RESEARCH NOTE

A novel splice-site mutation in *ATP6V0A4* gene in two brothers with distal renal tubular acidosis from a consanguineous Tunisian family

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Introduction

Primary distal renal tubular acidosis (dRTA) is a genetically heterogeneous rare disease caused by an impaired excretion of hydrogen ions (H^+) by intercalated cells in the collecting ducts (Alper 2010a; Batlle and Haque 2012). The H^+ secretion, which is required for the final urinary excretion of fixed acids, is impaired. Clinical and biological features include hyperchloremic acidosis with inappropriately alkaline urine, hypercalciuria, nephrolithiasis and/or nephrocalcinosis were found in the patients. Affected infants are presented with polyuria, dehydration and failure to thrive (Kamoun *et al*. 2013). Autosomal-recessive dRTA is associated with mutations in any of the genes *ATP6V0A4*, *ATP6V1B1* and *SLC4A1* (Alper 2010a), which encode for the a4 and b1 subunits of the V-ATPase and the AE1 bicarbonate/chloride exchanger, respectively (Alper 2010b). Several studies for the identification of splicing mutations in *ATP6V0A4* have been performed in different ethnic groups (Smith *et al.* 2000; Stover *et al.* 2002; Vargas-Poussou *et al.* 2006; Andreucci *et al.* 2009). The coexistence of deafness with the distal tubular acidosis was of particular interest due to the physiology of the normal inner ear. Previous studies reported an early sensorineural hearing loss (SNHL) observed in patients with mutations in the *ATP6V1B1* gene (Vargas-Poussou *et al*. 2006). Indeed, Stover *et al*. (2002) demonstrated that young adult patients with *ATP6V0A4* mutations could develop a mild SNHL in the long term (Stover *et al.* 2002). Some dRTA patients without early SNHL were also previously found to have mutations in *ATP6V1B1* (Vargas-Poussou *et al.* 2006; Elia *et al.* 2011).

In this work, we describe the clinical features and molecular characterization of a consanguineous Tunisian family suffering from dRTA. Direct sequencing of *ATP6V0A4* revealed a novel homozygous mutation of the donor splice site in intron 18 (c.2010 + 1G>T). The predicted effect of this variant is likely the creation of a new cryptic 5′ splice site. The predicted mutant enzyme was truncated by the loss of 171 carboxyl-terminal amino acids.

Materials and methods

A 25-day-old male, born to consanguineous parents with a normal neonatal weight of 3600 g was admitted for severe dehydration, diarrhea, vomiting and failure to thrive. The family originated from the Siliana region, northwestern Tunisia. His six-year-old brother was suffering from renal tubular acidosis. On admission, his weight was 2600 g. He had respiratory problem with a severe nongap metabolic acidosis (blood pH: 7.12; bicarbonate: 2.8 meq/L), hypokaliemia, renal failure and hypercalciuria. He also exhibited polyuria (100 mL/kg/h) and alkaline urine pH (pH = 7). Renal ultrasound showed bilateral nephrocalcinosis. Based on these data, the diagnosis of primary distal tubular acidosis was made and treatment with high doses of sodium bicarbonate and potassium was started. Family conditions were poor; the infant had not been observed regularly and the treatment was discontinued. This child was hospitalized several times for dehydration and severe acidosis. He

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developed short stature and rickets. At the age of seven, he showed enuresis and learning difficulties. The magnetic resonance imaging (MRI) was normal and audiometric evaluation revealed normal hearing. Molecular diagnosis was performed in the two siblings and this study was conducted with the parents' written informed consent. The study protocol was also approved by the Ethics Committee of the Université Tunis El Manar.

Genomic DNA was extracted from whole peripheral blood following the standard salting-out protocol. *ATP6V1B1* and *ATP6V0A4* exons and intron–exon junctions were amplified by polymerase chain reaction (PCR). The samples were later sequenced on an ABI Prism 3130×l Genetic Analyzer (Applied Biosystems, Foster City, USA). The reference sequences used for mutation reporting were NM_001692.3 and NM_020632.2 for *ATP6V1B1* and *ATP6V0A4*, respectively. The mutation nomenclature was designated according to the Human Genome Variation Society (http://www.hgvs.org) and confirmed by Mutalyser (https://mutalyser.nl, last released 20 Oct 2014). The DNA samples from 50 controls were investigated for the G nucleotide in position c.2010 + 1 using ASO-PCR.

