

Molecular Investigation of Distal Renal Tubular Acidosis in Tunisia, Evidence for Founder Mutations

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Background: Distal renal tubular acidosis (dRTA) is a rare genetic disease caused by mutations in different genes involved in the secretion of H⁺ ions in the intercalated cells of the collecting duct. Both autosomal dominant and recessive forms have been described; the latter is also associated with sensorineural hearing loss. **Methods:** Twenty-two Tunisian families were analyzed for mutations in the *ATP6V1B1* and *ATP6V0A4* genes by direct sequencing. Dating of the founder mutations was performed. **Results:** Two founder mutations in the *ATP6V1B1* gene were found in 16/27 dRTA cases. The p.Ile386Hisfs*56 founder mutation was estimated to be older than 2400 years and no correlations were found with deafness. For the remaining patients, two mutations in the *ATP6V0A4* gene, one of them being novel, were found in three Tunisian cases. The presence of a heterozygous missense mutation p.T30I, of the *ATP6V1B1* gene, was identified in six patients, while no mutations of the second gene were detected. No deleterious mutations of either *ATP6V1B1* or *ATP6V0A4* were found for the two probands. **Conclusion:** Our study gives evidence of phenotypic and genotypic heterogeneity of dRTA in the Tunisian population. Five different mutations were found, two of them were due to a founder effect, and screening of these mutations could provide a rapid and valuable tool for diagnosis of dRTA.

Introduction

DISTAL RENAL TUBULAR ACIDOSIS (dRTA) is a rare genetic disorder due to a defect in urinary acidification leading to alteration in the secretion of H⁺ by the intercalated cells in the collecting duct. It is clinically characterized by severe hyperchloremic hypokalemic metabolic acidosis, prominent renal tract calcification, and rickets (Halperin *et al.*, 1985; Rodriguez-Soriano, 2000). Primary dRTA is inherited either as an autosomal dominant or autosomal recessive trait (OMIM 179800, 267300, and 602722). The autosomal dominant forms have been linked to the *SLC4A1* gene encoding the basolateral membrane protein anion exchanger 1 (AE1) (Bruce *et al.*, 1997). The autosomal recessive forms have been associated with mutations in the *ATP6V1B1* gene in individuals with sensorineural hearing loss (SNHL) (Smith *et al.*, 2000) or with mutations in the

ATP6V0A4 gene in individuals either with late-onset SNHL or normal hearing (Smith *et al.*, 2000; Stover *et al.*, 2002). Nevertheless, several cases of *ATP6V1B1* mutation without SNHL have been described (Karet *et al.*, 1999; Vargas-Poussou *et al.*, 2006). Except for the hearing status, the two forms of recessive dRTA appear to be clinically similar.

The *ATP6V1B1* gene encodes for the B1 subunit of the H⁺ ATPase; it is expressed by interdental cells and endolymphatic sac epithelia, accounting for the associated hearing impairment (Stover *et al.*, 2002). The *ATP6V0A4* gene located in the 7q33-34 region has 23 exons, of which 20 encode the 840 amino acid transmembrane a4 subunit of the H⁺ ATPase. Recent studies demonstrated that several families with dRTA and deafness excluded 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 (Stover *et al.*, 2002; Vargas-Poussou *et al.*, 2006).

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A previous study shows the presence of the c.175-1 G>C and p.Ile386Hisfs*56 mutations in Tunisian and North African patients with dRTA, which may be due to a founder effect (Vargas-Poussou *et al.*, 2006). In our study, we report clinical and genetic investigation of dRTA in 22 Tunisian families. We determined the frequency of previously reported mutations and discuss the implications for molecular diagnosis of dRTA in North Africa. We aimed also to demonstrate the genetic link, thus identifying a common ancestor and to establish the age of the mutation.

Materials and Methods

Patients

We studied 27 probands from 22 families for whom dRTA diagnosis was established (Table 1). The majority of the cases were from the Center of Tunisia (15 families), 4 families from the North and 2 from the South. Clinical diagnosis was based on the presence of the following criteria: metabolic acidosis with a normal anion gap and urinary pH > 5.5 in a context of acidosis, hypercalciuria, and/or nephrocalcinosis. Additional optional criteria were hypokalemia, hypocitraturia, polyuria, and failure to thrive. Hearing was assessed by pure tone audiometry and/or auditory evoked responses. The SNHL was observed in 11 patients with different ages of onset.

Informed consent was obtained for blood collection from the venous puncture and genetic analysis from each subject. This work was held according to the Declaration of Helsinki.

DNA sequence and haplotype analysis of the *ATP6V1B1* and *ATP6V0A4* genes

After obtaining written informed consent, genomic DNA was extracted from peripheral blood leucocytes by salting-out procedures. The mutation screening was performed by direct sequencing of corresponding polymerase chain reaction (PCR) products. Primer sequences are available upon request. PCR products were purified on a QIAquick gel extraction purification kit (Qiagen, Hilden, Germany) and sequenced using the Big Dye terminator Kit (Applied Biosystems, Foster City, CA) and by using one of the PCR primers on an ABI prism 3130 *xl* DNA sequencer (Applied Biosystems) in accordance with the manufacturer's recommendations.

