Heterozygous non-synonymous *ROBO2* variants are unlikely to be sufficient to cause familial vesicoureteric reflux

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ROBO2, the receptor of SLIT2, is one of many genes/proteins that regulate the outgrowth of the ureteric bud, which is the first step in the development of the metanephric urinary system. Non-synonymous variants in *ROBO2* have been found in a small proportion of patients with primary vesicoureteric reflux (VUR) in various countries. Here we sequenced 1 kb of promoter and all exons of *ROBO2b* with intronic margins in 227 index cases with primary VUR in an Irish population and found 55 variants, of which 20 were novel. We assessed the variants for evolutionary conservation and investigated novel and uncommon known conserved variants in 23 further index cases and family members of all index cases (to check for segregation with VUR), and then in healthy controls if we found segregation of the variants with VUR. Apart from one non-synonymous variant that was previously found in controls, we did not find any of the six other previously reported non-synonymous variants, but found four new non-synonymous variants. Of those, only two segregated with the disorder (p.Pro522Thr and p.Val799Ile). The former was not present in any of 592 healthy controls; the latter was present in one control. There are now 35 reported non-synonymous coding variants of *ROBO2b*. The predicted pathogenicity of those that have so far been found exclusively in VUR patients does not differ from that predicted for those variants also found in controls. Thus, our finding does not completely rule out that some variants may be the sole cause of VUR, but it is clear from the overall frequency that most of them cannot be. However, it is possible that some of these variants may cause VUR in combination with a mutation in another gene.

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Primary vesicoureteric reflux (VUR), the retrograde flow of urine from the bladder toward the kidneys, is a common disorder with a high familial incidence, which is asymptomatic in many cases and may resolve spontaneously, but in some persists and is a common cause of hypertension and end-stage renal failure in children and adults.[1]

In the early embryo, the development of the metanephric urinary system begins with the outgrowth of the ureteric bud from the mesonephric (Wolffian) duct, stimulated by signals from the adjacent cells, known as the metanephric mesenchyme. Reciprocal signaling between these two causes the bud to elongate to form the ureter, and the metanephric mesenchyme to start forming the kidney, and the growing ureter to branch to form the calyces. Disruptions in this process can lead to VUR because of malformation of the ureterovesical valve, as well as to a range of other urinary tract anomalies including duplex systems and renal dysplasia, hypoplasia, or agenesis.[2,3] These anomalies are collectively known as CAKUT (congenital anomalies of the kidney and urinary tract).[4] Embryology and developmental genetics, much of it in mice, has revealed a complex arrangement of stimulatory and inhibitory signaling, which control the precise positioning and singularity of ureteric bud outgrowth, and the identity of many of the genes involved in this process.[4–10]

One of the inhibitory signals comes from the engagement of SLIT2 with its receptor ROBO2, and lack of either molecule leads to renal dysplasia and supernumerary ureteric budding.[11] The investigation of a 3;Y translocation in a patient with multiple congenital abnormalities including severe bilateral VUR and ureterovesical junction defects revealed that the breakpoint on chromosome 3 was in intron 2 of *ROBO2b*, and placed the promoter and first two exons upstream of exons 1d-6 of *PCDH11Y*.[12] The authors sequenced the 26 exons of *ROBO2b* in 124 British and Dutch families with VUR with potentially autosomal dominant inheritance, and found three non-synonymous variants. One was also present in 3/276 control subjects, but the other two were not found in their controls. In each of

the families with these variants, CAKUT beyond simple VUR was present (renal hypoplasia in one, duplex kidney in the other). They then constructed mice with random levels of reduced expression of full-length ROBO2, and the results suggested that reduced gene dosage can cause CAKUT. Subsequently, another group sequenced *ROBO2b* in 95 patients with VUR from a small region of Italy.[13] They found 24 variants, of which 5 were non-synonymous coding variants. One of these was the same one that had already been found in controls, but the other four, which all encoded evolutionarily conserved amino acids, segregated with VUR/CAKUT in the families in which they were found, and were not found in 190 controls from the same geographic area. A Swedish study of the same gene in 54 VUR families[14] found only six variants, all intronic, and none predicted to alter splicing.

