Whole Exome Sequencing of Patients with Steroid-Resistant Nephrotic Syndrome

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ABSTRACT (295/300 words)

Introduction: Steroid-resistant nephrotic syndrome overwhelmingly progresses to end-stage renal disease. More than 30 monogenic genes have been identified to cause steroid-resistant nephrotic syndrome. We previously detected causative mutations using targeted panel sequencing in 30% of patients with steroid-resistant nephrotic syndrome. Panel sequencing has a number of limitations when compared to whole exome sequencing. We employed whole exome sequencing to detect monogenic causes of steroid-resistant nephrotic syndrome in a large international cohort of 300 families.

Methods: 335 individuals with steroid-resistant nephrotic syndrome from 300 families were recruited from 4/1998 to 6/2016. Age of onset was restricted to under 25 years of age. Exome data were evaluated for 33 known monogenic steroid-resistant nephrotic syndrome genes.

Results: In 74/300 families (25%), we identified a causative mutation in one of 20 genes known to cause steroid-resistant nephrotic syndrome. In 11 families (3.7%), we detected a mutation in a gene that causes a phenocopy of steroid-resistant nephrotic syndrome. This is consistent with our previously published identification of mutations using a panel approach. We detected a causative mutation in a known steroid-resistant nephrotic syndrome gene in 38% of consanguineous families and in 13**%** of non-consanguineous families, and 48% of children with congenital nephrotic syndrome.

A total of 68 different mutations were detected in 20 of 33 steroid-resistant nephrotic syndrome genes. Fifteen of these mutations were novel. *NPHS1*, *PLCE1*, *NPHS2* and *SMARCAL1* were the most common genes in which we detected a mutation. In another 28% of families, we detected mutations in one or more candidate genes for steroid-resistant nephrotic syndrome.

Conclusions: Whole exome sequencing is a sensitive approach towards diagnosis of monogenic causes of steroid-resistant nephrotic syndrome. A molecular genetic diagnosis of steroid-resistant nephrotic syndrome may have important consequences for the management of treatment and kidney transplantation in steroid-resistant nephrotic syndrome.

2993/3000 words

INTRODUCTION

Nephrotic syndrome in childhood is characterized by proteinuria (>40 mg/m^2/hour), hypoalbuminemia, edema and hyperlipidemia. It can cause hypertension, severe infections and thrombotic events. Patients are classified by their response to steroid therapy. In children and young adults, about 80% of patients respond to standard steroid therapy and are termed steroid sensitive (1). In contrast, individuals with steroid-resistant nephrotic syndrome overwhelmingly progress to chronic kidney disease (CKD) and end-stage renal disease (ESRD). At this time, there is no effective therapy to curtail the relentless progression to ESRD.

The most frequent kidney histologic feature of steroid-resistant nephrotic syndrome is focal segmental glomerulosclerosis (FSGS). In patients with FSGS, the risk of recurrence after kidney transplantation is estimated to be ~33% (2-4). FSGS constitutes the third most prevalent cause of ESRD in the first 2 decades of life (5). To date, more than 30 monogenic causes of steroid-resistant nephrotic syndrome have been identified (6), many of which implicate the glomerular podocyte and slit membrane as the primary sites where the pathogenesis of steroid-resistant nephrotic syndrome unfolds (7). The majority of genes known to cause steroid-resistant nephrotic syndrome are recessively inherited. Patients with mutations in these genes manifest with steroid-resistant nephrotic syndrome in childhood and adolescence, whereas dominant steroid-resistant nephrotic syndrome genes often manifest later in life.

We showed previously by targeted panel sequencing of 27 known steroid-resistant nephrotic syndrome genes that in 30% of steroid-resistant nephrotic syndrome cases with onset before 25 years, a causative mutation can be detected (8). However, panel sequencing by multiplex polymerase chain reaction (PCR) is limited by requiring large numbers of Sanger sequencing to confirm individual genetic variants (8). Additionally, evaluation of genes by panel sequencing is limited to approximately 30 genes. With the growing number of genes available, we sought a mechanism by which we could evaluate a patient for a high number of steroid-resistant nephrotic syndrome genes, as well as detect novel causes of nephrosis should no known gene be identified.

In a cohort of patients with CKD and the phenotype of increased kidney echogenicity, we identified a causative mutation in 63% using whole exome sequencing (9). We evaluated here the utility of whole exome sequencing in an international cohort with steroid-resistant nephrotic syndrome. To date, this cohort is the largest to undergo whole exome sequencing (10). Given the very high rate of establishing an etiologic diagnosis and the significant implications for clinical management, pre-transplant and post-transplant care, whole exome sequencing should be considered in all individuals with steroid-resistant nephrotic syndrome diagnosed before age 25 years.

