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SHORT COMMUNICATION

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The *Alu* insertion in the *CLCN5* gene of a patient with Dent's disease leads to exon 11 skipping

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Abstract *Alu* sequences are short, interspersed elements that have generated more than one million copies in the human genome. They propagate by transcription followed by reverse transcription and integration, causing mutations, recombination, and changes in pre-mRNA splicing. We have recently identified a 345-bp long *Alu* Ya5 element inserted in codon 650 within exon 11 of the chloride channel ClC-5 gene (*CLCN5*) of a patient with Dent's disease. A microsatellite pedigree analysis indicated that the insertion occurred in the germline of the maternal grandfather. Dent's disease is an X-linked renal tubular disorder characterized by low-molecular-weight proteinuria, hypercalciuria, nephrolithiasis, and nephrocalcinosis. Here, we found, by RT-PCR amplification of RNA extracted from the patient's blood and subsequent DNA sequencing, that the *Alu* insertion led to an aberrant splicing of the *CLCN5* pre-mRNA that skipped exon 11. Using the ESE finder and RESCUE-ESE Web interfaces, we identified two high-score exonic splicing enhancer (ESE) sequences in the site of insertion. The functional significance of these ESE motifs is suggested by our observation that these sequences are highly conserved among mammal *CLCN5* genes. Therefore, we suggest that the *Alu* insertion causes exon skipping by interfering with splicing regulatory elements. The altered splicing would predict a truncated ClC-5 protein that lacks critical domains for sorting and chloride channel function.

Keywords *CLCN5* · Exon skipping · Chloride channel CBS motif · Renal tubulopathy

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Introduction

Alu elements are short sequences of DNA that generate new copies of themselves by reverse transcription of an *Alu*-derived RNA polymerase III transcript (Batzer and Deininger 2002). The human genome contains ~1.4 million *Alu* copies, which are commonly found in the noncoding regions of genes, such as 5′ and 3′ UTRs and introns (Jurka 1998; International Human Genome Sequencing Consortium 2001). Genetic diseases can result from different types of mutations caused by the insertion of an *Alu* sequence. Examples include neurofibromatosis, Apert syndrome, hemophilia, familial hypocalciuric hypercalcemia, breast cancer, hereditary nonpolyposis colorectal cancer, and Hunter's disease (Ostertag and Kazazian 2001; Ganguly et al. 2003; Ricci et al. 2003; Kloor et al. 2004). Recent work by Lev-Maor et al. (2003) has shown that intronic *Alu* sequences can be included into mature mRNAs via a splicing-mediated process termed exonization, which can result in human disease or contribute to human-specific protein diversity.

Recently, we identified an *Alu* insertion in exon 11 of the *CLCN5* gene in a patient with Dent's disease (Claverie-Martin et al. 2003). Sequence analysis showed that the *Alu* element is inserted in the same orientation as the *CLCN5* gene (Fig. 1a) and is closely related to the AluYa5 subfamily (Claverie-Martin et al. 2003). Dent's disease is a renal tubular disorder characterized by low-molecular-weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, rickets, and eventual renal failure (OMIM: 300009; Lloyd et al. 1996). This

