Spectrum of Mutations in Gitelman Syndrome

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ABSTRACT

Gitelman's syndrome (GS) is a rare, autosomal recessive, salt-losing tubulopathy caused by mutations in the *SLC12A3* gene, which encodes the thiazide-sensitive NaCl cotransporter (NCC). Because 18 to 40% of suspected GS patients carry only one *SLC12A3* mutant allele, large genomic rearrangements may account for unidentified mutations. Here, we directly sequenced genomic DNA from a large cohort of 448 unrelated patients suspected of having GS. We found 172 distinct mutations, of which 100 were unreported previously. In 315 patients (70%), we identified two mutations; in 81 patients (18%), we identified one; and in 52 patients (12%), we did not detect a mutation. In 88 patients, we performed a search for large rearrangements by multiplex ligation-dependent probe amplification (MLPA) and found nine deletions and two duplications in 24 of the 51 heterozygous patients. A second technique confirmed each rearrangement. Based on the breakpoints of seven deletions, nonallelic homologous recombination by *Alu* sequences and non-homologous end-joining probably favor these intragenic deletions. In summary, missense mutations account for approximately 59% of the mutations in Gitelman's syndrome, and there is a predisposition to large rearrangements (6% of our cases) caused by the presence of repeated sequences within the *SLC12A3* gene.

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Gitelman's syndrome (GS, MIM 263800) is a rare salt-losing tubulopathy[1] characterized by hypokalemic metabolic alkalosis, hypomagnesaemia, and hypocalciuria. Loss of function mutations in the *SLC12A3* gene encoding for the thiazide-sensitive NaCl cotransporter (NCC) are responsible for most of the cases.[2,3] GS is inherited as an autosomal recessive trait, and homozygous and combined heterozygous mutations are expected.[1] However, between 18 and 40% of patients with clinical GS are usually found to carry only one mutant allele after *SLC12A3* screening.[4–6] These incomplete genetic results raise the possibility of an excess of clinically suspected cases, genetic heterogeneity, or a failure in the mutation detection process. These issues have already been extensively discussed.[7,8] However, genetic heterogeneity also exists, and a minority of patients with the GS phenotype harbor mutations at the *CLCNKB* gene.[9,10]

An important cause of apparently negative genetic testing could be large genomic rearrangements that are missed by direct sequencing and that usually account for 5 to 15% of the molecular defects responsible for autosomal recessive diseases.[11,12] Several techniques exist for detecting these large deletions or insertions, such as the multiplex ligation-dependent probe amplification (MLPA) assay[11,13] or the quantitative multiplex PCR of short fluorescent fragments (QMPSF),[14] previously used in our laboratory.[15] However, to date, a systematic screening for large genomic rearrangements at the *SLC12A3* gene in GS is lacking, and only 1[16] of the 143 reported mutations in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) is a large genomic rearrangement.

Here we report the molecular analysis of the *SLC12A3* gene in a large cohort of 448 patients in whom GS was suspected. Direct sequencing showed homozygosity and combined heterozygosity in 70% of patients and only one mutated allele in 18% of patients, and a search for large genomic rearrangements was performed in 88 GS patients (51 heterozygous for point mutations 26 without mutation and 11 patients with homozygous mutations without consanguinity history). We show that large rearrangements may account for ≥6% of all mutations detected at the *SLC12A3* gene in GS patients and therefore could improve the sensitivity of genetic testing to >80%.

RESULTS

Results of the First Screen by Direct Sequencing Analysis

Of 448 index cases, two affected alleles were identified in 315 patients (70%): 79 of them were homozygous (25%) and 236 were compound heterozygous (74.9%). Only one mutant allele was detected in 81 patients (18%), and the wild-type genotype was detected in 52 patients (11.6%). The list of point mutations found is given in Supplementary Table 1: 172 different mutations were detected, spread throughout the gene. These included 64% missense, 14% frameshift, and 2% in-frame small deletions or insertions and 14% splice and 6% nonsense mutations. Figure 1 shows the distribution of the 711 mutated alleles along the 26 exons of the *SLC12A3* gene. There were

Table 1. Rearrangements detected by MLPA in 24 GS patients with one heterozygous mutation in the *SLC12A3* gene detected by direct sequencing

Patient	Nucleotide*	Protein	Exon/ Intron	Reference	MLPA Heterozygous del or dup	Second Technique for Confirmation
BT038	c.3077C>T	p.Thr1026Ile	26	18	E2_E3del	Long range PCR and breakpoint sequencing
BT213	c.1046C>T	p.Pro349Leu	8	1	E1_E7del	SNPs analysis and oligo array comparative genomic hybridization
BT231	c.1195C>T	p.Arg399Cys	10	16	E9del	Long range PCR
BT243	c.1519C>T	p.Arg507Cys	12	This study	E4_E5del	Long range PCR and breakpoint sequencing + E6del by QMPSF
BT247	c.938C>T	p.Ala313Val	7	16	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT004	c.1664C>T	p.Ser555Leu	13	16	E19_E23del	QMPSF, Long range PCR and breakpoint sequencing
GT034	c.965–2_965–1dup	Splice defect	7	This study	E4_E5del	Long range PCR and breakpoint sequencing + E6del by QMPSF
GT059	c.2891G>A	p.Arg964Gln	25	1	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT121	c.2981G>A	p.Cys994Tyr	26	29	E18del	QMPSF, Long range PCR and breakpoint sequencing
GT122	c.2965G>A	p.Gly989Arg	21	19	E2_E3del	QMPSF, Long range PCR and breakpoint sequencing
GT137	c.2576T>C	p.Leu859Pro	22	1	E1_E3dup	QMPSF
GT142	c.2687G>A	p.Arg896Gln	23	30	E14del	QMPSF, Long range PCR and breakpoint sequencing
GT165	c.2576T>C	p.Leu859Pro	22	1	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT185	c.1825 + 1del	Splice defect	14	This study	E1_E7del	QMPSF for exons 1 to 3 and 6
GT187	c.2576T>C	p.Leu859Pro	22	1	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT196	c.473G>A	p.Arg158Gln	3	29	E24_E25del	Long range PCR and breakpoint sequencing
GT243	c.2981G>A	p.Cys994Tyr	26	29	E4_E5del	Long range PCR and breakpoint sequencing + E6del by QMPSF
GT278	c.1387G>A	p.Gly463Arg	11	This study	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT281	c.626G>A	p.Arg209Gln	5	16	E26del	QMPSF, Long range PCR and breakpoint sequencing
GT285	c.2929C>T	p.Arg977X	25	1	E14del	Long range PCR and breakpoint sequencing
GT291	c.533C>T	p.Ser178Leu	4	16	E1_E4dup	QMPSF
B026	c.1095 + 4A>G	Splice defect	8	This study	E26del	QMPSF, Long range PCR and breakpoint sequencing
B099	c.2883 + 1G>T	Splice defect	24	1	E26del	QMPSF, Long range PCR and breakpoint sequencing
B104	c.1883C>G	p.Ser628Trp	15	This study	E26del	QMPSF, Long range PCR and breakpoint sequencing

Numbering is according to the cDNA sequence (GenBank : NM_000339.2). The A of the ATG of the initiator Methionine codon is denoted as nucleotide 1.

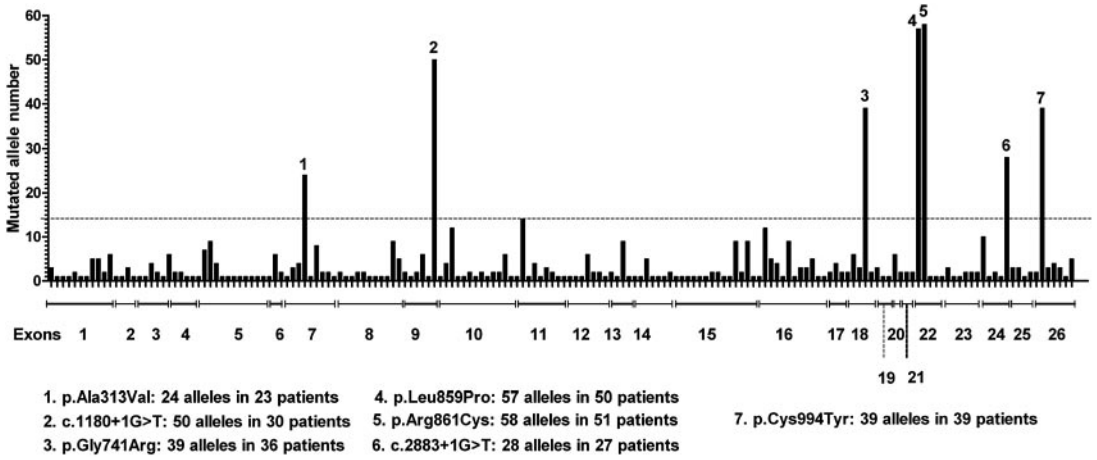

Figure 1. Frequency and distribution of the 172 detected mutations in 711 alleles. On the horizontal axis, each bar represents one mutation (there is no relation with the actual position in the exon). Dotted line corresponds to an allele frequency >2%.

seven recurrent mutations, recurrence being defined arbitrarly by an allele frequency >2%, and included five missense amino acid changes found in 217 alleles (199 patients) and two splice mutations detected in 78 alleles (57 patients). They were mainly found in heterozygous compound subjects, except for c.1180 + 1G>T, which is highly prevalent in the Gypsy population.[17] In the entire set of 172 point mutations, 100 have not been described before (53 missense, 19 frameshift, 16 splice, 6 nonsense, and 6 in-frame mutations; Supplementary Table 1, A–C). Supplementary Table 2 sums up the novel missense mutations and their *in silico* predictions.

Screening for Genomic Rearrangements in Simple Heterozygotes

We decided to screen for large rearrangements at the *SLC12A3* gene in patients with only one mutation detected by direct sequencing. Only 51 of the 81 samples were of sufficient DNA quality to be screened by MLPA or QMPSF. Rearrangements were found in 24 of them (47%, Table 1); single exon deletions were observed in 13 samples (E9del, $n = 1$; E14del, $n = 2$; E18del, $n = 1$; E26del, $n = 9$); deletions of two or more exons were detected in 9 samples (E1_E7del, $n = 2$; E2_E3del, $n = 2$; E4_E5del, $n = 3$; E19_E23del, $n = 1$; E24_E25del, $n = 1$); and duplications were detected in 2 samples (E1_E3dup and E1_E4dup). Two examples of these rearrangements are shown in Figure 2A.

