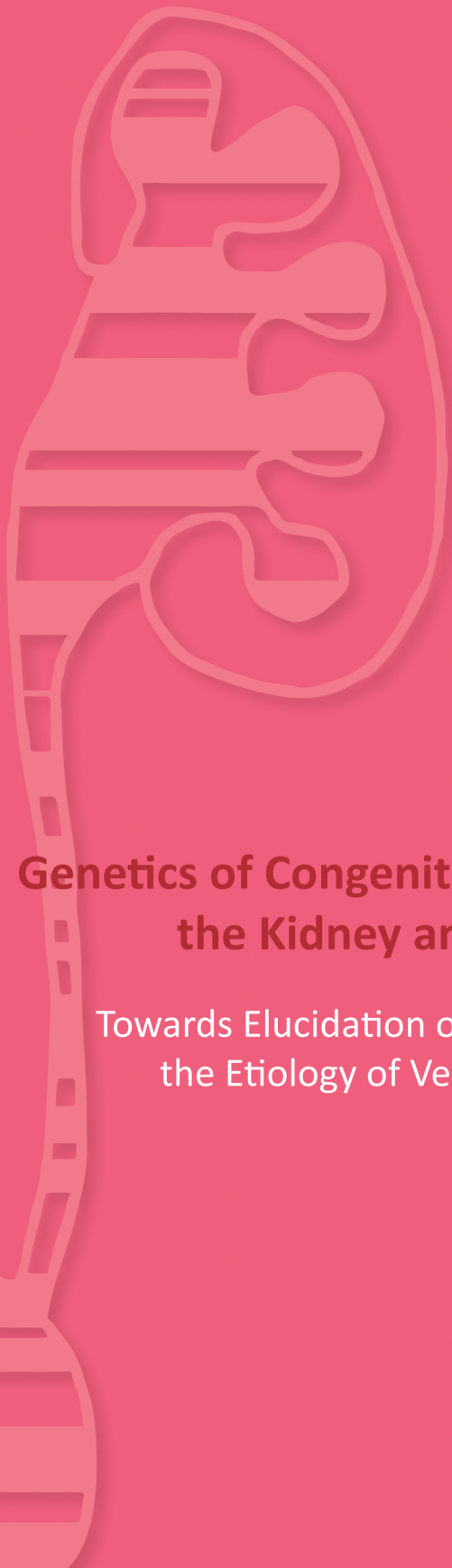

Genetics of Congenital Anomalies of the Kidney and Urinary Tract

Towards Elucidation of Genetic Factors in the Etiology of Vesico-Ureteral Reflux

Albertien M. van Eerde

Cover
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Albertien M. van Eerde

Thesis, Utrecht University, The Netherlands

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Genetics of Congenital Anomalies of the Kidney and Urinary Tract

Towards Elucidation of Genetic Factors in the Etiology of Vesico-Ureteral Reflux

Genetica van Aangeboren Afwijkingen van de Nieren en Urinewegen

Inzicht in Genetische Risicofactoren voor Vesico-Ureterale Reflux

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 8 juni 2011 des ochtends te 10.30 uur

door

Albertien Marjan van Eerde
geboren op 2 maart 1978 te Leiderdorp

Promotoren: Prof. dr. V.V.A.M. Knoers
Prof. dr. C. Wijmenga

Co-promotoren: Dr. J.C. Giltay
Dr. T.P.V.M. de Jong

CONTENTS

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thank you.

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CHAPTER 1

Introduction

PREVALENCE AND CLINICAL OVERVIEW OF VESICO-URETERAL REFLUX

Vesico-ureteral reflux [VUR (MIM 193000)] is the retrograde passage of urine from the bladder into the upper urinary tract. It is one of the most commonly detected congenital anomalies and probably has a conservatively estimated prevalence of 1%.[1, 2] The first detection of VUR is usually the result of either the work-up after a febrile urinary tract infection (UTI), or the work-up after the prenatal ultrasound detection of renal pelvis dilatation.

VUR has a primary and a secondary form. The studies in this thesis focus on primary VUR. Primary VUR is due to an incompetent valve mechanism at the uretero-vesical junction (Figure 1.1), while secondary VUR is due to a functional or anatomical urethral obstruction (Figure 1.2). The uretero-vesical junction normally is like a one-way valve. It allows urine to flow into the bladder and it closes during voiding, thereby preventing retrograde flow (Figure 1.1). Effective valve function is a result of the combination of several factors. These include the length of the submucosal or intramural ureter, the width of the ureteric opening, the muscles of the trigone and ureter, and coordinated ureteral peristalsis (Figure 1.1).[2] Concomitant symptoms of lower urinary tract (LUT) malfunction[3] are often present in VUR patients and can even cause mild VUR by themselves.

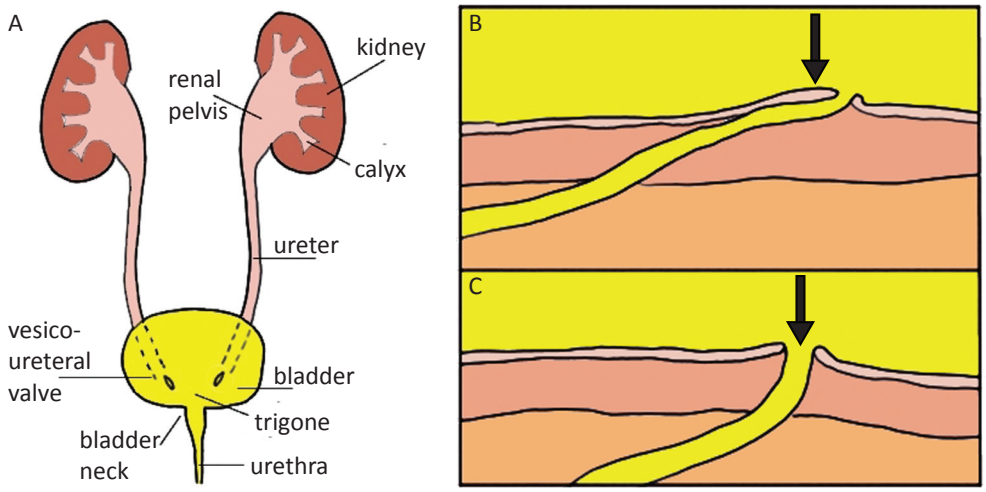

Figure 1.1 A) The urinary tract (normal situation), the ureteric orifice is the location of the vesico-ureteral valve. B/C) Factors involved in proper vesico-ureteral valve function include the length of the submucosal or intramural ureter and the width of the ureteric opening. B) Normal situation: when pressure (bladder lumen in yellow) builds up, the valve will close. C) Primary vesico-ureteral reflux: despite pressure build up, the valve will not close, and retrograde flow will occur. Illustration by Dr. Pieter Dik, pediatric urologist, UMC Utrecht.

VUR severity is graded (I to V) using the International Reflux Study (Figure 1.3).[4] The reference test for VUR is a voiding cystourethrogram (VCUG).[2] VUR is a developmental disorder, which may occur in isolation or as part of a multi-organ malformation syndrome. The Winter-Baraitser Dysmorphology Database currently lists 70 syndromes with 'urinary reflux'.[5] Examples of these syndromes are renal-coloboma syndrome (MIM 120330), branchiootorenal syndrome (MIM 113650) and Townes-Brocks syndrome (MIM 107480).

Intervention options for VUR include: watchful waiting for a UTI, continuous antibiotic prophylaxis (CAP), open surgical ureteral reimplantation or endoscopic injection of bulking agents. A choice is made based amongst others on VUR grade, age, presence of renal damage, evidence for breakthrough UTI under CAP, the presence of lower urinary tract symptoms (LUTS) or other congenital anomalies of the urinary tract and the personal preference of the parents and the treating (pediatric) urologist.[6]

Although most children grow out of the disorder without serious morbidity, a subset does develop long-term complications. In this group VUR is associated with renal damage, either as a result of ascending urinary tract infections (reflux nephropathy) or of renal hypo- or dysplasia, which is often associated with VUR. As such, in these two groups VUR accounts for 7.4% and 8.8%, respectively, of end-stage renal disease in Dutch children.[7]

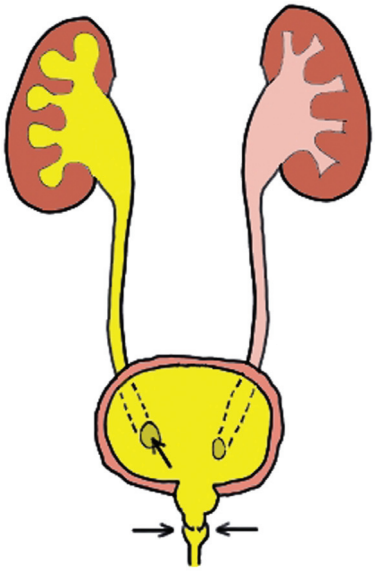

Figure 1.2 Secondary vesico-ureteral reflux. When urethral obstruction is present, in this example due to posterior urethral valves, high pressure will first destroy one uretero-vesical junction resulting in dilatation of this low pressure system. This unilateral VUR works as a pressure release mechanism (pop-off valve) and thus prevents reflux occurring on the other side. Illustration by Dr. Pieter Dik, pediatric urologist, UMC Utrecht.

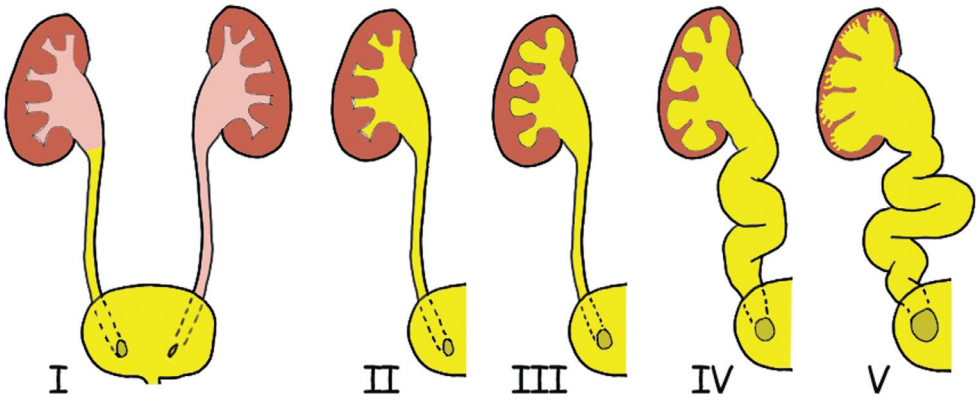

Figure 1.3 Grading of vesicoureteral reflux according to the International Reflux Study. Drawings illustrate the five grades (I-V) of vesicoureteral reflux. Grade I represents reflux into the ureter. Grade II represents reflux into a nondilated ureter and nondilated pelvicaliceal system. Grade III represents reflux into a mildly dilated ureter and pelvicaliceal system. The forniceal angles and papillary impressions remain distinct. Grade IV represents reflux into a tortuous ureter and dilated pelvicaliceal system. The forniceal angles become blunted while the papillary impressions remain distinct. Grade V represents reflux into a markedly dilated and tortuous ureter and marked dilation of the pelvicaliceal system with filling of the collecting ducts. Both the forniceal angles and the papillary impressions are obliterated. Illustration by Dr. Pieter Dik, pediatric urologist, UMC Utrecht.

EMBRYOLOGY, URETER BUDDING, VUR AND CAKUT

Human urinary tract development begins at the end of the fourth week of gestation. A signal from a group of cells called the metanephric mesenchyme induces outgrowth of the ureter bud from the mesonephric or Wolffian duct (Figure 1.4).[8, 9]

Reciprocal, inductive signals between cells in the ureteric bud and cells in the surrounding mesenchyme then propagate urinary tract development. The metanephric mesenchyme induces the ureteric bud to grow, bifurcate and branch further to form the collecting ducts. At the same time, the ureteric bud induces the mesenchyme to differentiate into nephrons, from glomeruli to distal tubules. The ureteric bud also gives rise to the renal pelvis, the ureter, and the bladder trigone.[10-12]

Since the development of the ureter bud and renal mesenchyme depend on mutually inductive interactions, it is not surprising that kidney malformations are often accompanied by lower urinary tract abnormalities. Ectopic ureteral budding can lead to a diverse spectrum of phenotypes known as “congenital anomalies of the kidney and urinary tract” or CAKUT (Figure 1.4). CAKUT include VUR, renal agenesis, hypo-/dysplastic kidneys, duplex collecting systems and other anomalies (Figure 1.5). Variable combinations of these phenotypes are seen in sibships (both in mice and humans) suggesting that the same genetic variation is causally involved in the whole CAKUT spectrum.[9, 13-15] For example, defects of the *Ret* and *Gdnf* genes have been shown to cause ectopic ureteral budding.[16, 17]

GENETICS OF VUR

Several studies support the notion that there is a strong genetic predisposition to primary VUR. These include demonstration of a substantially higher risk of VUR in first-degree relatives of patients[18] and the higher concordance of VUR in monozygotic (80%) compared with dizygotic (35%) twins.[19] The sibling recurrence risk is estimated at 27%, that of the offspring of an index case at 36%.[18] These recurrence risks should be weighed against the population prevalence.

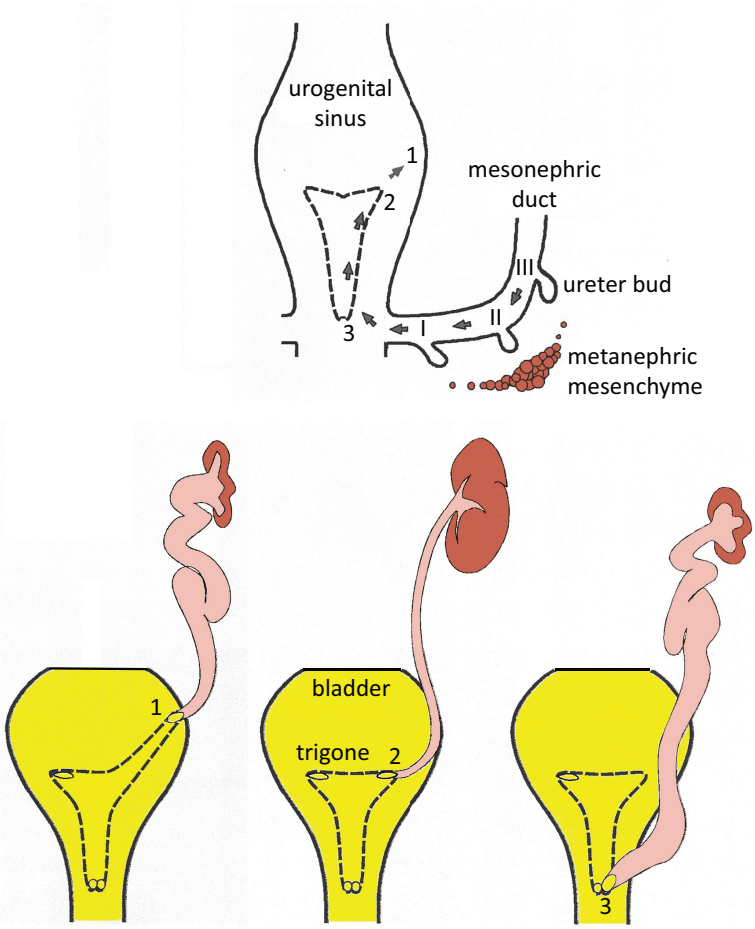

Figure 1.4 Kidney and ureteral development and the 'bud theory'. The orifices of the ureter in the bladder are located at the corner of the trigone of the bladder. This site results from the migration and incorporation of the terminal segment of the mesonephric duct into the urogenital sinus, ultimately forming the trigone of the developing bladder. The migration places the normal ureter bud orifice 'II' on the corner (site 2), and the ectopic bud orifice 'I' on the lateral and cranial end of the extended trigone (site 1). The vesico-ureteral junction from the 'I' ureteral bud results in VUR. When ureter budding occurs ectopically at 'III', the final site of the ureteral orifice will be at site 3, thereby often resulting in urinary outflow obstruction. The metanephric mesenchyme is well differentiated when interacting with a bud at the normal site 'II' but sparse and poorly differentiated around bud 'I' (and 'III'). Adapted by permission from Macmillan Publishers Ltd: Kidney International,[9] © 2002

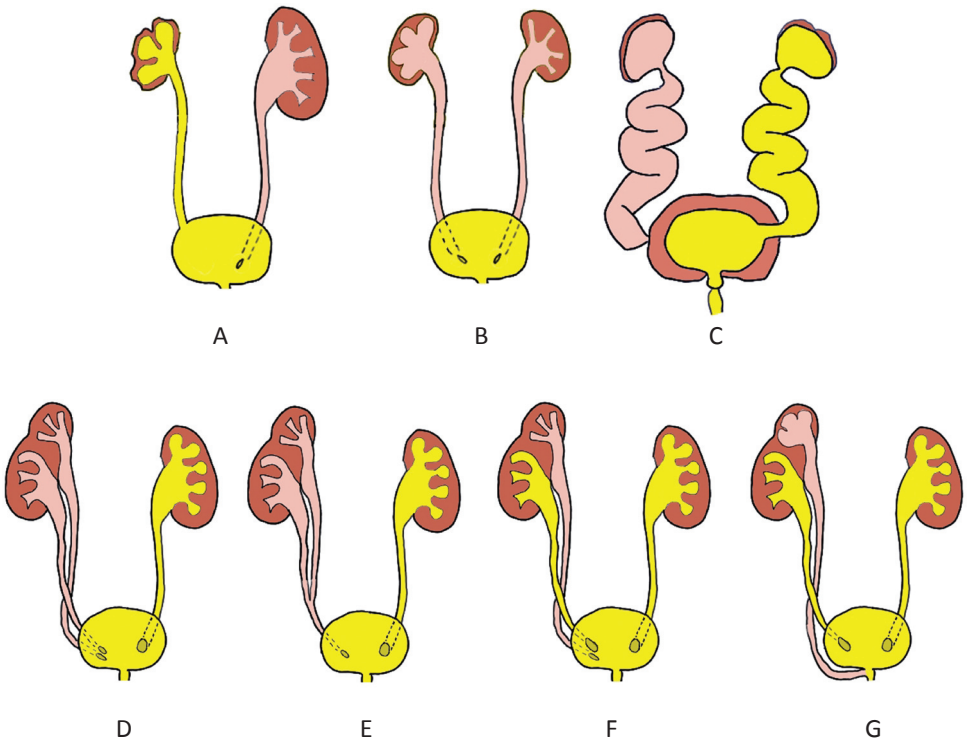

Figure 1.5 Examples of congenital anomalies of the kidney and urinary tract (CAKUT). A) unilateral reflux nephropathy; B) dysplastic left kidney and hypoplastic right kidney; C) Severe obstructive uropathy with malfunctional kidneys, tortuous megaureters and unilateral VUR; D) complete duplex collecting system with contralateral VUR; E) incomplete duplex collecting system with contralateral VUR; F) complete duplex collecting system with contralateral VUR and ipsilateral VUR in lower pole; G) complete duplex collecting system with urethral insertion of upper pole ureter and dysplasia of upper kidney pole. Illustration by Dr. Pieter Dik, pediatric urologist, UMC Utrecht.

This is 1% when conservatively estimated.[1] A prevalence of 10 to 20% (in asymptomatic children) might be more valid, but will never be properly documented in a large enough group of asymptomatic children for ethical reasons, since the reference test for VUR is a VCUG, which is an invasive diagnostic procedure.[2] Different patterns of inheritance (Box 1.1) have been suggested for isolated VUR.

In a subset of families, the segregation pattern suggests autosomal dominant inheritance with incomplete penetrance and variable expressivity of the phenotype.[20-23] Other inheritance patterns, including polygenic, have also been observed.[24-26]

Whether one views VUR as an autosomal dominant disorder with reduced penetrance, or as a complex genetic disorder, is mostly determined by the hypothesis that best suits the chosen study design.[22, 27] Since there indeed seems to be a spectrum from near-Mendelian to truly polygenic patterns in VUR, probably neither view is wrong.

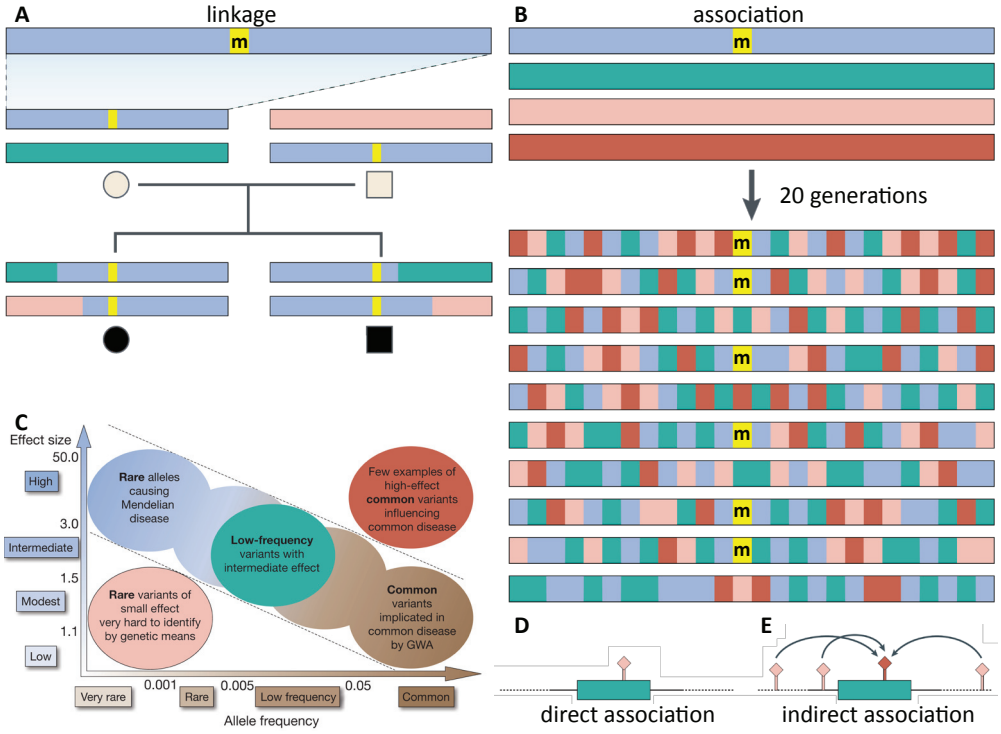

Figure 1.6 Principles of genetic linkage analyses and association studies. A/B) Genetic association and linkage analysis rely on similar principles. Both study the co-inheritance of adjacent DNA variants, with linkage (A) identifying regions that are inherited intact over several generations, and association (B) relying on the retention of adjacent DNA variants over many generations. Thus, association studies can be regarded as linkage studies of very large unobserved, hypothetical pedigrees. A mutation ('m') occurs on a background of other pre-existing DNA variants. Because linkage focuses on recent ancestry, in whom there have been relatively few opportunities for recombination to occur, identified candidate loci will often be large, and can encompass hundreds or even thousands of genes (A). By contrast, association studies draw from historic recombination, so disease-associated regions are (theoretically) small, encompassing only one gene or gene fragment (B). Through many generations, recombination will cause the disease mutation to be separated from its original background. Particular DNA variants can remain together on the ancestral background for many generations. This type of non-random association of alleles is known as linkage disequilibrium (LD). LD provides the genetic basis for most association strategies. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics,[53] © 2001. C) Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio). Most interest lies in identifying associations with characteristics shown within diagonal dotted lines. Adapted by permission from Macmillan Publishers Ltd: Nature,[54] © 2009. D/E: Testing SNPs for association by direct and indirect methods. (D) A case in which a candidate SNP (pink) is directly tested for association with a disease phenotype. For example, this is the strategy used when SNPs are chosen for analysis on the basis of prior knowledge about their possible function. (E) The SNPs to be genotyped (pink) are chosen on the basis of LD patterns to provide information about as many other SNPs as possible. In this case, the SNP shown in red is tested for association indirectly, as it is in LD with the other three SNPs. A combination of both strategies is also possible. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics,[55] ©2005.

Box 1.1 Glossary of genetic terms and genetic study designs used or discussed in this thesis

DNA: Deoxyribonucleid acid. Almost every cell in the human body contains a complete copy of the genetic make-up, 'written in' DNA.

Chromosome: The DNA is packaged in 23 different pairs of chromosomes: 22 autosomes, each present as two copies, and two sex chromosomes.

Gene: Part of the DNA that codes for a protein or another functional gene product.

Genome: the full DNA sequence of an organism.

Exome: The full DNA sequence of all coding parts ('exons') of genes in the genome.

Genetic markers: Landmarks in the DNA. The characteristic of any type of genetic marker is that it can vary between individuals. Two types of genetic markers were used in the studies in this thesis: 'microsatellite markers' in chapter 5 and 'single nucleotide polymorphisms' (SNPs) in chapter 6.

Genotype: The genetic code at a specific spot, for a specific individual.

Phenotype: Any disease, symptom, syndrome, or individual characteristic (like height or eye color) that is determined by the genetic make-up of an individual.

Mendelian phenotypes, inheritance patterns, diseases: The simplest phenotypes are the ones that are caused by a defect in one specific spot, often a gene, in the DNA. These characters are called Mendelian, because when they run in families they show specific inheritance patterns, which were first recognized by Gregor Mendel in 1866.[50] The types of patterns depend on whether the defect is located on an autosome or a sex chromosome and whether one or both chromosomes of a pair need to carry a defect in order to cause a phenotype. Depending on the specific Mendelian inheritance pattern of a disease, children in an affected family can be at 25 or 50% risk of the disease. 'Autosomal dominant' and 'autosomal recessive' inheritance patterns are examples of Mendelian inheritance patterns. Mendelian diseases are quite rare in a population.

Penetrance: When in a family with a Mendelian disease, some persons who carry the disease genotype, do not develop the disease, there is reduced penetrance of the disease.

Genetic linkage analysis: To identify the gene that is responsible for a Mendelian disease, this analysis may be used.[51] With this approach one investigates with the use of genetic markers whether a specific region (a 'locus') in the genome co-segregates with a disease in a pedigree. If such a locus can be identified, it usually encompasses many genes and additional methods are necessary to identify the disease gene (Figure 1.6).

Multifactorial or complex diseases: Many common diseases in populations, like type 2 diabetes, are coined complex; they are caused by a combination of many genetic factors of modest effect size in concert with environmental factors.[51] Carrying one of these factors might mean an increase in risk to developing the common disease that is orders of magnitude smaller than the risks involved in Mendelian diseases (Figure 1.6). Common diseases cluster in families, but do not follow a Mendelian inheritance pattern. One of the main hypotheses of the past decade has been, that genetic risk factors for common diseases are common in the population.[52]

Genetic association studies: Studies that aim to test whether a specific genotype of a common genetic marker is significantly more often present in unrelated patients than in healthy controls.[51] This genetic marker is then pointing to a nearby causal genetic susceptibility variant (Figure 1.6).

Next Generation Sequencing: Until very recently it was not possible to 'read' the entire genome or exome, Next Generation Sequencing enables the scientific community to do exactly that. This means it is possible to identify causal variants directly (i.e. without the use of genetic markers) both in Mendelian and in complex diseases.

Given the assumed variability in the number and effect size of involved genetic variants, phenotypic variability is not surprising.[2] On top of that, genetic factors may also be influenced by environmental exposures, like teratogens or maternal diabetes.[10, 28, 29]
Evidence to support the heritability of VUR is convincing, yet it can be assumed that the mode of transmission, the number of required specific genetic variants required to express a phenotype, or the expressed phenotype itself will be different across different families.[2]

GENETIC STUDIES AND CANDIDATE GENES

Different lines of genetic investigation indicate that there is a heterogeneous genetic basis for VUR, implying that defects in many genes (Box 1.1) might cause or predispose to VUR. Examples of these approaches include:

1. The investigation of the urinary tracts of mice in which the function of a specific candidate gene or genes is artificially hampered. These candidate genes are derived from embryological or other pathways known from literature to possibly be involved in the etiology of VUR.[30] For instance, both *Agtr2*$^{-/-}$ and *Hoxb7/Ret*$^{+/-}$ mice have VUR and hypo- or dysplastic kidneys. These genes are part of the ureter budding pathway. [31-34] Uroplakin 3A (*UPK3A*) was put forward as a candidate gene for VUR/CAKUT based on a mouse model (*Upk3A*$^{-/-}$) with a VUR/CAKUT phenotype.[35] However, before the onset of the studies in this thesis no pathogenic mutations in *UPK3A* in VUR/CAKUT patients were demonstrated.[36, 37] Uroplakins are an important constituent of urothelial plaques, but a link to pathways known to be involved in renal organogenesis has not been clearly established.[38]
2. Studies following the discovery of a structural genomic variant in a patient with VUR/CAKUT. Either a part of a chromosome (Box 1.1) can be deleted (or duplicated), or displaced, or both.[39]
3. The elucidation of genetic causes in known monogenic multiorgan malformation syndromes with VUR. Examples of these syndromes are the renal-coloboma syndrome, caused by mutations in *PAX2*, a transcription factor gene, and the branchiootorenal syndrome caused by mutations in *EYA1*, *SIX1* and possibly *SIX5*, three genes encoding regulators of *GDNF*.[8, 40]
4. Hypothesis free approaches, such as genome wide linkage analysis and association studies (Box 1.1, Figure 1.6), aimed at identifying (new) candidate loci or genes for VUR. Linkage studies, mostly in families with presumed autosomal dominant inheritance, have revealed different loci linked to VUR, although most loci have not been convincingly replicated (see references [21, 22, 27, 41-43] and Appendix 1). Linkage studies have not revealed major susceptibility genes for human VUR.[21, 22, 27, 30, 41-43] Genome wide association studies (GWAS) have not yet been properly conducted in VUR patient cohorts. The association studies that have been performed, usually investigated association of few markers in few candidate genes with

VUR.[44-49] One exception is the study of Cordell et al.[27] in which 6 candidate genes for VUR were well covered with SNPs and furthermore a tentative association analysis was performed on the genome wide linkage SNP set. Although the SNPs in the genome wide set were too widely spaced to reach the quality standards for a GWAS, this is still the most elaborate hypothesis free association study performed so far (see Appendix 1 for a summary of results).[27]

AIM AND OUTLINE OF THIS THESIS

At the start of our research into genetic aspects of VUR, one linkage study in seven families had been published, the results implying heterogeneity in this disorder.[21] In this thesis, using different approaches, we aimed to identify genetic risk factors for VUR and/or CAKUT. We selected patients with high-risk genetic causal variants by collecting families with multiple affected cases through hospital records. In addition, since one of the signs of VUR can be renal pelvis dilatation, we evaluated whether prenatally detected cases of hydronephrosis were associated with a familial predisposition to VUR (chapter 2). We also collected isolated cases, in order to allow the identification of low-risk genetic variants that might influence the predisposition to VUR. In chapter 3 we clinically tested whether joint hypermobility may be associated with VUR susceptibility. In chapters 4, 5 and 6 we investigated candidate genes mainly involved in the ureter budding process for their role in the etiology of VUR with varied genetic approaches. In chapter 7 we broadened our scope towards renal adysplasia, another part of the CAKUT spectrum, and performed a candidate gene study in *UPK3A*. In chapters 8 and 9 our work is discussed and summarized and some recurring themes and ideas for future studies are highlighted.

CHAPTER 2

Vesico-ureteral reflux in children with prenatally detected hydronephrosis: a systematic review

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**Both authors contributed equally to this study*

ABSTRACT

Objective

To investigate the value of prenatally detected hydronephrosis (PNH) as a prognostic factor for vesicoureteral reflux (VUR).

Methods

The MEDLINE database was searched for articles on PNH and VUR published between 1980 and 2004. A total of 18 studies were identified and reviewed for various aspects. Results were separated for primary and/or secondary VUR whenever possible, because of the different underlying pathogenic mechanisms.

Results

There was considerable variation between the different studies with respect to methodology and study design. One of the main discrepancies was the way in which postnatal abnormalities were ascertained: by postnatal ultrasound, voiding cystourethrogram (VCUG) alone, or combined or sequential ultrasound and VCUG. Taking these limitations into account, the published data showed there to be a mean prevalence of 15% for postnatal primary VUR after PNH. Of all patients with PNH, 53% had no postnatal anomalies, whereas 29% had other anomalies, such as duplex collecting systems.

Conclusions

Of all infants with PNH, 15% had primary VUR proven postnatally and 53% had no other anomalies detected. We suggest a standardized protocol for future studies, to enable better comparison of follow-up protocols.

INTRODUCTION

Vesico-ureteral reflux (VUR) has an estimated prevalence of approximately 1% in Caucasian populations[1, 57] and is one of the most commonly detected congenital anomalies. It can be primary (incompetent valve mechanism at the ureterovesical junction) or secondary (due to an obstruction in the urinary tract and high detrusor pressures). Primary VUR is often a genetic disorder.[19] Together with urinary tract infections, VUR can cause significant kidney damage and hypertension if not recognized early and treated appropriately. Severe primary VUR can concur with congenital renal insufficiency based on hypoplasia/dysplasia of one or both kidneys.[58, 59] It is important to detect VUR as early as possible in order to minimize renal damage and associated morbidity. Unfortunately, many patients are only identified after they have had urinary tract infections and renal damage has already occurred.[60]

Although prenatal hydronephrosis (PNH) may be an isolated finding, with no postnatal consequences,[61-63] congenital urinary tract anomalies, including VUR, may be present in some patients. Thus, at least the VUR patients presenting with prenatal anomalies (PNH) can be followed and treated appropriately. No study has reported exact information on the proportion of patients with VUR who, in retrospect, also had PNH. We therefore evaluated published, prospective studies on patients with PNH and postnatal VUR. This information could be helpful in counseling pregnant women on the postnatal impact of PNH.

METHODS

We searched MEDLINE for articles published between 1980 and 2004, using the following search strategies:
(1) 'renal AND pelvis AND dilatation', (2) '''Hydronephrosis''[MeSH] AND ''Vesico-Ureteral Reflux'' [MeSH]''' and (3) '(pyelectasis OR pyelectasia) AND ''Vesico-Ureteral Reflux''[MeSH]''', combined with 'prenatal(ly)' and 'antenatal(ly)'. These combinations yielded 311 articles, of which we screened the title and abstract. Papers on PNH were selected only if a voiding cystourethrogram (VCUG) had been performed to diagnose VUR and there was a clear description of how many patients with PNH had been included and how many patients with VUR were detected. Only 17 studies met these criteria.[61-77] For two papers we used only the abstracts because the main text was in Spanish[77] or Italian,[64] but these contained the appropriate information. From another study, by Damen-Elias *et al*.,[78]a subset of patients with both PNH and a postnatal VCUG was included after we had obtained extra information from the author (H. Damen-Elias, Utrecht, The Netherlands, pers. comm.). Four papers reported on primary VUR,[64, 70-72] and six reported on both primary and secondary VUR.[62, 65, 67, 68, 74, 77] Eight papers did not make a distinction,[61, 63, 66, 69, 73, 75, 76, 78] so we interpreted these data as if they included both primary and secondary VUR.
The following aspects were reviewed: number of pregnant women screened, inclusion criteria, effect of cut-off anteroposterior diameter (APD) of fetal renal pelvis, method of postnatal follow-up (VCUG either in all cases of PNH or only after an abnormal postnatal ultrasound result), number of patients with VUR (primary and secondary), number of patients with other congenital anomalies of the urogenital tract, uni-or bilateral occurrence of VUR, gender, long-term follow-up data and family history of VUR. Our results are specified for VUR (primary and secondary; VUR_{p+s}), primary VUR (VUR_p) and secondary VUR (VUR_s) whenever possible, because of the different pathogenic mechanisms underlying VUR_p and VUR_s. We use 'hydronephrosis' to indicate renal pelvis dilatation with an APD > 4 mm, with or without caliectasis. Chi-square tests were used for statistical analyses and $P < 0.05$ was considered statistically significant.

RESULTS

Of the 18 studies on PNH, 16 studies investigated children with PNH detected by routine ultrasound screening of pregnant women. Damen-Elias *et al*.[78] and Stocks *et al*.[73] included only cases that were referred to a tertiary center because of a medical indication, but did not specify the precise indications. The 18 studies reported a total of 2232 infants with PNH who had had a postnatal VCUG. The data on these infants were analyzed in detail.

Eight studies reported the size of the population screened prenatally[61-63, 65, 66, 69, 74, 75] (Table 2.1 and Figure 2.1); in total, this comprised 69 079 women, with 1178 fetuses found to have PNH. Of these, 104 fetuses were diagnosed with VUR_{p+s}. The overall prevalence of VUR_{p+s} can be estimated from Figure 2.1 as follows: assuming that a routine postnatal VCUG result is the best available indicator, the ratio of the number of patients with VUR (n = 34) found in the group of patients screened by VCUG (n = 155) is 0.22 (34/155). This would mean that 226 (0.22 × (1178 − 148)) patients would have been found in the PNH group (corrected for loss to follow-up) and the prevalence in the total prenatal screening group (also corrected for loss to follow-up) would have been 0.33% (226/(69079 − 148), i.e. if every prenatally detected case had been followed up by ultrasound and VCUG.

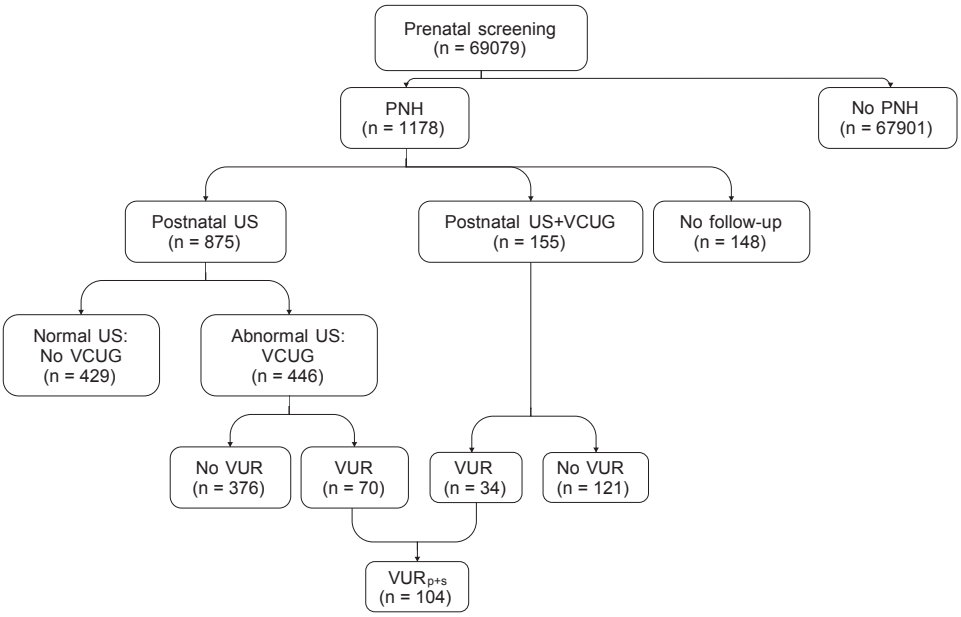

Figure 2.1 Flow chart from prenatal screening to VUR_{p+s}, showing the numbers of individuals and steps involved as reported in eight studies.[61-63, 65, 66, 69, 74,75] PNH, prenatal hydronephrosis; US, ultrasound examination; VCUG, voiding cystourethrogram; VUR, vesico-ureteral reflux ($_p$, primary; $_s$, secondary).

Table 2.1 Prenatal screening: prevalence of prenatal hydronephrosis (PNH) and of postnatal vesico-ureteral reflux (VUR) as reported in the literature (eight studies, 1980-2004)

Study	Fetuses screened (n)	PNH cases (n (%))	VCUG performed (n)	VUR_{p+s} (n(%))
Anderson et al. (1997)[65]	9,800	426 (4.3)	264	38 (0.4)
Brogan and Chiyende (2000)[66]	3,397	29 (0.9)	18	6 (0.2)
Dudley et al. (1997)[62]	18,766	100 (0.5)	48	12 (0.1)
Gloor et al. (2002)[69]	5,432	40 (0.7)	33	5 (0.1)
Gunn et al. (1988)[63]	3,228	62 (1.9)	15	2 (0.1)
Jaswon et al. (1999)[61]	7,000	139 (2.0)	104	23 (0.3)
Persutte et al. (1997)[75]	5,529	306 (5.5)	84	12 (0.2)
Walsh and Dubbins (1996)[74]	15,927	76 (0.5)	35	6 (0.04)
Total	**69,079**	**1,178**	**601**	**104**

VCUG, voiding cystourethrogram; VUR_{p+s}, primary and secondary vesico-ureteral reflux

A postnatal ultrasound examination (varying from 48 h to 6 weeks after birth) was carried out in the children with PNH in all the studies. In six of the 18 studies (Group A), a VCUG was performed only when hydronephrosis was still present on the postnatal ultrasound exam.[62-65, 74, 76] In the other 12 studies (Group B), a VCUG was performed in all the children with PNH, irrespective of the outcome of the postnatal ultrasound exam.[61, 66-73, 75, 77, 78] In most studies, the VCUG was performed within 3 months after birth. In both follow-up groups, we investigated the mean prevalence of VUR_p and VUR_s if they were reported separately, or of VUR_{p+s}. Prevalences and statistical differences were calculated (Table 2.2).

The mean prevalence of VUR_{p+s} in infants with PNH was 21.7% (14 studies; 275/1265 cases; range, 13-37%), that of VUR_p was 14.9% (10 studies; 262/1757 cases; range, 11-20%) and that of VUR_s was 8% (five studies; 63/790 cases; range, 2–18%) (Table 2.3). The mean prevalence of anomalies other than VUR (e.g. duplex systems, stenosis of the ureteropelvic junction (UPJ), posterior urethral valves, mega-ureter) was 28.6% (390/1366 cases; range, 5-54%). On average, 53% of the infants with PNH had no anomaly detected after birth (724/1366 cases; range, 24–81%).

Table 2.2 Reported prevalence of vesico-ureteral reflux (VUR) according to follow-up protocol

	VCUG after abnormal ultrasound		VCUG in all cases		
	Prevalence (% (n))	References	Prevalence (% (n))	References	*P*
VUR_{p+s}	16 (58/362)	62, 63, 65, 74	24.8 (207/835)	61, 66-69, 73, 75, 78	0.0008
VUR_p	12.7 (44/347)	62, 65, 74	17.4 (77/443)	67, 68	0.069
VUR_s	3.5 (12/347)	62, 65, 74	11.5 (51/443)	67, 68	0.00003

VCUG, voiding cystourethrogram; VUR, vesico-ureteral reflux ($_p$, primary; $_s$, secondary).

Different APD cut-offs were used to define the presence of PNH in the 18 studies. Some used a cut-off of APD ≥ 4 or 5 mm (week of gestation not specified),[61, 62, 65, 66, 69, 72-75] some used APD ≥ 4 mm in the second trimester and/or ≥ 7 mm in the third trimester,[70, 71, 76] one study used APD ≥ 4 mm before 32 weeks' gestation and > 10 mm thereafter,[78] and some did not report the APD cut-off used.[63, 64, 67, 68, 77] Five studies excluded fetuses with APD ≥ 10 or ≥ 15 mm,

Table 2.3 Postnatal outcome for prenatal hydronephrosis (PNH) as reported in the literature (18 studies, 1980–2004)

Reference	PNH (n)	VURp+s (n (%))	VURp (n (%))	VURs (n (%))	Other anomalies (n (%))[a]	No other anomalies (n (%))	Follow-up group[b]
Arena et al (2001)[64]	382	NRep	68 (17.8)	NSpec	NSpec	NSpec	A
Anderson et al. (1997)[65]	264	38 (14.4)	33 (12.5)	5 (1.9)	27 (10.2)	199 (75.4)	A
Dudley et al. (1997)[62]	48	12 (25)	6 (12.5)	6 (12.5)	NSpec	NSpec	A
Gunn et al. (1988)[63]	15	2 (13.3)	NSpec	NSpec	5 (33.3)	8 (53.3)	A
Walsh and Dubbins (1996)[74]	35	6 (17.1)	5 (14.3)	1 (2.9)	7 (20)	22 (62.9)	A
Adra et al. (1995)[76]	68	10 (14.7)	NSpec	NSpec	20 (29.4)	38 (55.9)	A
Angulo et al. (1991)[77]	163	61 (37.4)	32 (19.6)	29 (17.8)	58 (35.6)	44 (27)	B
Jaswon et al. (1999)[61]	104	23 (22.1)	NSpec	NSpec	NSpec	NSpec	B
Brogan and Chitayende (2000)[66]	18	6 (33.3)	NSpec	NSpec	7 (38.9)	5 (27.8)	B
Brophy et al. (2002)[67]	191	40 (20.9)	27 (14.1)	13 (6.8)	NSpec	NSpec	B
Devausssuzenet et al. (1997)[68]	89	27 (30.3)	18 (20.2)	9 (10.1)	38 (42.7)	24 (27)	B
Gloor et al. (2002)[69]	33	5 (15.2)	NSpec	NSpec	NSpec	NSpec	B
Ismaili et al. (2003)71	213	NRep	23 (10.8)	NSpec	109 (51.2)	81 (38)	B
Ismaili et al. (2002)[70]	264	NRep	34 (12.9)	NSpec	34 (12.9)	196 (74.2)	B
Phan et al. (2003)[72]	108	NRep	16 (14.8)	NSpec	NSpec	NSpec	B
Stocks et al. (1996)[73]	26	6 (23.1)	NSpec	NSpec	12 (46.2)	8 (30.8)	B
Persutte et al. (1997)[75]	84	12 (14.3)	NSpec	NSpec	4 (4.8)	68 (81)	B
Damen et al. (2005)[78]	127	27 (21.3)	NSpec	NSpec	69 (54.3)	31 (24.4)	B
Total	**2,232**	**275/1,265 (21.7)**	**262/1,757 (14.9)**	**63/790 (8)**	**390/1,366* (28.6)**	**724/1,366* (53)**	

[a]'Other anomalies' includes anomalies of the urinary tract other than VUR, such as ureteropelvic junction stenosis and posterior urethral valves. [b]Follow-up Group A had a voiding cystourethrogram (VCUG) only when hydronephrosis was still present on the postnatal ultrasound exam; follow-up Group B had a VCUG in all cases. *1366 as a total in these two columns is a result of summing all patients in studies that actually mentioned other anomalies. For this calculation either VUR_{p+s} (references 63, 65, 66, 68, 73-78), when available, or VUR_p (70, 71) was added to the numbers of patients with and without other anomalies. NRep, not reported; NSpec, not further specified; VUR, vesico-ureteral reflux ($_p$, primary; $_s$, secondary).

because they were only investigating mild PNH.[68, 69, 71, 75, 76] We looked at the prevalence of VUR_{p+s} and VUR_p in infants with APD < 10 mm and in infants with APD ≥ 10 mm in studies in which the data were stratified by APD.[65, 69, 72, 74, 78] These studies included 567 infants with PNH. VUR_{p+s} was diagnosed in 14% (37/262) of infants with APD < 10 mm and in 20% (39/197) of infants with APD ≥ 10 mm. VUR_p was diagnosed in 14% (38/275) of infants with APD < 10 mm and in 12% (16/132) of infants with APD ≥ 10mm.

Ten of the 18 studies reported data on bilateral occurrence of VUR.[62, 64, 68, 69, 71-74, 77, 78] VUR_{p+s} was bilateral in 47% (67/144) of affected infants, and VUR_p was bilateral in 56% (60/107).

Gender was reported in six studies, which included 581 males and 231 females with PNH (Table 2.4).[65, 67-69, 72, 78] The mean prevalence of VUR_{p+s} in males was 19% (96/512) and in females it was 21% (41/192). The mean prevalence of VUR_p in males was 8% (20/256) and in females it was 25% (29/116).

Long-term follow-up data were available for 79 infants.[63, 67, 69, 73, 78] In 37 cases, the VUR resolved spontaneously (47%). In 42 cases it persisted or surgery was necessary. The mean follow-up period (2 years) was only mentioned in two studies.[67, 69] Gender differences were not reported in these studies. No data on familial occurrence of VUR were given in any of the 18 articles.

Table 2.4 Prenatal hydronephrosis (PNH) and primary vesico-ureteral reflux (VUR): stratification by gender as reported in six studies

	PNH (n)		**VUR_{p+s} (n)**		**VUR_p (n)**	
Reference	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Anderson et al. (1997)[65]	187	77	20	18	16	17
Brophy et al. (2002)[67]	144	47	34	6	NSpec	NSpec
Damen-Elias et al. (2005)[78]	95	32	22	5	NSpec	NSpec
Devaussuzenet et al. (1997)[68]	63	26	16	11	NSpec	NSpec
Gloor et al. (2002)[69]	23	10	4	1	NSpec	NSpec
Phan et al. (2003)[72]	69	39	NRep	NRep	4	12
Total	**581**	**231**	**96/512 (19%)**	**41/192 (21%)**	**20/256 (8%)**	**29/116 (25%)**

NRep, not reported; NSpec, not further specified; $_p$, primary; $_s$, secondary.

DISCUSSION

The prevalence of VUR in the general population estimated in the literature is approximately 1%.[1, 57] The real prevalence may be lower, because to our knowledge this figure is not supported by reports of VCUG in asymptomatic children.[79] Still, it appears that this prevalence is three times higher than that (0.3%) found in the prenatal screens reported in the studies we included. Since not all patients with VUR have PNH, this is not unexpected.[80] Furthermore, the size of the fetal renal pelvis can vary with bladder volume.[81] When kidneys are observed by ultrasound for only a short time, infants with PNH due to VUR can be missed if their bladder is empty during the examination. Thus, a negative prenatal screening does not exclude VUR later in life.