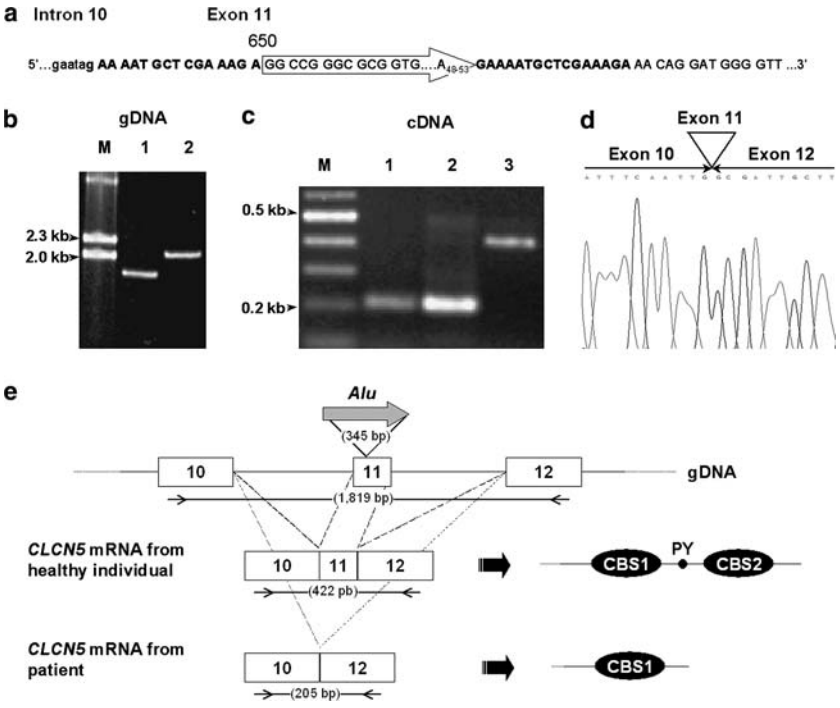

Fig. 1 Analysis of the effect of the *Alu* insertion. **a** Scheme of the *CLCN5* region containing the *Alu* insertion in codon 650. Intron 10 and exon 11 sequences are in *lowercase* and *uppercase*, respectively. The *arrow* indicates the *Alu* insertion, flanked by the target-site duplication (*bold*). **b** Agarose gel electrophoresis of PCR products amplified from genomic DNA with primers specific to exons 10 and 12. *Lane M* molecular-weight marker, *lane 1* healthy individual, *lane 2* patient. **c** Agarose gel electrophoresis of RT-PCR products from *CLCN5* exons 10–12. *Lane M* molecular-weight marker, *lane 1* patient, *lane 2* patient's mother, *lane 3* healthy individual. **d** Partial patient's cDNA sequence showing the skipping of *CLCN5* exon 11. **e** Schematic representation of the *Alu* insertion, *CLCN5* mRNAs, and ClC-5 carboxy terminus. The size of the amplified fragments is shown in *parentheses*

disease is caused by mutations in the *CLCN5* gene that encodes the voltage-gated chloride channel ClC-5 (Fisher et al. 1995; Lloyd et al. 1996; Carballo-Trujillo et al. 2003) although recent findings have shown that mutations in the *OCRL1* gene, also involved in proximal tubular function, are responsible for the disease phenotype in some patients (Hoopes et al. 2005). Studies with ClC-5 knockout mice showed that loss of ClC-5 function results in an endosomal acidification defect and reduced proximal tubular protein reabsorption, which explain the symptoms of Dent's disease (Piwon et al. 2000).

Based on the results obtained with the *Alu* insertion mutation in *BRCA2* (Miki et al. 1996), we suggested that the *Alu* insertion in *CLCN5* might cause skipping of exon 11 (Claverie-Martin et al. 2003). In this study, we show that the *CLCN5 Alu* insertion resulted in altered splicing that skipped exon 11 in the patient's blood cells. Analysis of the insertion site suggests that the *Alu* element disturbs two highly conserved exonic splicing enhancer (ESE) motifs.

Material and methods

The clinical and biochemical characteristics of the patient have been previously described (Claverie-Martin et al. 2003). The diagnosis of Dent's disease was based on the presence of hypercalciuria and microproteinuria and was then confirmed by the identification of the mutation in the *CLCN5* gene. To date, the patient does not show nephrolithiasis or nephrocalcinosis and is being treated with hydrochlorothiazide and amiloride. Analysis of the patient's pedigree indicated that apart from the patient, only the patient's mother carries the mutation (Claverie-Martin et al. 2003). Furthermore, a polymorphism analysis indicated that the *Alu* insertion found in *CLCN5* was originated in the germline of the maternal grandfather (Claverie-Martin et al. 2003). Both of his parents and his sister had hypercalciuria, and his mother had moderate proteinuria, but there was no history of urolithiasis or Dent's disease in the family, including the maternal grandfather (Claverie-Martin et al. 2003). As the proband was a minor, parents gave informed consent for this study.

