ORIGINAL ARTICLE

Novel *ATP6V1B1* and *ATP6V0A4* mutations in autosomal recessive distal renal tubular acidosis with new evidence for hearing loss

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Autosomal recessive distal renal tubular acidosis (rdRTA) is characterised by severe hyperchloraemic metabolic acidosis in childhood, hypokalaemia, decreased urinary calcium solubility, and impaired bone physiology and growth. Two types of rdRTA have been differentiated by the presence or absence of sensorineural hearing loss, but appear otherwise clinically similar. Recently, we identified mutations in genes encoding two different subunits of the renal α-intercalated cell's apical H^+-ATPase that cause rdRTA. Defects in the B1 subunit gene *ATP6V1B1*, and the a4 subunit gene *ATP6V0A4*, cause rdRTA with deafness and with preserved hearing, respectively.

We have investigated 26 new rdRTA kindreds, of which 23 are consanguineous. Linkage analysis of seven novel SNPs and five polymorphic markers in, and tightly linked to, *ATP6V1B1* and *ATP6V0A4* suggested that four families do not link to either locus, providing strong evidence for additional genetic heterogeneity.

In *ATP6V1B1*, one novel and five previously reported mutations were found in 10 kindreds. In 12 *ATP6V0A4* kindreds, seven of 10 mutations were novel. A further nine novel *ATP6V0A4* mutations were found in "sporadic" cases.

The previously reported association between *ATP6V1B1* defects and severe hearing loss in childhood was maintained. However, several patients with *ATP6V0A4* mutations have developed hearing loss, usually in young adulthood. We show here that *ATP6V0A4* is expressed within the human inner ear. These findings provide further evidence for genetic heterogeneity in rdRTA, extend the spectrum of disease causing mutations in *ATP6V1B1* and *ATP6V0A4*, and show *ATP6V0A4* expression within the cochlea for the first time.

Acid-base regulation by the kidney is tightly controlled through the coupled processes of acid secretion and bicarbonate reabsorption via intercalated cells of the nephron's collecting duct segment. The result is regulated secretion into the urine of the net acid load provided by the human diet. The main proton pump responsible for urinary acidification by α-intercalated cells, the apical H^+-ATPase, is a multi-subunit structure with a "head and stalk" configuration. The V_1 (head) and V_0 (membrane anchored) domains are responsible for ATP hydrolysis and transmembrane proton translocation respectively.[1] So far, novel genes encoding two H^+-ATPase subunits specific to intercalated cells have been identified, termed *ATP6V1B1* and *ATP6V0A4*.[2 3] (In May 2002, the official symbols for these genes were altered by the HUGO Gene Nomenclature Committee from *ATP6B1* and *ATP6N1B* respectively.) They encode the B1 subunit in the catalytic head and the a4 subunit at the pump's base, which co-localise apically in α-intercalated cells and are distinct from the genes for the corresponding subunits (B2 and a1) in the ubiquitously expressed H^+-ATPases present in intracellular organelles. Destructive mutations in both these kidney subunit genes are associated with autosomal recessive distal renal tubular acidosis (rdRTA).

RdRTA is characterised clinically by impaired urine acidification leading to severe hyperchloraemic hypokalaemic metabolic acidosis, prominent renal tract calcification, and rickets. About one third of patients with rdRTA have progressive and irreversible sensorineural hearing loss evident in childhood. Except for the hearing status, the two forms of recessive disease appear to be clinically similar. Studies to date have shown that mutations in *ATP6V1B1* cause rdRTA with sensorineural hearing loss (SNHL), whereas rdRTA with preserved hearing is caused by mutations in *ATP6V0A4*.

A genome wide linkage screen of a set of 31 kindreds with rdRTA resulted in the identification of *ATP6V1B1* as the first gene associated with this disorder.[4] This gene is encoded in 14 exons, yielding the B1 subunit of 513 amino acids. It is expressed by interdental cells and endolymphatic sac epithelia, accounting for the associated hearing impairment. In vitro studies have previously shown that the B subunit is necessary, but not sufficient, for ATP hydrolysis.[5]

Genome wide screening of a hearing cohort identified a linked locus at 7q33-34[6] that proved to contain the novel *ATP6V0A4* gene.[3] *ATP6V0A4* has 23 exons, of which 20 encode the 840 amino acid transmembrane a4 subunit, whose function in the pump is currently unknown. By analogy with

Abbreviations: rdRTA, autosomal recessive distal renal tubular acidosis; SNP, single nucleotide polymorphism; SNHL, sensorineural hearing loss; WT, wild type; DHPLC, denaturing high performance liquid chromatography; RT, reverse transcription; SSCP, single strand conformation polymorphism