A total of 148 patients were lost to follow-up. In all 18 studies, the children with PNH had a postnatal ultrasound examination to determine whether hydronephrosis had persisted after birth. In some studies, it was decided to investigate all PNH cases with a VCUG, while others reserved a VCUG for those with an abnormal postnatal ultrasound exam (i.e. persisting hydronephrosis). These strategies affected the outcome. Anderson *et al*.,[65] who performed a VCUG in all cases, detected eight children with VUR out of 88 with a normal postnatal ultrasound result. They therefore concluded that a VCUG should always be performed, regardless of the outcome of the postnatal ultrasound exam. This conclusion seems to be in agreement with the results of our review, at least with respect to VUR_{p+s}. However, with respect to postnatal screening strategy, other aspects such as the APD in PNH (see below), should be taken into account.

We found a similar proportion of VUR_p in Groups A and B, which was unexpected, suggesting that the increased prevalence of VUR_{p+s} (when both an ultrasound examination and a VCUG are carried out routinely) stems from an increase in VUR_s. This is counterintuitive because sonography is commonly believed to detect VUR_s better than it does VUR_p due to the concurrence of anatomical anomalies and VUR in VUR_s. These findings may well be due to operator-dependent differences in performing and interpreting ultrasound examinations.

The reported prevalences of VUR_{p+s} and VUR_p varied widely. This may be due to the small populations studied and the different APD cut-offs used for inclusion. Not all the studies reported clearly whether they investigated primary or secondary VUR, or both. We may therefore have classified some of the results as VUR_{p+s} when the authors in fact investigated VUR_p. In addition to VUR, the reported prevalence of other urogenital tract anomalies after birth varied widely in the PNH group. One explanation could be that some studies paid extra attention to the urinary tract when performing the prenatal ultrasound examination and therefore found more anomalies.[65, 71, 75] Secondly, the week of gestation in which the prenatal investigation is carried out can influence results. Feldman *et al*.[82] evaluated PNH, concluding that most cases of mild PNH are resolved before delivery and attributing this to the relatively high production of urine at 20 weeks' gestation. Therefore, more patients with PNH (but

fewer urinary tract anomalies) should be found on prenatal sonography at 20 compared with at 35 gestational weeks. Thirdly, Damen-Elias *et al*.[78] and Stocks *et al*.[73] investigated pregnant women with a medical indication for a prenatal ultrasound exam. We think that this group of patients has more fetal anomalies compared with the general population and that they therefore found a higher percentage of urinary tract anomalies. Taking into account the heterogeneity of the studies, the figures we have obtained on the consequences of PNH can still be useful for counseling pregnant women.

It is evident that in using an APD cut-off of ≥ 4mm rather than ≥ 10 mm, more patients with VUR will be detected. Limiting the study group to patients with mild PNH only will underestimate the total prevalence of VUR in a prenatally screened population. With respect to VUR_p, an equal number of patients were found above and below an APD of 10 mm. Thus, VUR_p is not characterized by a large APD. Kapadia *et al*.[83] showed that children with PNH and an APD of < 10 mm have a low risk of urinary tract infections and renal morbidity. They stated that it is not necessary to perform diagnostic procedures other than a postnatal ultrasound exam in these patients. Since the reviewed articles yielded only 79 patients with long-term follow-up, our data do not contribute to this discussion. However, based on data collected in our own patients, we do not perform postnatal follow-up for PNH detected with an APD of < 10 mm.[84]

VUR_p was found to be bilateral more often than was VUR_{p+s}. This is to be expected because VUR_p is a genetic disorder in many cases and it is more prone to occurring bilaterally than is VUR_s. When there is a urethral obstruction (secondary VUR), one ureterovesical junction is more likely to work as a pressure release mechanism (pop-off valve) and thus prevent reflux also occurring on the other side.[85]

Ismaili *et al*.[70] stated that primary VUR affects more boys than girls. However, we conclude that, with respect to VUR_{p+s}, boys and girls were equally affected, whereas for VUR_p, three times more girls than boys were affected. This is in agreement with the fact that anomalies causing secondary reflux occur predominantly in boys, and the higher prevalence of VUR in girls detected later in childhood.[86]

Kamil's study[87] reported that 80% of patients with VUR_p will outgrow the condition and need no surgical intervention. This percentage is higher than our finding (47%), which can be explained by the fact that most of the studies investigated VUR_{p+s}. VUR_s needs intervention more frequently because of additional pathology.

We have discussed discrepancies in follow-up protocols and recording of data in this article. In Figure 2.2 we propose a flow diagram for a standard follow-up protocol and adequate recording of data. This diagram is based mainly on previous work in our hospital.[84] Although we are aware of the fact that many clinicians will find performing a standard VCUG (and ultrasound exam) too invasive, we think that evidence supporting that opinion needs to be produced.

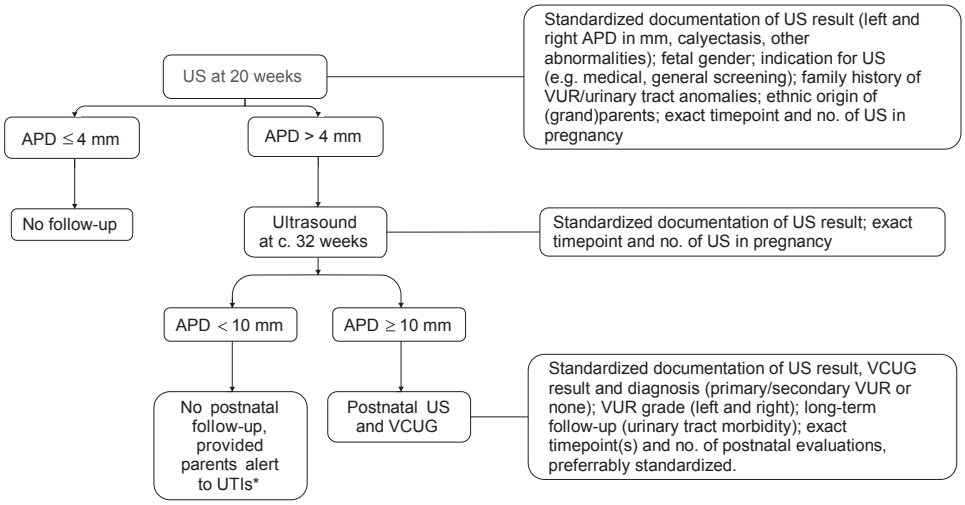

Figure 2.2 Flow diagram for adequate data collection (left) and proposal for a standard follow-up protocol in future studies of prenatal hydronephrosis patients (right). *For research purposes, this group should be followed up long-term also. APD, anteroposterior diameter; US, ultrasound; UTI, urinary tract infection; VCUG, voiding cystourethrogram; VUR, vesico-ureteral reflux.

To summarize, of all infants with PNH, 15% will have VUR_p (22% VUR_{p+s}) and 26% will have other urogenital anomalies like duplex systems, UPJ stenosis, urethral valves and mega-ureter. In 53%, no apparent anomalies of the urogenital tract will be detected. Surprisingly, VUR_p could be detected in a similar number of cases by postnatal ultrasound and by VCUG. We suspect this may be due to operator-dependent differences. In contrast, in VUR_{p+s}, routine postnatal VCUG had a higher detection rate than had VCUG performed only after an abnormal postnatal ultrasound exam. The number of patients found with VUR varied with the APD cut-off used. Below an APD of 10 mm an equal number of VUR_p patients was found as above. However, the low grade VUR found in these patients probably has no clinical significance and would not require follow-up. Because the methods used in these studies varied in several aspects (e.g. inclusion criteria, APD cut-off, postnatal follow-up), they were difficult to compare, and it is likely that this has influenced the results. If investigations were carried out following a standard protocol (Figure 2.2), the data could be compared more easily and more definite conclusions could be drawn on the prevalence of VUR in a population with PNH and on the value of a postnatal ultrasound exam in determining whether an infant has VUR.

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CHAPTER 3

Joint hypermobility is associated with vesico-ureteral reflux

Assessment of joint hypermobility in 50 VUR patients

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SUMMARY

Objective

To assess whether there is an increased prevalence of joint hypermobility in VUR patients. Vesico-ureteral reflux (VUR) is one of the most commonly detected congenital anomalies. Since clinical examinations in VUR patients repeatedly reveal hypermobile joints, we hypothesized that the same differences in composition of the extracellular matrix that lead to (asymptomatic) joint hypermobility might contribute to the development of clinically relevant VUR.

Materials and methods

We studied 50 primary VUR patients and matched controls drawn from a reference population. Joint mobility was assessed with the hypermobility score according to Bulbena.

Results

When we applied the diagnostic cut-offs for hypermobility according to the Bulbena score, we identified significantly more VUR patients with generalized hypermobility compared to controls (24.0% versus 6.7%, $p = 0.007$).

Conclusion

These findings confirm our clinical observation of an increased rate of joint hypermobility in VUR patients. We speculate that an altered composition of the connective tissue may contribute to the severity of the (pre-existing) VUR phenotype.

INTRODUCTION

Vesico-ureteral reflux (VUR), the retrograde flow of urine from the bladder into the ureter, is one of the most commonly detected congenital anomalies. With an estimated prevalence of approximately 1% in Caucasians,[1] VUR can be primarily caused by an incompetent valve mechanism at the uretero-vesical junction, or be secondary to a functional or anatomical urethral obstruction. Of patients with VUR and urinary tract infections (i.e. clinically manifest VUR), 39% additionally have signs of dysfunctional voiding and/or dysfunctional defecation.[88] Renal damage accounts for 16.2 - 23.4% of end-stage renal disease in Dutch children, either as a result of ascending urinary tract infections (reflux nephropathy, 7.4 - 9.6%) or due to renal hypo- or dysplasia (8.8 - 13.8%) often associated with VUR.[7]

VUR is a complex genetic developmental disorder, which may occur isolated or as part of a syndrome (e.g. renal-coloboma syndrome, MIM 120330[89]). The prevalence of primary VUR in siblings and offspring of VUR patients is as high as 30%.[90] Hypothesis-based (genetic)

research into the etiology of VUR is mainly focused on the embryological processes of ureter budding, outgrowth and interaction with the nephrogenic mesenchyme. This has resulted in the detection of *ROBO2* as a risk factor in a subset of VUR patients.[30, 91]
Other genetic or environmental factors than the genes involved in these embryological processes are likely to contribute to the severity of the VUR phenotype.
The present study is based on our clinical observation that VUR patients often seem to have increased joint hypermobility, which often coincides with ultrasound findings of a marked descent of the pelvic floor while straining. A previous study showed that symptoms of voiding and defecation dysfunction are more prevalent in children with generalized joint hypermobility (GJH).[92] Recently, in a group of male patients with slow transit constipation, a higher prevalence of GJH compared to controls was demonstrated.[93]
We hypothesized that the same (as yet unknown), subtle differences in components of the extracellular matrix (ECM) that lead to (asymptomatic) joint laxity also lead to (subtle) differences in connective tissue composition of the bladder wall and the UVJ. This could influence the balance between factors promoting or preventing the development of VUR in the maturing UVJ. Thus, in patients with a genetic predisposition for VUR, an altered connective tissue composition could lead to joint laxity and contribute to the severity of the VUR phenotype. In order to substantiate our clinical observation of hypermobility in VUR patients and, in the light of recent studies addressing the same issue in related disorders,[92, 93] we assessed whether VUR patients have a higher prevalence of joint hypermobility than matched controls.

MATERIALS AND METHODS

PARTICIPANTS

Cases

113 patients aged 8 - 10 years and 12 - 14 years who had been previously treated in our hospital for non-syndromal primary VUR were invited to participate. These age categories were chosen to match the children in the control groups available.[94, 95] 50 patients (response rate: 44%) were eventually included in the study. Table 3.1 shows a comparison of the clinical characteristics of responders and non-responders.
Our institution's medical ethics committee approved the study and informed consent was obtained.

Controls

As a reference, we selected 50 controls matched for gender, age (+/- 6 months) and body mass index (BMI) (+/- 1 kg/m^2) from a reference group of 200 healthy primary school

prepubertal pupils and healthy secondary school adolescents from the city of Zeist, the Netherlands (8 - 10 years: n = 117 and 12 - 14 years: n = 83 controls).[94, 95] The reference group contained no children with known signs of rheumatic, neurologic, skeletal, metabolic, or collagen disease or a reported delay in motor performance. No specific information on urological history was obtained. From the 200 controls, we drew 50 matched to the patients for gender, age (+/- 6 months) and body mass index (BMI) (+/- 1 kg/m^2).

Table 3.1. Summary of clinical characteristics of responders and non-responders in our study

	responders, n=50 (number of patients/ ureters for which data available)	**non-responders, n=63 (number of patients/ ureters for which data available)**	***p*-value***
Gender (% girls)	54.0 (50)	68.3 (63)	0.1
Age (years)	11.1 (50)	11.0 (63)	0.8
Median VUR grade (per ureter)	3.0 (75)	3.0 (97)	0.3
Percentage voiding signs/ symptoms of any kind (%)	53.8 (39)	41.1 (55)	0.7
Average age of first recorded VUR diagnosis in hospital information system, if available (years)	1.9 (50)	2.5 (63)	0.2
Average age of first recorded surgical reflux treatment, if performed (years)	2.8 (50)	3.4 (63)	0.3
Average age of first recorded urethrocystoscopy, if performed (years)	2.7 (50)	3.2 (63)	0.3

*Mann-Whitney test

PHYSICAL EXAMINATION

Cases and controls

Joint mobility measurements and physical examination in cases was performed according to the same standardized protocol that was previously used in the reference population.[94, 95] The VUR patients were all examined by VJMV. VJMV was trained and supervised by RHHE, who also supervised the examinations in the reference group studies.[94, 95] Because of subtle differences in the protocols used to characterize the two reference groups, the Bulbena mobility score[96] was measured in 30 of 50 matched controls.

Intraobserver reproducibility

Before our study, the (intraobserver) reproducibility of the Bulbena score was assessed by twice examining five subjects bilaterally, resulting in 10 double measurements of separate joints per subject. None of the 50 second measurements (absence or presence of hypermobility per joint) deviated from the first. The reproducibility of the Bulbena score was considered to be high.

Protocol

Body height and weight were measured without shoes or heavy clothing, to the nearest 1 centimeter and 100 grams. Body mass index was calculated as weight (kg) divided by the square of body length (m^2). Joint mobility was assessed with Bulbena's hypermobility score (Table 3.2).[96, 97] Generalized hypermobility of joints was present if the Bulbena score (range: 0 - 10) was ≥ 5 in girls and ≥ 4 in boys.[96] Local hypermobility of the joints was considered to be present if the Bulbena score was 1 - 4 in girls and 1 - 3 in boys.[94, 97, 98] The Bulbena score has a high concurrent validity (Spearman's rho correlation > 0.85) with the other internationally accepted score for joint hypermobility the Beighton score, and a high test-retest reliability kappa > 0.9).[96]

Parental questionnaire

A parental questionnaire provided information concerning the child's health status, presence of possible symptoms of connective tissue disease (e.g. ecchymoses, fractures, subluxations, abnormal scarring, heart defects, visual problems, striae), hours per week spent on sports activities, complaints regarding the musculoskeletal system, defecation and voiding pattern, presence of familial hypermobility, and the presence of familial urinary tract anomalies.

Table 3.2. Bulbena Criteria for the Clinical Assessment of Joint Hypermobility* extracted from Bulbena et al.[12]

	Joint	Excursion
Upper extremity	Thumb	Passive apposition of the thumb to the flexor aspect of the forearm
	Metacarpophalangeal joint	Passive dorsiflexion of the fifth finger > 90°
	Elbow	Passive hyperextension of the elbow > 10°
	Shoulder**	Passive exorotation > 85°
Lower extremity: supine position	Hip**	Passive hip abduction of both legs > 85°
	Patella	Excessive passive movement of the patella in lateral and medial direction
	Ankle	Passive dorsiflexion > 20° of the ankle joint
	Metatarsophalangeal joint	Passive dorsal flexion of the first toe > 90°
Lower extremities: prone position	Knee**	Knee (hyper)flexion allows the heel to make contact with the buttock
Ecchymoses		Appearance of ecchymoses after minimal trauma
Scoring	1 point each positive criterium; cut-off for generalized hypermobility: ♂ ≥ 4 points, ♀ ≥ 5 points, cut-off for localized hypermobility: ♂ 1-3, ♀ 1-4.	

*Passive movements, measured unilaterally

**Joints most frequently involved in VUR patients

Statistics

Central estimators of all relevant variables were calculated as means (standard error of the mean) or medians (minimum, maximum, interquartile range (IQR)) when appropriate.

Due to skewness in the distribution of parameters, differences were analyzed non-parametrically, using the Wilcoxon signed ranks test for differences between patients and control subjects and the Mann-Whitney test for differences within the patient group and between responders and non-responders. Since the Bulbena score was available for 30 of 50 matched control subjects, the Bulbena score in available controls was compared with both the total group of cases and the subset of 30 cases that matched to the available controls.

All analyses were performed with SPSS version 16.0 for Windows and results were considered statistically significant when the corresponding p value was < 0.05.

RESULTS

The baseline characteristics of patients and controls were comparable (see Table 3.3). Table 3.4 summarizes the results of the joint mobility measurements. Bulbena score was available for 30 of 50 matched controls. The 50 VUR cases had a significantly higher median Bulbena score than the 30 controls (3.0 (IQR: 2.0 - 4.0) versus 0.5 (IQR: 0.0 - 2.0), $p < 0.0001$). When comparing the Bulbena score of the 30 cases and the 30 controls for which the score was available, we also found a significantly higher median Bulbena score in the VUR patients (4.0 (IQR: 3.0 - 4.25), $p < 0.0001$). 76% of VUR patients had a Bulbena score consistent with "local hypermobility"(Bulbena score in girls: 1 - 4, in boys: 1 - 3). When we applied the Bulbena diagnostic criteria, we identified significantly more VUR patients with "generalized hypermobility" than controls (24.0% versus 6.7%, $p = 0.007$, see figure 3.1).

Table 3.3. Baseline data for 50 VUR patients and controls matched for gender, age, and BMI

	Cases				Controls				Case / control comparison
	Mean/%	**SD**	**IQR**	**Range**	**Mean/%**	**SD**	**IQR**	**Range**	***p*- value***
Gender (% girls)	54.0%	-	-	-	54.0%	-	-	-	-
Age (mean, years)	11.1	2.2	9.4-13.1	8.0-14.8	11.1	2.2	9.4-13.3	8.1-14.8	0.632
BMI (mean, kg/m2)	18.1	2.7	16.2-19.6	13.8-24.9	18.2	2.5	16.4-19.3	14.0-25.4	0.828
Ethnicity (%)**	96.0%	-	-	-	96.0%	-	-	-	0.083
Mean hours of sports activity (mean, hours/ week)	5.2	2.8	3.4-6.0	0.0-13.5	4.6	1.5	3.0-6.0	2.0-7.0	0.263

*Wilcoxon signed ranks test, **Assessed differently in both groups – cases: % of grandparents (max. 4) born in the Netherlands, controls: % Caucasians

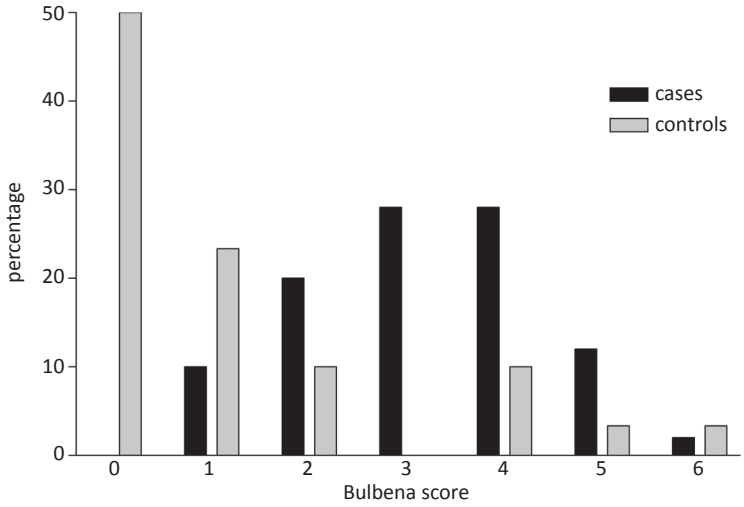

Figure 3.1. Distribution of Bulbena scores in the VUR patient group and the control group. Since Bulbena scores were available for 30/50 matched controls (and 50/50 cases), the Y-axis represents percentages.

The hip- (98%), shoulder- (86%), and knee-joint (62%) were the hypermobile joints most frequently seen in VUR patients (Table 3.2). Commonly VUR grade 3-5 is considered to be severe and grade 1-2 is considered to be mild. When we dichotomized the 1-2 ureters of the 50 patients in ureters with a VUR grade of ≥ 3 (n=45) or ureters with VUR grade < 3 (n=30) and assessed the patient Bulbena score per individual ureter (data available for 75 ureters), we found a statistically significant (p = 0.008, Mann-Whitney test) 1,0 higher median Bulbena score for ureters with a VUR grade of ≥ 3 (median Bulbena score = 4.0) compared to the ureters with a VUR grade < 3 (median Bulbena score = 3.0).

Table 3.4. Results of measurements of joint mobility in 50 VUR patients and controls matched for gender, age, and BMI

	Cases				**Controls**				**Case / control comparison**
	Mean/ median/%	**SD**	**IQR**	**Range**	**Median/ mean**	**SD**	**IQR**	**Range**	***p*-value* (number of cases versus controls)**
Bulbena score (median, max. 10 points), 50 cases vs. 30 controls	3.0	-	2.0-4.0	1.0-6.0	0.5**	-	0.0-2.0	0.0-6.0	<0.0001 (50 vs. 30)
Bulbena score (median, max. 10 points), 30 cases vs. 30 controls	4.0	-	3.0-4.25	2.0-6.0					<0.0001 (30 vs. 30)
GJH according to Bulbena score (%, ♂≧4, ♀≧5), 50 cases	24.0	-	-	-	6.7**	-	-	-	0.007 (50 vs. 30)

GJH generalized joint hypermobility, *Wilcoxon signed ranks test, **Bulbena score was measured in 30 of 50 matched controls, because of subtle differences in the protocols used to characterize the two reference groups.

The questionnaire did not reveal any (objective or subjective) signs of connective tissue disease or hypermobility. Splitting the case-group in patients with or without reported voiding symptoms, revealed no differences in hypermobility score (data not shown).

DISCUSSION

We assessed joint mobility as a parameter of connective tissue status in VUR patients and compared these findings to a reference population. The Bulbena score[96] was significantly increased in patients compared to controls and when we applied the diagnostic criteria we detected significantly more VUR patients with "generalized" as well as "local hypermobility" than controls. This finding confirms our initial observation of an increased rate of joint hypermobility in VUR patients. As hypothesized, patients with higher grades of VUR showed a significantly higher Bulbena score. Possibly, an altered composition of connective tissue may contribute to the severity of a (pre-existing) VUR phenotype.

There are some limitations to this study. Firstly, the assessor (VJMV) was not blinded for our hypothesis. She was however blinded for VUR phenotype details and severity and she was trained by the same person who supervised the measurements in the controls.
Secondly, the response rate for patients (44%) was not high. The patients had to be informed about the hypothesis before giving their informed consent for the investigations. A differential response of the patients (for constitutional laxity) is not likely. Furthermore, we analyzed the clinical characteristics of responders and non-responders, which were not significantly different.
Finally, the Bulbena score was available in 30 of 50 control subjects. When analyzing the data for the 30 cases that match to these controls specifically, the median Bulbena score was even higher and the difference even more significant, indicating that the difference observed is indeed true.

Benign Joint Hypermobility Syndrome (BJHS) is present in symptomatic hypermobile patients when symptoms match the so-called Brighton criteria.[99] BJHS and hypermobility type Ehlers Danlos syndrome (EDS) (type III) are considered to be indistinguishable.[99] One study of pediatric patients with BJHS reported a prevalence of VUR of 3% (compared to 1% in the Caucasian population) and of urinary tract infections in 6% (males) to 13% (females).[100] This suggests that these patients have a relative risk of 3 of having VUR. In adult patients with the hypermobility type of EDS, there are reports of lower urinary tract pathology: these patients have increased numbers of urinary tract infections, incontinence, voiding problems, and uterine prolapse and bladder diverticuli.[101, 102] This could be due to a structural change in the connective tissue of the pelvic floor and bladder wall.[103] Possibly, the occurrence of VUR in

other hereditary disorders of connective tissue such as EDS (other than hypermobility type), Marfan's syndrome and osteogenesis imperfecta is underestimated because of other more severe symptoms that attract attention in these patients. Even in asymptomatic hypermobile (pediatric) subjects (prevalence: 10 - 25% depending on age, gender and race[94]), the skin is significantly more extensible than in controls.[97] So even these subjects (reminiscent of our VUR patient population) show signs of constitutional laxity.

Recent studies have already shown associations between generalized joint hypermobility and voiding and defecation dysfunction and GJH and slow transit constipation.[92, 93] We now show an association between GJH and VUR, a different disorder that often co-occurs with voiding and defecation dysfunction. For this study we retrospectively selected primary VUR patients in specific age groups. Even this selected patient group was still somewhat heterogeneous, for instance 54% (at most) of the patients, with girls in the majority, also had some kind of voiding or defecation dysfunction (at any time). Although patients with other congenital anomalies of the urinary tract were excluded, there are still some subtle differences in urinary tract anatomy of the patient group, such as the degree of displacement of ureteral orifices (assessed at cystoscopy). So there will have been differences in the importance of the respective factors contributing to the development of clinically relevant VUR in individual patients. We do not know of any study on primary VUR patients that has prospectively or retrospectively assessed the patients in such anatomical or clinical detail as suggested above. This could be considered in future VUR research. In the light of studies by De Kort et al.[92] and Reilly et al.[93] we analyzed our findings in the subgroup of patients with voiding symptoms. We found that the results were similar to our whole patient group (data not shown), so we could not replicate the link between voiding symptoms and joint mobility that was suggested by their studies.[92, 93]

At the basis of our hypothesis lies the assumption that connective tissue composition differences in general may lead to constitutional laxity resulting in joint hypermobility, but also in differences in connective tissue composition of the UVJ and bladder wall. Similarities in main collagen components (predominantly type I but also type III) between ligaments and bladder tissue support this.[104, 105] Previously, skin extensibility, bone density, collagen degradation products in urine, blood and pulse pressure (as parameters for arterial stiffness) have also been studied in combination with joint mobility to test similar hypotheses.[94, 95, 97]

There are few studies investigating extracellular matrix (ECM) composition in uretero-vesicular junction (UVJ) specimens from VUR patients. These studies have somewhat contradictory results.[106-109] In UVJs from pediatric patients with persistent VUR, collagen type I seems to be increased, while the number of smooth muscle cells is decreased.[106] Another study on ureteral wall thickness and collagen thickness of refluxing ureters however, showed decreased collagen

thickness in the VUR group.[109] We do not know of any study describing ECM composition in bladder tissue (as opposed to UVJ tissue) of VUR patients.

To further explore the relevance of our findings, one line of future investigation could be the assessment of associaton of variants in genes coding for ECM components and VUR. It would also be interesting to investigate the constitutional laxity hypothesis more extensively in VUR patients (by measuring i.e. skin extensibility, bone density, collagen degradation products in urine).

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CHAPTER 4

Disruption of *ROBO2* is associated with urinary tract anomalies and confers risk of vesicoureteral reflux

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ABSTRACT

Congenital anomalies of the kidney and urinary tract (CAKUT) include vesicoureteral reflux, or VUR (OMIM 193000). VUR is a complex, genetically heterogeneous developmental disorder characterized by the retrograde flow of urine from the bladder into the ureter and is associated with reflux nephropathy, the cause of 15% of end-stage renal disease in children and young adults. We investigated a male with a *de novo* translocation, 46,X,t(Y;3)(p11;p12)dn, who exhibits multiple congenital abnormalities including severe bilateral VUR with uretero-vesical junction defects. This translocation disrupts *ROBO2*, encoding a transmembrane receptor for SLIT ligand, and produces dominant negative ROBO2 proteins that abrogate SLIT-ROBO signaling *in vitro*. In addition, we identified two novel *ROBO2* intracellular missense variants that segregate with CAKUT and VUR in two unrelated families. Adult heterozygous and mosaic mutant mice with reduced *Robo2* gene dosage also exhibit striking CAKUT-VUR phenotypes. Collectively, these results implicate the SLIT-ROBO signaling pathway in the pathogenesis of a subset of human VUR.

INTRODUCTION

CAKUT is a family of diseases with a diverse anatomical spectrum, including kidney anomalies (*e.g.*, renal dysplasia, duplex kidney, hydronephrosis), and ureter anomalies (*e.g.*, vesicoureteral reflux, megaureter, ureterovesical junction obstruction).[110, 111] In particular, vesicoureteral reflux (VUR), a polygenic genetic disorder with an incidence of approximately 1 in 100 infants,[21, 79] is one of the most common clinical manifestations of CAKUT. VUR is characterized by reflux of urine from the bladder into the ureters and sometimes the kidneys, and is a risk factor for urinary tract infection (UTI).[112] In combination with intrarenal reflux, the resulting inflammatory reaction may result in renal injury or scarring, also called reflux nephropathy (RN).[113] Extensive renal scarring impairs renal function and may predispose to hypertension, proteinuria, and renal insufficiency. Reflux nephropathy accounts for as much as 15% of end-stage renal disease (ESRD) in children and young adults.[114] Primary VUR results from a developmental defect of the uretero-vesical junction (UVJ)[8] and is known to occur in families. In siblings and offspring of affected patients, the prevalence is as high as 50%.[115, 116] Despite its high incidence in the pediatric population, the genetic basis of VUR remains to be elucidated.

Human *ROBO1-4* encode homologs of *Drosophila* Roundabout, a transmembrane receptor that binds SLIT ligand and transduces a signal to prevent axons from re-crossing the CNS midline.[119] Based on the mouse *Robo1* mutant phenotype, *ROBO1* is a candidate gene for pulmonary hypoplasia and adenocarcinoma,[120, 121] and also for dyslexia (DYX5).[122] Mutations in *ROBO3* result in horizontal gaze palsy with progressive scoliosis (HGPSS),[118] while zebrafish

Robo4 is implicated in angiogenesis.[123] *Robo2* loss-of-function mutations in zebrafish and mice result in retinal and commissural pathfinding defects, respectively.[124, 125] Interestingly, *Robo2* and *Slit2* mouse mutants reveal an additional key role for SLIT-ROBO signaling in regulating the metanephric expression of *GDNF*, which in turn induces ureteric bud outgrowth from the nephric duct and restricts it to a single site.[126] However, a role for *ROBO2* in human disease has not been identified previously.

MATERIAL AND METHODS

FISH and Array Comparative Genomic Hybridization Analyses

Metaphase FISH was performed by standard methods. RP11 BAC clones were obtained from BAC/PAC Resources, labeled as FISH probes and hybridized to metaphase chromosomes prepared from a t(Y;3)(p11;p12)dn lymphoblastoid cell line established from DGAP107. Array CGH experiments were performed by Spectral Genomics (SpectralChip 2006 array) and Agilent Technologies (Human Genome CGH Microarray Kit 44A).

Southern and RT-PCR analyses

Southern and RT-PCR analyses were performed by routine protocols. RT-PCR primers used to amplify the 2.8 kb *ROBO2* cDNA were *ROBO2-F1* and *ROBO2-R1*; those to amplify ROBO2-PCDH11Y fusion transcripts were *ROBO2-F2* (same for all transcripts), and *PCDH11Y-R1, PCDH11Y-R2*, and *PCDH11Y-R3*; those to amplify wild-type *ROBO2* transcripts were *ROBO2-F2* and *ROBO2-qR*; and those to amplify *PCDH11Y* cDNA were *PCDH11Y-rtF1* and *PCDH11Y-rtR1* (Appendix C). All primer sequences are listed in Appendix G.

Quantitative real-time PCR analyses

PCR primers and TaqMan fluorogenic probes for analysis of the *ROBO2* non-translocated allele and the *Fu-129* and *Fu-153* fusion transcripts were designed using Primer Express software (Applied Biosystems, Foster City, CA). TaqMan primers and probes for *Gapdh* and *ß-actin* were used for normalization. Probe Tms were ~7-10°C higher than for the matching primer pair. HPLC-purified fluorogenic probes contained covalently attached 5'-FAM reporter and 3'-BHQ1 quencher dyes. Sequences of TaqMan primer and probe sets are listed in Appendix G. RT-PCR reactions were performed using an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA). SuperScript One-step RT-PCR with Platinum Taq kits (Invitrogen, Carlsbad, CA) were used for triplicate RT-PCR amplifications, each 50 µl reaction containing 200 ng total RNA, 5 mM $MgSO_4$, 500 nM forward and reverse primers and 200 nM fluorogenic probe. Controls included no reverse transcriptase, or substitution of H_2O for RNA for each primer and probe set. The one-step RT-PCR protocol was 15 min at 50°C, 5 min at 95°C, followed by 45 cycles each consisting of 15 sec at 95°C and 1 min at 60°C. IQ Supermix reagent

for real-time PCR (Bio-Rad) was used for two-step RT-PCR. Relative gene expression was analyzed using standard curve and comparative C_T methods.

Fusion proteins

cDNA sequences for Fu-129 and Fu-153 were amplified by PCR using forward primer 5'-hR2(E1), reverse primers 3'-LEVA(X1) and 3'-SRSC(X1) (Appendix G) and cloned into *Eco*R1 and *Xho*1 sites in pcDNA3 under control of the CMV promoter. To express yellow fluorescent protein (YFP)–ROBO2-PCDH11Y fusions, the YFP (Venus) coding sequence was cloned into *Bam*HI and *Eco*RI sites in pCS, whereas *ROBO2-PCDH11Y* coding sequences were inserted inframe with YFP at *Eco*RI and *Xho*I sites. eYFP-ROBO2-PCDH11Y fusion proteins were expressed under the control of the SCMV IE94 promoter. Myc-SLIT and HA-RoboN (Robo1-N) constructs have been previously described.[117]

Neuronal migration assay

The *in vitro* neuronal migration assay using postnatal anterior subventricular zone (SVZa) cells was previously described.[118] Briefly, P1-6 Sprague-Dawley rat brains devoid of meninges were placed in 10% fetal calf serum (FCS) in DMEM and embedded. 300 µm coronal sections were prepared by vibratome and tissues within the SVZa borders dissected to make SVZa explants of 200-300 µm diameter. Explants were embedded together with human embryonic kidney (HEK) cell aggregates in collagen and matrigel (3:2:1 collagen:matrigel:medium), and cultured in DMEM at 37°C, 5% CO_2 for 24 hours. Co-cultured cells were washed in PBS for 10 min and fixed in 4% paraformaldehyde at 4°C overnight.

To make cell aggregates, HEK cells were transiently transfected to express mouse Slit2, RoboN, or DGAP107 Fu-129, or Fu-153, or pcDNA3 or Semaphorin 3A expression vectors as negative controls using Effectene Transfection Kit (Qiagen, Valencia, CA). After 24 hrs, transfected HEK cells were detached, collected by brief centrifugation and cell pellets resuspended in an equal volume of DMEM. 10 µl of suspended cells were hung from the dish cover at 37°C, in 5% CO2 for 1-2 hrs to form aggregates. Aggregated cells were washed in DMEM and squared with a needle.

Western blot

HEK cells were transfected to express eYFP-ROBO2-PCDH11Y-Fu-129, eYFP-ROBO2-PCDH11Y-Fu-153, Myc-Slit, HA-RoboN. Cells transfected with pCS2 vector were used as a negative control. 48 hrs post-transfection, media were collected and centrifuged for 20 min at 4°C to remove cell debris. Supernatants were diluted with 6-fold protein loading buffer and heated at 95°C for 20 min. Cells were lysed (1× PBS, 0.5% Triton X-100, 1× protease inhibitor), diluted with 6-fold protein loading buffer and heated at 95oC for 20 min. Proteins were resolved by 12% SDS PAGE, with eYFP (Venus) fusion proteins detected by monoclonal anti-GFP antibody that detects eYFP (Clontech, Mountain View, CA); Myc-Slit2 and HA-RoboN were blotted with monoclonal antibodies against Myc and HA respectively.

Mutation analysis

ROBO2 mutation screening employed PCR amplification of each of the 26 human *ROBO2* exons and intron-exon boundaries, followed by purification and bi-directional DNA sequencing. Sequences of the *ROBO2* PCR primer sets are listed in Appendix G. Sequence data were analyzed using Lasergene (DNAStar) sequence analysis software. DNA samples with sequence changes were confirmed by re-sequencing. NCBI RefSeq *ROBO2* cDNA sequence NM_002942 (GI:109254774) and protein sequence NP_002933 (GI:61888896) were used to calculate the nucleotide and amino acid positions.

Preparation of Robo2^{flox} and Robo2^{del5} alleles

Robo2^{flox} mice were produced using homologous recombination in 129 ES cells and blastocyst injection. After germline transmission, mice were backcrossed to C57BL/6 and analyzed thereafter in a mixed C57BL/6-129/Sv background. The *Robo2*^{flox} allele was genotyped by PCR amplification followed by *SpeI* restriction digestion using PCR primers *Ro2-MEBAC15F* and *Ro2-MEBAC15R* (Appendix G), which amplify an 1100 bp fragment for both wild-type and *Robo2*^{flox} alleles. After SpeI digestion, the *Robo2*^{flox} amplicon remains uncut while the wild-type amplicon yields 750 and 350 bp products. *Robo2*$^{flox/+}$ mice were bred with *Tg*$^{EIIa\text{-}Cre}$ (Stock no. 003724, Jackson Laboratory, Bar Harbor, ME) to produce the *Robo2*^{del5} allele. The *Robo2*^{del5} allele was amplified by primers *Robo2koF* and *Robo2R*, which produces an 1100 bp fragment. The wild-type allele was amplified by primers *Robo2wtF* and *Robo2R*, which yields a 1390 bp fragment. F2 *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic mice were prepared as described (Appendix F) and analyzed for the presence of urinary tract phenotypes. To examine the ureter and kidney defects, *Hoxb7-GFP* transgenic mice (gift from Dr. Frank Costantini, Columbia University) were bred with *Robo2* mutants. GFP fluorescence was monitored and photographed using a Nikon SMZ-1500 epi-fluorescence stereomicroscope.

Human and animal studies

All human studies were performed under informed consent protocols approved by the Partners HealthCare System Human Research Committee (Boston), the Human Research Ethics Committee (Institute of Child Health, University College London), or the University Medical Center (Utrecht). Mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School or Boston University Medical Center, with additional approval from Kings College, London.

RESULTS

The Developmental Genome Anatomy Project (DGAP) is a collaborative effort to use chromosomal rearrangements associated with developmental disorders to identify the

underlying genetic etiology. DGAP107 is an 18 years old male with a 46,X,t(Y;3)(p11;p12)dn whose phenotype includes bilateral high-grade VUR and right megaureter at the ureterovesical junction (UVJ) (Figure 4.1A-D, Appendix A). He required ureteral reimplantation surgery at age nine and was found to have wide-open right and left ureteral orifices due to bilateral absence of intravesical ureteral segments. Normally, these submucosal ureteral segments obliquely traverse the muscular layers of the bladder to prevent retrograde flow of urine by a flap valve mechanism. By metaphase FISH, we identified a BAC clone (RP11-54A6) that crosses the 3p12 breakpoint, which disrupts intron 2 of *ROBO2*, composed of 26 exons and spanning ~606 kb of genomic DNA (Figure 4.1E, F).

We cloned and sequenced the breakpoints on the der(3) and der(Y) chromosomes (Appendix B). In addition to disruption of *ROBO2* at 3p12, the protocadherin gene *PCDH11Y* at Yp11 was also disrupted by the translocation. A contribution of *PCDH11Y* disruption to the VUR phenotype in DGAP107 is unlikely, however, as *PCDH11Y* expression has only been detected in placenta, brain, retina and testis,[127] and was not detected in embryonic kidney (Appendix C). By array CGH and FISH, we also identified a 3.4 Mb interstitial deletion at 17p11.2 in DGAP107.

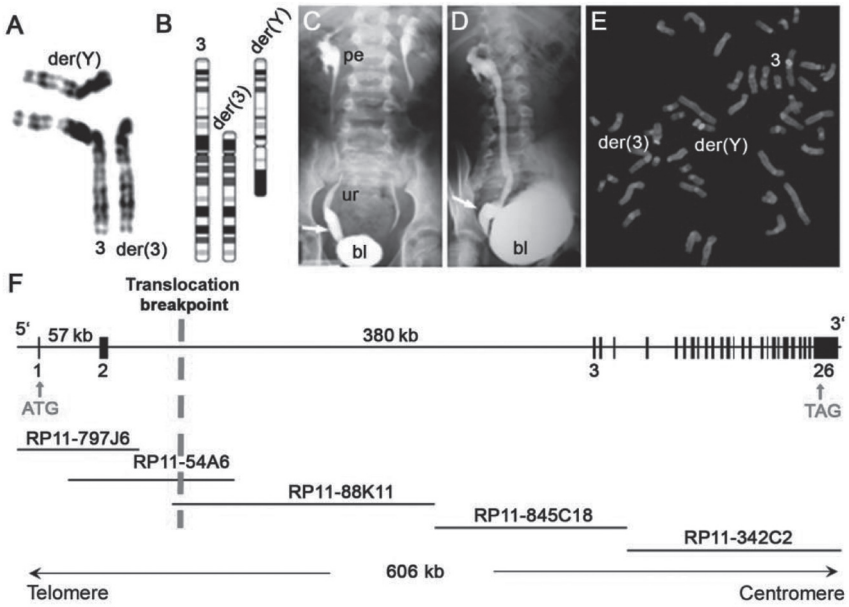

Figure 4.1 *ROBO2* disrupted in DGAP107. Partial karyogram (*A*) and idiogram (*B*) for 46,X,t(Y;3)(p11;p12)dn is shown. VCUG of DGAP107 shows anterior-posterior (*C*) and lateral (*D*) views of bilateral grade IV VUR and megaureter at the right UVJ (*arrows*). bl = Bladder; pe = renal pelvis; ur = ureter. *E*, FISH analysis showing BAC RP11-54A6 (*green*), which hybridizes to normal chromosome 3, der(3), and der(Y) and crosses the 3p12 breakpoint. *F*, Intron-exon structure of *ROBO2*, with select exons numbered and the relevant BAC contig. The location of the 3p12 translocation breakpoint is indicated by a red dotted vertical line. See page 196 for a full-color representation of this figure.

This region is within the common microdeletion region pathogenetic in Smith-Magenis Syndrome, SMS (OMIM 182290), a mental retardation syndrome associated with behavioral and sleep disturbances and craniofacial and skeletal anomalies.[128] Thus, a role for del(17)(p11.2) in the sleep, behavioral and cognitive deficits in DGAP107 seems likely. The del(17)(p11.2) could also contribute to the pathogenesis of VUR in DGAP107. However, for reasons described below and elsewhere (Appendix D), we conclude that *ROBO2* disruption alone is sufficient to account for the VUR phenotype observed in DGAP107.

The t(Y;3) in DGAP107 juxtaposes *ROBO2* and *PCDH11Y* in the same transcriptional orientation. On the der(Y), the promoter and first two exons of *ROBO2* reside upstream of exons 1d-6 of *PCDH11Y* (Figure 4.2A). From RT-PCR experiments using DGAP107 lymphoblast RNA, we identified two *ROBO2-PCDH11Y* fusion transcripts driven by the *ROBO2* promoter (Figure 4.2B).

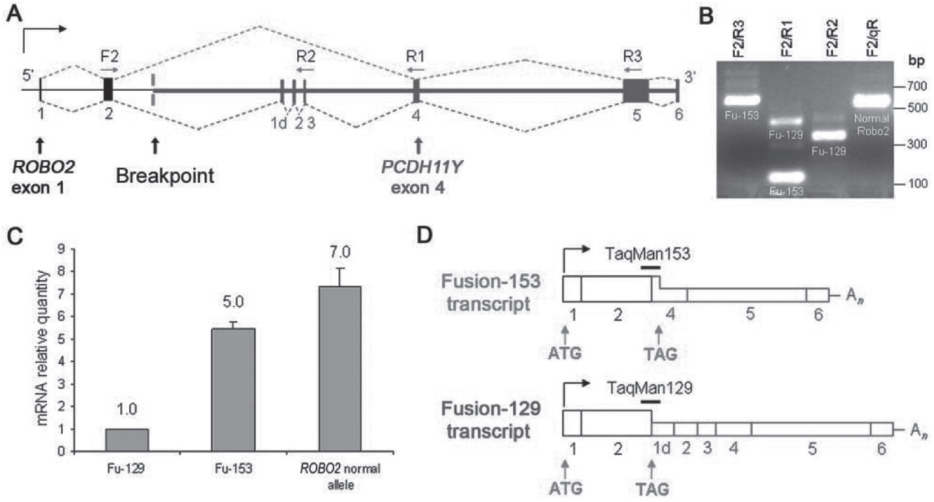

Figure 4.2 The t(Y;3) translocation in DGAP107, which generates novel *ROBO2* fusion transcripts. *A*, *ROBO2* and *PCDH11Y* intron-exon structure surrounding the der(Y) breakpoint. The forward primer F2 in *ROBO2* exon 2 (*black bar*) was used in RT-PCR with three reverse primers-R2, R1, and R3-in *PCDH11Y* exons 3, 4, and 5, respectively (*blue bars*). Dotted lines indicate the observed splicing patterns of the two fusion transcripts. The red splicing pattern generates Fu-153, which encodes 153 aa, and the blue pattern generates Fu-129, which encodes 129 aa. *B*, RT-PCR fusion transcript amplification. *Lane 1*, F2/R3 primers amplify Fu-153 (641 bp) and Fu-129 transcripts; only the shorter Fu-153 amplicon is shown. *Lane 2*, F2/R1 primers amplify transcripts for both Fu-129 (456 bp) and Fu-153 (122 bp). *Lane 3*, F2/R2 primers amplify only Fu-129 transcripts (347 bp). *Lane 4*, F2/qR primers amplify only transcripts from the wild-type nontranslocated *ROBO2* allele (606 bp). qR primer is located in exon 7 of *ROBO2*. C, Real-time RT-PCR quantitation of *ROBO2* fusion transcripts Fu-129 and Fu-153 (detected by TaqMan probes shown in panel D) and of *ROBO2* nontranslocated allele transcripts (detected by TaqMan probe across *ROBO2* exons 2 and 3) in DGAP107 lymphoblast RNA. *D*, Exon structure of Fu-153 and Fu-129. Horizontal bars indicate TaqMan probes used to quantify fusion transcripts. Black boxes indicate *ROBO2* exons; blue boxes, *PCDH11Y* exons; fullheight boxes, coding exons; and half-height boxes, noncoding exons. See page 197 for a full-color representation of this figure.

Each transcript contains the first two exons of *ROBO2* spliced out-of-frame to *PCDH11Y* downstream exons, resulting in premature stop codons shortly after *ROBO2* exon 2. When assayed by real time RT-PCR, these fusion transcripts, denoted Fu-129 and Fu-153, are expressed at somewhat reduced levels compared to the wild-type *ROBO2* transcripts derived from the non-translocated allele (Figure 4.2C); the wild-type transcripts contained no detectable mutations. Fu-129 and Fu-153 encode 129 and 153 residue polypeptides containing the first ROBO2 extracellular Ig domain, but they are truncated before the transmembrane and cytoplasmic domains required for SLIT-ROBO signal transduction (Figure 4.2D).
When expressed without transmembrane and cytoplasmic domains, the soluble extracellular Ig domains of ROBO, denoted RoboN, are able to bind SLIT ligand.[129] Moreover, the first ROBO Ig domain is necessary and potentially sufficient for SLIT binding.[129] RoboN isoforms can thus inhibit SLIT-ROBO signaling by competing with wild-type ROBO for SLIT binding.[130] We therefore hypothesized that the truncated proteins encoded by the ROBO2-PCDH11Y fusions might act in a dominant-negative manner to block endogenous SLIT-ROBO signaling. To test this hypothesis, we performed an *in vitro* neuronal migration assay[131] in which anterior subventricular zone (SVZa) explants were cultured in proximity to human embryonic kidney (HEK) cell aggregates secreting Slit2, or Slit2 and either RoboN, Semaphorin 3A, Fu-129 or Fu-153 (Figure 4.3A). We then examined the directionality of neuronal migration away from the SVZa explants (Figure 4.3B-F). HEK cell aggregates alone have no effect on SVZa explants, resulting in a radially symmetric pattern of neuronal outgrowth.[130]
When they were cultured with HEK cell aggregates transfected with vectors expressing Slit2 only, or Slit2 and Semaphorin 3A (a molecule having no effect on Slit function, as a control), the Slit2-expressing cell aggregates acted upon the SVZa explants to repel SVZa neuronal outgrowth (Figure 4.3B, C). In contrast, in aggregates co-expressing Slit2 and either RoboN, Fu-129 or Fu-153, the latter molecules abrogated the chemorepulsive effect of Slit2 on the SVZa explant and significantly increased the number of neurons able to migrate towards the Slit2 source, resulting in a radially symmetric pattern of neuronal outgrowth (Figure 4.3D-F). These results indicate that the ROBO2-PCDH11Y fusion proteins that result from the t(Y;3) can act as dominant-negative molecules to block SLIT-mediated chemorepulsive function. The fusion proteins could further compromise ROBO2 function in DGAP107, who retains only hemizygous *ROBO2* expression from the non-translocated allele.

Family studies indicate that primary VUR frequently segregates with autosomal dominant inheritance and incomplete penetrance.[20, 115] To test whether mutations in *ROBO2* are associated with CAKUT and VUR in the general population, we sequenced the 26 exons and intron-exon boundaries of ROBO2 in 124 VUR families with potential autosomal dominant inheritance.[21, 36] One sequence change, c.2436T>C, I598T, was observed in exon 12 in the ROBO2 extracellular domain, but was also identified in three control DNAs (see below). It therefore most likely represents a sequence polymorphism, and was discounted from further

study. In contrast, two novel *ROBO2* intracellular coding sequence changes were identified that were not found in 276 controls (see below). These produce non-conservative amino acid substitutions in two independent CAKUT-VUR families (Figure 4.4; Appendix E). In CAKUT-VUR family 2559x, the affected daughter 25592 has bilateral VUR, hypoplastic kidneys and nephropathy, while her mother 25593 required ureteral re-implantation due to severe VUR (Figure 4.4A-C). Both individuals have a heterozygous T→C change at position 3477 in coding exon 19 (c.3477T>C) that would cause a non-conservative missense I945T substitution in the ROBO2 intracellular domain (Figure 4.4D, E).

