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Rasmussen, M; Sunde, L; Nielsen, M L; Ramsing, M; Petersen, A; Hjortshøj, T D; Olsen, T E; Tabor, A; Hertz, J M; Johnsen, I; Sperling, L; Petersen, O B; Jensen, U B; Møller, F G; Petersen, M B; Lildballe, D L

Published in:
Clinical Genetics

DOI (link to publication from Publisher):
[10.1111/cge.13185](https://doi.org/10.1111/cge.13185)

Publication date:
2018

Document Version
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

Citation for published version (APA):
Rasmussen, M., Sunde, L., Nielsen, M. L., Ramsing, M., Petersen, A., Hjortshøj, T. D., Olsen, T. E., Tabor, A., Hertz, J. M., Johnsen, I., Sperling, L., Petersen, O. B., Jensen, U. B., Møller, F. G., Petersen, M. B., & Lildballe, D. L. (2018). Targeted gene sequencing and whole-exome sequencing in autopsied fetuses with prenatally diagnosed kidney anomalies. *Clinical Genetics*, 93(4), 860-869. Advance online publication. <https://doi.org/10.1111/cge.13185>

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Targeted Gene Sequencing and Whole-Exome Sequencing in Autopsied Fetuses with Prenatally Diagnosed Kidney Anomalies

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Acknowledgments

This study was supported by grants from Danish Society of Nephrology; The Regional Postgraduate Medical Education Office Research Fund; Carl and Ellen Hertz' grant; Maria Dorthea and Holger From, Haderslevs Foundation; Director Jacob Madsen and his wife, Olga Madsen's foundation; and Aarhus University, Health.

Conflicts of interest

None declared.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cge.13185

Identification of fetal kidney anomalies invites questions about underlying causes and recurrence risk in future pregnancies. We therefore investigated the diagnostic yield of next-generation sequencing in fetuses with bilateral kidney anomalies and the correlation between disrupted genes and fetal phenotypes.

Fetuses with bilateral kidney anomalies were screened using an in-house-designed kidney-gene panel. In families where candidate variants were not identified, whole-exome sequencing was performed. Genes uncovered by this analysis were added to our kidney-panel.

We identified likely deleterious variants in 11 of 56 (20%) families. The kidney-gene analysis revealed likely deleterious variants in known kidney developmental genes in six fetuses and *TMEM67* variants in two unrelated fetuses. Kidney histology was similar in the latter two fetuses – presenting a distinct prenatal form of nephronophthisis. Exome sequencing identified *ROBO1* variants in one family and a *GREB1L* variant in another family. *GREB1L* and *ROBO1* were added to our kidney-gene panel and additional variants were identified.

Next-generation sequencing substantially contributes to identifying causes of fetal kidney anomalies. Genetic causes may be supported by histological examination of the kidneys. This is the first time that SLIT-ROBO signaling is implicated in human bilateral kidney agenesis.

Keywords: NGS, kidney anomalies; kidney agenesis; kidney dysplasia; prenatal screening; CAKUT

Introduction

Fetal kidney anomalies are diagnosed more frequently now than earlier owing to the wider use of second-trimester ultrasound screening. Although prenatal ultrasound scans can identify kidney agenesis or cystic, enlarged, hypoplastic, and/or echogenic kidneys, these findings do not point toward a specific underlying cause. Furthermore, kidney anomalies may be accompanied by non-urinary disease, like blindness, diabetes, and learning disability, which are un-detectable on a prenatal scan. Consequently, no valid overall prognosis can be made. Moreover, the diagnosis of severe bilateral kidney anomalies often invites considerations regarding termination of pregnancy and inevitably raises questions about recurrence risk in future pregnancies.

A growing number of monogenic causes of kidney anomalies are discovered, each however accounting for only a minority of cases. Furthermore, many genetic underlying causes have shown reduced penetrance and variable expressivity (1,2). Also, environmental factors like maternal diabetes and drug exposures may increase the risk of fetal kidney anomalies (3,4). These complexities have limited recognition of novel underlying causes. However, identification of underlying genetic causes may significantly improve the quality of prenatal counseling.

Previous studies on kidney anomalies using next-generation sequencing (NGS) have focused on specific disease entities like nephronophthisis or used broadly defined cohorts of patients with congenital anomalies of the kidney and urinary tract (CAKUT), also including cases with only minor anomalies (5-7). It is estimated that variants in protein coding sequence or copy number variants (CNVs), which likely explain the phenotype, can currently be identified in 10-16% of CAKUT patients (8).

In this study, we investigate the diagnostic yield of targeted-gene sequencing and whole-exome sequencing in a well-defined cohort of autopsied fetuses with severe prenatally diagnosed bilateral

kidney anomalies in which previous genetic analyses have not uncovered an underlying cause; and we correlate our genetic findings with detailed phenotypic information, including post-mortem kidney histology.

