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Title: A novel, dominant disease mechanism of distal renal tubular acidosis with specific variants in *ATP6V1B1*

Authors

Myrte Daenen[1]*, Marguerite Hureaux[2]*, Emma Ashton[3], Francesca Becherucci[4], Marcus Benz[5], Anna Bjerre[6], Andrew Buckton[3] , Richard Caswell[7], Celia Duff-Farrier[7], Joseph Mcallister[8], Anna Moczulska[9] , Viviana Palazzo[4], Hitesh Prajapati[10] , Moin Saleem[11], Karl-Peter Schlingmann[12], Francisco Telma[13], Francesco Emma[14], Detlef Bockenhauer[1,15]

*: contributed equally

Affiliations

1) Paediatric nephrology, UZ Leuven and Department of Cellular and Molecular Physiology, KUL,Leuven, Belgium
2) XXX Hopital Europeen Georges Pompidou
3) XXX
4) Xxx
5) Xxx
6) Xxx
7) Exeter Genomics Laboratory, Royal Devon University Healthcare NHS Foundation Trust, Exeter, UK
8) Xxx
9) Xxx
10) Xxx
11) Xxx
12) Xxx
13) Xxx
14) xxx
15) Department of Renal Medicine, UCL and Paedaitric Nephrology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

Corresponding authors

Marguerite Hureaux
xxxx

Detlef Bockenhauer
Paediatric nephrology
UZ Leuven
Herestraat 48
3000 Leuven
Belgium
Detlef.bockenhauer@uzleuven.be

Abstract (297 of max 300 words)

Background and hypothesis: *ATP6V1B1* encodes a subunit of the vacuolar H^+-ATPase and pathogenic variants are associated with autosomal recessive distal renal tubular acidosis (dRTA) with deafness. Heterozygous variants predicted to affect a specific amino acid, Arg394, have been recurrently reported in dRTA but their significance has been unclear. We hypothesised that these variants are associated with a dominant disease mechanism.

Methods: Retrospective analysis of cases identified in our genetic laboratories and through European nephrology organisations. Data regarding demographics, clinical presentation, laboratory findings, hearing and imaging studies of kidneys were collected from the index patient and, if available, from other family members. The potential disease mechanism was investigated through structural modelling.

Results: Twenty index patients in total were included, of which 19 carried the variant c.1181G>A; p.(Arg394Gln) and one c.1180C>G; p.(Arg394Gly). In 7 families, more than one member was affected and the variant segregated with the disease in those with available information (13 affected, 6 unaffected), except for the unaffected mother of 2 affected children, who was mosaic. In no patient was a second causative variant *in trans* identified. In 8 sporadic patients and 1 affected parent, the variant was confirmed to be *de novo*. Both variants are absent in gnomAD. Sensorineural hearing loss was reported in 8 of the 22 patients with available information. Structural modelling supports a crucial role for Arg394 in nucleotide binding.

Conclusion: We provide strong evidence for the pathogenicity of heterozygous variants affecting Arg394 and thus a novel inheritance modus for *ATP6V1B1*-associated dRTA. Clinically, this form differs from the recessive one by the lower prevalence of hearing loss. The prominent position of Arg394 in the nucleotide binding fold of the H^+-ATPase structure is consistent with a dominant negative mechanism. Our findings inform the diagnosis and management of patients with dRTA and variants in Arg394.

Introduction

Distal renal tubular acidosis (dRTA) refers to the impairment of acid excretion by the α-intercalated cells in the collecting duct [1]. Primary dRTA can be caused by variants in *SLC4A1, ATP6V1B1, ATP6V0A4, WDR72* and *FOXI1* [2]. Except for *SLC4A1*, variants in which can cause autosomal dominant as well as recessive dRTA, all other genes are only recognised as recessive disease genes. *ATP6V1B1* was identified in 1999 as a causative gene for dRTA with sensorineural deafness (MIM#267300) [3]. Yet, sporadic cases with only heterozygous *de novo* variants predicted to change a specific amino acid, Arg394 (based on reference sequence NP_001683.2) have been recurrently reported, but their clinical significance has been unclear: were these simply cases in whom a second variant on the other allele had been missed or could these variants have a dominant negative effect so that they would be pathogenic in heterozygosity [4-8]. Accurate classification of identified variants is important as the finding of a causative variant allows a genetic diagnosis with all its benefits, such as precise genetic counselling and information about prognosis. In contrast, erroneous labelling of an identified variant as causative may establish an erroneous diagnosis and misinform patient management [9]. We therefore aimed to collect evidence for better assessment of the association between variants of Arg394 and dRTA.