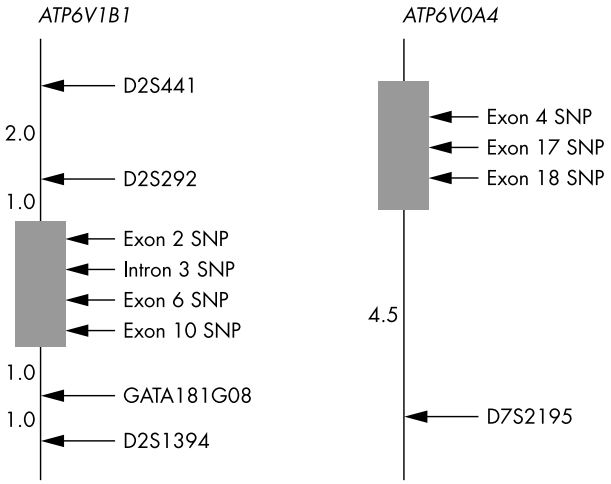

Figure 1 Polymorphic markers used for linkage analysis of the *ATP6V1B1* and *ATP6V0A4* loci. Genetic distances (cM) are shown.

yeast, it may be involved in H^+ translocation or transport and/or assembly of the H^+-ATPase.[7]

Importantly, some families with rdRTA and deafness have been identified that exclude linkage to *ATP6V1B1*, and some with rdRTA and normal hearing are not linked to *ATP6V0A4*, suggesting that there may be additional genes involved in rdRTA. The initial aims of this study were to survey the spectrum of mutations in *ATP6V1B1* and *ATP6V0A4* and to identify additional families unlinked to either of these loci that might be used to help discover new dRTA genes. As a result of our finding, presented below, that some *ATP6V0A4* mutations are associated with older onset SNHL, we also assessed *ATP6V0A4* expression in the human inner ear.

METHODS

RdRTA kindreds and controls

RdRTA kindreds were recruited by ascertainment of affected index cases. Kindreds were defined as recessive when both parents were unaffected and were either consanguineous or had multiple affected offspring. Single affected offspring of unrelated, unaffected parents were classified as sporadic. Control subjects consisted of unrelated, unaffected subjects from similar ethnic backgrounds (13 each of Saudi Arabian, Turkish, and Spanish origin).

DNA from all subjects was isolated from whole blood by standard methods,[8] and quantified using the PicoGreen® kit (Molecular Probes). Biochemical data sought from referring physicians included serum sodium, potassium, chloride, bicarbonate, pH, calcium, magnesium, phosphate, and urine pH and calcium. Differences among groups were sought using the T or Mann-Whitney test as appropriate. The presence of renal tract calcification, chronic renal impairment, and rickets was also reported. Hearing was assessed by pure tone audiometry and/or auditory evoked responses according to age. Kindreds were classified as having rdRTA associated deafness if the affected subjects had bilateral sensorineural hearing loss of >16 dB (500-2000 Hz average) and no apparent alternative causes of hearing loss. Severity of decibel loss was quantified according to standard criteria (16-25: slight; 25-40: mild; 41-55: moderate; 56-70: moderately severe; 71-90: severe; >90: profound).[9]

Single nucleotide polymorphism (SNP) characterisation

Four intragenic SNPs in *ATP6V1B1* and three in *ATP6V0A4*, previously identified in Turkish and Saudi Arabian patients during mutation screening, were characterised for use in genotyping (fig 1). Using appropriate enzymes for RFLP analysis (table 1), allele frequencies in 50 chromosomes of unrelated Turkish and Saudi control subjects were analysed. Primer sequences were as previously published.[3 4]

Genotyping and linkage analysis

Genotyping was performed by PCR amplification and either RFLP analysis of SNPs (table 1) or microsatellite analysis using fluorescently end labelled primers or [^{32}P]-dCTP incorporation, under conditions previously described[10 11] (fig 1). Evidence for linkage to *ATP6V1B1* and *ATP6V0A4* was assessed, qualitatively by seeking homozygosity by descent in consanguineous families or shared haplotypes in affected members of outbred kindreds, and quantitatively using the GENEHUNTER program[12 13] under models previously described.[6]

Mutation detection

Individual *ATP6V1B1* and *ATP6V0A4* exons and flanking intronic sequences were amplified from genomic DNA, using primers and conditions previously reported,[3 4] with a mixture of AmpliTaq Gold® and PfuTurbo® DNA polymerases (Applied Biosystems and Stratagene) and 38-40 PCR cycles. Double stranded DNA duplexes were formed by denaturing and slowly reannealing a 1:2 mixture of known wild type (WT) and sample DNA. The resulting samples were analysed using denaturing high performance liquid chromatography (DHPLC) (WAVE™ system, Transgenomic Inc).[14] All identified WAVE variants were directly sequenced on both strands using the ABI PRISM® BigDye™ Terminator method (Applied Biosystems) according to standard protocols.

