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Localization of a Gene for Autosomal Recessive Distal Renal Tubular Acidosis with Normal Hearing (*rdRTA2*) to 7q33-34

Fiona E. Karet,[1] Karin E. Finberg,[2,3] Ahmet Nayir,[5] Aysin Bakkaloglu,[6] Seza Ozen,[6] Sally A. Hulton,[7] Sami A. Sanjad,[8] Essam A. Al-Sabban,[9] Juan F. Medina,[10] and Richard P. Lifton[2,3,4]

[1]Cambridge Institute for Medical Research, University of Cambridge, Cambridge; [2]Howard Hughes Medical Institute and Departments of [3]Genetics and [4]Medicine, Yale University School of Medicine, New Haven; [5]Department of Pediatric Nephrology, University of Istanbul, Istanbul; [6]Department of Pediatric Nephrology, Hacettepe University, Ankara; [7]Department of Nephrology, Birmingham Children's Hospital, Birmingham, United Kingdom; [8]Department of Pediatrics, American University of Beirut Medical Center, Beirut; [9]Department of Pediatrics, King Faisal Specialist Hospital and Research Centre, Riyadh; and [10]Department of Medicine and Liver Unit, University of Navarra, Pamplona, Spain

Summary

Failure of distal nephrons to excrete excess acid results in the "distal renal tubular acidoses" (dRTA). Early childhood features of autosomal recessive dRTA include severe metabolic acidosis with inappropriately alkaline urine, poor growth, rickets, and renal calcification. Progressive bilateral sensorineural hearing loss (SNHL) is evident in approximately one-third of patients. We have recently identified mutations in *ATP6B1*, encoding the B-subunit of the collecting-duct apical proton pump, as a cause of recessive dRTA with SNHL. We now report the results of genetic analysis of 13 kindreds with recessive dRTA and normal hearing. Analysis of linkage and molecular examination of *ATP6B1* indicated that mutation in *ATP6B1* rarely, if ever, accounts for this phenotype, prompting a genomewide linkage search for loci underlying this trait. The results strongly supported linkage with locus heterogeneity to a segment of 7q33-34, yielding a maximum multipoint LOD score of 8.84 with 68% of kindreds linked. The LOD-3 support interval defines a 14-cM region flanked by *D7S500* and *D7S688*. That 4 of these 13 kindreds do not support linkage to *rdRTA2* and *ATP6B1* implies the existence of at least one additional dRTA locus. These findings establish that genes causing recessive dRTA with normal and impaired hearing are different, and they identify, at 7q33-34, a new locus, *rdRTA2*, for recessive dRTA with normal hearing.

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Address for correspondence and reprints: Dr. Fiona E. Karet, Cambridge Institute for Medical Research, Room 4.2, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2XY, United Kingdom. E-mail: fek1000@hermes.cam.ac.uk

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Introduction

In adult humans, the net result of catabolization of an omnivorous diet is the production of a nonvolatile acid load of ~70 mmol H^+/d. To preserve acid-base homeostasis, which maintains arterial pH very close to 7.40, the kidney must excrete this acid. This function is critical for optimal activity of a wide variety of physiological pathways.

The mechanism for urinary acidification is tightly regulated in the distal part of the nephron (Alpern and Rector 1996). In a process coupled to bicarbonate reabsorption into the bloodstream, hydrogen ions are secreted into the urine by specialized polarized α-intercalated cells in the collecting duct (fig. 1). At the apical surface of these cells, the main secretory complex is the multisubunit proton pump (H^+-ATPase) (Gluck 1992). In response to a drop in arterial pH, this pump can increase urinary H^+ concentration by some 10,000 fold. At the basolateral surface of these same cells, bicarbonate is reabsorbed, in exchange for chloride, by the *AE1* gene product. Proper α-intercalated cell function is important not only for maintenance of correct body-fluid pH but also for the solubility of calcium in urine and for the stability of calcium in bone.

In type I, or distal, renal tubular acidosis (dRTA), there is inadequate urinary H^+ secretion by these α-intercalated cells. The result of this defect (and a biochemical hallmark of dRTA) is that the urine is inappropriately alkaline (pH >5.5) in the presence of metabolic acidosis, whether spontaneous or induced by acid challenge (Lightwood 1935; Butler et al. 1936). In primary dRTA, metabolic acidosis of varying severity is usually accompanied by hypokalemia and hypercalciuria. If left untreated, the acidosis leads to leaching of calcium and phosphate from bone, resulting in osteomalacia, rickets, and growth impair-

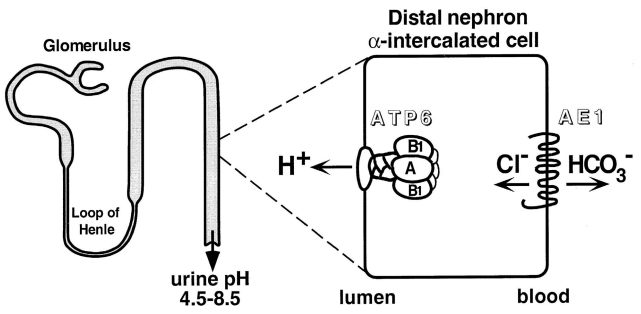


Figure 1 Urine acidification in the human collecting duct. A schematic drawing of a nephron is shown (*left*). To achieve alterations in urine H^+ concentration of ≤10,000 fold, specialized α-intercalated cells of the collecting duct (*right*) secrete H^+ ions across their apical surface via the multisubunit H^+-ATPase pump (ATP6). This molecule comprises an ATP-catalytic "head" of 3A- and 3B1-subunits and a membrane-anchored "stalk" of at least eight other subunits. H^+ secretion is coupled to absorption of HCO_3^- across the basolateral surface, by way of AE1, a 12–14-transmembrane-spanning Cl^-/HCO_3^- exchanger. Other transporters probably contribute to H^+ and HCO_3^- transport in this cell type, but their significance in humans remains unclear.