MATERIALS AND METHODS

Human subjects. The study was approved by the institutional review board of the University of Michigan and Boston Children's Hospital. From April 1998 to June 2016, patients were enrolled after obtaining informed consent. Inclusion criteria were: onset of symptoms before 25 years AND a clinical diagnosis of steroid-resistant nephrotic syndrome (e.g. proteinuria, hypoalbuminemia, edema) OR nephrotic range proteinuria with kidney histology of FSGS or diffuse mesangial sclerosis (Supplementary Table 1). 335 individuals from 300 families were enrolled. Prior to December 2013, enrolled individuals were screened for mutations in *WT1* and *NPHS2.* Those that screened positive were not included in this study.

Whole exome sequencing and variant calling. Whole exome sequencing and variant burden analysis were performed as previously described (11-13). Genomic DNA was isolated from blood lymphocyte or saliva samples and subjected to exome capture using Agilent SureSelect™ human exome capture arrays (Life technologies™) followed by next generation sequencing on the Illumina HighSeq™ sequencing platform. Sequence reads were mapped to the human reference genome assembly (NCBI build 37/hg19) using CLC Genomics Workbench™ (version 6.5.2) software (CLC bio, Aarhus, Denmark). Following alignment to the human reference genome (GRCh37/hg19), variants were filtered for most likely non-deleterious variants as previously described (8, 11). Variants with minor allele frequencies >1% in the dbSNP (version 142) or in the 1,000 Genomes Project (1,094 subjects of various ethnicities; May, 2011 data release) databases were excluded as they were unlikely to be deleterious. We used manual inspection for the p.Arg229Gln mutation in the *NPHS2* gene which is reported to be deleterious with other variants, which would be filtered out using this method (14). Synonymous variants and intronic variants that were not located within splice site regions were excluded. Remaining variants included non-synonymous variants and splice site variants.

Mutation calling in known SRNS genes. We evaluated whole exome sequencing data for causative mutations in 33 steroid-resistant nephrotic syndrome genes known at the time (Supplementary Table 2). Mutation calling was applied as stated above, followed by filtering remaining variants for changes in the regions of the 33 genes. Remaining variants were ranked based on their probable impact on the function of the encoded protein considering evolutionary conservation among orthologues using ENSEMBL Genome Browser and assembled using Clustal Omega, as well as PolyPhen-2 (15), SIFT (16), and MutationTaster (17). Mutations were designated as likely pathogenic based on criteria given by Supplementary Table 3. Mutation calling was performed by clinician scientists/geneticists, with knowledge of the clinical phenotypes and pedigree structure, as well as experience with homozygosity mapping and whole exome sequencing evaluation. Remaining variants were confirmed in patient DNA by Sanger sequencing as previously described (8). Whenever parental DNA was available, segregation analysis was performed.

If no causative mutation was identified, we evaluated for mutations in genes that may represent phenocopies of steroid-resistant nephrotic syndrome (Supplementary Table 2). Variants were evaluated as above. Correlation of genotype and phenotype was examined and, if matching, the genetic variant was deemed a causative mutation.

Mutation calling to identify novel causes of steroid-resistant nephrotic syndrome.

If no causative mutation was found in a known steroid-resistant nephrotic syndrome gene and a family had homozygosity (>100Mbp) detected following homozygosity mapping, we then evaluated whole exome sequencing data for homozygous variants. Single heterozygous variants were excluded. We applied homozygosity mapping in consanguineous families or linkage analysis in sibling cases to filter variants (18). Remaining variants were ranked as described above. Variants were confirmed as described above.

Homozygosity mapping and linkage analysis. Prior to 2014, for genome-wide homozygosity mapping, the GeneChip® Human Mapping 250k *d* Array (Affymetrix) was used. Alternatively, homozygosity mapping data was generated from whole exome sequencing data. Non-parametric logarithm (base 10) of odds (LOD) scores were calculated using a modified version of the program GENEHUNTER2.1 (19, 20) through stepwise use of a sliding window with sets of 110 SNPs and the program ALLEGRO (21) in order to identify regions of homozygosity as described (18, 22) using a disease allele frequency of 0.0001 and Caucasian marker allele frequencies. To generate homozygosity mapping after 2014, downstream processing of aligned

BAM files was done using Picard and SAMtools4 (23). Single nucleotide variants calling was performed using Genome Analysis Tool Kit (GATK) (24) and the generated VCF file was subsequently used in Homozygosity Mapper (25).