The sequences were aligned against the reference sequence (*ATP6V1B1* NG_008016.1 and *ATP6V0A4* NG_008145.1) using BioEdit software (Hall, 1999).

To confirm the novel mutation (c.1345 C>T; p.R449C) detected by sequencing, allele-specific oligonucleotides (ASO) were designed to perform an ASO-PCR screening for one of the mutations. The DNA samples from 50 blood donors were analyzed by an ASO-PCR assay for the T nucleotide at the position 1345. Primers used to amplify the mutant sequence were (PCR conditions: by request) *ATP6V0A4-EX14-R* (5'-CCCCAACCATGAAAACAGTC-3') and *ATP6V0A4-EX14-F-ASO1345-m* (5'-ACACCTTCTTCCACGGGT-3').

Primers used to confirm the presence of the normal sequence were ASO1345-S and *ATP6V0A4-EX14-R* (5'-GTGCAAAGGAAGGAAGAGCTTC-3').

For haplotype analysis, one patient and his/her parents were chosen per family, who clearly had the founder muta-

tion (c.1155dup C; p.Ile386Hisfs*56) in the *ATP6V1B1* gene. Five polymorphic microsatellite markers flanking the *ATP6V1B1* gene at a distance ranging from 72.12 Mb 3' to 70.52 Mb 5' were selected and analyzed at 3130*xl* automated sequencer, as shown in Table 2. Data were analyzed using ABI GeneMapper software v 3.2.

Dating the origin of I385fsX441 mutation

To estimate the age of the Tunisian *ATP6V1B1* p.Ile386Hisfs*56 mutation, two approaches were applied. The first method implemented using the formula described by Risch *et al.*: generations = $\log \delta / \log (1 - \theta)$ (Risch *et al.*, 1995; Colombo, 2000).

The genetic distance between the mutation and the markers was calculated by linear regression analysis of genetic versus the physical map position using the eight markers (D2S136, D2S1336, D2S2152, D2S292, D2S2977, D2S2109, D2S1394, and D2S2116) in proximity to the *ATP6V1B1* gene with known genetic and physical distances (Marshfield map; UniSTS—NCBI) and Mb (Ensembl).

Kosambi function was applied to convert the genetic map distance (cM) into a recombination fraction (θ) (Devlin and Risch, 1995). The linkage disequilibrium (LD or δ) index was calculated using the Bengtsson and Thomson (1981) formula: $\delta = (P_d - P_n) / (1 - P_n)$, where P_d and P_n are the frequencies for the marker allele on mutation-bearing and normal chromosomes, respectively. Allele frequencies in unrelated normal chromosomes were found by genotyping 30 unrelated individuals of the general population.

To verify the accuracy of our approximation with the methods presented above, we also used the DMLE+2.3 program, (www.dmle.org) (Reeve and Rannala, 2002), for re-estimating the age of the founder mutation by using data from five microsatellites examined in 10 cases presenting the founder mutation and 30 controls.

This program was designed for high-resolution mapping of disease mutations and for estimating their age. It is based on the observed LD between a mutation and linked markers tested in DNA samples of unrelated affected individuals and controls (Rannala and Reeve, 2001; Reeve and Rannala, 2002). The parameters used were as follows: genotypes of microsatellites in patients of the p.Ile386Hisfs*56 mutation and control chromosomes; chromosome map distances. For the population growth rate, we considered data on the population size from the National Institute of Statistics, Tunisia (INST, www.ins.nat.tn).

The population growth rate (r) was estimated using formula $p_1 = p_0 \times r^g$. In this study, g is the number of generations, p_1 and p_0 are the population sizes at different times, and the growth rate is e^r -fold per generation. From INST, in the year 2011, there were 10.6738 million Tunisian individuals and there were 1.1 million in the year 1860 (Seklani, 1974). Therefore, the growth rate was ~ 1.122 -fold per generation (assuming 20 years per generation) over this period.

The proportion of population sampled is $0.000431 = 23/53369$. This estimate is based on the assumption that 0.5% of the Tunisian population carries the p.Ile386Hisfs*56 mutation, and hence, $10.6738 \text{ million} \times 0.5\%$ is 53369. There were 12 cases identified, 11 of whom were homozygous for the mutation.