ment. Intrarenal deposition of calcium salts as stones or nephrocalcinosis commonly occurs. Simple alkali replacement suffices to correct the systemic metabolic defects, but it does not appear to reverse nephrocalcinosis (McSherry and Morris 1978).

Inherited dRTA may be autosomal dominant (MIM 179800), in which case the disease is usually mild, or autosomal recessive (MIM 267300 and MIM 602722), for which severe early presentation is the rule. Among patients with recessive dRTA, a substantial fraction have progressive and irreversible bilateral sensorineural hearing loss (SNHL) (Zakzouk et al. 1995). Mutations in *AE1* are responsible for dominant dRTA in all kindreds thus far reported (Bruce et al. 1997; Jarolim et al. 1998; Karet et al. 1998), but recessive dRTA is rarely the result of mutations in this gene (Karet et al. 1998; Tanphaichitr et al. 1998). Recently, recessive dRTA with SNHL has been shown to be caused by mutations in *ATP6B1,* which encodes the B-subunit of the α-intercalated cell's apical proton pump (Karet et al. 1999). Hearing loss seen in patients with mutations in this gene can be attributed to the loss of H^+ secretion into endolymph by ATP6B1-containing H^+-ATPase within the cochlea, which is required for maintenance of appropriate endolymph pH.

It is presently unknown whether recessive dRTA with normal hearing is also attributable to mutations in *ATP6B1.* In this report, we show that dRTA with normal hearing is not accounted for by mutation in *ATP6B1,* and we identify linkage of this trait to a new locus, *rdRTA2,* on chromosome 7q33-34.

Families, Material, and Methods

Classification of Families

Thirteen kindreds with autosomal recessive dRTA and normal hearing were ascertained via affected index cases, as previously reported (Karet et al. 1999). All individuals were classified as being affected if they demonstrated inappropriately alkaline urine in the presence of metabolic acidosis but had otherwise normal renal function and absence of secondary causes of dRTA. Inheritance was prospectively ascribed as being recessive either when affected offspring were the result of a consanguineous union of unaffected parents or when the union of unrelated unaffected parents resulted in at least two affected offspring. This interpretation is supported by the observed high penetrance of dominant disease; moreover, almost all the patients in the current study were diagnosed by age 2 years, which is typical of recessive disease, in contrast with patients who have dominant disease, which is usually diagnosed later in life. The research protocol was approved by the Yale University Human Investigation Committee.

Phenotypic Data

With the use of age-appropriate normal ranges for each referring hospital, the biochemical data obtained included measurement of serum sodium, potassium, creatinine, calcium, phosphate, and magnesium; measurement of arterial blood pH; and determination of urinary calcium, pH, electrolytes, and protein. Hearing status was determined by pure-tone audiometry and/or by brain-stem auditory evoked responses. Renal calcification was assessed by ultrasonography and/or radiography.

Linkage Studies

Genomic DNA was prepared, by standard procedures (Bell et al. 1981), from whole blood of study members. Genotyping of a total of 270 polymorphic loci spanning all autosomes, as well as those in intervals suggestive of linkage and the previously described *ATP6B1* locus, was performed by PCR with a customized set of primers, as described elsewhere (Craig et al. 1998). PCR products were labeled either by incorporation of $\alpha[^{32}P]$-dCTP or by fluorescent end-labeling of oligonucleotide primers, with analysis performed on an ABI 377 instrument equipped with GENESCAN 2.1 and GENOTYPER 1.1.1 software (Applied Biosystems). All genotypes were scored independently by two investigators blinded to affection status. Marker order was obtained from databases at the Whitehead Institute for Biomedical Research/MIT Center for Genome Research, The Cooperative Hu-

man Linkage Center, and The Genome Database. Homozygosity mapping (Lander et al. 1987) and analysis of linkage were performed by use of the GENEHUNTER program (Kruglyak et al. 1996) run on a Sun SPARCstation. Regions suggestive of linkage were refined by typing these and additional markers in all available family members.

Since only nuclear families were genotyped, and given the ethnic variation among kindreds, each marker locus was specified as having four alleles of equal frequency, approximating 75% heterozygosity. The actual average heterozygosity observed for these markers was 83%. Models of the trait locus specified recessive transmission, allowing for locus heterogeneity, with a disease-gene frequency of 1/500, 99% penetrance, and a 1% phenocopy rate. No substantial differences in LOD scores were obtained when these parameters were varied.