Table 1 Single nucleotide polymorphisms in *ATP6V1B1* and *ATP6V0A4* used for genotyping. Each had been identified during previous mutation screening and typed in 50 control chromosomes. All were found to be in Hardy-Weinberg equilibrium

	Amino acid position and change	Nucleotide alteration	% allele frequency (cut:uncut)	Enzyme	Product sizes before & after digestion
ATP6V1B1					
Exon 2	S46S	138T>C	36:64	*Tsp*RI	282 → 123+159
Intron 3		IVS3+117C>A	46:54	*Hpy*CH4IV	470 → 237+233
Exon 6	E161K*	481G>A	90:10	*Ava*I	251 → 66+185
Exon 10	R334R	1002C>T	28:72	*Bst*UI	267 → 120+147
ATP6V0A4					
Exon 4	A2V†	5C>T	72:28	*Bgl*I	299 → 149+150
Exon 17	F554F	1662T>C	68:32	*Xcm*I	262 → 157+105
Exon 18	H604H	1812C>T	68:32	*Bsa*AI	323 → 174+149

*Conserved in all species except *Neurospora*.
†Not evolutionarily conserved.

Table 2 Diagnostic clinical, biochemical, and sequencing features of index cases where mutations in *ATP6V1B1* were found. Residues numbered according to the sequence in Genbank NM_001692

Kindred/ sex	Age	Origin	Age at dx	Consang ?	RTA associated SNHL?	Mutation		Na	K	Cl	HCO_3^-	Arterial pH	Urine pH
						DNA	Protein						
*203/F	15 y	Saudi	5 y	Y	Y†	1155–1156insC	I386fsX441	136	3.4	112	16.0	NA	7.5
225/F	41 y	Morocco	2 mth	Y	Profound‡	1155–1156insC	I386fsX441	141	2.7	NA	NA	NA	NA
*227/F	10 y	Sicily	3 mth	Y	Severe	1155–1156insC	I386fsX441	147	3.2	122	11.1	7.31	8.0
*214	NA	Saudi	NA	Y	Y	497delC	T166fsX174	NA	NA	NA	NA	NA	NA
212/F	6 y	Pakistan	7 mth	Y	Profound	IVS8+1G>A	Exon 8 skip?[19]	139	3.7	112	12.0	NA	7.0
*208/F	6 y	Macedonia	4 y	N	Severe	242T>C	L81P	137	3.2	110	8.0	7.16	7.0
223/M	10 y	Turkey	3 y	Y	NA	469C>T	R157C	135	4.3	104	17.4	7.27	6.5
91/F	25 y	Italy	3 y	Y	Severe	1037C>G	P346R	140	1.8	119	NA	7.12	7.0
*204/F	11 y	Saudi	Birth	Y	Y	1037C>G	P346R	132	3.3	114	15.0	NA	7.0
*205/M	10 y	Saudi	14 mth	Y	Y	1037C>G	P346R	139	2.9	115	11.0	7.20	7.5
Sporadics													
201/M	7 y	Turkey	5 mth	N	NA	368G>T	G123V	133	2.6	110	16.6	7.30	7.0
211/M	28 y	Turkey	6 y	N	Severe	[91C>T + 823A>C]	R31X + T275P	142	2.4	114	15.4	7.15	7.8
							Mean	138	3.0	113	13.6	7.22	7.2
							SEM	1	0.2	2	1.1	0.03	0.1

Consang = consanguineous parental union; dx = diagnosis; * = additional affected with mutation; NA = not available or not tested; fs = frameshift; del = deleted; ? = unknown; SEM = standard error of mean.
†SNHL detected by auditory evoked responses in infancy, and/or not quantified.
‡Standard criteria for hearing loss.[9]

All novel missense mutations were subjected to RFLP analysis using the appropriate restriction enzyme or allele specific PCR[15] to assess their frequencies in all 39 control subjects.

RT-PCR studies of *ATP6V0A4* in inner ear epithelia

With local ethical approval, discarded fragments of epithelia from adult human inner ear (each approximately 2 mg, n=3) were snap frozen in liquid nitrogen at the time of surgery for unrelated disorders. Total RNA was extracted using Trizol solution (GibcoBRL) as per the manufacturer's protocol, and reverse transcription performed with oligo-$dT_{12\text{-}18}$ by standard methods. One tenth of each reverse transcription (RT) reaction was used as template for 36 cycles of amplification of separate segments of *ATP6V0A4* coding sequences that spanned intron 4 (forward: ttgtcacaactgtttctccaggtg, reverse: aggattctctccagtgattcacac), introns 9-11 (forward: gttactgtggcgaatctgccgag, reverse: cttctgcaccttgatgagccag), or intron 22 (forward: gctgtcctgacagtagccatc, reverse: caaagtccttcctctgacgtg), thus providing internal controls for genomic DNA amplification.

For fetal cochlea RNA,[16] RT was performed with SuperScript Reverse Transcriptase (Invitrogen) following the manufacturer's instructions, using 1 μg of total RNA as template and gene specific primers (sequences available on request). Reactions were performed with and without enzyme. One tenth of the RT reaction was used as a template for PCR. Two primer pairs were used in nested amplification of *ATP6V0A4* or *ATP6V1B1* (as positive control) using Platinum *Taq* (Invitrogen) according to the manufacturer's instructions, 20 cycles each round, with the second round using 1/100 dilution of the first round product as template. Internal *ATP6V1B1* primers spanning intron 7 were forward: ctcagatctgccgccaggcg and reverse: gaagaatctggctgtctccatg.