In CAKUT-VUR family B5, the proband has bilateral VUR and a right duplex collecting system and kidney, as do her mother and two aunts, while her grandmother has a unilateral small kidney (Figure 4.4F-I). All five family members carry a heterozygous G→A sequence alteration at position 4349 in coding exon 23 (c.4349G>A) that would cause a non-conservative missense amino acid substitution, A1236T, in the ROBO2 intracellular domain (Figure 4.4J, K).

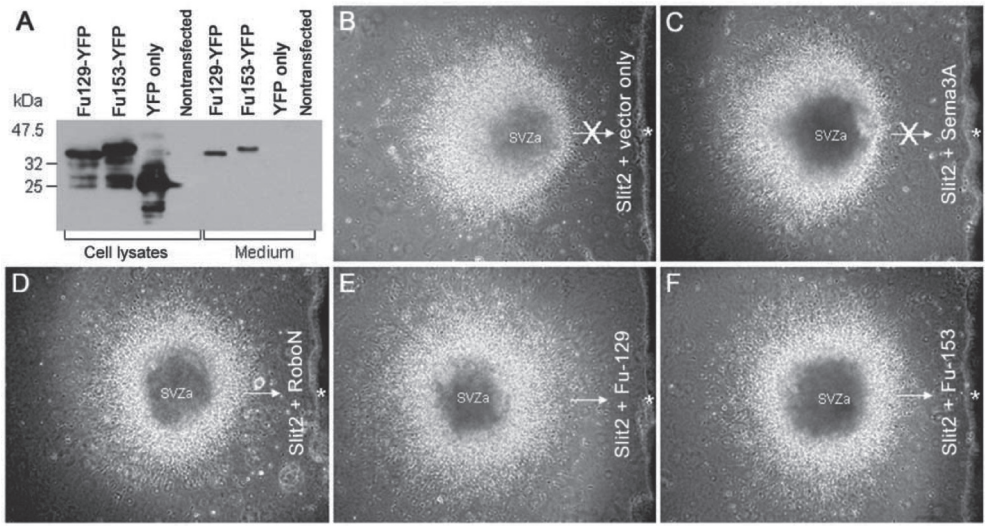

Figure 4.3 ROBO2 fusion proteins inhibiting SLIT chemorepulsion. *A*, YFP-tagged ROBO2 fusion proteins (Fu129-YFP [40 kDa] and Fu153-YFP [42 kDa]) detected by an anti-YFP antibody, expressed in HEK cell lysates, and secreted into the medium. In the presence of aggregated cells transfected with Slit2 plus empty vector (*B*) or Slit2 plus Sema3A (Semaphorin 3A, with no effect on Slit2 repulsive activity) (*C*), cells migrate out of SVZa explants and away from the Slit2-expressing cell aggregate (*asterisk*). In the presence of aggregated cells transfected with Slit2 plus RoboN (the Robo extracellular domain, which inhibits Slit repulsive activity), cells migrate out of SVZa explants symmetrically in all directions (*D*) including toward (*arrow*) the Slit2 and RoboN-expressing cell aggregate (*asterisk*). Fu-129 and Fu-153 also effectively block Slit2 repulsive activity (*E* and *F*), allowing symmetrical neuronal migration out of SVZa explants and toward (*arrows*) Slit2 and Fu-129 or Slit2- and Fu-153-expressing cell aggregates (*asterisks*). See page 198 for a full-color representation of this figure.

An additional family member, uncle A1, also has this alteration but did not exhibit an ultrasonographically detectable renal phenotype; however, non-penetrance of VUR is common.[115] Both I945 and A1236 are evolutionarily conserved in all mammals, and only slightly divergent in birds and fish, organisms that lack a urinary bladder and UVJ (Figure 4.4E, K).

To assess further the likelihood that these sequence changes represent functional missense variants as opposed to rare neutral variants found in the general population, we sequenced *ROBO2* exons 12, 19 and 23 in 180 unrelated normal controls of ethnic backgrounds similar to those of the affected individuals. Two occurrences of c.2436T>C in exon 12 were detected, but no nucleotide changes were identified in exons 19 or 23.

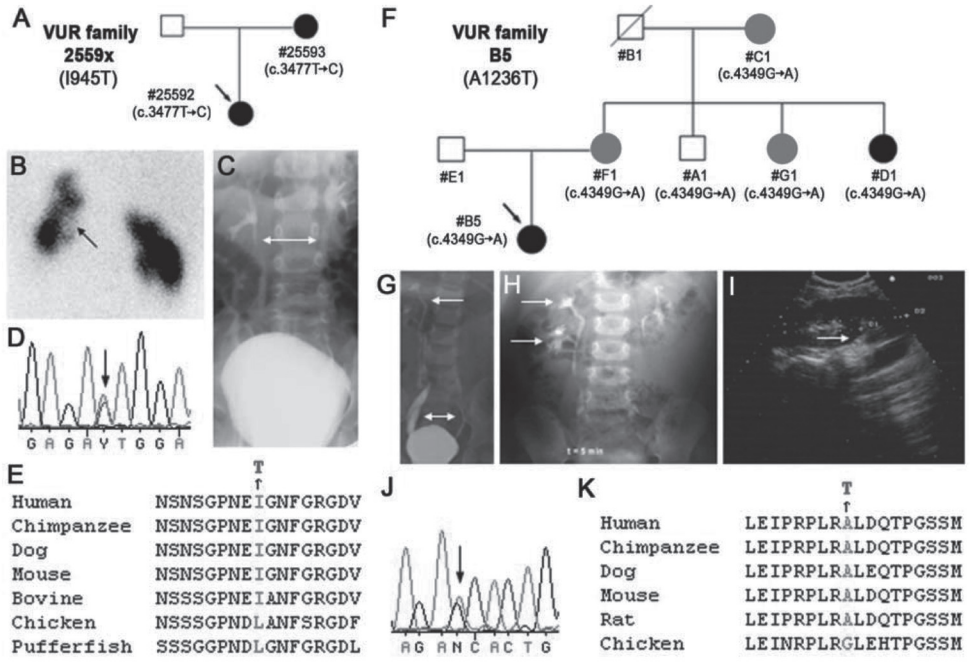

Figure 4.4 *ROBO2* missense mutations in familial CAKUT and VUR. *A*, Family 2559x with CAKUT-VUR and exon 19 (c.3477TrC) mutation. Arrow indicates the proband. Blackened and gray symbols indicate patients with CAKUT-VUR and family members with urinary tract symptoms and radiological evidence of CAKUT. Nucleotide changes are shown under each individual. *B*, 99mTc-dimercaptosuccinic acid renogram of proband 25592 showing bilateral renal parenchymal defects (*arrow*). *C*, VCUG of proband 25592 showing bilateral reflux (*bidirectional arrow*). Chromatograms show TrC change (*arrow*) in exon 19 of family 2559x (*D*) and amino acid conservation across species (*E*). *F*, Family B5 with CAKUT-VUR and exon 23 (c.4349GrA) mutation. *G*, VCUG showing bilateral VUR (*bidirectional arrow*) and right duplex kidney (*arrow*) in proband B5. *H*, IVP detecting right duplex kidney (*arrows*) in proband B5. *I*, US showing suspected duplex (*arrow*) in upper pole of the right kidney in D1, an asymptomatic aunt of proband B5. Chromatograms show GrA change (*arrow*) in exon 23 of family B5 (*J*) and amino acid conservation across species (*K*). See page 199 for a full-color representation of this figure.

In addition, to determine the full spectrum of *ROBO2* sequence variation, we re-sequenced the 26 *ROBO2* exons and intron-exon boundaries in an additional 96 controls. We found only one reoccurrence of c.2436T>C. Of several non-validated putative synonymous and non-synonymous *ROBO2* cSNPs listed in Ensembl v39, we detected only one, c.737C>A, R32R, in our own sequencing efforts. Moreover, this apparent change was found to represent a sequencing artifact. Thus, in sum, these results suggest that the two sequence changes in the intracellular domain identified in familial VUR are deleterious missense variants that contribute to the CAKUT-VUR phenotype.

Both I945T and A1236T could alter the function of the ROBO intracellular domain (ICD), which regulates actin polymerization and cellular migration,[132] by creating novel threonine phosphorylation sites or by influencing the binding of proteins that interact with the ROBO ICD.[133] For example, the SH3 domain of srGAP1 binds to the ROBO1 ICD CC3 subdomain,[132, 133] which is partly conserved in ROBO2. The ROBO2 CC3 subdomain (residues 1193-1201) resides close to A1236, and an extended ROBO1 CC3 peptide binds the srGAP1 SH3 domain much more strongly than does the isolated CC3,[132] suggesting that residues outside CC3 also mediate srGAP binding. I945T and A1236T may act as either dominant gain- or loss-of-function mutations that influence protein binding to the ROBO2 ICD.

To establish further the involvement of *ROBO2* in the pathogenesis of the CAKUT-VUR phenotype postnatally, we next generated and analyzed a conditional *Robo2* mouse mutant. A previously described homozygous *Robo2* null mouse with a targeted deletion of exon 1 exhibits a multiple ureter phenotype and fails to survive after birth.[127] To determine whether heterozygosity for Robo2 loss-of-function could produce an abnormal urinary tract phenotype and recapitulate human CAKUT-VUR, we prepared a mouse *Robo2* floxed allele, *Robo2*^{flox}, containing *loxP* sites flanking *Robo2* exon 5 (Figure 4.5A). *Robo2*$^{flox/flox}$ homozygotes are viable, fertile and lack urinary tract abnormalities. We then produced *Robo2*$^{del5/+}$ mice that lack exon 5 by crossing the floxed allele with the *Tg*$^{EIIa\text{-}Cre}$ deletor strain.[134] The deletion of *Robo2* exon 5 causes a reading frameshift. RT-PCR and *in situ* hybridization experiments showed that *Robo2*^{del5} transcripts were expressed at markedly reduced levels compared to transcripts from the wild-type allele. We thus conclude that *Robo2*^{del5} is effectively a null allele. Consistent with results reported for the previously described *Robo2* null allele,[126] *Robo2*$^{del5/del5}$ homozygotes uniformly died shortly after birth with multiplex, dysplastic kidneys and short ureters (Figure 4.5B-E). However, whereas no heterozygous phenotype was described for the existing *Robo2* null allele,[126] when a *Hoxb7-GFP* reporter transgene that specifically identifies the ureteric epithelium[135] was introduced into the *Robo2*$^{del5/+}$ background, 4 of 26 (15%) *Robo2*$^{del5/+}$ heterozygous newborns exhibited a unilateral CAKUT-VUR phenotype (Figure 4.5F, G). This heterozygous phenotype included both massive and lesser degrees of megaureter and a wide-open UVJ, similar to the pathology identified in DGAP107.

To test whether further reductions in *Robo2* gene dosage could increase CAKUT-VUR penetrance, we took advantage of the variable expression of the *EIIa*-directed Cre

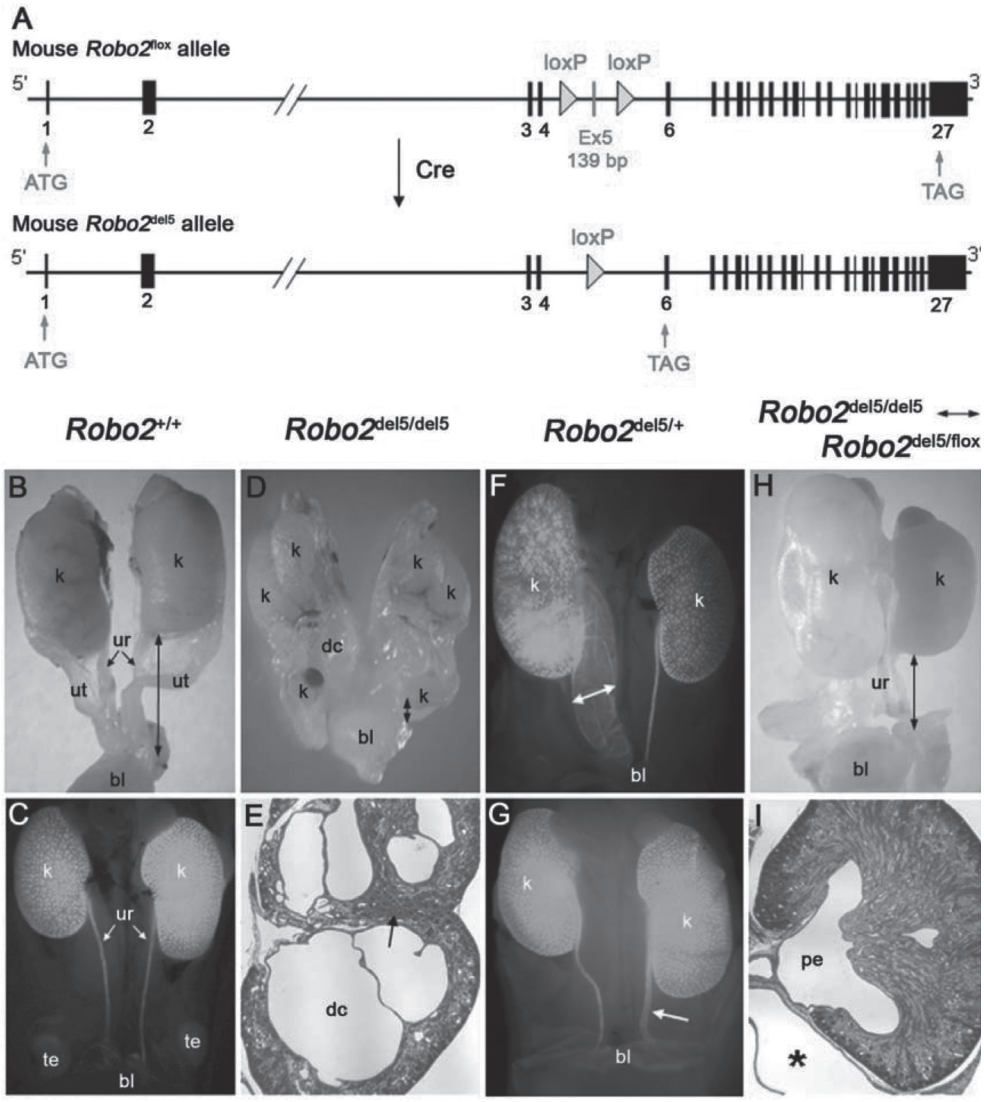

Figure 4.5 *Robo2*$^{del5/del5}$ homozygous, *Robo2*$^{del5/-}$ heterozygous, and *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic newborn mice expressing striking CAKUT phenotypes. *A*, Structures of the mouse *Robo2*^{flox} and *Robo2*^{del5} alleles. The *Robo2*^{flox} allele encodes a wild-type, full-length 1,470-aa Robo2 protein but contains two *loxP* sites flanking exon 5. The *Robo2*^{del5} allele is generated from *Robo2*^{flox} by Cre, which deletes *Robo2* exon 5 to produce an aberrant transcript expressed only at low levels. *B* and *C*, Wild-type female (*B*) and male (*C*) newborn mouse excretory system. The male excretory system in panel C is illuminated by the *Hoxb7-GFP* transgene. k = kidney; bl = bladder; ur = ureter; ut=uterus; te=testis. Black bidirectional arrows indicate ureter length in panels B and D. *Robo2*$^{del5/del5}$ newborn homozygotes display multiplex dysplastic kidneys (*D*) and, at 25# magnification (*E*), reveal dysplastic cysts (dc) in the calyces and an internalized nephrogenic zone (*arrow*). *Hoxb7-GFP* transgene-positive *Robo2*$^{del5/-}$ heterozygous newborns show megaureter dilation (*F*) (*bidirectional arrow*) and early ureter dilatation (*G*) (*arrow*). Robo2$^{del5/del5}$↔Robo2$^{del5/flox}$ mosaic newborns show hydronephrosis in the left kidney (*H*). At 25# magnification (*I*), they show megaureter (*asterisk*). Black bidirectional arrows indicate ureter length in panel H. pe = pelvis. See page 200 for a full-color representation of this figure.

recombinase in the early pre-implantation embryo[136] to generate mosaic progeny that consisted of admixtures of *Robo2*$^{del5/del5}$ (null) and *Robo2*$^{del5/flox}$ (haploinsufficient) cells. This mosaicism originates from the incomplete, stochastic action of the *EIIa-Cre* transgene upon the *Robo2*^{flox} allele in the early embryo prior to implantation.[136] Remarkably, 4 of 10 (40%) *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic newborns (resulting from the union of *Robo2*^{del5};*Tg*$^{EIIa\text{-}Cre}$ and *Robo2*^{flox} gametes) exhibited unilateral urinary tract defects including short ureter, megaureter and hydronephrosis (Figure 4.5H, I).

To determine whether *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic newborns with urinary tract defects could survive after prolonged reflux and obstruction, we followed another cohort of these mice to adulthood. Seven of 10 (70%) *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ adult mosaics, ranging in age from 45 to 77 days, manifested defects involving the uretero-vesical junction (UVJ) (Figure 4.6). These UVJ defects were bilateral and especially notable in that one UVJ was typically located laterally and cephalad in the bladder (Figure 4.6A-D), a location commonly associated with reflux in humans,[137] while the contralateral UVJ was located caudad in the bladder or even ectopically in the urethra. The caudal UVJ location was associated with obstruction, resulting in megaureter and severe hydronephrosis (Figure 4.6C, E). In some male *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mice, the ureter was connected to the vas deferens, resulting in massive hydronephrosis (Figure 4.6F). The *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mouse model is thus consistent with the frequent co-existence of reflux and obstruction in the same VUR patient.[138] During embryonic development, the nephric duct undergoes apoptosis, transposing the ureter orifice from the nephric duct to the urogenital sinus epithelium to form the UVJ.[11] Because mutation in mouse *Robo2* causes abnormal sites of ureteric bud outgrowth,[126] this provides a developmental explanation for the ectopic UVJ sites frequently observed in VUR.

DISCUSSION

Collectively, these results demonstrate that reduced *Robo2* gene dosage can contribute to the pathogenesis of CAKUT-VUR (Table 4.1, Appendix F). In human primary VUR, linkage studies have produced inconsistent results,[41] underscoring the need for other methods to identify the responsible genes. This problem is especially acute for VUR, as the manifestations may vary during life, progressing or resolving spontaneously.[139] Furthermore, because asymptomatic individuals cannot be classified as unaffected, linkage studies may be inconclusive or yield false negative results unless confined to affected individuals.[139] Lastly, intra-familial phenotypic variability and genetic heterogeneity also exist.[21, 41] Since we identified only two coding region changes segregating with VUR in 124 VUR families, alterations in *ROBO2* itself are likely to account for a small subset of VUR. Interestingly, however, recent studies indicate that *ROBO2* resides in an inherently unstable genomic region,

3p12.3, that is prone to evolutionary chromosomal rearrangements and to LOH in human cancers.[140] This raises the yet untested possibility that *ROBO2* may be subject to frequent rearrangement or microdeletion and duplication at either the organismal or cellular level, which could be missed by direct sequencing. In addition, variants in other genes whose products function in the ROBO2 signal transduction pathway may be implicated in the molecular pathogenesis of renal dysplasia and VUR, which may co-exist due to inter-related pathophysiology, a common underlying genetic abnormality, or due to both mechanisms.

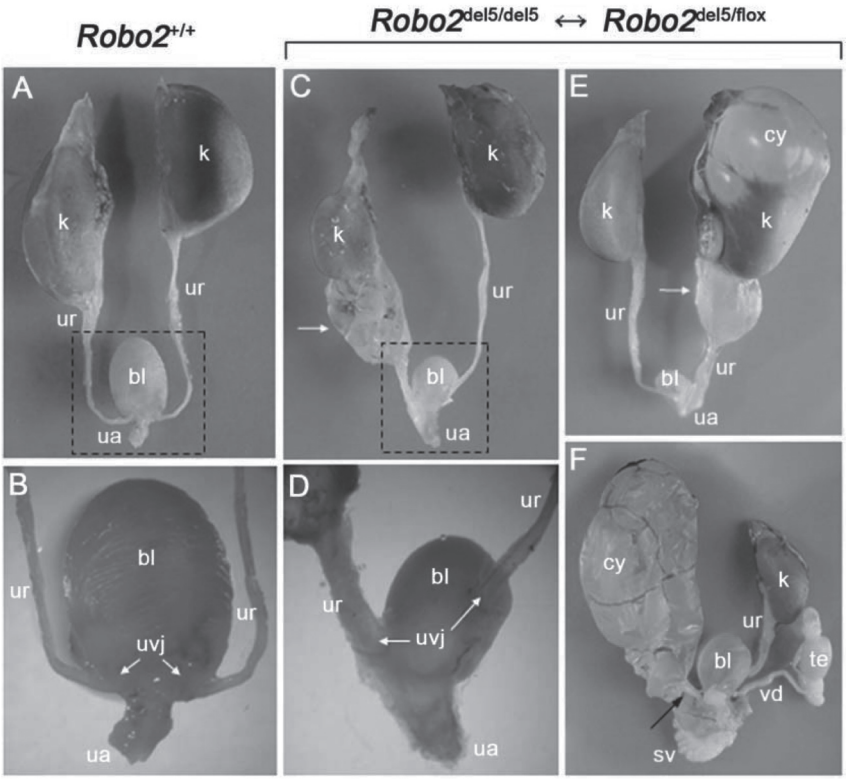

Figure 4.6 Adult *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaics exhibiting megaureter, hydronephrosis, and UVJ defects. *A–D*, Ventral views. k = kidney; ur = ureter; bl = bladder; ua = urethra. *A*, Urinary tract in a wild-type mouse aged 87 d. *B*, Higher magnification of boxed region in panel A, indicating normal position of the UVJ. *C*, Right megaureter (*arrow*) in a *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic aged 45 d. *D*, Higher magnification of boxed region in panel C, demonstrating abnormal bilateral UVJ. The obstructed right UVJ connects to a caudal site in the bladder close to the urethra, causing megaureter. The left UVJ is located laterally in the bladder, a site commonly associated with human VUR. *E* and *F*, Dorsal views. *E*, Right megaureter (*arrow*) and hydronephrosis in a mosaic aged 45 d. Hydronephrosis replaces the normal renal parenchyma (k), causing an upper pole cyst (cy). *F*, Left ureter of a male mosaic mouse aged 77 d that remains connected to the vas deferens (vd) (*arrow*), resulting in obstruction and severe hydronephrosis. The left kidney has lost all parenchyma and is replaced by a large cyst. The right kidney, ureter, and vas deferens are normal in appearance. sv = seminal vesicle; te = testis. See page 201 for a full-color representation of this figure.

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APPENDICES TO CHAPTER 4

Appendix A: DGAP107 phenotype

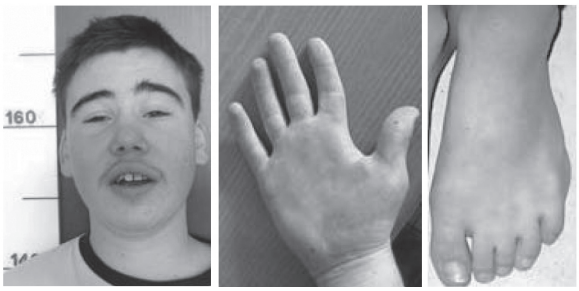

Figure 4.A1. Facial and limb abnormalities in the DGAP107 proband. Note the low set, dysplastic ears and subtle membranous syndactyly and clinodactyly. Blepharophimosis is also present. See page 202 for a full-color representation of this figure.

Table 4.A1. Clinical findings in the DGAP107 subject at age 14

Characteristic	Patient Phenotype
Weight (kg)	40 (10th percentile)
Height (cm)	146 (<5th percentile)
Visual disorders	Daltonism, strabismus, hypermetropia
Limb defects	Mild syndactyly, clinodactyly, brachymetacarpia
Urinary tract defects	Vesicoureteral reflux grade IV, bilateral UVJ defects, unilateral megaureter
Learning disabilities	Global verbal retardation, verbal IQ: 69, performance IQ: 46
Facial features	Blepharophimosis, low set and dysplastic ears
Dental anomalies	Malformed lower incisor (“T” shape)
Genital anomalies	Complete left testicular agenesis
Neurological defects	Seizures, hyperactivity, sleep disorder
Orthopedic abnormality	Hyperlordosis
Growth retardation	Delayed puberty
Other abnormalities	Bilateral inguinal hernia

Appendix B: The DGAP107 3p12 breakpoint disrupts *ROBO2*

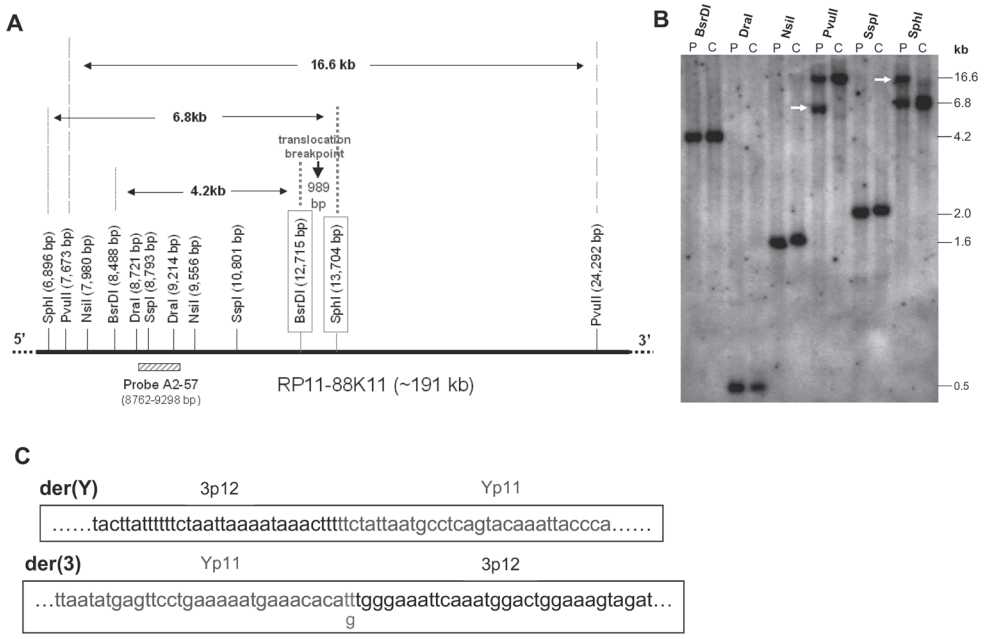

Figure 4.B1. *ROBO2* disrupted in DGAP107, with the breakpoint lying within intron 2. *A*, Restriction map surrounding the 3p12 breakpoint. The base-pair position of BAC RP11-88K11 (AC131005, within intron 2 of *ROBO2* [see BAC contig in fig. 4.1*F*]) was used to calculate the distance between restriction enzyme sites. RP11-88K11 overlaps with BAC clone RP11-54A6 used in FISH and also contains the breakpoint, which is between boxed *Bsr*DI and *Sph*I sites, on the basis of the aberrant bands detected by Southern blot analysis. *B*, Southern blot analysis of DGAP107 (P) and unaffected control (C) genomic DNA, with use of the designated restriction enzymes and the probe A2-57 shown in panel A. Aberrant bands (*white arrows*) are present only in DGAP107 DNA digested with *SphI* and *PvuII*. *C*, Breakpoint cloning showing the sequence of the junction fragment from der(3) with a 1-bp deletion (g [*green*]) and a 2-bp insertion (tt [*pink*]). There is no gain or loss of nucleotides at the der(Y) breakpoint. See page 203 for a full-color representation of this figure.

Appendix C: *ROBO2* and *PCDH11Y* expression in human adult and fetal tissues

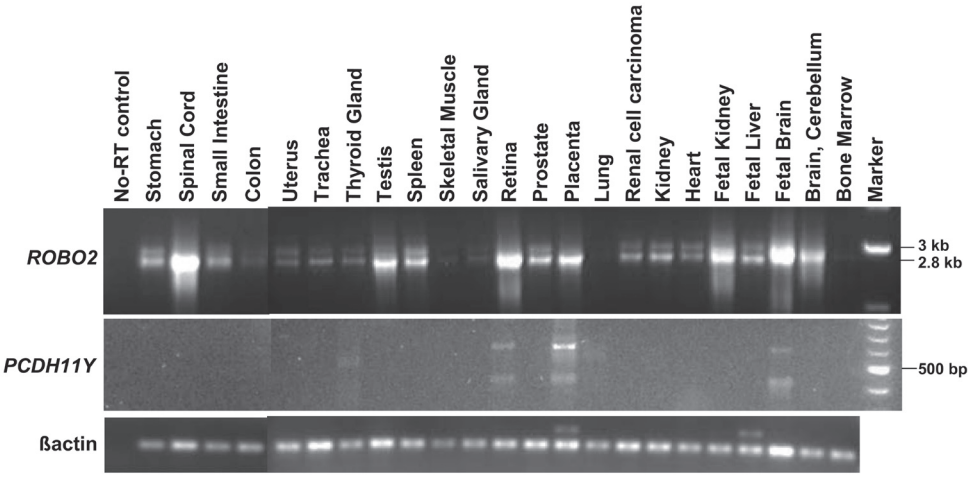

Figure 4.C1. Expression of *ROBO2* and *PCDH11Y* in human tissues. RT-PCR amplified 2.8-kb *ROBO2* cDNAs with the use of primers *ROBO2-F1* and *ROBO2-R1*. The 3-kb *ROBO2* cDNA product (*upper band of doublet*) contains an alternatively spliced exon 24B. RT-PCR amplification of 404-bp and 620-bp *PCDH11Y* cDNAs used primers *PCDH11Y-F1* and *PCDH11Y-R1*. The intensity of the fragments indicates the approximate expression level of *ROBO2* and *PCDH11Y* in these tissues. Notably, there is no expression of *PCDH11Y* in the fetal kidney. *b-actin* was used as a cDNA loading control.

Appendix D: Analysis of the DGAP107 del(17)(p11.2) microdeletion

The pleiotropic nature of the DGAP107 phenotype suggests that both the t(Y;3)(p11;p12)dn translocation and the del(17)(p11.2) microdeletion may contribute to the overall DGAP107 phenotype. However, we parse the contribution of the del(17)(p11.2) to the VUR phenotype as follows. Point mutations in *RAI1* that resides in the Smith-Magenis Syndrome (SMS) critical deletion region suggest that *RAI1* haploinsufficiency accounts for many features of SMS.[141] Less frequently observed cardiac, renal and other defects may reflect hemizygosity for other genes in the SMS common deletion region.[142] Of note, *RAI1* is included in the 3.4 Mb del(17)(p11.2) in DGAP107, the boundaries of which were defined by FISH experiments (not shown). It seems likely that the del(17)(p11.2) microdeletion contributes to some aspects of the sleep, behavioral and cognitive deficits in DGAP107, because similar phenotypes are observed in SMS patients who exhibit 17p11.2 deletions or *RAI1* haploinsufficiency. Thus, *ROBO2* disruption, 17p11.2 microdeletion, or both could theoretically account for VUR in DGAP107. Our human and mouse experimental results indicate that *ROBO2* disruption can contribute substantially to the VUR phenotype. Conversely, our analyses also suggest that while the 17p11.2 microdeletion in DGAP107 may contribute to the pathogenesis of VUR in DGAP107, it is not likely to play a primary role. There are three bases for this conclusion.

First, although we note an isolated case report describing VUR in SMS[143], re-examination of the frequency of renal defects in SMS indicates that these are relatively infrequent and less frequent than originally believed.[128, 144]

Second, in the context of this study, we examined and observed no kidney or collecting system defects in three age-matched genetically engineered mouse models of SMS: *SMS*$^{df(11)17/+}$ (Δ2Mb), *SMS*$^{df(11)17\text{-}l/+}$ (Δ500kb) and *Rai1*$^{-/-}$.[145-147] The first two of these represent deletion alleles that eliminate 2 Mb and 500 Kb respectively, from mouse chromosome 11. These regions are homologous to and share conservation of synteny with the 17p11.2 region that is involved in SMS. Although *SMS*$^{df(11)17}$ and $^{SMSdf(11)17\text{-}l}$ homozygotes die prior to nephrogenesis (E9.5), examination of 20 *SMS*$^{df(11)17\text{-}l/+}$ mice in the context of this study revealed no UVJ or other urinary tract defects. In addition, *Rai1*$^{-/-}$ are not reported to exhibit any evidence for renal or ureteral defects,[145] and our own analysis of these mice confirms this finding.

Third, analysis of the urinary tract in five *SMS*$^{df(11)17\text{-}l/+}$; *Robo2*$^{del5/+}$ trans-heterozygotes, the genotype of which nominally approximates the DGAP107 genotype, revealed no phenotype compared to genetic background and age-matched littermate controls. Thus, while the 17p11.2 microdeletion could be a contributory factor, it seems unlikely to play a major role in the pathogenesis of CAKUT and VUR phenotypes in DGAP107.

Appendix E: Clinical Data for Families 2559x and B5

Clinical Data for Family 2559x

2559x is a British Caucasian family. The index patient (25592) has bilateral VUR and bilateral nephropathy. She presented at 3 years of age with a symptomatic, documented *E. coli* urinary tract infection at which time ultrasound (US) revealed two small kidneys (each 6.0 cm long; normal mean for age, 7.0 cm). An indirect isotope cystogram with mercaptoacetyltriglycine (MAG3) showed bilateral VUR. She was treated long-term with antibiotics. At age 4, her plasma creatinine was 107 µmol/l (normal mean for age, 56 µmol/l). Her formal EDTA-glomerular filtration rate was 31 ml/min/1.73m^2 (normal mean >90 ml/min/1.73m^2), and a formal voiding cystourethrogram (VCUG) confirmed bilateral VUR. A repeat US confirmed two small kidneys with dimensions 4.7 cm left and 6.0 cm right (50th percentile for age, 7.2 cm), both with scarred upper poles. At age 9, she had progressive renal failure with a plasma creatinine of 306 µmol/l. By age 12, her plasma creatinine had risen to 407 µmol/l. Her mother (25593) has a history of VUR that required ureteral re-implantation surgery.

Clinical Data for Family B5

This is a Dutch Caucasian family. By VCUG and IVP, the proband B5 had bilateral VUR and a right kidney duplex system. Ultrasound investigations further documented the proband's double collecting system on the right side, and a single system on the left, with a slightly dilated upper pole system. Renal US studies were also performed in all other family members except the grandfather B1, who is deceased. The US findings for family B5 include: A1 (uncle): normal kidneys; right kidney, 11.0 cm; left kidney 12.5 cm (normal, 11.5±1.0 cm). C1 (grandmother): right kidney, small at 9.6 cm; left kidney, 11.4 cm, thin collecting system on both sides with a normal cortico-medullary ratio, no signs of a duplex system and normal flow in the right renal artery. D1 (aunt): right kidney, 11.8 cm with an upper pole duplex system (column of Bertini), left kidney, 11.0 cm with normal cortico-medullary ratio and no dilatation; no urologic complaints. E1 (father): both sides, thin collecting system with normal kidneys, small peripelvic cyst on the right side, no urologic complaints. F1 (mother): left kidney with fetal lobulation on the right side; no visible column from the renal parenchyma to the hilus; a duplex system can neither be proven nor excluded; collecting system is thin on both sides; febrile UTI history. G1 (aunt): no dilatation on either side of kidney; the upper pole of the left kidney contains a cyst (4.8 x 3.5 cm); pyelonephritis at age 8.

Controls

All controls for genetic studies on Families 2559x and B5 were unrelated. 180 control samples were from Americans of European Caucasian descent (CEPH). The remaining 96 control samples were from Caucasians in the same geographic region as Family B5. These latter 96 samples were subjected to both targeted sequencing of exons 12, 19 and 23 and to complete resequencing of all 26 coding *ROBO2* exons.

Appendix F: Analysis of *Robo*^{del5} mutant mice

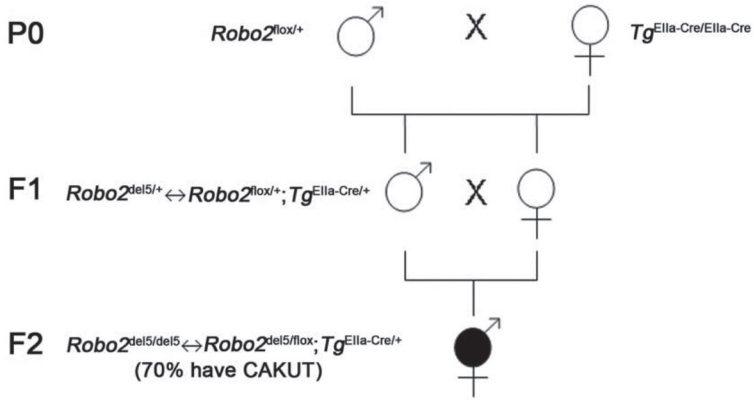

Figure 4.F1. Generation of F2 *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaics

Table 4.F1. Phenotype-genotype correlation in F2 *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaics

The following F2 offspring were obtained:			
Identification number	**Age (d)**	**Sex**	**Phenotype**[a]
3235	45d	F	L and R megaureter, hydronephrosis
3239	45d	F	R: megaureter, hydronephrosis; L: normal
404	62d	M	L: megaureter, hydronephrosis with complete loss of renal parenchyma; R: normal
3218	77d	M	R: megaureter, hydronephrosis with complete; loss of renal parenchyma; L: normal
3236	77d	F	No discernible gross phenotype
403	62d	M	No discernible gross phenotype
3372	27d	M	No discernible gross phenotype
3964	20d	F	L: megaureter, hydronephrosis; R:normal
3963	20d	F	L: megaureter, hydronephrosis; R: normal
3962	20d	F	L and R: megaureter, hydronephrosis

Note: Genotyping was performed using PCR of mouse tail DNA. All mosaics were positive for *Robo2*^{del5}, *Robo2*^{flox}, and Tg$^{Ella-Cre}$. [a] L = left; R = right

Notes on the breeding scheme

In the P0 generation, *Robo2*$^{flox/+}$ heterozygotes were crossed to *Tg*$^{EIIa\text{-}cre/EIIa\text{-}cre}$ homozygotes. The 50% of the progeny that receive *Tg*$^{EIIa\text{-}cre}$ and *Robo2*^{flox} gametes constitute the F1 generation shown. When transmitted from males, the *Tg*$^{EIIa\text{-}cre}$ is variably expressed in the F1 embryos after fertilization and prior to implantation[134,135], resulting in recombination in some embryonic cells and not in others. Thus, the resulting F1 progeny are mosaic, and contain mixtures of *Robo2*$^{flox/+}$ (no recombination) and *Robo2*$^{del5/+}$ (recombination) cells. These mosaics are genotypically denoted *Robo2*$^{flox/+}$↔*Robo2*$^{del5/+}$;*Tg*$^{EIIa\text{-}cre/+}$. PCR experiments demonstrated variable ratios of the *Robo2*^{flox} and the *Robo2*^{del5} alleles, confirming the mosaicism of the F1 mice (not shown). As expected, ten F1 mosaic mice examined exhibit no phenotype, because heterozygous *Robo2*$^{del5/+}$ mice exhibit a phenotype at low percentage (i.e., 15%), and even at the most extreme degree of mosaicism (100% *Robo2*$^{del5/+}$, 0% *Robo2*$^{flox/+}$), the overall reduction in *Robo2* gene dosage would not surpass that in *Robo2*$^{del5/+}$ heterozygotes.

The F2 generation was generated by intercross of the subset of F1 germline mosaics that also carried the *Tg*$^{E11a\text{-}cre/+}$ transgene. The F2 generation thus also consisted of mosaic progeny, due to the union of *Robo2*^{del5}; *Tg*$^{EIIa\text{-}cre}$ and *Robo2*^{flox} gametes. This particular combination of gametes results in a subset of F2 mosaics that contain both *Robo2*$^{del5/del5}$ and *Robo2*$^{del5/flox}$ cells; i.e., a mixture of heterozygous and homozygous cells. Cells carrying the *Robo2*$^{del5/del5}$ genotype in these *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaics derive from the action of Cre in cells that commence embryogenesis with the *Robo2*$^{del5/flox}$ genotype; *Robo2*$^{del5/flox}$ cells result when Cre activity in those cells is insufficient to effect recombination. As described in the text and in Table 4.F1, 70% of these mosaics exhibit striking CAKUT phenotypes, while 30% exhibit no phenotype. The presence of a CAKUT phenotype presumably correlates with the percentage of *Robo2*$^{del5/del5}$ cells that comprise the urinary tracts of these mosaics.

The mosaic nature of the F2 mice (at least in the germline) was established by further intercross of a subset of F2 *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$;*Tg*$^{EIIa\text{-}cre}$ mosaics to generate an F3 generation. In this case, a high percentage of *Robo2*$^{del5/del5}$ cells in the F2 germline result in a preponderance of gametes carrying the *Robo2*^{del5} mutant allele. The union of these *Robo2*^{del5} gametes results in an increased proportion of *Robo2*$^{del5/del5}$ mice (90% of progeny) in the F3 generation that die at birth from CAKUT phenotypes (not shown). The remaining 10% retain the *Robo2*$^{del5/flox}$ allele and survive. The non-Mendelian ratio of these resulting F3 genotypes confirms germline mosaicism in the F2 mice.

Appendix G: PCR Primers

Table 4.G1. PCR primers and probesPrimer

Primer or Probe Used[a]	Type	Location	Sequence
RT-PCR analyses:			
2.8-kb *ROBO2* cDNA[b]:			
ROBO2-F1	Forward	Exon 9 of *ROBO2*	5'-GAGCAAGGCACACTGCAGATTA-3'
ROBO2-R1	Reverse	Exon 26 of *ROBO2*	5'-AGTCATCACTTCCATGAGTCCG-3'
404-bp and 620-bp *PCDH11Y* cDNA[b]:			
PCDH11Y-F1	Forward	Exon 1 of *PCDH11Y*	5'-CAGAAACAACCTCAGCGACTCC-3'
PCDH11Y-R1	Reverse	Exon 4 of *PCDH11Y*	5'-GAACACCACGCATACTAGCAGG-3'
***ROBO2-PCDH11Y* fusion transcripts[c]:**			
ROBO2-F2	Forward	Exon 2 of *ROBO2*	5'-GCAGTGAGTCGAAATGCGTC-3'
PCDH11Y-R1	Reverse	Exon 4 of *PCDH11Y*	5'-GAACACCACGCATACTAGCAGG-3'
PCDH11Y-R2	Reverse	Exon 3 of *PCDH11Y*	5'-CCCACTGTCATTCAGTCCTCAT-3'
PCDH11Y-R3	Reverse	Exon 5 of *PCDH11Y*	5'-TGTTTCAATGACATCGAGGCC-3'
606-bp *ROBO2* nontranslocated allele[c]:			
ROBO2-F2	Forward	Exon 2 of *ROBO2*	5'-GCAGTGAGTCGAAATGCGTC-3'
ROBO2-qR	Reverse	Exon 7 of *ROBO2*	5'-TGGCCGAACCACAAACTGT-3'
Quantitative real-time PCR analyses:			
***ROBO2* nontranslocated allele[d]:**			
ROBO2-F2	Forward	Exon 2 of *ROBO2*	5'-GCAGTGAGTCGAAATGCGTC-3'
ROBO2-qR2	Reverse	Exon 3 of *ROBO2*	5'-GCTCTCCAGCTGCCACTACAA-3'
ROBO2-FAM2	TaqMan probe	*ROBO2* exon 2-exon 3 junction	5'-CTGGAAGTGGCATTGTTACGAGATGACTTCC-3'
Fu-129 transcript[d,e]:			
ROBO2-F2	Forward	Exon 2 of *ROBO2*	5'-GCAGTGAGTCGAAATGCGTC-3'
PCDH11Y-qR1	Reverse	Exon 1d of *PCDH11Y*	5'-TATTCCATCCTCTTCCATCCATTC-3'
TaqMan 129	TaqMan probe	*ROBO2* exon 2 and *PCDH11Y* exon 1d junction	5'-CTGGAAGTGGCATAGGGTGCTTAAAAAGTACAGA-3'
Fu-153 transcript[d,e]:			
ROBO2-F2	Forward	Exon 2 of *ROBO2*	5'-GCAGTGAGTCGAAATGCGTC-3'
PCDH11Y-qR2	Reverse	Exon 4 of *PCDH11Y*	5'-ATGTACGTCCCGGACAACAAA-3'
TaqMan 153	TaqMan probe	*ROBO2* exon 2 and *PCDH11Y* exon 4 junction	5'-TGGAAGTGGCATTGTTGTGCGGGT-3'
Genotyping of *Robo2*^{flox} and *Robo2*^{del5} mice:			
***Robo2*^{flox} allele[f]:**			
Ro2-MEBAC15F	Forward	Intron 5 of *Robo2*	5'-CCAATCATAGTCTCTCCACG-3'
Ro2-MEBAC15R	Reverse	Intron 5 of *Robo2*	5'-CCTCTGATTCAATGAGATGC-3'

1,180-bp fragment from *Robo2*^{del5} allele:			
Robo2koF	Forward	Intron 4 of *Robo2*	5'-CCACTATGCTGGCTCTGTCTCACAC-3'
Robo2R	Reverse	Intron 5 of *Robo2*	5'-GGTTTTGGAGGTCTTACTACGTAGC-3'
1,390-bp fragment from *Robo2*$^{+}$ wild-type allele:			
Robo2wtF	Forward	Intron 4 of *Robo2*	5'-CAACTTTTCCTTTTCCGGGAGG-3'
Robo2R	Reverse	Intron 5 of *Robo2*	5'-GGTTTTGGAGGTCTTACTACGTAGC-3'
Synthesis of fusion protein constructs:			
Fu-129 *ROBO2-PCDH11Y* fusion cDNA (129-aa fusion protein):			
5’-hR2(E1)	Forward	Exon 1 of *ROBO2* with 5' EcoRI linker	5'-ATCGAATTCATGAGTCTGCTGATGTTTACACAACTACTG-3'
3’-LEVA(X1)	Reverse	Intron 1d of *PCDH11Y* with 5' XhoI linker	5'-TTACTCGAGCTATGCCACTTCCAGAGACGCATTTCG-3'
Fu-153 *ROBO2-PCDH11Y* fusion cDNA (153-aa fusion protein):			
5'-hR2(E1)	Forward	Exon 1 of *ROBO2* with 5' EcoRI linker	5'-ATCGAATTCATGAGTCTGCTGATGTTTACACAACTACTG-3'
3'-SRSC(X1)	Reverse	Intron 4 of *PCDH11Y* with 5'XhoI linker	5'-CCACTCGAGCTAGCAGGACCGCGAAAATGTACGTCC-3'

[a] Primers and probes were used for amplification and quantitation.
[b] As shown in figure 4.C1.
[c] As shown in figure 4.2B.
[d] As shown in figure 4.2C.
[e] As shown in figure 4.2D.
[f] PCR amplifies a 1,100-bp fragment from both wild-type and Robo2^{flox} alleles. After *Spe*I digestion, *Robo2*^{flox} allele will not cleave (1,100-bp product),whereas the wild-type allele will be cleaved by SpeI to yield two smaller products of 750 bp and 350 bp.