TABLE 1. CLINICAL AND BIOCHEMICAL FEATURES AT DIAGNOSIS OF THE PATIENTS WITH DISTAL RENAL TUBULAR ACIDOSIS

Family	Patients	Age	Origin	Age at diagnosis	Gender	Consanguinity	SNHL	Blood			K ⁺ (mmol/l)	Urine pH	Nephrocalcinosis	Identified mutation	
								pH	HCO ₃ ⁻	pH				ATP6V1B1 gene	ATP6V0A4 gene
dRTA-1	dRTA-1-1	14 months	Kasserine	14 months	M	1°	Yes	7.23	9.5	3	8	Yes		p.R6X (HMZ)	
	dRTA-1-2	3 months		3 months	M	1°	Yes	7.18	9.5	1.7	8.5	Yes		p.R6X (HMZ)	
dRTA-2	dRTA-2-1	6 months	Nfidha	6 months	M	No	No	7.48	18.1	5.2	6	Yes	NF	NF	
dRTA-3	dRTA-3-1	4 years	Sousse	2 months	M	3°	No	7.29	13.3	3	7.8	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-4	dRTA-4-1	4 years	Nfidha	6 months	M	2°	No	7.28	12.2	2.6	8	Yes	p.Ile386Hisfs*56 (HMZ)		
	dRTA-4-2	3 months		4 months	F	2°	Yes	7.32	11.3	1.7	7	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-5	dRTA-5-1	3 months	Kairouan	3 months	M	2°	Yes	7.34	9.5	2.8	7.5	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-6	dRTA-6-1	3 months	Mahres	18 days	M	1°	ND	7.23	8.2	4.1	6	Yes	c.175-1G>C (HTZ)		
dRTA-7	dRTA-7-1	6 months	Djbeniana	4 months	F	1°	No	ND	15	5.2	7	No	p.T30I (HTZ)		
dRTA-9	dRTA-9-1	1 year	Sfax	25 days	M	1°	No	7.22	6.4	3.1	6.5	Yes	—	p.R449C (HMZ)	
dRTA-10	dRTA-10-1	4 years	Khmiss	3 months	M	1°	Yes	7.19	10.9	3.21	7.6	Yes	c.175-1G>C (HMZ)		
dRTA-11	dRTA-11-1	Died	Sfax	6 months	M	1°	ND	ND	ND	ND	ND	ND		p.Ile386Hisfs*56 (HMZ)	
dRTA-13	dRTA-13-1	13 years	Kebili	4 months	F	2°	Yes	7.30	14.4	3.2	7	Yes	c.175-1G>C (HMZ)		
dRTA-14	dRTA-14-1	2 years	Sousse	7 months	M	No	No	7.38	10	4	6	Yes	NF	NF	
dRTA-15	dRTA-15-1	5 years	Fahes	2 month	F	1°	Yes	7.30	12.3	2.75	7.5	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-17	dRTA-17-1	5 years	Khmiss	6 months	F	No	Yes	7.34	15.8	3.8	7	Yes	c.175-1G>C (HMZ)		
dRTA-18	dRTA-18-1	1 year	ND	2 months	M	No	No	ND	ND	ND	ND	Yes	p.T30I/— (HTZ)		
dRTA-19	dRTA-19-1	6 years	Sousse	1 month	M	2°	Yes	7.35	14	2.5	6.5	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-20	dRTA-20-1	1 year	Bir Ali	1 day	M	1°	No	7.44	20	5.3	7.5	Lithiasis	p.Ile386Hisfs*56 (HMZ)		
dRTA-21	dRTA-21-1	1 month	Gabes	1 month	M	1°	Yes	7.30	16.6	2.4	7.5	Yes	p.Ile386Hisfs*56 (HMZ)		
dRTA-22	dRTA-22-1	1.5 years	Boumerdes-Mahdia	1 month	M	2°	No	7.10	12	2.1	7.8	Yes (with urolithiasis)	p.Ile386Hisfs*56/p.T30I		
dRTA-23	dRTA-23-1	15 years	Braadaa-Mahdia	4 months	M	1°	No	7.30	18.1	4.2	6.7	Lithiasis	p.T30I (HTZ)		
	dRTA-23-2	12 years		1 month	F	1°	Yes	7.40	18.3	4.5	7	Lithiasis	p.T30I (HTZ)		
	dRTA-23-3	7 years		1 month	F	1°	No	7.41	20.3	ND	6	Lithiasis	p.T30I (HTZ)		
dRTA-24	dRTA-24-1	2 years	Kef	6 months	M	1°	ND	7.25	13.1	3.1	8	Medullar	—	p.R6X (HMZ)	
dRTA-25	dRTA-25-1	1 year	Bouselem-Jendouba	5 months	M	1°	Yes	7.12	5.7	2.8	6	No	p.Ile386Hisfs*56 (HMZ)		
	dRTA-25-2	Fœtus		—	—	—	—	—	—	—	—	—	—	p.Ile386Hisfs*56 (HMZ)	

dRTA, distal renal tubular acidosis; F, female; HMZ, homozygous; HTZ, heterozygous; M, male; ND, not done; NF, not found; SNHL, sensorineural hearing loss.