Locus heterogeneity and multilocus LOD scores were calculated as described elsewhere (Ott 1991). The likelihood ratio LR(α,θ_1) for linkage either to the *rdRTA2* locus (θ_1) or to another locus in each kindred is αLR(θ_1) + (1-α), where α is the proportion of families linked to θ_1. Multilocus likelihood ratios for linkage to *rdRTA2* or *ATP6B1* (θ_2) were calculated, in each kindred, as αLR(θ_1) + (1-α)(LRθ_2). Multilocus likelihood ratios for linkage to *rdRTA2,* to *ATP6B1,* or to a third, unknown locus were calculated, in each kindred, as αLR(θ_1) + βLR(θ_2) + γ, where β and γ represent the proportion of families linked to *ATP6B1* or to the unknown locus, respectively. The posterior probability of linkage to *rdRTA2* versus linkage to additional unknown loci was calculated, in each kindred, as αLR(θ_1)/[αLR(θ_1)+(1-α)].

Candidate-Gene Analysis

SLC4A2 was identified as a potential candidate because of its prior localization to chromosome 7q. It was further localized on the human genetic map by radiation-hybrid mapping, with primers 4F and 4R (table 1) used to amplify exon 4 of this gene by means of PCR and with DNA of the 93 hybrid cell lines in the Genebridge-4 radiation-hybrid panel (Research Genetics) used as a template. Products were visualized on a 2% agarose gel. Results were submitted to the Whitehead Institute for Biomedical Research/MIT Center for Genome Research Web site for analysis.

Primers lying within introns were designed from genomic *SLC4A2* sequence (GenBank accession numbers U76667–U76669, U62290) and were used separately to amplify all 23 exons by PCR. Products were denatured and were subjected to SSCP analysis under two different nondenaturing conditions, as described elsewhere (Shimkets et al. 1994). Primer sequences are listed in table 1. Identified variants were eluted from gel and were reamplified by PCR, and the DNA sequence of both strands was determined, by use of the dideoxy termination method, on an ABI 377 instrument. An analogous protocol was used to both amplify and sequence all exons and exon-intron boundaries of *ATP6B1* from the genomic DNA of patient RTA28-1, as previously described (Karet et al. 1999; see also the Results section below).

Table 1

Primer Sequences for *SLC4A2* Screening by SSCP

Exon	Primer Sequence (5′→3′) Forward	Reverse
1	gttgagttgggagaagttgg	tacccgctcgctctggacttc
2	cagcgcagcctgcgcgtg	ggacccttggccggagcac
3	ctctggcactgggaagggtgag	ggcctcctgtaggatctcctc
4	ggacactgtgcctgccacag	gccctgccgacattccctag
5	ctcctagggaatgtcggcag	ctgctaggatagtgaggtgatg
6	gggaggaggccaggtttctc	ctcactcctttgcccaccag
7	ctggtgggcaaaggagtgag	gagattacagctggtctgagag
8	gctcgctccggctctgtac	gtgggagaatttcctggggac
9	catcccatctcctgccactg	caggtgggctgaagtgtcatc
10	gatgacacttcagcccacctg	cttcctcagccctgccgccag
11	ctggcggcagggctgaggaag	ctcaaggcctggccaccaag
12	gtcctgctgctctgctcttg	ggatgggctccaggaagcag
13	cagccctcttctttgtgctctc	gaggcagccctggccccag
14	gaggtgggcaagaggggctcgtg	gtggccaggccctgggcag
15	gtctcctgccatcacctttg	ggaggggcggctggagatg
16	catccccacccagagctcag	cctggcccagctctcgcag
17	gtccagctgatggaggccgtg	aacccccttctggcctttaag
18	ggcaggactctgagcgctg	cttaaggacaggctgggtttg
19	caccttaacccttgtccctac	ctggggctctggcctctcac
20	ccagcctgggcctcagcag	ggaatccactggaagggaaag
21	cggggccaactctttctcctg	gaggcacacccctccggtg
22	gcaggctagggaggaagctg	cacagggaggcaagaggaac
23	cccttcttgaccgccacctc	ggagggaggggcatgggag

Results

Recessive dRTA Kindreds with Normal Hearing

The kindreds enrolled in this linkage study comprised 13 apparently unrelated families with autosomal recessive dRTA, in which the hearing of all affected individuals is normal (table 2). Of these families, 10 were from different parts of Turkey, 2 were from Saudi Arabia, and 1 originated in Pakistan. In all but one family, affected individuals were the offspring of consanguineous union; union was between first cousins in nine kindreds, between second cousins in two kindreds, and between third cousins in the other kindred (fig. 2).

Clinical and biochemical data are summarized in table 2. All patients presented with dRTA in early childhood; in 14 of the 17 living affected patients, the diagnosis was made at $\leq$2 years of age. All patients presented with severe metabolic acidosis (mean arterial pH 7.18 $\pm$ 0.06), with accompanying urine pH $\geq$6.5 and hypokalemia; renal function was otherwise normal. All but

Table 2

Clinical and Biochemical Features at Diagnosis of Index Patients with dRTA and Normal Hearing

