Evaluating *PVALB* as a candidate gene for *SLC12A3*-negative cases of Gitelman’s syndrome

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Abstract

**Background.** Loss-of-function mutations in *SLC12A3* coding for the thiazide-sensitive NaCl cotransporter (NCC) cause Gitelman’s syndrome (GS), a recessively inherited salt-losing tubulopathy. Most GS patients are compound heterozygous. However, up to 30% of GS patients carry only a single mutant allele, and a normal *SLC12A3* screening is also observed in a small subset of patients. Locus heterogeneity could explain the lack of detection of mutant *SLC12A3* alleles in GS patients. The renal phenotype of the parvalbumin knockout mice pointed to *PVALB* as a candidate gene for GS for *SLC12A3*-negative cases.

**Methods.** PCR and direct sequencing of *PVALB* was performed in 132 GS patients in whom only one or no (N = 79) mutant *SLC12A3* allele was found. The possible interference of biallelic SNPs (single nucleotide polymorphisms) on normal transcription or normal splicing was investigated. Genotyping of 110 anonymous blood donors was performed to determine the allelic frequency in the normal population.

**Results.** No sequence variants resulting in amino acid substitution or truncated protein within the *PVALB* gene were found in the 264 chromosomes tested. Ten biallelic SNPs, including six novel polymorphisms, were identified: five in the 5′ UTR, none of them affecting predicted regulatory elements; three in the coding region, without alteration of the consensus splice sites, and two in the 3′ UTR. The observed allelic frequencies did not differ significantly between GS patients and controls.

**Conclusion.** Our results strongly suggest that mutations in the *PVALB* gene are not involved in GS patients who harbour a single or no mutant *SLC12A3* allele.

**Keywords:** distal convoluted tubule (DCT); NCC; parvalbumin; sodium-chloride cotransporter; thiazide

Introduction

Gitelman’s syndrome (GS; MIM 263800) is a salt-losing tubulopathy associated with hypokalaemic alkalosis, hypomagnesaemia and hypocalciuria. Loss-of-function mutations in the *SLC12A3* gene that encodes the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) were found to be responsible for the disease [1]. NCC is expressed in the cells lining the distal convoluted tubule (DCT) of the kidney, a segment responsible for the reabsorption of 5–10% of total filtered NaCl [2]. The incidence of GS may be as high as 1/1000 births in some populations [3], and it is characterized by a variable expression in terms of age at presentation, and nature and severity of the biochemical abnormalities and clinical manifestations. This significant phenotypic heterogeneity is observed not only between all patients harbouring *SLC12A3* mutations but also among family members or patients with identical mutations [4,5].

Although GS is transmitted as an autosomal recessive trait, up to 30% of GS patients are found to carry only one mutant allele by classical *SLC12A3* screening [6]. In addition, no mutations are detected in some patients presenting all clinical features of GS [7]. Genetic heterogeneity could be one explanation for this failure in the detection of *SLC12A3* mutant alleles.

Our group has recently demonstrated that mice lacking parvalbumin (PV) harbour several manifestations similar to GS [8]. PV is a cytosolic protein that is encoded by the *PVALB* gene located on the long arm of chromosome 22 (22q13.1). In the mammalian kidney, PV is restricted to the DCT where it co-distributes with NCC [8,9]. Detailed
one mutant allele was detected. The study group included 132 unrelated patients clinically diagnosed as having GS, originating from the St-Luc Academic Hospital (Brussels), the Hôpital Européen George Pompidou (Paris), the Radboud University Nijmegen Medical Centre (Nijmegen) and the University Children’s Hospital (Marburg). All patients met the classical diagnostic criteria for GS including hypokalaemia due to renal potassium wasting associated with metabolic alkalosis, hypomagnesaemia, either hypo- or normocalciuria, normal (or low) blood pressure and normal renal function [11,12]. A similar SLC12A3 molecular testing by sequence analysis of the entire coding region (26 exons, with ~50 nucleotides in the 5’ and 3’ part of each exon) was routinely performed in each laboratory. The present screening did not include gene dosage, regulatory region sequencing or transcript analysis. There were no related individuals included in the analysis. Based on SLC12A3 genotyping, two groups were then distinguished: a group of 53 simple heterozygous patients, i.e. in which only one mutant SLC12A3 allele could be detected, and another group of 79 patients in which the screening for SLC12A3 mutations was strictly negative, i.e. in which no mutant allele was identified. DNAs from 110 healthy blood donors served as normal controls.