The effect of the novel mutation was evaluated *in silico* using the following five different online splicing prediction tools: fruitfly (NNSPLICE) (http://www.fruitfly.org/) (Reese *et al.* 1997), human splicing finder (HSF) (http://www.umd.be/HSF/) (Desmet *et al.* 2009), gene splicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml) (Pertea *et al.* 2001), MaxEntScan (MaxEnt) (http://genes.mit.edu/) (Yeo and Burge 2004) and splice site finder (SSF) (http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html) (Zhang 1998).

Results and discussion

The diagnosis of dRTA in the child described in this study was suspected by clinical features like dehydration with polyuria, nongap severe metabolic acidosis and inability to renal acidification, as shown by alkaline urine, hypokalemia and nephrocalcinosis. Direct sequencing analysis of *ATP6V0A4* showed a novel G to T substitution at 1-bp downstream of exon 18 (c.2010 + 1G>T) in homozygous state in the two patients (figure 1). Their

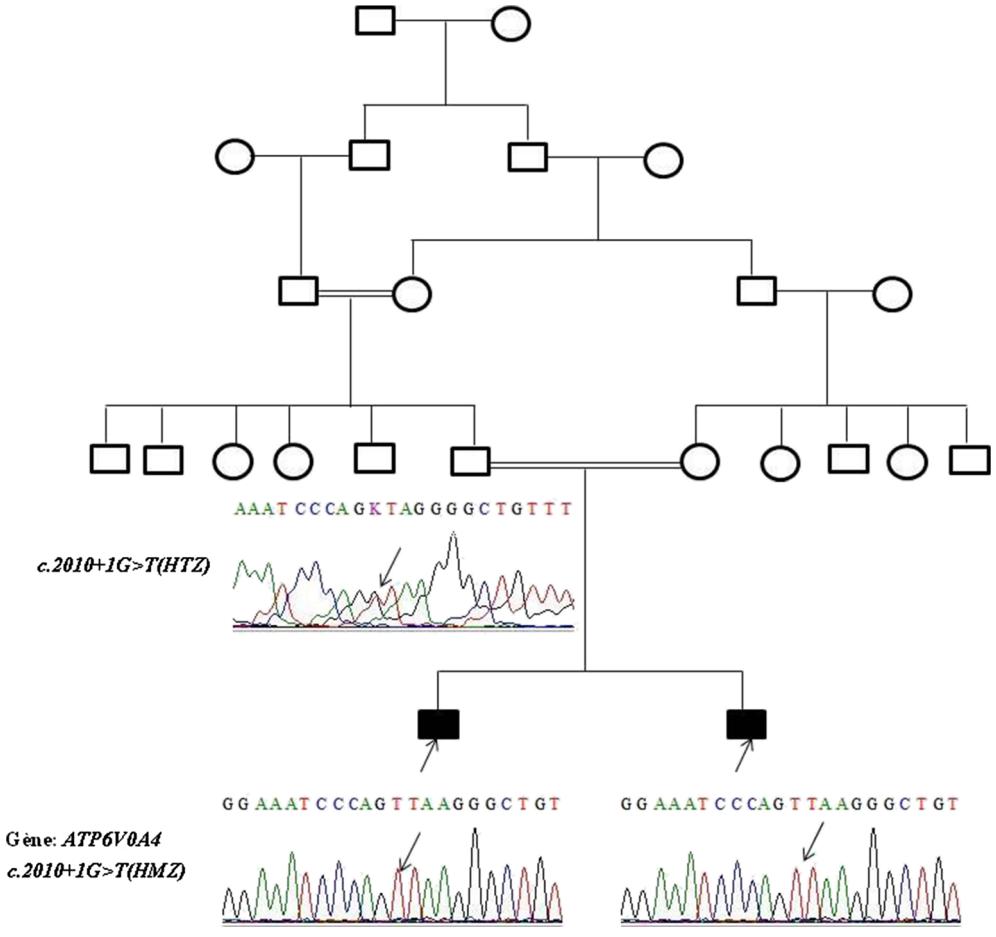

Figure 1. Novel splice site mutation in the *ATP6V0A4* gene detected by direct sequencing analysis in both the patients and their parents. A G to T transition at position 2010 in exon 18 is shown.

