Vesicoureteral reflux (VUR) (OMIM %193000), a common cause of childhood renal failure, is strongly influenced by hereditary factors. Familial VUR most closely conforms to autosomal-dominant inheritance, but because of variable penetrance and expressivity, large multigenerational pedigrees tractable to linkage analysis have been difficult to ascertain. A single genome-wide study of familial VUR has demonstrated linkage to chromosome 1p13, with 78% locus heterogeneity. Previous studies in humans have also suggested loci on chromosomes 6p21, 10q26, and 19q13, whereas mutations in a single genome-wide study of familial VUR has demonstrated linkage to chromosome 1p13, with 78% locus heterogeneity. Previous studies in humans have also suggested loci on chromosomes 6p21, 10q26, and 19q13, whereas mutations in 6p21 (ROBO2 gene) (13–17). Because mutations in

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Address correspondence to: Dr. Ali G. Gharavi, Department of Medicine, Division of Nephrology, Columbia University College of Physicians and Surgeons, 630 W 168th Street, P&S 10-432 New York, NY 10032. Phone: 212-342-1277; Fax: 212-305-3475; E-mail: ag2239@columbia.edu

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PAX2 on 10q24 cause the renal coloboma syndrome, a rare autosomal-dominant disease with kidney anomalies that includes VUR (15), this gene has also been proposed as a candidate. Linkage studies have also suggested the HLA locus on 6p21 in some families with VUR, UPJ obstruction, or renal dysplasia (13,18). Finally, Lu et al. have recently reported mutations in ROBO2 in some VUR patients (19).

Linkage to many of these loci (particularly the 1p13 interval) has not been replicated in an independent, well-powered cohort, leaving open the question of how applicable these findings are to other kindreds with VUR. Lander and Kruglyak have suggested that linkage should be replicated in an independent cohort to verify positive findings, to assess the level of heterogeneity of the trait, and to evaluate the potential for type I error in the initial reports (20). In such a replication test, because one only tests linkage to a single chromosome segment rather than the entire genome, significance at a point-wise $P$ of 0.05 should provide significant evidence of linkage. Lander and Kruglyak proposed the more stringent threshold $P$ of 0.01 to correct for multiple testing across a 20-cM interval (20).

We identified seven new multigenerational VUR families with multiple affected members and tested whether they demonstrate linkage to the candidate loci reported for humans on chromosomes 1, 3, 6, 10, and 19. Despite considerable power, we were unable to replicate linkage to any of the published intervals, suggesting that heterogeneity of this trait may be more significant than previously appreciated.

**Materials and Methods**

**Patients**

We recruited seven pedigrees with multiple individuals affected with VUR (Figure 1). All families were ascertained through an index case with VUR documented by a voiding cystourethrography (VCUG). Family members were considered as affected based on the presence of reflux documented by VCUG (29 cases), the diagnosis of childhood ESRD in absence of obvious causes such as glomerulonephritis (two cases) or documented recurrent urinary tract infections in a male child before age 9 (one case). Two family members in K114 were diagnosed by renal sonogram with UPJ obstruction but never underwent VCUG testing. Because VCUG is an invasive test, our study could not recommend this procedure to determine whether these two patients had concurrent VUR. Nevertheless, these two individuals were considered as affected because UPJ obstruction is a clear abnormality, and previous studies have suggested that VUR and UPJ obstruction have a common genetic cause (4,21). Affected individuals had no evidence of secondary causes of VUR or syndromic abnormalities. Current practice involves screening siblings of affected individuals. Thus, many members of the youngest generations in this study had been previously screened by VCUG. However, because VUR often improves or resolves with age, only individuals younger than 6 years with a negative VCUG study were classified as unaffected. All other individuals were classified as phenotype unknown. All individuals gave informed consent and the study protocol adhered to the Declaration of Helsinki and was approved by the Western Institutional Review Board and ethics committees at the University of Brescia and the Gaslini Institute.