Materials and methods

Study population

Eligible fetuses were identified by systematically searching local registers at departments of clinical genetics and departments of pathology in Aalborg, Aarhus, Odense, and at Rigshospitalet (Copenhagen), Denmark. To be included, the fetus should prenatally have been diagnosed with bilateral kidney anomalies, or unilateral kidney anomalies in combination with oligohydramnios or anhydramnios, indicating that the kidney affection most likely was bilateral. The pregnancy should be terminated upon parental request approved by a regional abortion committee. Subsequently, the fetuses should have undergone post-mortem examination. DNA extracted from fresh tissue or cultured cells from the fetus or the pregnancy should be available from at least one fetus from each family. From some affected fetal siblings, DNA was recovered from formalin-fixed, paraffin-embedded tissue.

Fetuses with additional other organ abnormalities, like major heart malformations, neural tube defects, and/or missing extremities, were excluded. Fetuses with anal atresia, genital malformations, or minor vertebra defects were not excluded as we hypothesized that the implicated organs belong to the same developmental field as the kidneys and that malformations in these organs may be caused by variants in kidney developmental genes. Moreover, we excluded fetuses already known to harbor genetic variants likely explaining the kidney phenotype, viz. monosomy X, 22q11 deletion, 17q12 deletion, *HNFI1B* variant, and *PKHDI* variants, among others.

Families with two affected fetuses whose fetal kidney anomalies remained unsolved after kidney-gene analysis were recruited for whole-exome sequencing. Blood samples were obtained from the parents and other adult family members. Buccal smear samples were obtained from affected and non-affected live-born siblings for Sanger sequencing of candidate variants if requested by the parents.

Novel genes identified by whole-exome sequencing were added to our kidney-gene panel and selected samples were re-analyzed.

Kidney gene panel

The kidney gene panel included 108 genes chosen because of their potential involvement in embryonic kidney development, cystic kidney disease, nephrotic syndrome, or the renin-angiotensin system. The list of panel genes is available in Supporting Information I.

Sequencing and data analyses

Genomic DNA was extracted from fetal tissue, chorion villi, amnion fluid, fibroblasts, formalin-fixed paraffin-embedded tissue, whole blood, or buccal smear samples using standard methods.

Kidney-gene sequencing

A library for Illumina paired-end sequencing was constructed using the KAPA HTP Library Preparation Kit following the manufacturer's instructions (KAPA Biosystems Inc., Wilmington, MA, USA). The libraries were enriched for regions of interest using the customized targeting probe set (SeqCap EZ Choice, Roche Nimblegen, Inc., Madison, WI, USA). Samples were sequenced on a NextSeq 500 Sequencer (Illumina, San Diego, CA, USA) generating paired-end reads that were aligned to the human genome (hg19), and variants were called and annotated in coding exons ± 10 bp using Biomedical Genomics Workbench v.2 (CLC bio-Qiagen, Aarhus, Denmark). The average

coverage was 698 (range: 133-1543), and the average percentage of bases covered >30X was 98.2% (range: 95.4%-99.9%).

Copy number variant analysis was performed using the built-in CNV detection tool in the Biomedical Genomics Workbench v.2, comparing local coverage in each patient data set to local coverage in a set of four controls.

Whole-exome sequencing

Libraries were prepared as described above. The libraries were enriched for the exome using the SeqCap EZ MedExome Kit following the manufacturer's instructions (Roche NimbleGen Inc., Madison, WI, USA). Samples were sequenced on a NextSeq 500 Sequencer (Illumina, San Diego, CA, USA). The reads obtained from sequencing were aligned to the human genome (hg19) and variants were called and annotated in coding exons ± 10 bp using Biomedical Genomics Workbench v.2 (CLC bio-Qiagen, Aarhus, Denmark).

Across eight samples from two families, we achieved average targeted exome coverage of 119X with an average mapping quality of 63.6 for calling high-quality variants (Supporting Information II).

Sanger sequencing

Significant NGS findings were confirmed by Sanger sequencing using BigDye® Terminator v1.1 Cycle Sequencing Kit following the manufacturer's description (Applied Biosystems, Life Technology) and analyzed using ABI 3500xl Genetic Analyzer (Applied Biosystems, CA, USA). Relatives were also tested by Sanger sequencing if samples were available. Primer sequences and other PCR details are available upon request.

Variant filtering

Kidney-gene panel

Synonymous variants were removed except for those located ± 2 bp off exon boundaries. Also, all intronic variants >10 bp from exon boundaries were removed. Furthermore, variants with a minor allele frequency (MAF) $>1\%$ in public databases (the Exome Aggregation Consortium (ExAC) database, the 1000 Genomes Project, or whole-exome sequencing of 2,000 Danish individuals) were removed as were variants with an MAF $>5\%$ in our in-house database. However, all frame-shift variants and variants affecting stop codons were retained irrespectively of their MAF. The functional significance of the retained variants was evaluated *in silico* using the online prediction software Polyphen, Mutation Taster, Provean, Sift, CADD if applicable.