TABLE 2. HAPLOTYPE ANALYSIS OF FAMILIES LINKED TO THE *ATP6V1B1* GENE WITH P.ILE386HISFS*56 MUTATION

Marker	<i>D2S2113</i>	<i>D2S443</i>	<i>D2S2604</i>	<i>ATP6V1B1</i>	<i>D2S1581E</i>	<i>D2S291</i>	<i>D2S2977</i>	
Physical position (Mb) ^a	70.52 Mb	70.79 Mb	70.95 Mb	71.19 Mb	71.37 Mb	71.93 Mb	72.12 Mb	
Families with p.Ile386Hisfs*56 founder mutation	dRTA-3-1	200	236	246	p.Ile386Hisfs*56	528	186	232
		200	236	246	p.Ile386Hisfs*56	528	186	232
	dRTA-4-1	198	236	246	p.Ile386Hisfs*56	528	186	236
		198	236	246	p.Ile386Hisfs*56	528	186	236
	dRTA-5-1	198	236	246	p.Ile386Hisfs*56	528	186	232
		198	236	246	p.Ile386Hisfs*56	528	186	232
	dRTA-11-1	198	246	246	p.Ile386Hisfs*56	528	186	236
		198	246	246	p.Ile386Hisfs*56	528	186	232
	dRTA-15-1	190	224	246	p.Ile386Hisfs*56	528	186	204
		190	224	246	p.Ile386Hisfs*56	528	186	204
	dRTA-19-1	202	236	246	p.Ile386Hisfs*56	528	186	232
		202	236	246	p.Ile386Hisfs*56	528	186	232
dRTA-20-1	192	240	246	p.Ile386Hisfs*56	528	192	224	
	192	228	246	p.Ile386Hisfs*56	528	180	212	
dRTA-21-1	200	236	246	p.Ile386Hisfs*56	528	186	232	
	200	236	246	p.Ile386Hisfs*56	528	186	232	
dRTA-25-1	198	236	246	p.Ile386Hisfs*56	528	186	236	
	198	236	246	p.Ile386Hisfs*56	528	186	236	
dRTA-22-1	200	236	246	p.Ile386Hisfs*56	528	186	232	
	188	244	230	-	528	184	228	
θ		0.00814	0.00188	-	0.00243	0.00796	0.00796	
δ		0.5495				0.8804	0.5338	
G		129.019					136.078	

The parts of the ancestral haplotype shared homozygously by all patients are indicated in bold and those shared by a subset of patients are indicated in italics.

^aPhysical position in Mb obtained from www.ncbi.nlm.nih.gov

Gray Shade: common haplotypes shared between patients.

θ: is the recombination rate between *ATP6V1B1* and the markers; δ: The linkage disequilibrium index was calculated using the Bengtsson and Thomson formula (1981) (see Materials and Methods section) and G: is number of generation.

Results

Genetic screening

On the basis of family history and clinical presentation, 22 families were classified as having autosomal recessive dRTA. Their clinical and biologic characteristics are reported in Table 1. Most of these families were of Central Tunisian origin, 17 of the 22 families were consanguineous. Taking into account the clinical features, early age of onset, and consanguinity profile of our patients, as well as SNHL comorbidity, we have chosen to first screen for mutations in the *ATP6V1B1* gene in all patients. Previously reported mutations and novel ones were detected in 22 patients. The studied variants were found in a homozygous state in 14 probands and in a heterozygous state in six patients (Table 1).

The *ATP6V1B1*-p.Ile386Hisfs*56 mutation was identified in 11 consanguineous patients in a homozygous state and in one patient from family 22 in a heterozygous state (Table 1). This insertion leads to a frame shift mutation and a premature translation termination of the encoded protein at position 441 (p.Ile386Hisfs*56). Six patients had a bilateral hearing loss, which manifested itself very early (3 months on average), and one of these patients (dRTA-11) died at the age of 6 months of severe hypotrophy and stage III dehydration.

Direct sequencing of the *ATP6V1B1* gene also revealed the presence of the splice site mutation in intron 2 (c.175-1 G>C) in four patients. Three of them were homozygous and one was heterozygous (Table 1).

In our cohort, the c.89 C>T (p.T30I) mutation was detected always in a heterozygous state in four patients and this variation is in LD with two polymorphisms, c.89C>T (rs17720303) and c.1002C>T (p.R334R) (rs2072462), in the promoter region and the exon 10, respectively. Direct sequencing for these patients shows no other variation for the remaining exons of the *ATP6V1B1* gene.

No mutation in the *ATP6V1B1* gene was found in six patients from families 1, 2, 9, 14, and 24. For these patients, analysis of the nucleotide sequence of the entire coding region and intron-exon junctions of the *ATP6V0A4* gene from 6 unrelated index cases revealed the presence of two different mutations, one of them is novel (Table 1).