All three previous teams of investigators[12–14] sequenced 26 *ROBO2* exons; however, there are actually 28. Like *ROBO1*, *ROBO2* has two isomorphs, *ROBO2a* and *ROBO2b*.[15] They share 25 exons, but the first two exons of the 'a' isomorph, which are about 1 Mb from the rest of the gene, replace exon 1 of isomorph b. The proteins differ mainly in their signal peptide sequences. ROBO2a has 36 N-terminal amino acids that differ from the first 20 of ROBO2b, but after cleavage of the signal peptides the difference is only that the mature 'a' protein has an additional 4 N-terminal amino acids compared with the 'b' form and is otherwise identical. This was published before the first study[12] but *ROBO2a* was not shown in the genome browsers at the time (and is still not correctly displayed in the UCSC Genome Browser at the time of submission of this paper) and this may be the reason why the first two exons escaped notice. However, we do not think this compromises their results in relation to VUR because: (1) *ROBO2* and *ROBO1* are syntenic in all vertebrates studied but it was found that there is a break in synteny between the human and mouse chromosomes not far upstream of the start of *ROBO2a*. (2) It was shown that the expression of Robo2a/ROBO2a in the brain differs between mouse and man, being strongly expressed in human fetal brain but very little in adult brain, whereas in mouse it is strongly expressed in adult brain. (3) It was suggested that the chromosomal rearrangement during evolution may have resulted in *ROBO2a* having different long-range gene regulation upstream, or a different local chromatin environment in the two species.[15] (4) If so, it would be right to concentrate on *ROBO2b*, because VUR occurs in mice as well humans. (5) Experiments have not so far resolved this. Mouse knock-outs displaying kidney and urinary tract malformations are null mutants and null/wt mosaic mice and lack or have reduced expression of both isoforms[11,12] and GUDMAP (genitourinary development molecular anatomy project),[16,17] which holds data on expression patterns of genes during mouse urinary tract development, does not acknowledge the existence of two different isoforms of *Robo2* yet either. However, it is known that expression of the 'a' isoform in adult kidney is extremely low in mice and humans compared with the 'b' isoform.[15]

We have therefore also sequenced the 26 exons of *ROBO2b* along with intronic margins (but with the addition of over 1 kb of upstream sequence) in VUR index cases, and have checked the segregation of those variants that are in evolutionarily conserved positions in the families of all those carrying them, and where relevant in control subjects. We also consider the non-synonymous variants found in VUR in all studies with the many now discovered in controls in the light of improved pathogenicity predictors.

RESULTS

Sanger sequencing of 1 kb of promoter sequence and all 26 exons of *ROBO2b*, including 3′ and 5′ untranslated regions yielded 55 single-nucleotide variants. We screened these for evolutionary conservation. Fish and amphibia have mesonephric (not metanephric) urinary systems, and birds and many reptiles do not have a urinary bladder, so we selected variants on the basis of conservation in mammals only. There are various methods for doing this but they give very similar results although their statistical bases are different,[18,19] and we chose genomic evolutionary rate profiling (GERP),[20–22] which generates rejected substitution (RS) scores. Variants in genomic positions with RS scores >4 are the most likely to be pathogenic,[20] but to reduce our chance of missing a variant-causing VUR, we investigated all variants in positions with RS >0 (fewer substitutions than expected) using the original GERP scoring that was available at the time from the SeattleSeq website (range -11.6 to $+5.82$). We used PCR, high-resolution melting-curve analysis and Sanger sequencing to check for the presence of these variants in 23 index cases not previously investigated and in the family members of all index cases having the variant, usually as a heterozygote. We then screened 592 Irish control subjects for variants that segregated with VUR in all families in which they were found. The results are shown in Table 1. In our experience, a small proportion of variants discovered by sequencing of whole-genome amplified DNA, which look absolutely real on chromatograms, prove not to be real when genomic DNA from the same individual is sequenced. For this study, we only investigated the variants that were evolutionarily conserved. Therefore, a few of the variants that we report (that were not further investigated) may not be real. Most of the variants that we did not investigate are either validated by having been reported before, or by having been found in PCRs from more than one individual, or in more than one PCR from the same individual. The remaining 11 are presented separately in Appendix 1 and, of these, possibly 1 or 2 may not be real, based on our experience of investigating variants in this and other genes.

Before submitting our work for publication, the GERP scoring available through the SeattleSeq website was updated (new range -12.3 to $+6.17$) and we resubmitted the positions of our variants in order to quote the latest scores. Five of the uncommon variants that we had not investigated now have scores >0, and two that we had investigated now have a score <0. However, they are all non-coding, and four