We classified variants identified by the kidney-gene panel as likely deleterious if the MAFs were extremely low, they were predicted as deleterious by the majority of the prediction softwares applied, and the fetal phenotype had similarities with the phenotypes previously reported for variants in that gene. Variants thought to be benign were not reported and variants appearing in heterozygous form in genes associated with autosomal recessive disease were reported only if an additional likely deleterious variant was identified in the same gene.

Whole-exome sequencing

Variants identified by whole-exome sequencing were analyzed in QIAGEN's Ingenuity® Variant Analysis™ (www.qiagen.com/ingenuity) software. Exome data from each family were analyzed separately based on the family history.

Variant calls were removed based on default coverage and quality thresholds. Only variants outside very variable regions were retained. We removed variants with an MAF $\geq 0.1\%$ in the 1000 Genomes Project, the National Heart Lung Blood Institute Exome Sequencing Project (NHLBI-

ESP), or the Allele Frequency Community (AFC). Variants listed in HGMD were retained. Variants predicted to be deleterious, frame-shift variants, in-frame indel variants, missense variants, and variants affecting start/stop codons were retained. We also retained variants causing splice site loss ≤ 2 bases into introns or predicted to affect splice sites according to MaxEntScan (9).

In Family 1 (Fig. 1a), an autosomal recessive disease entity was suspected as both fetuses had a syndromic presentation and both parents were unaffected. Filtering was applied to retain variants present in homozygous or compound heterozygous form in the affected fetus with each parent being a carrier. Subsequently, filtering was applied to detect variants present in heterozygous form in the affected fetus but not in the parents, assuming one parent has germline mosaicism. In Family 2 (Fig. 1b), an autosomal dominant disease entity was suspected due to the observation of an affected mother and two affected fetuses. Filtering was applied to retain variants present in heterozygous form in the affected mother and the second affected fetus. Subsequently, filtering was applied to retain variants present in heterozygous form only in affected but not in unaffected family members.

For variants identified by exome sequencing, we classified variants as likely deleterious if the MAFs were extremely low, they were predicted as deleterious by the majority of the prediction softwares applied, the affected gene had a known function in kidney development, and all affected individuals in the family under study harboured the variant.

Kidney histology

Post-mortem examination was performed with informed consent in all cases. Hematoxylin and eosin-stained kidney and liver sections from fetuses in which we identified a likely deleterious variant were all re-evaluated by a fetal pathologist.

Ethics

The study was approved by the Danish Data Protection Agency (1-16-02-26-12).

The Central Denmark Regional Committee of Ethics approved the kidney gene-targeted analysis study (1-10-72-169-14). The National Committee of Ethics approved the whole-exome sequencing study (1504797). Written informed consent for whole-exome sequencing was obtained from all included parents and adult family members. Whole-exome sequencing in live-born children was not approved.

Results

Kidney gene panel

We included samples from 62 fetuses from 56 families. Prenatal kidney anomalies included: kidney agenesis, kidney hypoplasia, classic multicystic dysplastic kidneys, dysplastic kidneys, cystic kidneys, duplex kidney, kidney parenchymal thinning, and echogenic kidneys (Table 1). The male/female fetal ratio was 2.1. Eleven families had a family history of kidney anomalies. None of the parents were known to be consanguineous.

Detailed information about prenatal findings, post-mortem findings, source of DNA, and previous genetic analyses in each fetus is presented in Supporting Information III.

Kidney-gene analysis in fetuses from the 56 families revealed likely deleterious variants in eight fetuses from seven families (Table 2). No disease-associated CNVs were identified.

The unrelated fetus 9 and fetus 53 were both affected by enlarged cystic kidneys and ductal plate malformations. We identified two *TMEM67* variants in each. Unfortunately, it could not be confirmed that the variants were located on separate chromosomes.

Fetus 11 and fetus 12 were dizygotic twins both affected by bilateral kidney hypoplasia with cystic dysplasia. We identified the same missense variant in *PAX2* in both. The father has unilateral

kidney agenesis, the paternal grandfather has an unknown kidney disease. Unfortunately, they were not available for genetic testing.

In fetus 16, affected by isolated bilateral kidney agenesis, we identified a missense variant in *WNT4*.

In fetus 19, post-mortem examination revealed isolated kidney affection with unilateral duplex kidney and cystic kidney dysplasia of the upper kidney as well as bilateral hydronephrosis. A missense variant in *SIX2* was identified in fetus 19.

In fetus 29, a nonsense variant was previously identified in *RET*: c.3148C>T p.(Arg1050*), but was not considered the cause of bilateral kidney agenesis at the time of identification as it was inherited from the healthy father. The fetus was therefore included in the present study. Next-generation sequencing showed no evidence of *RET* variant mosaicism in blood obtained from the father.

Kidney ultrasound of the father revealed no abnormalities. He had no symptoms of Hirschsprung disease or multiple endocrine neoplasia type 2, nor had his immediate relatives.

In fetus 43, affected by bilateral kidney agenesis and hypertrophic heart, we identified a missense variant in *ROBO2* as well as a missense variant in *SLIT2*.

Whole-exome sequencing

Families with two affected fetuses were contacted for permission to perform exome sequencing.