	Features at Diagnosis of Index Patients with dRTA and Normal Hearing									
	Clinical Data				Biochemical Data[a]					
		Patient Age							Urine Ca^{2+}	
Patient (Sex), Age at Study (years)	Origin	At Diagnosis	At Audiometry (Years)	Rickets?	Arterial pH	Serum HCO_3^- (mM)	Serum K^+ (mM)	Urine pH	mg/kg/d	mg/mg Creatinine
4-1 (F), 5.5	Turkey	3 wk	4.5	No	7.10	5.5	2.4	8.0	6.6	
5-1 (F), 9	Turkey	1 year	9	No	7.12	11.0	3.1	7.0	6.0	
11-1 (M), 10	Turkey	5 years	9	Yes	7.20	12.0	1.6	7.5	20.0	
14-1 (F), 22.5	Turkey	6 years	22	Yes	7.20	13.0	3.5	7.2	8.0	
17-1[b] (F), 21	Pakistan	1 wk	4	Yes	7.25	12.0	2.7	7.6		.4
23-1 (M), 13	Turkey	3 mo	12	No	7.10	12.0	2.8	7.5	5.7	
28-1 (F), 5	Turkey	14 mo	4	Yes	7.23	14.0	2.8	6.5		1.2
34-1 (M), 3	Turkey	8 mo	2.5	Yes	7.23	16.0	2.1	6.8	5.5	
37-1 (F), 10	Turkey	3 mo	9	No	7.10	5.5	2.2	7.0		.1
49-1 (M), 12	Saudi Arabia	5 mo	NA[c]	No	7.21	14.0	2.8	7.5		1.3
62-1[b] (F), 7	Turkey	3 years	7	No	7.20	7.6	3.0	7.0		.3
63-1 (M), 2	Turkey	6 mo	1	Yes	7.10	6.1	2.1	7.0	3.1	
75-1 (M), 12.5	Saudi Arabia	2 years	3	Yes	7.25	11.4	3.3	7.0		.7
Overall mean ± SEM[d]					7.18 ± .06	10.8 ± 3.3	2.6 ± .5	7.2 ± .4		
Mean ± SEM for *ATP6B1* mutants[e]					7.20 ± .09	10.9 ± 2.5	3.1 ± .7	7.3 ± .6		

[a] Normal ranges for biochemical data are as follows: arterial pH, 7.36–7.44; serum HCO_3^-, 20–28 mM; serum K^+, 3.5–5.0 mM; urine pH, <6.0 in the setting of metabolic acidosis; urine calcium, <4.0 mg/kg/d or .2 mg/mg creatinine.

[b] Kindred has additional living affected member(s).

[c] NA = not available.

[d] Mean ± standard error of the mean (SEM) for this study.

[e] Mean ± SEM for 19 index patients with *ATP6B1* mutations (Karet et al. 1999).

two index patients had elevated urinary calcium, and nephrocalcinosis was present in all at diagnosis. None had hypomagnesemia. Rickets was documented in the affected members of seven kindreds. All affected patients have undergone pure-tone audiometric evaluation, auditory evoked–response measurements, or both, with normal results. In no case has delay in the development of speech been reported.

