ROBO2 Gene Variants Are Associated with Familial Vesicoureteral Reflux

Aida M. Bertoli-Avella,* Maria Luisa Conte,*† Francesca Punzo,† Bianca M. de Graaf,* Giuliana Lama,† Angela La Manna,† Cesare Polito,† Carolina Grassia,† Bruno Nobili,† Pier Francesco Rambaldi,‡ Ben A. Oostra,* and Silverio Perrotta†

*Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, Netherlands; and †Department of Pediatrics, and ‡Department of Radiological Sciences, Nuclear Medicine, Second University of Naples, Naples, Italy

ABSTRACT

The SLIT2 receptor ROBO2 plays a key role in the formation of the ureteric bud, and its inactivation in mice leads to supernumerary ureteric bud development, lack of ureter remodeling, and improper insertion of the ureters into the bladder. Recently, two heterozygous ROBO2 missense mutations were identified in two families with primary vesicoureteral reflux occurring in combination with congenital anomalies of the kidney and urinary tract (VUR/CAKUT). This study investigated a possible causal role of ROBO2 gene variants in 95 unrelated patients with primary VUR (n = 78) or VUR/CAKUT. Eighty-two percent of all patients had a family history of genitourinary anomalies. Twenty-four ROBO2 gene variants were identified by direct sequencing of all 26 exons and the exon-intron boundaries. Of these, four led to amino acid substitutions: Gly328Ser, Asn515Ile, Asp766Gly, and Arg797Gln. When the families were examined, the missense variants co-segregated with VUR (three families) or VUR/CAKUT (one family). These variants were not found in 190 control subjects, and the affected amino acids have been conserved through evolution. In conclusion, a relatively high frequency of ROBO2 variants (5.1%) was found in familial cases; however, functional studies and validation in other cohorts are warranted.


Primary vesicoureteral reflux (VUR; OMIM 193000) is a common and complex disorder, occurring with an incidence of approximately one in 100 infants. The disease is characterized by the retrograde flow of urine from the bladder into the upper urinary tract and into the kidney. Renal defects associated with VUR are usually known as reflux nephropathy (RN). These defects may result from either congenital dysplasia, as a result of a ureteral bud abnormality occurring during the embryogenesis, or postinfectious damage. RN may lead to hypertension, proteinuria, and renal insufficiency. Moreover, RN accounts for up to 25% of end-stage renal failure (ESRF) in children.

VUR may be primary or secondary and may occur isolated or in combination with other congenital abnormalities of the kidney/urinary tract (VUR/CAKUT). A high familial occurrence has been described with a prevalence of 27 to 50% among siblings and offspring of patients. This is compatible with autosomal dominant inheritance with reduced penetrance. Early segregation studies have pointed to the role of a single major locus/gene, with a dominant mutant allele.

Nowadays, it is evident that VUR is a genetically heterogeneous disorder. We might be studying a disease spectrum that varies from “sporadic” patients in whom the disease is caused by a combination of frequent genetic
variants with low phenotypic effect (multifactorial or polygenic) to relatively large families carrying more rare gene variants with a strong phenotypic impact (monogenic disease).

Recently, Lu et al. described a de novo human translocation [46,X,t(Y;3)(p11;p12)] in an individual who exhibited multiple congenital anomalies including severe bilateral VUR with ureterovesical junction defects. The translocation disrupts ROBO2 (roundabout, axon guidance receptor, homolog 2 [Drosophila]), an ideal functional candidate gene for VUR/CAKUT. The gene was initially known as an axon guidance receptor and gatekeeper controlling axon midline crossing. Recently, it was shown that SLIT2 and its receptor ROBO2 also play a key role in controlling the ureteric bud (UB) formation, a process of critical importance for the normal kidney development. Inactivation of either Slit2 or Robo2 in mice leads to supernumerary UB development, lack of ureter remodeling, and improper insertion into the bladder.