Materials and methods

Patient identification

We retrospectively searched for subjects with a phenotype of dRTA and a variant in *ATP6V1B1* predicted to alter the amino acid arginine at position 394 (Arg394) through the records of the genetic testing laboratories in Paris (Hopital Europeen Georges Pompidou) and London (Great Ormond Street Hospital). In addition, a survey was sent through the working groups inherited kidney diseases and tubulopathies from the European Society for Paediatric Nephrology (ESPN), the European Renal Association (ERA) and the European Rare Kidney Disease Reference Network (ERKNet) to canvas members for further cases.

Sequencing of index patients was done according to the methods used at the respective clinical laboratories and included full sequencing of the coding and adjacent intronic sequences of *ATP6V1B1* and other dRTA disease genes.

Clinical information

Clinicians were asked to provide data regarding demographics (date of birth/ age at first presentation, height and weight), specific genetic variant information, clinical presentation (biochemistry of blood and urine), imaging studies of the kidneys (evidence of nephrocalcinosis, stones or cysts) and hearing studies on the index patient and, if available on family members. For subjects with missing or unclear data, clinicians were contacted again via e-mail and were asked to complete or verify their data.

Statistics

Statistical significance with regards to clinical severity was assessed using Chi-square (https://www.socscistatistics.com/tests/chisquare/default2.aspx) and a p-value <0.05 was considered significant.

Structural modelling

In silico analysis was performed in PDB 7u8q, the structure of porcine kidney V-ATPase in complex with SidK, in rotary state 2 [10]. The human and porcine B1 subunits share 94.5% amino acid identity overall, rising to 98.6% over the P-loop NTPase domain (residues 114-399) and 100% identity over residues 350-404, allowing the structure of the porcine protein to be used directly as a proxy for the human orthologue. The Arg394Gln missense variant was introduced by in silico mutagenesis following repair of PDB 7u8q using the FoldX modeling suite [11]. All structures were visualized in PyMOL (PyMOL Molecular Graphics System, Version 3.0.2, Schrödinger LLC; New York, NY, USA).

Results

Patients

Twenty index patients in total were included in this study, of which 13 were sporadic cases and seven had at least one additional affected family member. Clinical and biochemical details are presented in Table 1. In addition, eight family members carrying the Arg394 variant were identified (Figure 1).

Genetics

Nineteen of the index patients carried the variant c.1181G>A; p.(Arg394Gln) and only one (patient O1) the variant c.1180C>G; p.(Arg394Gly). Both variants are absent in gnomAD (https://gnomad.broadinstitute.org., accessed 03 July 2024)

All index patients had undergone full sequencing of *ATP6V1B1* and in none was a second causative variant *in trans* identified. In addition, all index patients had undergone panel testing of dRTA disease genes and no other causative variants had been identified. In ten patients (eight sporadic cases and one affected parent), the variant was confirmed to be *de novo*. Parents were not available for testing in the other cases. In the seven affected families, a total of thirteen additional family members (seven affected and six unaffected) were tested and the variant segregated with the disease in all, but one. This subject (I.2 in family D, Figure 1) was clinically unaffected. Genetic testing had been done by Sanger sequencing in this individual, which showed expression of the mutant allele at around 35%, indicative of mosaicism.

Clinical phenotype- acidosis

Most index patients presented in the first year of life with a median age at presentation of 6 months (Table 1). For patient O1, age at presentation could not be determined as she first presented to medical attention age 24 years, when she immigrated to Europe. At that time, she was acidotic and her height of 145 cm (-2.4SD) suggests that she had been affected already for a long time.