Two families gave informed consent.

In Family 1, two pregnancies were terminated due to bilateral kidney agenesis. In both, post-mortem examination confirmed the absence of both kidneys and revealed female fetuses with genital hypoplasia, hypoplasia of halluces, intestinal malrotation, and anteriorly displaced anus. In addition, one fetus was diagnosed with corpus callosum agenesis. Kidney ultrasound examinations in both parents and prenatal ultrasound examinations in the two live-born brothers identified no

abnormalities. Exome sequencing in the second affected fetus, the mother, and the father revealed two novel likely deleterious variants in *ROBO1* in the fetus. One of the variants, *ROBO1* c.4823C>G p.(Ser1608*), was also identified in the mother; the other variant, *ROBO1* c.526C>T p.(Pro176Ser), was also identified in the father. Subsequently, Sanger sequencing identified both *ROBO1* variants in the first affected fetus. Buccal swab samples were obtained from the live-born brothers. Surprisingly, also the older brother harbored both *ROBO1* variants. However, a detailed anamnesis and medical chart review revealed that he has dysmorphic features (frontal bossing, curled ears, and curled retinal arteries), chronic constipation requiring medication, nocturnal and diurnal enuresis, delayed motor development (he was 2 years and 3 months before walking) as well as delayed social and emotional development. He follows the -3 standard deviation reference curve for height and weight although the parents are of normal height. Array-cgh analysis and kidney ultrasound have been normal. The younger brother, only harbouring one *ROBO1* variant, has normal growth and has reached developmental milestones at average age (Table 2, Fig. 1a).

In Family 2, two pregnancies were terminated due to bilateral kidney agenesis. Post-mortem examination confirmed isolated absence of both kidneys in a female and in a male fetus, respectively. Kidney ultrasound in the parents revealed left-sided kidney agenesis in the mother and normal kidneys in the father. Kidney ultrasound in the maternal grandparents and maternal uncle disclosed no abnormalities. Kidney ultrasound examinations of the three live-born brothers were unremarkable. Exome sequencing in the second affected fetus, the mother, the father, and the maternal grandparents revealed a novel likely deleterious variant in *GREB1L* (c.5608+1del) in the mother and the second affected fetus. The same variant was identified in the first affected fetus and in the two eldest live-born brothers using Sanger sequencing (Table 2, Fig. 1b).

Re-analyzing samples

As *ROBO1* and *GREB1L* seemed to be novel genes associated with bilateral kidney agenesis, we added these genes to our kidney-gene panel and re-analyzed samples from the eight fetuses with bilateral kidney agenesis that remained unsolved after the initial kidney gene-targeted analysis. We identified two *ROBO1* variants in a fetus with bilateral kidney agenesis, hydrocephalus, and shortening of 1. metacarpals as well as another *GREB1L* variant in two fetal siblings with isolated bilateral kidney agenesis (Table 4). The father of these fetal siblings has unilateral kidney agenesis. Unfortunately, none of the parents were available for genetic testing.

Kidney histology

Hematoxylin and eosin staining of kidneys and liver of fetuses harboring likely deleterious variants were compared with a control fetus terminated 21+3. Fetus 9 and fetus 53 had similar histologic presentations of kidney and liver, showing a narrow abrupt nephrogenic zone, plenty of immature stroma, and medullary kidney cysts and hepatic ductal plate malformations (Fig. 2). In the remaining mutant fetuses, kidney histology fulfilled the criteria of kidney dysplasia and liver histology was unremarkable (data not shown) (10).

Discussion

In this cohort of fetuses in which previous genetic analyses had been unable to detect an underlying genetic cause, we applied kidney gene-targeted analyses and whole-exome sequencing (Table 2 and Table 3), revealing likely deleterious variants in 15 out of 62 (24%) fetuses, which is equivalent to 11 of 56 (20%) families.

Variants in *TMEM67* (* 609884)

The presence of similar kidney histology in two unrelated fetuses with *TMEM67* variants supports the presence of a common underlying cause. Also, similarly to the findings in fetus 9 and fetus 53, it has been reported that most patients with *TMEM67* variants have liver affections (Figure 2) (11).

Gunay-Aygun et al. reported that *TMEM67*-associated disease may phenocopy the presentation of autosomal recessive polycystic kidney disease (ARPKD) (12). In fact, one of the fetuses with *TMEM67* variants was suspected of ARPKD due to the combination of enlarged cystic kidneys and ductal plate malformation. However, the kidney cysts did not appear radically in the parenchyma as usually seen in ARPKD.

TMEM67 encodes a cilia-related protein and is mutated in Meckel syndrome type 3 (# 607361), Joubert syndrome type 6 (# 610688), and nephronophthisis type 11 (# 613550) (13). As no structural cerebral abnormalities were observed in fetus 9 and fetus 53, the most likely clinical diagnosis is nephronophthisis type 11. Clinically, three forms of nephronophthisis have been described: an infantile, a juvenile, and an adolescent form. In the infantile form, end-stage renal disease is reached before the age of 2 years, and kidney histology is characterized by cortical microcysts and cystic dilatation of Bowman's space (14). However, here we report two cases of severe nephronophthisis, histologically characterized by a narrow, abrupt nephrogenic zone, plenty of immature stroma, and medullary cysts, but not cystic dysplasia as seen in the lethal Meckel syndrome. This distinct presentation of nephronophthisis seems to be a novel prenatal form.

