(42)	(43)	(44)	(6)	(45)	(46)	(7)
Cohort characteristics												
Total patients	53	125	2016	81	187	302	335	135	193	21	50	49
Number of family	53	110	1783	76	N/A	N/A	300	135	179	7	24	N/A
Number of family with history of renal diseases	2/53 (3.8%)	24/110 (21.8%)	N/A	24/76 (31.6%)	22/187 (11.8%)	58/183 (31.7%)	126/300 (42.0%)	0/135 (0%)	29/179 (16.2%)	7/7 (100%)	N/A	20/48 (41.7%)
Asian	53/53 (100%)	N/A	159/1783 (8.9%)	1/76 (1.3%)	24/187 (12.8%)	28/150 (18.7%)	136/300 (45.3%)	N/A	24/193 (12.4%)	0/21 (0%)	N/A	N/A
Consanguinity	0/53 (0%)	7/110 (6.4%)	372/1783 (20.9%)	N/A	13/187 (7.0%)	17/141 (12.1%)	146/300 (48.6%)	0/135 (0%)	N/A	6/7 (85.7%)	2/24 (8.3%)	N/A
Adult patients (age > 18 years)	35/53 (66.1%)	48/110 (43.6%)	28/1783 (1.6%)	70/81 (86.4%)	0/187 (0%)	62/302 (20.5%)	8/335 (2.4%)	135/135 (100%)	N/A	N/A	24/24 (100%) In index cases	49/49 (100%)
FSGS pathology	53/53 (100%)	108/125 (86.4%)	N/A	73/81 (90.1%)	98/187 (52.4%)	115/160 (71.9%)	153/223 (58.3%)	100/135 (74.1%)	148/148 (100%)	11/21 (52.4%)	N/A	49/49 (100%)
SRNS	30/53 (56.6%)	78/125 (62.4%)	1783/1783 (100%)	N/A	181/187 (96.8%)	255/302 (84.4%)	205/300 (68.3%)	N/A	N/A	21/21 (100%)	N/A	N/A
Mutation detection rate												
Overall	6/53 (11.3%)	37/110 (33.6%)	526/1783 (29.5%)	10/76 (13.2%)	49/187 (26.2%)	71/302 (23.5%)	74/300 (24.7%)	16/135 (11.8%)	20/179 (11.2%)	7/7 (100%)	7/24 (29.2%)	21/49 (42.9%)

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Among patients with FSGS pathology	6/53 (11.3%)	20/108 (18.5%)	N/A	9/75 (12.0%) FSGS/SRNS combined	N/A	N/A	40/153 (26.1%)	14/100 (14.0%)	20/179 (11.2%)	N/A	N/A	21/49 (42.9%)
Among patients with SRNS	2/31 (6.5%)	18/78 (23.1%)	526/1783 (29.5%)		48/181 (26.5%)	54/255 (21.2%)	57/205 (27.8%)	N/A	N/A	21/21 (100%)	N/A	6/13 (42.6%)
Among adult patients (age >18 years)	4/35 (11.4%)	7/48 (14.6%)	6/28 (21.4%)	9/69 (13.0%)	N/A	19/62 (30.6%)	2/8 (25.0%)	16/135 (11.8%)	N/A	N/A	N/A	21/49 (42.9%)

ESRD = end-stage renal disease, FSGS = focal segmental glomerulosclerosis, N/A = not available, SRNS = steroid-resistant nephrotic syndrome

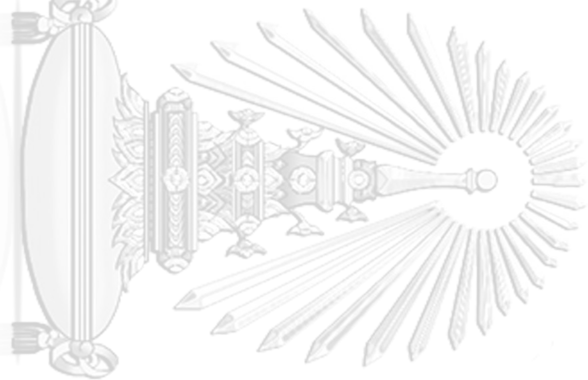

Table 16: ***Clinicopathological data of LMX1B-associated nephropathy (c.737G>A, p.Arg246Gln).***