As a second screening, we performed QMPSF analysis targeting the exon 6 of the *SLC12A3* gene, not included in MLPA kit. This screening allowed the detection of E6del in only the three patients harboring the E4_E5del, thus extending the deletion to exon 6 (E4_E6del). QMPSF was also used to confirm E26del detected in nine patients, E19_E23del, and E1_E3dup (Figure 2B).

Clinical and biologic features of patients harboring one heterozygous rearrangement are shown in Table 2. All had a profound hypokalemia accompanied with metabolic alkalosis and usually hypomagnesaemia.

The updated spectrum of mutations detected in our cohort of GS patients, including genomic rearrangements, is shown in Figure 3. Whereas missense mutations were the majority (59%), small insertions or deletions detected by direct sequencing represent 14% and large rearrangements detected by MLPA and QMPSF accounted for 6% of the entire set of mutations.

Further Genetic Screening in Subjects with No *SLC12A3* Mutation

Because the phenotype caused by *CLCNKB* mutations may vary from the various types of Bartter syndrome to GS, we first analyzed the *CLCNKB* gene (sequencing and MLPA or QMPSF) in 49 of the 52 patients who tested negative for the *SLC12A3* gene. Mutations were detected in 14 patients; Supplementary Table 4, A and B, summarizes the mutations detected and clinical data of these patients. We performed a search for genomic rearrangements in *SLC12A3* gene by MLPA and QMPSF in 26 of those 38 remaining patients for whom the DNA quality was sufficient. No abnormality was detected, suggesting either another mutation at the *SLC12A3* gene that was not detected by our screening or further genetic heterogeneity. Patients without mutations had similar features to the *SLC12A3* mutated patients (Supplementary Table 3).

Further Genetic Screening in Homozygous Subjects without Notion of Consanguinity

In a subset of 28 patients belonging to the group of homozygous patients, the SNPs detected along the gene were homozygous, despite the absence of consanguinity. To exclude one heterozygous deletion, we were able to perform MLPA in 11 of them. Neither deletions nor duplications were detected in this group.

Characterization of the Deletion Breakpoints

Long-range PCR completed by direct sequencing of the abnormal allele was first performed in nine patients (Supplementary Figure 1). Six different deletions (from 1.1 to 10.7 kb size) were found (Figure 4A): E2_E3del in two patients (c.282 + 667_c.506–205del and c.283–273_c.506–213del), the same E4_E6del

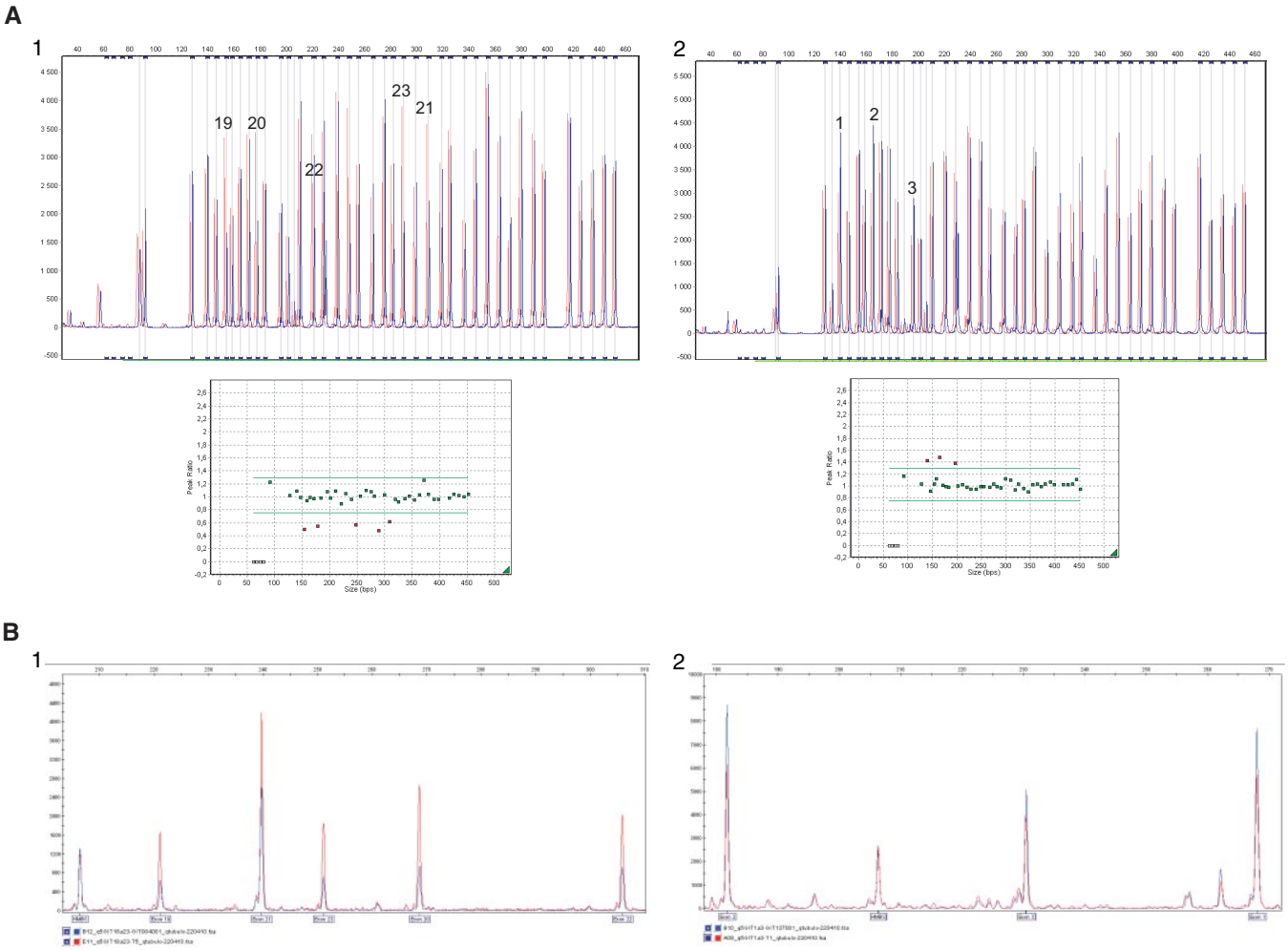

Figure 2. MLPA and QMPSF electropherograms for *SLC12A3* from two patients. For MLPA, each peak represents one exon of the *SLC12A3* gene and 13 control probes. For QMPSF, each peak represents one analyzed exon and the HMBS internal control. Control samples are shown in red and patients' samples in blue. (A1) The MLPA half doses for exons 19 to 23 in patient GT004 and peak height ratio showing the deleted exons with ratio <0.7. (A2) MLPA duplication of exons 1 to 3 in patient GT137 and peak height ratio showing the duplicated exons with ratio >1.3. (B1) QMPSF half doses for exons 19 to 23 in patient GT004. (B2) QMPSF duplication of exons 1 to 3 in patient GT137.

(c.506–315_852 + 185del) in three patients, the same E14del (c.1169 + 773_c.1825 + 247del) in two other patients, a 1.3-kb E18del (c.2178 + 269 c.2285 + 685del) in one patient, and a 10.7-kb E24_E25del (c.2748–324_c.2952–505) in one patient.

We also paid particular attention to a patient and his family with a large deletion containing the 5′ part of the gene up to exon 7 (Figure 5A). First, single nucleotide polymorphism (SNP) segregation analysis was performed by genotyping 16 SNPs at the SLC12A3 locus. The proband was homozygous for 14 of the16 SNPs, whereas his mother was heterozygous for 4 of the 7 SNPs lying in the intergenic region proximal to the *SLC12A3* gene and for 7 of 9 SNPs in the *NUP93* gene (Figure 5B1). Thus, the deletion mapped in an 89.9-kb region between rs2043635 (intron 5 of *NUP93* gene; 56,818,987 Mb [hg19]) and rs11640954 (intron 8 *SLC12A3* gene; 56,908,884 Mb [hg19]). To define this more precisely, we performed comparative genomic hybridization microarray analysis that allowed limiting the deletion size to 13,090 bp (Figure 5B2). On the centromeric side (q-arm), the closest probe that tested positive was mapped at position 56,899,150 Mb within the 5′UTR of *SLC12A3*. On the telomeric side, the closest probe that tested positive was located in exon 9 of *SLC12A3* at position 56,912,240 Mb.

High Number of Low Copy Repeats Sequences Favor Most Intragenic Deletions

Sequence alignment using RepeatMasker allowed identification of the presence of 41 *Alu* and 11 *LINEs* sequences within the *SLC12A3* gene. Interestingly, the breakpoints of all of the genomic rearrangements identified in our patients contained low copy repeats (LCRs; Figure 4, A and B). For example, the 3342- and 2720-bp E2_E3del probably originated from the

Table 2. Clinical and biological characteristics of GS patients with one heterozygous mutation in the *SLC12A3* gene and a large heterozygous rearrangements at the same gene