RESULTS

rdRTA kindreds

Twenty-six rdRTA kindreds (23 consanguineous) and 13 sporadic dRTA cases are reported in this study. Affected subjects presented with failure to thrive, dehydration and vomiting, and/or growth impairment at under 9 years of age. The diagnosis of primary dRTA was made by the combination of inability to acidify urine (pH >5.5) in the setting of a normal anion gap and spontaneous systemic metabolic acidosis, hypokalaemia, otherwise normal renal function, and no evidence of secondary causes of dRTA. Calcium, phosphate, and magnesium levels were normal. Biochemical data and other clinical parameters for the affected index cases of kindreds and sporadic cases are shown in tables 2 and 3.

Linkage analysis

Fig 1 shows the markers used for genotyping at the loci of *ATP6V1B1* and *ATP6V0A4* on chromosomes 2p13 and 7q33-34, respectively. The intragenic SNPs previously identified are listed in table 1. All were common, and were found to be in Hardy-Weinberg equilibrium in 50 control chromosomes, and were considered to be neutral biallelic polymorphisms. GENEHUNTER analysis (data not shown) and inspection of haplotypes were used to classify kindreds as probably linked to *ATP6V1B1*, *ATP6V0A4*, both, or neither (seven, eight, seven, and four kindreds respectively), as shown in fig 2. Kindreds were defined as unlinked where lod scores were ⩽−2, and/or there was heterozygosity in affected offspring of a consanguineous union and/or non-identical haplotypes were observed among affected sibs.

The data followed the expected pattern of hearing impaired kindreds showing evidence for linkage to *ATP6V1B1* and normal hearing kindreds being probably linked to *ATP6V0A4*. Notably, four families (two with normal audiometry, one with SNHL, and one of unknown hearing status) were not linked to either gene, providing strong evidence for additional genetic heterogeneity in dRTA.

Mutation detection

DHPLC (Transgenomic WAVE™) analysis was performed for all coding exons and flanking intronic segments in *ATP6V1B1* and/or *ATP6V0A4* as dictated by the linkage results. This method proved 100% sensitive in this cohort in that, in all linked kindreds, variants were identified that led to detection of a mutation or polymorphism. Other studies have concluded that DHPLC is superior in sensitivity to SSCP.[17] Owing to parental consanguinity, most of the mutations were predicted to be homozygous, but this method detects heterozygous changes by virtue of altered HPLC mobility. Analysis was therefore carried out using a mixture of patient and known

Table 3 Diagnostic clinical, biochemical, and sequencing features of index cases where mutations in *ATP6V0A4* were found. Residues numbered according to the sequence in Genbank NM_020632

Kindred/ sex	Age	Origin	Age at dx	Consang ?	RTA associated SNHL?	Mutation		Na	K	Cl	HCO_3^-	Arterial pH	Urine pH
						DNA	Protein						
76/F	Died 6 mth	Turkey	3 mth	Y	NA	1072C>T	Q358X	143	3.5	125	7.3	7.12	7.0
206/M	10 y	Saudi	2 mth	Y	N	2257C>T	Q753X	NA	NA	NA	16.0	NA	7.6
217/F	NA	Saudi	NA	Y	NA	2257C>T	Q753X	NA	NA	NA	NA	NA	NA
*228/F	17 y	Sicily	1 y	N	?†	[2521T>C + 2332delG]	X841Q + V778fsX788	141	3.1	110	17.8	7.4	7.0
*213/F	6 y	Iran	1 mth	Y	N	338delA	N113fsX117	140	2.8	118	8.6	7.14	5.6
209/F	3 y	Lebanon	11 mth	Y	N	709–711delAAG	K237del	139	2.1	118	7.0	7.13	8.0
83/F	15 y	Canada	2 mth	Y	?	IVS6-1G>A	?§	131	3.7	114	7.8	7.16	7.0
220/M	10 y	Turkey	1 mth	Y	Mild‡	IVS12-2A>C	?	147	2.2	110	11.5	7.23	7.0
221/F	2 y	Turkey	4 mth	Y	N	IVS12-2A>C	?	142	2.8	115	16.0	7.30	7.5
25/F	18 y	Pakistan	2 mth	Y	Moderate	[IVS17+1G>A]	Exon 17 skip?[19]	136	2.4	114	15.0	7.31	NA
*60/M	12 y	Turkey	3 mth	Y	NA	1346G>A	R449H	145	2.5	128	NA	NA	7.5
226/F	4 y	Turkey	Birth	Y	N	1346G>A	R449H	141	2.3	111	10.0	7.2	7.0
Sporadics													
53/M	13 y	Turkey	3 mth	N	Mild	[580C>T + 243–244insTC]	R194X + E82fsX87	137	3.4	112	9.2	7.21	7.1
72/M	34 y	Spain	1 mth	N	Moderate	1506T>A	Y502X	142	3.6	120	11.0	7.99	7.3
67/M	15 y	Spain	18 d	N	N	[IVS17+2–+3insT]	Exon 17 skip?	139	4.4	113	16.2	7.37	7.2
73/M	10 y	Spain	1 mth	N	N	[1506T>A + IVS17+2–+3insT]	Y502X + exon 17 skip?	135	2.5	116	16.1	7.32	7.2
74/F	9 y	Spain	1 mth	N	N	[1506T>A + IVS17+2–+3insT]	Y502X + exon 17 skip?	136	3.3	122	4.0	6.72	7.4
66/M	26 y	Spain	8 mth	N	N	306–307insA	L103fsX139	138	4.4	126	12.8	7.21	7.3
65/M	5 y	Spain	3 mth	N	N	1185delC	P395fsX407	140	3.4	118	10.0	7.12	7.4
3/M	10 y	Turkey	6 wk	N	N	1832–1833delAGinsT	S611fsX648	140	2.2	125	9.7	7.22	6.5
41/F	7 y	Turkey	6 wk	N	N	IVS12-2A>C	?	141	2.5	127	8.1	7.08	7.5
68/M	30 y	Spain	1 mth	N	N	[524G>A + IVS17+2–+3insT]	G175D + exon 17 skip?	142	4.3	116	10.7	7.19	7.0
70/M	26 y	Spain	2 mth	N	Severe	2420G>A	R807Q	138	1.3	112	5.3	7.14	7.5
							Mean	140	3.0	118	11.0	7.23	7.2
							SEM	1	0.2	1	0.9	0.05	0.1