Patient	Family number	Sex	Ethnicity	Age of onset	Familial/ Sporadic	Clinical presentation	Clinical diagnosis	Renal pathology	ESRD (Y/N)	Time from onset to ESRD (yr)	Age of ESRD (yr)	Extra-renal manifestations	Reference
1 (III-2)	1	M	Thai	8	F	B	2	FSGS	Y	6.4	15	None	Our report
2 (II-2)	1	M	Thai	33	F	B	3	NA	Y	0.8	34	None	Our report
3	2	M	European	36	F	NA	3	Advanced CKD	Y	22	58	NA	(34)
4	2	F	European	22	F	NA	1	FSGS	N	-	-	NA	(34)
5	2	M	European	6	F	B	2	FSGS	Y	48	54	None	(34)
6	2	F	European	26	F	B	2	FSGS	N	-	-	None	(34)
7	2	M	European	7	F	A	1	NA	N	-	-	None	(34)
8	3	F	European	22	F	NA	NA	FSGS	N	-	-	None	(34)
9	3	F	European	17	F	NA	NA	FSGS	N	-	-	None	(34)
10	4	F	Japanese	6	S	A	1	MCD	NA	-	-	None	(47)
11	5	F	Japanese	6	F	A	1	MCD	Y	32	38	None	(48)
12	5	F	Japanese	13	F	A	1	FSGS	N	-	-	None	(48)
13	5	F	Japanese	1	F	A	1	MCD	N	-	-	None	(48)
14	5	F	Japanese	11	F	A	1	NA	N	-	-	None	(48)
15	5	F	Japanese	1	F	A	1	NA	N	-	-	None	(48)
16	6	F	White	15	F	B	2	FSGS	N	-	-	None	(41)
17	7	F	Chinese	1	F	NA	1	NA	N	-	-	None	(49)
18	8	M	Chinese	5	F	NA	1	MsPGN	N	-	-	None	(49)

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Patient	Family number	Sex	Ethnicity	Age of onset	Familial/ Sporadic	Clinical presentation	Clinical diagnosis	Renal pathology	ESRD (Y/N)	Time from onset to ESRD (yr)	Age of ESRD (yr)	Extra-renal manifestations	Reference
19	9	M	NA	21	F	B	2	FSGS	Y	18	39	None	[50]
20	10	F	NA	17	S	B	2	FSGS	Y	-	-	None	[50]
21	11	F	Turkish	8	F	NA	2	FSGS	Y	1	9	None	[51]
22	12	M	European	4	S	NA	2	FSGS	Y	39	43	None	[51]
23	13	F	European	18	S	NA	NA	FSGS	Y	25	43	None	[51]
24	14	F	East Asian	11	S	NA	NA	NA	NA	-	-	NA	[6]
25	15	F	European	24	S	NA	NA	NA	NA	-	-	NA	[6]
26	16	F	Japanese	3	F	B	2	FSGS	N	-	-	None	[52]
27	17	F	Hispanic	3	F	NA	1	FSGS	N	-	-	None	[53]
28	18	F	Hispanic	29	F	NA	1	FD	N	-	-	None	[53]
29	18	M	Hispanic	9	F	NA	1	NA	N	-	-	None	[53]
30	19	F	Hispanic	5	S	B	2	FSGS	Y	30	35	None	[54]
31	20	F	White	58	F	B	1	FD	Y	13	71	None	[55]

Abbreviations: CKD = chronic kidney diseases, eGFR = estimated glomerular filtration rate, ESRD = end-stage renal disease, F = familial, FD = Fabry disease, FSGS = focal segmental glomerulosclerosis, MCD = minimal change disease, MsPGN = mesangial proliferative glomerulonephritis, N = no, NA = not available, S = sporadic, Sex:M = male, Sex:F = female, Y = yes, yr = year

Clinical presentation: A = asymptomatic, case detected by program screening or check-up, B = symptomatic such as edema, dyspnea, nephrotic syndrome or CKD, NA = not available

Clinical diagnosis: 1 = non-nephrotic proteinuria with normal renal function, 2 = steroid-resistant nephrotic syndrome, 3 = chronic kidney disease (CKD) or end-stage renal disease (ESRD) at presentation, NA = not available