Patient	Age at Diagnosis (years)	Gender	Clinical Presentation	Plasma Laboratory Findings							Urinary Laboratory Findings	
				Na (mmol/L) (reference range, 135 to 145)	K (mmol/L) (reference range, 3.5 to 4.5)	Cl (mmol/L) (reference range, 95 to 107)	HCO_3 (mmol/L) (reference range, 22 to 28)	Mg (mmol/L) (reference range, 0.60 to 1.05)	Renin	Aldosterone	K (mmol/L)	Ca/creat (reference range, 0.04 to 0.37)
BT038	26	F	Fortuitous diagnosis	138	2.7	99	27	0.40	High	High	90	ND
BT213	16	M	Growth retardation	137	1.3	94	24	0.50	High	Normal	53	ND
BT231	2	M	Severe dehydration after diarrhea and growth retardation	141	3.5	100	27	ND	ND	High	185	ND
BT243	32	M	Paresthesias and constipation	138	2.6	102	26	0.84	Normal	Normal	35	ND
BT247	31	F	Growth retardation cramps, tetany, paresthesias	139	2	98	27	0.44	High	Normal	51	0.03
GT004	53	F	Fortuitous diagnosis	ND	2.5	97	31	0.40	High	Normal	68	0.15
GT034	30	F	Fortuitous diagnosis	ND	2.8	98	ND	0.56	ND	ND	59	0.29
GT059	28	M	Fortuitous diagnosis	137	2.4	97	30	0.55	High	High	59	0.28
GT121	49	M	Cramps, malaise with syncope	142	2.9	100	33	0.62	High	High	62	ND
GT122	13	F	Cramps, polyuria	ND	2.8	100	ND	0.70	High	Normal	23	0.01
GT137	37	M	Fortuitous diagnosis	139	3.1	94	36	0.41	ND	ND	72	ND
GT142	25	F	Asthenia, chondrocalcinosis	139	2.3	ND	26	0.34	High	Normal	96	ND
GT165	37	F	Fortuitous diagnosis	139	2.5	96	29	0.50	High	Normal	32	0.01
GT185	21	M	Fatigue, cramps	143	2.6	ND	31	0.53	High	Normal	ND	0.27
GT187	23	F	Palpitations, lypothymia, paresthesias	139	2.7	102	32	0.57	High	Normal	34	ND
GT196	12	M	Growth retardation	136	2.7	97	29	0.71	ND	High	47	ND
GT243	5	M	Growth retardation	ND	2.9	102	26	0.67	High	Normal	134	0.03
GT278	5	M	Fortuitous diagnosis	140	1.9	100	28	0.62	Normal	Normal	265	ND
GT281	15	M	Thoracic pain and palpitations	ND	2.8	100	23	0.68	High	High	106	0.05
GT285	32	F	Chondrocalcinosis	139	2.5	94	30	0.54	ND	ND	86	0.08
GT291	47	M	Right hemiparesis	142	1.7	98	35	0.58	Normal	Normal	7	ND
B026	48	M	Familial hypokalemia	142	3	99	21	0.64	High	Normal	97	0.09
B099	2	M	Abdominal pain	138	2.6	97	27	0.60	High	Normal	52	0.3
B104	6	F	Abdominal pain	133	2	91	33	0.83	High	Normal	94	0.28

F, female; M, male; ND, not determined.

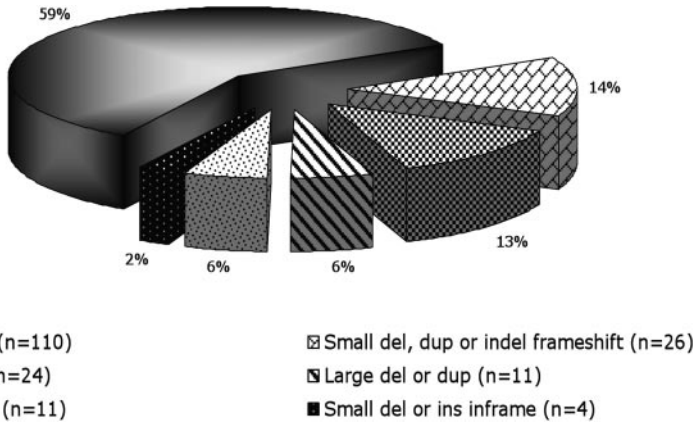

Figure 3. Pattern of mutations by type including genomic rearrangements at the *SLC12A3* gene.

crossing over between two different *AluSx* repeats in intron 1 (312 and 237 bp) and the same *AluSx* repeat in intron 3 (256 bp). Similarly, the breakpoints for the 1508-bp deletion, which includes exons 4 to 6, were inside a sequence shared by the 256-bp *AluSx* repeat in intron 3 and the 309-bp *AluSx* repeat in intron 6. The breakpoints of the 1355-bp deletion were inside a sequence shared by the 297-bp *AluSx* repeat in intron 17 and the 308-bp *AluY* repeat in intron 18.

For two other deletions, the 1.1-kb E14del and the larger 10.7-kb E24_25del, the breakpoints were within nonhomologous LCRs: an *AluY* and a *MER115* repeat in introns 13 and 14 and *L2a_LINE* and *L2b_Line* repeats in introns 23 and 25, respectively (Figure 4A). The sequence alignments at the breakpoint junctions for these two deletions showed six nucleotides with microhomology for the 10711-bp deletion and three nucleotides with microhomology and GAG rearrangement-promoting elements for the 1183-bp deletion (Figure 4C, deletions 2 and 4). These data strongly suggest nonhomologous end-joining as the causal mechanism for these deletions, facilitated by the presence of rearrangement-promoting elements inside the LCRs.

A Particular Rearrangement Was Observed for the Recurrent E26del

Taking into account the data obtained in the characterized deletions, we searched whether LCRs could also explain the recurrent E26del. Indeed, four *AluSx* and two *AluJb* are dispersed in intron 25 and one *AluSx* ~500 bp after the stop codon. Long-range PCR amplification performed with a forward primer in exon 25 and a reverse primer distal to *AluSx* in the 3′ breakpoint resulted in the amplification of two bands in the nine patients with E26del detected by MLPA (Supplementary Figure 2). Direct sequencing of the short product showed the same complex rearrangement in the nine unrelated patients: a 2412-bp deletion with a 25-bp insertion (c.2952–1593_*677delins25). The 5′ breakpoint is 50 bp after an *ALuJb* repeat in intron 25, and the 3′ breakpoint is inside an *AluSx* repeat in the 3′UTR region (Figure 4A and 4C).

DISCUSSION

Based on a complete molecular investigation of the *SLC12A3* gene on the largest cohort reported thus far, we showed the value of combining several techniques to finally achieve an 91% mutation detection rate in GS. Next to the identification of missense mutations (59% of the cases) and small insertions or deletions (14%) by direct sequencing, we used MLPA and QMPSF, which allowed the detection of large rearrangements in 24 of 51 GS patients (47%) known to be heterozygous for a point mutation. This suggests that almost one half of the patients suspected to have GS with only one mutated allele detected by direct sequencing (18% of our series and of the smaller series described by Ji *et al.*[6]) have a large genomic rearrangement on the other allele.

To date, >180 mutations in *SLC12A3* have been reported, about 70% of which being missense mutations. In this study, direct sequencing allowed the detection of 110 different missense mutations in 290 subjects (64% of the mutations found), 51 of them being novel. Supplementary Table 2 sums up *in silico* predictions for these changes. None of these amino acid changes are known SNPs, and they were not detected in 200 control chromosomes. Furthermore, they were not detected either in 220 control chromosomes in another European study,[5] and only one (p.Gly779Glu) was detected (allelic frequency of 0.02%) in 1985 unrelated subjects from the Framingham cohort.[6] This missense change was predicted as functional and was associated with lower BP. Taken together, the novel 51 missense mutations described in this paper are rare variants very likely to be pathogenic.

Five previously described missense mutations were particularly frequent in our unrelated patients (Figure 1), raising the possibility of neutral polymorphisms. Two of them, p.Gly741Arg and p.Cys994Tyr detected in 36 and 38 patients, respectively, led to a total or partial loss of function when expressed *in vitro* into *Xenopus laevis* oocytes.[18,19] Mutations p.Leu859Pro and p.Arg861Cys have not been expressed *in vitro*; however, three *in silico* methods predicted these amino acid changes to be pathogenic. Thus, there are strong arguments for p.Gly741Arg, p.Cys994Tyr, p.Leu859Pro, and p.Arg861Cys being hotspot mutations. In contrast, the previously reported p.Ala313Val mutation was predicted *in silico* as nondeleterious. This variant was detected in 19 GS patients: it was associated with a second pathogenic mutation in 18 patients and was homozygous in 1 patient, suggesting that it is deleterious.

All mutations described in this study, including large rearrangements and the 100 novel point mutations, will be available online at the European Network for the Study of Orphan Nephropathies website (http://www.eunefron.org/).