Consang = consanguineous parental union; dx = diagnosis; * = additional affected with mutation; NA = not available or not tested; fs = frameshift; del = deleted; ins = inserted; SEM = standard error of mean.
†Hearing impairment not definitely attributable to dRTA.
‡Standard criteria for hearing loss.[9]
§Effect of splice acceptor loss unknown.

wild type PCR products to ensure the creation of heteroduplexes. The correspondence among linkage results, gene mutation, and hearing status for all kindreds is shown in fig 2. No significant differences in means (or medians where data were not normally distributed) for biochemical parameters were found among the groups of patients harbouring mutations in each gene (tables 2 and 3), nor the group of four kindreds unlinked to either gene (data not shown).

ATP6V1B1

ATP6V1B1 mutations were identified in the affected members of 10 kindreds, nine of them deaf and one with indeterminate hearing status, as shown in table 2 and fig 3. All of these kindreds had shown evidence of linkage to *ATP6V1B1*, and hearing loss was evident in childhood in all. The mutations were homozygous in all cases, even where there was no known parental consanguinity. Identified sequence alterations included two frameshifts that resulted in the generation of premature stop codons after short segments of novel triplets, one consensus splice site, and three missense alterations. In two sporadic patients, an additional three mutations (two missense, one nonsense) were identified, one homozygously.

Two novel *ATP6V1B1* mutations (R157C and G123V) were identified. Neither was present in the 39 controls, and these residues are entirely conserved across numerous species including yeast. By contrast, missense alteration T30I, which is not evolutionarily conserved, was found heterozygously in two sporadic cases, and also in 18 of the 78 control chromosomes, in Hardy-Weinberg equilibrium. As a result we designated it a novel neutral polymorphism (table 4).

ATP6V0A4

ATP6V0A4 mutations were identified in affected members of 12 kindreds, as listed in table 3 and fig 3. All of these kindreds had shown evidence of linkage to *ATP6V0A4*. These mutations comprised two different nonsense, two frameshift, three consensus splice site, one amino acid deletion, and two missense mutations. In one of these, the normal termination codon was altered to glutamine, extending the encoded protein by 52 residues. In a further 11 sporadic cases we identified another nine different mutations. Four sporadic cases were compound heterozygotes and the remainder homozygotes, again without previous evidence of consanguinity. In contrast to *ATP6V1B1* mutations, the great majority observed in *ATP6V0A4* were novel. There was geographical evidence for a founder effect for several of these, such as Y502X in subjects from northern Spain and loss of the intron 12 splice acceptor site in patients from Turkey.

Also clustered among subjects from northern Spain was the intron 17 donor splice site change inserting a T at position +3. This mutation was present both homozygously and in compound heterozygosity with a second severe mutation in a total of four kindreds. It was absent from controls, including an extended panel of 30 unrelated unaffected Spaniards.