**Patients and methods**

**Patients’ recruitment**

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**PVALB mutation analysis**

Total DNA was extracted from peripheral blood leukocytes according the manufacturer’s instructions (Genta Systems, Puregene, Minneapolis, USA). Five primer pairs were designed using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) to amplify by PCR the coding region and flanking intronic sequences of the human PVALB gene and part of its 5’ and 3’ UTR (Table 1). Thirty cycles of PCR were performed in the presence of 1.5 μl MgCl2 25 mM using AmpliTaq Gold (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The PCR products were then directly sequenced with the Big Dye terminator kit (Perkin Elmer Applied Biosystems). Sequence reactions were purified with MultiScreen SEQ384 Filter Plate (Millipore, Billerica, MA, USA) and Sephadex™ G-50 DNA Grade Fine (Amersham Biosciences, Piscataway, NJ, USA) dye terminator removal, before analysis on an ABI3100 capillary sequencer (Perkin Elmer Applied Biosystems). PVALB mutation analysis was performed in 46 patients by single-strand conformation analysis (SSCP) prior to direct sequencing, using the same primers as those amplifying the coding sequences and the intron/exon boundaries. Abrerrant bands, after separation on polyacrylamide gels, were directly sequenced on both strands.

Splice site prediction analyses were performed using Automated Splice Site Analyses (https://splice.cmnh.edu) [13]. Predictions of putative promoter regions were evaluated using PROSCAN version 1.7 (http://bimas.dcrt.nih.gov/molbio/proscan/) [14] and Promoter 2.0 prediction server (http://www.cbs.dtu.dk/services/Promoter/) [15]. UCSC Genome Browser (http://genome.ucsc.edu/) was used to evaluate the presence of risk factors for genomic rearrangements through the RepeatMasker program.

Polymorphisms within the coding region are described at the cDNA level, based on the reference sequence NM_002854, being nucleotide 1 being the first adenine of the translation initiation codon. Those polymorphisms located at the 5’ or 3’ UTR are described at the genomic level, based on the reference contig NT_011520.11 between 16 586 000 and 16 606 532, minus strand.

**Data analysis**

Comparisons between groups were made by the $\chi^2$ test. The significance level was set at $P < 0.05$.

**Results**

One hundred and thirty-two individuals with a clinical and biochemical diagnosis of GS were screened for mutations in the entire coding region of the human PVALB gene and flanking intronic sequences, using PCR amplification and direct sequencing. Among these 132 individuals, there were 53 heterozygotes for a disease-causing mutation in SLC12A3 and 79 with a normal SLC12A3 sequence analysis. In the 264 chromosomes tested, analysis of PVALB failed to reveal sequences that could result in an amino acid substitution or in a truncated protein.