Table 4.G2. PCR Primer Sets Used to Amplify 26 Human *ROBO2* Exons and Intron-Exon Boundaries in Mutation Analysis

Exon and Primer		Amplicon size (bp)	Location in *ROBO2*	Sequence
1:	Forward	286	5'-UTR	5'-TTTGCTCTTCTTGACTTTAATTAGTATCTAGG-3'
	Reverse		Introns 1–2	5'-TATAACCCACATCAAATTCAAAAAGAAAT-3'
2:	Forward	501	Introns 1–2	5'-CGAAGAGTTTAATTTCCCCATCA-3'
	Reverse		Introns 2–3	5'-GCGTCTATGGGAACACATCAAAA-3'
3:	Forward	264	Introns 2–3	5'-TTGTACAACAAAAAGCCTAAGTTACTGTC-3'
	Reverse		Introns 3–4	5'-AAAATTCAATCTCTCTGGGCCAT-3'
4:	Forward	280	Introns 3–4	5'-TAATGACCTTTATTTTCTATTCTGTTCCTTT-3'
	Reverse		Introns 4–5	5'-TTATATGGCCCAGTTTTTAATGTTAGTAATACT-3'
5:	Forward	291	Introns 4–5	5'-CTTTTTTCATAATGTACTTAAAGCATGCA-3'
	Reverse		Introns 5–6	5'-GTGGCATTGTAGCTGTCCTTTTATT-3'
6:	Forward	427	Introns 5–6	5'-TTGCACTTTGTGGCTGATTTG-3'
	Reverse		Introns 6–7	5'-TAATTTTATTTCAACTAATGATAGAGAGGACAC-3'
7:	Forward	289	Introns 6–7	5'-CAACATAGTACCATATTTTCTCCTTGACATA-3'
	Reverse		Introns 7–8	5'-AAGCAAGGCAAGCTTTCAGG-3'
8:	Forward	336	Introns 7–8	5'-CCCACTGTATTCCTTAATTGTAGTAGCTT-3'
	Reverse		Introns 8–9	5'-TCCACATGGTTAACGTGTATCTAGAAA-3'
9:	Forward	351	Introns 8–9	5'-TTCAGTGTCAATATATCAAGCCTACTGA-3'
	Reverse		Introns 9–10	5'-CACTATGCAATTTTTCCATAGAGCAG-3'
10:	Forward	231	Introns 9–10	5'-TGGCTGTCATTGAGTAATTATTCTGC-3'
	Reverse		Introns 10–11	5'-TCCCCCCTTAACTTATTATTTGATATTG-3'
11:	Forward	343	Introns 10–11	5'-CTGTCTAGGTCAGGTCCTTTAGTAGACTG-3'
	Reverse		Introns 11–12	5'-CAGCAGGATAGTTCAGGTGACATT-3'
12:	Forward	324	Introns 11–12	5'-AACCTTTGTCATTGATACCCAACTC-3'
	Reverse		Introns 12–13	5'-TCCTCATCAAGCCCCTCGT-3'
13:	Forward	298	Introns 12–13	5'-AGTTCTAAAGACATGAGGTTGATTTACATAA-3'
	Reverse		Introns 13–14	5'-CACTCTTTGTTCATTTGCATTTTCC-3'
14:	Forward	409	Intron 13–14	5'-AGGACAGAAATGGGACAAATGAA-3'
	Reverse		Intron 14–15	5'-TTCTAAGGAAGATAACAAATAGGTACTGTAACA-3'
15:	Forward	294	Introns 14–15	5'-AGTCTCCTGCAACTTGTCTTTATACTCAT-3'
	Reverse		Introns 15–16	5'-TCATTCGTGAGACACTGAGATTTCT-3'
16:	Forward	321	Introns 15–16	5'-AATATTTGATCAGTTACAGTAGTCTCGTTACC-3'
	Reverse		Introns 16–17	5'-TGCAAAATCATCATCCACCTTG-3'
17:	Forward	338	Introns 16–17	5'-TCTTCATTTTTGATGCACCATGT-3'
	Reverse		Introns 17–18	5'-TTTCTGTTCCTTCCATTCATTTCAT-3'
18:	Forward	211	Introns 17–18	5'-CCTCAGCTCTAAACTAAGGGCCA-3'
	Reverse		Introns 18–19	5'-TCTTACTATAGAGTTTCCCCAGTCCTG-3'
19:	Forward	240	Introns 18–19	5'-AAATCTTCCATTTCTTAACGCTTTATATTG-3'
	Reverse		Introns 19–20	5'-AAAAACACAACTTACCTCCACGG-3'

20:	Forward	432	Introns 19–20	5'-GATAGTTTTGGGCTTCCGGTG-3'
	Reverse		Introns 20–21	5'-TGAATCACTAAGTCAAACAACAAATACTAATT-3'
21:	Forward	306	Introns 20–21	5'-CATAAATACACCTTGCCATCTGATG-3'
	Reverse		Introns 21–22	5'-TGGCAAAAATGAACAACAGAGAG-3'
22:	Forward	411	Introns 21–22	5'-TGCATGTATGTGTATATGTATTTGTGTCA-3'
	Reverse		Introns 22–23	5'-TGTAGTTCTATCAGAATCTCTTGTGAATTTATT-3'
23:	Forward	379	Introns 22–23	5'-AAGACAGTATGAGTTACTATAGCATGCATTT-3'
	Reverse		Introns 23–24	5'-GGAAGTAGTTGACTTTTGATGCATTTTA-3'
24:	Forward	325	Introns 23–24	5'-AGGTAGATTTACAGGTTAGTCATAGTGCA-3'
	Reverse		Introns 24–25	5'-CATGGAGCACGTCTCTTCAGC-3'
25:	Forward	351	Introns 24–25	5'-TGGTAAAGTAGGCCATTCACAGTG-3'
	Reverse		Introns 25–26	5'-CAAGATTCTTTCTGAATCACGATAGC-3'
26:	Forward	574	Introns 25–26	5'-TCACAAACTCATCTATCTGAAGACCTTAT-3'
	Reverse		3'-UTR	5'-AAAATTGCAGTGCAAAATTTAAACA-3'

WEB RESOURCES

Accession numbers and URLs for data presented herein as follows:
BAC/PAC Resources, http://bacpac.chori.org/
DGAP, http://dgap.harvard.edu
Ensembl, http://www.ensembl.org/Homo_sapiens/index.html
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *ROBO2* [accession number NM_002942] and *PCDH11Y* [accession number NM_032971])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for VUR and SMS)

Table 4.1. Penetrance of CAKUT in Robo2^{del5} mutant mice

Genotype	***Robo2*$^{del5/+}$ newborn**	***Robo2*$^{del5/flox}$ mosaic newborn**	***Robo2*$^{del5/flox}$ mosaic adult**	***Robo2*$^{del5/del5}$ newborn**
CAKUT penetrance (%)	15	40	70	100
Total number observed	26	10	10	20

CHAPTER 5

Linkage study of 14 candidate genes and loci in four large Dutch families with vesico-ureteral reflux

Previously published in Pediatric Nephrology,[148]
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A.M. van Eerde, B.P. C. Koeleman, J.M. van de Kamp, T.P.V.M. de Jong, C. Wijmenga, J.C. Giltay

ABSTRACT

Vesico-ureteral reflux (VUR) is a major contributing factor to end-stage renal disease in paediatric patients. Primary VUR is a familial disorder, but little is known about its genetic causes. To investigate the involvement of 12 functional candidate genes and two reported loci in VUR, we performed a linkage study in four large, Dutch, multi-generational families with multiple affected individuals. We were unable to detect linkage to any of the genes and loci and could exclude the *GDNF, RET, SLIT2, SPRY1, PAX2, AGTR2, UPK1A* and *UPK3A* genes and the 1p13 and 20p13 loci from linkage to VUR. Our results provide further evidence that there appears to be genetic heterogeneity in VUR.

INTRODUCTION

Vesico-ureteral reflux [VUR (MIM 193000)], the retrograde passage of urine from the bladder, is one of the most commonly detected congenital anomalies. With a prevalence of approximately 1%,[1] VUR can be primary, due to an incompetent valve mechanism at the uretero-vesical junction, or secondary, due to a functional or anatomical urethral obstruction. VUR is often accompanied by nonneuropathic bladder/sphincter dysfunction (NNBSD). This complex is a major cause of urinary tract infections in children[149] and the sometimes resulting reflux nephropathy is the cause of approximately 7% of end-stage renal disease in paediatric patients in the Netherlands.[7] Severe primary VUR can concur with congenital renal insufficiency based on hypoplasia/dysplasia of one or both kidneys. Genetic factors play an important role in the aetiology of primary VUR, since siblings of affected children have a 32% risk of VUR,[150] and since there is 80% concordance between monozygotic twins.[19] VUR may occur in isolation or as part of a syndrome, such as renal-coloboma syndrome. Apart from the recently published involvement of *ROBO2*[91] little is known about the genetic causes of isolated primary VUR in humans. The aim of the present study was to confirm linkage to published candidate loci and genes. So far, only one genome wide linkage study has been reported, which showed significant linkage to a 17 cM locus on chromosome 1p13 in five Caucasian families and suggestive linkage to chromosome 20p13.[21] To date, these results have not been replicated.[41] Embryonal ectopic ureteral budding has been proposed to be a mechanism for the development of VUR.[9, 151] Defects of the *RET* and *GDNF* genes have been shown to cause ectopic ureteral budding.[16, 17] Hence, these and other genes involved in the *RET/GDNF* pathway are obvious functional candidate genes for VUR. Genes involved in syndromal VUR and genes derived from mouse models with urinary tract abnormalities (such as *AGTR2*) are also attractive functional candidate genes for VUR. The aim of this study was to assess the 1p13 and 20p13 loci and appropriate candidate genes (Table 5.1) for their role in the Dutch VUR population by performing linkage analysis in four large families.

METHODS

DNA of four unrelated Dutch VUR families was collected (Figure 5.1), which had been ascertained as part of a previous study.[36] Of a total of 51 samples there were 21 affected individuals. The families provided moderate power to detect linkage as calculated with SLINK (probability of obtaining LOD scores of at least 1.0, 2.0, or 3.0 was 74%, 49% and 18%, respectively). An affected phenotype for index patients was based on their having been treated for primary VUR, while for family members it was based on having a positive case history (of actual VUR, or multiple urinary tract infections with high fever as a child, or evidence of reflux nephropathy, such as requiring renal replacement therapy without obvious other causes) (see also Figure 5.1). All other family members were classified as "unknown", despite negative imaging results at a young age in some of them. Dutch paediatric urologists consider the use of voiding cysto-urethrography (VCUG) in asymptomatic children just for research purposes inappropriate. Therefore, we could not classify family members as "not affected".

Some of the candidate genes play roles in congenital anomalies of the kidney and urinary tract (CAKUT) phenotypes (such as VUR, duplex collecting system and renal hypoplasia in mice).[9, 31]

Table 5.1. Genes tested in linkage study of four large multi-generational VUR families (LOD logarithm of the odds, NPL non-parametric linkage, HLOD heterogeneity LOD, A ureteral budding, B *RET/GDNF* pathway, C mouse and human phenotype, D linkage study, E in urothelial plaque with UPK3A (mouse model), F mouse model)

Gene	Relevance	Chromosome	Location (cM)	Multi-point LOD score at the gene location	NPL	NPL *p*-value	alpha[b]	HLOD[b]	Reference
GDNF	A/ B	5	54	-2.03	0.70	0.22	0.10	0.01	161
RET	A/ B	10	66	-2.55	-0.85	0.80	0.00	0.00	161
SLIT2	A/ B	4	34	-2.15	0.35	0.32	0.15	0.80	162
SPRY1	A/ B	4	126	-3.25	-0.98	0.86	0.00	0.00	162
PAX2	A/ B	10	124	-3.43	-0.49	0.63	0.00	0.00	161
AGTR2	A/ C	X	71	-3.81	-1.18	0.88	0.00	0.00	31
HLADRB1	D	6	46	-1.84	0.25	0.35	0.20	0.16	163
UPK1A	E	19	61	-2.90	-0.62	0.69	0.00	0.00	35, 164
UPK1B	E	3	138	0.15	1.24	0.12	0.65	0.43	35, 164
UPK2	E	11	115	-1.50	-0.22	0.52	0.00	0.00	35, 164
UPK3A	A/F	22	53	-3.40[a]	-1.20	0.80	0.00	0.00	35, 164
UPK3B	E	7	89	-1.08	0.59	0.25	0.00	0.00	35, 164

[a] Two-point analysis of marker D22S928; 0.5 cM away from *UPK3A*.
[b] Alpha: estimated proportion of families linked to result in corresponding heterogeneity LOD (HLOD). HLOD analyses were performed, but did not contribute and are not discussed.

Therefore, families in which one or more patients had these kinds of phenotypes (and VUR) were not excluded. All participants gave their informed consent, and the Medical Ethics Committee of the University Medical Centre Utrecht approved the study.

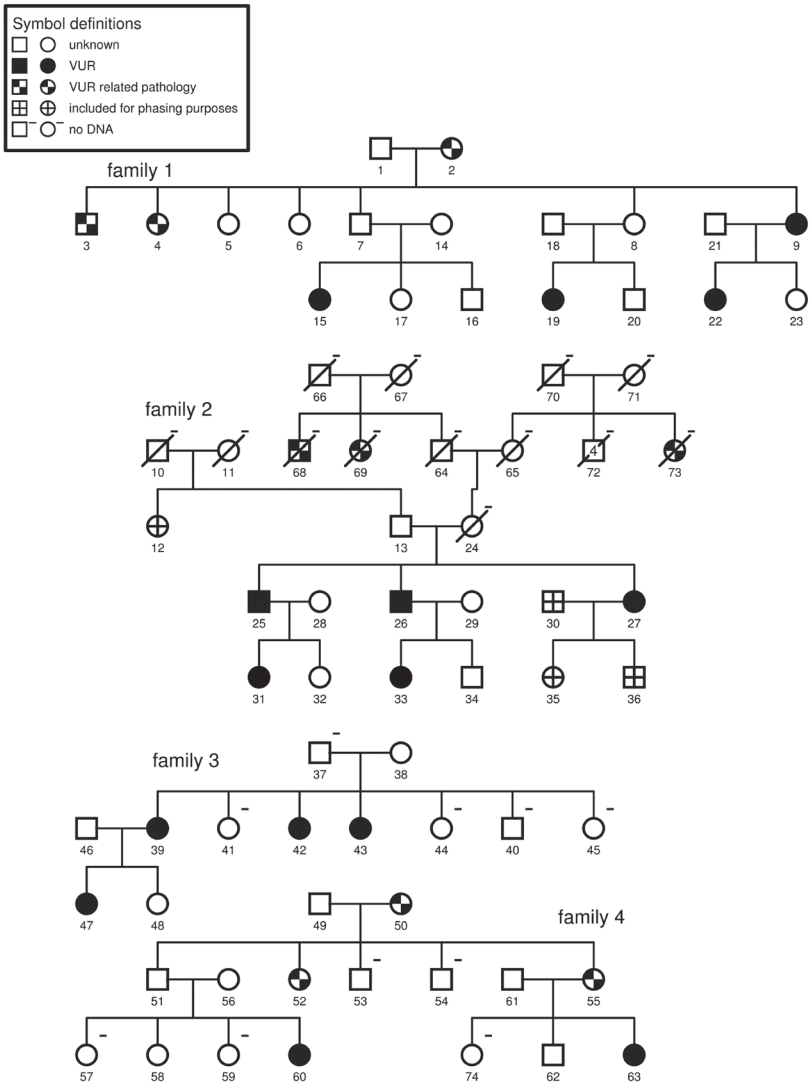

Figure 5.1. VUR family pedigrees (21 affected individuals in 51 samples). Family 1: 2 left kidney had to be removed at 5 years; 3, 4 end-stage renal disease (ESRD); 9 VUR and ESRD; 15 VUR and duplex collecting system; 19 VUR; 18 VUR and nephropathy. Family 2: 68, 69, 73 not included in analyses; 25, 26, 30 and 31 VUR; 33 VUR and dysfunctional voiding. Family 3: 39, 42, 43 VUR and dysfunctional voiding symptomatology; 47 VUR, dysfunctional voiding, meatal stenosis. Family 4: 50 and 52 recurrent urinary tract infections (UTIs) as a child, duplex collecting system; 55 UTIs and urinary tract operation; 49 VUR; 51 VUR and dysfunctional voiding symptomatology

For the 1p13 locus, we started out with the markers flanking the reported 1p13 linkage peak (D1S1653 and GATA176C01).[21] Both markers now have different map locations if one is reviewing the most recent updates of the Ensembl (v38) and Marshfield databases. In fact, the telomeric marker GATA176C01 (D2S2972) even maps to a different chromosome (2q11). The centromeric marker D1S1653, which in our query result has roughly the same genetic position (164.09-166 cM) as previously published,[21, 41] localizes on chromosome 1q23. Therefore, we tested both the entire 1p13–1q23 and 2q11 loci for linkage to VUR. A total of 11 short tandem repeat polymorphism (STRP) markers for 1p13–1q23 and seven STRP markers for 2q11 (with an average intermarker distance of 5 cM) were chosen to saturate the regions spanning 55.3 Mb on chromosome 1 and 46.3 Mb on chromosome 2. For 20p13, five STRP markers were selected, spanning 12.0 Mb (Supplementary Table 5.1).
For the candidate genes, we aimed to cover the specific location with an average intermarker distance of 2 cM (Supplementary Table 5.1).
Markers were genotyped as described elsewhere[152] in the 51 family members, together with three Centre d'Etude Polymorphism Humaine (CEPH) reference samples and three negative controls. The polymerase chain reactions (PCRs) were carried out on a GeneAmp PCR system 9700 machine (Applied Biosystems). The PCR products were separated on an ABI 3730 DNA sequencer (Applied Biosystems). The output was analysed with Genemapper 3.5 software (Applied Biosystems). Two investigators checked all the genotypes, and we verified the identity of the markers by comparing genotypes of the CEPH reference samples with the CEPH genotype database. A Mendelian inheritance check was performed with PedCheck 1.1 software,[153] and samples with Mendelian errors were excluded from the linkage analysis.
Multi-point (both parametric and non-parametric) analyses were performed for all markers with GENEHUNTER (version 2.1_r2 beta), or GENEHUNTER PLUS (for Xlinked dominant calculations in *AGTR2*).[154] We assumed an autosomal dominant model with reduced penetrance (0.8) for the parametric analyses, similar to the parameters previously described.[20, 21] This mode of inheritance agreed most with our pedigrees (Figure 5.1). The phenocopy rate was equal to the population frequency of VUR (0.01). Disease allele frequency was assumed to be 0.01. Regions with a parametric LOD score ≤ −2 were defined as exclusion regions.[155] All significance levels applied in this study were based on previously proposed thresholds.[155, 156]

RESULTS

Twelve functional candidate genes were screened for linkage to VUR. The multi-point LOD score obtained for each of the 12 genes (at the genetic location of the gene) is shown in Table 5.1, together with the non-parametric linkage (NPL) score and corresponding *P* value. Multi-point LOD scores with NPL score and corresponding *P* value for all markers are shown in

Supplementary Table 5.1. Eight of the functional candidate genes (*GDNF, RET, SLIT2, SPRY1, PAX2, AGTR2, UPK1A* and *UPK3A*) were completely excluded. For the other four, the results were inconclusive, although linkage is highly unlikely.

For the reported linkage regions, no significant linkage was detected either. One of the markers reported to be on chromosome[121] appeared to reside on chromosome 2 (see Methods section). Therefore, both the original locus on chromosome 1p13 and the "new" locus on chromosome 2q11 were tested. The chromosome 1 locus was completely excluded, as the multi-point LOD score was below −2 for the entire region. Sixty-one percent of the locus on chromosome 2 could be totally excluded. The locus on chromosome 20p13 was completely excluded (Figure 5.2).

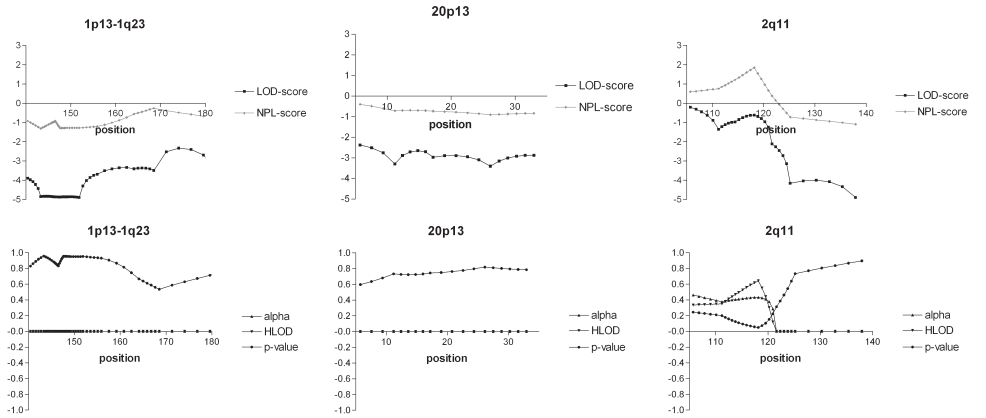

Figure 5.2. Multi-point LOD plots for the reported loci tested in the linkage study of four large multi-generational VUR families. Because one of the flanking markers of the 1p13 locus proved to actually map to chromosome 2, we also tested the chromosome 2q11 region. HLOD (heterogeneity LOD) analyses did not contribute. Positions in cM

DISCUSSION

We performed a comprehensive screen of 12 functional candidate genes and two reported loci (which later proved to be three separate regions). All the genes, except *HLADRB1*, had, in some way, been proven to play a role (indirectly) in ectopic ureteral budding and were thought likely to play a role in human primary VUR. However, we did not detect linkage to any of them. We were able to exclude eight genes (*GDNF, RET, SLIT2, SPRY1, PAX2, AGTR2, UPK1A* and *UPK3A*) as major players in these Dutch VUR families. *ROBO2*, the receptor of SLIT2,[126] had already been ruled out, since it had been sequenced in the four probands in a parallel study; no mutations were detected,[91] therefore it was not included in the present study. Nevertheless, these genes may still be involved in the mechanism causing VUR. The moderate power these pedigrees provided to reveal linkage means that it is possible that the

genes that showed inconclusive results might have yielded positive results in a more highly powered study. Since the *RET/GDNF* pathway plays such a central role in ureteral budding, it is plausible that these genes are, indeed, causative factors for VUR but that the pathogenetic variants lie in upstream or downstream regulatory elements. Other genes that are more or less directly involved in the *RET/GDNF* pathway or in ureteral budding in general, such as *EYA1, GATA3, WT1* or *BMP4*,[157] may also contribute to VUR.

We could not confirm linkage for any of the reported loci, and we were even able to exclude completely the 1p13 and 20p13 loci. This is the second non-replication of the 1p13 region.[41] Our data show that the 1p13 locus resides either on chromosome 1p13 to 1q23 or on 2q11. We also excluded linkage to most of this 2q11 locus.

We realize that these families show some intra-familial and inter-familial heterogeneity. This heterogeneity, however, is similar to that described in VUR families by others.[21] In older generations (Figure 5.1) it is impossible to have more data than self-reported history, but the described phenotypes are very likely to be caused by VUR. Therefore, we did assign the affected status to these patients, but we are aware of the fact that this is one of the limitations of the study.

One of the major obstacles for linkage studies in VUR is the relative rarity of large pedigrees, which is due to many children growing out of the disorder, the reduced penetrance of the trait, and the locus heterogeneity.[41] Furthermore, when doing linkage studies in relatively few families, one assumes a large effect of one or few genes. Maybe the genes of interest do play a role, but their effect is too small to be picked up. Therefore, studies like ours and those published[21, 41] are useful to search for one or more major genes. For this reason, association studies with large sample sizes may offer a better approach for unravelling the genetics of VUR. Both a hypothesis-free (genome wide) approach and a more elaborate candidate gene study would be interesting follow-up studies. Alternatively, it might be interesting to study the role of copy number variants (CNVs) in VUR, since, recently, such CNVs were proposed to be involved in the mechanism underlying a number of complex disorders.[158, 159] Apart from those in a recent study by Lu et al.,[91] no genes have been published that appear to be directly involved in primary VUR in humans, and no replication of the linkage peak on 1p13[21] has been reported. Our results provide further evidence for genetic heterogeneity in VUR. We hypothesize that several genes, which still have to be identified but which are likely to affect ureteral budding, will each play a role in the pathogenesis of VUR.

ACKNOWLEDGEMENTS

We would like to thank Jackie Senior for critically reading the manuscript. Our gratitude goes out to Arend Bökenkamp for referring new patients in family 1. A.M.v.E. was supported by a grant from the Dutch Kidney Foundation (no. C02.2009).

Supplementary Table 5.1. Markers used in linkage study of four large, Dutch, multi-generational VUR families. MPL: Multi-point LOD score

Locus/ gene	Chromo-some	Marker	Position (cM)	Position (bp)	Forward primer sequence
1p13-1q23	1	D1S3723	140.39	[b]	ATCTCATCAAGGTCAAATCCC
	1	D1S2695	143.31	110497630	GGAGGGCACTGGCTACA
	1	D1S187	145.45	112162152	AGGTGTGAGCTGTTCTCATA
	1	D1S502	146.53	[b]	GGGTCACCTCTGAGGA
	1	D1S2746	147.60	112827308	TAGCCTGGGCAACATAGATA
	1	D1S1675	149.20	114541676	CTAGCCAAGGCAGGTCTGTA
	1	D1S534	151.88	119479787	AGCACATAGCAGGCACTAGC
	1	D1S2612	155.89	146252391	GCTGTTCTTAGGGCTTTTCC
	1	D1S1653	164.09	156199398	GGAAAGCCTGTAGGAAGAGG
	1	D1S1167	168.52	158275688	TCTGGGGGCTTAGAATAGAC
	1	D1SDVCAG1[a]	182.4	165776203	GTAGAAGCACATGGTGTGG
20p13	20	D20S103	2.13	507263	GTTCATAGAGGGACAAGACACAGT
	20	D20S116	11.20	4001387	TGACCACAGGGGTTAATG
	20	D20S905	17.19	5811629	AGCTTGAGGAGCAGTGTCTT
	20	D20S901	26.13	9989532	CTACTGCGCTCCATGAGA
	20	D20S604	32.94	12532311	TGAGTGAATTTTTCTAATAAATCCC
2q11	2	D2S1790	105.7	84928800	ACATGTCGATCTCAGCGTTC
	2	D2S113	111.21	96641286	GCTTGTTTCATCTCACCCTG
	2	D2S2972	114.42	101938508	TCTCACTACACCACCTGGGT
	2	D2S436	118.16	[b]	GATATGGGAGCAACATGAGC
	2	D2S1888	121.63	111111607	TTTGAAGTTTGGTGTCTGTG
	2	D2S410	125.18	115957399	ATGTAGAAGAGTCAGAATTTCAACC
	2	D2S1260	137.93	131307274	AGGTGAGATGGTGCCGCTG
GDNF	5	D5S2023	53.67	37349306	GAGCACTTGAGCCTGG
	5	D5S1964	54.79	37895179	TGTGACACAAAAGCACACAG
RET	10	D10S1793	68.63	49806348	AGGGGTCACGGTCCAT
	10	D10S1220	70.23	52348570	GATTGCTTTAGAAGTGGGCA
SLIT2	4	D4S2946	33.42	17362511	GTCAAGAGGGCTGATTCTG
	4	D4S2397	42.74	26866965	CATGCACACCAAAACAAGAA
SPRY1	4	D4S1612	124.45	122544018	AAGGCTTTATTCNCTTATTGTT
	4	D4S430	126.15	123827098	CTGTATATGTTAATGTGC
	4	D4S1615	128.31	128429186	CCTTGGGTCAGCCACATATC
PAX2	10	D10S1709	119.70	99475362	GTGAGTCCAGAATCACCCC
	10	D10S184	121.98	100121771	AAAATGAGGGAAGTTGGGA
	10	D10S1239	125.41	103186343	CCCTAGCTAATGTTAAAATATCACG

Reverse primer sequence	MPL	MPL fam 1	MPL fam 2	MPL fam 3	MPL fam 4	NPL	NPL *P*-value
TTAGCATCCAATCAGAGAAGC	-3.89	-0.15	-1.12	-0.92	-1.71	-0.92	0.829
TGCTGGCTCAGGGGAC	-4.85	-0.14	-2.04	-0.88	-1.79	-1.31	0.957
GCAAGACAGCTGCCTCATA	-4.84	-0.12	-2.01	-0.92	-1.79	-1.06	0.883
CTGGGCAAAAAGTGGA	-4.87	-0.12	-2.04	-0.92	-1.79	-0.93	0.831
CTGGGAGCTCTATTCTGTCC	-4.88	-0.14	-2.02	-0.92	-1.79	-1.30	0.954
GCCTAGACAATGGGAGAGGT	-4.86	-0.15	-2.03	-0.90	-1.78	-1.28	0.950
CGATTGTGCCACTACACAGT	-4.89	-0.16	-2.02	-0.93	-1.78	-1.28	0.951
AACTTGGGCTTCTCTGCTTC	-3.69	-0.4	-1.13	-0.92	-1.24	-1.20	0.931
CCTGGATGACAGAGTGCTCT	-3.41	-0.35	-1.61	-0.92	-0.53	-0.57	0.666
TCAGTGTGTAGGGACAAGATG	-3.49	-0.11	-1.99	-0.93	-0.46	-0.26	0.534
CTGGAGTAAATGCAGCTCAG	-3.48	-0.68	-1.12	-0.83	-0.86	-0.76	0.756
CCATGATGTTTGGTTAATCACA	-2.37	-0.19	-0.65	-0.78	-0.75	-0.26	0.537
CAGGACTCAGTGCACCAG	-3.30	-0.42	-1.10	-0.93	-0.85	-0.72	0.733
TCAGCAGATCCCACCA	-2.97	-0.38	-1.10	-0.64	-0.84	-0.74	0.744
CTGGTTGGTGGTCAACATA	-3.41	-0.38	-1.11	-1.12	-0.80	-0.91	0.819
AAGCAATCTCATTTATTTTTACACA	-2.88	-0.36	-1.15	-0.51	-0.85	-0.83	0.785
GAGTTTTATTGGCCAAAGCA	-0.21	-0.92	-0.42	0.41	0.72	0.59	0.245
CTGTTGTTTTTTAGGTGGGAG	-1.36	-1.53	-1.13	0.49	0.80	0.76	0.198
CTCTATCTCTATCTCTGTCTCTGCC	-0.97	-1.31	-1.13	0.51	0.96	1.21	0.109
GGAATCAACTTTCAGTATAAACCC	-0.62	-1.12	-1.13	0.52	1.11	1.85	0.049
TGAAGTCCCTTGGAAATGTT	-2.11	-1.09	-1.12	0.48	-0.39	0.37	0.312
CAGACACAAATGCACACACA	-4.16	-1.77	-1.09	-0.91	-0.39	-0.72	0.733
CAGGAGGAGGCAATGCCCA	-4.89	-1.99	-1.12	-0.93	-0.85	-1.09	0.896
CATAAAATGAAGTAGGAGTGGTGTA	-2.15	-1.98	-0.69	0.53	0.00	0.69	0.215
TTATCACCAAATAGGCAGGA	-1.80	-1.75	-0.58	0.53	0.00	0.70	0.214
CTGCTTCCCAAGGTCAGG	-3.09	-0.37	-0.92	-0.93	-0.87	-0.94	0.830
TGTAGGTTTTCACATGTTCAGC	-3.12	-0.37	-1.12	-0.75	-0.87	-1.02	0.864
ACCTGTCTGAACTTGCGTG	-2.34	-1.39	0.82	-0.93	-0.84	0.33	0.326
GCAACAAACCTGCACATTCT	-0.57	-0.84	0.76	0.11	-0.60	0.66	0.227
GGTCCAAAGACAGGTCAAA	-3.21	-1.39	-1.32	0.00	-0.50	-0.95	0.841
GGACCCAGTCTTGCTATG	-3.25	-1.28	-1.34	0.00	-0.64	-0.98	0.863
CACTCAGAACAGAAACTTGGGT	-3.08	-1.27	-0.85	0.00	-0.95	-1.05	0.887
CAGTGGAATGGCTCATTTG	-3.20	-0.21	-2.05	-0.93	-0.02	-0.45	0.614
TCTTTTCTTGCCTCTTCCAT	-3.32	-0.32	-1.97	-0.93	-0.10	-0.45	0.615
CAGAGTGAGACCCTGTTTCA	-3.59	-0.39	-1.90	-0.91	-0.39	-0.52	0.645

Locus/ gene	Chromo-some	Marker	Position (cM)	Position (bp)	Forward primer sequence
AGTR2	X	DXS6797	67.12	107367721	TTCCCTCTCTCCCTCTGTCT
	X	DXS6804	68.74	111999363	CCCAGATATTTTGACCACCA
	X	DXS1001	75.79	119720696	TACAAGTAACCCTCGTGACA
HLADRB1	6	D6S1281	44.41	25405006	GATGCCACGTTTTAAAATGC
	6	D6S291	49.50	36373494	CTCAGAGGATGCCATGTCTAAAATA
UPK1A	19	D19S220	62.03	43123394	GTGTCTTATGTTCAGAAAGGCCATGTCATTTG
	19	D19S400	64.70	46219373	CGGTATGTCTTTATCAGCAG
UPK1B	3	D3S3665	129.73	115686173	GGGCCTCAAAGCACTTC
	3	D3S2460	134.64	118885068	ATTGCTTCCCCTTTACCTGA
	3	D3S3606	143.94	128682900	AAAATTCCCTGCAGTGGGA
UPK2	11	D11S4104	113.93	118140629	GGAGAATGGCCTGAACCTG
	11	D11S924	115.53	118943143	ATTGAACTCCAGCCCG
UPK3A	22	D22S928	52.08	43854113	TGCAAAGTGCTGGAGG
UPK3B	7	D7S1870	86.12	73764649	TTCACTCAGGAAGTGGC
	7	D7S2204	90.95	77964859	TCATGACAAAACAGAAATTAAGTG

[a] self-designed marker, [b] is not mapped to the assembly in Ensembl v38.

Reverse primer sequence	MPL	MPL fam 1	MPL fam 2	MPL fam 3	MPL fam 4	NPL	NPL *P*-value
ACACACACCCAAAACCAGAT	-4.20	-0.62	-1.41	-1.08	-1.09	-1.34	0.929
GGCATGTGGTTGCTATAACC	-4.04	-0.62	-1.24	-1.08	-1.10	-1.34	0.929
GTTATGGAATCAATCCAAGTG	-3.72	-0.13	-1.41	-1.08	-1.10	-0.86	0.804
AGAAGCAGCTGTGCTTTGTT	-1.79	-0.4	-1.05	-1.30	0.96	0.35	0.321
GGGGATGACGAATTATTCACTAACT	-2.64	-1.12	-1.12	-1.31	0.90	-0.05	0.455
TCCCTAACGGATACACAGCAACAC	-3.30	-0.37	-2.25	-0.11	-0.56	-0.65	0.703
ATGACAGCTCTAGGAAGGC	-4.30	-0.38	-1.88	-0.93	-1.11	-0.93	0.825
CCATCTTCAGGCAGTAAAGC	0.65	-0.82	0.66	0.53	0.28	1.80	0.056
GACAGGAGACAGAATGTTATAAGTT	0.33	-1.08	0.62	0.54	0.26	1.65	0.067
GGGGCTCGAAAGACAGTAAA	-2.14	-1.23	-1.01	0.48	-0.39	0.54	0.257
ATCTCTATCATGGGCAATTTGG	-1.63	-0.24	-0.56	0.02	-0.85	-0.11	0.477
CCTCATTGGGCCACTC	-1.46	-0.23	-0.50	-0.03	-0.70	-0.28	0.539
TGAAGATGGCTAGTACGGG	-3.66	-0.49	-0.93	-1.12	-1.12	-1.23	0.923
TGGTGATGTGCTTTACTACG	-1.25	-0.91	0.00	0.48	-0.82	0.38	0.309
AGTAAATGGAATTGCTTGTTACC	-1.03	-1.02	0.00	0.37	-0.38	0.75	0.199

WEB RESOURCES

Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm. nih.gov/entrez/query.fcgi?db=OMIM

Ensembl v38 http://ensembl.org

Marshfield (last revised 14 March 1999) http://research.marshfieldclinic. org/genetics[160]

CEPH genotype database, version V10.0 http://www.cephb.fr/cephdb/

CHAPTER 6

Genes in the ureter budding pathway: Association study on vesico-ureteral reflux patients

Submitted

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ABSTRACT

Vesico-ureteral reflux (VUR) is the retrograde passage of urine from the bladder to the urinary tract and causes 8.5% of end-stage renal disease in children. It is a complex genetic developmental disorder, in which ectopic embryonal ureter budding is implicated in the pathogenesis. VUR is part of the spectrum of Congenital Anomalies of the Kidney and Urinary Tract (CAKUT).

We performed an extensive association study for primary VUR using a two-stage, case-control design, investigating 44 candidate genes in the ureter budding pathway in 409 Dutch VUR patients. The 44 genes were selected from the literature and a set of 567 single nucleotide polymorphisms (SNPs) capturing their genetic variation was genotyped in 207 cases and 554 controls. The 14 SNPs with $p < 0.005$ were included in a follow-up study in 202 cases and 892 controls. Of the total cohort, ~50% showed a clear-cut primary VUR phenotype and ~25% had both a duplex collecting system and VUR. We also looked for association in these two extreme phenotype groups. None of the SNPs reached a significant *p*-value. Common genetic variants in four genes (*GREM1, EYA1, ROBO2* and *UPK3A*) show a trend towards association with the development of primary VUR (*GREM1, EYA1, ROBO2*) or duplex collecting system (*EYA1* and *UPK3A*). SNPs in three genes (*TGFB1, GNB3* and *VEGFA*) have been shown to be associated with VUR in other populations. Only the result of rs1800469 in *TGFB1* hinted at association in our study. This is the first extensive study of common variants in the genes of the ureter budding pathway and the genetic susceptibility to primary VUR.

INTRODUCTION

Vesico-ureteral reflux [VUR (MIM 193000)] is the retrograde passage of urine from the bladder into the upper urinary tract. It is one of the most commonly detected congenital anomalies and probably has a conservatively estimated prevalence of 1%.[1, 2] It has a primary and a secondary form: primary VUR is due to an incompetent valve mechanism at the uretero-vesical junction, while secondary VUR is due to a functional or anatomical urethral obstruction. VUR is a developmental disorder, which may occur in isolation or as part of a Mendelian or other syndrome. The Winter-Baraitser Dysmorphology Database lists 68 syndromes with 'urinary reflux'.[165]

Although most children grow out of the disorder without serious morbidity, a subset does develop long-term complications. In this group VUR results in renal damage, either as a result of ascending urinary tract infections (reflux nephropathy) or of renal hypo- or dysplasia, which is often associated with VUR. As such, in these two groups VUR accounts for 7.4 - 9.6% and 8.8 - 13.8%, respectively, of end-stage renal disease in Dutch children.[7]

Clinical observations and the results of many studies support the notion that there is a

heterogeneous genetic basis for VUR. The incidence of VUR is increased in first-degree relatives of patients[115, 150, 166] and there is 80% concordance between monozygotic twins.[19] In a subset of families, the segregation pattern suggests autosomal dominant inheritance with variable penetrance.[20-22] Other inheritance patterns, including polygenic, have also been observed.[24-26] Linkage studies have revealed different loci linked to VUR, although most loci have not been convincingly replicated.[21, 22, 27, 41-43, 148] Work in knock-out mice has confirmed the importance of genetic factors in the etiology of VUR.[30] Evidence for a continuous distribution of anatomic parameters associated with VUR suggests that these parameters are quantitative traits encoded by multiple genes.[30] In common complex diseases, common genetic variants are thought to be part of the genetic disease component.[52, 167] Because of their modest individual effect size, common variants are not detected by a linkage approach.

To date, no major susceptibility genes have been identified for VUR.[21, 22, 27, 30, 41, 42, 91, 148] However, since embryonal ectopic ureteral budding has been proposed as a mechanism for the development of VUR,[9, 151] genes involved in this process are considered to be potential candidate genes for VUR susceptibility (Figure 6.1). In particular, ectopic ureteral budding can lead to a diverse spectrum of phenotypes known as "congenital anomalies of the kidney and urinary tract" or CAKUT. CAKUT include VUR, hypo-/dysplastic kidneys and duplex collecting systems. Variable combinations of these phenotypes are seen in sibships (both in mice and humans) suggesting that the same genetic variation is causally involved in the whole CAKUT spectrum.[9] For example, defects of the *RET* (ENSG00000165731) and *GDNF* (ENSG00000168621) genes have been shown to cause ectopic ureteral budding.[16, 17] *ROBO2* (ENSG00000185008) regulates the expression of *GDNF*[126] and was shown to be mutated in a small number of VUR/CAKUT patients.[168] Genes involved in the *RET/GDNF* pathway are obvious functional candidate genes for VUR, while genes involved in syndromal VUR are often also implicated in the ureteral budding pathway and thus attractive candidate genes as well. Hence, we hypothesize that common variants in genes in the ureter budding pathway contribute to the genetic susceptibility for primary VUR.

We performed an exploratory candidate-pathway, genetic association screen for VUR using a two-stage, case-control design. We aimed to investigate both the main genes in the ureter budding pathway and those more peripherally connected to this pathway.

RESULTS

In stage one, we successfully genotyped 567 (out of 758) SNPs (single nucleotide polymorphisms) in 44 genes (Supporting Tables 6.1, 6.2 and 6.4) for association analysis in a cohort of 207 primary VUR patients and 572 controls (Figure 6.2). We also performed a subset analysis in two extreme phenotype subgroups: (1) a group of 111 clear-cut primary VUR cases

(e.g. patients with mild dysfunctional voiding, a minor relative meatal stenosis, or insignificant urethral valves were excluded) and (2) a group of 47 patients with VUR and a uni- or bilateral, complete or incomplete, duplex collecting system.

We set out to replicate the top 14 SNPs, which mapped to six different genes, from the three combined association analyses (Table 6.1). This corresponded with a *p*-value threshold for the first stage of 0.005. The replication cohort of 202 cases and 892 controls was also used for the subset analysis (87 clear-cut primary VUR cases, and 58 cases with a duplex collecting system and VUR) (Figure 6.2).

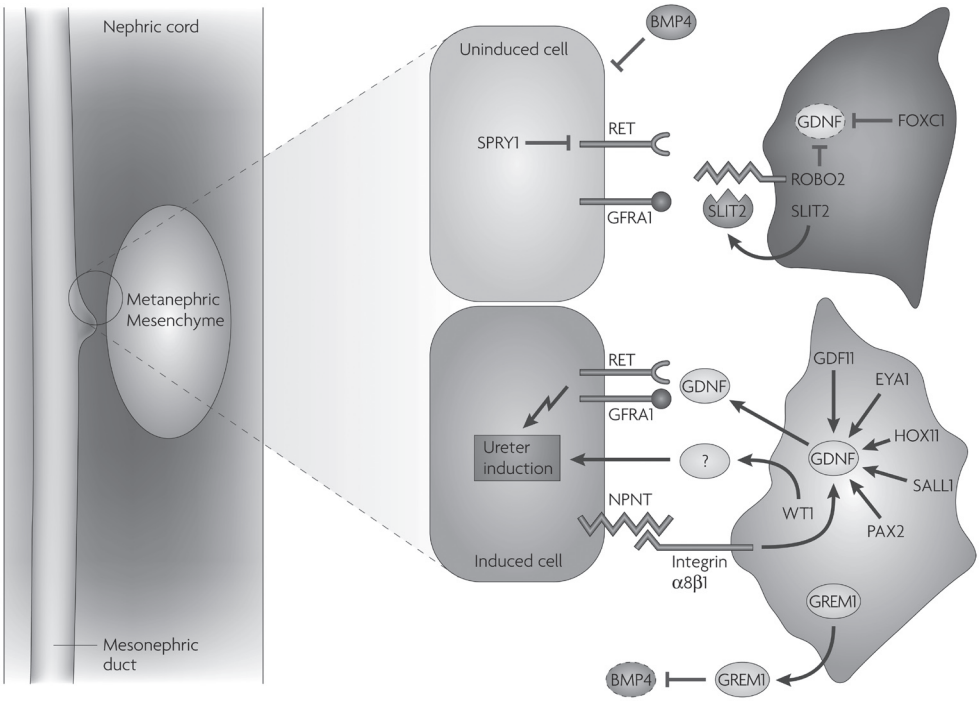

Figure 6.1. Molecular pathways that control kidney induction. Mesenchymal cells at the caudal end of the nephrogenic cord (in light blue) express various factors that activate expression of glial-derived neurotrophic factor (GDNF). In addition, mesenchymal cells release gremlin 1 (GREM1), an inhibitor of bone morphogenetic protein (BMP) signaling, and other unidentified factors. Released GDNF binds to RET and GDNF-family receptor a1 (GFRA1) receptors that are presented by epithelial cells of the mesonephric duct (in red) The combination of these signals induces ureteric budding. Mesenchymal cells at a more rostral level (colored dark blue) express forkhead box protein C1 (FOXC1), Slit homologue 2 (SLIT2) and its receptor Roundabout homologue 2 (ROBO2), leading to a repression of GDNF. In epithelial cells of the mesonephric duct, the tyrosine kinase inhibitor sprouty 1 (Spry1) suppresses RET activation. Finally, BMP4 also inhibits ureter outgrowth. EYA1, Eyes-absent homologue 1; GDF11, growth differentiation factor 11; HOX11, homeobox protein 11; NPNT, nephronectin; WT1, Wilms tumour transcription factor. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Genetics.[8] See page 204 for a full-color representation of this figure.

The results of the joint analyses of the discovery (stage one) and the replication (stage two) cohorts, including the analyses in the two phenotype subsets, did not reach significant *p*-values when corrected for multiple testing ($p < 9*10\text{-}5$). Analyses of the permuted datasets did not yield significant *p*-values either (data not shown). The results of the stage two cohort in themselves do not replicate the stage one *p*-values.

Scrutinizing the results for interesting trends, revealed eight SNPs (and three perfect proxies) in six genes that had a 95% confidence interval (95% CI) for the odds ratio (OR) that was not equal to one. Five of these SNPs (and the three perfect proxies), in four genes, hinted at association with $p < 0.01$ (Table 6.1). For two genes, *GREM1* (OR 0.77 (95% CI 0.65 - 0.91)) and *ROBO2* (OR 0.80 (95% CI 0.69 - 0.94)), it was mainly the primary VUR cases that contributed to the overall trend. For *EYA1*, (OR 1.27 (95% CI 1.07 - 1.51)) the trend that would support our hypothesis ($p<0.05$), was visible in the joint (stage one combined with stage two) results from both phenotype subgroups. The trend in the *UPK3A* gene (OR 1.52 (95% CI 1.11 - 2.06)) only showed in the subgroup with duplex collecting systems and VUR. In *ROBO2, EYA1* and *UPK3A*, more than one SNP showed in the best results list and linkage disequilibrium plots showing the allelic association between the SNPs are shown in Supporting Figure 6.1.

Although the result in *UPK3A* was not significant, it was intriguing given the limited sample size of the subgroup of duplex collecting system patients. This gene was subsequently sequenced in all duplex collecting system patients and we identified three inherited missense mutations that were not present in 96 control chromosomes. *In silico* analysis suggested that these amino acid substitutions have, at most, a mild effect on the protein (Supporting Table 6.3).

<table>
<tr><th></th><th colspan="2">stage one cohort</th><th colspan="2">stage two cohort</th></tr>
<tr><th></th><th>cases</th><th>controls</th><th>cases</th><th>controls</th></tr>
<tr><td>before quality control</td><td>n=215</td><td>n=572</td><td>n=208</td><td>n=1033</td></tr>
<tr><td rowspan="3">phenotype (sub-) groups after quality control</td><td>all cases : n=207</td><td rowspan="3">n=554</td><td>all cases : n=202</td><td rowspan="3">n=892</td></tr>
<tr><td>clear cut primary VUR : 54% (n=111)</td><td>clear cut primary VUR : 43% (n=87)</td></tr>
<tr><td>VUR + duplex collecting system : 23% (n=47)</td><td>VUR + duplex collecting system : 29% (n=58)</td></tr>
<tr><td>gender</td><td>female: 58%</td><td>female: 42%</td><td>female 69%</td><td>female: 44%</td></tr>
<tr><td>mean year of birth (interquartile range)</td><td>1992 (1988-1996)</td><td>1957 (1947-1966)</td><td>1999 (1997-2002)</td><td>1948 (1940-1955)</td></tr>
<tr><td rowspan="4">provenance</td><td>UMC Utrecht: 64% (n=132)</td><td rowspan="4">Utrecht and Amsterdam bloodbank</td><td>UMC Utrecht: 56% (n=113)</td><td rowspan="2">Utrecht and Amsterdam bloodbank n=144</td></tr>
<tr><td>UMC St Radboud: 34% (n=71)</td><td>UMC St Radboud: 25% (n=50)</td></tr>
<tr><td>Erasmus MC Rotterdam: 0.5% (n=1)</td><td>Erasmus MC Rotterdam: 19% (n=38)</td><td rowspan="2">ALS controls cohort n=892</td></tr>
<tr><td>other: 1% (n=3)</td><td>other: 0.5% (n=1)</td></tr>
<tr><td>DNA derived from</td><td>whole blood</td><td>whole blood</td><td>whole blood or saliva</td><td>whole blood (amplified DNA for ALS cohort)</td></tr>
</table>

Figure 6.2. Detailed overview of the two Dutch case-control cohorts and two phenotype subgroups in which the association study was performed.

Table 6.1. Results of the joint, stage one and stage two analyses of the 14 SNPs tested for association in stage two

SNP	gene	chromosome	basepair position	minor allele (major allele)	MAF (controls; joint unless not genotyped in stage two)	MAF (all cases; joint unless not genotyped in stage 2)	stage 1 *p*-value all cases *	stage 2 *p*-value all cases *	joint *p*-value all cases **	OR all cases
rs6780105	*RARB*	3	25278709	G (C)	0.18	0.15	0.0024#	0.765	0.019	0.77 (0.62 - 0.96)
rs755661	*RARB*	3	25447044	A (G)	0.45	0.46	0.3350	0.179	0.763	1.02 (0.88 - 1.20)
rs4476545$	*ROBO2*	3	77193190	C (G)	0.15	0.17	0.0402	0.879	0.136	1.18 (0.95 - 1.46)
rs1666130$	*ROBO2*	3	77681633	G (A)	0.49	0.44	0.0042#	0.321	0.007	0.80 (0.69 - 0.94)$
rs1403848***	*ROBO2*	3	77692345	C (A)	0.50	0.41	0.0025#	***	***	***
rs10103397****	*EYA1*	8	72274153	G (A)	0.26	0.32	0.0075	****	****	****
rs9298164****	*EYA1*	8	72289193	A (G)	0.24	0.32	0.0047#	****	****	****
rs3735935$	*EYA1*	8	72290318	A (C)	0.25	0.30	0.0046#	0.306	0.007	1.27 (1.07 - 1.51)$
rs1481800	*EYA1*	8	72293980	A (G)	0.23	0.27	0.0128	0.746	0.051	1.20 (1.00 - 1.43)
rs11197571	*GFRA1*	10	117932624	G (A)	0.13	0.16	0.0143	0.468	0.026	1.28 (1.03 - 1.59)
rs7497354$	*GREM1*	15	30802694	G (A)	0.39	0.33	0.0030#	0.148	0.002	0.77 (0.65 - 0.91)$
rs1057353$	*UPK3A*	22	44061968	G (C)	0.22	0.24	0.6806	0.293	0.294	1.10 (0.92 - 1.33)
rs1135360	*UPK3A*	22	44063666	G (A)	0.41	0.45	0.2655	0.175	0.080	1.15 (0.98 - 1.35)
rs3788643	*UPK3A*	22	44064754	A (G)	0.15	0.16	0.7451	0.291	0.320	1.12 (0.90 - 1.38)

SNP= single nucleotide polymorphism, MAF= minor allele frequency, OR = odds ratio, *RARB*=retinoic acid receptor beta, *ROBO2*=roundabout axon guidance receptor homolog 2 (Drosophila), *EYA1*=eyes absent homolog 1 (Drosophila), *GFRA1*=GDNF family receptor alpha 1, *GREM1*=gremlin 1 cysteine knot superfamily homolog (Xenopus laevis) , *UPK3A*=uroplakin 3A. # top 14 *p*-value in the combined association results of all cases and the two endo-phenotype groups (clear cut primary VUR and duplex collecting system + VUR); SNP was analysed in stage 2 because of this result. $ results of the joint analysis showing a trend towards association; warrant replication. * CHI2 test, ** Cochran-Mantel-Haenszel test, *** rs1403848 is in strong linkage disequilibrium (D'=1 and r^2>=0.95) with rs1666130. Since rs1666130 is a perfect proxy for rs1403848, rs1403848 was not genotyped in stage two. **** rs10103397 and rs9298164 are in strong linkage disequilibrium (D'=1 and r^2>=0.95) with rs3735935. Since rs3735935 is a perfect proxy for both rs10103397 and rs9298164, they were not genotyped in stage two.