Variants in *PAX2* (* 167409) and *HNF1B* (* 189907)

Transcription factor *PAX2* is involved in capmesenchymal progenitor cell condensation, mesenchymal-epithelial transition, and cell polarization in the developing metanephric kidney (15).

PAX2 variants cause autosomal dominant renal coloboma syndrome (# 120330) and have previously been reported to cause fetal bilateral kidney hypoplasia (16-18). Madariaga et al. reported pathogenic *PAX2* variants in 4 of 75 fetuses with prenatally diagnosed severe kidney anomalies and pathogenic *HNF1B* variants in 12 of 90 fetuses with prenatally diagnosed severe kidney anomalies¹⁶. In consistency with their findings, we identified a *PAX2* missense variant in

dizygotic twins with kidney hypoplasia with cystic dysplasia from a family, suggesting autosomal dominant inheritance of the kidney affection.

No *HNF1B* variants were identified in our cohort. However, we excluded two fetuses previously reported to have a *HNF1B* missense variant and a fetus known to harbor a 17q12 deletion encompassing the *HNF1B* gene identified by chromosomal microarray analysis (19,20).

Variants in *WNT4* (* 603490)

Wnt4 is essential for generation of pretubular aggregates, leading to renal vesicles and further nephron morphogenesis (21-23). A *Wnt4* knockout mouse model shows kidney hypodysplasia and defective sexual differentiation only in female mice (24, 25). Previously, *WNT4* variants were reported to cause renal hypodysplasia in two brothers and unilateral kidney agenesis and uterovaginal agenesis in a hyperandrogenic female (26, 27). The male fetus 16 with a missense variant in *WNT4* accordingly showed no defects in sexual differentiation. However, the kidney phenotype was more severe than previously reported.

Variant in *SIX2* (* 604994)

Six2 expressed by the capmesencyme is important for maintaining the nephron progenitor cell population causing severe kidney hypoplasia in *Six2* mutant mice (28, 29). *SIX2* variants in humans have previously been reported to be a rare cause of isolated kidney anomalies. The human mutants have shown renal hypodysplasia, renal cysts, vesicoureteric reflux, and posterior urethral valve (18, 30, 31). In accordance with this, fetus 19 showed a similar isolated kidney phenotype.

Variant in *RET* (+ 164761)

RET variants are a frequent cause of Hirschsprung disease and the underlying cause of multiple endocrine neoplasia type 2 (MEN2). *RET* variants have previously been reported in patients harboring both Hirschsprung disease and kidney anomalies or a combination of MEN2A and kidney

anomalies (32, 33), which underlines that the same *RET* variant may cause multiple-organ manifestations.

Heterozygosity for *RET* variants was previously reported in 9 of 29 fetuses with either bilateral or unilateral kidney agenesis. None of the parents were tested for the *RET* variants (34). In a subsequent study, *RET* variants were found in only 7 of 105 fetuses with severe bilateral kidney anomalies. In four of the seven fetuses, the variant's parental origin was determined and identified in four healthy fathers (35). The paternally inherited *RET* variant c.3148C>T p.(Arg1050*) has previously been reported in a patient with Hirschsprung disease (36). Unfortunately, colon tissue from fetus 29 had not been archived, and it could therefore not be determined if enteric ganglia were present. Although we found no evidence of *RET* being imprinted (www.geneimprint.com/site/genes-by-species, igc.otago.ac.nz), it is possible that expression of the paternal allele is particularly important in early embryonic kidney development.

Variants in *ROBO2* (* 602431), *SLIT2* (* 603746), and *ROBO1* (* 602430)

In mice, Slit2-Robo2 signaling is shown to restrict Gdnf-Ret signals to the site of the ureteric bud outgrowth preventing formation of multiple ureteric buds. *Robo2* and *Slit2* mice mutants show multiplex kidneys due to multiple ureteric bud formation. Multiplex kidneys are similar to duplex kidney, except that they result from fusion of multiple kidneys (37, 38).

In humans, heterozygosity for *ROBO2* variants has been reported as a rare cause of kidney anomalies (18, 39, 40). Recently, variants in the gene encoding the receptor ligand *SLIT2* were also reported to cause kidney anomalies (39). It seems plausible that kidney development could be disrupted completely by the accumulated effect of a variant in the gene encoding the ligand and a variant in the gene encoding the receptor, as seen in fetus 43. Digenic combination of variants has previously been reported to exacerbate a kidney phenotype (41). However, it has to our knowledge not been reported within the *CAKUT* disease spectrum. In addition to bilateral kidney agenesis, fetus 43 was also diagnosed with a hypertrophic heart. Interestingly, Slit-Robo signaling has also been implicated in murine heart morphogenesis (38, 42).