Lu et al. identified two novel ROBO2 heterozygous missense changes in two unrelated families (British and Dutch ancestry), in which the variants co-segregated with the VUR/CAKUT phenotype. Whereas homozygous Robo2-null mice exhibit a multiple ureter phenotype and dysplastic kidneys and fail to survive after birth, heterozygous and mosaic mutant mice show a CAKUT/VUR phenotype with megaureter, wide-open ureteropelvic junction, and hydrenephrosis. These findings implicated for the first time the SLIT-ROBO2 signaling pathway in the pathogenesis of a subset of patients with VUR/CAKUT.

We evaluated a possible role of ROBO2 mutations in 95 unrelated Italian patients who had primary VUR or VUR/CAKUT; most of them had a positive family history. We found a relatively high frequency of missense variants in this cohort, indicating the importance of evaluating this gene especially when dealing with familial cases.

RESULTS

Clinical Characterization
A summary of the clinical findings in 95 patients included in this study is presented in Table 1. Seventy-eight (82%) patients had familial VUR, and 17 (18%) were classified as sporadic because no evidence of renal diseases was found across their relatives. Fifty-seven (60%) patients were detected as having RN; 43 of them were familial cases. The majority of the cases (n = 78) showed an isolated primary VUR, whereas 17 patients displayed additional renal/urinary tract abnormalities in combination with VUR (i.e., duplex collecting system, ureterocele, renal agenesis).

Additional family members, when available and recruited in the study, were considered as “affected” on the basis of the presence of reflux documented by voiding cystourethrogram (VCUG)/direct radionuclide cystography (RNC; see the Concise Methods section) and/or the diagnosis of RN, or the detection of ESRF/renal replacement in absence of other known causes. Because VUR may spontaneously disappear during childhood and adolescence, the finding of scintigraphic signs of RN in relatives of patients with VUR strongly suggests the previous occurrence of reflux.

ROBO2 Sequence Analysis
Table 2 summarizes all sequencing findings in this study and includes several previously described variants (NCBI-SNP database). We found 24 DNA variants in the coding region and ex-intron boundaries of the ROBO2 gene, including 17 novel variants. From them, six were located within the coding region: four nucleotide changes led to an amino acid change (Gly328Ser, Asn515Ile, Asp766Gly, and Arg797Gln), and two were synonymous, or “silent,” changes (Val745Val and Gly1352Gly). All base changes were found in heterozygous state. In two of our patients, we also found a missense variant, Ile598Thr (exon 12, 1793T→C), previously suggested to represent a polymorphism.

We then sequenced exons 7, 11, 12, 15, and 16, in which missense changes were found, in a set of 190 Italian control subjects (380 alleles). None of the variants was present in the control group. Furthermore, we found a total of 16 intronic variants located in the proximity of exons, 11 of which were novel variants (Table 2). We investigated nine of these intronic variants located within 50 bases from the exons to exclude a possible effect on gene splicing (see the Concise Methods section); however, none of the splicing programs predicted an effect of these variants on gene splicing.

Table 1. Patients’ clinical characteristics

<table>
<thead>
<tr>
<th>Index Patients (n = 95)*</th>
<th>Familial Cases (n = 78; 82%)</th>
<th>Sporadic Cases (n = 17; 18%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with VUR and RN (n = 57; 60%)</td>
<td>43 (75%)</td>
<td>14 (25%)</td>
</tr>
<tr>
<td>Patients with VUR and no RN (n = 38; 40%)</td>
<td>35 (92%)</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>Patients with isolated VUR (n = 78; 82%)</td>
<td>66 (85%)</td>
<td>12 (15%)</td>
</tr>
<tr>
<td>Patients with VUR and additional abnormalities (n = 17; 18%)</td>
<td>12 (71%)</td>
<td>5 (29%)</td>
</tr>
</tbody>
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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>duplex collecting system (n = 9)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>renal agenesis (n = 2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>ureterocele (n = 2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>renal crossed ectopy (n = 1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>multicystic kidney (n = 1)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>UPJO (n = 1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>hypospadias (n = 1)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Phenotype of family members is not included.
Four Novel Likely Pathogenic Candidate Mutations

Gly328Ser.