The reported frame shift mutation in exon 3 (c.16 C>T, p.R6X) was found in three patients at the homozygous state, both parents were heterozygous. One patient (dRTA-9) presents a novel missense mutation c.1345 C>T (p.R449C) in exon 13, caused by a cytosine to thymine substitution at position 1345 of the coding sequence of the *ATP6V0A4* gene (Fig. 1). At this position, the nucleotide and the amino acid

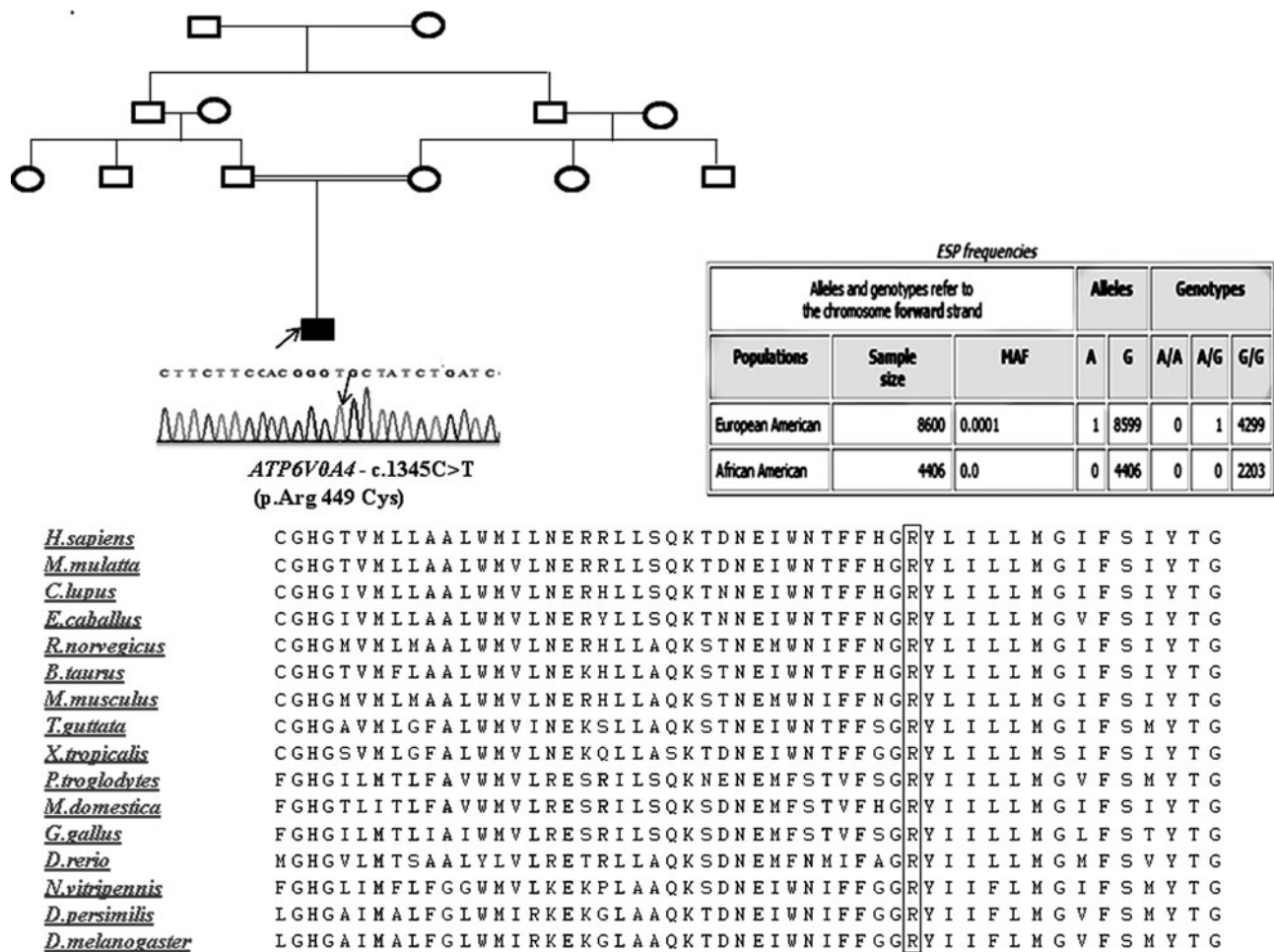


FIG. 1. Pedigree for the Tunisian family dRTA-9 with the novel mutation c.1345 C>T in the *ATP6V0A4* gene. White circles, unaffected females; white squares, unaffected males; black symbols denote affected individuals; conserved amino acid “R” in all species is outlined.

are highly conserved (phyloP score: 6.18 [−14.1; 6.4]) (Pollard *et al.*, 2010). The physicochemical characteristics show a large difference between arginine and cystidine (Grantham’s distance: 180 [0–215]).

Bioinformatics analysis with Align GVGD (<http://agvvd.iarc.fr/>), SIFT (<http://sift.jcvi.org/>), and Mutation Taster (www.mutationtaster.org) confirms the deleterious effect of this variation (GV: 353.86—GD: 0.00, SIFT: score: 0.00, median: 3.11, and Mutation Taster shows a *p*-value: 1.0) (Fig. 1).