Compound heterozygosity for two novel *ROBO1* variants was identified in three siblings; two fetuses with syndromic bilateral kidney agenesis and one live-born brother challenged in multiple ways (Fig. 1a and Table 3). Re-analysis of the samples identified another fetus with two *ROBO1* variants. This fetus also had syndromic bilateral kidney agenesis.

ROBO1 variants have not previously been associated with human kidney anomalies. However, *Robo1* has been shown to be expressed in the developing murine kidney along with the expression of the putative ligand, *Slit2*, and the receptor, *Robo2* (43). Also, at least one *Robo1* mice mutant develops multiplex kidneys (44). Furthermore, *in situ* hybridization analysis revealed *Robo1* to be expressed in mice 14.5 dpc in a variety of tissues, including brain, spinal cord, eye, alimentary system, skeletal muscles, and limbs (www.eurexpress.org). Post-mortem examination of the two fetuses with biallelic *ROBO1* variants revealed similar syndromic presentations. Also, one of the fetuses with *ROBO1* variants was diagnosed with corpus callosum agenesis. Actually, the *Drosophila robo1* was originally identified in a large-scale mutant screen of genes controlling midline axonal crossing in the central nervous system (45). Moreover, the live-born brother

harboring both *ROBO1* variants seemed to have a syndromic presentation with the brain, eye, and alimentary system being affected.

Nicolaou et al. reported four patients with duplex collecting system, vesicoureteral reflux, posterior urethral valve, and kidney dysplasia, harboring candidate variants in *ROBO1* for further research, but none of the patients were homozygous or compound heterozygous for *ROBO1* variants (6). Our observations suggest that *ROBO1* variants are implicated in a novel autosomal recessive syndrome that may include bilateral kidney agenesis. However, our findings do not support a disease-causing effect of heterozygosity for *ROBO1* variants.

Variants in *GREB1L*

We identified a variant in *GREB1L*; yet the literature contained no indication of an association with kidney anomalies. However, we came across a report on a family in Iowa with kidney agenesis harboring a *GREB1L* variant. Our Family 1 (Fig. 1b and Table 3) and the Iowa family are reported by Brophy et al. who presents an argument for the implication of *GREB1L* in kidney morphogenesis through steroid hormone/retinoic acid receptor target activation (46). The combination of unilateral kidney agenesis and bilateral kidney agenesis in the same pedigree and the incomplete penetrance of autosomal dominant inherited kidney agenesis has previously been described (47).

We subsequently added *GREB1L* to our kidney gene panel and identified another *GREB1L* variant in two fetal siblings with isolated bilateral kidney agenesis. Similarly to our first family harboring a *GREB1L* variant, one parent had unilateral kidney agenesis.

In conclusion, targeted next-generation sequencing contributes substantially to identifying potential underlying causes of fetal kidney anomalies. We observed two unrelated fetuses with variants in

TMEM67 affected by a prenatal form of nephronophthisis type 11 presenting with a similar distinct histological picture. Digenic combination of variants in *ROBO2* and *SLIT2* was associated with bilateral kidney agenesis and hypertrophic heart. Furthermore, we propose that *ROBO1* variants are a cause of a novel autosomal recessive syndrome that may include bilateral kidney agenesis. This is the first time that SLIT-ROBO signaling is implicated in human bilateral kidney agenesis.

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Table 1

Prenatal ultrasound diagnoses in fetuses included in the study.

Prenatal kidney diagnoses	Number
Bilateral kidney agenesis	14
Bilateral MCDK †/kidney dysplasia/adysplasia/cystic kidneys	22
Bilateral enlarged kidneys	2
Bilateral hypoplastic kidneys	1
Bilateral echogenic kidneys	16
Bilateral duplex kidney	1
Bilateral parenchymal thinning	1
Unilateral echogenic kidney and oligohydramnios	1
Unilateral echogenic kidney and contralateral agenesis	3
Unilateral parenchymal thinning and contralateral echogenic kidney	1
Total number of fetuses	62

†multicystic dysplastic kidney

Table 2

Likely deleterious variants identified by our in-house-designed kidney gene-targeted analyses and the prenatal and post-mortem fetal phenotypes.

