Novel ATP6V1B1 and ATP6VOA4 mutations in autosomal recessive distal renal tubular acidosis with new evidence for hearing loss


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\(_h\) (head) and V\(_o\) (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 ATP6VOA4.\(^2\) \(^3\) (In May 2002, the official symbols for these genes were altered by the HUGO Gene Nomenclature Committee from ATP6BI 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. Descriptive 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 ATP6VOA4.

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 intertubular 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\(^*\) that proved to contain the novel ATP6VOA4 gene.\(^6\) ATP6VOA4 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

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

Importantly, some families with dRTA and deafness have been identified that exclude linkage to ATP6V1B1, and some with dRTA and normal hearing are not linked to ATP6V0A4, suggesting that there may be additional genes involved in dRTA. 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 unrelated Turkish and Saudi control subjects were analysed.

**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 [32P]-dCTP incorporation, under conditions previously described10,11 (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 program12,13 under models previously described.5

**Mutation detection**

Individual ATP6V1B1 and ATP6V0A4 exons and flanking intronic sequences were amplified from genomic DNA, using primers and conditions previously reported,14 with a mixture of AmpliTaq Gold® and PfuTurbo® DNA polymerases (Applied Biosystems and Stratagene) and 38-40 PCR cycles. Two 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). 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

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino acid alteration</th>
<th>% allele frequency cut/uncut</th>
<th>Enzyme</th>
<th>Product sizes before &amp; after digestion</th>
</tr>
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<tbody>
<tr>
<td>ATP6V1B1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>S465</td>
<td>138T&gt;C</td>
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<td>TspRI</td>
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<td>Intron 3</td>
<td>IVS3+117C&gt;A</td>
<td>46.54</td>
<td></td>
<td>HpyCH4IV</td>
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<td>481G&gt;A</td>
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<td>Avai</td>
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<td>ATP6V0A4</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Exon 4</td>
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<td>3C&gt;T</td>
<td>72.28</td>
<td>BglII</td>
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<tr>
<td>Exon 17</td>
<td>F554F</td>
<td>1662T&gt;C</td>
<td>68.32</td>
<td>XcmI</td>
</tr>
<tr>
<td>Exon 18</td>
<td>H604H</td>
<td>1812C&gt;T</td>
<td>68.32</td>
<td>BsaAI</td>
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</tbody>
</table>

*Conserved in all species except Neurospora.
†Not evolutionarily conserved.
were used in nested amplification of these segments of the ATP6V0A4 coding sequences that spanned intron 4 (forward: tttctgaaatggcttcgctggtctcagctattgctgacactacagcagagccagagccagtgg, reverse: aggattctctccagtgattcacac), introns 9-11 (forward: gttactgtggcagagcttta and reverse: gacgtg), thus providing internal controls for genomic DNA amplification.

For fetal cochlea RNA, 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, 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

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

<table>
<thead>
<tr>
<th>Kindred/sex</th>
<th>Age</th>
<th>Origin</th>
<th>Age at dx</th>
<th>Consang</th>
<th>RTA-associated SNHl</th>
<th>DNA</th>
<th>Mutation</th>
<th>Protein</th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
<th>HCO3</th>
<th>Arterial pH</th>
<th>Urine pH</th>
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</thead>
<tbody>
<tr>
<td>*203/F</td>
<td>15  y</td>
<td>Saudi</td>
<td>5 y</td>
<td>Y</td>
<td>1155-1156insC</td>
<td>I3865X441</td>
<td>136</td>
<td>3.4</td>
<td>112</td>
<td>16.0</td>
<td>NA</td>
<td>7.5</td>
<td></td>
<td></td>
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<tr>
<td>225/F</td>
<td>41 y</td>
<td>Morocco</td>
<td>2 mth</td>
<td>Y</td>
<td>1155-1156insC</td>
<td>I3865X441</td>
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<td></td>
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<td>I3865X441</td>
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<td>112</td>
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<td>7.3</td>
<td>8.0</td>
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<td>NA</td>
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<td>497delC</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
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<td>IVS5+1G&gt;A</td>
<td>Exon B skip?</td>
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<td>Macedonia</td>
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<td>L81P</td>
<td>137</td>
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<tr>
<td>223/M</td>
<td>10 y</td>
<td>Turkey</td>
<td>3 y</td>
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<td>R157C</td>
<td>135</td>
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<tr>
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<td>P346R</td>
<td>140</td>
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<tr>
<td>*204</td>
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<td>Y</td>
<td>1037C&gt;G</td>
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<tr>
<td>*205/M</td>
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<td>14 mth</td>
<td>Y</td>
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<td>P346R</td>
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<tbody>
<tr>
<td>201/M</td>
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<td>I3865X441</td>
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<td>3.0</td>
<td>113</td>
<td>13.6</td>
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<td>6 y</td>
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<td>Severe</td>
<td>[91C&gt;T + 823A&gt;C]</td>
<td>G123V</td>
<td>133</td>
<td>2.6</td>
<td>110</td>
<td>16.6</td>
<td>7.30</td>
<td>7.0</td>
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</tr>
<tr>
<td>201/M</td>
<td>7 y</td>
<td>Turkey</td>
<td>5 mth</td>
<td>N</td>
<td>1155-1156insC</td>
<td>I3865X441</td>
<td>138</td>
<td>3.0</td>
<td>113</td>
<td>13.6</td>
<td>7.22</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

TSNHl detected by auditory evoked responses in infancy, and/or not quantified.