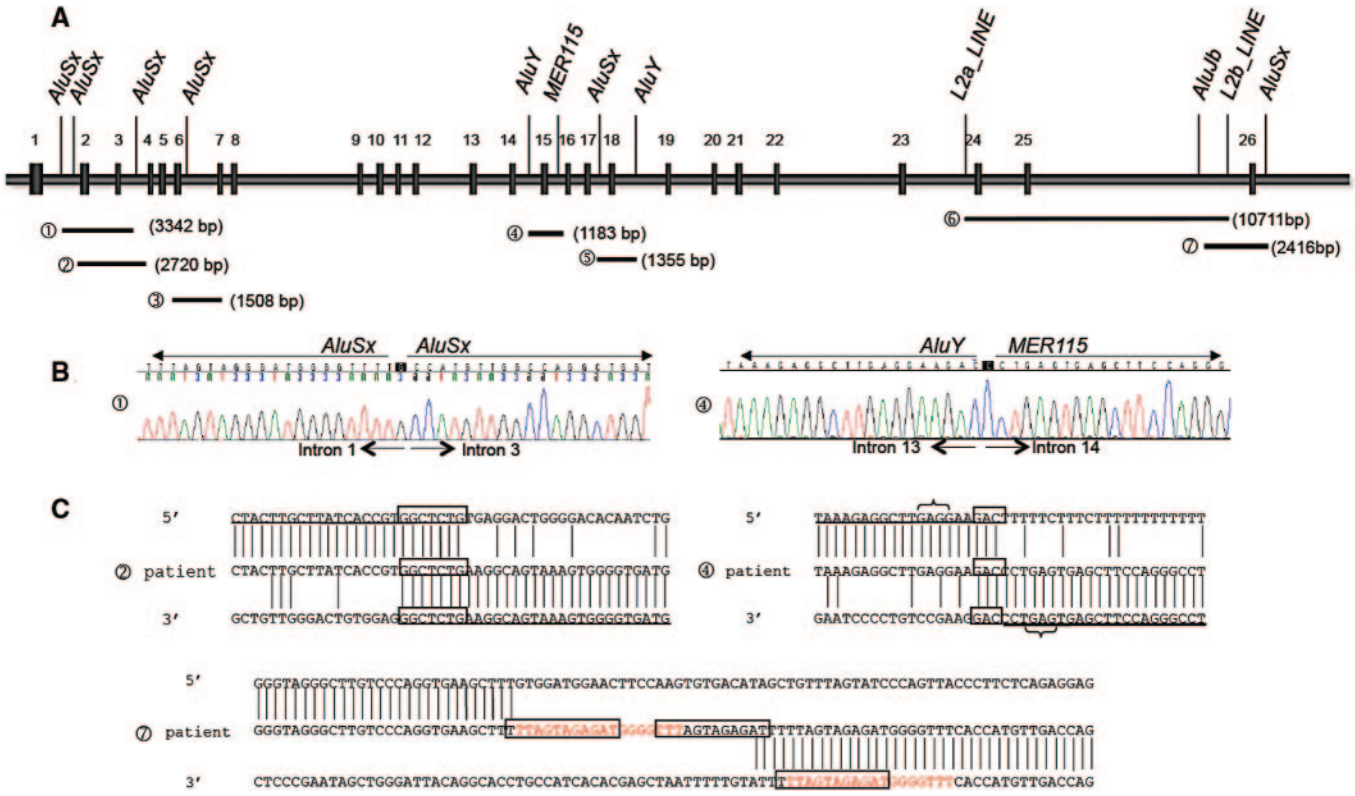

Figure 4. Mapping and characterization of breakpoints of *SLC12A3* heterozygous deletions. (A) Schematic representation of the genomic organization of the 26 exons of the *SLC12A3* gene and the location of breakpoints of 7 deletions: (A1) E2_E3del: 3342 bp deletion (c.282 + 667_c.506–205del). (A2) E2_E3del: 2720 bp deletion (c.283–273_c.506–213del). (A3) E4_E6del: 1508 bp deletion (c.506–315_852 + 185del). (A4) E14del: 1183 bp deletion (c.1169 + 773_c.1825 + 247del). (A5) E18del: 1355 bp deletion (c.2178 + 269_c.2285 + 685del). (A6) E24_E25del: 10711bp deletion (c.2748–324_c.2952–505). (A7) E26del: 2416bp deletion (c.2952–1593_*677delins25). The LCRs on or near the breakpoints are indicated. (B) Sequence analysis showing breakpoints of deletions 1 and 4. Breakpoints of deletion 1 are inside *Alu* repeats, suggesting a nonallelic homologous recombination. Breakpoints of deletion 4 are inside nonhomologous LCRs. (C) Sequence alignments at the breakpoints of two *SLC12A3* heterozygous deletions probably originated from nonhomologous end-joining and one complex rearrangement. For deletions 2 and 4, boxes indicate the nucleotide microhomology. Brackets depict short motifs that might have facilitated the rearrangements. For deletion 7, boxes indicate the repetitive motifs; the read sequence is the 18-bp repetition that is present in inserted sequence and in the 3′UTR breakpoint.

One of our major findings was the detection of large rearrangements in GS patients, representing about one half of the heterozygous patients tested. The breakpoints of these large-scale mutations were found to correspond to a high frequency of repetitive sequences within the *SLC12A3* gene. More than a million of *Alu* sequences are dispersed throughout the genome, and regions with high *Alu* repeat content are prone to nonallelic homologous recombination, which may cause inherited diseases.[20] The *SLC12A3* gene contains 41 *Alus* and 11 *LINEs* dispersed in intronic regions corresponding to 22.5 and 6% of the gene sequence, respectively. Four of seven deletions were the consequence of *Alu*-mediated recombination: *AluSx* sequences involved in E2_E3del and in E4_E6del in two and three patients, respectively, and the *AluSx* and *AluY* sequences involved in E18del share >80% identity. The breakpoints of two other deletions were located in nonhomologous LCRs: *AluY* and *MER115* for E14del and *L2a* and *L2b_LINE* for E24_E25del. However, we observed nucleotide microhomology and, in one case, the presence of the recombinant-promoting element GAG at breakpoint junctions. These data correspond to the characteristics observed in the nonhomologous end-joining mechanism.[21] This mechanism, used by eukaryotic cells to repair double-strand DNA breaks, could be stimulated by genomic architecture (*i.e.*, LCRs and sequence motifs).

The breakpoints analysis of the recurrent E26del detected in probands of nine unrelated families showed the presence of a complex indel mutation (2412-bp deletion and 25-bp insertion). The 5′ breakpoint was in close proximity to *AluJb* and the 3′ breakpoint was inside an *AluSx*. Repetitive motifs were detected in the insertion and in the 3′ breakpoint (Figure 4C, deletion 7). Similar indels have been described in the *NF1* and *CFTR* genes.[22,23] A multistep mechanism facilitated by the presence of LCRs is likely to favor them, as has been suggested for other complex rearrangements.[24] The deletions and duplications detected have not been described as copy number

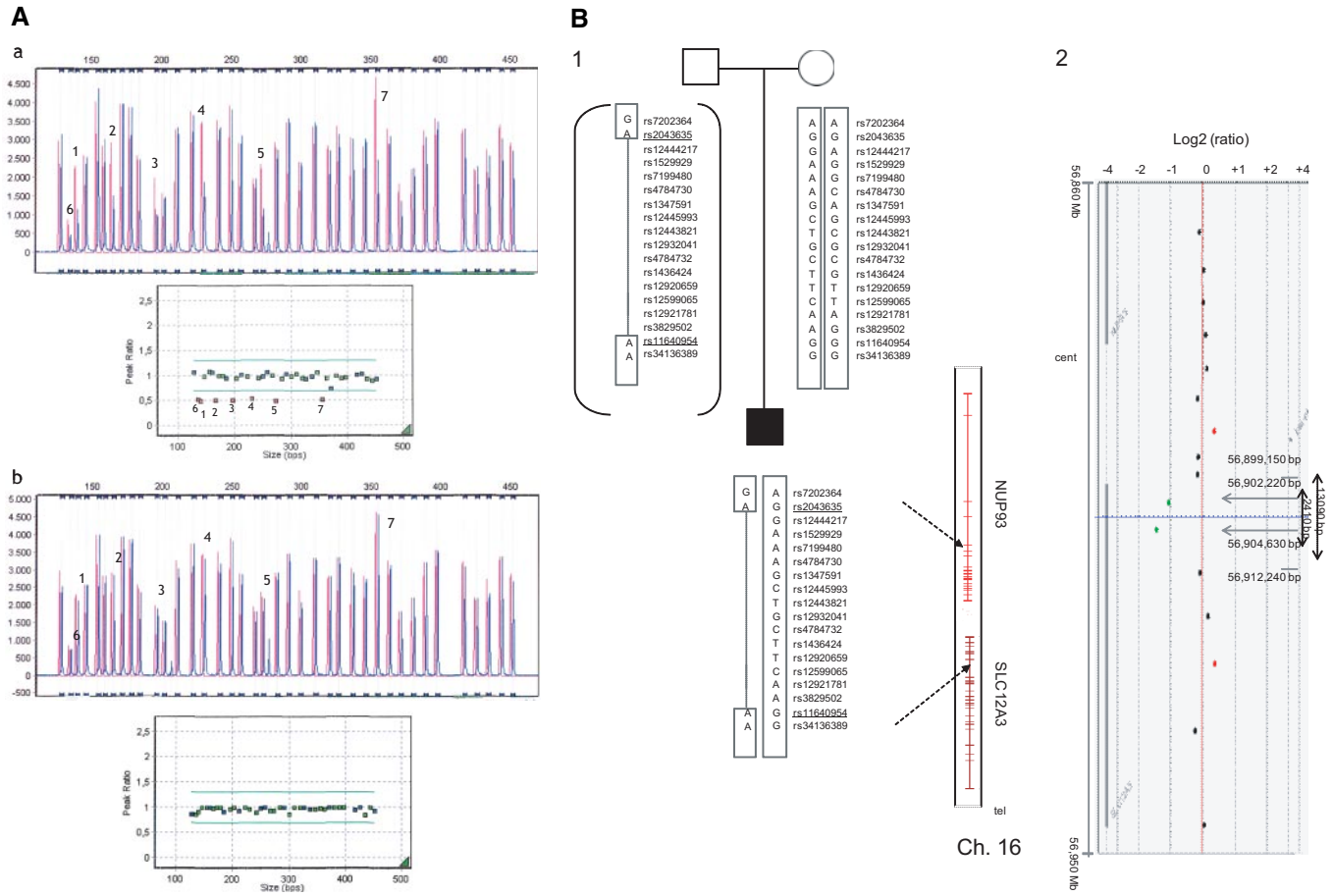

Figure 5. Characterization of *SLC12A3* E1_E7del for patient BT213. (A) MLPA electropherograms for the proband and his mother. Each peak represents one exon of the *SLC12*A3A gene and 13 control probes. (Aa) The electropherogram of the proband shows abnormal height peaks (in blue) in seven exons (numbered) compared with a normal control (in red). The peak ratio graph shows the 7 deleted exons for which the ratio was <0.7. (Ab) The electropherogram of the mother showing normal height peaks for all of the exons (blue) compared with a normal control (red). Normal peak ratios revealed an equivalent number of copies. (B1) Pedigree showing the constructed haplotypes resulting from biallelic SNP genotyping. The proband is shown in black. The SNPs covering the approximately 16-kb region upstream from the *SLC12A3* gene are shown, including the two SNPs mapped to intron 8 of the *SLC12A3* gene. The two deletion-flanking SNPs, covering a region of 89.9 kb, are underlined. Chromosome 16 showing both *SLC12A3* and *NUP93* (on the right). Cen, centromere; Tel, telomere. (B2) Results of molecular karyotyping. Array-comparative genomic hybridization log 2 ratio plot of chromosome 16 of the proband from the Agilent 244k, showing a 13-kb deletion from the probe lying within the 5′UTR of the *SLC12A3* gene (56,899,150 Mb; hg19) to the probe that is specific to exon 9 (56,912,240 Mb; hg19). The deleted probes are in green, and unaltered probes (gain or loss) are in black.

variants in healthy controls (Database of Genomic Variants at http://projects.tcag.ca/variation/, Copy number variation project at the Children's Hospital of Philadelphia at http://cnv.chop.edu, and 1000 Genomes at http://browser.1000genomes.org/index.html).

Of note, large-scale mutations were not associated with a more severe phenotype when clinical and biochemical data from patients harboring a heterozygous deletion were compared with those from subjects homozygous or compound heterozygous for two missense mutations.

This was true for the age at diagnosis (17 [range, 2.25 to 63] *versus* 25.5 [range 2 to 53] years) or the importance of hypokalemia (2.62 [range, 1.8 to 3.4] *versus* 2.60 [range, 1.3 to 3.5] mmol/L). Also, the heterozygous E1_E7del was detected in two patients: one with a relatively severe phenotype and the other one with a mild phenotype. This deletion was identified previously in one homozygous and one heterozygous member of an Amish kindred,[16] who were not mentioned as having a particularly severe phenotype compared with the 48 other GS patients. Thus, a null allele does not seem to be more detrimental than a punctual loss of function mutation, although the nature and position of *SLC12A3* mutations have been thought to influence the severity of GS.[5]

Concerning the group of patients without mutations, most of them have a GS-like phenotype. Only three of them had an early presentation (BT100, B001, and B059 in Supplementary Table 2). In these patients, another cause of severe hypokalemic metabolic alkalosis such as congenital chloride diarrhea could be considered.