Hearing status among *ATP6V0A4* mutants

Of the 23 rdRTA cases reported here where *ATP6V0A4* mutations were found, information about hearing status is available for 20. Notably, most of these were recorded to

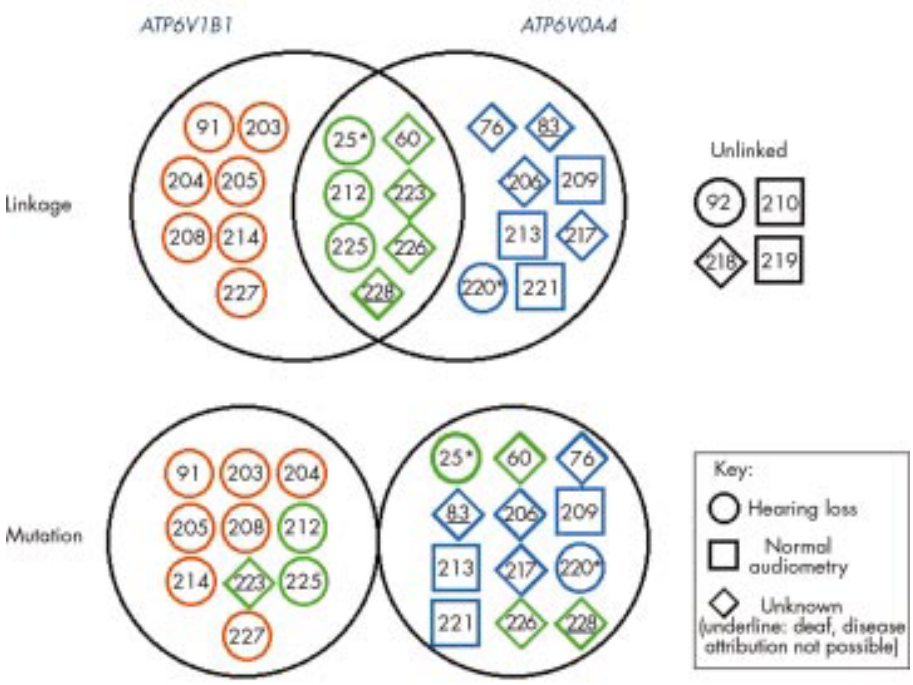

Figure 2 Results of linkage analysis and mutation detection in 26 rdRTA kindreds. The upper panel summarises the linkage results, in which evidence for sole linkage to *ATP6V1B1* or *ATP6V0A4* was obtained from seven and eight kindreds respectively, whereas in four kindreds, linkage to both was excluded. All those linking to *ATP6V1B1* alone were deaf, and all where audiometry was normal linked to *ATP6V0A4*. The lower panel illustrates the distribution of mutations (tables 2 and 3). Notably, the association of deafness with *ATP6V1B1* was strongly maintained, while hearing loss has recently been found in cases 25-1 and 220-1 who harbour *ATP6V0A4* mutations (asterisked).

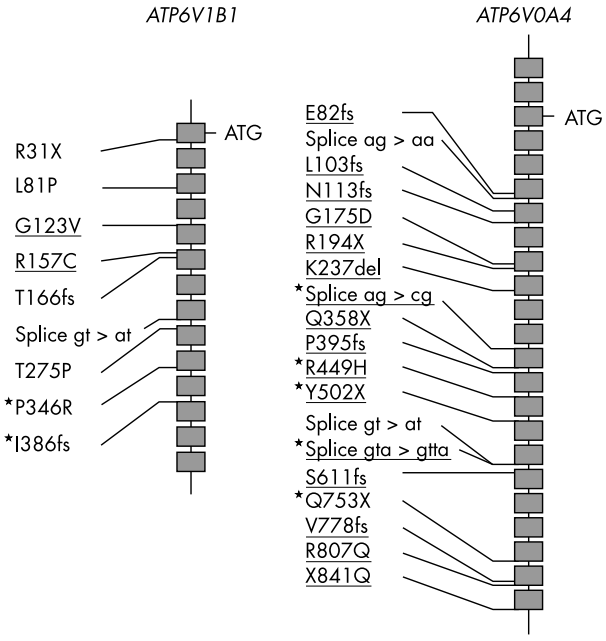

Figure 3 Schematic of *ATP6V1B1* and *ATP6V0A4* mutations identified. Grey boxes represent exons. A variety of nonsense, frameshift, missense, and splice site alterations were found. Mutations not previously reported are underlined. Asterisks indicate mutations observed in multiple kindreds. fs = frameshift.

have normal audiograms when they were referred for study, in contrast to the *ATP6V1B1* cohort. Currently, patients 83-1, 228-1, 25-1, 53-1, 70-1, 72-1, and 220-1 from this cohort have hearing impairment. Patient 83-1 has a deaf mother and therefore may have a different, non-syndromic dominant cause for her hearing loss. Hearing loss in 228-1 has been attributed to ototoxic antimicrobial exposure in infancy. Thus, in neither case can SNHL definitely be associated with the dRTA. In four of the remaining five, hearing loss is either mild to moderate, or became evident only in the second to fourth decade, or both. For example, we have recently learned that 72-1 has been found to have SNHL for the first time at the age of 33 years. He and 25-1 have had previous audiograms that were within normal limits. By contrast, 70-1 has the most marked hearing loss and now requires hearing aids. We have consequently reviewed the hearing status of those in our original *ATP6V0A4* cohort[3] and found that one (17-1) has developed mild SNHL at the age of 22.