MAF (clear cut primary VUR cases; joint unless not genotyped in stage 2)	stage 1 *p*-value clear cut primary VUR cases *	stage 2 *p*-value clear cut primary VUR cases *	joint *p*-value clear cut primary VUR cases **	OR clear cut primary VUR cases	MAF (case duplex collecting system; joint unless not genotyped in stage 2)	stage 1 *p*-value duplex collecting system+VUR cases *	stage 2 *p*-value duplex collecting system+VUR cases *	joint *p*-value duplex collecting system+VUR cases **	OR duplex collecting system+VUR cases
0.15	0.0435	0.979	0.134	0.80 (0.60 - 1.07)	0.11	0.0155	0.237	0.012	0.57 (0.37 - 0.89)
0.47	0.7664	0.424	0.448	1.09 (0.88 - 1.34)	0.42	0.0052#	0.223	0.344	0.87 (0.66 - 1.16)
0.20	0.0020#	0.381	0.005	1.48 (1.13 - 1.94)$	0.13	0.9889	0.574	0.658	0.91 (0.60 - 1.38)
0.43	0.0257	0.201	0.012	0.76 (0.61 - 0.94)$	0.49	0.8533	0.874	0.996	1.00 (0.75 - 1.33)
0.41	0.0199	***	***	***	0.48	0.6920	***	***	***
0.36	0.0020#	****	****	****	0.31	0.2554	****	****	****
0.34	0.0025#	****	****	****	0.31	0.1691	****	****	****
0.30	0.0023#	0.752	0.043	1.27 (1.01 - 1.61)$	0.31	0.2107	0.116	0.045	1.36 (1.01 - 1.85)$
0.26	0.0052#	0.217	0.224	1.16 (0.91 - 1.49)	0.29	0.4201	0.100	0.076	1.33 (0.97 - 1.81)
0.16	0.0542	0.975	0.163	1.23 (0.92 - 1.66)	0.18	0.0037#	0.909	0.063	1.42 (0.98 - 2.06)
0.33	0.1936	0.055	0.024	0.77 (0.62 - 0.97)	0.33	0.0798	0.501	0.096	0.78 (0.58 - 1.05)
0.21	0.2785	0.680	0.619	0.94 (0.72 - 1.21)	0.30	0.0020#	0.421	0.008	1.52 (1.11 - 2.06)$
0.43	0.6395	0.141	0.509	1.08 (0.87 - 1.33)	0.47	0.0023#	0.588	0.103	1.26 (0.95 - 1.67)
0.15	0.1938	0.064	0.718	1.06 (0.79 - 1.42)	0.20	0.0011#	0.838	0.020	1.51 (1.07 - 2.15)

Five SNPs in three genes (*TGFB1, GNB3* and *VEGFA*) were included in the stage one study because they were associated with VUR in other populations.[44-46, 161, 162, 169] The SNP in *VEGFA* did not pass quality control criteria. Only rs1800469 in *TGFB1* showed a marginal effect in stage one (OR 1.32; 95% CI 1.03-1.70; $p = 0.028$) but it did not reach the threshold for inclusion in stage two.

DISCUSSION

A cohort of VUR patients was screened for association with tag SNPs covering 44 candidate genes (Supporting Table 6.1) that are related to ureter budding function (Figure 6.1). No significant associations were detected in this exploratory, candidate pathway association study. The best results of the study show common genetic variants in *GREM1, EYA1* and *ROBO2* in the subgroup with isolated primary VUR and of genetic variants in *EYA1* and *UPK3A* in the subgroup with duplex collecting systems. Association studies such as this, in common complex diseases, are suited to detecting common genetic variants with modest individual effect sizes.[52, 167] Earlier studies have shown that pathogenic mutations in three of these genes cause human urinary tract malformations or syndromes. Mutations and microdeletions of *EYA1* cause Branchiootorenal Syndrome (BOR, MIM 113650)[170] and branchiootorenal spectrum disorders.[171] Among other congenital anomalies, BOR is characterized by renal anomalies in 38.2% of mutation carriers.[172] These anomalies typically include renal agenesis, hypoplasia or dysplasia, but VUR is also part of the phenotypic spectrum.[171] *ROBO2* was shown to be mutated in a small number of (familial) VUR/CAKUT patients.[91, 168, 173] Mutations in *UPK3A* are a cause for renal adysplasia, a phenotype within the CAKUT spectrum.[174, 175] Mouse models for all four genes show phenotypes reminiscent of VUR/CAKUT.[35, 91, 176, 177]

Since VUR, both with and without a duplex collecting system, can occur within the same family, the phenotypes may partly be caused by the same underlying genetic factors, as previously discussed by Kelly et al.[43] For this reason we also included cases with a duplex collecting system in our study. Nevertheless, for the analyses, we also analyzed the two extreme phenotype subgroups (i.e. clear-cut primary VUR cases and cases with a duplex collecting system and VUR) separately. In one of the four genes (*EYA1*), the joint ORs in both groups showed a trend supporting our hypothesis of contribution of common genetic variants to the genetic susceptibility for VUR.

The subgroup association analysis identified *UPK3A* as a probable risk factor for the duplex collecting system phenotype alone. On sequencing the complete coding region of *UPK3A* in this subgroup, we identified three inherited amino acid substitutions, which may represent susceptibility alleles. In one of the parents with the mutation there is an indication of the presence of a duplex collecting system on renal ultrasound (Supporting Table 6.3). This family will be followed up in a separate study. Mutations in *UPK3A* are known to cause renal

adysplasia,[174, 175] but were not detected in VUR patients so far.[36, 37, 178] One, albeit weak, association between VUR and a missense polymorphism in *UPK3A* has been published.[37] It is known from other diseases that different risk variants with diverse effects in the same gene can contribute to both Mendelian (syndromal) and multifactorial phenotypes.[179] Future studies will reveal whether mildly pathogenic mutations and/or common genetic variants in *UPK3A* contribute to the duplex collecting system subphenotype, or also to VUR, in general.

The trends in *GREM1* and *ROBO2* in this study are mainly derived from the clear-cut primary VUR cases. Interestingly, in one of the families in which a *ROBO2* mutation was previously identified as cause of the phenotype,[91] duplex collecting systems were also part of the phenotype. Our study only had power to detect association in the duplex collecting system subgroup with common variants with a relatively large effect size. It is therefore possible that a milder effect in this subgroup from variants in *ROBO2* remained undetected.

It appears from the linkage disequilibrium (LD) plots in Supporting Figure 6.1a that the three SNPs in *ROBO2* that reached the cut-off for stage two of our study, might represent two independent effects. Two SNPs are part of an LD block so the likely risk factor may be a variant anywhere in that block. In *EYA1* (Supporting Figure 6.1b), all four SNPs are part of the same LD block. The SNPs in *UPK3A* are not in LD. The SNP (rs1057353) that shows a trend for association with the duplex collecting system phenotype is a non-synonymous coding SNP and part of an LD block, so again the likely causative locus may be anywhere in the block, or we may have picked up an effect of this specific SNP.

As a by-product of their linkage study Cordell et al. recently performed an association scan for six candidate genes, five of which we also studied in our two cohorts.[27] Two of these genes (*ROBO2* and *UPK3A*) were included in the top results in our Dutch combined cohorts.None of the genes were significantly associated with VUR in Cordell et al.'s study. They also tested their genome wide linkage SNP set (~140,000 SNPs) for association with VUR. The SNPs with the most promising *p*-values were not located in genes related to the ureter budding pathway; they were therefore not studied in our cohort. Other SNPs in genes in the ureter budding pathway were not reported, but since coverage may not have been adequate, we cannot rule out that these genes play a role in that study.[27]

SNPs in three genes (*TGFB1, GNB3* and *VEGFA*) were previously shown to be associated with VUR in other populations and therefore included in our study.[44-46, 161, 162, 169] Only rs1800469 in *TGFB1* showed a marginal trend towards association in our Dutch cases.

Our study provides no conclusive evidence for association of common genetic variants in the ureter budding pathway with VUR in the Dutch population. This study had 80% power to detect an effect size of >1.57 (or a protective effect of < 0.64). Either we did not detect a larger (>1.57) effect (20% chance), or effects of genetic variants in ureter budding genes are more moderate (< 1.57) and therefore not significantly detected by our study. The null hypothesis (no association of common genetic variants in the genes in the ureter budding pathway) cannot be discarded based on our results. Hence, our reported findings should be

interpreted cautiously and warrant replication in other, preferably larger, cohorts.
Implication of genes involved in the ureter budding pathway in multifactorial, isolated primary VUR remains to be established. Based on the large body of evidence from human and mouse studies (see references in Introduction and for Supporting Table 6.1), we believe there is also a role for these genes in the pathogenesis of isolated VUR. Association studies in larger cohorts will elucidate the role of common genetic variants with small effect sizes. Furthermore, as shown for *ROBO2*,[91, 173] it may well be that rare as well as common genetic variants explain part of the heritability of VUR. Future 'exome sequencing' studies in well-characterized multiplex families as well as sporadic cases may shed light on this alternative hypothesis.[180, 181] It is also possible that common or rare genetic variants in as yet undiscovered genes in this or another pathway will prove to be key players in the development of VUR.
In conclusion, this was the first extensive association study of the ureter budding pathway in VUR patients and controls and it provides no conclusive evidence for association of common variants in genes in the ureter budding pathway with VUR.

METHODS

Study Design
We used a two-stage approach. In stage one, SNPs in 44 genes were genotyped in 207 unrelated cases and 554 controls. The SNPs with the 14 lowest *p*-values (*p*-value cut-off: 0.005) for association in either the whole group or a subgroup were genotyped in stage two in a second cohort of 202 cases and 892 controls. Allelic association *p* values were calculated per stage (chi^2 test for independence) and combined (Cochran-Mantel-Haenszel) in PLINK.[182] The datasets were also permuted 10,000 times and analyzed in PLINK. Deviations from Hardy Weinberg equilibrium in the controls were tested with a chi2 goodness-of-fit test in PLINK (cut-off: 0.001).

Cases and Controls
The total case population consisted of 409 VUR patients of Dutch descent (see Figure 6.2 for detailed information). All patients were diagnosed and treated in pediatric urology clinics of the participating Dutch university medical centers. Medical records were reviewed in order to ensure the correct diagnosis of VUR.
We performed both overall and endo-phenotype analyses (Figure 6.2). The first endo-phenotype group consisted of clear-cut primary VUR patients, i.e. with no other mild urological findings, like mild dysfunctional voiding, a relative meatal stenosis, or insignificant urethral valves (n=111/207 and 87/202). The second endo-phenotype group consisted of VUR patients with only complete or incomplete duplex collecting systems (n=47/207 and 58/202).

The control group comprised two independent cohorts (Figure 6.2) in order to obtain a larger sample size and more power. The first were 554 healthy Dutch donors from the blood banks in Amsterdam and Utrecht.[183] The second group were 338 healthy Dutch volunteers recruited for an unrelated study on amyotrophic lateral sclerosis.[184] Controls in stage one were entirely from the blood donor group, while controls in stage two were from both groups.
All patients and controls gave their informed consent and the study was approved by the ethics review committees of each of the participating hospitals (UMC Utrecht Institutional Review Board protocol 00-103/K).

Gene Selection
For stage one, initially 52 candidate genes were selected based on at least one of the following criteria (Supporting Table 6.1):
(a) direct involvement in the ureter budding pathway as reviewed by Schedl[8] (Figure 6.1); (b) evidence from the literature for implication in the ureter budding pathway; (c) involvement in human syndromes associated with VUR or VUR-related phenotypes; (d) five SNPs in three genes were included because they showed association with VUR or VUR-related phenotypes in other studies (the genes were not tagged, only the specific genetic variations were included for replication), (e) 8 "wildcard genes" were included that showed co-expression with the core group of candidate genes as reviewed by Schedl[8] (Figure 6.1), in an online database of co-expression ('Gemma', http://www.chibi.ubc.ca/Gemma/). SNPs in 44 genes passed our quality control criteria (see 'Quality Control').

SNP Selection
For stage one, 634 tag SNPs were selected with the Tagger program for the following parameters: $r^2 > 0.8$, minor allele frequency (MAF) > 0.1, pairwise or aggressive tagging. Each tagged locus included the coding part of the gene and at least 3 kb of the promoter region and 2 kb of the 3' end. If only a few tag SNPs were available at suboptimal parameters, all the known SNPs were included. Furthermore, by using FastSNP,[185] where possible we added SNPs with a predicted functional effect in the chosen genes (n=124). This second SNP category was allowed to have a MAF < 0.1. For 7 of 52 genes, there were no tagging SNPs available, so only functional SNPs were included for these (see Supporting Table 6.4).

Genotyping
DNA samples for stage one were derived from whole blood. In stage two, DNA samples from cases were either derived from whole blood or Oragene saliva kits (DNA Genotek, Ottawa, Canada), but in controls they originated from whole blood. DNA of part of the stage two control samples had been previously amplified (REPLI-G, Qiagen, Valencia, CA, USA).

SNP genotyping for the discovery cohort was performed with a GoldenGate assay on an Illumina BeadStation 500GX per the manufacturer's protocol (Illumina, San Diego, USA). Raw data were analyzed with Bead Studio software (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Clustering for all SNPs was checked manually and any dubiously clustered SNPs were removed.

Genotyping of the 14 SNPs in the replication cohort was performed with TaqMan probes and primers and an ABI 7900HT system (Applied Biosystems). Assay IDs are provided in Supporting Table 6.5. Clustering for all SNPs was checked manually. As it proved difficult to genotype rs1057353 satisfactorily with a TaqMan assay, it was partly genotyped via Sanger sequencing. See Supporting Table 6.6 for primer details. Because of linkage disequilibrium (D' = 1 and r^2 ≥ 0.95) between rs1666130 and rs1403848 in *ROBO2* and rs3735935, rs9298164 and rs10103397 in *EYA1*, we included one SNP from each set (rs1666130 and rs3735935) for genotyping in stage two. These two SNPs were perfect proxies for the three that were not actually genotyped. We consequently genotyped 11 SNPs. LD plots for the genes that showed the best results were created with Haploview version 4.2 and based on HAPMAP CEU data. *UPK3A* was sequenced using Sanger sequencing in the endo-phenotype subgroup of VUR patients with complete or incomplete duplex collecting systems, and 96 control chromosomes. A margin of at least 143 basepairs was observed surrounding the coding regions. See Supporting Table 6.6 for primer details. *In silico* analysis of mutations was performed with Alamut version 1.4 from Interactive Biosoftware (Rouen, France).

Quality Control

As stage one quality-control measures, duplicate samples were removed, sample call rate, genotype call rate and Hardy-Weinberg equilibrium (HWE) within controls were determined. Initially, 758 SNPs were included in this study. Only samples with a call rate above 90% were included in further analyses (Figure 6.2). SNPs with a minor allele frequency (MAF) of < 0.1 (188 SNPs) or a genotyping call rate of less than 90% (64 SNPs) were excluded. Four SNPs showed strong deviation from Hardy- Weinberg equilibrium in the controls (p_{HWE} < 0.001) and were discarded from further analysis. After quality control, 567 successfully genotyped SNPs were used for further analysis. For the 42 genes for which tagging SNPs were included, the median percentage of tagging SNPs passing our quality criteria was 90%. For 8 of 10 genes that had only some or all functional SNPs included, these SNPs did not pass the quality control. So effectively, SNPs in 44 genes were tested for association with VUR (see Supporting Tables 6.1 and 6.4).

For the stage two cohort, we determined sample call rate, genotype call rate, MAF and HWE. Only samples with a call rate > 90% were included in further analyses (Figure 6.2). In stage two, all SNPs satisfied the quality control criteria (genotyping rate > 90%, MAF > 0.1, p_{HWE} > 0.001 in controls).

Power Estimation

The power to detect an effect in the joint cohorts under the assumption of an additive model was estimated using the Genetic Power Calculator[186] (Supporting Figure 6.2). We assumed a prevalence of 0.01, a high risk allele frequency (A) of 0.25, a disease allele frequency of 0.25 and D' of 1. This study had 80% power to detect a heterozygote relative risk of 1.57 (or a protective effect of 1/1.57=0.64) at a significance level of $8.6*10^{-5}$ (=0.05/(567+14) tests).

DISCLOSURE

We report not having competing financial interests.

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Supporting Table 6.1. Genes selected for vesico-ureteral reflux association study.

HGNC symbol	MIM gene ID	chromosome, band	(a) direct involvement in the ureter budding pathway as reviewed by Schedl[8] (Figure 6.1)	(b) evidence for implication in the ureter budding pathway (human or mouse, genetic, functional or expression studies)	(c) implicated in human syndromes associated with VUR or other urinary tract (congenital) anomalies
*AGTR2**	MIM 300034	Xq23		x	
BMP4	MIM 112262	14q22.2	x	x	
CTNNB1	MIM 116806	3p22.1			
*E2F4**	MIM 600659	16q22.1			
EMX2	MIM 600035	10q26.11		x	
EYA1	MIM 601653	8q13.3	x	x	x
FGF10	MIM 602115	5p12			
FGF7	MIM 148180	15q21.1			
FOXC1	MIM 601090	6p25.3	x	x	
*FOXC2**	MIM 602402	16q24.1		x	x
FSTL1	MIM 605547	3q13.33			
GATA3	MIM 131320	10p14			x
*GDF11**	MIM 603936	12q13.2	x	x	
GDNF	MIM 600837	5p13.2	x	x	
GFRA1	MIM 601496	10q25.3	x	x	

(d) association of single or few genetic polymorphisms in the gene with VUR or VUR-related phenotypes (these genes were not tagged, only the specific genetic variations were included for replication)	(e) "wildcard genes" that showed co-expression with the core group of candidate genes as reviewed by Schedl[8] (Figure 6.1) according to 'Gemma' (online database of coexpression, http://www.chibi.ubc.ca/Gemma/)	(f) other reasons	all OMIM morbid IDs with known gene(s) that have a urinary tract congenital anomaly in "clinical synopsis"	7 key references that review the ureteric budding / early kidney induction pathway	miscellaneous references
				161, 162	
			OMIM 607932;MICROPHTHALMIA, SYNDROMIC 6	8, 157, 161, 162	
		in early embryonic kidney development		8	
	x				
				157, 161, 162	
			OMIM 113650; BRANCHIOOTORENAL SYNDROME 1 / OMIM 113620; BRANCHIOOCULOFACIAL SYNDROME; BOFS / OMIM 610896; BRANCHIOOTORENAL SYNDROME 2; BOR2	8, 157, 161, 162, 187, 188	171
		in early embryonic kidney development	OMIM 149730; LACRIMOAURICULODENTODIGITAL SYNDROME		189
		in early embryonic kidney development		161	190
				8, 157, 161, 162, 188	
				157, 161, 162	191
	x				
			OMIM 146255; HYPOPARATHYROIDISM, SENSORINEURAL DEAFNESS, AND RENAL DISEASE	157, 162	192
				8, 162, 188	
				8, 157, 161, 162, 188, 193	
				8, 157, 162, 193	

HGNC symbol	MIM gene ID	chromosome	(a) in pathway[8]	(b) evidence for pathway	(c) human syndromes
GNB3	MIM 139130	12p13.31			
GREM1	MIM 603054	15q13.3	x	x	
HOXA11	MIM 142958	7p15.2	x	x	
HOXC11	MIM 605559	12q13.13	x	x	
HOXD11	MIM 142986	2q31.1	x	x	
IGHMBP2	MIM 600502	11q13.2			
ITGA8	MIM 604063	10p13	x	x	
KIAA0241		7p14.3			
*LHX1**	MIM 601999	17q12			
*MXRA8**		1p36.33			
NPNT	MIM 610306	4q24	x	x	
OSR1	MIM 608891	2p24.1		x	
PAX2	MIM 167409	10q24.31	x	x	x
RARA	MIM 180240	17q21.2		x	
RARB	MIM 180220	3p24.2		x	
RARG	MIM 180190	12q13.13		x	
RET	MIM 164761	10q11.21	x	x	
ROBO2	MIM 602431	3p12.3	x	x	
SALL1	MIM 602218	16q12.1	x	x	x
*SIX1**	MIM 601205	14q23.1		x	x
SIX2	MIM 604994	2p21		x	
SLIT2	MIM 603746	4p15.31	x	x	
SPRY1	MIM 602465	4q28.1	x	x	
SPRY2	MIM 602466	13q31.1		x	

(d) association	(e) wildcard	(f) other reasons	OMIM morbid IDs	key references	miscellaneous references
x				162	44
				8	
				8, 157, 161, 162, 187, 188	
				8, 157, 162, 187, 188	
				8, 157, 161, 162, 187, 188	
	x				
				8, 157, 161	
	x				
		in early embryonic kidney development		8, 157, 161	
	x				
				8	
				8, 187	
			OMIM 120330; PAPILLORENAL SYNDROME	8, 157, 161, 162, 187, 188, 193	194
			OMIM 182290; SMITH-MAGENIS SYNDROME	157, 161, 162	195
				157, 161, 162	195
				161	195
				8, 157, 161, 162, 187, 188, 193	
			OMIM 610878; VESICOURETERAL REFLUX 2	8, 157, 162, 193	42, 91
			OMIM 107480; TOWNES-BROCKS SYNDROME	8, 157, 161, 162, 188	196
			OMIM 608389; BRANCHIOOTIC SYNDROME 3	8, 157, 162, 187, 188	171
				8, 187, 188	
				8, 157, 162, 193	
				8, 162, 193	
				193	

HGNC symbol	MIM gene ID	chromosome	(a) in pathway[8]	(b) evidence for pathway	(c) human syndromes
TGFB1	MIM 190180	19q13.2			
THRA	MIM 190120	17q21.1			
TNRC6B	MIM 610740	22q13.1			
UPK1A	MIM 611557	19q13.12			
UPK1B	MIM 602380	3q13.32			
UPK2	MIM 611558	11q23.3		x	
UPK3A	MIM 611559	22q13.31		x	
UPK3B	MIM 611887	7q11.23			
*VEGFA**	MIM 192240	6p21.1			
WNT11	MIM 603699	11q13.5			
WNT9B	MIM 602864	17q21.32		x	
WT1	MIM 607102	11p13	x	x	x
ZIC1	MIM 600470	3q24			

* SNPs in these genes (functional SNPs, since no tagging SNPs were available) did not pass the quality control criteria. These genes were therefore not included in the analysis, but they were part of our initial selection of candidate genes. (a) to (f) denote selection criteria.

(d) association	(e) wildcard	(f) other reasons	OMIM morbid IDs	key references	miscellaneous references
x				161, 162	45, 46, 169
	x				
	x				
		in urothelial plaque with UPK2 and 3A			35, 164
		in urothelial plaque with UPK2 and 3A			35, 164
				162	35, 164
			OMIM 191830; RENAL ADYSPLASIA	162	35, 164
		in urothelial plaque with UPK2 and 3A			35, 164
x				161	45
		in early embryonic kidney development		8, 157	
		in early embryonic kidney development		8	
			OMIM 194080; DENYS-DRASH SYNDROME / OMIM 136680; FRASIER SYNDROME / OMIM 137357; GENITOURINARY DYSPLASIA COMPONENT OF WAGR SYNDROME / OMIM 256370; NEPHROTIC SYNDROME, EARLY-ONSET, WITH DIFFUSE MESANGIAL SCLEROSIS / OMIM 194070; WILMS TUMOR 1 / OMIM 194072; WAGR SYNDROME / OMIM 256370; NEPHROTIC SYNDROME, EARLY-ONSET, WITH DIFFUSE MESANGIAL SCLEROSIS	8, 157, 161, 162, 187, 188	197
	x				

Supporting Table 6.2. 567 SNPs in the VUR association study that passed our quality control criteria

SNP	gene	Chr	Illumina ID
rs13011502	*OSR1*	2	rs13011502-127_B_R_1500219473
rs5013667	*OSR1*	2	rs5013667-127_T_F_1503075764
rs1900000	*OSR1*	2	rs1900000-127_B_F_1526341739
rs2290030	*SIX2*	2	rs2290030-127_T_R_1526341992
rs921117	*SIX2*	2	rs921117-127_B_R_1502489506
rs847148	*HOXD11*	2	rs847148-127_B_F_1503918569
rs847146	*HOXD11*	2	rs847146-127_B_F_1503961555
rs12152294	*RARB*	3	rs12152294-127_B_R_1527437869
rs978142	*RARB*	3	rs978142-127_B_R_1528547463
rs931697	*RARB*	3	rs931697-127_T_F_1528547460
rs1499644	*RARB*	3	rs1499644-127_B_R_1528547501
rs2363594	*RARB*	3	rs2363594-127_T_F_1527437876
rs6765179	*RARB*	3	rs6765179-127_T_F_1527437918
rs6780105	*RARB*	3	rs6780105-127_B_R_1528547491
rs1483851	*RARB*	3	rs1483851-127_B_F_1527437867
rs13077354	*RARB*	3	rs13077354-127_T_R_1527437894
rs1872144	*RARB*	3	rs1872144-127_T_R_1527437864
rs322679	*RARB*	3	rs322679-127_B_R_1527437901
rs322677	*RARB*	3	rs322677-127_B_R_1528547489
rs6810082	*RARB*	3	rs6810082-127_B_R_1527437911
rs977224	*RARB*	3	rs977224-127_T_R_1527437896
rs2363596	*RARB*	3	rs2363596-127_T_R_1528547494
rs322668	*RARB*	3	rs322668-127_B_F_1527437885
rs4681045	*RARB*	3	rs4681045-127_T_F_1500007022
rs17016178	*RARB*	3	rs17016178-127_T_R_1528547456
rs1561116	*RARB*	3	rs1561116-127_B_R_1527437865
rs6550962	*RARB*	3	rs6550962-127_T_F_1527437924
rs4681047	*RARB*	3	rs4681047-127_B_R_1527437889
rs1483831	*RARB*	3	rs1483831-127_T_R_1527437874
rs6550967	*RARB*	3	rs6550967-127_B_F_1527437891
rs9310778	*RARB*	3	rs9310778-127_B_R_1526505349
rs7614710	*RARB*	3	rs7614710-127_B_R_1528547465
rs2195919	*RARB*	3	rs2195919-127_B_R_1528547457
rs7628351	*RARB*	3	rs7628351-127_B_F_1528547461
rs11714772	*RARB*	3	rs11714772-127_B_F_1528547467
rs1843028	*RARB*	3	rs1843028-127_B_F_1527437861
rs12330681	*RARB*	3	rs12330681-127_B_F_1528547499
rs2116701	*RARB*	3	rs2116701-127_T_F_1527437922
rs6550971	*RARB*	3	rs6550971-127_T_R_1528547454
rs4395373	*RARB*	3	rs4395373-127_T_R_1527437920
rs6783726	*RARB*	3	rs6783726-127_B_R_1527437899
rs4681060	*RARB*	3	rs4681060-127_T_R_1527437898
rs7620529	*RARB*	3	rs7620529-127_B_R_1529565199
rs1483827	*RARB*	3	rs1483827-127_T_R_1529565198
rs6805482	*RARB*	3	rs6805482-127_T_F_1527345492
rs6778608	*RARB*	3	rs6778608-127_T_R_1527345938
rs17016408	*RARB*	3	rs17016408-127_B_R_1527345783
rs922939	*RARB*	3	rs922939-127_T_R_1527345508
rs12630664	*RARB*	3	rs12630664-127_T_R_1527345584
rs755661	*RARB*	3	rs755661-127_T_R_1526093680
rs7620632	*RARB*	3	rs7620632-127_B_F_1527345475
rs9871002	*RARB*	3	rs9871002-127_B_F_1526103479
rs6550975	*RARB*	3	rs6550975-127_T_R_1527345764
rs6800566	*RARB*	3	rs6800566-127_T_F_1527345736
rs4681063	*RARB*	3	rs4681063-127_T_R_1527345526
rs6777544	*RARB*	3	rs6777544-127_T_R_1527346184
rs6550980	*RARB*	3	rs6550980-127_T_F_1500538390
rs6793694	*RARB*	3	rs6793694-127_B_R_1526093919
rs7629902	*RARB*	3	rs7629902-127_B_R_1527345485
rs6550981	*RARB*	3	rs6550981-127_T_R_1527346364
rs7616467	*RARB*	3	rs7616467-127_T_R_1527345920
rs2033447	*RARB*	3	rs2033447-127_T_R_1527345440
rs4681064	*RARB*	3	rs4681064-127_B_F_1527345773
rs871963	*RARB*	3	rs871963-127_B_F_1503919236
rs7629478	*RARB*	3	rs7629478-127_T_R_1526101754
rs4607073	*RARB*	3	rs4607073-127_T_R_1526099952
rs11715516	*RARB*	3	rs11715516-127_T_F_1526090716
rs6775425	*RARB*	3	rs6775425-127_T_R_1526101752
rs10212330	*RARB*	3	rs10212330-127_B_R_1527346235
rs6796669	*RARB*	3	rs6796669-127_B_R_1526101739
rs1286654	*RARB*	3	rs1286654-127_T_R_1526091672
rs1299407	*RARB*	3	rs1299407-127_T_F_1527345582
rs1997352	*RARB*	3	rs1997352-127_B_R_1527345725
rs1406575	*RARB*	3	rs1406575-127_B_R_1527345643
rs1153584	*RARB*	3	rs1153584-127_B_F_1527345515
rs1881706	*RARB*	3	rs1881706-127_T_R_1527345560
rs1286750	*RARB*	3	rs1286750-127_B_R_1527346037
rs1153589	*RARB*	3	rs1153589-127_B_R_1500539600
rs1153591	*RARB*	3	rs1153591-127_B_R_1503288678
rs1286756	*RARB*	3	rs1286756-127_B_R_1503309332
rs13099641	*RARB*	3	rs13099641-127_T_R_1527346344
rs17525900	*RARB*	3	rs17525900-127_T_R_1527345944
rs1153606	*RARB*	3	rs1153606-127_B_R_1527345617
rs1286665	*RARB*	3	rs1286665-127_T_R_1527345552
rs1881703	*RARB*	3	rs1881703-127_T_F_1503260937
rs7616062	*RARB*	3	rs7616062-127_T_R_1527346290
rs1286772	*RARB*	3	rs1286772-127_T_R_1500536398
rs1286769	*RARB*	3	rs1286769-127_T_R_1500537809
rs17016718	*RARB*	3	rs17016718-127_T_R_1527346228
rs1286761	*RARB*	3	rs1286761-127_B_R_1503289171
rs10510568	*RARB*	3	rs10510568-127_T_F_1527345524
rs17016773	*RARB*	3	rs17016773-127_T_R_1527346008
rs17016778	*RARB*	3	rs17016778-127_T_F_1527345532
rs17016781	*RARB*	3	rs17016781-127_T_F_1527345622
rs1286729	*RARB*	3	rs1286729-127_B_F_1526088869
rs1286738	*RARB*	3	rs1286738-127_B_F_1527345519
rs1656463	*RARB*	3	rs1656463-127_T_F_1503032738
rs1730221	*RARB*	3	rs1730221-127_B_F_1527346165
rs9809535	*RARB*	3	rs9809535-127_B_R_1527345555
rs4681028	*RARB*	3	rs4681028-127_T_R_1527346272
rs7620852	*RARB*	3	rs7620852-127_T_R_1527346124
rs7621140	*RARB*	3	rs7621140-127_B_F_1527346031
rs2164360	*RARB*	3	rs2164360-127_B_F_1503361240
rs1798802	*CTNNB1*	3	rs1798802-127_T_F_1526233190
rs4135385	*CTNNB1*	3	rs4135385-127_B_R_1503910162
rs3923745	*ROBO2*	3	rs3923745-127_B_R_1528580403
rs6784307	*ROBO2*	3	rs6784307-127_B_R_1503326009
rs3934881	*ROBO2*	3	rs3934881-127_T_R_1528583630
rs11712939	*ROBO2*	3	rs11712939-127_B_R_1529565165
rs6779154	*ROBO2*	3	rs6779154-127_T_R_1500382203
rs4476545	*ROBO2*	3	rs4476545-127_B_F_1529565179
rs7640155	*ROBO2*	3	rs7640155-127_T_R_1500225375
rs4459934	*ROBO2*	3	rs4459934-127_B_R_1529565273
rs7628843	*ROBO2*	3	rs7628843-127_T_F_1500225374
rs4624600	*ROBO2*	3	rs4624600-127_T_F_1500035705
rs9859970	*ROBO2*	3	rs9859970-127_B_F_1500225605

SNP	gene	Chr	Illumina ID
rs13322517	*ROBO2*	3	rs13322517-127_B_R_1528580543
rs4645165	*ROBO2*	3	rs4645165-127_B_F_1500029520
rs4475074	*ROBO2*	3	rs4475074-127_B_R_1529565243
rs12496474	*ROBO2*	3	rs12496474-127_T_F_1529565264
rs7618319	*ROBO2*	3	rs7618319-127_B_F_1529565171
rs4077419	*ROBO2*	3	rs4077419-127_T_F_1500029459
rs7432300	*ROBO2*	3	rs7432300-127_B_F_1500225684
rs13097139	*ROBO2*	3	rs13097139-127_B_R_1529565257
rs9852548	*ROBO2*	3	rs9852548-127_B_R_1500034878
rs11709672	*ROBO2*	3	rs11709672-127_B_F_1500027429
rs4684028	*ROBO2*	3	rs4684028-127_T_F_1500224927
rs7432274	*ROBO2*	3	rs7432274-127_B_R_1500225318
rs12487684	*ROBO2*	3	rs12487684-127_T_F_1500020771
rs17769969	*ROBO2*	3	rs17769969-127_B_R_1528582541
rs6772736	*ROBO2*	3	rs6772736-127_B_R_1503961664
rs12491865	*ROBO2*	3	rs12491865-127_B_R_1500381684
rs879728	*ROBO2*	3	rs879728-127_T_R_1529565174
rs4072806	*ROBO2*	3	rs4072806-127_B_R_1500224845
rs7430171	*ROBO2*	3	rs7430171-127_T_R_1500029853
rs1470203	*ROBO2*	3	rs1470203-127_T_F_1528582876
rs17822079	*ROBO2*	3	rs17822079-127_B_F_1529565185
rs6776288	*ROBO2*	3	rs6776288-127_T_R_1529565254
rs13087163	*ROBO2*	3	rs13087163-127_B_R_1529565147
rs11928406	*ROBO2*	3	rs11928406-127_T_F_1529565266
rs998037	*ROBO2*	3	rs998037-127_B_F_1500025182
rs9873219	*ROBO2*	3	rs9873219-127_T_F_1529565270
rs12637117	*ROBO2*	3	rs12637117-127_B_F_1529565245
rs9820365	*ROBO2*	3	rs9820365-127_T_R_1502589545
rs1978940	*ROBO2*	3	rs1978940-127_B_R_1500020996
rs2028512	*ROBO2*	3	rs2028512-127_B_F_1500035439
rs9818075	*ROBO2*	3	rs9818075-127_T_F_1529565122
rs10511055	*ROBO2*	3	rs10511055-127_B_R_1528581879
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rs7639939	*ROBO2*	3	rs7639939-127_B_R_1529565247
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rs12492221	*ROBO2*	3	rs12492221-127_T_F_1500020785
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rs9830337	*ROBO2*	3	rs9830337-127_T_F_1500024965
rs6777631	*ROBO2*	3	rs6777631-127_T_F_1500225661
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rs7651943	*ROBO2*	3	rs7651943-127_T_R_1502519293
rs2121817	*ROBO2*	3	rs2121817-127_T_R_1529565252
rs2166802	*ROBO2*	3	rs2166802-127_T_R_1529565260
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rs12487172	*ROBO2*	3	rs12487172-127_B_R_1503369132
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rs876675	*ROBO2*	3	rs876675-127_B_R_1501188725
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rs9832079	*UPK1B*	3	rs9832079-127_B_R_1526361431
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rs10934486	*UPK1B*	3	rs10934486-127_B_R_1500027353
rs6772525	*UPK1B*	3	rs6772525-127_T_R_1526361216
rs9878996	*UPK1B*	3	rs9878996-127_B_F_1526361355
rs7628485	*UPK1B*	3	rs7628485-127_B_R_1526361383
rs13098659	*UPK1B*	3	rs13098659-127_T_F_1526361226
rs7622158	*UPK1B*	3	rs7622158-127_T_R_1500220725
rs1700	*FSTL1*	3	rs1700-127_T_F_1500033881
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rs1147700	*FSTL1*	3	rs1147700-127_B_R_1528587787
rs4676778	*FSTL1*	3	rs4676778-127_B_R_1500041841
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rs13097755	*FSTL1*	3	rs13097755-127_B_F_1500220955
rs1259294	*FSTL1*	3	rs1259294-127_B_F_1500020803
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rs1402372	*FSTL1*	3	rs1402372-127_T_R_1529246900
rs9879383	*FSTL1*	3	rs9879383-127_T_R_1529248534
rs1259329	*FSTL1*	3	rs1259329-127_T_R_1500384338
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rs564041	*SLIT2*	4	rs564041-127_T_R_1503244372
rs7655084	*SLIT2*	4	rs7655084-127_T_R_1503308195
rs17534458	*SLIT2*	4	rs17534458-127_T_F_1529565234
rs500869	*SLIT2*	4	rs500869-127_T_F_1529565192
rs6447952	*SLIT2*	4	rs6447952-127_B_R_1503294884
rs7680377	*SLIT2*	4	rs7680377-127_T_R_1503107972
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rs13435197	*SLIT2*	4	rs13435197-127_T_F_1529565168
rs12646142	*SLIT2*	4	rs12646142-127_B_F_1500418530
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rs10938795	*SLIT2*	4	rs10938795-127_B_F_1529564983
rs10938796	*SLIT2*	4	rs10938796-127_B_R_1529565205
rs6447955	*SLIT2*	4	rs6447955-127_T_F_1503324475
rs4406013	*SLIT2*	4	rs4406013-127_B_F_1529565221
rs4552454	*SLIT2*	4	rs4552454-127_T_F_1503373648
rs13142826	*SLIT2*	4	rs13142826-127_B_R_1500189167
rs9996157	*SLIT2*	4	rs9996157-127_T_R_1500189561
rs17537997	*SLIT2*	4	rs17537997-127_B_R_1529565213
rs16869595	*SLIT2*	4	rs16869595-127_T_F_1529565182
rs6849536	*SLIT2*	4	rs6849536-127_T_R_1502557447
rs11933090	*SLIT2*	4	rs11933090-127_T_F_1529565210

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rs11733205	*SLIT2*	4	rs11733205-127_B_F_1500001926
rs1033111	*SLIT2*	4	rs1033111-127_T_R_1503348581
rs491795	*SLIT2*	4	rs491795-127_B_F_1500364352
rs11932685	*SLIT2*	4	rs11932685-127_B_R_1529565225
rs17612037	*SLIT2*	4	rs17612037-127_T_F_1528586114
rs525684	*SLIT2*	4	rs525684-127_T_F_1529565224
rs12374415	*SLIT2*	4	rs12374415-127_T_R_1529564982
rs490478	*SLIT2*	4	rs490478-127_B_R_1502496707
rs556816	*SLIT2*	4	rs556816-127_B_F_1502558120
rs6825654	*SLIT2*	4	rs6825654-127_B_R_1500190014
rs519813	*SLIT2*	4	rs519813-127_T_F_1503278111
rs16869706	*SLIT2*	4	rs16869706-127_T_F_1529564988
rs7690660	*SLIT2*	4	rs7690660-127_B_F_1502509622
rs3775815	*SLIT2*	4	rs3775815-127_B_F_1502546164
rs2168801	*SLIT2*	4	rs2168801-127_T_F_1529564994
rs2322557	*SLIT2*	4	rs2322557-127_B_R_1529565187
rs573118	*SLIT2*	4	rs573118-127_B_F_1503364183
rs3775816	*SLIT2*	4	rs3775816-127_T_R_1503293615
rs3775820	*SLIT2*	4	rs3775820-127_B_R_1528593929
rs675465	*SLIT2*	4	rs675465-127_B_R_1529565207
rs7690492	*SLIT2*	4	rs7690492-127_T_R_1529564976
rs7669094	*SLIT2*	4	rs7669094-127_T_R_1529565232
rs3775824	*SLIT2*	4	rs3775824-127_T_R_1529565228
rs649424	*SLIT2*	4	rs649424-127_T_R_1529565230
rs2292439	*SLIT2*	4	rs2292439-127_T_F_1503103810
rs10516357	*SLIT2*	4	rs10516357-127_B_R_1500189046
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rs644607	*SLIT2*	4	rs644607-127_T_R_1502507922
rs7666974	*SLIT2*	4	rs7666974-127_B_F_1503315095
rs2168802	*SLIT2*	4	rs2168802-127_B_F_1503370986
rs17620814	*SLIT2*	4	rs17620814-127_T_F_1529565218
rs17621372	*SLIT2*	4	rs17621372-127_B_F_1500419020
rs1379659	*SLIT2*	4	rs1379659-127_B_R_1502542514
rs6823809	*NPNT*	4	rs6823809-127_B_F_1526341403
rs4600917	*NPNT*	4	rs4600917-127_T_R_1503040448
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rs7677557	*NPNT*	4	rs7677557-127_B_F_1526341407
rs7680832	*NPNT*	4	rs7680832-127_T_F_1526341338
rs6817700	*NPNT*	4	rs6817700-127_B_R_1503917505
rs159762	*SPRY1*	4	rs159762-127_B_R_1527230141
rs300574	*SPRY1*	4	rs300574-127_B_F_1503925150
rs3749692	*GDNF*	5	rs3749692-127_T_F_1529565048
rs7731209	*GDNF*	5	rs7731209-127_B_F_1529565001
rs2973050	*GDNF*	5	rs2973050-127_T_R_1528595520
rs1549250	*GDNF*	5	rs1549250-127_T_R_1528595518
rs2973043	*GDNF*	5	rs2973043-127_T_R_1529565064
rs884344	*GDNF*	5	rs884344-127_T_F_1528586868
rs12521946	*GDNF*	5	rs12521946-127_B_R_1529564999
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rs10941370	*GDNF*	5	rs10941370-127_T_R_1526511372
rs3812047	*GDNF*	5	rs3812047-127_B_R_1528585525
rs2975100	*GDNF*	5	rs2975100-127_B_F_1528585427
rs1011814	*FGF10*	5	rs1011814-127_T_F_1526340210
rs11743802	*FGF10*	5	rs11743802-127_B_F_1526340385
rs2121875	*FGF10*	5	rs2121875-127_T_R_1526340392
rs11750845	*FGF10*	5	rs11750845-127_B_F_1526340247
rs1384449	*FGF10*	5	rs1384449-127_T_F_1526340388
rs10512849	*FGF10*	5	rs10512849-127_B_F_1526340349
rs984253	*FOXC1*	6	rs984253-127_B_F_1503910605
rs6968828	*HOXA11*	7	rs6968828-127_T_R_1528626850
rs17427875	*HOXA11*	7	rs17427875-127_B_F_1529056509
rs12673952	*KIAA0241*	7	rs12673952-127_B_F_1500456181
rs6959234	*KIAA0241*	7	rs6959234-127_B_F_1529248465
rs6967167	*KIAA0241*	7	rs6967167-127_B_R_1529247185
rs2290214	*KIAA0241*	7	rs2290214-127_B_F_1529246297
rs17365249	*KIAA0241*	7	rs17365249-127_T_R_1529248246
rs1993050	*KIAA0241*	7	rs1993050-127_B_R_1529246221
rs6462381	*KIAA0241*	7	rs6462381-127_B_F_1529246227
rs4431500	*KIAA0241*	7	rs4431500-127_T_R_1500469742
rs34778959	*KIAA0241*	7	rs34778959-127_B_F_1529247449
rs3801332	*KIAA0241*	7	rs3801332-127_T_R_1524234030
rs2110465	*UPK3B*	7	rs2110465-127_T_R_1526361316
rs4072400	*EYA1*	8	rs4072400-127_T_F_1503035014
rs10103397	*EYA1*	8	rs10103397-127_B_R_1529565109
rs9298164	*EYA1*	8	rs9298164-127_B_F_1528642799
rs3735935	*EYA1*	8	rs3735935-127_T_F_1503243325
rs4738118	*EYA1*	8	rs4738118-127_B_R_1529565117
rs1481800	*EYA1*	8	rs1481800-127_T_F_1503270360
rs4738119	*EYA1*	8	rs4738119-127_T_F_1529565112
rs16937518	*EYA1*	8	rs16937518-127_B_R_1529565113
rs13274732	*EYA1*	8	rs13274732-127_T_F_1527363860
rs13262002	*EYA1*	8	rs13262002-127_T_R_1500333989
rs17782527	*EYA1*	8	rs17782527-127_T_R_1527363854
rs1900078	*EYA1*	8	rs1900078-127_B_R_1503937327
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rs7004007	*EYA1*	8	rs7004007-127_T_R_1503377679
rs2380716	*EYA1*	8	rs2380716-127_B_F_1502514741
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rs7011671	*EYA1*	8	rs7011671-127_T_R_1529565106
rs733745	*EYA1*	8	rs733745-127_B_R_1503275770
rs6990158	*EYA1*	8	rs6990158-127_B_F_1503248387
rs8181006	*EYA1*	8	rs8181006-127_T_F_1526516196
rs10092844	*EYA1*	8	rs10092844-127_T_R_1500095739
rs11993876	*EYA1*	8	rs11993876-127_B_F_1527363819
rs1031178	*EYA1*	8	rs1031178-127_B_R_1503239185
rs10095184	*EYA1*	8	rs10095184-127_T_R_1528644378
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rs7008755	*EYA1*	8	rs7008755-127_B_R_1502518267
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rs10957546	*EYA1*	8	rs10957546-127_T_R_1529565120
rs4738128	*EYA1*	8	rs4738128-127_B_F_1500096315
rs17712160	*EYA1*	8	rs17712160-127_B_R_1527363843
rs17712165	*EYA1*	8	rs17712165-127_T_F_1527363840
rs3779748	*EYA1*	8	rs3779748-127_T_R_1503363314
rs1900077	*EYA1*	8	rs1900077-127_B_R_1503370502
rs4738129	*EYA1*	8	rs4738129-127_B_R_1503292983
rs6983443	*EYA1*	8	rs6983443-127_T_R_1527363814
rs6983593	*EYA1*	8	rs6983593-127_B_F_1527363833
rs2218488	*EYA1*	8	rs2218488-127_T_R_1503241469
rs6992926	*EYA1*	8	rs6992926-127_B_R_1502497650
rs1822917	*EYA1*	8	rs1822917-127_T_R_1502583181
rs11776476	*EYA1*	8	rs11776476-127_T_R_1527363026
rs2275806	*GATA3*	10	rs2275806-127_T_F_1503252834
rs1399180	*GATA3*	10	rs1399180-127_T_R_1502589696
rs3781093	*GATA3*	10	rs3781093-127_T_F_1503910117

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rs570613	*GATA3*	10	rs570613-127_T_F_1503373793
rs569421	*GATA3*	10	rs569421-127_B_F_1503910301
rs528778	*GATA3*	10	rs528778-127_B_F_1503910281
rs263419	*GATA3*	10	rs263419-127_B_R_1529564977
rs878321	*ITGA8*	10	rs878321-127_T_R_1502499796
rs878319	*ITGA8*	10	rs878319-127_T_R_1528660618
rs11813586	*ITGA8*	10	rs11813586-127_T_R_1529565240
rs7910674	*ITGA8*	10	rs7910674-127_B_R_1503962716
rs1349534	*ITGA8*	10	rs1349534-127_T_F_1502522682
rs17137440	*ITGA8*	10	rs17137440-127_T_F_1529565238
rs11259736	*ITGA8*	10	rs11259736-127_B_R_1529565135
rs10752399	*ITGA8*	10	rs10752399-127_T_F_1507483251
rs2277204	*ITGA8*	10	rs2277204-127_T_R_1503913433
rs10906931	*ITGA8*	10	rs10906931-127_T_R_1529565044
rs1451668	*ITGA8*	10	rs1451668-127_T_R_1529564992
rs1057969	*ITGA8*	10	rs1057969-127_B_F_1529565045
rs980712	*ITGA8*	10	rs980712-127_T_R_1503069219
rs1319614	*ITGA8*	10	rs1319614-127_T_F_1529565094
rs1451667	*ITGA8*	10	rs1451667-127_B_R_1529564989
rs4747247	*ITGA8*	10	rs4747247-127_B_R_1529565137
rs2282384	*ITGA8*	10	rs2282384-127_B_F_1529564995
rs7083600	*ITGA8*	10	rs7083600-127_B_F_1500145621
rs7079006	*ITGA8*	10	rs7079006-127_T_R_1500146042
rs12414926	*ITGA8*	10	rs12414926-127_B_F_1529565041
rs1376690	*ITGA8*	10	rs1376690-127_T_F_1503072104
rs7918309	*ITGA8*	10	rs7918309-127_B_R_1529565131
rs12774861	*ITGA8*	10	rs12774861-127_T_F_1529565176
rs11253593	*ITGA8*	10	rs11253593-127_T_F_1529565178
rs2275617	*ITGA8*	10	rs2275617-127_B_F_1502504680
rs1341106	*ITGA8*	10	rs1341106-127_B_F_1501190690
rs6602051	*ITGA8*	10	rs6602051-127_T_R_1529565134
rs3765584	*ITGA8*	10	rs3765584-127_B_R_1502545713
rs1341099	*ITGA8*	10	rs1341099-127_B_F_1500183385
rs1891050	*ITGA8*	10	rs1891050-127_T_F_1529565140
rs11253615	*ITGA8*	10	rs11253615-127_T_F_1529565142
rs9333260	*ITGA8*	10	rs9333260-127_T_R_1529565104
rs2506011	*RET*	10	RS2506011-127_T_R_1503381609
rs2506021	*RET*	10	rs2506021-127_T_R_1529564964
rs12247456	*RET*	10	rs12247456-127_B_R_1529565037
rs2435347	*RET*	10	rs2435347-127_T_F_1528665464
rs2505535	*RET*	10	rs2505535-127_T_R_1503050136
rs1800858	*RET*	10	rs1800858-127_B_R_1503909600
rs3026737	*RET*	10	rs3026737-127_B_F_1503949769
rs2505515	*RET*	10	rs2505515-127_B_F_1503913913
rs1800860	*RET*	10	rs1800860-127_B_R_1503909601
rs741968	*RET*	10	rs741968-127_B_R_1529565077
rs2742234	*RET*	10	rs2742234-127_T_R_1529565036
rs1800861	*RET*	10	rs1800861-127_B_R_1529564967
rs1800863	*RET*	10	rs1800863-127_T_R_1503956374
rs715106	*RET*	10	rs715106-127_B_R_1503255536
rs2565201	*RET*	10	rs2565201-127_T_F_1529565072
rs17028	*RET*	10	rs17028-127_T_R_1528665258
rs4917908	*PAX2*	10	rs4917908-127_B_F_1503085986
rs4278455	*PAX2*	10	rs4278455-127_T_F_1503959892
rs6421335	*PAX2*	10	rs6421335-127_T_R_1500465103
rs4244341	*PAX2*	10	rs4244341-127_T_R_1500138182
rs11599767	*PAX2*	10	rs11599767-127_B_F_1529565145
rs10786607	*PAX2*	10	rs10786607-127_T_F_1507483487
rs11190695	*PAX2*	10	rs11190695-127_B_F_1500493227