Median dose of alkaline supplementation for those with available data (N=14) at last follow-up was 2.3 mEq/kg/d.

Information on nephrocalcinosis was available for 22 patients and was present on ultrasound in 19 (86%).

Clinical phenotype- Sensorineural hearing loss

Sensorineural hearing loss (SNHL) was reported in 8 out of the 22 (36%) patients with available information. This is significantly different (p=0.000023) from the prevalence reported with recessive ATP6V1B1 disease (88%) in a large European cohort [12]. Moreover, two out of these 6 patients presented with hearing loss only at follow up appointments in adulthood and in Patient L1, SNHL was only unilateral, first noted at age 6.5y.

Evolutionary Conservation and structural modelling

Arg394 is located in the cytoplasmatic V1 domain, which is involved in adenosine triphosphate (ATP) binding and hydrolysis and thus provides the energy for the proton secretion into the tubular lumen. The amino acid is highly conserved with a VarSite conservation score of 0.95, with arginine present in 196/199 structural homologues. In the structure of the porcine V-ATPase, Arg394 has a prominent position in the nucleotide binding fold of the V1 domain, with

its positively-charged side chain oriented towards the surface of the binding pocket and forming hydrogen bonds to adenosine diphosphate (ADP) (Figure 2)

Discussion

Variant pathogenicity

We here provide strong evidence for a dominant disease mechanism for variants in *ATP6V1B1* predicted to affect Arg394. In total we have identified twenty index patients plus seven affected family members and in those with available information (N=16), the variant had either arisen *de novo* or was inherited in an apparent autosomal dominant fashion (Table 1 and Figure 1). In the seven investigated families, the variant segregated with the disease with but one exception, individual D1.2, where analysis of the electrophoretic traces indicates mosaicism. This sequencing was performed on DNA obtained from peripheral white blood cells and thus we do not know about expression of the variant allele in the intercalated cells in the collecting duct, but presumably they express the wild type.

All of the affected subjects carried the variant c.1181G>A (p.(Arg394Glu)), except for subject O1 (table 1) who carries the c.1180C>G (p.(Arg396Gly)), thus the genetic evidence for this latter variant is much weaker. However, given that it affects the same amino acid and also changes its charge, it is highly likely that it is associated with the same disease mechanism.

Disease mechanism

Considering that heterozygous predicted null variants (e.g. nonsense, frameshift or splice variants) in *ATP6V1B1* are not associated with dRTA, haplotype insufficiency can be excluded as a disease mechanism for the Arg394 variants, and a dominant negative mechanism appears most likely. The structure of porcine ATPase shows that Arg394 is involved in nucleotide binding, mediated both by hydrogen bonding and electrostatic interaction between the arginine sidechain and phosphate groups of the nucleotide, and may also be involved in

stabilisation of the transition state during ATP hydrolysis, as has been postulated from observations of the crystal structure of a bovine V-ATPase homologue [13]. During normal function, ATP hydrolysis proceeds around the V_1 head group, comprised of a trimer of A and B1 heterodimers, in a processive, unidirectional manner, driving rotation of the rotor and the membrane-embedded V_0 sub-complex which in turn mediates proton pumping (Figure 2) [10]. We postulate that variants at Arg394 result in proteins that can be incorporated into the V-ATPase complex but are unable to carry out ATP hydrolysis such that the complex becomes stalled, thus providing a molecular basis for the dominant negative impact of these substitutions.

Clinical phenotype

For most disease genes that can be inherited in either autosomal dominant or recessive fashion, the phenotype is typically milder in the dominant form, including for instance the dRTA disease gene *SLC4A1* [14]. However, with respect to the acidosis associated with the Arg394 variants, severity appears to be very similar to that reported with recessive variants in *ATP6V1B1*, when assessed using commonly used retrospective markers [5, 8, 12]: median (IQR) age at onset was 0.5 (0.3-1) years and thus very similar to that of 0.5 (0.1-1.9) years reported in the large European cohort that included 59 subjects with recessive *ATP6V1B1* disease [12]. Similarly, the median (IQR) amount of alkali prescribed in this cohort (2.3 mEq/kg/d) to treat the acidosis is very similar to that in the European recessive cohort of 1.7 (1.1-2.3) mEq/kg/d [12]. Of note. the severity of acidosis was variable, with one patient showing (GII.1) only borderline abnormalities when screened at the age of 2 years because of the family history of dRTA in the father, whereas others presented in the first few months of

life with severe acidosis. A similar variability has also been reported with recessive *ATP6V1B1*-associated dRTA [5, 8, 12].