Web Resources

UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway;

1000 Genomes Browser http://browser.1000genomes.org;

Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustal;

Ensembl Genome Browser, http://www.ensembl.org;

Exome Variant Server, http://evs.gs.washington.edu/EVS;

Exome Aggregation Consortium, exac.broadinstitute.org;

HGMD Professional 2016.3, https://portal.biobase-international.com/hgmd;

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org;

Polyphen2, http://genetics.bwh.harvard.edu/pph2;

Sorting Intolerant From Tolerant (SIFT), http://sift.jcvi.org;

MutationTaster http://www.mutationtaster.org

RESULTS

Identification of causative mutations in one of 20 steroid-resistant nephrotic syndrome *genes in 25% of* steroid-resistant nephrotic syndrome *cases*

Whole exome sequencing was performed in 335 individuals from 300 families and evaluated for mutations in the 33 steroid-resistant nephrotic syndrome genes known at the time (Table 1). In 74 families (25%), a causative mutation in one of 20 known steroid-resistant nephrotic syndrome genes was detected (Figure 1, Table 1). *NPHS1* (13 families)*, PLCE1* (11 families)*, NPHS2* (8 families)*,* and *SMARCAL1* (8 families) were the most common genes in which mutations were identified, comprising 51% of all mutations identified (Figure 1, Table 1).

94 of the 300 families studied by whole exome sequencing have been previously studied using Fluidigm panel sequencing. The overlap between cohorts is given in Supplementary Table 4. We found that whereas in 20/74 (27%) families the causative mutation was previously detected using panel sequencing, 9/74 (12%) had a diagnosis made by whole exome sequencing and not by panel sequencing. In an additional 28% of families, we detected a likely causative mutation in one or more potential novel SRNS genes (Figure 1), given in Supplementary Table 5.

Novel mutations detected in known steroid-resistant nephrotic syndrome genes

We detected 68 different mutations in 20 of 33 known steroid-resistant nephrotic syndrome genes, 53 of which had previously been reported in the literature (Table 1). 15 novel mutations have not been reported previously (Table 1). Individual families in whom a causative mutation was detected are described in Supplementary Table 9.

Whole exome sequencing identifies phenocopies in 11 of 90 families with a causative mutation detected

We detected a causative mutation in 11 of 300 families with steroid-resistant nephrotic syndrome (3.7%) (Figure 1, Table 1). Mutations were found in 8 phenocopy genes, specifically *COL4A5*, *COL4A3*, *CLCN5*, *GLA*, *AGXT*, *CTNS, FN1* and *WDR19*. A total of 10 different mutations were detected, 5 of which are novel (Table 1).

Novel candidate genes are identified using whole exome sequencing

In 61/146 (42%) consanguineous families with no causative mutation found in a known steroid-resistant nephrotic syndrome gene, one or more candidate genes were detected using homozygosity mapping (Figure 2, Supplementary Table 5). In non-consanguineous families >1 individual affected, linkage analysis was used to identify a potentially causative mutation in 18 of 135 families (13%).

Description of cohort

Onset of illness ranged from birth to 24 years of age (Figure 3A and Supplementary Table 6). The median age in individuals in whom a causative mutation was detected in a steroid-resistant nephrotic syndrome gene was 1.7 years versus 4 years in those without a causative mutation identified, which was statistically significant (Figure 3B).

146/300 (49%) of families were consanguineous, 56 (38%) of whom we detected a causative mutation in a steroid-resistant nephrotic syndrome gene (Figure 4, Supplementary Table 7). In 56/147 of families with >100 Mbp of homozygosity on mapping (38%), a causative mutation was detected in a steroid-resistant nephrotic syndrome gene. In contrast, in 17/135 (13%) of non-consanguineous families and 18/153 (12%) of families with <100 Mbp of homozygosity (non-homozygous) on mapping, a causative mutation was detected in a steroid-resistant nephrotic syndrome gene (Figure 4). The difference in mutation detection between consanguineous and non-consanguineous families and between homozygous and non-homozygous families was statistically significant using a chi-squared test ($p<0.001$) (Figure 4). There was no significant difference in the rate of mutation detection when comparing families with 1 affected individual versus 2 affected individuals or ≥ 3 affected individuals (Figure 4).

In 24% of those with additional systemic manifestations in addition to kidney disease, a causative mutation was detected in an steroid-resistant nephrotic syndrome gene, compared to 27% of those with no extra-renal manifestations with a causative mutation detected in a steroid-resistant nephrotic syndrome gene (Supplemental Figure 2, Supplemental Table 8). This difference was not statistically significant.