Fetus	Ultrasound findings	Outcome and findings at post-mortem examination	NGS findings	Prediction Polyphen Mutation Taster Sift Provean CADD	MAF 1000Genomes ExAC 2000DK
9	Bilateral, enlarged cystic kidneys	TOP 14+4. Bilateral, enlarged cystic kidneys and ductal plate malformation in the liver. Abnormal lung lobulation.	<i>TMEM67</i> [NM_153704.5] c.1027T>G p.(Phe343Val)	-probably damaging -disease causing -damaging -deleterious -PHRED 29.3	- - -
			<i>TMEM67</i> [NM_153704.5] c.1715C>T p.(Ala572Val)	-benign -disease causing -damaging -deleterious -PHRED 31	- 0.0008% -
53	Bilateral enlarged cystic kidneys and oligohydramnios	TOP 19+5. Bilateral, enlarged cystic kidneys and ductal plate malformation in the liver. Bicorn uterus and oligohydramnios sequence.	<i>TMEM67</i> [NM_153704.5] c.175G>C p.(Ala59Pro)	-benign - polymorphism -damaging -deleterious -PHRED 14.1	- - -
			<i>TMEM67</i> [NM_153704.5] c.1063C>T p.(Gln355*)	-NA -disease causing -NA -NA -PHRED 41	- - 0.03%
11†	Bilateral small MCDK and oligohydramnios	TOP 18+1. Bilateral kidney hypoplasia with cystic dysplasia and mild oligohydramnios sequence.	<i>PAX2</i> [NM_003990.3] c. 254G>C p.(Gly85Ala)	-probably damaging -disease causing -damaging -deleterious -PHRED 26.8	- - -
12†	Left small MCDK, right kidney	TOP 18+1. Bilateral kidney	<i>PAX2</i> [NM_003990.3]	-probably damaging	- -

	agenesia and oligohydramnios	hypoplasia with cystic dysplasia and mild oligohydramnios sequence.	c. 254G>C p.(Gly85Ala)	-disease causing -damaging -deleterious -PHRED 26.8	-
16‡	Bilateral kidney agenesis and anhydramnios	TOP 20+4. Bilateral kidney agenesis and oligohydramnios sequence.	WNT4 [NM_003391.2] c.1027C>T p.(Arg343Trp)	-probably damaging -disease causing -damaging -deleterious -PHRED 34	- - -
19	Bilateral echogenic kidneys and hydronephrosis	TOP 21+3. Unilateral duplex kidney with upper dysplastic kidney and bilateral hydronephrosis.	SIX2 [NM_016932.4] c.283G>A p.(Ala95Thr)	-probably damaging -disease causing -damaging -deleterious -PHRED 32	- - -
29	Bilateral MCDK and anhydramnios	TOP 21+2. Bilateral MCDK, bladder hypoplasia, and oligohydramnios sequence.	RET [NM_020975.4] c.3148C>T p.(Arg1050*)	-NA -disease causing -NA -NA -PHRED 48	- - - HGMD: CM1110093 PMID: 22174939 (Hirschsprung disease)
43	Bilateral kidney and bladder agenesis. Enlarged heart and oligohydramnios.	TOP 18+4. Bilateral kidney and bladder agenesis. Hypertrophic heart.	ROBO2 [NM_002942.4] c.2005C>G p.(Arg669Gly)	-probably damaging -disease causing -damaging -deleterious -PHRED 26.1	- - -
			SLIT2 [NM_004787.3] c.1022C>T p.(Pro341Leu)	-benign -disease causing -damaging -deleterious -PHRED 23.4	- - -

MCDK, multicystic dysplastic kidneys; MAF, minor allele frequency; TOP, termination of pregnancy; 1000Genomes, 1000 Genomes Project; ExAC, Exome Aggregation Consortium database; 2000DK, Whole-exome sequencing of 2,000 Danish individuals.

† Fetus 11 and 12 were twins, the father has left unilateral kidney agenesis, the paternal grandfather had an unknown kidney disease.

‡ The mother's half-sister also had a TOP because of fetal bilateral kidney agenesis.

Table 3

Likely deleterious variants identified by whole-exome analyses and the prenatal and post-mortem fetal phenotypes.

Family	Fetuses	Ultrasound findings	Outcome and findings at post-mortem examination	NGS findings	Prediction Polyphen Mutation Taster Sift Provean CADD	MAF 1000Genomes ExAC 2000DK
1	17	Left small, cystic echogenic kidney, right enlarged echogenic kidney, and anhydramnios.	TOP 17+5. Bilateral kidney agenesis, genital hypoplasia, hypoplasia of halluces, intestinal malrotation, anteriorly displaced anus, corpus callosum agenesis, and oligohydramnios sequence.	ROBO1 [NM_002941.3] c.526C>T p.(Pro176Ser)	- probably damaging -disease causing - damaging - deleterious -PHRED 24.7	- - -
				ROBO1 [NM_002941.3] c.4823C>G p.(Ser1608*)	-NA -disease causing -NA -NA -PHRED 50	- - -
	18	Bilateral kidney agenesis	TOP 19+0. Bilateral kidney agenesis, genital hypoplasia, hypoplasia of halluces, intestinal malrotation, anteriorly displaced anus, and temporal polymicrogyria.	ROBO1 [NM_002941.3] c.526C>T p.(Pro176Ser)	- probably damaging -disease causing - damaging - deleterious -PHRED 24.7	- - -
				ROBO1 [NM_002941.3] c.4823C>G p.(Ser1608*)	-NA -disease causing -NA	- - -

					-NA -PHRED 50	
2	61	Bilateral kidney agenesis and anhydramnios	TOP 18+0. Bilateral kidney and bladder agenesis. Undifferentiated external genitalia.	GREBIL [NM_001142966.2] c.5608+1del p.?	-NA -NA -NA -NA -PHRED 25.8	- - -
	24	Bilateral kidney agenesis	TOP 15+5. Bilateral kidney and bladder agenesis.	GREBIL [NM_001142966.2] c.5608+1del p.?	-NA -NA -NA -NA -PHRED 25.8	- - -

Table 4

After adding *GREB1L* and *ROBO1* to the kidney-gene panel, eight samples were re-analyzed.