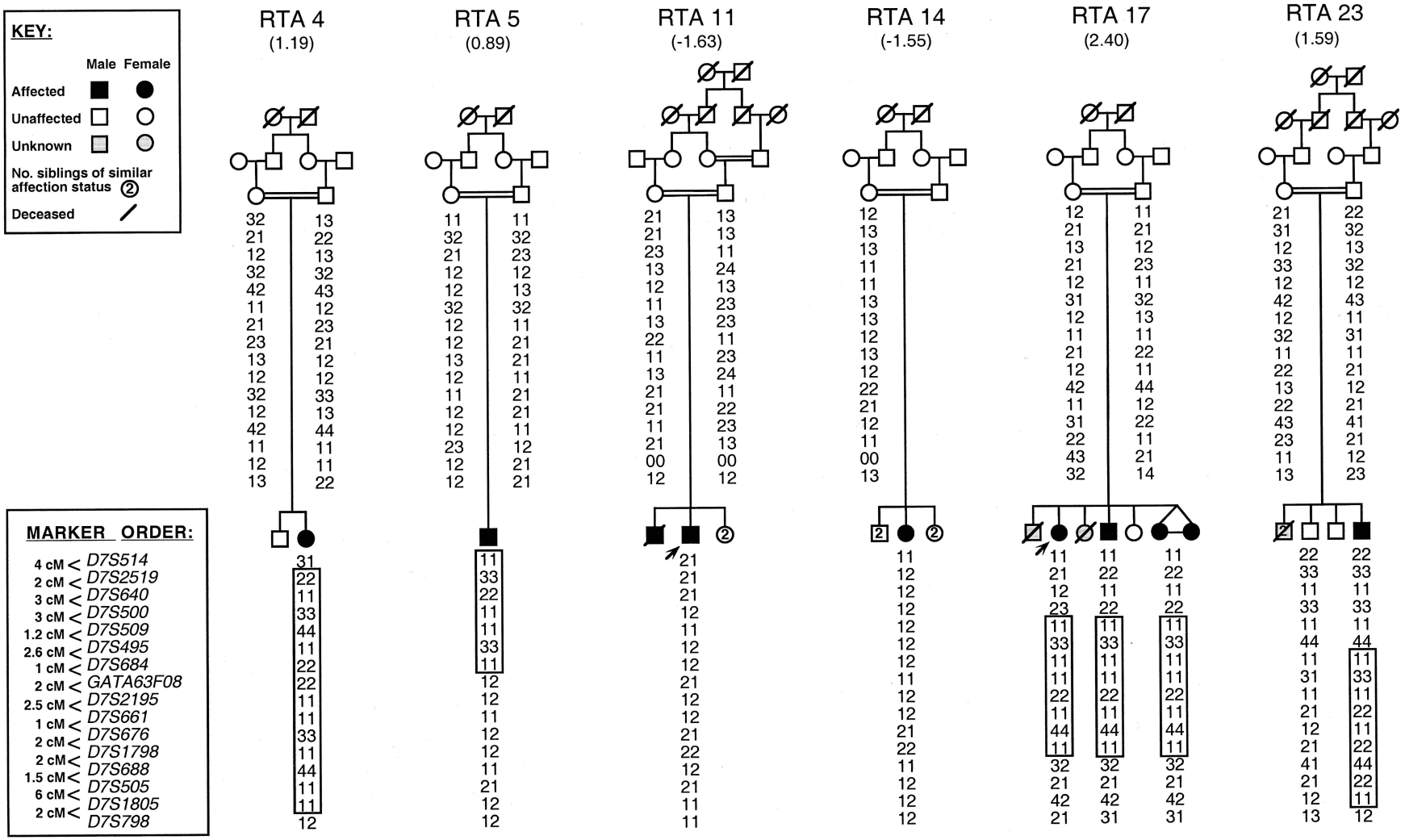
Candidacy of ATP6B1

Other than the difference in hearing status, there were no significant differences in severity of acidosis or biochemical parameters between these 13 kindreds and those previously reported to have mutations in *ATP6B1* (Karet et al. 1999). To test whether mutations in *ATP6B1* might account for a significant fraction of these cases with normal hearing, genotyping of the polymorphic loci tightly linked to this gene on chromosome 2p13 was performed in all available family members. Analysis of the results yielded a maximum multipoint LOD score (Z_{max}) of −21.1 at the *ATP6B1* locus, with a model of genetic homogeneity. With the use of models allowing locus heterogeneity, the Z_{max} was .69, with an estimated 18% of kindreds having linkage at this locus (fig. 3*A*). Additionally, all exons and intron-exon boundaries of *ATP6B1* have been screened by SSCP analysis done under two different conditions in all 13 kindreds, with the use of the same primers and conditions that identified mutations in 91% of hearing-impaired, apparently linked kindreds (Karet et al. 1999). No mutations altering the encoded gene product were identified. Taken together, these findings indicate that mutations in *ATP6B1* do not commonly cause dRTA with normal hearing.

Linkage of Recessive dRTA with Normal Hearing to 7q33-34

These results indicated that mutations in at least one gene other than *ATP6B1* cause recessive dRTA with normal hearing. To map a further gene or genes, a genomewide screen was performed, as described in the Families, Material, and Methods section. Analysis of the results of the initial screen identified seven intervals, on chromosomes 5–8, 16, and 18, with multipoint LOD scores >1.

Further polymorphic markers were genotyped in each


KEY:
Male Female
Affected
Unaffected
Unknown
No. siblings of similar affection status
Deceased
MARKER ORDER:
4 cM < D7S514
2 cM < D7S2519
3 cM < D7S640
3 cM < D7S500
1.2 cM < D7S509
2.6 cM < D7S495
1 cM < D7S684
2 cM < GATA63F08
2.5 cM < D7S2195
1 cM < D7S661
2 cM < D7S676
2 cM < D7S1798
2 cM < D7S688
1.5 cM < D7S505
6 cM < D7S1805
2 cM < D7S798
RTA 4
(1.19)
RTA 5
(0.89)
RTA 11
(-1.63)
RTA 14
(-1.55)
RTA 17
(2.40)
RTA 23
(1.59)