Table 1. Splicing predictions and the effect of c.2010 + 1 G>T mutation.

	SSF (0–100)	MaxEnt (0–12)	NNSPLICE (0–1)	GeneSplicer (0–15)	HSF (0–100)
Threshold	≥70	≥0	≥0.4	≥0	≥65
Exon 18 – c.1932					= 74.21
Exon 18 – c.1993					= 65.16
Exon 18 – c.2009					– ⇒ 65.55
Exon 18 – c.2010 N	94.98 ⇒ –	11.08 ⇒ –	1.00 ⇒ –	4.04 ⇒–	98.07 ⇒ –
Intron 18 – c.2010 + 9					= 68.79
Intron 18 – c.2010 + 43	= 72.64	= 2.76			= 78.68
Intron 18 – c.2010 + 47					= 67.32
Intron 18 – c.2010 + 70					= 65.24

–, absent or below threshold; ⇒, wild type to mutated change; gene, *ATP6V0A4*; transcript, NM_020632.2; variant, c.2010 + 1G>T; analysis range, c.1911 (exon 18) – c.2010+101 (intron 18).

parents were also carrying this mutation at the heterozygous state (figure 1). However, analysis of 100 chromosomes from healthy individuals revealed no carriers for this mutation.

To evaluate the impact of the novel mutation, we performed *in silico* analysis using bioinformatic online resources. Five different splicing prediction algorithms were used to predict the effect of this variation. All these programmes indicated that the novel mutation substantially decreased the recognition of the intron 18 donor site (table 1). The silent G>T transversion at this nucleotide might therefore affect the normal splicing process and result in missplicing. Computation of the strength of the wild-type *ATP6V0A4* intron 18 donor site using splice site prediction by neural network indicated that it is a weak splice site (score = 1.0) and that the c.2010 + 1 G>T transversion reduces this score below the threshold (score = 0.10).

The hereditary forms of dRTA have received increased attention due to the advances in the understanding of the molecular mechanism, whereby mutations in the main proteins involved in acid–base transport result in impaired acid excretion. Dysfunction of intercalated cells in the collecting tubules accounts for all the known genetic causes of dRTA. These cells secrete protons into the tubular lumen through H^+ATPase functionally coupled to the basolateral anion exchanger 1 (AE1). Mutations in *ATP6V1B1*, which encodes the B-subtype unit of the apical H^+ATPase, and *ATP6V0A4*, which encodes the A-subtype unit, lead to the loss of functions of the apical H^+ATPase and are usually responsible for patients with autosomal recessive dRTA, often associated with early or late sensorineural deafness (Batlle and Haque 2012). The child described in this study, had an early and severe manifestation. Knowing that the deafness related to *ATP6V0A4* mutations has a late onset and since our patient is still young we cannot confirm his status.

We report a novel splice site mutation c.2010 + 1G>T detected in homozygous state in *ATP6V0A4* (Smith *et al.* 2000; Stover *et al.* 2002; Andreucci *et al.* 2009). Only four splice