SNP	gene	Chr	Illumina ID
rs2863046	*PAX2*	10	rs2863046-127_B_R_1503054136
rs10883543	*PAX2*	10	rs10883543-127_B_F_1528663499
rs2077642	*PAX2*	10	rs2077642-127_B_F_1528665165
rs1800898	*PAX2*	10	rs1800898-127_B_R_1500137251
rs996359	*PAX2*	10	rs996359-127_T_F_1503030975
rs11816136	*PAX2*	10	rs11816136-127_T_F_1500493858
rs1061413	*GFRA1*	10	rs1061413-127_T_R_1502571337
rs11598215	*GFRA1*	10	rs11598215-127_T_F_1529565010
rs3781504	*GFRA1*	10	rs3781504-127_B_R_1503959032
rs10490904	*GFRA1*	10	rs10490904-127_B_R_1528663543
rs7087152	*GFRA1*	10	rs7087152-127_T_R_1529565156
rs7087213	*GFRA1*	10	rs7087213-127_B_R_1529565029
rs11197523	*GFRA1*	10	rs11197523-127_B_F_1529565003
rs3901216	*GFRA1*	10	rs3901216-127_B_F_1526509357
rs7070180	*GFRA1*	10	rs7070180-127_B_F_1529565025
rs3781517	*GFRA1*	10	rs3781517-127_B_R_1529565151
rs3847476	*GFRA1*	10	rs3847476-127_B_F_1529565031
rs2694798	*GFRA1*	10	rs2694798-127_B_F_1502575164
rs3843600	*GFRA1*	10	rs3843600-127_T_R_1529565024
rs3781523	*GFRA1*	10	rs3781523-127_B_R_1503352656
rs3781531	*GFRA1*	10	rs3781531-127_B_R_1503025999
rs3781532	*GFRA1*	10	rs3781532-127_B_R_1503243867
rs10885864	*GFRA1*	10	rs10885864-127_B_R_1503288801
rs2420242	*GFRA1*	10	rs2420242-127_B_F_1528665329
rs10749189	*GFRA1*	10	rs10749189-127_T_R_1502551243
rs10885868	*GFRA1*	10	rs10885868-127_B_F_1529565011
rs4751955	*GFRA1*	10	rs4751955-127_B_R_1529565017
rs11197567	*GFRA1*	10	rs11197567-127_T_F_1528663522
rs7901076	*GFRA1*	10	rs7901076-127_B_R_1529565015
rs11197571	*GFRA1*	10	rs11197571-127_T_F_1529565154
rs7904143	*GFRA1*	10	rs7904143-127_B_F_1529565021
rs10787635	*GFRA1*	10	rs10787635-127_T_R_1500492485
rs11197576	*GFRA1*	10	rs11197576-127_T_R_1529565008
rs3781550	*GFRA1*	10	rs3781550-127_T_F_1503925506
rs1107030	*GFRA1*	10	rs1107030-127_B_F_1526513243
rs881726	*GFRA1*	10	rs881726-127_B_R_1503099342
rs10787637	*GFRA1*	10	rs10787637-127_T_F_1500492486
rs7907314	*GFRA1*	10	rs7907314-127_T_F_1529565020
rs3781556	*GFRA1*	10	rs3781556-127_T_R_1503293619
rs730357	*GFRA1*	10	rs730357-127_B_F_1503246500
rs7920266	*GFRA1*	10	rs7920266-127_B_R_1529565013
rs385209	*EMX2*	10	rs385209-127_B_R_1500184774
rs2240776	*EMX2*	10	rs2240776-127_T_R_1503301180
rs5030335	*WT1*	11	rs5030335-127_B_F_1528672271
rs5030320	*WT1*	11	rs5030320-127_T_F_1527354882
rs16754	*WT1*	11	rs16754-127_T_F_1529565100
rs1569776	*WT1*	11	rs1569776-127_B_F_1528674139
rs10767935	*WT1*	11	rs10767935-127_B_F_1528668337
rs3901671	*WT1*	11	rs3901671-127_T_R_1528668426
rs3858444	*WT1*	11	rs3858444-127_T_F_1527354988
rs3818055	*WT1*	11	rs3818055-127_T_F_1528672066
rs2900741	*WT1*	11	rs2900741-127_T_F_1527354886
rs7936152	*WT1*	11	rs7936152-127_T_F_1527355110
rs3930513	*WT1*	11	rs3930513-127_T_R_1527355122
rs5030141	*WT1*	11	rs5030141-127_T_F_1529564998
rs1799925	*WT1*	11	rs1799925-127_T_R_1529564948
rs520654	*IGHMBP2*	11	rs520654-127_B_F_1529245933
rs1249463	*IGHMBP2*	11	rs1249463-127_T_F_1529245900
rs560096	*IGHMBP2*	11	rs560096-127_B_F_1528489439
rs10896379	*IGHMBP2*	11	rs10896379-127_T_R_1529245726
rs10896380	*IGHMBP2*	11	rs10896380-127_T_F_1528666842

SNP	gene	Chr	Illumina ID
rs10896381	*IGHMBP2*	11	rs10896381-127_T_F_1529245786
rs637120	*IGHMBP2*	11	rs637120-127_T_R_1529245798
rs4930627	*IGHMBP2*	11	rs4930627-127_B_F_1529245859
rs674654	*IGHMBP2*	11	rs674654-127_B_R_1529245847
rs653264	*IGHMBP2*	11	rs653264-127_B_F_1529245767
rs2282504	*IGHMBP2*	11	rs2282504-127_B_R_1529245527
rs11228413	*IGHMBP2*	11	rs11228413-127_B_F_1529245971
rs598255	*IGHMBP2*	11	rs598255-127_T_R_1503037698
rs622082	*IGHMBP2*	11	rs622082-127_T_F_1528666788
rs2236654	*IGHMBP2*	11	rs2236654-127_T_F_1529245502
rs546382	*IGHMBP2*	11	rs546382-127_B_F_1529245803
rs17612126	*IGHMBP2*	11	rs17612126-127_B_R_1529245855
rs598143	*WNT11*	11	rs598143-127_T_F_1526162950
rs12277860	*WNT11*	11	rs12277860-127_T_R_1526161280
rs17749202	*WNT11*	11	rs17749202-127_B_F_1524942819
rs10899175	*WNT11*	11	rs10899175-127_T_R_1524942596
rs882151	*WNT11*	11	rs882151-127_B_F_1526094579
rs3781730	*WNT11*	11	rs3781730-127_T_R_1526155690
rs4944092	*WNT11*	11	rs4944092-127_T_F_1526150666
rs689095	*WNT11*	11	rs689095-127_T_F_1524942702
rs1790191	*UPK2*	11	rs1790191-127_B_R_1525050605
rs623500	*UPK2*	11	rs623500-127_T_F_1525130326
rs5443	*GNB3*	12	rs5443-127_B_F_1524468811
rs1554753	*RARG*	12	rs1554753-127_T_F_1525908398
rs6580936	*RARG*	12	rs6580936-127_B_R_1526093683
rs4512901	*HOXC11*	12	rs4512901-127_T_R_1529564970
rs12427129	*HOXC11*	12	rs12427129-127_B_F_1529564957
rs11911	*SPRY2*	13	rs11911-127_T_R_1500169961
rs504122	*SPRY2*	13	rs504122-127_B_F_1500040694
rs17563	*BMP4*	14	rs17563-127_B_F_1501192486
rs2071047	*BMP4*	14	rs2071047-127_T_R_1529564966
rs762642	*BMP4*	14	rs762642-127_B_F_1526093633
rs2761887	*BMP4*	14	rs2761887-127_B_R_1529564971
rs1528734	*GREM1*	15	rs1528734-127_B_R_1526338599
rs7497354	*GREM1*	15	rs7497354-127_T_F_1526340304
rs3743105	*GREM1*	15	rs3743105-127_B_R_1526340293
rs17228641	*GREM1*	15	rs17228641-127_B_R_1526339355
rs10318	*GREM1*	15	rs10318-127_T_R_1526340298
rs7176378	*GREM1*	15	rs7176378-127_B_R_1526338601
rs11070692	*FGF7*	15	rs11070692-127_T_R_1526186610
rs16962440	*FGF7*	15	rs16962440-127_T_R_1526186646
rs10519225	*FGF7*	15	rs10519225-127_B_R_1526183483
rs4338740	*FGF7*	15	rs4338740-127_T_R_1526183074
rs11070693	*FGF7*	15	rs11070693-127_T_F_1526186616
rs17400706	*FGF7*	15	rs17400706-127_B_R_1526186651
rs11634375	*FGF7*	15	rs11634375-127_B_F_1526183975
rs4480740	*FGF7*	15	rs4480740-127_B_R_1526183079
rs4407014	*FGF7*	15	rs4407014-127_T_F_1526186674
rs2413958	*FGF7*	15	rs2413958-127_B_F_1526088627
rs9972329	*FGF7*	15	rs9972329-127_T_R_1526183460
rs751979	*SALL1*	16	rs751979-127_T_F_1527981936
rs11645288	*SALL1*	16	rs11645288-127_B_R_1527959235
rs1965024	*SALL1*	16	rs1965024-127_B_F_1503024104
rs1015438	*SALL1*	16	rs1015438-127_B_F_1527969097
rs7502966	*THRA*	17	rs7502966-127_T_R_1524465306
rs1568400	*THRA*	17	rs1568400-127_T_F_1524465312
rs939348	*THRA*	17	rs939348-127_B_F_1524465307
rs2715553	*RARA*	17	rs2715553-127_T_R_1526099954
rs9303285	*RARA*	17	rs9303285-127_B_F_1527355019
rs482284	*RARA*	17	rs482284-127_T_F_1526094040
rs8074816	*WNT9B*	17	rs8074816-127_T_R_1526340330
rs2165846	*WNT9B*	17	rs2165846-127_T_F_1526340280
rs6504591	*WNT9B*	17	rs6504591-127_B_F_1526340271
rs4968281	*WNT9B*	17	rs4968281-127_B_F_1526093543
rs1530364	*WNT9B*	17	rs1530364-127_B_R_1526340269
rs2267584	*UPK1A*	19	rs2267584-127_B_R_1526360963
rs10413852	*UPK1A*	19	rs10413852-127_B_R_1526361243
rs2285421	*UPK1A*	19	rs2285421-127_T_R_1526360972
rs11882996	*UPK1A*	19	rs11882996-127_T_F_1526361218
rs1982073	*TGFB1*	19	rs1982073-127_B_F_1524232927
rs1800469	*TGFB1*	19	rs1800469-127_T_R_1503909587
rs1800468	*TGFB1*	19	rs1800468-127_B_R_1524837341
rs5995802	*TNRC6B*	22	rs5995802-127_T_F_1500073127
rs7288667	*TNRC6B*	22	rs7288667-127_T_F_1502498587
rs7292838	*TNRC6B*	22	rs7292838-127_T_F_1529246316
rs9611265	*TNRC6B*	22	rs9611265-127_B_R_1500325832
rs9611266	*TNRC6B*	22	rs9611266-127_B_F_1529246969
rs12628757	*TNRC6B*	22	rs12628757-127_B_F_1500337271
rs2413611	*TNRC6B*	22	rs2413611-127_B_F_1503271983
rs2143177	*TNRC6B*	22	rs2143177-127_T_R_1529246808
rs11089974	*TNRC6B*	22	rs11089974-127_B_F_1500072369
rs9611280	*TNRC6B*	22	rs9611280-127_T_F_1528769608
rs12628783a	*TNRC6B*	22	rs12628783-127_T_F_1529246754
rs138027	*TNRC6B*	22	rs138027-127_B_R_1503329426
rs12628042	*TNRC6B*	22	rs12628042-127_T_R_1529246552
rs9611302	*TNRC6B*	22	rs9611302-127_T_F_1500325833
rs763071	*TNRC6B*	22	rs763071-127_B_R_1502590645
rs713898	*TNRC6B*	22	rs713898-127_T_F_1502558372
rs139914	*TNRC6B*	22	rs139914-127_B_R_1502562293
rs1057353	*UPK3A*	22	rs1057353-127_T_F_1503031461
rs1135360	*UPK3A*	22	rs1135360-127_B_R_1526360985
rs3788643	*UPK3A*	22	rs3788643-127_T_F_1502575701
rs1057356	*UPK3A*	22	rs1057356-127_B_R_1503288866

Supporting Table 6.3. Three inherited *UPK3A* mutations identified in the duplex collecting system subgroup. Results of *in silico* analysis, online database queries, and renal ultrasound in parents.

Duplex collecting system and VUR case ID	location	position	amino acid	mutation (cDNA)	mutation (protein)	Sanger sequencing result in 96 control chromosomes	HGMD	ENTREZ SNP	domain	Grantham difference	align GVGD result (Grantham variation, Grantham Deviation)	previously published	renal ultrasound in parents
81079	exon 3	211	71	c.211A>G	p.Ile71Val	absent	not reported	not reported	luminal	29	ClassC0*, 179,47		no urinary tract abnormalities detected
27658	exon 6	811	271	c.811C>T	p.Arg271Trp	absent	not reported	not reported	cytoplasmic	101	Class C0*, 243,26		Parent that carries UPK3A mutation: central complex interrupted by a parenchymal ridge, suspect for a duplex collecting system. Other parent: no relevant abnormalities.
81152	exon 6	818	273	c.818C>T	p.Pro273Leu	absent	reported: accession# CM056713	not reported	cytoplasmic	98	Class C0*, 208,63	Jenkins et al.174: "probably behaves like wildtype"	no consent for renal ultrasound

* align GVGD class C0 means “probably neutral variant”

Supporting Table 6.4. Tagging and functional SNPs in this association study that passed our quality control criteria.

Gene	Percentage designed tag SNPs passed QC	Total nr of designed tag SNPs	Percentage of functional SNPs passed QC	Total nr of designed functional SNPs
HOXD11	100	2	n/a	0
BMP4	100	4	n/a	0
FSTL1	100	13	n/a	0
UPK3A	100	3	50	2
HOXC11	100	1	25	4
SALL1	100	3	20	5
FOXC1	100	1	0	2
UPK3B	100	1	0	4
CTNNB1	100	2	0	10
EMX2	100	2	0	2
SIX2	100	2	0	1
FGF10	100	6	0	1
GREM1	100	6	0	2
ITGA8	94	34	0	1
PAX2	93	14	n/a	0
ROBO2	93	81	0	6
FGF7	92	12	0	2
GDNF	92	13	0	2
RARB	91	105	n/a	0
IGHMBP2	90	10	47	17
GATA3	90	10	0	3
SLIT2	90	61	0	1
WNT11	89	9	n/a	0
UPK1B	89	19	0	1
EYA1	88	48	33	3
GFRA1	85	41	0	3
WNT9B	83	6	n/a	0
KIAA0241	80	10	67	3
WT1	80	15	25	4
TNRC6B	80	20	17	6
RET	78	18	15	13
SPRY1	67	3	0	1
SPRY2	67	3	0	2
UPK1A	67	6	0	3

Gene	Percentage designed tag SNPs passed QC	Total nr of designed tag SNPs	Percentage of functional SNPs passed QC	Total nr of designed functional SNPs
OSR1	60	5	0	2
RARA	60	5	0	2
THRA	60	5	0	2
ZIC1	50	2	n/a	0
HOXA11	50	4	n/a	0
RARG	50	4	0	1
NPNT	45	11	25	4
UPK2	33	3	50	2
*GNB3**	n/a	0	100	1
*TGFB1**	n/a	0	100	3
AGTR2	n/a	0	0	4
E2F4	n/a	0	0	2
FOXC2	n/a	0	0	2
GDF11	n/a	0	0	1
LHX1	n/a	0	0	2
MXRA8	n/a	0	0	1
SIX1	n/a	0	0	1
*VEGFA**	n/a	0	0	1

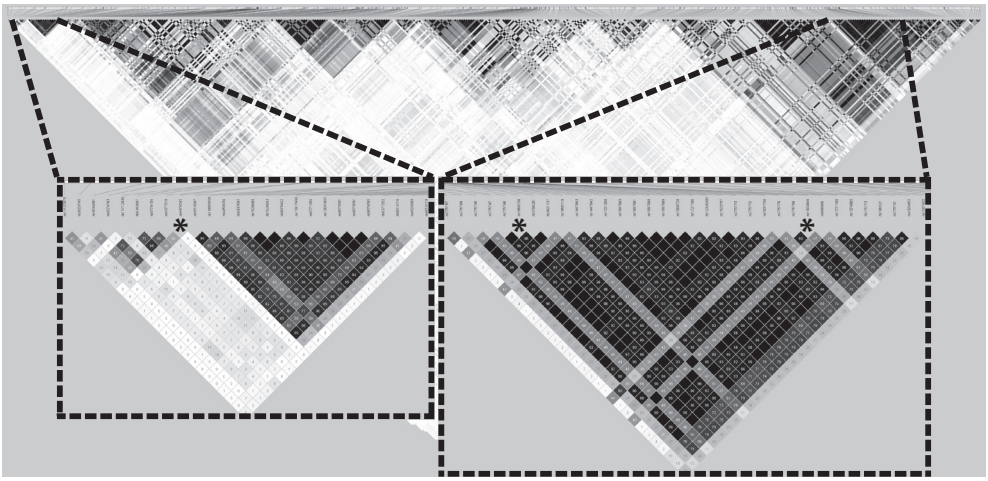
* Genes were not tagged, only specific SNPs, previously found to be associated with VUR, were included for replication.

Supporting Table 6.5. TaqMan assay IDs for SNPs genotyped in stage two (Applied Biosystems)

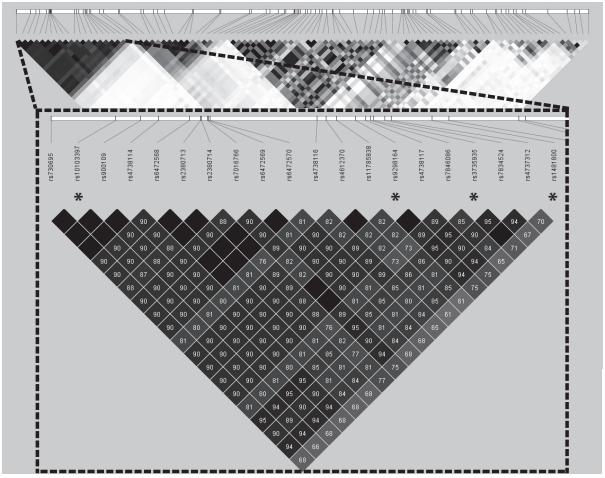
SNP	Gene	Assay ID
rs1481800	EYA1	C___7690584_10
rs3735935	EYA1	C__25804074_10
rs11197571	GFRA1	C__31975369_10
rs7497354	GREM1	C____466953_10
rs6780105	RARB	C__28985572_10
rs755661	RARB	C___1957990_20
rs1666130	ROBO2	C___8241262_10
rs1721175	ROBO2	C___2165875_10
rs4476545	ROBO2	C__30166401_20
rs1057353	UPK3A	C__11879932_20
rs1135360	UPK3A	C__11483267_1
rs3788643	UPK3A	C__27485900_10

Supporting Table 6.6. Primer sequences used for *UPK3A* sequencing and sequencing of rs1057353 (indicated with *).

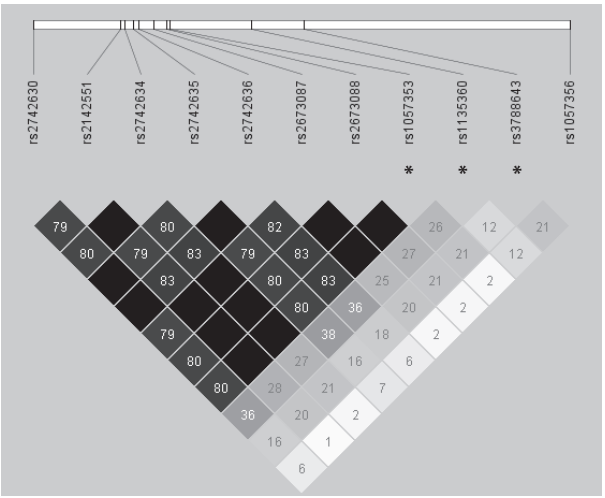
Exon_nr_strand	Sequence
exon_1_forw	ACACAGTAGGCGCTTTGAT
exon_1_rev	CGTAAACGTTGGCTATCACT
exon_2_forw	ATTTCTGAGCAGGATGACTG
exon_2_rev	TCCCTCACTAACTGGATGTC
exon_3_1_forw	CTGAGAGGGCAGAGACTAAG
exon_3_1_rev	TTTACACCCACCTGTACTCC
exon_3_2_forw*	GGCATTTGATGAATAACTGAG
exon_3_2_rev*	GCCTCTTCTGAACTTGAGG
exon_4_forw	CAGTAGCCGTCTACATTTCC
exon_4_rev	CCTGGCTACTTTTGTTTTTG
exon_5_forw	AAGTTGGAAAGTGGAATGTG
exon_5_rev	TGAGCAACTTGACTTTGATG
exon_6_1_forw	GTGGACCTCTTCCTTATTCC
exon_6_1_rev	TTTCACCTTCCCTGAAGTC
exon_6_2_forw	ATCACTCAGGAGGCTGTTC
exon_6_2_rev	CACGATCATAGCTCATTGC



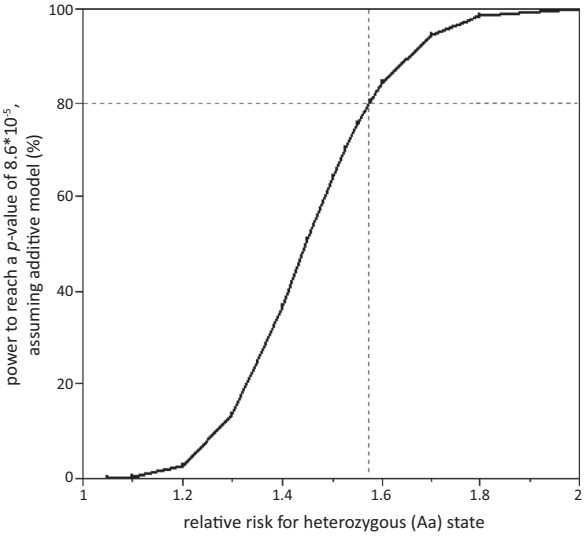
Supporting Figure 6.1. (a) LD plot (based on Hapmap r^2 data) for *ROBO2* (3 kb upstream and 2 kb downstream). SNPs that reached the cut-off for stage two of our study are highlighted (from left to right: rs4476545, rs1666130 and rs1403848; also see Table 6.1).



Supporting Figure 6.1. (b) LD plot (based on Hapmap r2 data) for *EYA1* (3 kb upstream and 2 kb downstream). SNPs that reached the cut-off for stage two of our study are highlighted (from left to right: rs10103397, rs9298164, rs3735935, rs1481800, also see Table 6.1).



Supporting Figure 6.1. (c) LD plot (based on Hapmap r2 data) for *UPK3A* (3 kb upstream and 2 kb downstream). SNPs genotyped in stage two of our study are highlighted (also see Table 6.1).



Supporting Figure 6.2. Power estimation. This study had 80% power to detect an association with a heterozygote effect size of 1.57.

CHAPTER 7

UPK3A and *FGF7* mutation analysis in Dutch renal adysplasia patients provides further evidence for the role of *UPK3A* in congenital anomalies of the kidneys and urinary tract (CAKUT) pathogenesis

Manuscript in preparation

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ABSTRACT

Renal adysplasia is part of the spectrum of congenital anomalies of the kidney and urinary tract (CAKUT) that forms a major cause of end-stage renal disease in children. Little is known about the origin of renal dysplasia, though it is anticipated that genetic and environmental factors are involved. There is a role for genes expressed during early nephrogenesis in CAKUT etiology. In this study, two genes, uroplakin 3A (*UPK3A*) and fibroblast growth factor 7 (*FGF7*), were screened for variants in a phenotypically diverse cohort of 19 Dutch renal adysplasia patients. Four novel, inherited, *UPK3A* mutations were identified in 3/19 (16%) patients with unilateral multicystic dysplastic kidney. The mutations - c.356T>C (p.Ile119Thr), c.418G>A (p.Gly140Arg), c.450C>A (p.Gly150Gly) and c.545G>A (p.Trp182X) - were not described before and not observed in 96 control chromosomes. As c.418G>A was detected in a patient with VACTERL association (Vertebral defects, Anal atresia or stenosis, Cardiac defects, Tracheo-Esophageal fistula, Radial defects and Renal anomalies, Limb defects), 25 additional DNA samples of VACTERL cases were screened; no mutations in *UPK3A* were detected. In *FGF7*, no likely pathogenic mutations were detected. This is the first time a stop-mutation in *UPK3A* is reported. All *UPK3A* mutations published so far were reviewed and *in silico* analyses are presented. This study revealed novel *UPK3A* mutations strengthening the position of variants in *UPK3A* in the etiology of renal adysplasia.

INTRODUCTION

Renal adysplasia [MIM:191830] is a congenital malformation with a prevalence of 1 in 1000 (unilateral) and 1 in 5000 (bilateral) in the general population.[10] Renal adysplasia is part of the spectrum of congenital anomalies of the kidney and urinary tract (CAKUT).[9, 10, 110] CAKUT also comprise duplex collecting system, uretero-pelvic junction obstruction, vesico-ureteric junction obstruction, vesico-ureteral reflux, ectopic ureter, bladder outflow obstruction, and posterior urethral valves.[198] These different anomalies can co-occur in one patient or in one family.

Renal adysplasia is a frequent cause of pediatric end stage renal disease (ESRD), accounting for 8.8% of Dutch pediatric ESRD.[7] The severity and clinical presentation of renal adysplasia vary widely from bilateral renal agenesis leading to Potter sequence, to unilateral renal hypoplasia detected by coincidence. Agenesis, dysplasia, and hypoplasia are different (histological) diagnoses, but in clinical practice, when histology is often not available, they are frequently difficult to distinguish.[199]

Renal adysplasia and CAKUT in general may either occur isolated or as part of a multiorgan malformation syndrome and can either be sporadic or familial.[200] Approximately 10% of patients with dysplastic kidneys have affected first-degree family members.[201-203] In many

cases there is no clear etiology, but known underlying causes of renal adysplasia include genetic defects, urinary tract obstruction and teratogens/drugs.[10] As such, the origin of renal adysplasia is anticipated to be complex in its nature, possibly covering monogenic inheritance, structural chromosomal variation, as well as multifactorial background.[10, 204, 205] During nephrogenesis, the complex interaction between the ureteric bud and metanephric mesenchyme is crucial.[8, 9, 110, 206] Genetic and environmental factors disrupting this interaction can contribute to the etiology of structural anomalies of both the upper and the lower urinary tract.[8, 207] Identification of genetic defects involved in renal adysplasia creates novel opportunities for DNA diagnostics in affected individuals and their relatives. In the current study, we focused on the identification of sequence variants in the uroplakin 3A (*UPK3A*) and fibroblast growth factor 7 (*FGF7*) genes in renal adysplasia patients.

Uroplakin 3a constitutes an important part of the urothelium. Uroplakin deficiency in mice compromises the urothelial permeability barrier via loss of urothelial plaques.[38] These mice also show enlarged ureteral orifices, vesico-ureteral reflux and hydronephrosis.[38] The mechanism through which *UPK3A* distorts nephrogenesis is not known. Uroplakin 3A is not known to be directly involved in the signaling cascade leading to ureter budding, interaction with the metanephric mesenchyme and development of the metanephros. It is conceivable that properly functioning urothelium is essential in renal organogenesis. The refluxing phenotype in the *Upk3a* knockout mouse model first suggested the human *UPK3A* to be a candidate gene for CAKUT and vesico-ureteral reflux (VUR) specifically.[208] Several studies showed VUR patients not to carry *UPK3A* mutations.[36, 37, 178] In renal adysplasia patients, however, heterozygous *UPK3A* mutations have been identified.[174, 175] In a ureter budding pathway association study that we performed recently, we found a trend towards association of common single nucleotide polymorphisms (SNPs) in *UPK3A* with VUR in combination with duplex collecting systems, another anomaly in the CAKUT spectrum. In this study, we also detected three inherited missense mutations in *UPK3A* in 105 patients with VUR and concomitant duplex collecting systems (Chapter 6).

FGF7 was previously implicated in ureter branching morphogenesis and early development of the human metanephros.[190, 209-211] *Fgf7* knockout mice were shown to have smaller kidneys and a reduced number of nephrons compared to wild-type mice.[190] This mouse phenotype in combination with the results of a pilot association experiment (unpublished data) conducted in parallel with the abovementioned association study strongly suggested *FGF7* to be a candidate gene for human renal adysplasia. To our knowledge, this gene has not been investigated in renal adysplasia patients before.

In the present study we screened the *UPK3A* and *FGF7* genes for mutations in a cohort of 19 Dutch well-characterized renal adysplasia patients showing the full extent of the phenotype.

PATIENTS AND METHODS

Patients

This was a collaborative study of the Dutch University Medical Centers of Utrecht and Nijmegen. In total 19 patients were included; 10 patients displayed (multi-)cystic renal dysplasia (multicystic dysplastic kidney; MCDK), 4 patients presented with oligohydramnion (Potter) sequence and 5 patients were diagnosed with non-cystic renal adysplasia. At least five patients had multiple features suggestive of a syndromal phenotype, including one case of suspected VACTERL association (Vertebral defects, Anal atresia or stenosis, Cardiac defects, Tracheo-Esophageal fistula, Radial defects and Renal anomalies, Limb defects). For the patient samples in which sequence variants were identified, parental DNA was available to investigate inheritance patterns. Table 7.1 represents an overview of phenotypic characteristics of the cases included in this study. For subsequent *UPK3A* mutation analysis, DNA samples of 25 additional VACTERL association cases with a renal adysplasia phenotype were selected in Nijmegen. Control DNA samples (n=48) were derived from healthy Dutch blood donors from Amsterdam and Utrecht.[183]

The Utrecht cases were retrospectively selected. Case-records of patients with a registered diagnosis of renal adysplasia for which a DNA-sample or cultured fibroblasts were available at the Clinical Genetics Department, were extensively reviewed. Patients were included based on the presence of renal agenesis, renal hypoplasia or (multicystic) renal dysplasia and the absence of an established diagnosis unequivocally accounting for the renal phenotype, such as a mutation of the *HNF1B* gene. Previous analysis of *HNF1B* and/or *RET* (other genes involved in renal adysplasia) was however not mandatory for inclusion. We prospectively included the Nijmegen cases in the AGORA (Aetiological research into Genetic and Occupational/environmental Risk factors for congenital Anomalies in children) project. Case-records were reviewed, and cases were included in this study according to the same criteria as mentioned above. In both hospitals this study was covered by standing ethics review committees' approval for genetic studies of CAKUT phenotypes.

Methods

DNA samples were isolated from whole blood or cultured fibroblasts. The full coding regions and intron-exon boundaries of *UPK3A* and *FGF7* were analyzed by conventional Sanger sequencing. Primer sequences were based on NCBI genome build 36 and are depicted in Table 7.2. DNA samples were subjected to whole genome amplification if DNA was limited (REPLI-G, Qiagen, Valencia, CA, USA) and reported variants were confirmed in non-amplified DNA. Sequencing failed for exons 2 and 4 of *FGF7* in sample 080082. *In silico* analysis of the identified and previously published mutations and SNPs was performed with Alamut version 1.5 (Interactive Biosoftware, Rouen, France) and was based on NCBI genome build 36. The online databases that were queried through Alamut are specified

in Table 7.3. Parents (n=4) of two out of three patients in whom (inherited) coding variants in *UPK3A* were detected, consented to and underwent renal ultrasound investigations.

RESULTS

Identification of *UPK3A* sequence variants

We identified four heterozygous *UPK3A* sequence variants in three patients (case 30499, 30529 and 36322), encompassing one truncating mutation (c.545G>A, p.Trp182X), two missense mutations (c.418G>A, p.Gly140Arg and c.356T>C, p.Ile119Thr) and one single base change not leading to an amino acid change (synonymous change; c.450C>A, p.Gly150Gly). These sequence variants were not identified before and not reported in online databases; details are displayed in Table 7.3.[212, 213] None of these variants were observed in our set of 96 control chromosomes. *In silico* analysis of the two identified missense mutations predicted these variants to have at most, a modest effect on the protein due to either a small physico-chemical difference (Grantham distance) and/or poor evolutionary conservation (Table 7.3). *UPK3A* mutations from the literature and known missense SNPs are also summarized in Table 7.3. *UPK3A* sequence analysis was performed in parental DNA samples: all variants were inherited from one of the parents, as depicted in Table 7.1. The patient (30490) with two *UPK3A* sequence variants inherited these variants from the same parent; both variants are thus located on the same allele. We did not perform MLPA analysis in order to check for small copy number variations, from the heterozygous SNPs that were detected no exonic deletions were to be expected (data not shown). Parents from two (30499 and 36322) out of three patients consented to having a renal ultrasound performed; no evidence for CAKUT was detected.

***UPK3A* involved in VACTERL association?**

One of the patients (36322) with a missense mutation in the *UPK3A* gene had presented with additional congenital anomalies collectively suggestive of VACTERL association. Therefore, we also performed *UPK3A* mutation analysis in 25 DNA samples of patients with renal adysplasia as part of VACTERL association. No sequence variants were detected in the additional VACTERL patients.

Novel variant in the *FGF7* gene

One sequence variant in *FGF7* (c.*182G) was identified in patient 36322, 182 bp downstream of the natural stop codon (Table 7.3). Interestingly, this region showed strong nucleotide conservation. We could not exclude an effect on the mRNA stability of the *FGF7* gene, although the *in silico* prediction did not reveal the induction of a novel splice site.

Table 7.1 Overview of the case cohort in which the *UPK3A* and *FGF7* sequencing study was performed.

sample ID	sequence variant *UPK3A* or *FGF7* (number / inherited / synonymous or non-synonymous)	sex	urinary tract phenotype
30490/30499 (two samples)	*UPK3A* (2 / yes / non-synonymous) c.356T>C, p.Ile119Thr and c.545G>A, p.Trp182X*	m	Unilateral afunctional multicystic dysplastic kidney
30529	*UPK3A* (1 / yes / synonymous) c.450C>A, p.Gly150Gly*	m	Unilateral multicystic dysplastic kidney, ipsilateral absence of ureteral ostium
36322	*UPK3A* (1 / yes / non-synonymous)* and *FGF7* (1 / unknown /synonymous) *UPK3A*: c.418G>A, p.Gly140Arg and *FGF7*: c.*182G>A*	m	Unilateral multicystic dysplastic kidney
080078/080088 (two samples)	no	m	Bilateral multicystic dysplasia
29651	no	m	Unilateral dysplastic afunctional kidney, contralateral VUR
32031	no	m	Unilateral renal agenesis
36726	no	f	Unilateral dysplastic kidney, ipsilateral VUR
080073	no	m	Unilateral cystic dysplastic kidney, contralateral VUR
080076/080087 (two samples)	no	m	Unilateral multicystic dysplastic kidney, contralateral PUJ obstruction
080090	no	f	Unilateral multicystic dysplastic kidney
29717	no	f	Unilateral multicystic dysplastic (horseshoe) kidney. Normal function on contralateral side
34124	no	m	Unilateral multicystic dysplastic kidney
41640	no	m	Unilateral multicystic dysplastic kidney, contralateral moderate hydronephrosis
080077	no	f	Bilateral hypo-/dysplastic kidneys with unilateral VUR
36286	no	m	Bilateral renal dysplasia, unilateral hydronephrosis and grade IV VUR
080074	no	m	Bilateral renal agenesis. Oligohydramnion (Potter) sequence
080081/080086 (two samples)	no		Oligohydramnion (Potter) sequence, bilateral renal and bladder agenesis
080082/080084 (two samples)	no	m	Oligohydramnion (Potter) sequence, bilateral renal and bladder agenesis
080083/080085 (two samples)	no	f	Oligohydramnion (Potter) sequence, bilateral renal agenesis

* See Table 7.3 for mutation details. Abbreviations: CAKUT, congenital anomalies of the kidney and urinary tract; DM1, Type 1 Diabetes Mellitus; *FGF7*, fibroblast growth factor 7;FISH, fluorescence in situ hybridization; MRI, magnetic resonance imaging; n/a, not applicable; *PAX2*, paired box 2; PUJ obstruction, pelvi-ureteric junction obstruction; *UPK3A*, uroplakin 3A; VACTERL, Vertebral defects, Anal atresia or stenosis, Cardiac defects, Tracheo-Esophageal fistula, Radial defects and Renal anomalies, Limb defects; VUR, vesico-ureteral reflux.

other relevant phenotypes	other genetic testing/ family history, if available
n/a	Renal ultrasound in parents: no evidence for CAKUT.
n/a	No clinical geneticist involved. No consent for renal ultrasound in parents.
Esophageal atresia, bilateral inguinal hernia, atrial septum defect, sacrococcygeal vertebral defects. Clinical geneticist: VACTERL association suspected	Postnatal karyotyping: 46,XY. No family history of congenital malformations. Parents: normal renal ultrasound.
some dysmorphic features, no lung hypoplasia	Normal postnatal karyotyping: 46,XY. Parents: normal renal ultrasound; negative family history for urinary tract phenotypes.
learning problems, gastro-esophageal reflux disease, orchidopexy	No clinical geneticist involved.
n/a	No clinical geneticist involved.
Postnatal feeding problems,tricuspid valve insufficiency	Postnatal karyotyping: 46,XX. Mother: DM1.
Mental retardation, seizures, dysmorphic features	Postnatal karyotyping: 46,XY; terminal deletion chromosome 22q13 (*UPK3A* not included). Family history positive for duplex collecting system.
n/a	Sibling with unilateral multicystic dysplastic kidney and contralateral hydronephosis and dysplasia.
n/a	n/a
n/a	No clinical geneticist involved.
dysmaturity	No clinical geneticist involved.
twin (dizygous)	No clinical geneticist involved.
slightly dysplastic retinal papillae	Postnatal karyotyping: 46,XX. *PAX2* sequencing: normal. Sibling with bilateral hypo-/dys-plastic kidneys. Parents: normal renal ultrasound.
Facial dysmorphisms, mental retardation, abnormality on brain MRI, hypotonia	Prenatal and postnatal karyotyping: 46,XY. High resolution banding for chromosome 22: normal. FISH 7q11.23 (Williams syndrome): normal
n/a	Normal postnatal karyotype. Negative family history for urinary tract phenotypes. Parents: normal renal ultrasound.
Prematurity (32 weeks)	Postnatal karyotyping: 46,XY. Renal ultrasound in parents: normal. Family history: negative.
n/a	Postnatal karyotyping: 46,XY. Family history: negative.
Anal atresia, rib abnormalities	Postnatal karyotyping: 46,XX. Family history: negative.

Table 7.2 Primer sequences used for *UPK3A* and *FGF7* sequencing.

Gene	Exon	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')
UPK3A	1	ACACAGTAGGCGCTTTGAT	CGTAAACGTTGGCTATCACT
	2	ATTTCTGAGCAGGATGACTG	TCCCTCACTAACTGGATGTC
	3 part 1	CTGAGAGGGCAGAGACTAAG	TTTACACCCACCTGTACTCC
	3 part 2	GGCATTTGATGAATAACTGAG	GCCTCTTCTGAACTTGAGG
	4	CAGTAGCCGTCTACATTTCC	CCTGGCTACTTTTGTTTTTG
	5	AAGTTGGAAAGTGGAATGTG	TGAGCAACTTGACTTTGATG
	6 part 1	GTGGACCTCTTCCTTATTCC	TTTCACCTTCCCTGAAGTC
	6 part 2	ATCACTCAGGAGGCTGTTC	CACGATCATAGCTCATTGC
FGF7	1	CCAATGAGGCAGCAAAGGT	AAATGTCAGGATTGCTCTGGA
	2	GGCTAACAATTTGGAAAGAGC	TTTTAGGGGCATAGTTCAACATAG
	3	CCATTCGTGGATCATTTGC	TTTTCAGAAAGGTGAGATATTTTGAC
	4	TTAAGAAAAAAATTACTG	TATATAACAACATCTGT

The full coding regions and intron-exon boundaries of *UPK3A* and *FGF7* were analyzed by conventional Sanger sequencing. A margin of at least 143 basepairs was observed surrounding the coding regions. Abbreviations: *FGF7*, fibroblast growth factor 7; *UPK3A*, uroplakin 3A.

Known SNPs

Both in *UPK3A* and *FGF7*, multiple known SNPs were identified in our cohort of renal adysplasia patients (Table 7.4). One of these SNPs (rs1057353) in *UPK3A* leads to an amino acid change (Alanine to Proline) and was previously shown to be tentatively associated with VUR with concomitant duplex collecting system. This does not however imply that it is pathogenic itself (Table 7.3). Its population frequency is 0.29 (Chapter 6).

DISCUSSION

In this candidate gene study of *UPK3A* and *FGF7* in a Dutch cohort of nineteen renal adysplasia patients, we identified four novel inherited mutations in *UPK3A* and one sequence variant downstream of *FGF7*. This is the first time a stop mutation in *UPK3A* (exon 4) is described. In the same patient, we detected an additional missense mutation in exon 3 on the same allele. In two more patients we detected inherited mutations; one missense, one synonymous. All mutations were detected in patients with a multicystic dysplastic kidney (MCDK) phenotype. This pilot experiment revealed novel *UPK3A* mutations in renal adysplasia patients, strengthening the role of variants in *UPK3A* in the etiology of this disorder.

The *UPK3A* stop-mutation (c.545G>A, p.Trp182X) is predicted to have a clear pathogenic effect on the protein, because of the presence of a partly assembled protein or nonsense mediated RNA decay. The detection of a stop-mutation puts a different perspective on the

previously raised suggestion that major *UPK3A* mutations might not be tolerated in humans.[38] In contrast, the clinical significance of the two amino acid changes (c.356T>C, c.418G>A) and the synonymous change (c.450C>A) in *UPK3A* is uncertain at present. As the variants were not detected in 96 control chromosomes, we can exclude that they are common polymorphisms in our population. They may represent pathogenic mutations or rare neutral changes. Given the fact that in previous studies very similar mutations in *UPK3A* were detected in renal adysplasia/CAKUT patients and not in controls, we think the latter is unlikely (Table 7.3). It is also possible that these changes are low-intermediate risk factors, which would fit with a oligogenic/multifactorial model with several risk factors, each conferring a moderate risk that can cumulatively cross a certain threshold in order for nephrogenesis to be disrupted. When considering the collected evidence for pathogenicity of all *UPK3A* mutations published so far (Table 7.3), our result is comparable to previous studies. Including the stop-mutation in our study, in total two mutations (c.545G>A and c.605G>A) are predicted to have a truly damaging effect on protein function.[175] The predicted effect of the other mutations is at most moderate. Although the evidence from mouse studies for *Upk3A* to play a role in nephrogenesis is strong, the working mechanism is not quite understood. One of the other uroplakins, also part of urothelial plaques, is an E. Coli adhesion factor.[38] Recurrent urinary tract infections can be one of the presenting features of CAKUT, and maybe variants in uroplakins can contribute to infection susceptibility and thus bias towards clinical presentation. As Uroplakin 3A seems not to be an E. Coli adhesion factor,[38] and our patients with *UPK3A* variants were already detected antenatally, a direct effect on nephrogenesis seems most likely. Functional studies are needed to resolve whether and how these new *UPK3A* variants affect expression of the protein and nephrogenesis as a whole.

The detection of inherited as opposed to the previously published de novo *UPK3A* mutations[174, 175] can imply several things. First, finding both fits with renal adysplasia occurring sporadically but also in families displaying an autosomal dominant inheritance pattern with variable expression and reduced penetrance.[202, 204, 205] Second, inherited mutations might have a milder effect on protein function and therefore act as low-intermediate risk factors in these patients. The obvious advantage of non-invasiveness of renal ultrasound examination as a screening method for the parents is weighed down by a lower sensitivity compared with more invasive imaging techniques. Therefore evidence for mild CAKUT in the parents also carrying mutations may have been missed.

In *FGF7*, one sequence variant was identified with no clear pathogenic effect, although based on our results an effect on mRNA stability could not be excluded. This variant downstream of the *FGF7* coding region was not further investigated, because there was no RNA available for follow-up studies.

The VACTERL association is a well known combination of phenotypes that occurs sporadically. The etiology is largely unknown.[214] The identification of a *UPK3A* sequence variant in a patient with a VACTERL phenotype including MCDK led us to perform sequence analysis of *UPK3A*

Table 7.3 Overview of mutations in *UPK3A* and *FGF7* detected in this study, a summary of *UPK3A* mutations in renal adysplasia/CAKUT from the literature and known missense SNPs.

Gene	Patient	This study, or previously published	Phenotype	Type of mutation	Basepair change	Amino acid change	Location	Inherited
UPK3A	n/a	Van Eerde et al. @	duplex collecting system and VUR	missense	c.211A>G	p.Ile71Val	exon 3	y
UPK3A	n/a	known SNP rs6006979 (EntrezSNP)	n/a	missense	c.272A>T	p.Gln91Leu	exon 3	n/a
UPK3A	30490/30499	this study	MCDK	missense	c.356T>C	p.Ile119Thr	exon 3	y
UPK3A	36322	this study	MCDK	missense	c.418G>A	p.Gly140Arg	exon 3	y
UPK3A	30529	this study	MCDK	synonymous	c.450C>A	p.Gly150Gly (p.=)	exon 3	y
UPK3A	n/a	known SNP rs1057353 (EntrezSNP) / Van Eerde et al. @	tentatively associated with duplex collecting system and VUR	missense	c.460G>C	p.Ala154Pro	exon 3	n/a
UPK3A	n/a	Schönfelder et al.$	MCDK	non coding	c.489-12G>A	n/a	intron 3	probably (deceased parent, siblings with same sequence variant)
UPK3A	30490/30499	this study	MCDK	stop / nonsense	c.545G>A	p.Trp182X	exon 4	y
UPK3A	n/a	Schönfelder et al.$	MCDK	missense	c.605G>A	p.Gly202Asp	exon 5	n
UPK3A	n/a	Van Eerde et al. @	duplex collecting system and VUR	missense	c.811C>T	p.Arg271Trp	exon 6	y
UPK3A	n/a	Jenkins et al.# and Van Eerde et al.@	bilateral renal adysplasia (w/o obstruction) and duplex collecting sytem and VUR	missense	c.818C>T	p.Pro273Leu	exon 6	n
UPK3A	n/a	Jenkins et al.#	bilateral renal adysplasia (w/o obstruction)	non coding	c.*67T>G	n/a	3'UTR	n
UPK3A	n/a	Jenkins et al.#	bilateral renal adysplasia (w/o obstruction)	non coding	c.*107T>C	n/a	3'UTR	n
FGF7	36322	this study	MCDK	non coding	c.*182G>A	n/a	3'UTR	not investigated

Present in 96 (Dutch) control chromosomes	In EntrezSNP	In public HGMD: accession number	Grantham distance (Scale: 0-215) [&]	Align GVGD: class [& ~]	Align GVGD: Grantham variation; Grantham deviation [&]	Nucleotide conservation [&]	Amino-acid conservation [&]	Effect on splicing [& %]	Conclusion (Comments)
n	n	n/a	29	C0	179; 0	weak	weak	see comments	unclassified variant (Effect on splicing cannot be excluded. Although splice acceptor (SA) site of exon 3 is predicted to be unaffected, cryptic SA site located 10 nucleotides downstream from natural SA seems to improve slightly because of the A>G base substitution.)
n	y	n/a	113	C15	87.17; 87.63	weak	high	n	SNP known in other than Caucasian population (Amino acid change at an evolutionary conserved position. Unlikely to interfere with normal protein function due to small physicochemical difference between Gln and Leu.)
n	n	n/a	89	C25	28.68; 69.84	weak	weak	n	unclassified variant
n	n	n/a	125	C0	152.17; 0	weak	moderate	n	unclassified variant
n	n	n/a	0	n/a	n/a	strong	n/a	n	unclassified variant
y	y	n/a	27	C0	26.87; 0	high	weak	n	known common polymorphism in Caucasian population
n	n	n/a	n/a	n/a	n/a	weak	n/a	n	unclassified variant
n	n	n/a	n/a	n/a	n/a	n/a	n/a	n/a	pathogenic mutation (Influence on protein function has not been investigated.)
n	n	CM063226	94	C65	0; 93.77	weak	high	n	pathogenic mutation (Influence on protein function has not been investigated.)
n	n	n/a	101	C0	243; 69	high	moderate	n	unclassified variant
n	n	CM056713	98	C0	208.63; 94.04	weak	moderate	n/a	unclassified variant (Detected in two unrelated patients. Protein function was investigated in COS1 celline: mutant protein protein was transported to cell membrane similar to wildtype.#)
n	n	CR057477	n/a	n/a	n/a	weak	n/a	n	unclassified variant
n	n	CR057476	n/a	n/a	n/a	weak	n/a	n	unclassified variant
n/a	n	n/a	n/a	n/a	n/a	high	n/a	n	unclassified variant (Effect on RNA stability cannot be excluded.)