We found a heterozygous nucleotide change (982G→A) in exon 7 of the ROBO2 gene, leading to the missense change Gly328Ser (nonpolar amino acid to uncharged polar) (Figure 1, C and D). The variant was found in a patient with a right ureteropelvic junction obstruction (UPJO) and left cystic dysplastic kidney (family A, III-14; Figure 1A). A renal radionuclide scan with mercaptoacetyltriglycine displayed the right UPJO (delayed washout and no response to furosemide) and the nonfunctioning left kidney (Figure 1B). RNC performed at the age of 5 yr did not show presence of VUR. This patient underwent the removal of the left kidney at age of 5 yr and surgical correction of right UPJO at age of 6 yr. Her mother was carrying the same missense variant; she was asymptomatic and had a normal 99Tcm-dimercaptosuccinic acid (DMSA) scan.

Individual III-13, the asymptomatic older sister of the proband (20 yr of age), was detected as a carrier of the 982G→A change as well; therefore, after informed consent, she was investigated with RNC and was found to have right moderate VUR. Individual III-15, the younger sister of the proband (7 yr of age) and asymptomatic, too, did not carry the variant, and RNC did not show evidence of reflux. The same missense variant was also found in a maternal uncle (II-9) and his son (III-19), affected with right RN and right VUR, respectively.

Asn515Ile.

We found a heterozygous base change, 1544A→T, located in exon 11, leading to a nonconservative amino acid change (uncharged polar, hydrophilic to nonpolar, hydrophobic) (Figure 2, C and D). This change was found in one patient (Family P, IV-10; Figure 2A) and in her affected twin brother (IV-9). They showed primary bilateral moderate VUR (Figure 2B) and left VUR (grade III) without RN, respectively. The asymptomatic mother (III-7) carried the same missense variant, and her sister (III-8) showed a bilateral slight pelvic dilation. The maternal grandmother (II-4, deceased) and her sister (II-5) both had ESRF. Unfortunately, no DNA was available from any of these patients.

Asp766Gly.

In exon 15, we found a heterozygous 2297A→G missense change coding for the amino acid glycine (nonpolar, hydrophobic) instead of aspartic acid (charge polar, hydrophilic) at position 766 of ROBO2, corresponding to the third fibronectin type 3 domain (Fn3) of the protein (Figure 3, C and D). The proband was diagnosed as having bilateral VUR (moderate at left and severe at right kidney) at the age of 15 mo after pyelonephritis (Family G, II-4; Figure 3, A and D). The same change was also found in her mother (I-2), affected with right VUR, and in the asymptomatic sister (II-
Individual II-3 was investigated with RNC at the age of 7 yr, but no evidence of reflux was found. The missense 2390G→A heterozygous change in exon 16 of ROBO2 leads to a nonconservative change of Arg797Gln (charge to uncharged polar) in the Fn3 domain of the ROBO2 protein (Figure 4, C and D). This variant was found in patient IV-11 of Family C (Figure 4A), who received a diagnosis of left mild VUR at the age of 1 mo after pyelonephritis. DMSA scan showed a left RN (Figure 4B). His father (III-9), carrying the same mutation, was found to have bilateral VUR grade 4 at the age of 18 yr. Then, he developed ESRD and received a transplant. His sister (III-8) also showed the same DNA variant. She had a history of microscopic hematuria and urinary tract infections, but she did not consent to RNC or DMSA scan. The same missense variant was also found in the paternal grandfa-
ther (II-4), who was asymptomatic but had a brother (deceased) with ESRF.

We carried out an “electronic” analysis of these missense changes to predict the impact of the amino acid allelic variants on the protein structure and function based on multiple protein alignment and three-dimensional structures (PolyPhen program). All but Asn515Ile (predicted to be “benign”) were classified as “possibly damaging” changes.