**Microsatellite Genotyping**

Total genomic DNA was isolated from peripheral white blood cells of the patients and relatives using standard procedures. We genotyped the patients for 35 microsatellite markers located within candidate regions reported (Table 1). These markers were selected from the genome-wide panel of the Marshfield Weber set 13 (http://www.marshfieldclinic.org), but additional markers were added to increase information, particularly on chromosomes 1 and 3. Altogether, we had an average marker spacing of 8.1 cM at the candidate intervals, with an average marker heterozygosity of 0.76.

Fluorescent dye-labeled primers were used to direct PCR at microsatellite loci. PCR products were resolved on a capillary sequencer (SpectruMedix LLC, State College, PA), and genotypes were scored using the GenoSpectrumV220 software (SpectruMedix LLC).

**Analysis of Linkage**

We performed pair-wise and multipoint analysis of linkage using the FASTLINK 4.1 (22,23) and ALLEGRO 1.2c (24) programs, respectively. Parametric analysis was performed under the model previously used to map the first locus for isolated VUR (12): Affected-only analysis with an autosomal-dominant inheritance, disease gene frequency of 0.01, and a phenocopy rate of 0.01. Allele frequencies were calculated based on the frequencies observed in the data set. We computed LOD scores under models of genetic homogeneity (i.e., all families link to the same locus) and genetic heterogeneity (a fraction of families link to the locus tested). Calculation of LOD scores under genetic heterogeneity (HLOD) enables detection of linkage in situations in which the same phenotype is
produced by mutations in different genes (locus heterogeneity), such that the genetic cause of disease differs between families. Linkage analysis under heterogeneity is performed assuming that a proportion of families (\(\alpha\)) is linked to the marker tested, whereas the remaining families are unlinked. The LOD score for each family is therefore maximized over two parameters (\(\alpha\) and the recombination fraction, \(\theta\)), yielding the HLOD. The total HLOD at a given locus is calculated by the sum of the HLOD from all of the kindreds studied. This analytic method results in minimal expenditure of degrees of freedom and has been shown to be a powerful tool for detection of linkage (25). In addition, to avoid erroneous exclusion of a locus because of mis-specification of the disease model, we performed nonparametric, allele-sharing analysis using permissive scoring functions (exponential model, with the “all” scoring function and equal weighting between families) as implemented in ALLEGRO. Published thresholds for genome-wide significance (LOD \(\geq 3.3\)), replication (\(P < 0.01\)), and exclusion of linkage (LOD \(\leq -2\)) were used (20). Studies exploring the properties of the HLOD statistic suggested that an HLOD of approximately 1.2 corresponds to \(P = 0.01\) (25).

Because these thresholds have been obtained assuming analysis of a large number of small pedigrees with an infinitely dense map, they may not be pertinent to data sets that vary in pedigree structure and information content. We therefore performed simulations to evaluate the power of our pedigrees to detect linkage and obtain the empiric thresholds for significance of our findings. We performed simulations of genotypes with ALLEGRO, using the same family structure and affection status as our VUR pedigrees, and specifying a 20-cM genetic map with marker spacing and heterozygosities equivalent to those used at our candidate intervals. To obtain the null distribution for our linkage statistics, we first performed simulations of genotypes under

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</table>

cM locations are based on the Marshfield map. LOD indicates logarithm of odds; Chr, chromosome.
the hypothesis of no disease locus in the interval. We performed 1000
simulations to provide a solid estimate of the empiric LOD score
threshold corresponding to $P \leq 0.01$. The simulated data were then
analyzed with ALLEGRO under the same model previously delineated
and HLOD were determined for each run. The HLOD calculated from
these 1000 simulated runs provide the distribution of the HLOD sta-

tistic under the hypothesis of no disease locus in the interval, the null
hypothesis. The HLOD in the top 1% and 5% of the distribution define
the point-wise empiric thresholds for type I error at $P \leq 0.01$ and $P \leq
0.05$, respectively, and therefore constitute our thresholds for replica-
tion of linkage at $P \leq 0.01$ and $P \leq 0.05$ significance levels.