(previous page)
Legend Table 7.3
Jenkins et al.[174]; $ Schonfelder et al.[175]; @ Chapter 6; & *in silico* predictions, retrieved through Alamut; % To predict effect on splicing, Alamut accesses four (online) prediction tools: SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer. We report an effect when a potential effect on splicing is predicted by three out of four tools.; ~ Seven classes based on multiple sequence alignment of eight vertebrate species, from human to frog: Homo sapiens, Pan troglodytes, Rattus norvegicus, Mus musculus, Canis familiaris, Felis catus, Bos taurus, Xenopus tropicalis. Classifiers range from C0 (least likely to interfere with function) to C65 (most likely to interfere with function). Abbreviations: 3'UTR, 3'untranslated region; COS1 cells, CV-1 (simian) Origin SV40 (kidney) cells; *FGF7*, fibroblast growth factor 7; MCDK, multicystic dysplastic kidney; RNA, ribonucleic acid; SA, splice acceptor; UPK3A, uroplakin 3A; VUR, vesico-ureteral reflux; w/o, without.

in 25 additional VACTERL patients with a renal adysplasia phenotype. In these VACTERL patients no *UPK3A* variants were detected. It is conceivable however that in patient 36322, and maybe in a minority of other VACTERL patients, *UPK3A* mutations can predispose to development of MCDK/CAKUT and therefore contribute to the renal phenotype in these complex patients.

Our work yielded *UPK3A* sequence variants in 4 of 19 patients (16% when corrected for detecting two variants on the same allele in one patient). This yield could have been even higher when taking into consideration that some likely syndromal cases (with possibly different genetic etiology) were included in our cohort. Jenkins et al. detected *UPK3A* sequence variants in 4 bilateral renal adysplasia patients, in a cohort of 17 patients with bilateral renal adysplasia, 19 patients with MCDK, and 6 patients with bilateral renal adysplasia and obstruction.[174] Schönfelder et al. investigated *UPK3A* in a cohort of 24 patients with MCDK and 81 bilateral and 60 unilateral renal adysplasia patients. They detected sequence variants in 2 MCDK patients.[175] *UPK3A* sequence variants in renal adysplasia are detected in a minority of patients.

The *UPK3A* sequence variants detected by Schönfelder et al occurred in two patients with unilateral MCDK and none were found in the other renal adysplasia patients.[175] The results of our study replicate this finding as all *UPK3A* variants were identified in MCDK patients. In the other study investigating *UPK3A* in renal adysplasia patients,[174] no sequence variants were detected in 19 MCDK patients. Our own data imply that investigating *UPK3A* in this subgroup of patients may reveal causal or predisposing genetic variants in some of them. Therefore, *UPK3A* is an important candidate gene suitable for screening in all renal adysplasia patients, and possibly duplex collecting system and other CAKUT patients. The availability of (diagnostic) sequencing of *UPK3A* will aid in clarifying in which specific CAKUT phenotypes variations in this gene are involved, either as causative or as risk variant. These findings will in the future aid in the genetic counseling of parents and patients.

Besides *UPK3A*, other genes are implied in the pathogenesis of structural renal anomalies. *HNF1B*, *RET* and *PAX2* mutations can give rise to renal adysplasia, the first often in combination with diabetes (MODY5), the latter often in combination with sometimes subtle ocular phenotypes.[10, 205, 215-219] We cannot exclude that mutations in one of these genes or other nephrogenesis genes are causing renal adysplasia in one or more of our patients. A next-

Table 7.4 Overview of known single nucleotide polymorphisms genotyped in our study population

Gene	Chromosome	Basepair position[#]	Snp	Consequence to transcript*	Minor allele / major allele	Minor allel frequency in our study population
UPK3A	22	44059488	rs2255432	upstream	A/G	0.24
UPK3A	22	44061700	rs3747234	intronic	C/G	0
UPK3A	22	44061910	rs2673088	synonymous coding	C/T	0.26
UPK3A	22	44061968	rs1057353	non-synonymous coding	G/C	0.22
UPK3A	22	44063666	rs1135360	synonymous coding	A/G	0.39
UPK3A	22	44063852	rs28561700	intronic	G/T	0.18
UPK3A	22	44070258	rs1057356	synonymous coding	G/A	0.37
FGF7	15	47504137	rs16962440	intronic	T/C	0.42
FGF7	15	47563723	rs12438444	intronic	A/G	0.28

[#] based on Ensembl v.54 (NCBI36) * concerning *UPK3A* based on canonical isoform 1 according to uniprot (http://www.uniprot.org/uniprot)

generation sequencing approach in a larger cohort allowing for many genes to be investigated at once would be a good way to proceed. Larger cohorts, being the result of an international effort, will also allow for association studies to establish whether there is a role for common genetic variants in renal adysplasia.

In conclusion, in this exploratory sequencing study of *UPK3A* and *FGF7*, for the first time we described a truncating (stop-)mutation in *UPK3A* in a renal adysplasia (MCDK) patient. We additionally detected three novel sequence variants in patients displaying MCDK. We detected *UPK3A* variants in 16% of our cohort. *FGF7* cannot be ruled out as candidate gene for renal adysplasia, but we did not detect clearly pathogenic mutations in our cohort. Expression studies and the screening of larger renal adysplasia patient cohorts for sequence variants in *UPK3A* and nephrogenesis genes, by means of a high throughput sequencing approach, will give further insight into genotype-phenotype correlations and the pathogenesis of human CAKUT. Exome sequencing studies may identify new CAKUT genes. This study supports the heterogeneous nature of renal adysplasia and the need for further genetic research of this disorder, also in the Dutch population.

ACKNOWLEDGEMENTS

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CHAPTER 8

Discussion

The main findings in this thesis are that *ROBO2* mutations can contribute to VUR and/or CAKUT in a subset of familial cases, that common SNPs in *GREM1, EYA1, ROBO2* and *UPK3A* might contribute to the genetic susceptibility for VUR, and that *UPK3A* mutations can also contribute to renal adysplasia in the Dutch population. Possibly, *UPK3A* mutations also contribute to the development of duplex collecting systems. A clinical study was performed investigating joint mobility in VUR patients. The results show that constitutive connective tissue laxity might contribute to VUR susceptibility. In this chapter I will highlight a few recurring themes and ideas for future studies from different perspectives.

PERSPECTIVE 1: PATIENTS

The results presented in this thesis as such modestly contribute to genetic counseling of a subset of CAKUT patients. *ROBO2* mutations are detected in 5% or less of familial VUR/CAKUT patients (chapter 4).[168, 173] *UPK3A* mutations are detected in a subset of Dutch renal adysplasia patients, but may also contribute to the development of duplex collecting systems and VUR, since in some patients with VUR and duplex collecting systems genetic variants in *UPK3A* were detected (chapter 6). Future studies are needed to quantify the risk that can be attributed to mutations in these and other CAKUT genes.

With an established genetic defect, more detailed, personalized, genetic counseling can take place. Siblings and other family members can be tested to identify persons at risk. When a mutation is de novo, the recurrence risk is probably low. In rare instances a parent can carry more than one germcell with the mutation (so-called germline mosaicism) and, although the mutation will not be detectable in DNA from blood, this parent can have more than one affected child. Family members that are at risk (because they carry the mutation) can be followed up rigorously in order to detect early signs of renal damage and start preventive measures at that time point.[220, 221] This subgroup of patients might also undergo more invasive imaging procedures than just a renal ultrasound if additional benefit of such procedures can be demonstrated.

If the identified genetic defect is unlikely to be Mendelian and probably constitutes one of multiple unidentified low effect size risk factors, and the pedigree does not resemble one of the Mendelian inheritance patterns, the risk for family members most probably is low. With more and more diagnostic tests being performed, increasing numbers of these low-risk variants will be identified. In these cases, there is as yet no evidence for a particular screening approach in relatives. The genetic counseling in these cases can be challenging.

When a genetic risk factor in a family with a patient with end stage renal failure is known, it might also be added to the suitability screening methods for living related kidney donors. With the present knowledge, careful evaluation of the identified genetic risk factor(s) and estimation of usefulness of genetic and other screening options have to be done for each family separately,

by a well-informed specialist. In the Dutch healthcare system this specialist preferably is a clinical geneticist.

PERSPECTIVE 2: CAKUT ALSO AFFECT ADULTS

Many genetic studies on VUR/CAKUT including ours cite the relative contribution of VUR/CAKUT to pediatric end stage renal disease (ESRD).[30, 148, 222, 223] Although pediatric ESRD is a pressing problem, the absolute numbers of patients requiring renal replacement therapy (RRT) as minors are low (on average 30-35 cases/ year in the Netherlands from 2000-2009; www.renine.nl).

To our knowledge, details on relative CAKUT contribution were not readily available for the Dutch adult onset RRT population. Therefore, we requested the current prevalence numbers from the Dutch RRT Registration ('Renine'; www.renine.nl). Figure 8.1 is compiled from this dataset. This figure shows the relative contribution of VUR and other congenital urinary tract anomalies in the population of Dutch RRT patients, stratified by age at onset of renal replacement therapy. Figure 8.1 shows that on Jan 1st 2010, for at least 35% of the patient population that started renal replacement therapy as a minor, the registered primary diagnosis was consistent with CAKUT.

This percentage is comparable to the percentage over 1987-2001[7] and is roughly comparable to numbers cited in other study populations.[224-226] With regard to adult age at onset of RRT in the Netherlands it is apparent that in the age groups of 18-30 yrs and 31-60 yrs the relative contribution of congenital anomalies is at least 20% and 8%, respectively. At least 10% of the RRT population as a whole has a primary diagnosis compatible with CAKUT. Two percent of the total RRT population has a registered primary diagnosis of VUR. It is conceivable that these are underestimations, since especially in the older part of the cohort, VUR or other not immediately clinically apparent congenital anomalies such as renal dysplasia might not have been diagnosed. Furthermore, we assumed that in this dataset 10% of non-specific diagnoses (like e.g. 'chronic renal failure, aetiology uncertain' and 'pyelonephritis/interstitial nephritis-cause not specified', see Figure 8.1) were in fact attributable to CAKUT. This probably is a conservative estimate. The fact that at least 10% of total ESRD is attributable to congenital anomalies and can likely be extrapolated to the larger population of patients with chronic renal disease not requiring RRT increases the impact of CAKUT even more.

In the group that was defined as likely CAKUT (Figure 8.1), the most prevalent diagnoses were: VUR, congenital obstructive uropathy, cystic kidney disease type unspecified, renal dysplasia, renal hypoplasia and medullary cystic disease (data not shown).

CAKUT being the primary cause in at least 10% of total renal disease makes it an important area of research.

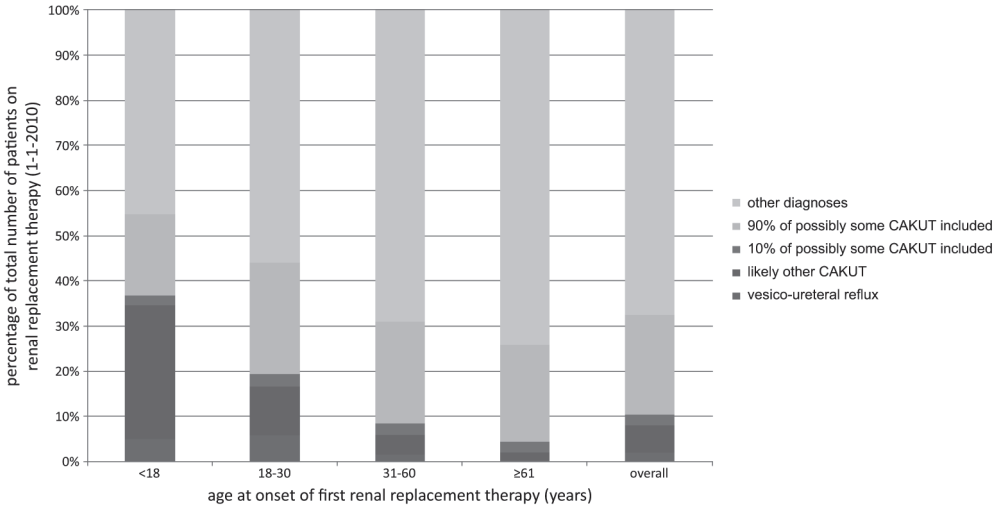

Figure 8.1 Relative contribution of VUR and/ or CAKUT as primary diagnoses in the population of patients on renal replacement therapy in the Netherlands divided by age category. Data provided by Renine (www.renine.nl). Primary diagnoses are grouped in the portrayed categories, the official primary diagnoses are the following: In blue: Pyelonephritis/Interstitial nephritis due to vesico-ureteric reflux without obstruction; in red: Pyelonephritis/Interstitial nephritis due to congenital obstructive uropathy with or without vesico-ureteric reflux / Cystic kidney disease-type unspecified / Polycystic kidneys, infantile (recessive) / Medullary cystic disease, including nephronophthisis / Cystic kidney disease-other specified type / Congenital renal hypoplasia-type unspecified / Oligomeganephronic hypoplasia / Congenital renal dysplasia with or without urinary tract malformation / Syndrome of agenesis of abdominal muscles (Prune Belly Syndrome); in light and dark green, diagnoses where possibly some CAKUT is included: Chronic renal failure, aetiology uncertain / Pyelonephritis/ Interstitial nephritis-cause not specified / Pyelonephritis or Interstitial nephritis due to other cause / Hereditary or Familial nephropathy-type unspecified / Hereditary nephropathy-other / Missing primary diagnoses. We assumed that in this dataset 10% of non-specific diagnoses (in dark green) were in fact attributable to congenital anomalies. See page 205 for a full-color representation of this figure.

PERSPECTIVE 3: GENETIC RESEARCH INTO VUR/CAKUT

Genetic technology and methodology in complex diseases have taken flight over the past years. The studies in this thesis are an excellent example. The approaches used range from linkage analysis in familial cases and sequencing a candidate gene that was serendipitously identified via a chromosomal aberrations in a syndromal patient, to association studies using large cohorts of unrelated (non-syndromal) cases.

First, selection of (familial) cases was aimed at identifying high risk (Mendelian) genetic variants, the large scale association studies were aimed at identifying common low risk genetic variants, in line with the common disease common variant hypothesis.[52] Although in other fields hypothesis-free genome wide association studies (GWAS) have been performed extensively, revealing over 1100 loci in over 165 diseases,[227] in the field of VUR/CAKUT hypothesis-free (genome wide) studies have been limited to linkage(-like) approaches in

familial cases.[21, 22, 27, 41-43] Chapter 6 is actually the first study that sets out to fully capture common genetic variation in a whole pathway in non-related VUR patients. The results of this study need to be replicated in other cohorts. The results of both genome wide human genetic studies and human genetic studies on >1 gene or locus are summarized in Appendix 1. Together with supplementary Table 6.1 in chapter 6 this summary can be used as a reference for future studies. Most identified loci await significant replication, but there are some regions that, albeit not significant, show peaks across multiple studies. These regions are located at 1p36, 3q12-q21 (in three separate studies), 3q26, 4q32, 6q23-q27, 10q25-q26, 13q33 (in three separate studies), 22q11-q12 and Xp11.

In order to obtain an adequate sample size to perform GWAS in this field, it will be necessary to establish international consortia, as has been done for other disorders.[228-231] After the first rush of GWAS in complex diseases, it has become apparent that a considerable proportion of the genetic component of these diseases still remains unexplained.[232]

With the advent of Next Generation sequencing the genetic community now turns again to studying rare monogenic diseases, but also to studying the contribution of rare variants to common complex genetic diseases.[180, 181, 233-237] Next Generation sequencing enables the generation of vast amounts of sequence data, for e.g. the entire genome, or exome (all genes), or large numbers of candidate genes, in one experiment. We intend to use these approaches in our future studies. The most challenging part of this new approach is the filtering of the true causal genetic variants from the total numbers of genetic variations detected.[181, 235]

Next Generation sequencing allows for large scale pathway testing: We intend to design a capture array with all genes known or suspected to be in or related to the ureter budding pathway. With this array we will not only be able to fully investigate our cohort of VUR/CAKUT patients for common and rare variations in these candidate genes, but we can also investigate samples of isolated or familial CAKUT cases where regular diagnostic analyses, like *PAX2*, *HNF1B* and *EYA1* molecular analysis, have not yielded a causal mutation. Limiting oneself to a predefined set of genes, leads to a raised coverage of the targeted sequences, and makes it financially possible to investigate large numbers of samples. In this way, association studies of putative low-risk genetic variants will gain power. Furthermore, the choice of analyzing a defined pathway eases the process of selecting true causal variants and interpreting their effects. The capture array results will be valuable in themselves, but they can also serve as a screening tool for samples before including them in exome- or even genome-sequencing experiments. Another set of genes that might be a good object for a targeted approach are the genes that code for extracellular matrix (ECM) proteins. The results of chapter 3 hint at the possible contribution of systemic connective tissue laxity to VUR susceptibility. If future studies can strengthen the hypothesis that persons with joint hypermobility also show differences in bladder or ureter connective tissue composition, the ECM will be a second focus of research.

Exome sequencing or whole genome sequencing will also be performed in subsets of our cohort. These subsets might constitute familial cases, or cases with a particular CAKUT endophenotype. With these approaches, there always is a chance of false negatives, and it will sometimes be difficult to interpret the data. In contrast to the targeted approach however, the great virtue of these methodologies is their intrinsic lack of prior hypothesis. This will enable us to identify new genes in the ureter budding pathway, or even previously unthought-of mechanisms involved in VUR/CAKUT development. At some point the costs per sample may be low enough that they become the method of choice for both ureter budding pathway and other analyses.

PERSPECTIVE 4: PHENOTYPE

In the course of collecting the Dutch VUR cohort and performing the studies in this thesis, several lessons were learned regarding the recording of the VUR phenotype.

The fact that adults can often only be assigned the unknown status, although they may have had VUR as a child, is at the expense of power in linkage studies. In the beginning of the project we aimed to collect VUR families. This approach did not yield enough families for a genome wide linkage approach. This was partly caused by the fact that there probably are not as many large families as was previously assumed. Also, when familial VUR was suspected, it often turned out to be a case of familial CAKUT, which at that point was decided to be too broad a phenotype to study in this way.

A confounding factor in assigning VUR affected status is the fact that concomitant symptoms of lower urinary tract (LUT) malfunction (chapter 3 and Neveus et al. 2006[3]) are often present and can even cause mild VUR by themselves. It is important to record LUT symptoms in order to be able to perform sub-group analyses in the future.

With the ureter budding paradigm in mind, it is not surprising that in a subset of VUR patients lateralized ureteral ostia are reported (personal observation).[9, 91, 137] These ureters are in fact ectopically inserted. At present, there is no quantitative measure for this displacement available. In addition, this observation is not recorded in every patient. It is generally accepted in urological clinical practice that lateralized ostia can be associated with primary VUR. One of the lines for future research might be to devise a preferably non-invasive, validated measure for ostia displacement and to prospectively record ostia displacement in at least all VUR cases in whom cystoscopy is performed. Ostia displacement might turn out to be a useful quantitative endophenotype for dissecting LUTS related VUR and more ectopic budding related VUR.

Our cohort was collected retrospectively, from patients who had usually undergone a cystoscopy for diagnostic or therapeutic reasons at least once. This enabled us to collect a well-characterized cohort. The downside of that approach is that phenotype recording was

based on non-standardized medical records, which undoubtedly introduced some noise in our dataset.

We designed a phenotype database for recording VUR phenotype; this database enabled us to extract subgroups of patients. Not all available data have been used for analyses yet, to avoid having to correct for an endless number of tests. To our knowledge, probably our cohort and the 150 affected sib-pair cohort in the study of Briggs et al.[22] are the cohorts that have been phenotyped in most detail. For future collection of large international cohorts an international research phenotyping standard could be designed by combining the database design of both studies.

Of the Dutch pediatric population on RRT, at least 35% has a registered primary diagnosis consistent with CAKUT. An aspect of CAKUT genetics, not addressed in this thesis, is the question why some CAKUT patients progress to end stage renal failure and others do not. Given the conservatively estimated prevalence of VUR in children of 1%,[1, 2] only a minor subset of these patients will eventually need RRT. Similarly to the *APOL1* risk allele in African Americans, that significantly increases the risk of developing nondiabetic ESRD after various primary diagnoses,[238, 239] ESRD predisposition risk factors may also exist in our population. These factors may e.g. influence the host defense mechanisms against (ascending) urinary tract mechanisms. To have adequate power for studies designed to identify genetic risk factors for the subset of CAKUT patients that progress to renal failure, a large sample size is necessary which will involve setting up international collaborations. Also, considerable attention will have to be given to consistently recording renal phenotypes in all cohorts.

PERSPECTIVE 5: THE FUTURE OF DIAGNOSING GENETIC CAUSES OF CAKUT

In a recent paper, Adalat et al.[240] describe the proceedings in a Clinic for Genetic Renal and Urinary Tract Malformations in London. The referral criteria for this clinic were (1) a child with a renal tract malformation (RTM) accompanied by syndromic features such as neuro-developmental delay, external dysmorphology, and malformations of nonrenal tract internal organs; and/or (2) a child with RTM with one or more siblings and/or a parent with a RTM.[240] In a three year period, 91 patients from 68 families were referred to the clinic. 27 patients from 20 families could be assigned to a recognized genetic syndrome and/or were found to have a mutation considered to be the cause of the renal tract malformation (RTM). This yields a per family diagnosis rate of almost 30% (20/68; A. Woolf, personal communication). Given the fact that research was not performed in this clinic and that only readily available genetic diagnostic tests like array-CGH and *HNF1B* and *PAX2* mutation analysis were performed this is a very respectable result. Within the Netherlands clinical genetics community, our group is on its way to become a CAKUT center of expertise. We intend to set up The Utrecht Clinic

for Genetic Renal and Urinary Tract Malformations, which will be a joint venture of the UMC Utrecht Department of Medical Genetics and the UMC Utrecht Pediatric Renal Center (see Figure 8.2). This clinic will most likely constitute a concept in which a clinical geneticist, a pediatric urologist and a pediatric nephrologist can be convened to discuss and sometimes re-evaluate qualifying cases (see Figure 8.2).

At the same time in the DNA diagnostic laboratory of the UMC Utrecht, we aim to offer parallel sequencing of increasing numbers of known CAKUT genes like *ROBO2, UPK3A, PAX2, HNF1B, RET* and *GDNF*. In this way we hope to increase the number of diagnosed cases and aid in the genetic counseling of a growing group of patients and their families. The samples submitted for diagnostics can, unless patients or parents object, anonymously be part of research and be used for detecting new CAKUT genes. Figure 8.2 shows the suggested follow-up in first degree relatives and genetic diagnostic flow for VUR patients.

Conclusion

The findings in this thesis will be of benefit in genetic counseling of a subset of CAKUT patients. At completion of this thesis, available CAKUT molecular diagnostics, especially in patients

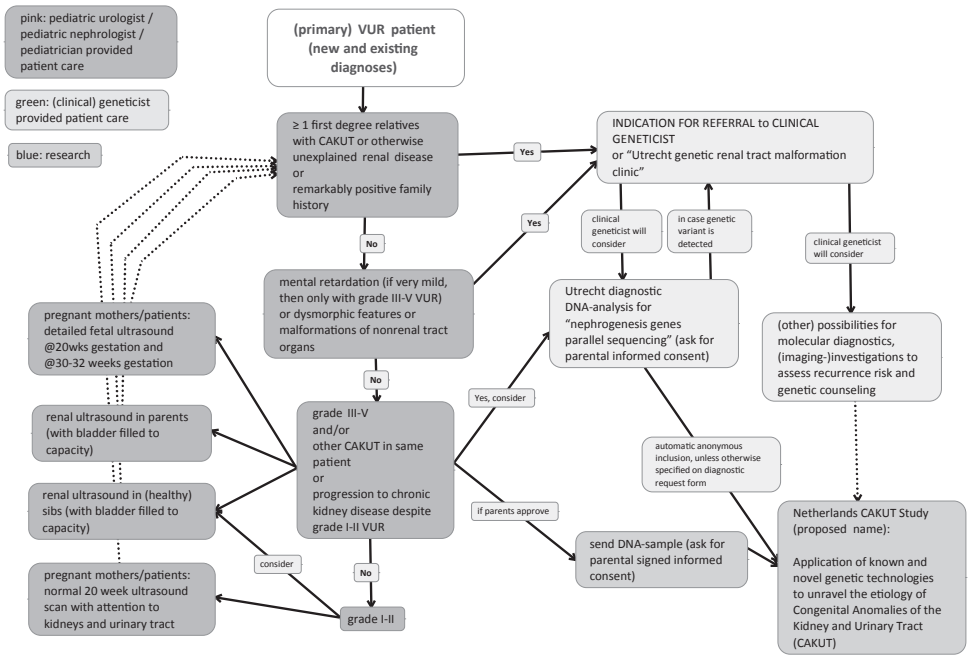

Figure 8.2 Flow-chart with the suggested follow-up in first degree relatives and genetic diagnostic flow for VUR patients. Recurrence risk and inheritance patterns are discussed in chapter 1. See page 206 for a full-color representation of this figure.

with no extrarenal features, is still quite limited. With the advent of the new massive parallel sequencing technologies we hope to in the near future be able to contribute to expanding the spectrum of genes that are offered in regular diagnostics. In this way a larger subset of CAKUT patients can be offered individualized genetic counseling.

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The author likes to thank Dr. Aline Hemke from Renine for kindly providing the data for Perspective 2.

CHAPTER 9

Summaries in English and Dutch

SUMMARY

Chronic renal failure and end stage renal disease (ESRD) can be life-threatening conditions. In a significant number of cases with ESRD the primary cause lies in congenital anomalies of the kidney and urinary tract (CAKUT). Vesico-ureteral reflux (VUR) is part of the CAKUT spectrum. VUR is the retrograde passage of urine from the bladder into the upper urinary tract. It is one of the most common congenital anomalies and has a conservatively estimated prevalence of 1%. It has a primary and a secondary form. Primary VUR is due to an incompetent valve mechanism at the uretero-vesical junction. Secondary VUR is due to an obstruction in the lower urinary tract. VUR may occur isolated or as part of syndromes; i.e., in combination with other congenital anomalies. The studies in this thesis focus on isolated primary VUR. Most children grow out of VUR without serious morbidity. A subset however does develop long-term complications. In this group VUR results in renal damage, either as a result of ascending urinary tract infections (reflux nephropathy) or of renal maldevelopment, which is often associated with VUR. These two groups together account for 15% of ESRD in Dutch children.

A genetic basis for VUR was recognized already several decades ago, based on pedigree analyses. Siblings and especially identical twins of patients with VUR have an increased prevalence of VUR. In some families children have almost 50% chance of inheriting VUR from an affected parent, suggesting that one genetic risk factor with a high effect causes VUR in these families. In other families, however, the inheritance pattern is much less clear. Most cases are sporadic cases, suggesting that a combination of multiple minor effect size genetic risk factors and environmental factors cause VUR in these patients.
An early disruption of the embryonal development of the kidneys and urinary tract is a mechanism for the development of VUR. Genes involved in this ureter budding process are considered to be potential candidate genes for VUR susceptibility. The effect size of variations in these genes may range from low to high. Disruption of this process (in mice) leads to the diverse spectrum of anomalies known as CAKUT. CAKUT include VUR, under- or maldeveloped kidneys and duplex collecting systems. Variable combinations of these anomalies are indeed seen in families (both in humans and mice) suggesting that the same genetic variation can be causally involved in the whole CAKUT spectrum. The studies presented in this thesis were aimed at identifying genetic risk factors for VUR and/or CAKUT.

Chapter 2 presents an in depth literature study regarding the relationship between prenatally detected hydronephrosis (accumulation of urine in the kidney; PNH) and postnatal VUR. As only a subset of cases is detected prenatally, we set out to evaluate whether these cases more often have VUR cases in their family and therefore a higher genetic predisposition. It appeared that in none of the studies family history was adequately reported. From the

summarized studies it was apparent that of patients with PNH, in 15% primary VUR will be detected, approximately 35% will appear to have other urogenital anomalies and 50% will have normal postnatal examinations.

To explore whether we could provide clinical evidence for a new hypothesis on a contributory constitutional factor to VUR predisposition, and therefore on VUR genetics, we evaluated joint hypermobility in 50 VUR patients (chapter 3). Indeed, VUR patients do show increased passive joint hypermobility when compared to controls. This finding suggests that patients with hypermobile joints may have underlying systemic laxity that might in turn contribute to VUR.

In chapters 4, 5 and 6 we investigated candidate genes mainly involved in the ureter budding process for their role in VUR development with varied genetic approaches.

For chapter 4 we studied a syndromal patient with severe VUR who had a complex chromosomal aberration disturbing the *ROBO2* gene. ROBO2 is a protein known to have an important role in the ureter budding pathway. Disruption in mice of Robo2 protein function resulted in VUR and/or CAKUT. This led us to search for mutations in this gene in an international cohort of VUR patients. Indeed mutations in *ROBO2* were detected in two familial cases. These findings were later replicated by other investigators.

In chapter 5 we performed a linkage study in four families. The study focused on a subset of genes in the ureter budding process and other candidate regions. In these four families, we could significantly exclude a role for the majority of genes under investigation.

In chapter 6 we performed an association study of common SNPs in 44 genes in the ureter budding pathway in > 400 Dutch VUR cases and > 1400 controls. None of the SNPs were significantly associated to VUR. Common SNPs in four ureter budding genes (*GREM1, EYA1, ROBO2* and *UPK3A*) did show a trend towards association. In a subset of VUR patients with duplex collecting systems we identified 3 patients with mutations in *UPK3A* that possibly contribute to their congenital anomaly.

In chapter 7 we broadened our scope towards another part of the CAKUT spectrum: renal adysplasia (under- or maldeveloped kidneys). We investigated a gene known to be involved in renal adysplasia in other populations (*UPK3A*) and a candidate gene (*FGF7*). We studied 19 patients and identified the first known stop-mutation in *UPK3A*.

In conclusion, the studies in this thesis show that *ROBO2* mutations can contribute to VUR and/or CAKUT in a subset of familial cases, that common SNPs in *GREM1, EYA1, ROBO2* and

UPK3A might contribute to the genetic susceptibility for VUR, and that UPK3A mutations can also contribute to renal adysplasia in the Dutch population. Possibly, UPK3A mutations also contribute to the development of duplex collecting systems. Future studies will shed further light on the impact of genetic variation in the ureter budding process on the development of CAKUT and on risk estimates involved in the detection of genetic variants in patients and their families.

SAMENVATTING IN HET NEDERLANDS

Terminaal nierfalen veroorzaakt een slechte kwaliteit van leven en heeft een hoge mortaliteit en comorbiditeit. In 10% van de gevallen van eindstadium nierfalen is de primaire oorzaak een aangeboren anatomische afwijking van de nieren of urinewegen. Vesico-ureterale reflux (VUR) is de terugstroom van urine van de blaas omhoog naar de nieren en is een veelvoorkomende aangeboren afwijking. Bij primaire VUR ontstaat de terugstroom door een disfunctionerend ventiel daar waar de urineleider in de blaas uitmondt. Secundaire VUR ontstaat als gevolg van een verstopping in de blaas of de urinebuis, waardoor de druk in de blaas toeneemt, en urine uiteindelijk richting de nieren stroomt. VUR kan geïsoleerd voorkomen, of in combinatie met aangeboren afwijkingen aan andere organen. De studies beschreven in dit proefschrift richten zich voornamelijk op geïsoleerde primaire VUR.
VUR komt voor bij minstens 1% van alle pasgeborenen. De meeste kinderen groeien over VUR heen zonder noemenswaardige restverschijnselen. Een deel ontwikkelt op de lange termijn echter nierfalen. In deze groep is VUR geassocieerd met nierschade door opstijgende urineweginfecties (‘refluxnefropathie’) of is er primaire onderontwikkeling van de nier (‘nier hypo- of dysplasie’).

Het was al jaren bekend dat erfelijke aanleg een rol speelt bij het ontstaan van VUR. Broers en zussen en in het bijzonder eeneiïge tweelingen van VUR patiënten, hebben een verhoogde kans op VUR. In sommige families lijken kinderen zelfs 50% kans te hebben om VUR van een aangedane ouder te erven. In deze families lijkt er één genetische risicofactor te zijn, die een hoge kans op VUR geeft. In andere families is het overervingspatroon minder duidelijk. Meestal is VUR niet erfelijk. Dit suggereert dat een combinatie van meerdere genetische risicofactoren, elk voor zich met een lage kans om VUR te veroorzaken, en omgevingsfactoren verantwoordelijk is voor het ontstaan van VUR bij deze patiënten.
De embryonale ontwikkeling van de nieren en van de urinewegen zijn sterk met elkaar verweven. Bij muizen kan een vroege verstoring van dit proces dan ook leiden tot een gevarieerd spectrum van afwijkingen aan zowel de nieren als urinewegen. Combinaties van dit soort afwijkingen worden ook gezien bij verschillende patiënten in dezelfde familie. Het lijkt dus waarschijnlijk dat genen die betrokken zijn bij deze embryonale ontwikkeling een rol spelen bij het ontstaan van VUR. De kans op een aangeboren afwijking in de nieren of urinewegen door een afwijking in deze genen zou kunnen variëren, afhankelijk van het specifieke gen en het soort afwijking. De studies in dit proefschrift richten zich op het identificeren van genetische risicofactoren voor het ontstaan van aangeboren nier- en urinewegafwijkingen in het algemeen en VUR in het bijzonder.

Hoofdstuk 2 betreft het verslag van een literatuuronderzoek naar de relatie tussen verwijding van de nierbekkens die voor de geboorte met echo-onderzoek ontdekt was en familiaire VUR

na de geboorte. Als deze relatie aantoonbaar zou zijn, zou men door patiënten te selecteren met prenataal ontdekte afwijkingen, een groep kunnen creëren waarbij de kans op het vinden van genetische risicofactoren met een hoge kans op de aandoening groter is. Het bleek dat in geen van de studies over prenataal ontdekte afwijkingen de familiegeschiedenis goed was gedocumenteerd. Uit de samengevatte studies bleek wel dat bij patiënten met prenataal ontdekte verwijding van de nierbekkens, in 15% na de geboorte VUR wordt vastgesteld, en dat in totaal 50% aangeboren afwijkingen van de nieren of urinewegen heeft. Bij de overige 50% werden na de geboorte geen afwijkingen vastgesteld.

Om te verkennen of er naast de embryonale aanleg theorie ook een ander mechanisme een rol zou kunnen spelen bij de aanleg voor VUR, hebben we onderzocht of kinderen met VUR leniger zijn dan kinderen zonder VUR. Dit bleek inderdaad het geval te zijn. (hoofdstuk 3). Mogelijk is er bij deze kinderen sprake van een onderliggende bindweefselzwakte die niet alleen in de gewrichten, maar ook in de urinewegen tot uitdrukking komt. Deze bindweefselzwakte zou kunnen bijdragen aan de gevoeligheid voor het ontwikkelen van VUR.

In de hoofdstukken 4, 5 en 6 is met verschillende genetische onderzoeksmethoden onderzocht of afwijkingen in genen die betrokken zijn bij de vroege embryonale ontwikkeling van de nieren en urinewegen een aanleg voor VUR veroorzaken.

Ten grondslag aan hoofdstuk 4 ligt het vinden van een complexe afwijking in het erfelijk materiaal van een patiënt met ernstige VUR. Deze complexe afwijking verstoorde het *ROBO2* gen. Uit muizenstudies bleek dat een dergelijke verstoring van *Robo2*, VUR en andere aangeboren afwijkingen van de urinewegen kon veroorzaken. In een groot internationaal cohort van VUR patiënten vonden we vervolgens twee patiënten (met familiaire VUR) met een afwijking in het *ROBO2* gen.

Hoofdstuk 5 beschrijft de resultaten van een genetische koppelingsstudie (linkage analyse) in vier grote families. Hierbij is gericht gezocht naar koppeling met genen betrokken bij de embryonale ontwikkeling en naar koppeling met regio's die bekend waren uit een eerdere Engelse studie. Er was geen koppeling aantoonbaar. Wel kon voor het merendeel van de genen en regio's worden aangetoond dat ze in deze families met grote zekerheid géén rol spelen.

Het doel van genetische associatiestudies is om na te gaan of vaak voorkomende genetische variaties geassocieerd zijn met risicofactoren met, ieder voor zich, een lage kans op VUR. In hoofdstuk 6 hebben we onderzocht of dit soort variaties in 44 genen betrokken bij de embryonale ontwikkeling van de nieren en urinewegen geassocieerd zijn met VUR. Deze

studie werd verricht in erfelijk materiaal van meer dan 400 VUR patiënten en meer dan 1400 controles. Hoewel er geen variaties waren die significant vaker voorkwamen bij de patiënten dan bij de controles, zagen we wel een trend richting associatie in vier van de onderzochte genen (*GREM1, EYA1, ROBO2* en *UPK3A*). Deze genen zullen moeten worden onderzocht in grotere cohorten, om na te gaan of ze daadwerkelijk een rol spelen. Onze resultaten wijzen erop dat variaties in het *UPK3A* gen alleen van belang zijn bij VUR patiënten met een extra aangeboren afwijking aan de hogere urinewegen, te weten een verdubbeld verzamelsysteem.

Hoofdstuk 7 beschrijft onderzoek van een andere afwijking uit het spectrum van aangeboren afwijkingen van de nieren en urinewegen. Renale a-dys-plasie is een verzamelterm voor het niet of onvoldoende ontwikkeld zijn van de nieren. In erfelijk materiaal van 19 patiënten met deze aandoening hebben we in twee kandidaatgenen onderzocht of er mutaties waren. Uit studies in andere populaties was al bekend dat mutaties in het *UPK3A* gen een rol kunnen spelen bij renale a-dys-plasie. Ook in ons cohort hebben we mutaties in *UPK3A* aangetoond. In het andere kandidaatgen, *FGF7*, zijn geen mutaties van betekenis aangetroffen.

Concluderend hebben de studies in dit proefschrift aannemelijk gemaakt dat mutaties in het *ROBO2* gen het ontstaan van VUR en/of aangeboren nier- en urinewegafwijkingen kunnen veroorzaken bij patiënten die ook aangedane familieleden hebben. Verder hebben we laten zien dat veelvoorkomende genetische variaties in de genen *GREM1, EYA1, ROBO2* en *UPK3A*, mogelijk bijdragen aan de genetische gevoeligheid voor VUR. Mutaties in *UPK3A* konden ook worden aangetoond in een klein Nederlands renale a-dys-plasie cohort en mogelijk dragen mutaties in *UPK3A* bij aan het ontstaan van verdubbelde verzamelsystemen. Nieuwe studies zullen het effect van genetische variatie in de embryonale ontwikkeling van de nieren en urinewegen verder ophelderen.

List of abbreviations

LIST OF ABBREVIATIONS

AGORA	Aetiological research into Genetic and Occupational/environmental Risk factors for congenital Anomalies in children
AGTR2	angiotensin II receptor, type 2
ALS	amyotrophic lateral sclerosis
APD	anteroposterior diameter
APOL1	apolipoprotein L, 1
ASP	affected sibpair study
BJHS	benign joint hypermobility syndrome
BMI	body mass index
BMP(4)	bone morphogenetic protein (4)
BOFS	branchiooculofacial syndrome
BOR	branchiootorenal syndrome
CAKUT	congenital anomalies of the kidney and urinary tract
CAP	continuous antibiotic prophylaxis
CC3 motif	conserved cytoplasmic motif 3
CEPH	Centre d'Etude Polymorphism Humaine
CEU	CEPH European
CGH	comparative genomic hybridization
CI	confidence interval
CMV	cytomegalovirus
CNS	central nervous system
COS1 cells	CV-1 (simian) Origin SV40 (kidney) cells
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
DGAP	Developmental Genome Anatomy Project
DM1	Type 1 Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSA renogram	dimercapto-succinic acid (DMSA) renogram
DNA	deoxyribonucleic acid
DTWD2	DTW domain containing 2
DYX5	dyslexia susceptibility locus 5
E2F4	E2F transcription factor 4, p107/p130-binding
ECM	extracellular matrix
EDS	Ehlers Danlos syndrome
EDTA	ethylenediaminetetraacetic acid
EMX2	empty spiracles homeobox 2
ES cells	embryonic stem cells
ESRD	endstage renal disease

EYA1	eyes absent homolog 1 (Drosophila)
FCS	fetal calf serum
FGF10	fibroblast growth factor 10
FGF7	fibroblast growth factor 7
FISH	fluorescence in situ hybridization
FOXC1	forkhead box C1
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1)
FSTL1	follistatin-like 1
GATA3	GATA binding protein 3
GDF11	growth differentiation factor 11
GDNF	glial cell derived neurotrophic factor
GFP	green fluorescent protein
GFRA1	GDNF family receptor alpha 1
GIDS	genetic information database system
GJH	generalized joint hypermobility
GNB3	guanine nucleotide binding protein (G protein), beta polypeptide 3
GREM1	gremlin 1
GWAS	genome wide association study
HEK cells	human embryonic kidney cells
HGNC	HUGO Gene Nomenclature Committee
HGPSS	horizontal gaze palsy with progressive scoliosis
HLA	major histocompatibility complex
HLADRB1	major histocompatibility complex, class II, DR beta 1
HLOD	heterogeneity logarithm of the odds ratio
HNF1B	HNF1 homeobox B
HOX11	homeobox 11
HOXA11	homeobox A11
HOXC11	homeobox C11
HOXD11	homeobox D11
HPLC	high-performance liquid chromatography
HWE	Hardy-Weinberg equilibrium
IACUC	Institutional Animal Care and Use Committee
ICD	intracellular domain
ID	identification
IGHMBP2	immunoglobulin mu binding protein 2
IQ	intelligence quotient
IQR	interquartile range
ITGA8	integrin, alpha 8
IVP	intravenous pyelogram

KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3
KIAA0241	AVL9 homolog (S. cerevisiase) (previously known as KIAA0241)
LD	linkage disequilibrium
LHX1	LIM homeobox 1
LOD	logarithm of the odds ratio
LOH	loss of heterozygosity
LUT(S)	lower urinary tract (symptoms)
MAF	minor allele frequency
MAG3	mercaptoacetyltriglycine
MAOA	monoamine oxidase A
MAOB	monoamine oxidase B
MC	medical center
MCDK	multicystic dysplastic kidney
MIM	Mendelian inheritance in man
MLPA	multiplex ligation-dependent probe amplification
MODY	maturity-onset diabetes of the young
MPL	multi-point LOD score
MRI	magnetic resonance imaging
MXRA8	matrix-remodelling associated 8
NCBI	National Center for Biotechnology Information
NNBSD	nonneuropathic bladder/sphincter dysfunction
NPL	non-parametric linkage
NPNT	nephronectin
OMIM	online Mendelian inheritance in man
OSBPL1A	oxysterol binding protein-like 1A
OSR1	odd-skipped related 1 (Drosophila)
PAX2	paired box 2
PBS	phosphate buffered saline
PCDH11Y	protocadherin 11 Y-linked
PCR	polymerase chain reaction
PNH	prenatally detected hydronephrosis
PUJ	pelvi-ureteric junction
QC	quality control
QQ-plot	quantile-quantile plot
RAI1	retinoic acid induced 1
RARA	retinoic acid receptor, alpha
RARB	retinoic acid receptor, beta
RARG	retinoic acid receptor, gamma
RET	ret proto-oncogene

RN	reflux nephropathy
RNA	ribonucleic acid
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)
ROBO2	roundabout, axon guidance receptor, homolog 2 (Drosophila)
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila)
RRT	renal replacement therapy
RTM	renal tract malformation
RT-PCR	reverse transcriptase PCR
SA	splice acceptor
SALL1	sal-like 1 (Drosophila)
SCMV	simian cytomegalovirus
SD	standard deviation
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3 domain	SRC homology 3 domain
SIX1	SIX homeobox 1
SIX2	SIX homeobox 2
SIX5	SIX homeobox 5
SLIT2	slit homolog 2 (Drosophila)
SMS	Smith-Magenis syndrome
SNP	single nucleotide polymorphism
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)
SPRY2	sprouty homolog 2 (Drosophila)
STRP	short tandem repeat polymorphism
SVZa	anterior subventricular zone
TDT	transmission disequilibrium test
TGFB1	transforming growth factor, beta 1
THRA	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)
TNRC6B	trinucleotide repeat containing 6B
TXNL4A	thioredoxin-like 4A
UCSC	University of California, Santa Cruz
UK	United Kingdom
UMC	University Medical Center
UPJ(O)	ureteropelvic junction (obstruction)
UPK1A	uroplakin 1A
UPK1B	uroplakin 1B
UPK2	uroplakin 2
UPK3A	uroplakin 3A
UPK3B	uroplakin 3B

US	ultrasound
USF2	upstream transcription factor 2, c-fos interacting
UTI	urinary tract infection
UTR	untranslated region
UVJ	uretero-vesicular junction
VACTERL	Vertebral defects, Anal atresia or stenosis, Cardiac defects, Tracheo-Esophageal fistula, Radial defects and Renal anomalies, Limb defects
VCUG	voiding cystourethrogram
VEGFA	vascular endothelial growth factor A
VUR	vesico-ureteral reflux
WAGR	Wilms tumor, Aniridia, Genitourinary anomalies and mental Retardation syndrome
WNT11	wingless-type MMTV integration site family, member 11
WNT9B	wingless-type MMTV integration site family, member 9B
WT1	Wilms tumor 1
WTCCC	Wellcome Trust case control consortium
YFP	yellow fluorescent protein
ZIC1	Zic family member 1 (odd-paired homolog, Drosophila)

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Appendix 1

Overview of results of human genetic VUR studies

Overview of results of human genetic VUR studies; genome wide linkage and association studies or studies covering more than one candidate gene. Completeness was favored over significance thresholds and/or statistical scrutiny: loci displayed are reported as interesting by original authors.