ASO-PCR with specific oligonucleotides for the normal C and mutant T sequences was designed to investigate the possibility of the p.R449C variant being a polymorphism rather than a mutation. The analysis of 100 control chromosomes showed the absence of the T variant sequence in the Tunisian population.

In addition to the disease-causing mutations, our genomic sequencing revealed the presence of two previously reported neutral polymorphisms in exons 2 (c.138 C>T, p.S46S) and 10 (c.1002 C>T, p.R334R) and four variations found in the promoter region and the exon 1 (c-89A>G, c-34C>T, c.2T>C (p.M1T), and c.27T>G), one of them c-155A>G has not been described before.

Two nonconsanguineous patients (dRTA-2 and dRTA-14) who are clinically confirmed for the dRTA without deafness

showed no variation in the exons and the exon–intron junctions in the two genes responsible for recessive form dRTA.

Our study showed that c.175-1G>C and p.Ile386Hisfs*56 mutations, in the *ATP6V1B1* gene, represent more than 55% of deleterious alleles. These two variations were usually found in the central region of Tunisia. However, the *ATP6V0A4*- p.R6X mutation (11.11%) was only found in the northwest region.

Founder effect and mutation dating

On the basis of the similar geographic origin of the patients, we hypothesized that there might be a founder effect for the p.Ile386Hisfs*56 mutation. We performed genotyping analysis of five microsatellite markers covering the shared region in all homozygous and compound heterozygous affected individuals (10 families), as well as 30 healthy control Tunisians.

A common DNA haplotype, namely, 236-246-186-232 at D2S443, D2S2604, D2S291, and D2S2977, respectively, was identified among mutated patients. Haplotypes of all control individuals were distinct from the ones associated to p.Ile386Hisfs*56 with statistically significant differences in allele frequencies of markers flanking the *ATP6V1B1* gene (Table 2).

TABLE 3. ALLELE FREQUENCIES IN CONTROLS AND dRTA PATIENTS CARRYING *ATP6V1B1*-p.Ile386Hisfs*56 MUTATION

Markers	Shared allele	Frequencies of shared allele (%) ^a		χ^2	df	p-Value
		Controls	Patients			
D2S2113	198	11.66	42.10	6.83	1	0.009
D2S443	236	21.66	68.42	14.29	1	0.000158
D2S2604	246	43.33	100	16.66	1	0.000045
D2S291	186	1.66	89.47	58.35	1	<10 ⁻⁷
D2S2977	232	15.00	52.63	11.19	1	0.000828

^aFrequency of the shared allele based on 30 matched Tunisian control individuals and 10 patients. df, degree of freedom.

The allele frequencies of markers ranging from the D2S2113 to D2S2977 of control subjects and patients carrying the p.Ile386Hisfs*56 mutation were significantly different ($p < 0.05$), which is consistent with a founder effect for the p.Ile386Hisfs*56 mutation (Table 3).

To date, the p.Ile386Hisfs*56 mutation, we used the D2S443 (5' of haplotype) and D2S2977 (3' of haplotype) with disease allele frequencies 0.2167 and 0.1167, respectively, among 30 healthy unrelated samples. Using linear regression, the p.Ile386Hisfs*56 mutation was estimated to be at position 89.025 cM.

Applying the Kosambi function and the LD equation by Bengtsson and Thomson (Risch *et al.*, 1995), we found the following values: D2S443-p.Ile386Hisfs*56: $\theta = 0.00814$, $\delta = 0.5495$ and p.Ile386Hisfs*56-D2S2977: $\theta = 0.00796$, $\delta = 0.5338$.

Using these data, the mutation is dated to be 129 generations old based on marker D2S443 and 136 generations old based on marker D2S2977. Assuming an average generation

length of 20 years and an average age of 2650 years, we can place the possible time period of the p.Ile386Hisfs*56 origin between 706 and 566 BC.

To calculate the posterior probability of the time of onset of the p.Ile386Hisfs*56 mutation, the Bayesian method for multipoint LD mapping incorporated in the DMLE+2.3 program was applied to the Tunisian haplotype frequency data for the 5 microsatellite markers.

The estimated age of p.Ile386Hisfs*56, assuming a growth rate of 1.122 per generation, was 102 to 139.5 generations and on an average of 116.5 generations (95% CI: 2040–2790 years, assuming a 20-year generation time) (Fig. 2).

Discussion

Our study deals with molecular and genetic investigation of dRTA in 22 Tunisian families, including 27 affected children. Our results showed that both c.175-1G>C and p.Ile386Hisfs*56 mutations were the most frequent (55.55% of tested patients).