Total RNA was isolated from whole blood samples using the PAXgene Blood RNA System (Qiagen), and digested with RNase-free DNase I. Kidney poly A^+ RNA (Clontech) and total RNA from blood were reverse transcribed with random hexamers using the First-strand cDNA synthesis kit (Roche). Two microliters of cDNA were then used as a template for PCR amplifications with the following profile: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 20 s, 60°C for 20 s, 72°C for 20 s), and an extension at 72°C for 5 min. Thermocycling was performed on a GeneAmp PCR system 9700 (Applied Biosystems). The

Table 1 Primer pairs used for cDNA amplifications

Primer name	Sequence (5′–3′)	Exon	Product size (bp)
CLCN5cDN 2F	GACTTCTTGGAGGAGCCAATC	2	441
CLCN5cDN 5R	GAGAGCCCAGAGGACGTACA	5	
CLCN5cDN 5F	GGCTCTCCTATTTGCCTTCC	5	751
CLCN5cDN 8R	GTCCAGAAGGCCACAGTCAT	8	
CLCN5cDN 8F	GGCAGCTGGCTTTAACACTC	8	985
CLCN5cDN 12R	TCCCGCTTTACATCCAGAAC	12	
CLCN510F	TACAGTGGCTTCCCAGTGGT	10	422
CLCN5cDN 12R	TCCCGCTTTACATCCAGAAC	12	

region spanning *CLCN5* exons 2–12 was amplified in three overlapping fragments using pairs of primers designed from the coding region (Table 1). To study the effect of the exon 11 *Alu* insertion on pre-mRNA splicing, we amplified a segment of *CLCN5* cDNA starting in exon 10 and finishing in exon 12 using primers CLCN510F and CLCN5cDN 12R (Table 1). These two primers were also used to amplify genomic DNA from blood of the patient and a healthy individual.

DNA sequencing of amplified PCR products was carried out directly on both strands using the Big Dye Terminator kit v 3.0 with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers used for sequencing were the same primers used for PCR amplification.

Both ESE finder (Cartegni et al. 2003) and RESCUE-ESE (Fairbrother et al. 2002) Web interfaces were used to search the human wild-type (NM_000084), mutant (Claverie-Martin et al. 2003), rabbit (AF195523), mouse (NM_016691), rat (NM_017106), and pig (NM_214139) exon 11 sequences for the presence of ESE motifs. ESE finder was used with a threshold value of 3.0 for all four types of ESE motifs.

Results and discussion

The entire coding region of *CLCN5* (exons 2–12) was amplified in three overlapping RT-PCR reactions using RNA isolated from blood of a healthy individual and kidney poly A^+ RNA (Clontech). The same expected-size products were observed in both the blood and the kidney RNA samples (results not shown). Forino et al. (2004) have also shown that the *CLCN5* splicing patterns in leukocytes and kidney biopsies of normal individuals are the same. We therefore considered that blood cells were a suitable source of RNA to study the effect of *CLCN5* mutations on pre-mRNA splicing.