Inadequate study power may also hinder replication of original
linkage findings. Therefore, we used simulations to assess the power
of our study sample to detect linkage under various levels of locus het-
erogeneity. We simulated genotypes of our pedigree sample (1000
replicates) with the assumption of linkage under each of the following
conditions: (1) genetic homogeneity; (2) 50% locus heterogeneity; and
(3) 28.6% locus heterogeneity (two of seven families linked). We then
analyzed this second set of simulated pedigrees with ALLEGRO to
obtain the maximum and average expected LOD (eLOD) scores with
the models above. The fraction of LOD scores exceeding the thresholds
for genome-wide significance and linkage replication provided our
power to detect linkage with various levels of heterogeneity.

Results

Patients

We studied seven Caucasian pedigrees from Italy (K108, K110, K114, K116) and the United States (K117, K118, K119). All
were ascertained via an index case with VUR documented by
VCUG. These seven multigenerational kindreds comprised 140
individuals (Figure 1); we collected DNA from 105 individuals.
In total, 41 individuals were classified as affected (36 based on
a positive VCUG, two based on the presence of ESRD, one by
UPJ obstruction documented by abdominal sonogram). Eight patients were unaffected based on unknown phenotype because they did not have a
clear phenotype (e.g., undocumented episodes of urinary tract infections or history of enuresis) or were asymptomatic and
were not investigated by VCUG. There were 14 males and 27
females affected (male/female ratio = 0.52). The age at the
diagnosis was $9.5 \pm 11$ yr (range, 1 mo to 45 yr). Among those
affected, 14 patients underwent corrective surgery, ESRD de-
veloped in two patients, and one of them underwent kidney
transplantation. Consistent with previous reports, some individ-
uals had associated urologic abnormalities: in K110, one
patient had renal stones; in K116, one patient had a pelvic
kidney; and in K117, one patient had a paraureteral diverticu-
llum. All of the affected individuals in K114 also had associated
UPJ obstruction documented by abdominal sonogram. Finally,
K117 is noteworthy because it has a theoretical maximal LOD score $>3$ and can alone enable gene localization with genome-
wide significance.

In these pedigrees, male-to-male transmission and a higher
ratio of females to males argued against X-linked inheritance.
The absence of consanguinity and occurrence of the disease in
multiple generations made recessive inheritance unlikely. Con-
sistent with the literature, the pattern of transmission was most
consistent with multifactorial inheritance or an autosomal-
dominant inheritance with reduced penetrance (7).

Simulation Studies

In addition to using published criteria for replication, we
used simulations to establish empiric thresholds for replication
of linkage. In 1000 simulations under the null hypothesis of no
disease locus in the interval, we observed an HLOD $\geq 0.84$ a
total of 10 times and an HLOD $\geq 0.51$ a total of 50 times. These
data established the empiric threshold for replication of para-
metric linkage at $P \leq 0.01$ and $P \leq 0.05$, respectively (20). The
nonparametric LOD scores corresponding to these thresholds
were 1.88 and 1.3, respectively.

To determine the power of our sample to detect linkage, we
next performed simulations under the assumption of linkage