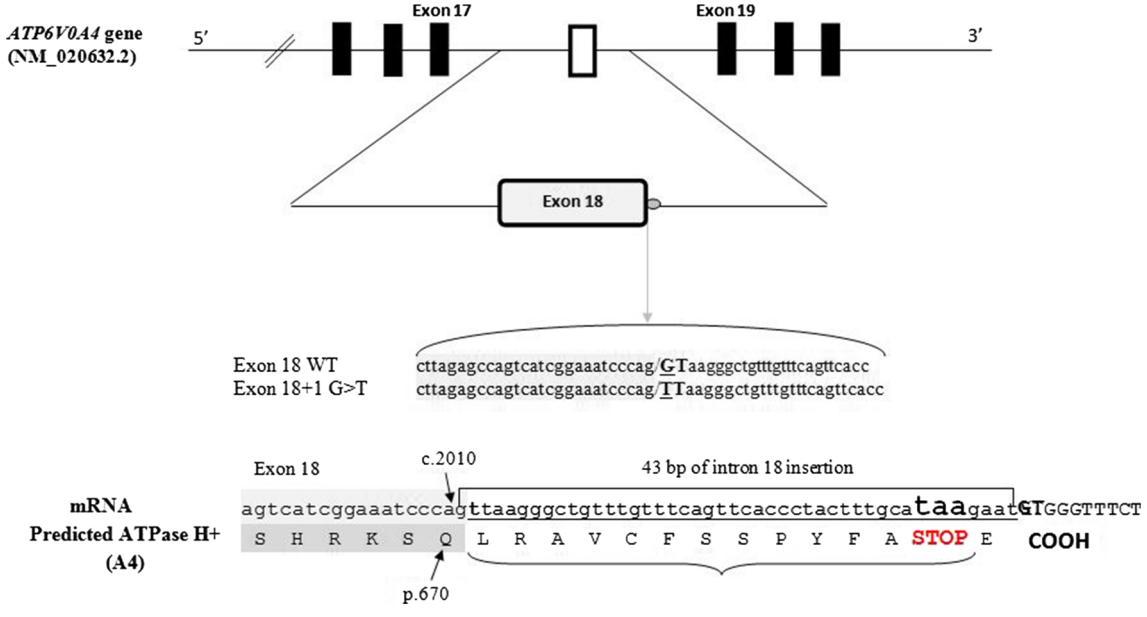

Figure 2. Schematic representation of the consequences of the donor splice-site mutation (c.2010+1 G>T).

site mutations have been reported so far in the *ATP6V0A4*. Previous studies on the effects of the splice-site mutations in human diseases showed that this type of mutation can result in either complete skipping of the exon, retention of the intron or the introduction of a new splice site within an exon or intron (Baralle and Baralle 2005).

In our patients, a G to T transition at −1 bp position downstream of exon 18 affecting the polypyrimide tract of intron 18 likely leads to the creation of a new 5′ splice site. The *in silico* studies of the effect of this change, show two possibilities: the creation of another donor site in intron 18 at position c.2010 + 43, which was determined from different prediction databases with a highly significant score (SSF = 72.64; MaxEnt = 2.76 and HSF = 68.79) (table 1). As a result, a 43 bp intronic sequence was inserted between exons 18 and 19 in the mRNA. This aberrant splicing is predicted to result in a truncated enzyme with loss of 23.33% of the whole enzyme structure, due to a premature termination at codon 683 owing to a frame shift (figure 2).

On the other hand, it is not ruled out that this creation of a premature stop codon results in the degradation of the mRNA by a nonsense-mediated decay mechanism. Unfortunately, this hypothesis could not be confirmed as RNA was unavailable and the patients are lost of sight.

In addition to the newly identified mutation, direct sequencing of the *ATP6V0A4* gene shows the existence of three polymorphisms in homozygous state: C/C in rs10258719, A/A in rs55843107 and T/T in rs1026435 and two SNPs in heterozygous state: C/T in rs149636333 and c.1030-5 del T/- in rs11324444.

Similar characteristics with early onset of nongap metabolic acidosis, hypokalemia and nephrocalcinosis were observed in patients carrying different mutations in both the genes. Previous studies show that there was no phenotype–genotype correlation between dRTA and SNHL (Elia *et al*. 2011). However, other studies confirmed association between *ATP6V1B1* mutations and dRTA with the early onset of SNHL, as well as between *ATP6V0A4* mutations and dRTA with normal hearing (at least until adulthood) (Karet *et al.* 1999; Smith *et al.* 2000; Hahn *et al.* 2003). In this case, the patients were young and currently did not suffer hearing loss. A follow-up of these two patients can confirm the severity of this novel mutation specifically for the development of hearing loss. Apart from dRTA, *ATP6V0A4* dysregulation was recently found to be implicated in kidney cancer (Batlle and Haque 2012; Wozniak *et al.* 2013; Zaravinos *et al.* 2014).

In conclusion, we have described patients with severe dRTA and a novel splicing mutation in the *ATP6V0A4* gene in a family originating from the Siliana region in northwestern Tunisia. Based on the history of Siliana settlements, the population could have mixed origins (i.e., Romans, Amazighs (Berbers), Phoenicians and Arabs). Although we could not rule out the fact that this new mutation could be a private mutation, we propose to screen the various Mediterranean and Middle East populations for the same.

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