Nevertheless, in these patients, there was no history of polyhydramnios, premature birth, or diarrhea. Furthermore, urinary electrolytes at diagnosis or on follow-up (5 to 10 years) showed sodium, potassium, and chloride wasting.

In conclusion, this molecular analysis of a large cohort of 448 GS patients, including the first search for large-scale mutations, showed that, despite the high efficiency of direct genomic sequencing in detecting the vast majority of the *SLC12A3* mutations found in this disorder, a complementary technique is necessary to achieve a high mutation detection rate, especially for those patients in whom only one mutation had been detected. We confirmed that MLPA is an efficient technique for analyzing large genomic rearrangements, which account for ≥6% of mutations detected in our patients with GS. Moreover, we showed that nonallelic homologous recombination by *Alu* sequences and nonhomologous end-joining are most likely to be responsible for intragenic deletions. Finally, we detected *CLCNKB* mutations in 3% and excluded mutations and large rearrangements of the *SLC12A3* gene in 8% ($n = 36$) of our GS patients, which questions the clinical diagnosis of GS and raises the possibility of genetic heterogeneity in this inherited tubulopathy.

CONCISE METHODS

Patients

Between January 2001 and August 2009, samples from 448 probands (219 males and 229 females) with a clinical diagnosis of GS were received at the Genetics Department at Hopital Européen Georges Pompidou, Paris. Most samples were sent from nephrology and endocrinology services thanks to the French Network for Tubulopathies and the European Network for the Study of Orphan Nephropathies (http://www.eunefron.org/). A few samples ($n = 6$) were also received from other countries (Austria, Canada, Luxemburg, and Portugal). Appropriate informed consent was obtained from all patients and their families. They were selected according to the classical criteria for GS: renal hypokalemia, metabolic alkalosis, hypomagnesaemia, hypocalciuria, and secondary hyperreninism and hyperaldosteronism. Nevertheless, because plasma renin and aldosterone were not measured in all cases at diagnosis and because the absence of hypomagnesaemia or hypocalciuria has been described in some genetically confirmed cases,[25,26] we did not systematically require all of the criteria to be met before performing the genetic testing.

Detection of Point Mutations

Total DNA was extracted from blood peripheral leukocytes by standard procedures. Mutation analysis was performed by PCR amplification and direct sequencing of exons and flanking intronic sequences of the *SLC12A3* gene, mainly as described previously[4] (primers available upon request), on an ABI Prism 3730XL DNA Analyzer Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA).

Two amino acid changes initially described as mutations and then as SNPs were considered as SNPs in this study: p.Arg913Gln (rs11643718) and p.Ala728Thr (rs61730207). In contrast, we considered the amino acid changes p.Arg209Trp (SNP rs28936388), p.Gly264Ala (SNP rs1529927), and p.Arg928Cys (SNP rs12708965) as loss of function mutations. Indeed, *in vitro* expression of p.Arg209Trp and p.Gly264Ala has been shown to produce a significant reduction in NCC activity,[18,27] and the p.Arg928Cys change is predicted *in silico* to be deleterious and is also considered disease-causing.[6] Eight novel missense changes detected in this study (p.Ala13Pro, p.Arg83Gln p.Val404Ile, p.Thr428Pro, p.Ser546Gly, pSer833Leu, p.Glu915Ala, and p.Gln1021Lys) were predicted *in silico* as nondeleterious (Supplementary Table 2) and were characterized as variants of unknown significance. The p.Ala322Val was also predicted as nondeleterious, but this change implicates the first nucleotide of exon 8 and may be considered as a splice mutation. Indeed, ESEfinder (at http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder) predicts a loss of two enhancer motifs (SRp55 and SF2/ASF).

MLPA Analysis

We used a commercially available kit, the SALSA MLPA P136 SLC12A3 Kit (MRC Holland, Amsterdam, The Netherlands) to detect large deletions or duplications in the *SLC12A3* gene. The P136 kit contains 38 probes: 25 probes for *SLC12A3* (one for each exon except exon 6) and 13 reference probes. The detailed procedure is described in Supplementary Materials.

QMPSF

We adapted the QMPSF method[28] to detect large deletions or duplications at the *SLC12A3* gene. Details of the procedure are given in Supplementary Materials and the corresponding primers in Supplementary Table 3. This method was used to amplify exon 6 of the *SLC12A3* gene, which is not included in the MLPA *SLC12A3* kit. It was also used to confirm deletions or duplications found by MLPA, especially when it was difficult to estimate the 3′ (exon 26) or 5′ breakpoints limits (duplication of exons 1 to 3 and 1 to 4) or if no amplification was obtained by long-range PCR (deletion of exons 19 to 23).

Mapping the Deletion Breakpoints by long-range PCR

Long-range PCR and sequencing analysis were performed in 10 patients to confirm the MLPA results and to determine the deletion breakpoints. Gene-specific primers located in proximal and distal nondeleted exons were designed for each type of deletion (Supplementary Table 4). Detailed of the procedure can be found in Supplementary Materials.

Characterization of Patient BT213's Deletion

1. Refinement of the deletion length by SNPs genotyping: a search for informative SNPs (with a minor allele frequency MAF >0.3) was performed on a region containing approximately 16 kb of sequence upstream from exon 1 of the *SLC12A3* gene. Sixteen pairs of primers allowed the genotyping of 10 informative SNPs plus 6 additional SNPs with lower MAF (Supplementary Table 5). Two SNPs located in intron 8 (rs11640954 and rs34136389) were also genotyped in the patient and his mother to confirm heterozygosity in this region.

2. Further analysis by comparative genomic hybridization: to analyze the deletion breakpoints, we performed a whole genome array compara-

tive genomic hybridization analysis using an Agilent 244k oligonucleotide array (Agilent, Santa Clara, CA). The complete procedure is given in Supplementary Materials.

Bioinformatic Analysis of Mutations

Mutation interpretation and amino acid conservation in orthologs were assessed using Alamut V.1.5 software (Interactive Biosoftware, Rouen, France; http://www.interactivebiosoftware.com/). For missense mutations, the Grantham chemical distance (Grantham R. 1974) between amino acids provided by Alamut software was used to test whether the changes between the residues were likely to affect physicochemical properties. Complementary analyses were performed with SIFT (Sorting Intolerant From Tolerant, http://www.Blocks.fhcrc.org/sift/SIFT.html), PolyPhen-2 (prediction of functional effects of human nsSNPs at http://genetics.bwh.harvard.edu/pph/), and Panther (evolutionary analysis of coding SNPs at http://www.pantherdb.org/tools/csnpScoreForm.jsp). LCRs in the *SLC12A3* gene were found with RepeatMasker software at http://www.repeatmasker.org/.

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DISCLOSURES

None.

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SUPPLEMENTARY TABLES

Table 1a. Patients Homozygous for mutation in the *SLC12A3* gene detected by sequencing

Patient	Sex	Nucleotide*	Protein	Exon/ Intron	References	Consanguinity
BT004	F	c.1196_1202dup	p.Ser402X	10	2	No
BT009¥	M	c.293_296dup	p.His99GlnfsX9	2	This study	Yes
BT013	M	c.83_84insGC	p.Ser28ArgfsX19	1	This study	No
BT044	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
BT045	M	c.2221G>A	p.Gly741Arg	18	1	No
BT060	F	c.2576T>C	p.Leu859Pro	22	1	No
BT064	F	c.1095+1G>A	Splice defect	8	This study	yes
BT083	F	c.2221G>A	p.Gly741Arg	18	1	ND
BT087¥	F	c.2581C>T	p.Arg861Cys	22	4	No
BT111	M	c.1390G>A	p.Ala464Thr	11	29	ND
BT113	F	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
BT114	M	c.2576T>C	p.Leu859Pro	22	1	No
BT118	M	c.2379dup	p.Phe794ValfsX2	20	6	ND
BT122§	M	c.1180+1G>T	Splice defect	9	17	Yes
BT144	M	c.2379dup	p.Phe794ValfsX2	20	6	No
BT151	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
BT158	M	c.2120C>T	p.Ala707Val	17	This study	ND
BT160	F	c.2581C>T	p.Arg861Cys	22	4	ND
BT161	M	c.2581C>T	p.Arg861Cys	22	4	ND
BT168	M	c.2581C>T	p.Arg861Cys	22	4	ND
BT170	M	c.1095+1G>T	Splice defect	8	This study	Yes
BT175	F	c.1956del	p.Asn653ThrfsX19	16	This study	Yes
BT179¥	M	c.1844C>G	p.Ser615Trp	15	29	Yes
BT187	M	c.1247G>C	p.Cys416Ser	10	This study	ND
BT202§	F	c.1095+1G>T	Splice defect	8	This study	Yes
BT205¥	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy

BT227	M	c.625C>T	p.Arg209Trp	5	1	ND
BT229§	F	c.1387G>A	p.Gly463Arg	11	This study	No
BT233	M	c.1095+1G>T	Splice defect	8	This study	No
BT241	M	c.2581C>T	p.Arg861Cys	22	4	ND
BT246	M	c.1763C>T	p.Ala588Val	14	1	Yes
GT008	F	c.1925G>A	p.Arg642His	15	4	Yes
GT010	F	c.2581C>T	p.Arg861Cys	22	4	Yes
GT012¥	F	c.1095+1G>T	Splice defect	8	This study	Yes
GT013	F	c.1924C>G	p.Arg642Gly	15	16	Yes
GT023	M	c.473G>A	p.Arg158Gln	3	29	Yes
		c.2782C>T	p.Arg928Cys	24	4	
GT033	M	c.1180+1G>T	Splice defect	9	17	Yes
GT046	M	c.1928C>T	p.Pro643Leu	16	16	No
GT053	M	c.1539C>A	p.Tyr513X	12	This study	No
GT055	F	c.1180+1G>T	Splice defect	9	17	Yes
GT064	M	c.1180+1G>T	Splice defect	9	17	Yes
GT065	M	c.1180+1G>T	Splice defect	9	17	Yes
GT077	M	c.1180+1G>T	Splice defect	9	17	Yes
GT083	F	c.947G>T	p.Gly316Val	7	29	No
GT84**	M	c.938C>T	p.Ala313Val	7	16	No
GT093**	M	c.2576T>C	p.Leu859Pro	22	1	No
GT098**	M	c.2576T>C	p.Leu859Pro	22	1	No
GT106	M	c.1946C>T	p.Thr649Met	16	6	Yes
GT119§	F	c.2221G>A	p.Gly741Arg	18	1	No
GT120**	F	c.1180+1G>T	Splice defect	9	17	No
GT140§	M	c.1939G>A	p.Val647Met	16	6	No
GT169**	F	c.3077C>T	p.Thr1026Ile	26	18	No
GT170	M	c.1180+1G>T	Splice defect	9	17	Yes
GT173**	M	c.539_543del	p.Thr180AsnfsX77	4	This study	No