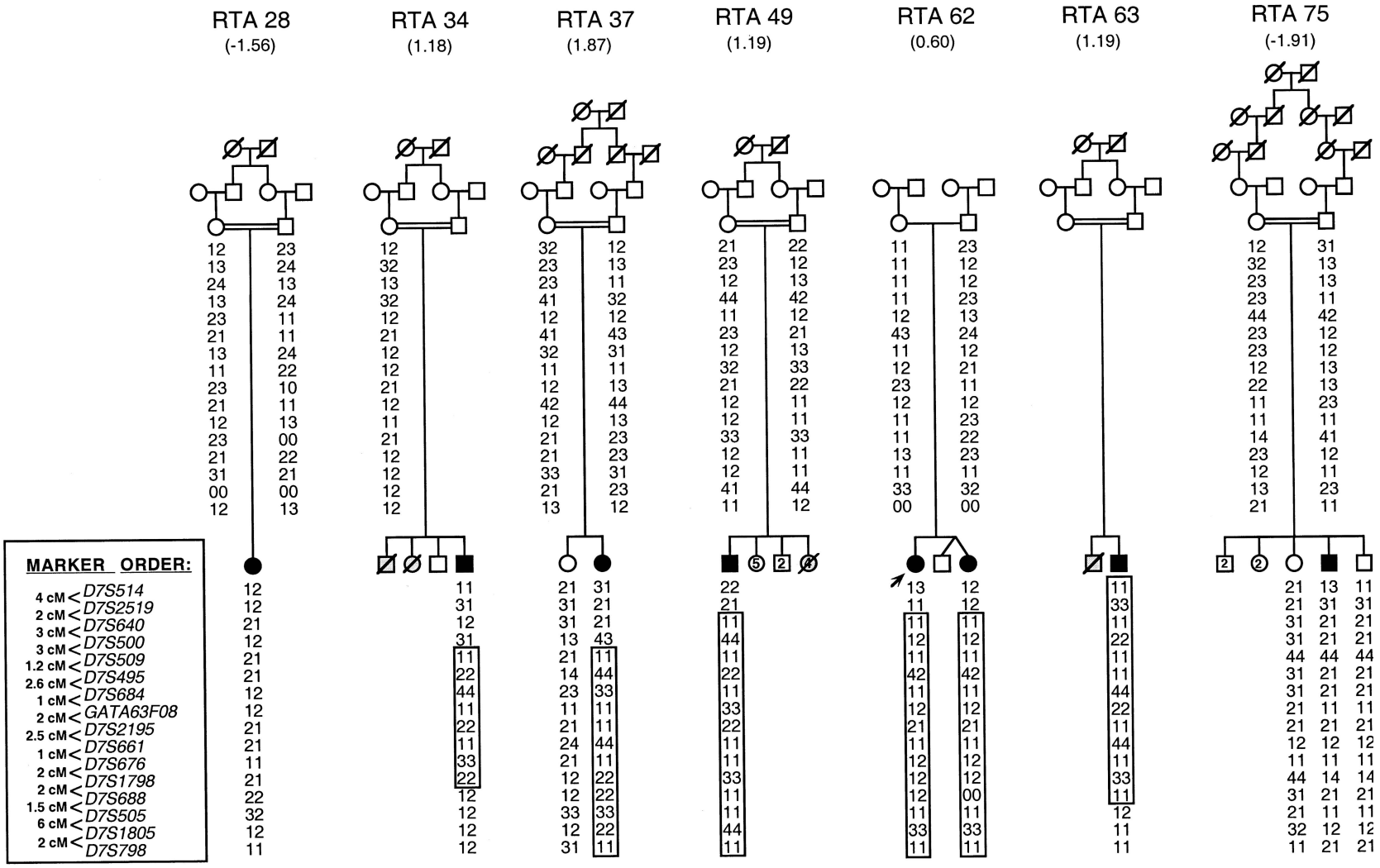


Figure 2 Haplotypes across the *rdRTA2* interval in kindreds with dRTA. Genotypes for polymorphic loci in their chromosomal order (*left*) are shown below the symbol for each available subject, as described in the Families, Material, and Methods section. Approximate genetic distances between adjacent loci are indicated. Genotypes enclosed by boxes indicate the maximum intervals across which affected patients are homozygous or concordant in consanguineous or outbred kindreds, respectively. LOD scores at Z_{max} for *rdRTA2* in each kindred are indicated below the pedigree numbers.

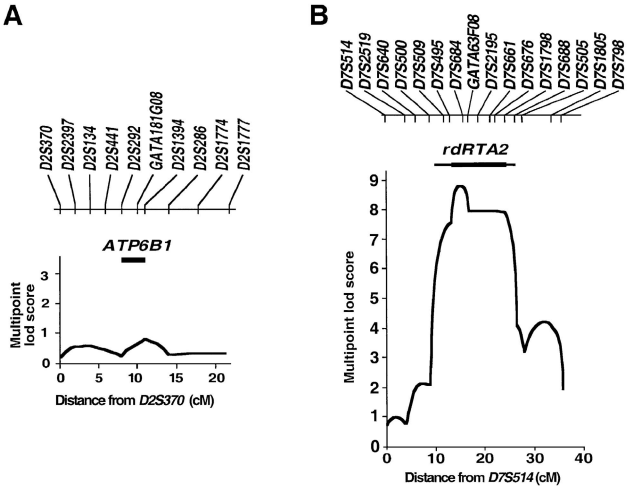


Figure 3 Multipoint analysis of linkage in 13 kindreds with recessive dRTA and normal hearing. The model of linkage allowed for locus heterogeneity and was as specified in the Families, Material, and Methods section. Multipoint LOD scores across intervals containing (*A*) the *ATP6B1* locus on chromosome 2p and (*B*) the newly identified *rdRTA2* locus on chromosome 7q are shown. The Z_{max} achieved at *ATP6B1* was 0.69, with $\alpha = .18$. Analysis of linkage, comparing segregation of *rdRTA2* with 16 linked marker loci, demonstrated a Z_{max} of 8.84. Approximate genetic distances between loci are shown infigure 2. Thick and thin bars indicate the LOD-1 and LOD-3 support intervals, respectively, for the location of *rdRTA2*.

of these intervals, resulting in the being reduced to <1 in all but one interval, a segment of chromosome 7q33-34, which had yielded the highest LOD score (3.90) in the initial genomewide screen.

Genotypes at 16 marker loci in this segment of chromosome 7 are displayed infigure 2. In eight consanguineous kindreds, segments of homozygosity were observed across genetic distances ⩾11 cM, incorporating at least seven consecutive homozygous loci in each kindred. In addition, affected siblings in outbred kindred RTA62 were concordant across 14 loci spanning 32 cM. All 16 loci across the 36-cM interval flanked by loci *D7S514* and *D7S798* gave positive LOD scores in pairwise analysis (data not shown).