Fetus	Ultrasound findings	Outcome and findings at post-mortem examination	NGS findings	Prediction Polyphen Mutation Taster Sift Provean CADD	MAF 1000Genomes ExAC 2000DK
6	Bilateral kidney agenesis and anhydramnios	TOP 16+0. Bilateral kidney agenesis, bladder hypoplasia, and oligohydramnios sequence	<i>GREB1L</i> [NM_001142966.2] c.371G>T p.(Gly124Val)	-probably damaging -disease causing -damaging -deleterious -PHRED 29.2	- - -
62	Bilateral kidney agenesis and anhydramnios	TOP 19+0 Bilateral kidney agenesis and oligohydramnios sequence	<i>GREB1L</i> [NM_001142966.2] c.371G>T p.(Gly124Val)	-probably damaging -disease causing -damaging -deleterious -PHRED 29.2	- - -
13	Bilateral kidney agenesis, anhydramnios, and cerebral ventriculomegaly	TOP 20+2. Bilateral kidney agenesis, bladder hypoplasia, hydrocephalus, and oligohydramnios sequence. Shortening of 1. metacarpals.	<i>ROBO1</i> [NM_002941.3] c.3685G>T p.(Glu1229*)	-NA -disease causing -NA -NA -PHRED 44	- - -
			<i>ROBO1</i> [NM_002941.3] c.4823C>G p.(Ser1608*)	-NA -disease causing -NA -NA -PHRED 50	- - -

Legends

Figure 1

Families subjected to whole-exome sequencing. Black-shaded symbols indicate bilateral kidney agenesis. Half-shaded symbol indicates unilateral kidney agenesis. Grey-shaded symbol indicates various features including delayed motor development, emotionally delayed development, mild dysmorphic features, and constipation requiring medication.

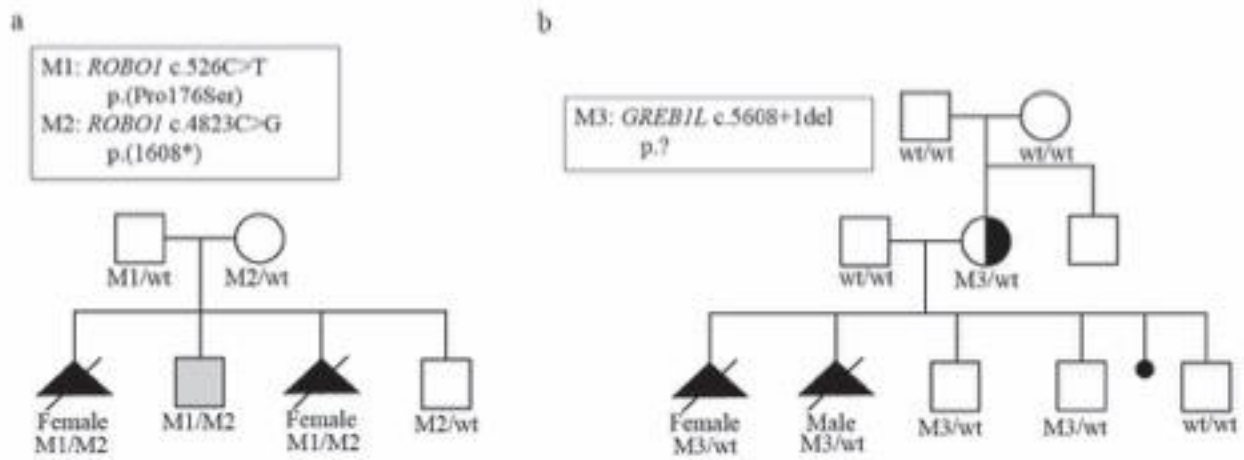


Figure 2

Hematoxylin- and eosin-stained mutant fetal kidneys and livers compared with a control fetus terminated at 21+3. Photomicrographs at lower magnification of the kidneys show a narrow and abrupt nephrogenic zone in fetus 9, fetus 53, and fetus 19, a disorganized nephrogenic zone with cysts in fetus 11 and fetus 12, and no nephrogenic zone in fetus 29. Photomicrographs at higher magnification of kidney medullas show plenty of immature stroma as well as cysts (cy) in fetus 9, fetus 53, and fetus 19. Furthermore, primitive ducts (pd) are seen in fetus 11, fetus 12, and fetus 19, immature glomeruli in fetus 19, and immature cartilage (ic) in fetus 29. Photomicrographs of livers show ductal plate malformations (dp) in fetus 9 and fetus 53.