Table 2. Multipoint LOD scores in VUR families

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<th>Candidate gene</th>
<th>Average LOD</th>
<th>LOD 1p13</th>
<th>LOD 3p12</th>
<th>LOD 6p21</th>
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<tr>
<td>K117</td>
<td>3.35</td>
<td>-1.43</td>
<td>-1.59</td>
<td>-1.24</td>
<td>-1.86</td>
<td>-0.53</td>
<td>-1.36</td>
<td>-0.76</td>
</tr>
<tr>
<td>K118</td>
<td>0.51</td>
<td>-0.59</td>
<td>-1.5</td>
<td>-0.57</td>
<td>-0.63</td>
<td>0.4</td>
<td>-0.33</td>
<td>0.07</td>
</tr>
<tr>
<td>K119</td>
<td>1.3</td>
<td>0.96</td>
<td>-1.06</td>
<td>-0.11</td>
<td>0.09</td>
<td>-0.41</td>
<td>-0.64</td>
<td>-0.11</td>
</tr>
<tr>
<td>Total LOD ($\alpha = 100%$)</td>
<td>9.49</td>
<td>-3.18</td>
<td>-8.14</td>
<td>-4.69</td>
<td>-3.84</td>
<td>-4.43</td>
<td>-3.13</td>
<td>-2.06</td>
</tr>
<tr>
<td>HLOD ($\alpha = 50%$)</td>
<td>3.45</td>
<td>-0.46</td>
<td>-1.37</td>
<td>-0.96</td>
<td>-0.69</td>
<td>-1.06</td>
<td>-0.86</td>
<td>-0.45</td>
</tr>
<tr>
<td>Nonparametric LOD</td>
<td>8.15</td>
<td>0.74</td>
<td>0.02</td>
<td>0.01</td>
<td>0.31</td>
<td>0.06</td>
<td>0.29</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Expected LOD (eLOD) for individual kindreds were obtained based on affected-only analysis with 1000 simulations,
assuming homogeneity. On chr01, LOD scores shown were obtained at the location with maximized HLOD (DIS1653, 164
cM). VUR indicates vesicoureteral reflux; HLOD, heterogeneity LOD.
with various levels of genetic heterogeneity (1000 replicates each). Under genetic homogeneity, the average parametric expected LOD for our kindreds was 1.4 (Table 2). For parametric analysis, simulations yielded a maximum expected LOD score of 11.14 (average 9.49 ± 1.65) under homogeneity, 11.14 (average 3.45 ± 2.34) under 50% locus heterogeneity, and 9.07 (average 1.71 ± 1.83) under 28.6% locus heterogeneity (two families out of seven linked). For nonparametric analysis, we obtained a maximum expected LOD scores of 12.89 (average 8.15 ± 1.52) under homogeneity, 9.96 (average 2.76 ± 2.11) with 50% heterogeneity, and 8.67 (average 1.56 ± 1.57) with 28.6% heterogeneity. The threshold for replication (at P ≤ 0.01) was exceeded in 100%, 85%, and 53% of simulated runs with homogeneity, 50% heterogeneity, and 28.6% heterogeneity, respectively. These data indicate that our sample had ample power not only to replicate linkage but also to achieve genomewide significance with at least 50% locus heterogeneity.

Analysis of Linkage

Tables 1 and 2 show the pair-wise and multipoint LOD scores at the candidate intervals, respectively. Under genetic homogeneity, multipoint LOD scores were strongly negative, excluding linkage to all candidate loci. In addition, our data do not support statistically significant linkage to these loci under a model of 50% genetic heterogeneity (negative HLOD at all intervals). Varying the percentage of linked families to maximize the HLOD also did not achieve statistical significance at any of these intervals, with a maximal HLOD of 0.55 on chromosome 19 (D19S246) and 50% percent of pedigrees linked. This peak score was obtained approximately 10 cM (16 Mb) away from our candidate gene (USF2) and is significantly lower than our empiric threshold for replication of linkage at the 1% level. These results did not change significantly with alternative analyses. As expected, lowering the disease allele frequency or the phenocopy rate strengthened the evidence against linkage at all intervals. Similarly, nonparametric analysis did not reveal significant linkage to any of the candidate loci (maximum nonparametric LOD of 0.74 on chromosome 1), demonstrating that absence of linkage cannot be attributed to mis-specification of disease model. Finally, secondary analyses with exclusion of K114, a pedigree with associated UPJ obstruction, did not sufficiently increase the LOD scores at candidate intervals to achieve statistical significance.