To assess the possible presence of mutations affecting regulatory regions in the 5’ UTR, putative promoter region analyses were performed on a fragment expanding 5 kb located immediately upstream of the translation start site using two different promoter prediction servers. Both resources predicted a region of ~300 bp containing several segments highly likely active in the regulation of transcription. This region was situated at position −2800 before the first translation initiation codon and included a CAAT box (CCAAAAT) at position −2546 and a TATA box (TATATA) at position −2524 (Figure 1). Ten biallelic SNPs were detected in the human PVALB gene: five in the 5’ UTR, three in the coding region and two in the 3’ UTR region (Figure 1). The first sequence variation found in the 5’ UTR region was a transition G to A located 2778 nucleotides before the translation initiation codon (g.191G > A). Two substitutions G to C were identified at position −2709 (g.260G > C) and −2634 (g.335G > C). One C to T transition was
Table 1. Human PVALB specific primers

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>5' UTR</td>
<td>CGCACAAAAGTGCTGAGTC</td>
<td>TCACACCGGAGGCGAAAGGA</td>
<td>685</td>
</tr>
<tr>
<td>Exon 2</td>
<td>ACTCGGACGCTGGTCATA</td>
<td>GACGAGGAGGAGGAGGT</td>
<td>191</td>
</tr>
<tr>
<td>Exon 3</td>
<td>CATCGGGAATCCATCAACGA</td>
<td>TGCTAAGAGCAGAGATGG</td>
<td>349</td>
</tr>
<tr>
<td>Exon 4</td>
<td>TACCTACCCAGGAAAGCACA</td>
<td>CACCAAGATCTCCAGATGC</td>
<td>345</td>
</tr>
<tr>
<td>Exon 5 and 3' UTR</td>
<td>AGAACCAGAGAGAGCACC</td>
<td>GATGCATCTGGAAGGCAATG</td>
<td>376</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic PVALB and the identified polymorphisms. (A) Genomic context of PVALB on chromosome 22. A region expanding 50 kb on chromosome 22q13.1 is represented. Genes located on the vicinity of PVALB are shown in their corresponding orientation (arrows). NC4, neutrophil cytosolic factor 4; FLJ90680, FLJ90680 protein; RABL4, RAB member of RAS oncogene family-like 4; CACNG2, calcium channel voltage-dependent gamma subunit 2. (B) PVALB gene structure comprising five exons (indicated by boxes, coding sequence in black). Dashed lines represent 5' and 3' UTR regions. Dotted box enclose the 300 bp putative promoter region including TATA and CAAT boxes. The location of the polymorphisms are indicated by asterisks, and their relative positions are shown.

Table 2. Variants detected in the human PVALB gene

| Location   | Nucleotide change | Allelic frequency GS patients (n = 132) | Allelic frequency normal controls (n = 110) | χ²   | P-value | Status
<table>
<thead>
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</thead>
<tbody>
<tr>
<td>5' UTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.191G &gt; A</td>
<td>G: 0.97</td>
<td>A: 0.03</td>
<td>G: 0.99 A: 0.01</td>
<td>1.10</td>
<td>0.29</td>
<td>Novel</td>
</tr>
<tr>
<td>g.260G &gt; C</td>
<td>G: 0.77</td>
<td>C: 0.23</td>
<td>G: 0.66 C: 0.34</td>
<td>1.87</td>
<td>0.17</td>
<td>rs2001064</td>
</tr>
<tr>
<td>g.335G &gt; C</td>
<td>G: 0.99</td>
<td>C: 0.01</td>
<td>G: 1 C: 0</td>
<td>1.93</td>
<td>0.16</td>
<td>rs5995346</td>
</tr>
<tr>
<td>g.361C &gt; T</td>
<td>C: 0.99</td>
<td>T: 0.01</td>
<td>C: 1 T: 0</td>
<td>1.93</td>
<td>0.16</td>
<td>Novel</td>
</tr>
<tr>
<td>g.407G &gt; A</td>
<td>G: 0.96</td>
<td>A: 0.04</td>
<td>G: 0.97 A: 0.03</td>
<td>0.27</td>
<td>0.60</td>
<td>Novel</td>
</tr>
<tr>
<td>IVS3</td>
<td>c.195-77G &gt; A</td>
<td>G: 0.99 A: 0.01</td>
<td>G: 1 A: 0</td>
<td>0.84</td>
<td>0.36</td>
<td>Novel</td>
</tr>
<tr>
<td>IVS4</td>
<td>c.305-24T &gt; C</td>
<td>T: 0.99 C: 0.01</td>
<td>T: 1 C: 0</td>
<td>0.84</td>
<td>0.36</td>
<td>Novel</td>
</tr>
<tr>
<td>3' UTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.1914T &gt; C</td>
<td>G: 0.55</td>
<td>T: 0.45</td>
<td>G: 0.55 T: 0.45</td>
<td>0.01</td>
<td>0.94</td>
<td>rs2001063</td>
</tr>
<tr>
<td>g.1920C &gt; T</td>
<td>C: 1</td>
<td>T: 0</td>
<td>C: 0 T: 0</td>
<td>0.70</td>
<td>0.40</td>
<td>Novel</td>
</tr>
</tbody>
</table>