PCR amplification of blood DNA from the patient with primers specific for exons 10 and 12 showed the presence of a 2,164-bp band instead of the normal 1,819-bp fragment, confirming the presence of the *Alu* insertion in the patient's *CLCN5* gene (Fig. 1b). RT-PCR analysis of the patient's RNA with the same primers showed a unique band of 205 bp instead of the full-length 422-bp band observed with the healthy individual, suggestive of an exon skipping event (Fig. 1c). A larger band(s) containing *Alu* sequences was not observed even after increasing the number of PCR cycles (results not shown). The same RT-PCR analysis using RNA from the heterozygous mother yielded the band corresponding to the truncated fragment, a faint 422-bp band (confirmed by further amplification and DNA sequencing as the wild-type fragment), and a another faint band of approximately 470 bp (Fig. 1c). Attempts to sequence this larger band were unsuccessful. We then sequenced the 205-bp RT-PCR products in order to identify which DNA sequences were missing. The results showed that the abnormal fragment lacked the entire exon 11 (217 bp) and contained the sequences expected from exons 10 and 12 precisely spliced together (Fig. 1d). This loss changes the open reading frame of the *CLCN5* transcript and adds 12 new codons (GDCLESLPKRMC) to the 3′ end of codon 644 followed by a premature stop codon. The predicted truncation removes 102 amino acids from the carboxy terminus of the ClC-5 protein, including the second CBS (cystathionine-beta-synthase) motif, CBS2, and the PY motif (Fig. 1e). Tandem pairs of conserved CBS motifs are found in a wide variety of proteins and associate to form Bateman domains (Bateman 1997). A recent study reveals that these domains bind adenosine derivatives (Scott et al. 2004), and the authors suggest that the binding of ATP to the CBS motifs of CLC chloride channels is necessary before the channels will open. Furthermore, heterologous expression studies in mammalian cells have shown that the CBS2 domain of ClC-1, a channel related to ClC-5, is critical for channel function (Hebeisen et al. 2004). The sequence located between the CBS domains of ClC-5 contains a PY sorting signal motif. Schwake et al. (2001) have found that alteration of this internalization motif leads to increased plasma membrane expression of ClC-5. These findings illustrate the importance of the carboxy terminus of CLC channels. Also, Hryciw et al. (2003) have found that the ClC-5 carboxy terminus binds to cofilin, a protein necessary for endocytosis.

The analysis of the *CLCN5* genomic region, including the *Alu* insertion, with three available programs to define protein coding regions, yielded different splicing patterns, but none of them predicted a complete skipping of exon 11 (results not shown). In order to explain the cause of exon skipping due to the *Alu* insertion, we searched the sequence of the 5′ end of exon 11 for the presence of potential ESE motifs. These motifs are