The most common clinical diagnosis was steroid-resistant nephrotic syndrome in 205/300 (68%) (Supplementary Figure 3, Supplementary Table 8). It was the most common clinical diagnosis in those families with a causative mutation identified (48/74 families, 65%) (Supplementary Figure 3, Supplementary Table 8).

In 24% of individuals with FSGS on biopsy and in 14% of individuals with diffuse mesangial sclerosis on biopsy, a causative mutation was detected in in steroid-resistant nephrotic syndrome gene (Supplementary Figure 4, Supplementary Table 8). 223/335 (66.6%) of individuals had kidney histology data available.

DISCUSSION

Summary and impact of this work

To date, this is the largest international cohort in which molecular causes of steroid-resistant nephrotic syndrome were evaluated using whole exome sequencing. Our rate of mutation detection is 25%, consistent with our previous work (8). Recently, in 187 individuals, a causative mutation was detected in one of 53 steroid-resistant nephrotic syndrome genes in 26% of individuals (10). We detected causative mutations in 20 of 33 known causes of steroid-resistant nephrotic syndrome, with a total of 68 different mutations, 15 of which have not been reported in the literature. To determine the pathogenicity of novel mutations in genes previously described to cause steroid-resistant nephrotic syndrome, we used a strict set of criteria separately for recessive or dominant. Criteria were based on evolutionary conservation, bioinformatic prediction programs of pathogenicity, and allele frequency in healthy control populations (Supplementary Table 3 and 9).

Prior to 2014, our lab screened for mutations in *NPHS2* and *WT1*, which may account for lower prevalence in our cohort. *NPHS2* and *WT1*, two of the three most commonly mutated genes in early-onset steroid-resistant nephrotic syndrome are underrepresented in the presented work, being together responsible for only 3.3% (Table 1) of 300 cases, while they were previously reported to be responsible for 15% of cases in 1783 cases (8). When all 1989 families studied in Sadowski, et al and in this study, are combined together, mutation rates for *NPHS2* and *WT1* become more representative of what has been previously published. *NPHS2* has a detection rate of 9.3% (185/1989) and *WT1* has a detection rate of 4.4% (87/1989). Mutation rates for *NPHS2* were previously 9.9% and for *WT1* were 4.8%.

We detected mutations in 8 genes that are phenocopies for steroid-resistant nephrotic syndrome, with 5 novel mutations and 5 mutations previously reported in the literature. As these genes may be excluded from panels that target steroid-resistant nephrotic syndrome specifically, these families may have been left without a molecular diagnosis.

Whole exome sequencing allows for the identification of novel genes using homozygosity mapping in consanguineous families and linkage analysis in related individuals. Panels are limited as to how many genes could be evaluated in a given experiment. However, whole exome sequencing allows for the evaluation of all genes, including those that may phenocopy steroid-resistant nephrotic syndrome and provide the opportunity for novel gene discovery.

Therapeutic implications

Identification of a monogenic cause of steroid-resistant nephrotic syndrome has significant therapeutic implications. i) In children, treatment often requires prolonged steroid exposure and potentially exposure to multiple immunosuppressant medications. All of these medications carry significant side effect profiles, including growth failure (steroids), bone marrow suppression (mycophenolate mofetil, tacrolimus, azathioprine), kidney dysfunction (tacrolimus), and unacceptable cosmetic effects (cyclosporine), amongst other side effects. This generates an indication for fast, efficient exome sequencing in order to avoid unfavorable side effects may be experienced while taking medications that may not provide clinical benefit. ii) Identification of a causative mutation may reveal that a potential therapy is available for some rare single-gene causes of nephrosis. For example, if a mutation in a gene of coenzyme Q_{10} biosynthesis (*COQ2, COQ6, ADCK4,* or *PDSS2*) is detected, treatment with coenzyme Q_{10} may be indicated (26-28). In the case of the individual with *COQ2* mutation, this individual was placed on COQ10 therapy and experienced a sustained remission of nephrosis. iii) Identification of causative mutations in *WT1* can also lead to surgical evaluation and intervention to remove streak gonads with potential for malignant transformation (29). iv) Genotype and phenotype correlations in the future may lead to stratification in clinical trials for novel therapeutics. In our study, we identify 5 families with the p.R1160* mutation in *NPHS1*. This mutation has been shown to cause congenital nephrotic syndrome; however, in some patients with this mutation, a milder phenotype has been reported (30). v) Furthermore, identification of mutations in genes that may represent phenocopies of steroid-resistant nephrotic syndrome, such as cystinosis, hyperoxaluria, or Fabry's disease can direct therapy. Mutations in these genes would be missed in panel sequencing, as they are not canonical nephrosis genes, but may present with proteinuria, edema, and chronic kidney disease. The ability to detect mutations in genes that represent phenocopies of nephrosis is a benefit of whole exome sequencing over panel sequencing.