To date, there have been 31 cases of *LMX1B*-associated nephropathy with the p.Arg246Gln in 20 different families, including ours (Table 16). Most of them (24/31 = 77.4%) are familial cases. The patients were reported across all ethnicities, including White, Hispanic, and Asian, with the majority female (22/31 = 71.0%). Remarkably, they had varied clinicopathological features, as shown in Table 16. Renal manifestations range from non-nephrotic proteinuria with normal renal function to CKD presenting with generalized edema and dyspnea. Among 17 cases with clinical information available, 7 patients (7/17 = 41.2%) were asymptomatic and 10 patients (10/17 = 58.8%) were symptomatic. Clinical diagnosis was reported in 26 patients. Fourteen patients (14/26 = 53.8%) were diagnosed as non-nephrotic proteinuria with normal renal function. Ten patients (10/26 = 38.5%) were diagnosed as steroid-resistant nephrotic syndrome. Two patients (2/26 = 7.7%) were diagnosed with CKD with unknown etiology. All cases were not correctly diagnosed the first time. Interestingly, none of the *LMX1B*-associated nephropathy cases with the p.Arg246Gln had typical extrarenal manifestations of nail-patella syndrome (NPS).

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Patient B presented with steroid-resistant nephrotic syndrome at the age of 8 and progressed to ESRD at 15 years, while his father presented with advanced CKD at 33. Different severity of the same mutation could be caused by several factors, including modifier genes and environmental factors. It has been demonstrated that renal

manifestations in NPS could be very different in the same family. A study in one pair of identical twins revealed rapidly progressive renal diseases to ESRD in one twin and non-nephrotic proteinuria in the other[56]. Modifier genes may play some roles in the difference in the severity of *LMX1B*-associated nephropathy[57]. There were reports that *CLIM2*, *COL4A3*, *COL4A4*, *COL4A5*, *LDB1*, and *PAX2* were the modifier genes of LMX1B mutation[58-61]. However, we did not find any pathogenic mutation in these genes.

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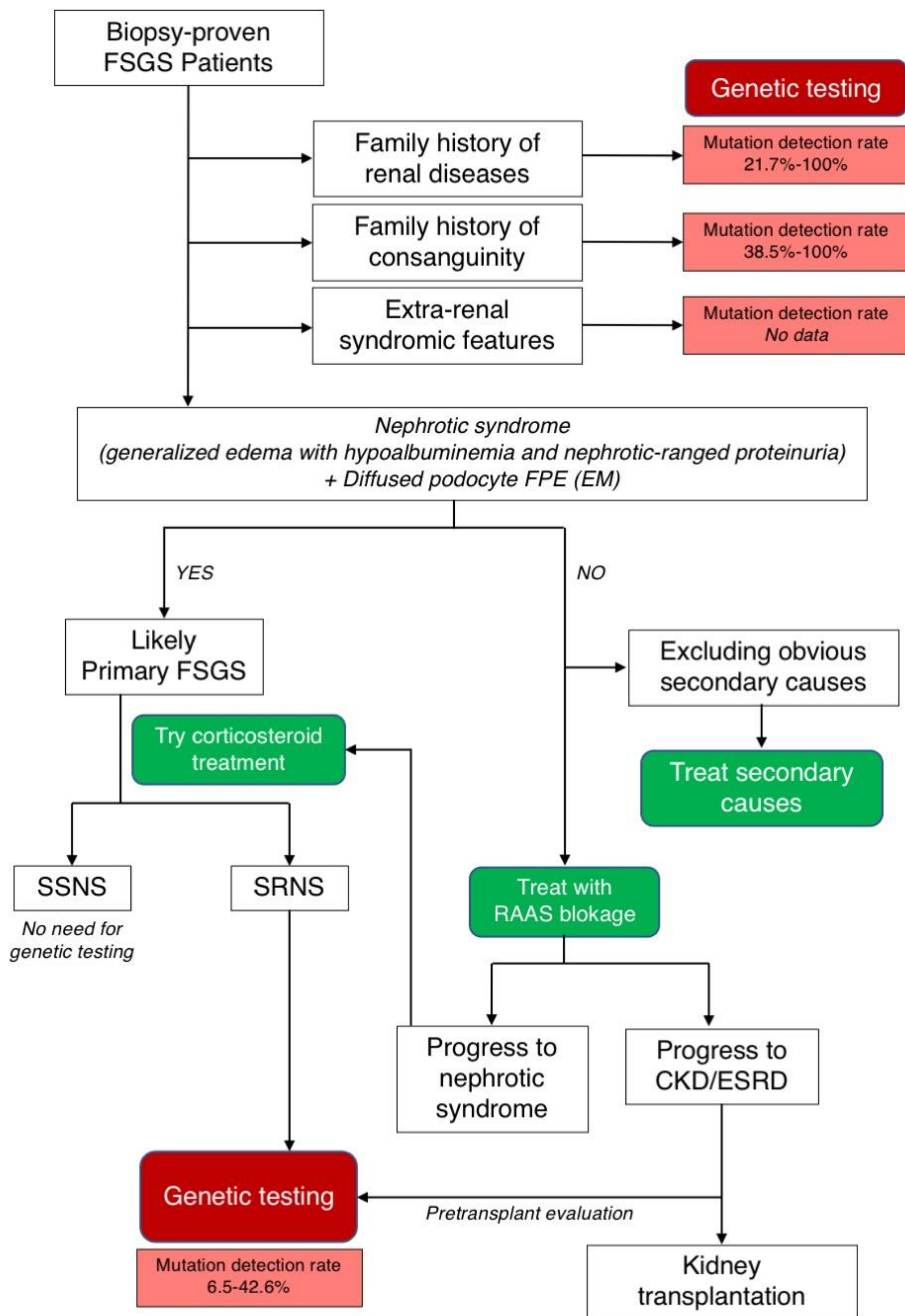