Expression of *ATP6V0A4* in inner ear epithelium

We therefore examined the expression of *ATP6V0A4* in the human inner ear. Suitable tissue is difficult to obtain in quantity from adults, and we have to date been limited to RT-PCR amplification of mRNA from tiny fragments of epithelia from the vestibular system, together with fetal cochlear cDNA, as we have previously reported for *ATP6V1B1*.[4] There was clear evidence for *ATP6V0A4* expression in both adult and fetal material, with the appearance of specific bands following amplification of exons 4-5, 9-12, and 22-23. Control amplification of *ATP6V1B1* was also positive in both. Representative results are displayed in fig 4.

DISCUSSION

In this study, linkage analysis results correlated well with the subsequently identified mutations. All kindreds that showed linkage to *ATP6V1B1* and could be excluded from linkage to *ATP6V0A4* had an *ATP6V1B1* mutation, and vice versa, and all kindreds showing evidence of potential linkage to both genes had a mutation in either *ATP6V1B1* or *ATP6V0A4*. These data

Table 4 Novel missense alterations in *ATP6V1B1* and *ATP6V0A4*. Each was typed in 78 control chromosomes; all except T30I were conserved across numerous species including yeast

	Amino acid position and change	Nucleotide alteration	% allele frequency in controls	Enzyme	Product sizes before & after digestion
ATP6V1B1					
Exon 1	T30I	90C>T	77:23	*Bst*EII	240 → 177+63
Exon 5	G123V	368G>T	100:0	*Bsu*36I	205 → 50+155
Exon 6	R157C	469C>T	100:0	*Hpy*CH4V	251 → 124+68+59 ('cut') or 183+68 ('uncut')
ATP6V0A4					
Exon 9	G175D	524G>A	100:0	*Msp*I	234 → 171+63
Exon 15	R449H	1346G>A	100:0	*Hha*I	295 → 190+105
Exon 22	R807Q	2420G>A*	100:0		
Intron 17		IVS17+2–+3insT	100:0	*Mse*I	240 → 190+50

*Detected by allele specific PCR.[15]

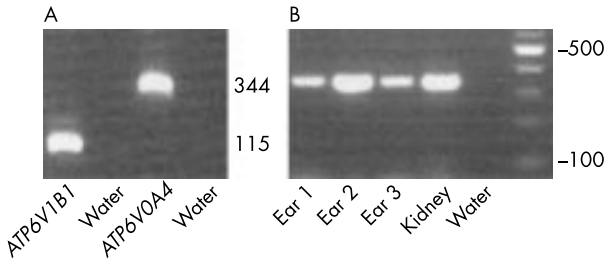

Figure 4 *ATP6V0A4* is expressed in human inner ear. PCR analysis of human cDNA from (A) fetal cochlea and (B) three samples of adult vestibular epithelium is shown. Positive controls were provided by amplification of the same four exons (9-12) of *ATP6V0A4* from kidney cDNA in the adult, and concomitant amplification of *ATP6V1B1*[4] from the same fetal cDNA. Water was used as template for negative controls. Product sizes (in bp) are shown, with 100 bp ladder on the right. See text for primer details.

show that analysis of linkage to these two loci is a good predictor of mutation status in these genes, which may be helpful for future screening and molecular diagnostic efforts, particularly since both genes are large. Moreover, in those kindreds linked to both loci, the presence of hearing loss at a young age provided good predictive evidence for the subsequent finding of a mutation in *ATP6V1B1*.

Our previous separate studies of *ATP6V1B1* or *ATP6V0A4* have each excluded some kindreds from linkage, but in neither case were they formally tested for linkage to the alternative locus. Thus, analysis of linkage under models of genetic heterogeneity in this study provides the first definite evidence for the existence of at least one additional rdRTA locus. Notably, the group of kindreds in which linkage to both *ATP6V1B1* and *ATP6V0A4* was excluded also had the same clinical profile as those with mutations in either gene. Therefore, the search for novel candidate genes can be focused by attempting to identify proteins that would produce the same physiological effects if their functions were abolished.

We have extended the spectrum of coding alterations in both of the kidney specific H^+-ATPase genes associated with rdRTA in this large cohort of both familial and sporadic cases, in the process identifying a number of novel mutations. Subjects from some geographical regions appear to share certain mutations suggestive of a founder effect (for example, Y502X and the intron 17 splice site mutation in subjects from Spain). There is also some evidence for geographical drift, for example, among the subgroup harbouring the insertion frameshift at position I386 in *ATP6V1B1*. These originate from North Africa, Saudi Arabia, and also Sicily, where there was Arab domination several centuries ago. Both insertion and deletion frameshifts in I386 were observed in three kindreds in our earlier study (one Swedish, two Spanish). It is also possible that I386 appears as a relative "mutation hotspot" owing to a string of seven cytosine residues at this position, suggesting that slippage may occur during replication. Similarly, we found a deletion at P395 in *ATP6V0A4*, where there is a row of five cytosines and an A insertion into a row of five at L103.