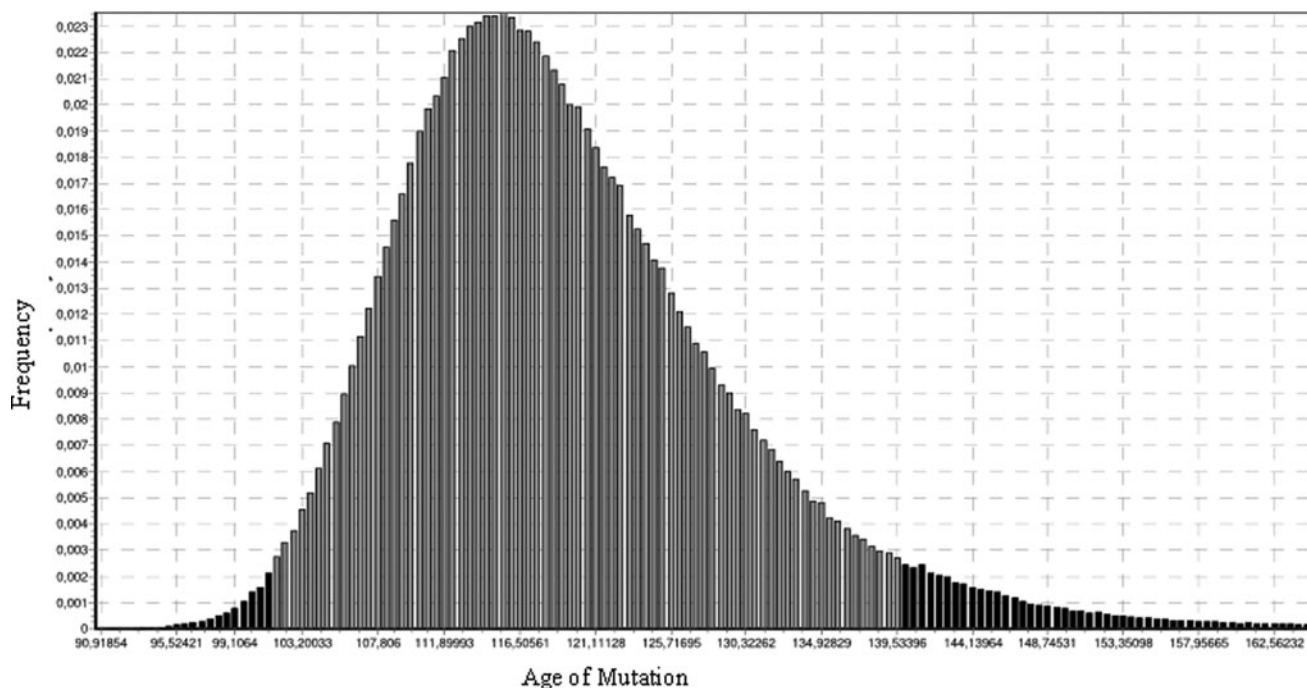


FIG. 2. Age estimates for the p.Ile386Hisfs*56 mutation. The posterior probability distribution plots of the mutation age (in generations), as estimated by the software DMLE+2.3, are shown when the population growth rate of 1.112-fold per generation (20 years) is assumed. Black and dark gray lines represent the average 95% credible set of values for posterior density.

The c.175-1 G > C mutation was identified in four families (13%). Three of them originated from the central east and one from the southeast of Tunisia.

The splice site mutation in intron 2 of the *ATP6V1B1* gene causes a loss of the splice acceptor site of exon 3 (Vargas-Poussou *et al.*, 2006), probably resulting in unstable mRNA or truncated protein (Mohebbi *et al.*, 2013).

This mutation was also identified in two Tunisian and Algerian patients with different haplotypes (Vargas-Poussou *et al.*, 2006, 2008). Genotyping analysis for each patient carrying the c.175-1 G > C mutation shows that these patients shared the same haplotype, suggesting a possible founder effect in the Tunisian population.

In addition, the p.Ile386Hisfs*56 mutation has been identified in 10 families (42.59%): Six originated from East-Central Tunisia, two from the north and one from the southeast of Tunisia (Table 1). This mutation was also identified in Tunisian, Algerian, and Moroccan patients with different haplotypes (Vargas-Poussou *et al.*, 2006). The presence of the same mutation on different haplotypes confirms that this mutation could be explained by recurrence or by an ancient founder mutation. Indeed, the corresponding haplotype diverged by the subsequent recombination events. In our study, we demonstrated that the *ATP6V1B1*_p.Ile386Hisfs*56 mutation shared by 10 families with dRTA is due to a common ancestor in Tunisia.