Figure 13: Algorithm for genetic testing and management of FSGS patients

Our findings, combined with reviewed data from previous studies (Table 15), emphasized the role of genetic testing in managing FSGS patients, as shown in Figure 13. The main indications for genetic testing include extrarenal syndromic manifestations, a history of consanguinity, and a family history of renal diseases. The positive rate among these patients is very high. Clinicians should note that a family history of dialysis or CKD could be a clue for genetic testing, as discussed in patients B and E in our study. It should be noted that clinical manifestations can be diverse and intrafamilial, and interfamilial variations have been described in families with genetic FSGS, as discussed in *LMX1B* gene and NPS. Our data also suggested that clinical syndrome and renal pathology should be used to guide treatment. Patients who did not have nephrotic syndrome or diffused podocyte FPE in EM should be evaluated for secondary causes. If secondary causes were found, treatment should be directed at the secondary causes. If the causes were unknown, these patients should be treated with renin-angiotensin-aldosterone system (RAAS) blockade. It has been demonstrated that proteinuria reduction with RAAS blockade was associated with slower CKD progression. For patients with nephrotic syndrome and diffused podocyte FPE who were likely to be diagnosed with primary FSGS, genetic testing would be recommended if they did not respond to corticosteroids before changing the medication to calcineurin inhibitors or cyclophosphamide. Giving immunosuppressive medications to these patients without genetic testing might lead to infection and other adverse events without any clinical benefits.

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After FSGS patients progress to CKD, genetic testing may be of benefit when the patients undergo KT. Recurrence of FSGS in the transplanted kidney is a troublesome condition that requires intense treatment with plasmapheresis and immunosuppression. Genetic FSGS is known to have a very low recurrence rate, reaching 0% in some studies; however, recurrence can be as high as 50% in primary FSGS(12, 13). Knowing the genetic causes before KT will be beneficial in managing after KT since recurrent FSGS can be presented as early as the first day after KT. In many countries, including Thailand, to prevent organ trafficking, living-donor KT is restricted only to family members. This might create a significant problem if the family had a genetic disorder without knowing the exact genetic defect. Taking one kidney from a donor with the variant causing FSGS could make a donor turn into CKD oneself.

The genetic test of choice in FSGS patients is currently WES. It has been demonstrated that most mutations causing genetic FSGS could be detected by WES. Compared with a comprehensive gene panel, a re-analysis of the WES data could be done. As new disease-associated genes have been increasingly identified, WES is better than gene panels in evaluating patients with FSGS.