In contrast, with regards to SNHL, the phenotype in this cohort is significantly milder than with recessive disease. *ATP6V1B1* was initially identified as the cause of "renal tubular acidosis with sensorineural deafness" and in large cohorts around 90% of patients are reported to have SHNL, which is typically severe, presenting in the first few years of life and treated with hearing aids and/or cochlear implants [3, 8, 12]. In contrast, in our cohort only 8 patients were reported to have SHNL, which was only noted in adulthood in two and unilateral in one. The use of hearing aids was reported in only 3 patients. While these results may have been affected by missing data (no data were available for 4 patients), there is no reason to assume that such bias would have affected this study more than the other retrospective cohort studies used for comparison here [8, 12]. This raises the obvious question of why there is such apparent discrepancy between kidney and inner ear manifestations when comparing dominant Arg394-associated disease with classical recessive dRTA caused by *ATP6V1B1* variants. While the subunit composition is not identical between the V-ATPases in kidney and inner ear, it is not clear why the presumed dominant negative effect of the Arg394 variants would have a differential impact in these tissues. Of course, it is possible that the markers used here to assess dRTA severity (age at diagnosis and alkali dosage) are too crude to detect subtle differences between dominant and recessive *ATP6V1B1*-associated manifestations in the kidney. Further investigations will be needed to get more clarity on this aspect.

Limitations

Our study has several limitations. Most notably, data collection was retrospective and incomplete in many patients, especially for adult subjects, where the data may date back decades and was no longer available. We also have no experimental data to assess the effect of the Arg394 variants on V-ATPase function, as there is no easily available model for the alpha-intercalated cells of human collecting duct [15].

Conclusions

In summary, we present strong genetic evidence for the pathogenicity of heterozygous variants of Arg394 in ATP6V1B1 and thus for an associated dominant inheritance. The observations from structural modelling are consistent with a dominant negative disease mechanism. These findings directly inform the diagnosis and management of patients with dRTA associated with such variant.

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Table 1: Clinical and biochemical features of patients with Arg394 variants in ATP6V1B1

Subject	Inheri-tance	Age at diagnosis [months]	K [mmol/L]	HCO_3 [mmol/L]	Urinary pH	Urinary Ca/Crea ratio [mol/mol]	Nephro-calcinosis	SNHL	Alkali treatment mEq/kg/day
AII.2 (index)	Dominant	4	7	13	N/A	1.5	Yes	Yes	1.8
AI.1 (father)	N/A	N/A	N/A	20*	N/A	N/A	N/A	No	0.5**
BII.1 (index)[%]	Dominant	6	2.6	13	N/A	N/A	Yes	No	1
BI.1 (father)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Yes	N/A
CIII.1 (index)	Dominant	6	2.6	13.6	N/A	N/A	Yes	No	N/A
CII.2 (mother)	*de novo*	N/A	N/A	N/A	N/A	N/A	Yes	No	N/A
DII.1 (index 1)	Dominant	4	2.3	12	N/A	N/A	Yes	Yes	1.1
DII.2 (index 2)	Dominant	2	1.7	<10	N/A	0.7	Yes	No	3.4
DLI.2 (mother)	mosaic					Clinically unaffected			
EII.1 (index)	Dominant	3	2	N/A	7	21	Yes	Yes (24y)	4.7
EI.2 (mother)[%]	N/A	48	N/A	N/A	N/A	N/A	Yes	No	1.9
FII.1 (index)	Dominant	20	3.8	13	N/A	N/A	Yes	No	2.4
FI.1 (father)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GI.1 (index)	N/A	N/A	N/A	N/A	N/A	N/A	Yes	N/A	0.1**
GII.1 (daughter)	Dominant	26	3.7	21.6	N/A	N/A	No	No	none
H1	De novo	26	3.3	12	7.6	3.22	Yes	No	2.6**
I1	De novo	5	3.1	12	8	1.9	Yes	No	6.9
J1	De novo	16	3	28.2	5	2	Yes	No	3.4
K1	De novo	3	2.7	13.6	7	11.57	Yes	No	3.3
L1	De novo	10	2.9	12.5	8	0.5	Yes (6.5y)	Yes (6.5y)[$]	2.2
M1	De novo	0.6	3.4	15	N/A	N/A	N/A	Yes	5-8
N1*	N/A	N/A	3.4	14	N/A	N/A	Yes	No	N/A
O1	N/A	12	0.8	12	N/A	N/A	N/A	N/A	N/A
P1	N/A	12	3.6	21	7	N/A	Yes	N/A	0.8**
Q1	N/A	6	2.7	11	N/A	N/A	No	No	N/A
R1	De novo	2.5	N/A	12.8	N/A	N/A	Yes	Yes	N/A
S1	N/A	1.2	1.7	16.3	N/A	N/A	No	N/A	N/A
T1	De novo	36	3.5	14.4	7.5	N/A	Yes	Yes (20y)	N/A