detected at position −2608 (g.361C > T). Finally, a substitution of G to A was identified at position −2562 (g.407G > A). Among them, the last four are included in the 300 bp region predicted to be highly likely active in the regulation of transcription. Within the coding region, the first nucleotide change was a G to A transition found in intron 3 (c.195-77G > A), whereas the last two were located at the end of intron 4, a T to C substitution (c.305-24T > C) and a C to T change (c.305-9G > T). In the 3' UTR region, two C to T substitutions were found, c.1914T > C and c.1920C > T, located 63 and 118 nucleotides downstream the TAA stop codon, respectively. A detailed search
in the dbSNP database (http://www.ncbi.nlm.nih.gov, reviewed on September 2007) showed that among the 10 variants identified in our study, 6 were unknown (Table 2).

We used Automated Splice Site analyses to assess whether the polymorphisms located in the flanking intronic sequences interfere with normal splicing of the pre-mRNA. None of them were considered to affect splicing, since the strength of the consensus splice sites did not undergo any change when the concerned nucleotide was replaced (data not shown). We observed that the two polymorphisms c.305-9G > T and g.19147C > T located upstream and downstream of the last exon and separated by 100 nucleotides are under perfect linkage disequilibrium, with a maximum expected $r^2$ value equal to 1. Next, we genotyped the 10 SNPs covering the PV ALB gene in 110 anonymous blood donors to determine the carrier frequency of each allele. The observed frequencies did not differ significantly between patients and controls (Table 2).

**Discussion**

In this study, we tested the hypothesis that mutations in the *PV ALB* gene could be involved in the pathogenesis of GS in those individuals in whom none or a single mutant *SLC12A3* allele was identified.

Accumulated genetic data indicate that two *SLC12A3* mutations inherited on both paternal and maternal alleles are found in the majority of the GS kindreds studied. However, a single heterozygous *SLC12A3* mutation is detected in $\sim$30% of GS patients [6,7,16]. In this group of individuals, the inheritance of GS could in principle be either recessive with an undetected second allele or digenic where *SLC12A3* deleterious change would interact with a mutation in another gene. Potential explanations for the apparent lack of detection include the presence of mutations in regulatory fragments of *SLC12A3* or in deeper intronic sequences which are not routinely screened, the involvement of unidentified large genomic rearrangements and the influence of epigenetic modifications and/or silent polymorphisms that could interfere in the function of the gene [16]. Locus heterogeneity could also be considered, especially in those GS individuals with normal *SLC12A3* sequence analysis. Indeed, other genes may also be involved in the pathogenesis of GS, particularly if the proteins they encode participate in the complex handling of ions in the DCT. A case in point is *CLCNKB*, the gene that codes for the chloride channel CIC-Kh. Mutations in *CLCNKB* are responsible for classic Bartter’s syndrome (cBS) that present during early childhood with failure to thrive, muscular weakness, marked hypochloremic alkalosis and hypokalaemia [17]. Of interest, *CLCNKB* mutations have also been detected in three unrelated patients [18] and in patients from a large inbred Bedouin family [19] presenting overlapping clinical features between cBS and GS. However, the reduced subset of patients harbouring *CLCNKB* mutations suggests that this gene plays a limited role for the pathogenesis of GS.