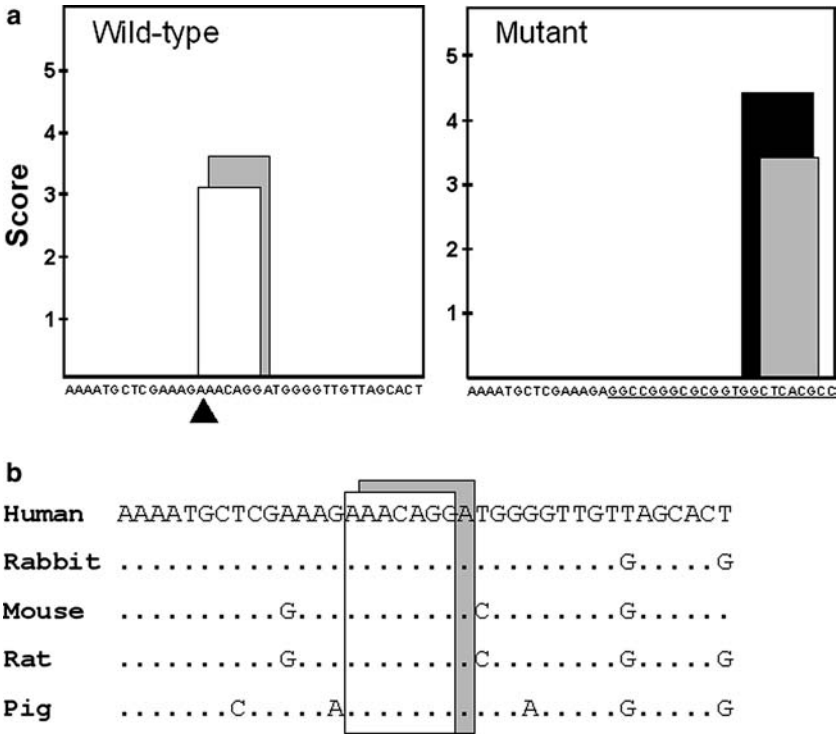

Fig. 2 Analysis of ESE sequences in exon 11 of *CLCN5*. **a** Potential ESE sequences within the first 38 nucleotides of the 5′ end of exon 11 shown as *histograms* of scores for SRp40 (*white*), SF2/ASF (*gray*), and SC35 (*black*) motifs. The *arrowhead* indicates the point of *Alu* insertion. Bases from the *Alu* sequence are *underlined*. **b** Partial sequence of human exon 11 compared with the corresponding sequence of other mammals. *Boxes* denote conserved potential ESE sequences for SRp40 (*white*) and SF2/ASF (*gray*) motifs. The *dots* indicate identical sequence

binding sites for essential splicing factors, the serine/arginine-rich (SR) proteins that play an important role in exon recognition during pre-mRNA splicing (Cartegni et al. 2002; Cáceres and Kornblihtt 2002). Using the ESE finder software, we found that the *Alu* element, inserted 15 nucleotides from the 5′ end of exon 11, is within a high-score ESE sequence (AAACAGG) that is likely bound by the SRp40 protein (Fig. 2a). Furthermore, the *Alu* insert creates a longer distance between an overlapping SF2/ASF ESE motif (AACAGGA) and the 5′ end of exon 11. Several studies suggest that functional ESEs occupy specific positions relative to the splice sites of an exon (Cartegni et al. 2002; Fairbrother et al. 2004). The alignment of *CLCN5* exon 11 from humans, rabbit, mouse, rat, and pig showed that the SRp40 and SF2/ASF ESE sequences close to the 5′ end are identical (Fig. 2b), indicating high conservation during evolution and a functional importance in splicing. The SRp40 and SF2/ASF ESE motifs strongly overlap (Fig. 2), being unlikely that the two SR proteins bind simultaneously. Therefore, only one of these two sequences may represent a functional ESE, or the motifs may act as enhancers in a mutually exclusive manner, depending on the individual pre-mRNA molecule or cell type (Cáceres and Kornblihtt 2002). On the other hand, RESCUE-ESE identified six overlapping candidate ESEs in the 5′ end of exon 11, five of which (AAAGAA, AAGAAA, AGAAAC, GAAACA and AAACAG) were disturbed by the *Alu* insertion. As the sequence AAACAG was identified with both approaches, we suggest that the *Alu* mutation causes exon 11 skipping by altering the interaction between SRp40 and this ESE sequence. We cannot exclude the possibility that the *CLCN5 Alu* sequence, which is inserted in the same orientation as the gene (Claverie-Martin et al. 2003), inhibits the pre-mRNA splicing by itself. However, a search in human genomic DNA libraries for sequences that inhibit splicing failed to find *Alu* sequences in the forward orientation (Fairbrother and Chasin 2000). An *Alu* insertion in exon 22 of *BRCA2* also results in exon skipping (Miki et al. 1996). However, in this case the *Alu* element is inserted just downstream from the 3′ end of two high-score ESE motifs, SRp40 and SF2/ASF (results not shown). Recently, Kloor et al. (2004) reported an *Alu* insertion in exon 6 of the *MSH2* gene in a patient with hereditary nonpolyposis colorectal cancer syndrome. They did not observe exon skipping in the RNA from the patient's peripheral blood cells, but an analysis of *MSH2* exon 6 with ESE finder showed that the *Alu* sequence was inserted within two overlapping high-score ESE motifs, SC35 and SRp40 (results not shown).

In conclusion, the *CLCN5 Alu* insertion caused skipping of exon 11 in pre-mRNA from blood as a result of the abnormal splicing, probably due to disruption of conserved ESEs. The altered splicing caused by the *Alu* insertion would predict a truncated ClC-5 protein that lacks part of the carboxy terminus, including the PY and CBS2 domains, critical for sorting and chloride channel function. Further investigations are needed to confirm the functional significance of the ESE in *CLCN5* exon 11 and that the skipping also takes place in kidney cells.

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