Table 1: Clinical and biochemical features of patients with Arg394 variants in ATP6V1B1

Shown are pertinent genetic and clinical features of the 19 index patients and variant-carrying relatives. Data are from 1st presentation, except otherwise indicated ($^{\%}$) and for alkali treatment dose, which is from last follow-up.

N/A: not available; *: patient carries variant c.1180C>G; p.(Arg394Gly); ** As a measured subject weight was not available, a weight of 70kg was assumed for calculation ; $: hearing loss is unilateral (right).

Figures

Figure 1: Pedigrees of familial cases with the p.(Arg394Gln) variant

A) Shown are the pedigrees of the 6 families with more than 1 member affected with dRTA associated with the p.(Arg394Gln) variant. Subjects that have been tested for the variant are denoted by “R394Q”, if they carry it and “WT” if they do not. Affected subjects are indicated with a filled symbol, unaffected with empty symbols. Note that the variant segregates with the disease, except for subject I.2 in family D, who was found to be mosaic for the variant.

B) Shown are the electrophoretic traces from individuals I.2 and II.1 of family D. Note that the “peaks” for guanine (black) and adenine (green) at position 1081 are at equal height in the affected son II.1, consistent with true heterozygosity, whereas the guanine peak is at lower (~35%) compared to adenine (~65%) in the unaffected mother I.2, indicative of mosaicism.

Figure 2: Arg394 is involved in nucleotide binding

A) Structure of porcine kidney V-ATPase in complex with SidK, in rotary state 2 (PDB 7u8q; Tan et al., 2022); subunits of the ATP6V1A-ATP6V1B1 heterohexamer, which form the V1 head group sub-complex, are shown as green and orange surfaces respectively, with a molecule of ADP bound at the closed interface; all other chains are shown in ribbon format, including the effector protein SidK (cyan); the grey box indicates the position of the vacuolar membrane, with orientation as indicated. B) Expanded view of the boxed region in A; proteins are shown in ribbon format with the sidechain of ATP6V1B1 Arg394 in stick format with carbon atoms coloured magenta; the ADP molecule is shown as space-filling spheres. C) Interaction of ATP6V1B1 Arg394 (magenta) with ADP at the closed interface, shown in the same orientation as in B but with the ATP6V1A subunit omitted for clarity; broken blue lines indicate hydrogen (H) bonds between the Arg394 sidechain and β-phosphate group of ADP, and internal H-bonds within the ADP ligand. D) As C, but rotated as shown. E) As D, but showing the predicted protein surface coloured by charge (blue, positive/basic; red, negative/acidic). F-H) As C-E but following introduction of the Arg394Gln variant by in silico mutagenesis; note the altered pattern of H-bonding to phosphate groups of ADP, and altered charge and topology in the phosphate binding pocket.