Although the third intronic base at the donor splice site is not fixed like the first and second, it is almost invariably A or G.[18] We predict that splicing will therefore be disrupted by the T insertion in intron 17 of *ATP6V0A4*, which also changes the relatively well conserved G at position +5 to A. Calculation of consensus values for similar mutations in previous studies suggests that exon skipping is highly likely, rather than cryptic splice site usage.[19] Indeed, identical intronic changes are reported to cause exon skipping in chronic granulomatous disease (+3 position)[20] and osteogenesis imperfecta (+5 position).[21] However, because of the tissue specificity of *ATP6V0A4* expression, we are unable to assess this effect directly in the absence of available kidney tissue from affected patients. A similar limitation applies to the identified intronic splice acceptor alterations, where aberrant splicing effects are less predictable.[19]

Some mutations identified in this study may in the future provide insight into a4 structure and function, which are presently unknown in mammals. For example, one patient (209-1) has a homozygous deletion of K236; this lysine is conserved across numerous species including yeast, suggesting that it may be a critical residue for the function, folding, or trafficking of this protein. In a similar vein, the R807Q substitution may be important, as yeast mutagenesis studies implicate the C-terminus in assembly and stability of the pump.[22]

This study confirms the association between *ATP6V1B1* mutations and rdRTA with deafness in childhood, and *ATP6V0A4* mutations with normal hearing (at least until young adulthood). Where hearing status was recorded in childhood in this cohort, none of the subjects with *ATP6V0A4* mutations had rdRTA associated SNHL, whereas all affected patients with *ATP6V1B1* mutations manifest SNHL in early life. With more recent clinical follow up, we have become aware that hearing loss may develop at an older age in a number of those with *ATP6V0A4* mutations, though it appears less severe in most cases. This led us to begin exploring the expression of *ATP6V0A4* in the human inner ear. Addressing this question is problematical as appropriate tissue samples are rarely available, and animal studies could be limited by species differences. However, while the results obtained from the small amounts of ex vivo human tissue we could assess must be regarded as preliminary, they clearly show that this gene is expressed by epithelia within the inner ear. It will require long term follow up to determine whether all those with *ATP6V0A4* mutations will eventually develop clinically significant hearing impairment.

These new findings raise the question of why mutations in different subunits of the same proton pump apparently have different effects on the severity of hearing loss. The lack of a severe auditory phenotype in patients with *ATP6V0A4* mutations could be simply explained if the ubiquitous a1 isoform encoded by *ATP6V0A1* (or another, as yet undiscovered, tissue specific isoform) were able largely to compensate for a4 function in the inner ear. Alternatively, α-intercalated cells may require an a4 function that is not as important in the ear. In terms of the polarisation of acid-base transporters and their functional roles, epithelial cells in the endolymphatic sac and cochlea have similar organisation to renal α-intercalated cells,[4 23] but molecular trafficking may differ at these sites. However, as the role of a4 within the proton pump complex has yet to be elucidated at either site, further studies will be required to understand the physiological basis for the differential effects of *ATP6V1B1* and *ATP6V0A4* mutations on the auditory system.

Such studies might have important clinical implications. Currently, rdRTA associated hearing loss is progressive and irreversible even when systemic alkali replacement that corrects the other biochemical abnormalities is provided.[24] Understanding the molecular mechanism of hearing loss in rdRTA may lead to new preventative or therapeutic options for patients with this disease.

With the larger cohorts of patients with *ATP6V1B1* and *ATP6V0A4* mutations available from this study, we were able to revisit the question of whether any differences in biochemical parameters exist between these two groups. We failed to find any significant differences in electrolytes, arterial pH, or urine pH at diagnosis, either in comparing the earlier studies of *ATP6V1B1* and *ATP6V0A4* or in the new cohort reported here. This indicates that, in contrast to their apparently distinct roles in the ear, both proteins probably influence intercalated cell function in a similar manner.

A comparison of age at diagnosis suggests that those with *ATP6V0A4* mutations were diagnosed at younger ages, which could reflect a more severe phenotype. However, this could also reflect ascertainment bias, differences in contact with the medical profession, or awareness of rdRTA among health care professionals in different societies.

In summary, these findings provide further evidence for genetic heterogeneity in rdRTA, extend the spectrum of disease causing mutations in *ATP6V1B1* and *ATP6V0A4*, confirm the association of *ATP6V1B1* with severe deafness in childhood, identify hearing loss as a feature associated with *ATP6V0A4* mutations, and show *ATP6V0A4* expression within the cochlea for the first time. The results will provide a focus for future structure-function correlations, prompt further assessment of proton pumps in the inner ear, and facilitate screens for new rdRTA genes.

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