**Frequency of the Variants**

We found a relatively high frequency of missense variants in ROBO2 in our cohort of patients: 4.2% for all patients with VUR (familial and sporadic). All four DNA variants were heterozygous missense changes, and they were found in the group VUR (familial and sporadic). All four DNA variants were heterozygous missense changes, and they were found in the group

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**Figure 3.** (A) Pedigree of Family G. Filled symbols indicate presence of VUR. The presence (+) or absence (−) of the missense variant is indicated. (B) Direct RNC showing a severe right and moderate left reflux in Family G, II-4. (C) Chromatogram showing the normal and mutated (2297A→G) sequences. Codons and corresponding amino acids are also displayed. (D) Protein conservation across various species.

**Figure 4.** (A) Pedigree of Family C. Filled symbols indicate presence of VUR. Gray symbols indicate other anomalies/symptoms of the genitourinary tract (III-6, stenosis of urethra; III-8, recurrent hematuria and urinary tract infections). (B) DMSA renal scintigraphy showing the left RN in Family C, IV-11. (C) Chromatogram showing the normal and mutated (2390G→A) sequences. Codons and corresponding amino acids are also displayed. (D) Protein conservation across various species.
of patients with familial VUR (three families) and familial VUR/CAKUT (one family). Thus, in the familial cases, missense variants in ROBO2 were found in 5.1% (four of 78) of the patients.

DISCUSSION

To investigate whether alterations in the ROBO2 gene play a causative role in our cohort of patients with VUR, we performed a systematic sequence analysis of all 26 exons of the gene. The majority of the patients belong to the familial primary VUR group followed by the familial VUR/CAKUT set.

We describe four novel missense changes in the ROBO2 gene, all of which were found in patients with a positive family history. When the families were examined, co-segregation of the missense variants with the VUR phenotype was observed. As reported before, asymptomatic individuals without any evidence of disease were also found to carry the sequence change. This observation is consistent with the reduced penetrance described in families with VUR. The presence of the missense variants in available affected family members and absence in control individuals, together with the fact that they affect amino acids conserved through evolution, suggests that these changes may be pathogenic (Figure 1D, 2D, 3D, and 4D); however, functional studies on these candidate mutations and replication in other cohorts are warranted.

ROBO family members act as transmembrane receptors and consist of an extracellular region with five predicted Ig and three Fn3 domains, a transmembrane domain, and an intracellular region with three cytoplasmic motifs.13,15 All four missense changes described here are located within the ROBO2 protein extracellular region: Gly328Ser is located at the beginning of the fourth Ig domain, Asn515Ile is between the last Ig and the first Fn3 domain, and Asp766Gly and Arg797Gln are within the third Fn3 domain. Members of the ROBO family (ROBO1 and ROBO2) are able to interact homophilically and heterophilically.16 A reduction in homophilic binding has been observed when any of the Ig or all Fn domains are missing; therefore, these domains are important for the homophilic binding of molecules such as ROBO1 and ROBO2. Moreover, extracellular Ig domains 1 and 2 of ROBO1 are shown to be important for ROBO-SLIT interaction.17

Lu et al.12 also described a nonconservative change in exon 12 of ROBO2 leading to a change in amino acid (Ile598Thr). This rare variant was found in 1.09% of the control subjects (n = 276). We found the same change in two of our patients with VUR and none from the control group (n = 190). The amino acid isoleucine 598 is located within the first Fn3 domain of the protein and is also conserved in mammals and birds (chicken). Whether the Ile598Thr change is a rare (innocent) polymorphism or confers a higher risk in some VUR cases deserves further investigation in other cohorts of patients and control subjects.

We found an unexpectedly high frequency of ROBO2 missense variants in our cohort of patients: 5.1% of all familial cases. Moreover, we showed that missense changes in ROBO2 are associated not only with VUR/CAKUT phenotype but also with familial VUR. In fact, most of the patients who were found to carry a ROBO2 missense variant had isolated VUR.