GT177**	F	c.815T>C	p.Leu272Pro	6	6	No
GT186	F	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
GT191	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
GT195	F	c.1387G>A	p.Gly463Arg	11	This study	Yes
GT197	M	c.1145C>T	p.Thr382Met	9	6	Adopted
GT199**	M	c.1939G>A	p.Val647Met	16	6	No
GT201§	F	c.1180+1G>T	Splice defect	9	17	Yes
GT206**	F	c.2576T>C	p.Leu859Pro	22	1	ND
GT216§	F	c.2576T>C	p.Leu859Pro	22	1	ND
GT219	F	c.1180+1G>T	Splice defect	9	17	Yes
GT228**	F	c.1387G>A	p.Gly463Arg	11	This study	No
GT238	F	c.488C>T	p.Thr163Met	3	29	Yes
GT244	M	c.1028T>A	p.Met343Lys	8	6	Yes
GT270	F	c.1924C>G	p.Arg642Gly	15	16	Yes
GT282	F	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
GT286**	M	c.2576T>C	p.Leu859Pro	22	1	No
GT287	F	c.625C>T	p.Arg209Trp	5	1	ND
B041	F	c.1925G>A	p.Arg642His	15	4	Yes
B069	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
B082	M	c.237_238dup	p.Arg80ProfsX35	1	2	Yes
B088	M	c.1180+1G>T	Splice defect	9	17	Yes/Gypsy
B094§	M	c.2581C>T	p.Arg861Cys	22	4	No
B096	M	c.1180+1G>T	Splice defect	9	17	Yes
B097	M	c.2883+1G>T	Splice defect	24	1	No
B108	M	c.3053G>A	p.Arg1018Gln	26	This study	Yes

*Numbering is according to the cDNA sequence (GenBank : NM_000339.2). The A of the ATG of the initiator methionine codon is denoted as nucleotide 1.
**No deletions detected by MLPA in these patients
§Parental heterozygosity confirmed for these patients
¥ Heterozygosity confirmed for one of the parents
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Table 1b. Patients compound heterozygous for mutation in the *SLC12A3* gene detected by direct sequencing

Patient	Sex	Nucleotide*	Protein	Exon/ Intron	Reference	Nucleotide*	Protein	Exon/ Intron	Reference
H112	M	c.947G>C	p.Gly316Ala	7	This study	c.1489A>T	p.Lys497X	12	16
H116§	M	c.1924C>G	pArg642Gly	15	16	c.1928C>T	p.Pro643Leu	16	16
BT002¥	F	c.2285+1G>A	splice defect	18	This study	c.2548+1G>T	splice defect	21	6
BT003	M	c.1928C>T	p.Pro643Leu	16	16	c.2221G>A	p.Gly741Arg	18	1
BT010	F	c.1964G>A	p.Arg655His	16	1	c.2221G>A	p.Gly741Arg	18	1
BT012	F	c.2221G>A	p.Gly741Arg	18	1	c.2883+1G>T	splice defect	24	1
BT015§	M	c.947G>T	p.Gly316Val	7	29	c.2191G>A	p.Gly731Arg	18	2
BT017	M	c.2687G>A	p.Arg896Gln	23	30	c.2981G>A	p.Cys994Tyr	26	29
BT018§	F	c.1387G>A	p.Gly463Arg	11	This study	c.2883+1G>T	splice defect	24	1
BT019¥	M	c.1456G>A	p.Asp486Asn	12	1	c.1924C>G	p.Arg642Gly	15	16
BT021	M	c.1928C>T	p.Pro643Leu	16	16	c.2191G>A	p.Gly731Arg	18	2
BT023	F	c.2576T>C	p.Leu859Pro	22	1	c.2890C>T	p.Arg964Trp	25	This study
BT025	M	c.696_707del	p.Met233_Val236del	5	This study	c.1852G>T	p.Ala618Ser	15	This study
BT026	M	c.602-1G>A	splice defect	4	This study	c.626G>A	p.Arg209Gln	5	16
BT028	M	c.473G>A	p.Arg158Gln	3	29	c.1836G>C	p.Trp612Cys	15	This study
BT029	M	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
BT030**	F	c.247C>T	p.Arg83Trp	1	This study	c.791G>C	p.Gly264Ala	6	31
		c.1901T>G	p.Val634Gly	15	This study				
BT032	M	c.1924C>G	p.Arg642Gly	15	16	c.2576T>C	p.Leu859Pro	22	1
BT034	M	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
BT035	F	c.1387G>A	p.Gly463Arg	11	This study	c.1946C>T	p.Thr649Met	16	6
BT039	F	c.658G>A	p.Gly220Ser	5	This study	c.1928C>T	p.Pro643Leu	16	16
BT040	M	c.1262G>A	p.Cys421Tyr	10	This study	c.2221G>A	p.Gly741Arg	18	1
BT041	M	c.644T>C	p.Leu215Pro	5	4	c.1315G>A	p.Gly439Ser	10	2

BT046	F	c.20_21del	p.Thr7ArgfsX22	1	This study	c.237_238dup	p.Arg80ProfsX35	1	2
BT048¥	M	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
BT049	M	c.2576T>C	p.Leu859Pro	22	1	c.2890C>T	p.Arg964Trp	25	This study
BT051	F	c.2827C>T	p.Arg943Trp	24	This study	c.791G>C	p.Gly264Ala	6	31
BT052	F	c.1145C>T	p.Thr382Met	9	6	c.2877_2878del	p.Arg959SerfsX11	24	32
BT055	F	c.689G>A	p.Gly230Asp	5	33	c.1844C>T	p.Ser615Leu	15	16
BT056	F	c.1287_1296del	p.Glu429AspfsX16	10	This study	c.2191G>A	p.Gly731Arg	18	2
BT058	F	c.1664C>T	p.Ser555Leu	13	16	c.1928C>T	p.Pro643Leu	16	16
BT059	F	c.434G>A	p.Arg145His	3	33	c.1145C>T	p.Thr382Met	9	6
BT062	F	c.2301del	p.Phe767LeufsX8	19	This study	c.2576T>C	p.Leu859Pro	22	1
BT065	M	c.2576T>C	p.Leu859Pro	22	1	c.3006G>C	p.Trp1002Cys	26	This study
BT066	M	c.1946C>T	p.Thr649Met	16	6	c.2891G>A	p.Arg964Gln	25	1
BT067	F	c.293_296dup	p.His99GlnfsX9	2	This study	c.2221G>A	p.Gly741Arg	18	1
BT069	F	c.1743del	p.Met581IlefsX30	14	This study	c.2883+1G>T	splice defect	24	1
BT070	M	c.1143G>A	p.Trp381X	9	This study	c.1432A>G	p.Lys478Glu	11	2
BT074	F	c.2221G>A	p.Gly741Arg	18	1	c.2981G>A	p.Cys994Tyr	26	29
BT076	M	c.514T>C	p.Trp172Arg	4	29	c.2581C>T	p.Arg861Cys	22	4
BT079	F	c.947G>T	p.Gly316Val	7	29	c.2581C>T	p.Arg861Cys	22	4
BT080	F	c.1925G>A	p.Arg642His	15	4	c.2782C>T	p.Arg928Cys	24	4
BT082	F	c.938C>T	p.Ala313Val	7	16	c.1424C>G	p.Ser475Cys	11	This study
BT084	F	c.433C>T	p.Arg145Cys	3	5	c.1924C>T	p.Arg642Cys	15	34
BT086§	M	c.1315G>A	p.Gly439Ser	10	2	c.2576T>C	p.Leu859Pro	22	1
BT088	M	c.247C>T	p.Arg83Trp	1	This study	c.1095+4A>G	splice defect	8	This study
BT089	F	c.1287_1296del	p.Glu429AspfsX16	10	This study	c.2581C>T	p.Arg861Cys	22	4
BT094	F	c.1963C>T	p.Arg655Cys	16	4	c.2981G>A	p.Cys994Tyr	26	29
BT101	M	c.1195C>T	p.Arg399Cys	10	16	c.1924C>T	p.Arg642Cys	15	34
BT106	F	c.911C>T	p.Thr304Met	7	This study	c.1751T>C	p.Leu584Pro	14	This study
BT107	F	c.1964G>A	p.Arg655His	16	1	c.2883+1G>T	splice defect	24	1

BT112	M	c.1664C>T	p.Ser555Leu	13	16	c.602-2A>G	splice defect	4	This study
BT116	M	c.1196_1202dup	p.Ser402X	10	2	c.2883+1G>T	splice defect	24	1
BT119	M	c.2089_2095del	p.Thr697GlyfsX2	17	29	c.2379dup	p.Phe794ValfsX2	20	6
BT120	F	c.2379dup	p.Phe794ValfsX2	20	6	c.1180+1G>T	splice defect	9	17
BT121**	F	c.83_84insGC	p.Ser28ArgfsX19	1	This study	c.2891G>A	p.Arg964Gln	25	1
		c.1928C>T	p.Pro643Leu	16	16				
BT123	M	c.1619A>G	p.Tyr540Cys	13	This study	c.1930del	p.Gln644SerfsX28	16	35
BT129§	F	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29
BT137	F	c.2221G>A	p.Gly741Arg	18	1	c.2581C>T	p.Arg861Cys	22	4
BT143	F	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
BT150	M	c.1387G>A	p.Gly463Arg	11	This study	c.2301del	p.Phe767LeufsX8	19	This study
BT152	F	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
BT154	M	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
BT155	F	c.1336-1G>C	splice defect	10	This study	c.2581C>T	p.Arg861Cys	22	4
BT157	F	c.1195C>T	p.Arg399Cys	10	16	c.2221G>A	p.Gly741Arg	18	1
BT162	F	c.443T>C	p.Leu148Pro	3	This study	c.626G>A	p.Arg209Gln	5	16
BT165	M	c.190_192del	p.Val64del	1	This study	c.1489A>T	p.Lys497X	12	16
BT178	M	c.160C>T	p.Arg54Cys	1	This study	c.2782C>T	p.Arg928Cys	24	4
BT183	M	c.2581C>T	p.Arg861Cys	22	4	c.3077C>T	p.Thr1026Ile	26	18
BT189	M	c.1487del	p.Gly496AlafsX33	12	This study	c.1925G>A	p.Arg642His	15	4
BT195	F	c.1095+1G>T	splice defect	8	This study	c.2581C>T	p.Arg861Cys	22	4
BT199§	F	c.2883+1G>T	Splice defect	24	1	c.3052C>T	p.Arg1018X	26	36
BT203	F	c.2581C>T	p.Arg861Cys	22	4	c.2883+1G>T	splice defect	24	1
BT204	M	c.1664C>T	p.Ser555Leu	13	16	c.2581C>T	p.Arg861Cys	22	4
BT208	F	c.2089_2095del	p.Thr697GlyfsX2	17	29	c.2576T>C	p.Leu859Pro	22	1
BT210	M	c.1946C>T	p.Thr649Met	16	6	c.2221G>A	p.Gly741Arg	18	1
BT212	F	c.1484T>C	p.Phe495Ser	12	This study	c.2221G>A	p.Gly741Arg	18	1
BT214¥	M	c.1946C>T	p.Thr649Met	16	6	c.2221G>A	p.Gly741Arg	18	1