One patient (dRTA_22-1) inherited this mutation in a compound heterozygous state with the p.T30I mutation in the *ATP6V1B1* gene. This variation (p.T30I) was identified in a heterozygous state in five other patients. The second altered allele was not detected in *ATP6V1B1* and no mutation was found in the *ATP6VOA4* gene. An impact of p.T30I on the disease expression is still to be confirmed. Indeed, a study done in the United Kingdom on patients from different origins (Saudi Arabian, Turkish, and Spanish) showed that this variant is not pathogenic; indeed, it was detected in 23% of controls (Stover *et al.*, 2002). Nevertheless, another study considers this variation as deleterious (Mohebbi *et al.*, 2013). Sequencing of all exons of both the *ATP6V1B1* and *ATP6VOA4* genes in our patients, in particular those with the p.T30I variation, allowed the identification of several polymorphisms in *ATP6V1B1*. The interaction between these polymorphisms might act as a modifier that changes a low-penetrant allele into a deleterious one. This situation has been already reported for Stargardt disease (Valverde *et al.*, 2006).

Direct sequencing of the *ATP6VOA4* gene for the remaining patients shows the presence of the p.R6X mutation at the homozygous state in three patients from two consanguineous families from the same geographic region; it was also found in two sisters from Pakistan (Vargas-Poussou *et al.*, 2006).

The fact that the mutation was shared between Tunisian and Pakistani patients could be due to a founder effect, as this has already been reported in other genetic diseases such as deafness due to the p.R34X mutation in the *TMC1* gene. This could be explained by the history of settlement of the two countries, which share in common a migratory wave of Hadramaout population during the 7th century (Hussain, 2005; Ben Said *et al.*, 2010).

Two nonconsanguineous patients (dRTA-2 and dRTA-14), showed no deleterious variation in the two genes responsible for the recessive form of dRTA. This result may be explained by the existence of another variation in the promoter region or in another gene.

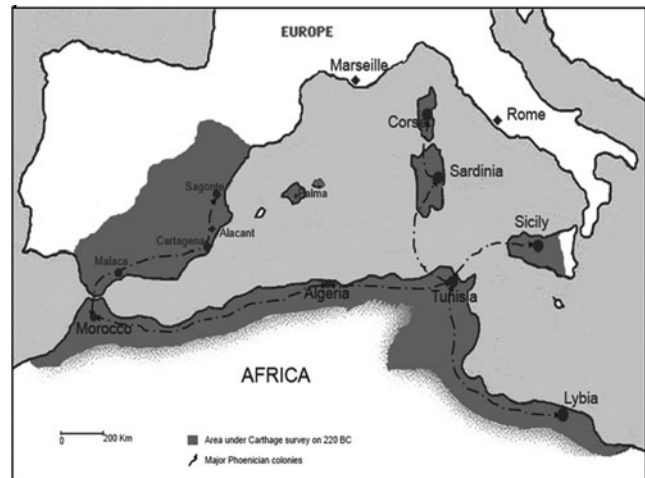


FIG. 3. Geographical extent of the Phoenician Empire around 220 BC. Source: Adapted from www.awesomestories.com

Haplotype analysis in cases carrying the p.Ile386Hisfs*56 mutation showed that 10 patients carried the same ancestral haplotype, this strongly suggesting a founder effect (Table 2). These patients that shared the ancestral haplotype are from different villages in the east of Tunisia, near the coast in general, with a higher frequency in the central part of the country. This strong geographic clustering in the formerly isolated population and the haplotype sharing is a very strong indication of the founder effect.

By performing mutation dating through two different methods, mutation p.Ile386Hisfs*56 dated between 636–316 BC, during a period when the Tunisian territory was mainly populated by autochthonous Amazigh (Berber) and Phoenicians (814–146 BC) (Fig. 3) who occupied the coastal areas of the country rather than the inland (Ibn_Khaldoun, 1925).

This founder mutation was previously described in families from North African populations like Algeria and Morocco who shared the same genetic background and historical invasions as Tunisia (Hoefler, 1865). This may support the hypothesis of an Amazigh origin of the described variant. However, the p.Ile386Hisfs*56 variant is also present in some populations on the northern side of the Mediterranean Sea like Sicily and Spain (Karet *et al.*, 1999; Stover *et al.*, 2002). Taking into account this geographic distribution and history of this era, the hypothesis of a Phoenician origin is more likely. Indeed, the genetic exchanges and migration of individuals around the Mediterranean Sea during the Phoenician period might account for the transfer of the p.Ile386Hisfs*56 mutation from Tunisia to other countries from North Africa and Southern Europe. The p.Ile386Hisfs*56 mutation may have been further dispersed even in Northern Europe through prospecting expeditions and trade exchanges of Phoenician explorers (Hoefler, 1865; Zalloua *et al.*, 2008).

In conclusion, our findings provide further evidence for genetic heterogeneity in dRTA. Indeed, five different mutations were found, two of them, c.175-1G > C and p.Ile386Hisfs*56, accounted for more than 55% of disease-causing mutations in our cohort. Identification of the mutation spectrum of dRTA allowed multiple genetic diagnostic tests, including genetic counseling and prenatal diagnosis. Based on common history

of settlement, the reported mutations should be prioritized in mutation screening in North Africa and the Middle East.

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