The selection of *PV ALB* as a candidate gene was based on its selective expression in the DCT of the human kidney and the recent observation that *PV ALB* inactivation in mouse results in a discrete salt-losing phenotype, with a significant decrease in the expression of NCC in DCT cells secondary to alterations in intracellular Ca$^{2+}$ signalling [8]. Furthermore, the PV KO mice showed increased tubular Ca$^{2+}$ reabsorption and higher bone density, similar to features observed in *Slc12a3* KO mice and GS patients [10–12].

To test whether mutations in the *PV ALB* gene are involved in GS, 53 GS patients with a single identified mutation in *SLC12A3* and 79 GS patients negative for the mutation screening were included in the study. The 132 individuals were screened for mutations in the entire coding region of the human *PV ALB* gene and flanking intronic regions. No disease-associated sequence variations were identified in the 264 chromosomes tested. The screening revealed the presence of 10 SNPs (including 6 novel) covering the *PV ALB* gene. Of them, three were found in intronic regions although none altered the strength of the consensus splice site when its possible interference with normal splicing of the pre-mRNA was tested, strongly suggesting that they are not disease-related variants.

After excluding the presence of disease-associated mutations in the coding region, we analysed the 5′ UTR region using two servers (PROSCAN and Promoter 2.0) designed to find putative eukaryotic promoter sequences in primary sequence data in order to define a region containing elements highly likely active in the regulation of transcription for its further sequencing. A CAAT box and a TATA box were predicted within a putative promoter region of 300 bp. Both elements coincide with those predicted by Berchtold et al. who determined the genomic organization of the rat PV gene that showed 94% of similarity with the human counterpart [20,21]. Five of the 10 SNPs are located in the 5′ UTR (4 of them into the predicted 300 bp region) but none of them affecting predicted regulatory elements, which very likely exclude their involvement in an abnormal transcription. The remaining two SNPs are located in the 3′ UTR region. We then compared the frequency of each allele among the GS patient group and the control population. No statistically significant difference was observed, strongly suggesting that none of the 10 polymorphisms is related to the pathophysiology of GS. Although regulatory or deeper intronic sequences were not completely screened, we consider it rather unlikely that pathogenic mutations occur exclusively in these regions.

The use of single-exon mutational screening methods, like the one used in this study, could mask the presence of genomic rearrangements, including deletions or duplications. Risk factors for these events are the presence of short repetitive sequences serving as a substrate for recombination events between partially homologous sequences [22,23]. A detailed search for such sequences, mainly the interspersed elements, revealed that only intron 4, the longest intron of the gene with 12.7 kb, contains some short interspersed nuclear elements (SINEs) also named Alu sequences (23 elements) and four long interspersed nuclear elements (LINEs). However, in view of the high frequency (~50%) of c.305-9G > T polymorphism (Table 2), common genomic deletions encompassing the 3′ UTR *PV ALB* region can be considered as an unlikely event.

The rationale to test the potential role of *PV ALB* in GS was based on the fact that PV regulates the expression of NCC in the mouse kidney. Our negative results, which
suggest that PV ALB is not involved in GS, could be explained by inter-species differences in the structure or function of the DCT. Reconstruction studies of the mouse nephron have shown special features in the length, transition and ultrastructure of segments including the DCT, which could have a physiologic importance [24]. Species differences in the response to thiazide diuretics have been observed [8]. In marked contrast to the GS in humans, the NCC-null mice showed no salt-losing phenotype and no disturbances in acid-base homeostasis [25]. Furthermore, the regulation of NCC in the DCT, in response to dietary NaCl content, angiotensin II or WNK1/4 kinases, could explain patients with hypokalemic salt-losing tubulopathies. Am J Med 2002; 112: 183–191

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