BT216	F	c.1387G>A	p.Gly463Arg	11	This study	c.1930del	p.Gln644SerfsX28	16	35
BT224	F	c.1964G>A	p.Arg655His	16	1	c.2576T>C	p.Leu859Pro	22	1
BT228	M	c.2576T>C	p.Leu859Pro	22	1	c.2981G>A	p.Cys994Tyr	26	29
BT232	M	c.2368+5G>T	splice defect	19	This study	c.2576T>C	p.Leu859Pro	22	1
BT237	F	c.2827C>T	p.Arg943Trp	24	This study	c.3077C>T	p.Thr1026Ile	26	18
BT238§	F	c.2747+1G>C	splice defect	23	This study	c.2883+1G>T	splice defect	24	1
BT242	M	c.971_984del	p.Phe325GlyfsX3	8	This study	c.2690T>C	p.Leu897Pro	23	This study
BT248	F	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
BT250	F	c.1055C>A	p.Thr352Lys	8	This study	c.2883+1G>T	splice defect	24	1
BT259	F	c.1256del	p.Leu419ArgfsX29	10	This study	c.2981G>A	p.Cys994Tyr	26	29
BT260§	M	c.1925+1G>A	splice defect	15	6	c.2581C>T	p.Arg861Cys	22	4
BT261	F	c.947G>T	p.Gly316Val	7	29	c.293_296dup	p.His99GlnfsX9	2	This study
BT262	M	c.742-1G>A	splice defect	5	This study	c.2221G>A	p.Gly741Arg	18	1
BT263§	M	c.1832del	p.Asn611IlefsX61	15	This study	c.2581C>T	p.Arg861Cys	22	4
BT266	M	c.938C>T	p.Ala313Val	7	16	c.1805_1806del	p.Tyr602CysfsX31	14	35
BT268	F	c.237_238dup	p.Arg80ProfsX35	1	2	c.2981G>A	p.Cys994Tyr	26	29
GT001	F	c.2576T>C	p.Leu859Pro	22	1	c.2748-2A>T	splice defect	23	This study
GT002	F	c.1289G>A	p.Cys430Tyr	10	This study	c.1924C>G	p.Arg642Gly	15	16
GT005	M	c.247C>T	p.Arg83Trp	1	This study	c.1124C>A	p.Thr375Asn	9	This study
GT006¥	M	c.293_296dup	p.His99GlnfsX9	2	This study	c.961C>T	p.Arg321Trp	7	16
GT011	F	c.2221G>A	p.Gly741Arg	18	1	c.2883+1G>T	splice defect	24	1
GT014	M	c.1840T>C	p.Ser614Pro	15	This study	c.2221G>A	p.Gly741Arg	18	1
GT015	M	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29
GT016	F	c.533C>T	p.Ser178Leu	4	16	c.2221G>A	p.Gly741Arg	18	1
GT020	M	c.1826-1>GA	splice defect	14	This study	c.2981G>A	p.Cys994Tyr	26	29
GT022	F	c.2301del	p.Phe767LeufsX8	19	This study	c.2576T>C	p.Leu859Pro	22	1
GT026	F	c.1000C>T	p.Arg334Trp	8	16	c.2221G>A	p.Gly741Arg	18	1
GT028	M	c.2929C>T	p.Arg977X	25	1	c.2981G>A	p.Cys994Tyr	26	29

GT029	M	c.1180+1G>T	splice defect	9	17	c.1367del	p.Leu456ArgfsX36	11	This study
GT031	F	c.1180+1G>T	splice defect	9	17	c.2576T>C	p.Leu859Pro	22	1
GT032	M	c.679A>G	p.Asn227Asp	5	This study	c.1664C>T	p.Ser555Leu	13	16
GT036	F	c.1180+1G>T	splice defect	9	17	c.2581C>T	p.Arg861Cys	22	4
GT037§	M	c.2191G>A	p.Gly731Arg	18	2	c.2981G>A	p.Cys994Tyr	26	29
GT039	M	c.2883+1G>T	Splice defect	24	1	c.2981G>A	p.Cys994Tyr	26	29
GT040	F	c.1432A>G	p.Lys478Glu	11	2	c.2782C>T	p.Arg928Cys	24	4
GT041	M	c.1664C>T	p.Ser555Leu	13	16	c.1930delC	p.Gln644SerfsX28	16	35
GT042	F	c.674_697delins9	p.Phe225_Met233delinsLeuHisThrVal	5	This study	c.2213T>G	p.Leu738Arg	18	4
GT045§	F	c.1881C>G	p.Tyr627X	15	This study	c.2221G>A	p.Gly741Arg	18	1
GT047	M	c.626G>A	p.Arg209Gln	5	16	c.2981G>A	p.Cys994Tyr	26	29
GT048	F	c.1315G>A	p.Gly439Ser	10	2	c.2883+1G>T	splice defect	24	1
GT049	F	c.2576T>C	p.Leu859Pro	22	1	c.2981G>A	p.Cys994Tyr	26	29
GT050	F	c.791G>A	p.Gly264Asp	6	This study	c.2686C>T	p.Arg896X	23	6
GT051	M	c.2981G>A	p.Cys994Tyr	26	29	c.3006G>C	p.Trp1002Cys	26	This study
GT052	M	c.965C>T	p.Ala322Val	8	This study	c.1946C>T	p.Thr649Met	16	6
GT054	F	c.184G>A	p.Asp62Asn	1	2	c.1289G>A	p.Cys430Tyr	10	This study
GT061	M	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
GT063	F	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
GT066	F	c.964+1G>T	splice defect	7	5	c.2576T>C	p.Leu859Pro	22	1
GT067	M	c.1036A>T	p.Ile346Phe	8	This study	c.2687G>A	p.Arg896Gln	23	30
GT068	M	c.938C>T	p.Ala313Val	7	16	c.2221G>A	p.Gly741Arg	18	1
GT069	F	c.247C>T	p.Arg83Trp	1	This study	c.911C>T	p.Thr304Met	7	This study
GT073	M	c.1387G>A	p.Gly463Arg	11	This study	c.2582G>A	p.Arg861His	22	This study
GT074	M	c.1315G>A	p.Gly439Ser	10	2	c.2221G>A	p.Gly741Arg	18	1
GT076	M	c.1928C>T	p.Pro643Leu	16	16	c.964+1G>T	splice defect	7	5
GT079§	M	c.1145C>T	p.Thr382Met	9	6	c.2221G>A	p.Gly741Arg	18	1
GT080	F	c.2221G>A	p.Gly741Arg	18	1	c.2581C>T	p.Arg861Cys	22	4

GT082	F	c.1925G>A	p.Arg642His	15	4	c.2883+1G>T	splice defect	24	1
GT088	M	c.1489A>T	p.Lys497X	12	16	c.2883+1G>T	splice defect	24	1
GT091	F	c.2221G>A	p.Gly741Arg	18	1	c.2581C>T	p.Arg861Cys	22	4
GT092	F	c.1196_1202dup	p.Ser402X	10	2	c.2576T>C	p.Leu859Pro	22	1
GT094§	F	c.1924C>G	p.Arg642Gly	15	16	c.2581C>T	p.Arg861Cys	22	4
GT095	M	c.514T>C	p.Trp172Arg	4	29	c.1927_1932dup	p.Pro643_Gln644dup	16	This study
GT096	F	c.2576T>C	p.Leu859Pro	22	1	c.2981G>A	p.Cys994Tyr	26	29
GT097	M	c.1262G>A	p.Cys421Tyr	10	This study	c.1424C>G	p.Ser475Cys	11	This study
GT099§	M	c.2191G>A	p.Gly731Arg	18	2	c.3053G>A	p.Arg1018Gln	26	This study
GT100§	F	c.910A>C	p.Thr304Pro	7	3	c.2581C>T	p.Arg861Cys	22	4
GT101	F	c.910A>C	p.Thr304Pro	7	3	c.2807_2810dup	p.Thr938GlyfsX17	24	This study
GT102§	F	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29
GT103	F	c.644T>C	p.Leu215Pro	5	4	c.1637G>T	p.Ser546Ile	13	This study
GT104	M	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
GT108	F	c.938C>T	p.Ala313Val	7	16	c.1564_1566del	p.Ile522del	12	This study
GT109	F	c.514T>C	p.Trp172Arg	4	29	c.3060C>A	p.Asn1020Lys	26	This study
GT112	F	c.1928C>T	p.Pro643Leu	16	16	c.2221G>A	p.Gly741Arg	18	1
GT113	F	c.1196G>C	p.Arg399Pro	10	This study	c.1930delC	p.Gln644SerfsX28	16	35
GT114	M	c.1145C>T	p.Thr382Met	9	6	c.2883+1G>T	splice defect	24	1
GT116§	M	c.938C>T	p.Ala313Val	7	16	c.1928C>T	p.Pro643Leu	16	16
GT118	M	c.403C>T	p.Arg135Cys	2	This study	c.1763C>T	p.Ala588Val	14	1
GT123	F	c.625C>T	p.Arg209Trp	5	1	c.1837G>A	p.Gly613Ser	15	4
GT124	M	c.938C>T	p.Ala313Val	7	16	c.2883+1G>T	splice defect	24	1
GT125§	F	c.1180+1G>T	splice defect	9	17	c.1930delC	p.Gln644SerfsX28	16	35
GT127	M	c.1196_1202dup	p.Ser402X	10	2	c.2576T>C	p.Leu859Pro	22	1
GT128	M	c.176A>T	p.Asn59Ile	1	This study	c.2981G>A	p.Cys994Tyr	26	29
GT129	F	c.2548+1G>T	splice defect	21	6	c.3052C>T	p.Arg1018X	26	36
GT130§	F	c.1669+1G>A	splice defect	13	This study	c.1928C>T	p.Pro643Leu	16	16