With models of locus homogeneity, multipoint analysis of linkage resulted in a LOD score of 5.48 for linkage of dRTA to this interval, with the Z_{max} lying in the 17-cM segment flanked by *D7S500* and *D7S688,* ~2 cM telomeric to *D7S495*. LOD scores for individual kindreds at this Z_{max} are shown in table 3. It is apparent that, although nine kindreds yielded LOD scores at or near their theoretical maxima, four gave negative LOD scores.

Further analysis allowing locus heterogeneity yielded a Z_{max} of 8.84 for linkage to the interval between *D7S495* and *D7S684,* with the Z_{max} lying in approximately the same position and with an estimated 68% of kindreds having linkage at this locus (fig. 3*B*). This model is supported with odds >1,000 : 1 compared with the single-locus model, providing significant evidence of linkage to 7q33-34, with locus heterogeneity. Changing estimates of marker-allele frequencies did not significantly alter the Z_{max}—for example, when average marker heterozygosities were specified as 90%, 67%, or 50%, LOD scores were 8.87, 8.79, and 8.24, respectively. These results identify, at 7q33-34, a locus for recessive dRTA with normal hearing, which we term "*rdRTA2.*" As shown in figure 3*B,* the LOD-3 support interval for the location of *rdRTA2* spans ~14 cM in the interval between loci *D7S509* and *D7S688,* whereas the LOD-1 support interval includes some 11 cM between *D7S495* and *D7S1798*.

We tested whether some of the four families who do not show linkage to *rdRTA2* might, in fact, show linkage to *ATP6B1*. LOD scores for linkage to *ATP6B1* in each kindred are shown in table 3. Only one of the kindreds with negative LOD scores at *rdRTA2* had a positive LOD score at *ATP6B1,* indicating that linkage at these two loci combined cannot account for all kindreds (the multilocus LOD score of 6.78 was significantly lower than that for linkage to *rdRTA2* plus additional unspecified loci). A further model, which allowed linkage to *rdRTA2,* to *ATP6B1,* or to a third, unidentified locus, gave a LOD score of 9.15, which was not significantly greater than that seen for the model that does not include linkage to *ATP6B1*. Finally, patient RTA28-1 represents the only individual with a posteriori evidence of linkage to *ATP6B1* but not to *rdRTA2* (table 3). Her genomic DNA was, therefore, directly sequenced across all exons and exon-intron boundaries of *ATP6B1,* to confirm the

Table 3

Multipoint LOD Scores, for Individual Families, at Z_{max} Positions for *rdRTA2* or *ATP6B1*

RTA Kindred	Theoretical Z_{max}	LOD Score		Posterior Probability[a]
		At *rdRTA2*	At *ATP6B1*	
17	2.4	2.4	−8.38	1.00
23	1.92	1.59	−.84	.99
37	1.92	1.87	−.84	.99
4	1.2	1.19	−1.59	.97
49	1.2	1.19	−1.64	.97
63	1.2	1.19	−1.77	.97
34	1.2	1.18	.98	.97
5	1.2	.89	1.17	.94
62	.6	.6	−4.79	.89
28	1.2	−1.56	1.19	.06
14	1.2	−1.55	−1.2	.06
11	1.12	−1.63	−1.5	.05
75	2.64	−1.91	−1.91	.03

[a] *rdRTA2* vs. unknown.

earlier negative SSCP results; no mutations were identified. Taken together, these findings do not support a significant fraction of kindreds with recessive dRTA and normal hearing that harbor mutations in *ATP6B1*.

Candidate-Gene Analysis

Screening of available databases showed that *AE2,* a member of the chloride-bicarbonate–exchanger gene family (*SLC4A2* [MIM 109280]), has been localized to the telomere of 7q (Palumbo et al. 1986). Because of the known role of *AE1* in dominant dRTA, *AE2* was pursued as a candidate for *rdRTA2*. Radiation-hybrid mapping placed this gene close to locus *D7S1798,* at the telomeric border of the linked interval. SSCP analysis of all 23 exons and exon-intron boundaries in all 13 kindreds demonstrated only two variants, both of which are single-base substitutions, that alter the encoded protein. Gly471→Glu occurred in patient RTA34-1 alone, whereas Gly29→Glu proved to be a common variant, observed in kindreds RTA17, 62, and 63 and in normal controls. Importantly, in two kindreds (RTA17 and RTA34) with long segments of homozygosity in the *rdRTA2* interval, the variant was heterozygous in affected family members and, moreover, was absent from patient RTA62-2. This finding places *SLC4A2* outside the linked interval in these kindreds, and these results make it unlikely that *SLC4A2* is the *rdRTA2* gene.

Discussion

The results of the genomewide linkage screen presented in this report identify a new locus, *rdRTA2,* for autosomal recessive dRTA. Significantly, in contrast to patients with dRTA resulting from a mutation in *ATP6B1,* all of these patients have normal hearing. For models allowing locus heterogeneity, a Z_{max} of 8.84 provides strong support for the location of *rdRTA2* as being 7q33-34. Importantly, only approximately two-thirds of this cohort of patients with dRTA and normal hearing have findings supporting linkage to this interval, indicating that recessive dRTA with normal hearing is genetically heterogeneous.