†Standard criteria for hearing loss.

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

RT-PCR studies of ATP6VOA4 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-dT12-18 by standard methods. One tenth of each reverse transcription (RT) reaction was used as template for 36 cycles of amplification of separate segments of ATP6VOA4 coding sequences that spanned intron 4 (forward: ttgtgcctcttcgctggtctcagctattgctgacactacagcagagccagagccagtgg, reverse: aggattctctccagtgattcacac), introns 9-11 (forward: gttactgtggcagagcttta and reverse: gacgtg), thus providing internal controls for genomic DNA amplification.

For fetal cochlea RNA, 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 ATP6VOA4 or ATP6V1B1 (as positive control) using Platinum Taq (Invitrogen) according to the manufacturer's instructions, 20 cycles each round, 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 ATP6VOA4 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. GENE-HUNTER analysis (data not shown) and inspection of haplotypes were used to classify kindreds as probably linked to ATP6V1B1, ATP6VOA4, 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 ATP6VOA4. 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 ATP6VOA4 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. Other studies have concluded that DHPLC is superior in sensitivity to SSCP. Owing to parental consangunuity, most of the mutations were predicted to be homozgyous, 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

<table>
<thead>
<tr>
<th>Kindred/sex</th>
<th>Age</th>
<th>Origin</th>
<th>Disease</th>
<th>Consang</th>
<th>RNA-associated SNHL†</th>
<th>Mutation</th>
<th>Protein</th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
<th>HCO₃⁻</th>
<th>Arterial pH</th>
<th>Urine pH</th>
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<tbody>
<tr>
<td>76/F</td>
<td>6 mth</td>
<td>Turkey</td>
<td>NA</td>
<td>Y</td>
<td>1072C&gt;T</td>
<td>ATP6V0A4</td>
<td>NA</td>
<td>143.3</td>
<td>125.7</td>
<td>7.3</td>
<td>7.12</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
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<td>2 mth</td>
<td>Saudi</td>
<td>N</td>
<td>Y</td>
<td>2257C&gt;T</td>
<td>ATP6V0A4</td>
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<td>120.0</td>
<td>7.3</td>
<td>7.6</td>
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</tr>
</tbody>
</table>

... (continued)

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, of nine 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 homozgyously.

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
have normal audiograms when they were referred for study, in contrast to the $\text{ATP6V1B1}$ 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 $\text{ATP6V0A4}$ cohort and found that one (17-1) has developed mild SNHL at the age of 22.

Expression of $\text{ATP6V0A4}$ in inner ear epithelium

We therefore examined the expression of $\text{ATP6V0A4}$ 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 $\text{ATP6V1B1}$. There was clear evidence for $\text{ATP6V0A4}$ 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 $\text{ATP6V1B1}$ 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 $\text{ATP6V1B1}$ and could be excluded from linkage to $\text{ATP6V0A4}$ had an $\text{ATP6V1B1}$ mutation, and vice versa, and all kindreds showing evidence of potential linkage to both genes had a mutation in either $\text{ATP6V1B1}$ or $\text{ATP6V0A4}$. These data
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 these 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 rRTA 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 rRTA mutations and hearing loss. Both insertion and frameshift at position 1386 in ATP6V0A4, 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 1386 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 1386 were observed in three kindreds in our earlier study (one Swedish, two Spanish). It is also possible that 1386 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. 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. Indeed, identical intronic changes are reported to cause exon skipping in chronic granulomatous disease (+3 position) and osteogenesis imperfecta (+5 position). 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.

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 implicated this C-terminus in assembly and stability of the pump.

This study confirms the association between ATP6V1B1 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 rRTA 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 problematic 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 α1 isoform encoded by ATP6V0A1 (or another, as yet undiscovered, tissue specific isoform) were able largely to compensate for α4 function in the inner ear. Alternatively, α-intercalated cells may require an α4 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, but molecular trafficking may differ at these sites. However, as the role of α4 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 ATP6V0B1 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. 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 ATP6V0B1 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 ATP6V0B1 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 summary, these findings provide further evidence for genetic heterogeneity in rdRTA, extend the spectrum of disease causing mutations in ATP6V0B1 and ATP6V0A4, confirm the association of ATP6V0B1 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 valuable insight into the cell biology underlying cell function, prompt further assessment of proton pumps in the inner ear, and facilitate screenings for new rdtRA genes.

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