chromosome	bp startposition (Ensembl release 60)	bp endposition (if applicable; Ensembl release 60)	publication	hypothesis / type of study	(upstream) marker/ SNP	downstream marker (if applicable)
1	3585005	37649717	A	genome wide parametric and non-parametric linkage	D1S468	D1S255
1	22960354		B	genome wide non-parametric linkage	rs665691	n/a
1	107154982	157932774	C	genome wide parametric and non-parametric linkage	GATA176CO1!	D1S1653
1	110696107	167729948	D	parametric and non-parametric linkage to candidate locus (replication)	D1S3723%	D1SDVCAG1^
1	111414360	157932774	E	parametric linkage to candidate locus (replication)	D1S2809	D1S1653
1	160009121	210522817	F	genome wide parametric and non-parametric linkage	rs1053074	rs946125
1	223816864	240876770	A	genome wide parametric and non-parametric linkage	D1S213	D1S2785
2	11808864		B	genome wide parametric and non-parametric linkage	rs4669767	rs101084321
2	26715509		B	genome wide non-parametric linkage	rs4665856	
2	28603479	60795786	A	genome wide parametric and non-parametric linkage	D2S165	D2S337%
2	185273128		B	genome wide non-parametric linkage	rs1304514	
2	234764497	242758942	F	genome wide parametric and non-parametric linkage	rs887062	rs16747
3	1538617	4593188	F	genome wide parametric and non-parametric linkage	rs1499260	rs902982

position (cM) (when and as reported by original authors)	parametric (heterogeneity) LOD score *	non parametric LOD score	NPL: *p*-value	association: OR	association: *p*-value	remarks
4–65	1.13	2.22	0.02			
41.418	not reported	1.064	0.0134			in UK population
149–166	3.12	5.94	0.0002			79% of families linked; probably the upstream marker was misspelled, D1S3723 / GATA176G01 does map to the right position on chromosome 1, but is not mapped to the current assembly. D1S248 was used to estimate upstream startposition
140.39-182.4	<-2; excluded	<0				for the upstream marker D1S2695 (the first marker tested downstream), and for the downstream marker D1S2750 was chosen to estimate physical position on current genome build
144-164	-3.18					excluded under homogenic model
152-203	1.45	2.10	0.0001			see tables in original article for possibly smaller boundaries; 29% of families linked
242–266	0.61	2.35	0.02			
29.73-33.27	3.02	2.334	0.0005			3.02 is highest HLOD with 40% of families linked, in a recessive model (UK population; rs4669767); HLODs for dominant model are also > 2 (rs645490)
49.453	not reported	1.276	0.0077			in Slovenian subset
not reported	1.3	3.05	0.062			suggestive in one family; D2S2160 was used to estimate the physical position of the downstream marker
189.214	not reported	1.268	0.0078			UK and Slovenian populations combined
243–261	2.90	4.10	0.00001			40% of families linked
3–12	-14.28	1.67	0.003			

chromosome	bp start	bp end	publication	study design	upstr. marker	downstr. marker
3	8185781	34624405	C	genome wide parametric and non-parametric linkage	GATA164B08%	D3S1768
3	68018646	79811728	E	candidate gene linkage	AAC023	D3S3681
3	73431830	125936487	F	genome wide parametric and non-parametric linkage	rs7064	rs713161
3	75955846	77699115	B	candidate gene association		
3	77110500		G	candidate gene association	rs4476545	
3	77598943		G	candidate gene association	rs1666130	
3	79811728	143371594	A	genome wide parametric and non-parametric linkage	D3S3681	D3S1569
3	100128691	167239700	C	genome wide parametric and non-parametric linkage	GATA128C02%	D3S1763
3	124225091		B	genome wide parametric linkage	rs484936	
3	171786094	177775345	F	genome wide parametric and non-parametric linkage	rs1563436	rs1489630
3	175013564		B	genome wide non-parametric linkage	rs4894708	
3	187110983		B	genome wide TDT: family based association	rs2102860	
3	187331590		B	genome wide non-parametric linkage	rs7635068	
4	10603017	20506101	F	genome wide parametric and non-parametric linkage	rs1981635	rs729918
4	20254883	20622184	D	candidate gene linkage		
4	120148181	169843146	A	genome wide parametric and non-parametric linkage	D4S402	D4S1597
4	124317950	124324910	D	candidate gene linkage		
4	156514725		B	genome wide non-parametric linkage	rs1123037	
5	25967703		B	genome wide non-parametric linkage	rs4307059	
5	37812779	37839788	D	candidate gene linkage		
5	81207272	86794495	H	genome wide non-parametric linkage (ASP)	rs1566629	rs878196
5	118216318		B	genome wide TDT: family based association	rs4895183	

position (cM)	(H)LOD	NPL	NPL *p*	asso-ciation: OR	asso-ciation: *p*-value	remarks
61–65	1.01	1.90	0.04			
108	-8.14					*ROBO2*: excluded under homogenic model
96–131	1.55	1.36	0.006			29% of families linked
						ROBO2: no evidence for association
				1.48	0.005	*ROBO2*: warrants replication in independent cohort
				0.8	0.007	*ROBO2*: warrants replication in independent cohort
109-158	2.69	2.96	0.008			76% of families linked; before finemapping: NPL 2.75 and HLOD 1.52; marker boundaries show initial region of interest, shared haplotype is between markers D3S3641 and D3S1764
114–177	1.89	3.79	0.004			D3S1544 was chosen to estimate physical startposition on current genome build
131.01	3.02					18% of families linked; recessive model; UK and Slovenian population combined
174–181	-10.39	1.60	0.0031			
180.583	not reported	1.552	0.0038			in Slovenian subset
197.67	not reported			3.8	7.43x10-7	more significant in subset with VUR only (instead of VUR or RN); UK and Slovenian populations combined;
197.985	not reported	1.411	0.0054			UK and Slovenian populations combined
18–30	1.64	1.33	0.007			29% of families linked
34	-2.15	0.32				*SLIT2*: excluded
117–169	1.31	2.15	0.02			suggestive in one family; that family showed excess allelesharing to another locus too (refer to original article)
126	-3.25	0.86				*SPRY1*: excluded
150.575	not reported	1.271	0.0078			in Slovenian subset
45.367	not reported	1.342	0.0065			in Slovenian population
54	-2.03	0.22				*GDNF*: excluded
98.58	not reported	4.46	0.00001			marker boundaries encompass entire region with NPL > 3; slightly larger region than 1-LOD drop
124.75				2.23	2.55x10-6	in Slovenian population; intronic SNP in *DTWD2*

chromosome	bp start	bp end	publication	study design	upstr. marker	downstr. marker
5	118240507		B	genome wide TDT: family based association	rs17144806	
5	132540109		B	genome wide parametric linkage	rs2162769	
5	n/a	n/a	F	genome wide parametric and non-parametric linkage	not reported	not reported
6	28644637	36632998	E	parametric linkage to candidate locus (replication)	D6S1022	D6S1051
6	41677196	48849865	E	parametric linkage to candidate locus (because of patients with deletions in that region)	D6S1017	D6S2410%
6	135373076	170850862	F	genome wide parametric and non-parametric linkage	rs1041480	rs756519
6	146347402		B	genome wide parametric and non-parametric linkage	rs863820	
6	164250674		B	genome wide non-parametric linkage	rs9364703	
6	169656248		B	genome wide non-parametric linkage	rs6605523	
7	150640285	155522126	F	genome wide parametric and non-parametric linkage	rs1547958	rs1343750
8	12835952	19786756	C	genome wide parametric and non-parametric linkage	D8S1106	D8S1145%
8	22786150		B	genome wide non-parametric linkage	rs2457426	
8	23753451		B	genome wide non-parametric linkage	rs819188	
8	72127764		G	candidate gene association	rs3735935	
8	136390515	138855902	I	genome wide parametric linkage	rs10505635	rs9324462
9	89208916		B	genome wide non-parametric linkage	rs12338022	
10	43572475	43625799	D	candidate gene linkage		
10	43572475	43625799	B	candidate gene association		
10	95964438	119340155	E	candidate gene linkage	D10S677	D10S1425
10	102505468	102589698	D	candidate gene linkage		
10	102505468	102589698	B	candidate gene association		

position (cM)	(H)LOD	NPL	NPL *p*	asso-ciation: OR	asso-ciation: *p*-value	remarks
124.76				2.39	5.81x10-7	in Slovenian population; intronic SNP in *DTWD2*
134.51	2.72					Slovenian population; recessive model; 33% of families linked
145	1.31	not reported				25% of families linked
45	-4.69					HLA: excluded under homogenic model : NB checken of zij verwijzen naar eerdere linkage studie
66	-3.84					excluded under homogenic model; D6S1280 was chosen to estimate physical endposition on current genome build
137–188	3.55	2.65	0.0002			see tables in original article for possibly smaller boundaries; 42% of families linked
147.51	3.12	2.35	0.0005			could correspond to peak in Kelly et al; UK population; when phenotype restricted to VUR only; parametric LOD: recessive model with 41% of families linked
176.067	not reported	1.200	0.0068			UK and Slovenian populations combined
187.786	not reported	2.213	0.0007			Slovenian population
164–179	1.84	2.19	0.0007			33% of families linked
24–30	0.86	2.57	0.01			
40.707	not reported	1.386	0.0058			UK and Slovenian populations combined
41.833	not reported	1.171	0.0101			UK population
				1.27	0.007	*EYA1*: warrants replication in independent cohort
	4.2	n/a	n/a	n/a	n/a	one Somalian CAKUT pedigree; recessive model; no mutations in *KHDRBS3* at this locus; phenotype: high grade VUR; UPJO; renal agenesis; incomplete duplex collecting system
85.707	not reported	1.058	0.0137			Slovenian population
66	-2.55	0.80				*RET*: excluded
						RET: no evidence for association
125	-4.43					*PAX2*: excluded under homogenic model
124	-3.43	0.63				*PAX2*: excluded
						PAX2: no evidence for association

chromosome	bp start	bp end	publication	study design	upstr. marker	downstr. marker
10	112053599	135053123	F	genome wide parametric and non-parametric linkage	rs1050755	rs880340
10	117923225		B	genome wide non-parametric linkage	rs4751955	
10	123436950		B	genome wide TDT: family based association	rs1696803	
10	127280375	129792021	B	genome wide parametric and non-parametric linkage	rs1368532	rs7904367
10	128566964		B	exploratory genome wide association	rs11599217	
10	129260749	131915365	E	parametric linkage to candidate locus (because of patients with deletions in that region)	D10S1222	D10S1248%
11	3060725		B	genome wide non-parametric linkage	rs451041	
11	23191372		B	exploratory genome wide association	rs17306391	
11	26105639		B	genome wide TDT: family based association	rs11029158	
11	64302526	83289994	B	genome wide parametric and non-parametric linkage	rs10792438	rs535809
11	80941126		B	genome wide non-parametric linkage	rs7933150	
12	3369174		B	genome wide non-parametric linkage	rs1860436	
12	27199180	51485234	J	genome wide parametric linkage	rs1388659	D12S361
12	128911838		B	genome wide non-parametric linkage	rs10773541	
13	47286938		B	genome wide non-parametric linkage	rs2762130	
13	91970363	112795433	C	genome wide parametric and non-parametric linkage	D13S793%#	D13S285

position (cM)	(H)LOD	NPL	NPL *p*	asso-ciation: OR	asso-ciation: *p*-value	remarks
119–165	1.78	2.89	0.00013			36% of families linked
135.942	not reported	1.413	0.0054			UK population
146.57				1.80	2.25x10-6	combined UK and Slovenian populations
154.661-160.38	2.44	2.32	0.0005			could correspond to peak in Kelly et al; NPL: both in UK and Slovenian population combined and in Slovenian population separately; NPL shown is for phenotype restricted to VUR (peak at rs7904367); parametric LOD: recessive model with 28% of families linked, in combined populations
158.28					3.96x10-7	UK VUR population and WTCCC controls; STATA *p*-value
165	-3.13					excluded, marker D10S505 was chosen to estimate physical endposition on current genome build
3.929	not reported	1.383	0.0058			Slovenian population
38.74					5.67x10-7	UK VUR population and WTCCC controls; STATA *p*-value
42.96				2.88	1.82x10-6	combined UK and Slovenian populations; *p*-value decreases to 10-7 with narrow VUR phenotype
86.984 -89.27	3.33	2.490	0.0004			in Slovenian population; parametric LOD for recessive model with 53% of families linked (rs2097171); also a peak in parametric dominant model at rs 535809
87.935	not reported	2.032	0.0011			UK and Slovenian populations combined; through the Affy-ID in the original paper it was apparent there was a typing error: rs9733150 should be rs7933150
not reported	not reported	2.02	0.001			UK popution positive for reflux nephropathy
	3.6					60% of families linked; LOD-1 interval displayed; recessive model
156.377	not reported	1.099	0.0122			UK and Slovenian populations combined
50.435	not reported	1.490	0.0044			UK population
(96–113)	2.08	2.37	0.02			D13S886 was chosen to estimate physical startposition on current genome build

chromosome	bp start	bp end	publication	study design	upstr. marker	downstr. marker
13	105018861	106907833	H	genome wide non-parametric linkage (ASP)	rs714668	rs2039120
13	106491736	108616491	F	genome wide parametric and non-parametric linkage	rs981900	rs1876723
14	25248293		B	genome wide non-parametric linkage	rs17795514	
15	33015402		G	candidate gene association	rs7497354	
16	82150293	90111279	F	genome wide parametric and non-parametric linkage	rs967955	rs8577
17	31312527		B	genome wide non-parametric linkage	rs9895463	
17	36046434	36105237	B	candidate gene association		
17	66780395	80822080	F	genome wide parametric and non-parametric linkage	rs755424	rs733342
18	21798850		B	genome wide TDT: family based association	rs11083021	
18	29950624		B	genome wide TDT: family based association	rs16963279	
18	43018264	44828935	H	genome wide non-parametric linkage (ASP)	rs1054986	rs1434511
18	77765220		B	exploratory genome wide association	rs12604993	
19	30417027	45330408	E	parametric linkage to candidate locus (because of patients with deletions in that region)	D19S433	D19S559
19	36157715	36169367	D	candidate gene linkage		
19	53149967		B	genome wide parametric linkage	rs475188	
20	559263	12584311	D	parametric and non-parametric linkage to candidate locus (replication)	D20S103	D20S604
20	559263	17372490	C	genome wide parametric and non-parametric linkage	D20S103	D20S470
20	11017796	18264029	F	genome wide parametric and non-parametric linkage	rs742920	rs761461
20	22412084	43768281	C	genome wide parametric and non-parametric linkage	D20S477	D20S481
21	36543928		B	genome wide non-parametric linkage	rs2834819	

position (cM)	(H)LOD	NPL	NPL *p*	asso-ciation: OR	asso-ciation: *p*-value	remarks
104.36	not reported	3.7	0.00001			marker boundaries: encompass entire region with NPL > 3; slightly larger region than 1-LOD drop; note: marker rs1339518 that the authors depict on the upstream boundary of the peak maps to chromosome 10; see original article figure 2
100–108	1.37	1.55	0.004			29% of families linked
19.591	not reported	1.923	0.0017			Slovenian population
				0.77	0.002	*GREM1*; warrants replication in independent cohort
102-123	-4.02	1.63	0.003			no HLOD reported
55.513	not reported	1.009	0.0155			Slovenian population
						HNF1B: no evidence for association
91–126	not reported	2.56	0.0003			
45.29				8.00	4.90x10-8	*p*-value for combined UK and Slovenian populations; top-hit in UK population; in intron 3 of *OSBPL1A*
54.84				4.88	3.13x10-6	intronic SNP in *FAM59A*; in Slovenian population
65.28	not reported	3.71	0.00002			note: refer to original article as 1-LOD drop was estimated from figure 2
121.56					2.38x10-7	in LD with *TXNL4A*; UK VUR population and WTCCC controls; SNPTEST *p*-value
59	-2.06					candidate gene: *USF2*; note: D19S559 currently also maps to chromosome 2
61	-2.9	0.69				*UPK1A*: excluded
92.61	2.87					combined UK and Slovenian populations; recessive model; 26% of families linked
2.13-32.94	<-2; excluded	<0				
2–36	1.22	1.57	0.02			
30–41	1.25	2.09	0.001			see tables in original article for possibly smaller boundaries
42–54	2.90	3.42	0.003			
41.632	not reported	1.092	0.0125			UK population

chromosome	bp start	bp end	publication	study design	upstr. marker	downstr. marker
21	37091275	38052785	B	genome wide parametric and non-parametric linkage	rs2835104	rs9977677
21	43841827	48062080	F	genome wide parametric and non-parametric linkage	rs876498	rs2256207
22	23068519	33209423	A	genome wide parametric and non-parametric linkage	D22S539%	D22S280
22	24037551	28856460	C	genome wide parametric and non-parametric linkage	GCT10C10 = D22S1685	D22S689
22	45680863	45691755	D	candidate gene linkage		
22	45680863	45691755	B	candidate gene association		
22	45683304		G	candidate gene association	rs1057353	
6/7?	n/a	n/a	B	genome wide non-parametric linkage	rs601223	
X	13931785		B	genome wide non-parametric linkage	rs11798108	
X	15323616	64655336	C	genome wide parametric and non-parametric linkage	GATA175D03= DXS9902	DXS7132
X	42848384		B	genome wide TDT: family based association	rs1983167	
X	42854606		B	genome wide TDT: family based association	rs7881785	
X	109197403		B	genome wide non-parametric linkage	rs2499416	
X	115301975	115306225	D	candidate gene linkage		
X	115301975	115306225	B	candidate gene association		

A Conte et al.[42]; B Cordell et al.[27]; C Feather et al. 2000[21]; D This thesis, chapter 5; E Sanna-Cherchi et al.[41]; F Kelly et al.[43]; G This thesis, chapter 6; H Briggs et al.[22]; I Ashraf et al.[223]; J Wenget al.[222]; *dominant model unless otherwise specified; ^ self-designed marker; # not in Marshfield map; ! maps to chromosome 2; % does not map to current assembly, nearby marker was chosen from Marshfield Genetic map to estimate band and physical position; WTCCC Wellcome Trust case control consortium

position (cM)	(H)LOD	NPL	NPL *p*	asso-ciation: OR	asso-ciation: *p*-value	remarks
42.21 - 43.627	3.21	2.637	0.0002			could correspond to peak on chromosome 21 in Kelly et al.; in combined UK and Slovenian populations; HLOD for recessive model with 28% of families linked at rs2835104; at rs9977677 dominant model HLOD peak of 3.11
52–58	-25.69	1.73	0.002			no HLOD reported
not reported	1.3	3.05	0.062			suggestive in one family; D22S686 was used to estimate the physical position of the upstream marker
24–28	0.3	1.9	0.04			
53	-3.4	0.80				*UPK3A*: excluded
						UPK3A: no evidence for association
				1.52	0.008	*UPK3A*: in subset with duplex collecting systems only; warrants replication in independent cohort; of note: also rare coding sequence variants detected in subset with duplex collecting systems
69.891	not reported	1.253	0.0082			UK and Slovenian populations combined; note: according to Ensembl (v60 and v54) this SNP maps to chromosome 6, while according to the original paper it maps to chromosome 7
27.452	not reported	2.012	0.0012			Slovenian population
74–84	0.89	2.04	0.03			
66.77				1.64	5.22x10-5	in LD with *MAOA*; combined UK and Slovenian populations; show clear departure from theoretical chi2 distribution (QQ-plot)
66.78				1.62	6.97x10-5	in LD with *MAOB*; combined UK and Slovenian populations; show clear departure from theoretical chi2 distribution (QQ-plot)
105.135	not reported	1.029	0.0147			UK population
71	-3.81	0.88				*AGTR2*: excluded
						AGTR2: no evidence for association

Appendix 2

Color figures

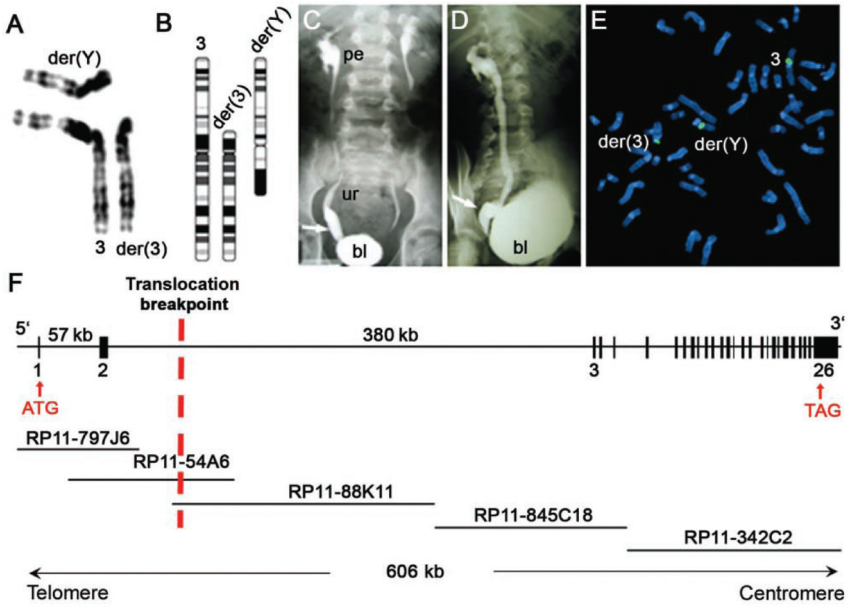

Figure 4.1 *ROBO2* disrupted in DGAP107. Partial karyogram (*A*) and idiogram (*B*) for 46,X,t(Y;3)(p11;p12)dn is shown. VCUG of DGAP107 shows anterior-posterior (*C*) and lateral (*D*) views of bilateral grade IV VUR and megaureter at the right UVJ (*arrows*). bl = Bladder; pe = renal pelvis; ur = ureter. *E*, FISH analysis showing BAC RP11-54A6 (*green*), which hybridizes to normal chromosome 3, der(3), and der(Y) and crosses the 3p12 breakpoint. *F*, Intron-exon structure of *ROBO2*, with select exons numbered and the relevant BAC contig. The location of the 3p12 translocation breakpoint is indicated by a red dotted vertical line.

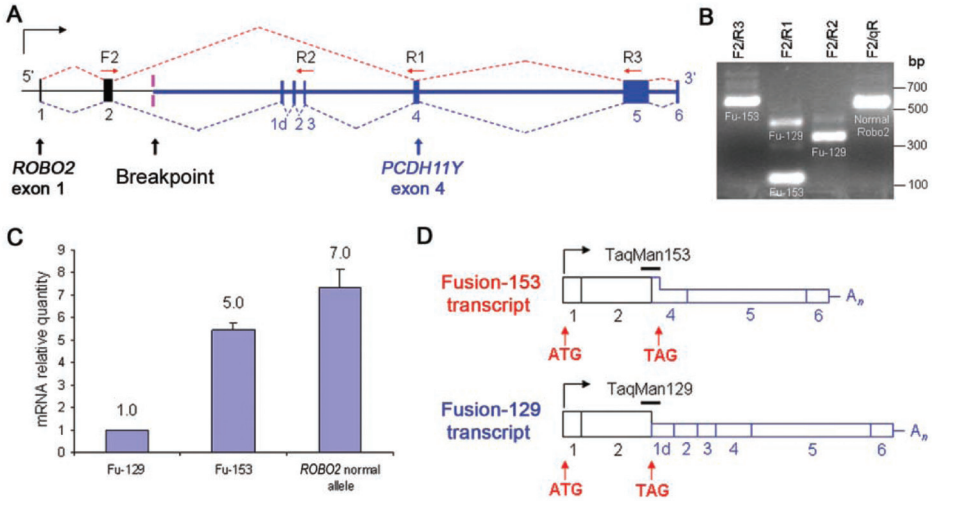

Figure 4.2 The t(Y;3) translocation in DGAP107, which generates novel *ROBO2* fusion transcripts. *A*, *ROBO2* and *PCDH11Y* intron-exon structure surrounding the der(Y) breakpoint. The forward primer F2 in *ROBO2* exon 2 (*black bar*) was used in RT-PCR with three reverse primers-R2, R1, and R3-in *PCDH11Y* exons 3, 4, and 5, respectively (*blue bars*). Dotted lines indicate the observed splicing patterns of the two fusion transcripts. The red splicing pattern generates Fu-153, which encodes 153 aa, and the blue pattern generates Fu-129, which encodes 129 aa. *B*, RT-PCR fusion transcript amplification. *Lane 1*, F2/R3 primers amplify Fu-153 (641 bp) and Fu-129 transcripts; only the shorter Fu-153 amplicon is shown. *Lane 2*, F2/R1 primers amplify transcripts for both Fu-129 (456 bp) and Fu-153 (122 bp). *Lane 3*, F2/R2 primers amplify only Fu-129 transcripts (347 bp). *Lane 4*, F2/qR primers amplify only transcripts from the wild-type nontranslocated *ROBO2* allele (606 bp). qR primer is located in exon 7 of ROBO2. C, Real-time RT-PCR quantitation of *ROBO2* fusion transcripts Fu-129 and Fu-153 (detected by TaqMan probes shown in panel D) and of *ROBO2* nontranslocated allele transcripts (detected by TaqMan probe across *ROBO2* exons 2 and 3) in DGAP107 lymphoblast RNA. *D*, Exon structure of Fu-153 and Fu-129. Horizontal bars indicate TaqMan probes used to quantify fusion transcripts. Black boxes indicate ROBO2 exons; blue boxes, *PCDH11Y* exons; fullheight boxes, coding exons; and half-height boxes, noncoding exons.

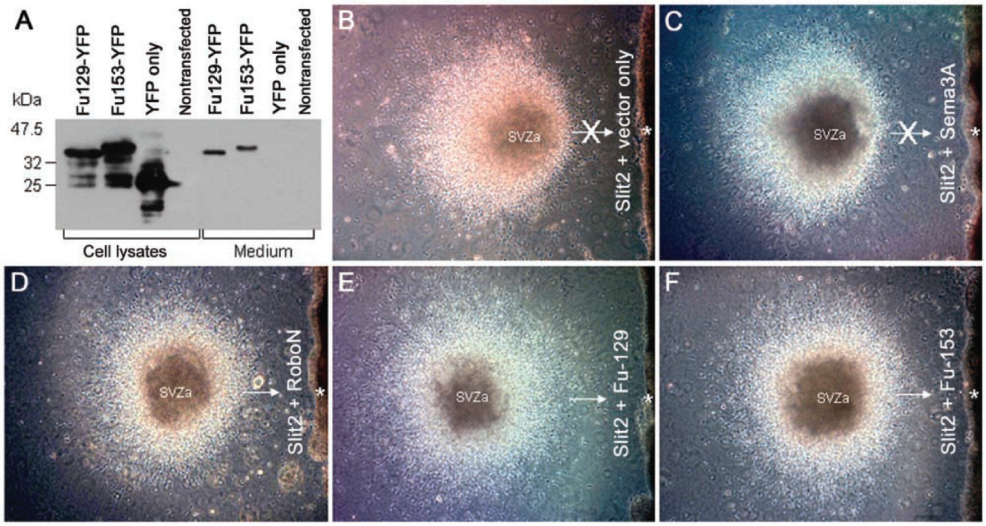

Figure 4.3 ROBO2 fusion proteins inhibiting SLIT chemorepulsion. *A*, YFP-tagged ROBO2 fusion proteins (Fu129-YFP [40 kDa] and Fu153-YFP [42 kDa]) detected by an anti-YFP antibody, expressed in HEK cell lysates, and secreted into the medium. In the presence of aggregated cells transfected with Slit2 plus empty vector (*B*) or Slit2 plus Sema3A (Semaphorin 3A, with no effect on Slit2 repulsive activity) (*C*), cells migrate out of SVZa explants and away from the Slit2-expressing cell aggregate (*asterisk*). In the presence of aggregated cells transfected with Slit2 plus RoboN (the Robo extracellular domain, which inhibits Slit repulsive activity), cells migrate out of SVZa explants symmetrically in all directions (*D*) including toward (*arrow*) the Slit2 and RoboN-expressing cell aggregate (*asterisk*). Fu-129 and Fu-153 also effectively block Slit2 repulsive activity (*E* and *F*), allowing symmetrical neuronal migration out of SVZa explants and toward (*arrows*) Slit2 and Fu-129 or Slit2- and Fu-153-expressing cell aggregates (*asterisks*).

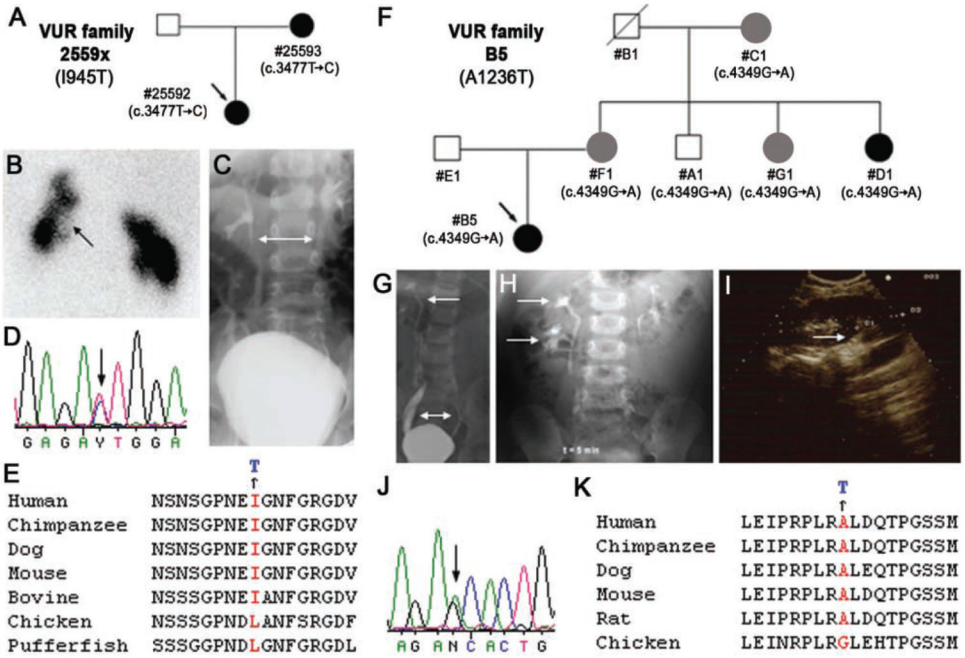

Figure 4.4 *ROBO2* missense mutations in familial CAKUT and VUR. *A*, Family 2559x with CAKUT-VUR and exon 19 (c.3477TrC) mutation. Arrow indicates the proband. Blackened and gray symbols indicate patients with CAKUT-VUR and family members with urinary tract symptoms and radiological evidence of CAKUT. Nucleotide changes are shown under each individual. *B*, 99mTc-dimercaptosuccinic acid renogram of proband 25592 showing bilateral renal parenchymal defects (*arrow*). *C*, VCUG of proband 25592 showing bilateral reflux (*bidirectional arrow*). Chromatograms show TrC change (*arrow*) in exon 19 of family 2559x (*D*) and amino acid conservation across species (*E*). *F*, Family B5 with CAKUT-VUR and exon 23 (c.4349GrA) mutation. *G*, VCUG showing bilateral VUR (*bidirectional arrow*) and right duplex kidney (*arrow*) in proband B5. *H*, IVP detecting right duplex kidney (*arrows*) in proband B5. *I*, US showing suspected duplex (*arrow*) in upper pole of the right kidney in D1, an asymptomatic aunt of proband B5. Chromatograms show GrA change (*arrow*) in exon 23 of family B5 (*J*) and amino acid conservation across species (*K*).

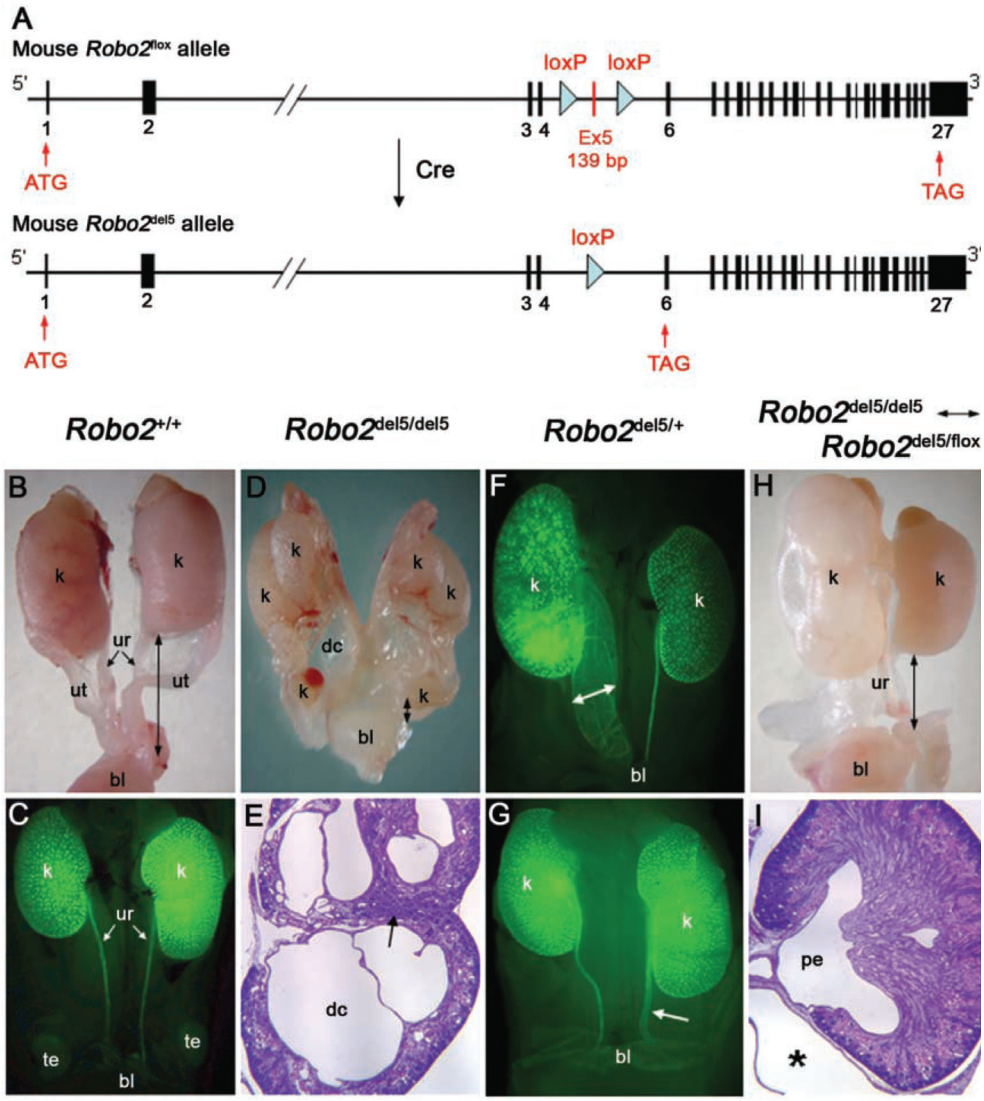

Figure 4.5 *Robo2*$^{del5/del5}$ homozygous, *Robo2*$^{del5/-}$ heterozygous, and *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic newborn mice expressing striking CAKUT phenotypes. *A*, Structures of the mouse *Robo2*^{flox} and *Robo2*^{del5} alleles. The *Robo2*^{flox} allele encodes a wild-type, full-length 1,470-aa Robo2 protein but contains two *loxP* sites flanking exon 5. The *Robo2*^{del5} allele is generated from *Robo2*^{flox} by Cre, which deletes *Robo2* exon 5 to produce an aberrant transcript expressed only at low levels. *B* and *C*, Wild-type female (*B*) and male (*C*) newborn mouse excretory system. The male excretory system in panel C is illuminated by the *Hoxb7-GFP* transgene. k = kidney; bl = bladder; ur = ureter; ut=uterus; te=testis. Black bidirectional arrows indicate ureter length in panels B and D. *Robo2*$^{del5/del5}$ newborn homozygotes display multiplex dysplastic kidneys (*D*) and, at 25# magnification (*E*), reveal dysplastic cysts (dc) in the calyces and an internalized nephrogenic zone (*arrow*). *Hoxb7-GFP* transgene-positive *Robo*$^{2del5/-}$ heterozygous newborns show megaureter dilation (*F*) (*bidirectional arrow*) and early ureter dilatation (*G*) (*arrow*). Robo2$^{del5/del5}$↔Robo$^{2del5/flox}$ mosaic newborns show hydronephrosis in the left kidney (*H*). At 25# magnification (*I*), they show megaureter (*asterisk*). Black bidirectional arrows indicate ureter length in panel H. pe = pelvis.

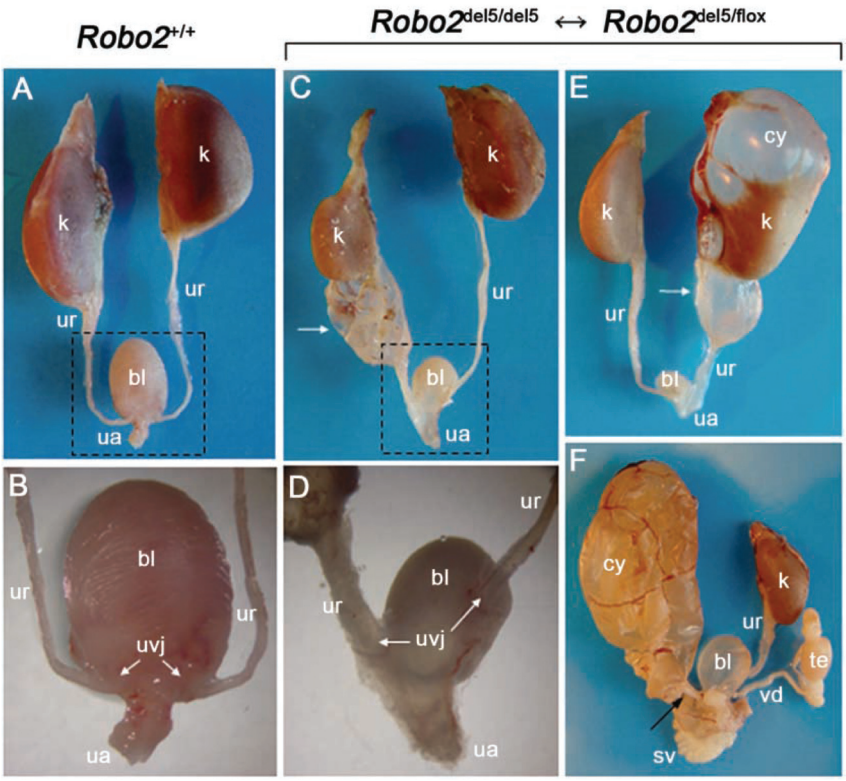

Figure 4.6 Adult *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaics exhibiting megaureter, hydronephrosis, and UVJ defects. *A–D*, Ventral views. k = kidney; ur = ureter; bl = bladder; ua = urethra. *A*, Urinary tract in a wild-type mouse aged 87 d. *B*, Higher magnification of boxed region in panel A, indicating normal position of the UVJ. *C*, Right megaureter (*arrow*) in a *Robo2*$^{del5/del5}$↔*Robo2*$^{del5/flox}$ mosaic aged 45 d. *D*, Higher magnification of boxed region in panel C, demonstrating abnormal bilateral UVJ. The obstructed right UVJ connects to a caudal site in the bladder close to the urethra, causing megaureter. The left UVJ is located laterally in the bladder, a site commonly associated with human VUR. *E* and *F*, Dorsal views. *E*, Right megaureter (*arrow*) and hydronephrosis in a mosaic aged 45 d. Hydronephrosis replaces the normal renal parenchyma (k), causing an upper pole cyst (cy). *F*, Left ureter of a male mosaic mouse aged 77 d that remains connected to the vas deferens (vd) (*arrow*), resulting in obstruction and severe hydronephrosis. The left kidney has lost all parenchyma and is replaced by a large cyst. The right kidney, ureter, and vas deferens are normal in appearance. sv = seminal vesicle; te = testis.

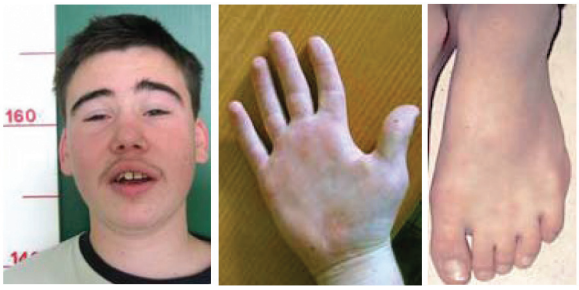

Figure 4.A1. Facial and limb abnormalities in the DGAP107 proband. Note the low set, dysplastic ears and subtle membranous syndactyly and clinodactyly. Blepharophimosis is also present.

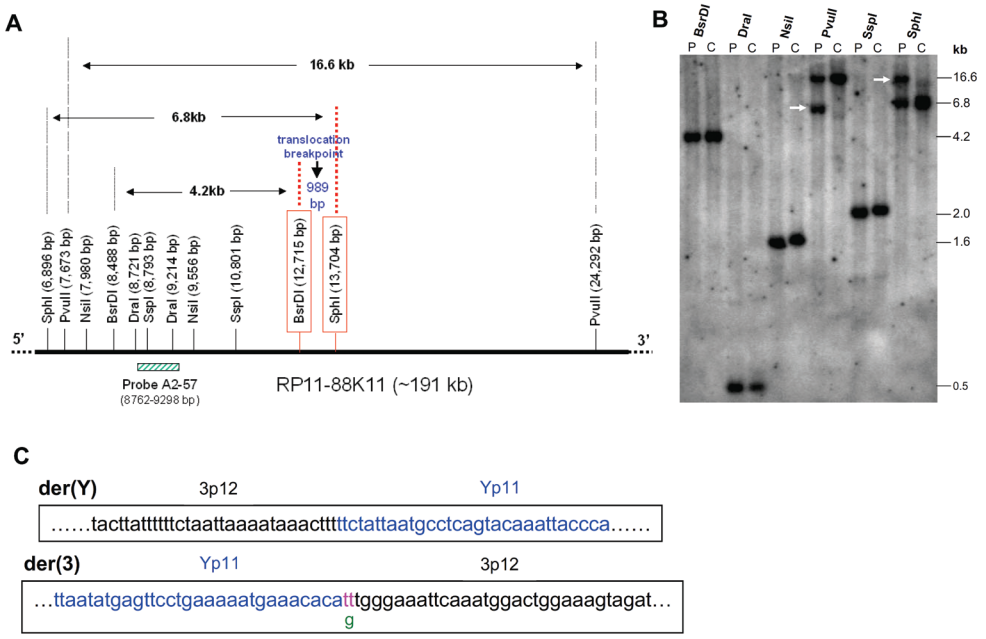

Figure 4.B1. *ROBO2* disrupted in DGAP107, with the breakpoint lying within intron 2. *A*, Restriction map surrounding the 3p12 breakpoint. The base-pair position of BAC RP11-88K11 (AC131005, within intron 2 of *ROBO2* [see BAC contig in fig. 4.1*F*]) was used to calculate the distance between restriction enzyme sites. RP11-88K11 overlaps with BAC clone RP11-54A6 used in FISH and also contains the breakpoint, which is between boxed *Bsr*DI and *Sph*I sites, on the basis of the aberrant bands detected by Southern blot analysis. *B*, Southern blot analysis of DGAP107 (P) and unaffected control (C) genomic DNA, with use of the designated restriction enzymes and the probe A2-57 shown in panel A. Aberrant bands (*white arrows*) are present only in DGAP107 DNA digested with *SphI* and *PvuII*. *C*, Breakpoint cloning showing the sequence of the junction fragment from der(3) with a 1-bp deletion (g [*green*]) and a 2-bp insertion (tt [*pink*]). There is no gain or loss of nucleotides at the der(Y) breakpoint.

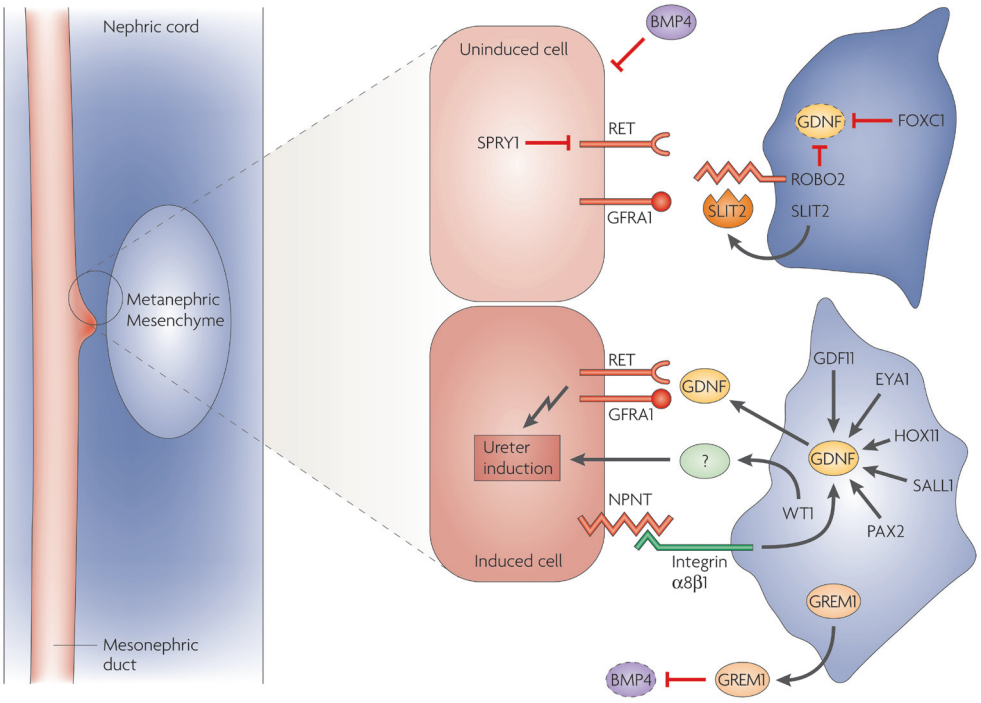

Figure 6.1. Molecular pathways that control kidney induction. Mesenchymal cells at the caudal end of the nephrogenic cord (in light blue) express various factors that activate expression of glial-derived neurotrophic factor (GDNF). In addition, mesenchymal cells release gremlin 1 (GREM1), an inhibitor of bone morphogenetic protein (BMP) signaling, and other unidentified factors. Released GDNF binds to RET and GDNF-family receptor a1 (GFRA1) receptors that are presented by epithelial cells of the mesonephric duct (in red) The combination of these signals induces ureteric budding. Mesenchymal cells at a more rostral level (colored dark blue) express forkhead box protein C1 (FOXC1), Slit homologue 2 (SLIT2) and its receptor Roundabout homologue 2 (ROBO2), leading to a repression of GDNF. In epithelial cells of the mesonephric duct, the tyrosine kinase inhibitor sprouty 1 (Spry1) suppresses RET activation. Finally, BMP4 also inhibits ureter outgrowth. EYA1, Eyes-absent homologue 1; GDF11, growth differentiation factor 11; HOX11, homeobox protein 11; NPNT, nephronectin; WT1, Wilms tumour transcription factor. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Genetics.[8]

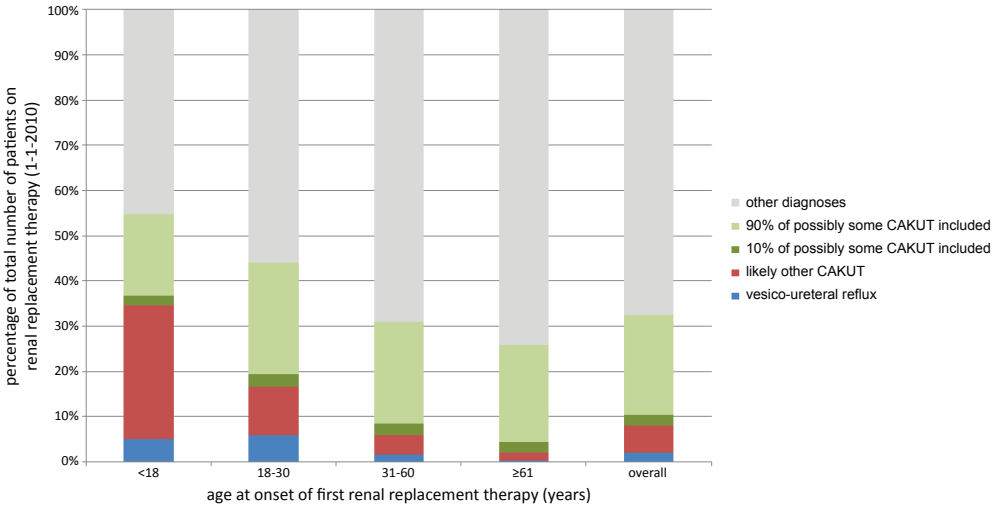

Figure 8.1 Relative contribution of VUR and/ or CAKUT as primary diagnoses in the population of patients on renal replacement therapy in the Netherlands divided by age category. Data provided by Renine (www.renine.nl). Primary diagnoses are grouped in the portrayed categories, the official primary diagnoses are the following: In blue: Pyelonephritis/Interstitial nephritis due to vesico-ureteric reflux without obstruction; in red: Pyelonephritis/ Interstitial nephritis due to congenital obstructive uropathy with or without vesico-ureteric reflux / Cystic kidney disease-type unspecified / Polycystic kidneys, infantile (recessive) / Medullary cystic disease, including nephronophthisis / Cystic kidney disease-other specified type / Congenital renal hypoplasia-type unspecified / Oligomeganephronic hypoplasia / Congenital renal dysplasia with or without urinary tract malformation / Syndrome of agenesis of abdominal muscles (Prune Belly Syndrome); in light and dark green, diagnoses where possibly some CAKUT is included: Chronic renal failure, aetiology uncertain / Pyelonephritis/Interstitial nephritis-cause not specified / Pyelonephritis or Interstitial nephritis due to other cause / Hereditary or Familial nephropathy-type unspecified / Hereditary nephropathy-other / Missing primary diagnoses. We assumed that in this dataset 10% of non-specific diagnoses (in dark green) were in fact attributable to congenital anomalies.

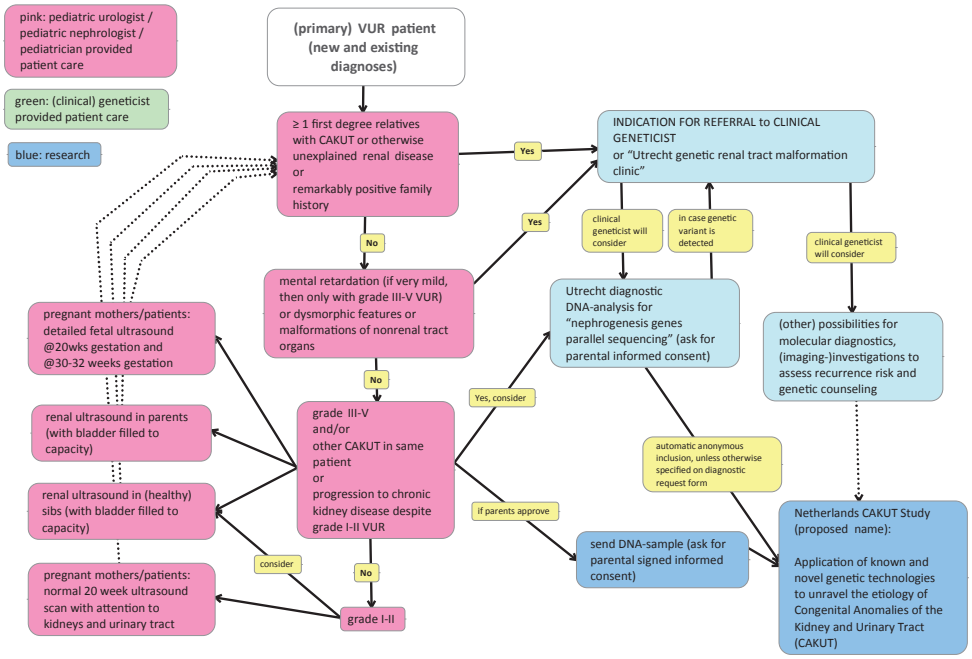

Figure 8.2 Flow-chart with the suggested follow-up in first degree relatives and genetic diagnostic flow for VUR patients. Recurrence risk and inheritance patterns are discussed in chapter 1.

Albertien van Eerde (Leiderdorp, 1978) started medical school at the University of Utrecht in 1996 after graduating from Leiden Grammar School cum laude. In 2001-2002 she was a visiting student-investigator to Dr. Laura Bull's lab at UC San Francisco (USA) where she participated in research into the genetics of Aagenaes syndrome. After graduating from medical school in 2003, Albertien was appointed at the Department of Medical Genetics of the UMC Utrecht. She combined her research (in part sponsored by the Dutch Kidney Foundation) that resulted in this thesis, with specialty training as a resident in clinical genetics (supervised by Profs. Frits Beemer, Dick Lindhout and Nine Knoers). Her graduation as a clinical geneticist is expected in the spring of 2013. Albertien lives in Utrecht with her husband Paul Krediet and their son Douwe.