Discussion

Replication studies are important components of genetic investigations because they help determine the level of heterogeneity of the trait and assess the potential for type 1 error (20,26). Thus, although the estimated probability of false-positive findings in genome scans is <0.05 when a threshold LOD score of 3.3 is used, Lander and Kruglyak emphasized that replication studies should be also performed (20). Replication studies may have difficulty in detecting linkage to reported loci because initial positive reports tend to overestimate the genetic effect (20,27). This bias occurs because initial reports tend to benefit from random fluctuations that push the LOD score above statistical significance, whereas follow-up studies regress to the true mean (20,27). Moreover, variation in methodology, ascertainment criteria, and study population may also complicate the ability to replicate initial findings (20,27). In the case of VUR, one of the major obstacles for linkage studies is the relative rarity of large pedigrees because of reduced penetrance of the trait (7). Reduced penetrance necessitates affected-only analysis and this constraint, combined with locus heterogeneity, diminishes the ability to assemble a well-powered cohort. Our study, however, benefited from relatively large kindreds, including one of the largest families reported to date (K117); this kindred alone should be sufficiently large to support gene localization at the genome-wide level. Moreover, our pedigrees resembled Caucasian cohorts previously described, particularly the one described by Feather et al. (12), and we used similar ascertainment criteria and linkage models described in their article. Our simulations indicated that we were well powered to replicate linkage or achieve significance at the genome-wide level, with a maximum expected LOD score of 11.14 under homogeneity (for comparison we estimate the maximum expected LOD in the pedigrees by Feather et al. at approximately 8.7) (12). Nevertheless, despite analyses under many alternative models, we found no evidence of linkage to loci previously implicated in humans. Our best interval barely reached the threshold for replication at P ≤ 0.05 on chromosome 19, but this maximal score was achieved 16 Mb distal to the candidate gene (USF2) and did not hold up to correction for multiple testing. These data highlight the need to use stringent criteria to avoid false replication of linkage.

The lack of replication is most likely caused by overestimation of the risk attributable to previously reported loci and substantial genetic heterogeneity of the trait. This heterogeneity is demonstrated by human and animal studies indicating that VUR can be caused by defects in a panel of genes expressed in the lower urinary tract. For example, loss of the uroplakin III gene causes VUR in the mouse (28); similarly, tissue-specific ablation of calcineurin causes ureteropelvic obstruction (29). Mice lacking either ROBO2 or its ligand, SLIT2, also have urogenital developmental abnormalities that include supernumerary ureteric buds that remain inappropriately connected to the nephric duct and failure of the ureters to connect to the bladder (30). The number of mutant mice exhibiting renal developmental defects is rapidly growing, reflecting the intricate biology of kidney development (31). These animal data highlight the potential for genetic heterogeneity of human disease and simultaneously provide a substantial list of candidate genes for human VUR.

The candidate gene approach had generally met with limited success in human VUR (e.g., screening of PAX2 or uroplakin genes) (32,33). Lu et al. have, however, recently reported disruption of the ROBO2 gene in one patient with a balanced translocation and a phenotype that included VUR; in addition, mutational screening of patients with nonsyndromic VUR revealed two nonconservative amino acid substitutions (19). The full description of these patients is not available at the time of writing this report, but these preliminary data indicate that ROBO2 accounts for a small fraction of VUR cases (19). Consistent with the rarity of ROBO2 mutation in humans, linkage...
to this locus was not supported in our seven kindreds. However, we expected to detect linkage to the 1p13 locus because it accounted for disease in the majority (78%) of the families Feather et al. studied (12). Among our kindreds, however, only K119 showed a positive LOD score on chromosome 1p13, a proportion (14.3%) insufficient to replicate linkage. Because we had 85% power to replicate with 50% locus heterogeneity, our data suggest that the chromosome 1p13 interval may account for disease in a smaller fraction of VUR kindreds, perhaps in as little as 15% and probably less than half of VUR families overall.

Together with the results of animal studies, our data raise the potential that mutations in different genes each account for disease in a small proportion of VUR families. In this scenario, the search for VUR genes may be very complicated and might have to rely on extensive screening of candidate genes in both familial and sporadic cases. Linkage studies of the trait would require a large number of average size pedigrees or would have to rely on pedigrees such as K117 that have a sufficiently large number of affected individuals to map a locus on their own. To be successful, comprehensive studies combining all approaches will likely be required. Accordingly, after testing the most promising candidate loci reported in humans, we are now proceeding with a genome-wide search to detect novel VUR loci in our kindreds.

Acknowledgments

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References

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