The enrichment of familial cases in our sample should not be the cause for the relatively high frequency of missense variants found. In the first study that implicated the ROBO2 pathway in the pathogenesis of a subset of VUR cases, 124 families were investigated and only two (1.6%) of them were found to have a missense variant.12 Whether these families presented with isolated VUR or VUR/CAKUT is not clear; however, VUR and VUR/CAKUT might be part of the same spectrum of urologic tract malformations.

Although in both studies the patients were of white origin, our sample comes specifically from a south European population. Thus, a possibility remains, as for other genetic disorders, that ROBO2 mutations are more common within certain geographic areas. Because all of the missense variants found are different (nucleotide positions and amino acid changes) and are not recurrent in unrelated patients, it is unlikely that we are facing a founder effect of an old mutational event. Our findings indicate that mutational screening of ROBO2 might offer physicians a future tool for the detection and management of familial VUR.

In conclusion, our study independently confirms that genetic alterations in ROBO2 are significantly associated with familial VUR/CAKUT. Furthermore, we showed that ROBO2 is implicated in the more common isolated VUR phenotype, especially in patients with a positive family history.

CONCISE METHODS

Patients

A cohort of 95 patients with VUR, originating from the same geographic region of South Italy, were available for the study. All patients were ascertained on the basis of the presence of VUR, isolated or in combination with additional renal/urinary tract abnormalities, documented by VCUG in male patients and direct RNC in female patients and family members when available. Five pediatric nephrologists and one radiologist assessed the patients. Grading of VUR detected with VCUG was made according to the International Grading System of Vesicoureteral Reflux.18 Reflux observed with RNC was graded as mild (reflux to ureter or renal pelvis), moderate (reflux to renal pelvis with mild to moderate dilation), or severe (distended redundant collecting system associated with ureteral dilation). RN was diagnosed by DMSA renal scintigraphy and defined as focal or multifocal defects of radionuclide uptake and/or as a split renal isotope uptake below 43%.19 In all 95 patients, secondary causes of VUR or presence of syndromic features were excluded. The majority of the patients (n = 78) had at least one affected relative (familial cases), showing a mode of inheritance compatible with an autosomal dominant pattern. In 17 patients, no evidence of renal disease could be found across their relatives (sporadic cases). Informed consent from the patients and families’ members (parents for their children) and
Sequencing Analysis

All 26 exons and exon-intron boundaries of the ROBO2 gene were amplified using newly design intronic primers (Supplementary Table 3). Direct sequencing of both strands was performed using the BigDye terminator chemistry (version 3.1; Applied Biosystems, Foster City, CA). Products were loaded on an ABI 3100 genetic analyzer and examined with the SeqScape software version 2.5 (Applied Biosystems). A negative control was included in all reactions.

The frequency of potential pathogenic variants was assessed by direct sequencing in a panel of 190 control individuals (380 alleles) from the same geographic area of Italy. In the familial cases, these novel variants were also tested in all available family members.

The program PolyPhen (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph/index.html) was used to predict the possible impact of an amino acid substitution on the structure and function of ROBO2 protein. This prediction is based on straightforward empiric rules that are applied to the sequence, phylogenetic, and structural information characterizing the substitution. The nonsynonymous changes are classified as benign, possibly or probably damaging, and unknown. Four different computer programs—NetGene2 (Center for Biological Sequence Analysis, Denmark), GeneSplicer (Center for Bioinformatics and Computational Biology, University of Maryland), SpliceView (Institute for Biomedical Technologies, National Research Council, Italy), and BDGP Splice Site Prediction (Berkeley Drosophila Genome Project)—were used to predict possible consequences on splicing of several novel intronic variants found in the proximity of the exon-intron boundaries.

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DISCLOSURES

None.

REFERENCES