This study is also the first to demonstrate IHC staining of α5(IV) collagen in FSGS patients with COL4A3/4/5 variants and the first to correlate IHC staining with WES. The IHC protocol could distinguish between positive IHC staining in normal renal allograft tissue and negative IHC staining in the AS case. We hypothesized that FSGS patients with

pathogenic and likely pathogenic variants would have negative IHC staining. However, both FSGS patients with pathogenic and likely pathogenic variants had positive IHC staining which mean IHC staining of α5(IV) collagen does not predict the expression of COL4A3/4/5 in the genomic variant of FSGS. IHC has been demonstrated to correlate with phenotypic severity in patients with X-linked AS who have mutation in the *COL4A5* gene. The expression of COL4A3/4/5 in GBM of FSGS patients who had a pathogenic variant in the *COL4A3/4/5*, which we hypothesized that there should be no expression, might be due to other reasons. First, all previous studies that reported the correlation between IHC and clinical severity of AS were exclusively studied in male X-linked AS with mutations in the *COL4A5* gene. A study with hemizygous female patients with mutations in the *COL4A5* gene was lacking. The study in *COL4A3* and *COL4A4* genes was also lacking, even in patients with clinical AS. Our patients are both female and had a heterozygous variant in the *COL4A4* gene; hence, IHC staining might not correlate with collagen protein expression because another normal allele might be able to express enough protein in GBM. Another possibility is that the IHC is not sensitive enough to detect the decrease in protein expression of COL4A3/4/5 in GBM. Therefore, other functional studies might be required for correlation between genomic variants and phenotype in FSGS patients with COL4A3/4/5, such as advanced renal pathology study by super-resolution imaging and airyscan laser scanning confocal microscope or cell-based study by split luciferase-based trimer formation assay.

This study is also the first study to use cell-based split-luciferase-based trimer formation assay of α345(IV) collagen for the study of *COL4A3/4/5* gene variants in FSGS patients. Our study was the first to use this method in evaluating the *COL4A4* gene, which successfully demonstrated decreased luminescence in all three P/LP variants in *COL4A4*. The decreased luminescence in all three P/LP variants in *COL4A4* was also correlated with the negative controls, which were the known nonsense and missense mutations. Therefore, we predicted these three variants in the *COL4A4* gene, which were c.1805G>A, p.Gly602Glu, c.2752G>A, p.Gly918Arg, and c.905delG, p.Gly302ValfsTer32, caused FSGS in patient C, patient D and patient E, respectively. However, as we only transfected the mutated *COL4A4* plasmids into HEK cells, this experiment might not reflect the situation in the real patients that had heterozygous mutation of *COL4A4* gene. The cell study might also be further improved by adding the controls with positive control, which might use the known benign variant. If the cells expressing known benign variant have the same luminescence as the cells expressing wild-type, this might further confirm that this cell-based split-luciferase-based trimer formation assay of α345(IV) collagen can be further used for the screening of COL4A3/4/5 variants found in FSGS and AS patients.

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Conclusion

A monogenic cause was identified in 11.3% of FSGS patients. Most of the causative variants were found in the *COL4A4* genes. This is the first study to evaluate the genetic etiology of patients with FSGS in the Thai population. The expression of COL4A3/4/5 by IHC staining was not correlated with the type of the *COL4A3/4/5* gene variants classified by ACMG criteria. However, cell-based split-luciferase-based trimer formation assay of α345(IV) collagen can be used as a functional study to evaluate the effect of *COL4A3/4/5* variants in FSGS.

Our study also emphasized the importance of WES in FSGS patients. FSGS who had the extra-renal syndromic manifestations, a family history of renal disease or consanguinity should undergo genetic testing regardless of other clinical data. After excluded three conditions above, clinicians should use clinicopathological data to classify FSGS patients into presumed primary FSGS group which should be treated with corticosteroids. Secondary causes should be sought in another group and treatment should be given without immunosuppressive medications. SSNS patients should not under go genetic testing; however, SRNS patients might benefit from genetic testing. Genetic testing should also be done in FSGS patients who will undergo KT as a part of pretransplant evaluation.

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AWARD RECEIVED	- Best Abstract of the Year Award, The Royal College of Physicians of Thailand, 2020 - Best Research Award, Thailand Society of Nephrology, 2018 - First place in “Medical Tournament” The Royal College of Physicians of Thailand, 2016

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