Implications for kidney transplant

Many patients progress to ESRD, requiring transplantation and dialysis. Given that ~30% of all cases of idiopathic FSGS can recur post-transplant, many centers employ increased immunosuppression prior to and after transplant to prevent recurrence (31, 32). Since monogenic forms of FSGS are unlikely to recur, young children could be spared exposure to aggressive immunosuppression and avoid many of the infectious complications seen in transplantation (10, 33). Patients with a monogenic cause of nephrosis identified are younger that those that do not have a causative mutation identified (Figure 3A and 3B, Supplementary Table 3), which puts them at greater risk for infections post-transplant, including Epstein-Barr virus and cytomegalovirus. A monogenic diagnosis provides the opportunity to reduce immunosuppression and reduce risk of infectious complication.

Furthermore, many pediatric patients receive a living donor kidney transplant from a close relative, such as a parent, and having a monogenic cause identified, such as *COL4A5,* may have implications on donor selection. Additionally, in families with *INF2* mutations, the parents and family members should be screened for *INF2* mutations, as this dominant disease has variable expressivity within families. Should a family member be positive for mutation, this would disqualify them from donation of a kidney for transplantation as they are at risk for developing proteinuria and kidney disease in the future.

Limitations

One limitation to our study is that ~70% of families remain undiagnosed. Our lab is currently performing trio analysis, in which both parents and the proband have sequencing performed, which allows for evaluation of non-consanguineous individuals. We anticipate that this may increase the number of candidate genes identified and lead to future molecular genetic diagnosis.

Cost of WES

Whole exome sequencing has several advantages over panel sequencing. It has the theoretical likelihood of 86% of detecting recessive disease mutations. Whole exome sequencing examines all exons in a genome, whereas panel sequencing typically examine only ~30. With falling costs of sequencing, a research exome is ~ $650, which ultimately is more cost effective than panel sequencing since hundreds of panels would be required to cover the whole exome and would not provide the opportunity to identify for novel causes of disease. Once an exome is performed, the data can be revisited as more genes are discovered. With the introduction of trio analysis, non-consanguineous families can be evaluated for novel genes potentially allowing for a conclusive monogenic diagnosis in the future.

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Table 1: Number and proportion of 300 families with steroid-resistant nephrotic syndrome, in whom causative mutations in one of 23 known monogenic causes of steroid-resistant nephrotic syndrome were detected. 54 of the mutations detected have previously been reported in BioBase, and 20 are novel (respective genes shown on a blue background). The most common genes to have a mutation detected in **steroid-resistant nephrotic syndrome** families were *NPHS1, PLCE1*, *NPHS2,* and *SMARCAL1* (51% of all **steroid-resistant nephrotic syndrome** gene mutations detected). In an additional 11 families, mutations were detected in 8 genes that cause a kidney disease that is a phenocopy of **steroid-resistant nephrotic syndrome** (respective genes shown on an orange background). Five of the mutations identified have previously been reported in BioBase, and 5 are novel.

SRNS Gene	**Number of families with causative mutation**	**Percent of families with gene**	**Number of mutations known from Biobase***	**Number of novel mutations**
NPHS1	13	18%	10	1
PLCE1	11	15%	8	2
NPHS2	8	11%	9	1
SMARCAL1	8	11%	5	1
LAMB2	6	8%	3	3
NUP93	4	5%	2	1
MYO1E	2	3%	1	1
SGPL1	3	4%	-	2
WDR73	3	4%	3	-
ITGA3	2	3%	2	-
LMX1B	2	3%	1	-
NUP205	2	3%	2	-
TTC21B	2	3%	1	1
WT1	2	3%	1	-
COQ2	1	1%	1	1
DGKE	1	1%	1	-
INF2	1	1%	-	1
KANK4	1	1%	1	-
PDSS2	1	1%	1	-
TRPC6	1	1%	1	-
TOTAL	**74**	**100%**	**53**	**15**
Phenocopy genes				
COL4A5	3	27%	2	-
AGXT	2	18%	0	2
CLCN5	1	9.1%	-	1
COL4A3	1	9.1%	-	1
CTNS	1	9.1%	1	-
FN1	1	9.1%	-	1
GLA	1	9.1%	1	-
WDR19	1	9.1%	1	-
Total	**11**	**100.0%**	**5**	**5**

*Biobase: https://portal.biobase-international.com/hgmd/pro; SRNS, steroid-resistant nephrotic syndrome