With the exception of their hearing status, the kindreds presented here do not appear to differ, either in their presentation or in the biochemical severity of their metabolic defect, from the cohort that we have reported that harbors mutations in *ATP6B1*. This indicates that, like ATP6B1, the *rdRTA2*-gene product is required for normal distal nephron acid secretion. However, in contrast to ATP6B1, these genetic data indicate that rdRTA2 does not play an indispensable role in inner-ear physiology.

In the assessment of the aberrant physiology characteristic of dRTA, the sole mapped gene that constituted a potential candidate for a primary role in acid-base homeostasis was *SLC4A2*. The absence of disruptive sequence alterations and the observation of intragenic heterozygosity in some kindreds in which homozygous mutations are expected effectively exclude *SLC4A2* as being the gene responsible for disease in this cohort. This finding also refines the map location of *SLC4A2* on chromosome 7, suggesting a location telomeric to *D7S1798*.

Although no other candidate genes are currently known to lie in the linked interval, the complex physiology of the α-intercalated cell suggests a number of possibilities for the identity of *rdRTA2*. First, rdRTA2 could, like ATP6B1, be part of the apical H^+-ATPase complex itself. This ATPase contains at least 10 different components (Stevens and Forgac 1997), not all of which have yet been cloned and/or characterized. Importantly, the B1-subunit isoform shows specificity for apical, as opposed to the ubiquitous vacuolar, H^+-ATPases. In the absence of more generalized clinical findings, we would anticipate that, if rdRTA2 is an H^+-ATPase subunit, it would also be specific for apical H^+-ATPases. Presently, the B-subunit is the only one known to have an apical pump–specific isoform (Nelson et al. 1992). However, given the contrast in hearing between patients with mutations in *ATP6B1* and *rdRTA2,* the *rdRTA2* gene product would be unlikely to form part of the cochlear apical H^+-ATPase. Alternatively, a defect in a gene whose product is required for trafficking of the proton pump to the apical membrane of the α-intercalated cell could also result in defective proton-pump activity, without altering any of the elements of the pump itself.

Third, defective H^+/K^+-ATPase function at the apical surface of these cells has been proposed as a potential mechanism of disease, consistent with the hypokalemia seen in affected patients (Kurtzman 1990). However, the observation that equally severe hypokalemia occurs, regardless of whether patients harbor mutations in *ATP6B1* or in *rdRTA2,* indicates that hypokalemia in dRTA does not require a defect in H^+/K^+-ATPase function. Moreover, the severity of acidosis in patients with mutations in *ATP6B1* implies that, in contrast to the results of studies in animals (Silver et al. 1997), H^+/K^+-ATPase activity cannot adequately compensate for diminished H^+-pump function, which suggests that this pathway plays a lesser role in pH homeostasis in humans.

Finally, defects in genes whose products are more indirectly required for H^+ secretion could result in the observed phenotype; these defects include those in mechanisms in α-intercalated cells necessary for generation of protons, absorption of bicarbonate, recycling of chloride, and maintenance of the electrochemical gradient across both apical and basolateral membranes. Identification of the *rdRTA2* gene will likely provide new in-

sight into the fundamental physiology of pH homeostasis in humans.

Our results will now direct further studies to narrow the linked interval, to permit identification of this gene. Since many of the kindreds reported here were recruited from Turkey, we have sought a possible founder effect, by examination of the original genotypes and haplotypes in the *rdRTA2* interval in this group. However, no evidence of linkage disequilibrium is present in the current data set, indicating either the absence of a founder effect or a founder more remote than that which can be detected by use of polymorphic microsatellite markers at the current density (data not shown).

Linkage to *rdRTA2* was not apparent in four families, and we found no evidence supporting mutation in *ATP6B1* in this group. These findings strongly suggest the existence of one or more additional loci in which mutation can produce the dRTA phenotype. Although the phenotypes of affected individuals from the four unlinked kindreds do not differ substantially from those linked to *rdRTA2* (table 2), the index patients do show a trend of greater urinary calcium loss, and all four have had rickets. It is possible that the aberrant gene or genes in these patients will exert a more seriously disruptive effect on calcium metabolism. However, separate analysis of the genomewide linkage data in these four kindreds alone has not yet provided significant evidence for linkage to an additional locus, and further studies, incorporating new kindreds, may be necessary to confer adequate power to detect additional dRTA loci. It was also notable that no positive LOD scores were obtained in the chromosome 17 interval containing *AE1,* thereby providing no support for a role of this gene in recessive dRTA, in contrast to its role in dominant disease (Karet et al. 1998).

Taken together, these results provide significant evidence that, in patients with recessive dRTA and normal hearing, the phenotype is attributable to *rdRTA2* and at least one additional locus and that these loci are different from the previously identified locus for recessive dRTA with hearing impairment. Meanwhile, the studies reported here provide a first step toward identification of the underlying disease genes.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Cooperative Human Linkage Center, The, http://lpg.nci.nih.gov/CHLC/ (for marker order)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for *AE2* [accession numbers U76667–U76669, U62290])

Genome Database, The, http://gdbwww.gdb.org/ (for marker order)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for autosomal dominant inherited dRTA [MIM 179800] and autosomal recessive inherited dRTA [MIM 267300 and MIM 602722])

Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www.genome.wi.mit.edu/ (for marker order)

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