GT131	M	c.1180+1G>T	splice defect	9	17	c.1763C>T	p.Ala588Val	14	1
GT132§	M	c.2883+1G>T	Splice defect	24	1	c.2981G>A	p.Cys994Tyr	26	29
GT133	F	c.1796_1797dup	p.Leu600SerfsX12	14	This study	c.2981G>A	p.Cys994Tyr	26	29
GT134	F	c.1963C>T	p.Arg655Cys	16	4	c.1964G>A	p.Arg655His	16	1
GT136	F	c.1196_1202dup	p.Ser402X	10	2	c.1881C>G	p.Tyr627X	15	This study
GT138	M	c.1964G>A	p.Arg655His	16	1	c.2551_2552dup	p.Thr852SerfsX24	22	This study
GT139	F	c.1315G>A	p.Gly439Ser	10	2	c.1664C>T	p.Ser555Leu	13	16
GT144	M	c.1315G>A	p.Gly439Ser	10	2	c.2221G>A	p.Gly741Arg	18	1
GT145	F	c.910A>C	p.Thr304Pro	7	3	c.2883+1G>T	splice defect	24	1
GT150	M	c.1664C>T	p.Ser555Leu	13	16	c.2883+1G>T	splice defect	24	1
GT151	F	c.1939delG	p.Val647CysfsX25	16	This study	c.2883+1G>T	splice defect	24	1
GT152	M	c.2186G>C	p.Gly729Ala	18	This study	c.2883+1G>T	splice defect	24	1
GT154	F	c.2089_2095del	p.Thr697GlyfsX2	17	29	c.2576T>C	p.Leu859Pro	22	1
GT156	F	c.644T>C	p.Leu215Pro	5	4	c.1946C>T	p.Thr649Met	16	6
GT157	M	c.911C>T	p.Thr304Met	7	This study	c.1095+1G>T	splice defect	8	This study
GT158	M	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
GT159	M	c.947G>T	p.Gly316Val	7	29	c.2576T>C	p.Leu859Pro	22	1
GT161	M	c.2883+1G>T	Splice defect	24	1	c.2965G>A	p.Gly989Arg	26	19
GT162§	M	c.1196_1202dup	p.Ser402X	10	2	c.2747+1G>A	splice defect	23	This study
GT164	F	c.938C>T	p.Ala313Val	7	16	c.2581C>T	p.Arg861Cys	22	4
GT166	F	c.911C>T	p.Thr304Met	7	This study	c.2981G>A	p.Cys994Tyr	26	29
GT168	M	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29
GIT171	F	c.626G>A	p.Arg209Gln	5	16	c.265C>G	p.Leu89Val	1	This study
GT174	M	c.2576T>C	p.Leu859Pro	22	1	c.2981G>A	p.Cys994Tyr	26	29
GT175	F	c.644T>C	p.Leu215Pro	5	4	c.1664C>T	p.Ser555Leu	13	16
GT176	F	c.1489A>T	p.Lys497X	12	16	c.1948G>A	p.Gly650Arg	16	This study
GT178	F	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29
GT179**	M	c.1191_1203del	p.Val398LeufsX4	10	This study	c.1204T>C	p.Ser402Pro	10	This study

		c.1946C>T	p.Thr649Met	16	6				
GT181§	F	c.514T>C	p.Trp172Arg	4	29	c.938C>T	p.Ala313Val	7	16
GT182	F	c.1387G>A	p.Gly463Arg	11	This study	c.2981G>A	p.Cys994Tyr	26	29
GT188	F	c.1143G>A	p.Trp381X	9	This study	c.2981G>A	p.Cys994Tyr	26	29
GT190**	F	c.322C>T	p.Arg108Trp	2	This study	c.1519C>T	p.Arg507Cys	12	This study
		c.2581C>T	p.Arg861Cys	22	4				
GT192	M	c.911C>T	p.Thr304Met	7	This study	c.2576T>C	p.Leu859Pro	22	1
GT194	F	c.2548G>C	p.Gly850Arg	21	37	c.2981G>A	p.Cys994Tyr	26	29
GT198	M	c.1967C>T	p.Pro656Leu	16	This study	c.3052C>T	p.Arg1018X	26	36
GT200	F	c.403C>T	p.Arg135Cys	2	This study	c.1180+1G>T	splice defect	9	17
GT202	F	c.1001G>C	p.Arg334Pro	8	This study	c.2899A>G	p.Arg967Gly	25	29
GT204	M	c.293_296dup	p.His99GlnfsX9	2	This study	c.1195C>T	p.Arg399Cys	10	16
GT205	M	c.938C>T	p.Ala313Val	7	16	c.2576T>C	p.Leu859Pro	22	1
GT208	F	c.938C>T	p.Ala313Val	7	16	c.2186G>C	p.Gly729Ala	18	This study
GT211	M	c.1387G>A	p.Gly463Arg	11	This study	c.1763C>T	p.Ala588Val	14	1
GT214	F	c.247C>T	p.Arg83Trp	1	This study	c.2221G>A	p.Gly741Arg	18	1
GT217	F	c.2221G>A	p.Gly741Arg	18	1	c.2576T>C	p.Leu859Pro	22	1
GT220	F	c.1085G>T	p.Gly362Val	8	This study	c.2576T>C	p.Leu859Pro	22	1
GT221	M	c.1180+1G>T	splice defect	9	17	c.403C>T	p.Arg135Cys	2	This study
GT225¥	M	c.1095+1G>A	Splice defect	8	This study	c.2581C>T	p.Arg861Cys	22	4
GT226	M	c.2213T>G	p.Leu738Arg	18	4	c.3006G>C	p.Trp1002Cys	26	This study
GT227	M	c.1925G>A	p.Arg642His	15	4	c.1963C>T	p.Arg655Cys	16	4
GT230	M	c.2089_2095del	p.Thr697GlyfsX2	17	29	c.2576T>C	p.Leu859Pro	22	1
GT231	F	c.1196_1202dup	p.Ser402X	10	2	c.1489A>T	p.Lys497X	12	16
GT232	M	c.961C>T	p.Arg321Trp	7	16	c.2710A>T	p.Ile904Phe	23	6
GT233	M	c.1001G>C	p.Arg334Pro	8	This study	c.2096G>A	p.Trp699X	17	This study
GT237	F	c.514T>C	p.Trp172Arg	4	29	c.938C>T	p.Ala313Val	7	16
GT239	F	c.2581C>T	p.Arg861Cys	22	4	c.2981G>A	p.Cys994Tyr	26	29

GT241	M	c.2576T>C	p.Leu859Pro	22	1	c.2581C>T	p.Arg861Cys	22	4
GT242	M	c.938C>T	p.Ala313Val	7	16	c.1424C>G	p.Ser475Cys	11	This study
GT248	M	c.2576T>C	p.Leu859Pro	22	1	c.2883+1G>T	splice defect	24	1
GT249	F	c.2213T>G	p.Leu738Arg	18	4	c.2890C>T	p.Arg964Trp	25	This study
GT252	F	c.2191G>A	p.Gly731Arg	18	2	c.2981G>A	p.Cys994Tyr	26	29
GT256	M	c.1826-1G>A	splice defect	14	This study	c.2576T>C	p.Leu859Pro	22	1
GT257	M	c.602G>T	p.Gly201Val	5	This study	c.1619A>G	p.Tyr540Cys	13	This study
GT267	F	c.2221G>A	p.Gly741Arg	18	1	c.2747+1G>C	splice defect	23	This study
GT273	F	c.1196_1202dup	p.Ser402X	10	2	c.2221G>A	p.Gly741Arg	18	1
GT275	M	c.248G>A	p.Arg83Gln	1	This study	c.626G>A	p.Arg209Gln	5	16
GT276¥	F	c.2221G>A	p.Gly741Arg	18	1	c.2981G>A	p.Cys994Tyr	26	29
GT277	M	c.2883+1G>T	Splice defect	24	1	c.2981G>A	p.Cys994Tyr	26	29
GT283	M	c.2221G>A	p.Gly741Arg	18	1	c.2981G>A	p.Cys994Tyr	26	29
GT284	M	c.1489A>T	p.Lys497X	12	16	c.2576T>C	p.Leu859Pro	22	1
GT288	F	c.947G>T	p.Gly316Val	7	29	c.2581C>T	p.Arg861Cys	22	4
GT289§	F	c.1196_1202dup	p.Ser402X	10	2	c.2576T>C	p.Leu859Pro	22	1
B063	M	c.1967del	p.Pro656ArgfsX16	16	This study	c.3052C>T	p.Arg1018X	26	36
B084	M	c.1925G>A	p.Arg642His	15	4	c.2285+1G>A	splice defect	18	This study
B090	M	c.506-1G>A	splice defect	3	This study	c.2981G>A	p.Cys994Tyr	26	29
B091	M	c.647G>A	p.Gly216Glu	5	This study	c.2221G>A	p.Gly741Arg	18	1

*Numbering is according to the cDNA sequence (GenBank : NM_000339.2). The A of the ATG of the initiator methionine codon is denoted as nucleotide 1.
** These patients have 3 mutations detected by direct sequencing.
§Parental heterozygosity confirmed for these patients
¥ Heterozygosity confirmed for one of the parents

Table 1c. Group of Patients with one heterozygous mutation in the *SLC12A3* gene detected by direct sequencing

Patient	Sex	Nucleotide*	Protein	Exon/ Intron	Reference
BT001	F	c.1924C>T	p.Arg642Cys	15	34
BT011	M	c.1095+4A>G	Splice defect	8	This study
BT016	F	c.1196_1202dup	p.Ser402X	10	2
BT038	F	c.3077C>T	p.Thr1026Ile	26	18
BT073	M	c.1196_1202dup	p.Ser402X	10	2
BT084	F	c.433C>T	p.Arg145Cys	3	5
BT092	F	c.791G>C	p.Gly264Ala	6	31
BT096	M	c.1394C>A	p.Thr465Asn	11	This study
BT097	F	c.2981G>A	p.Cys994Tyr	26	29
BT098	M	c.2581C>T	p.Arg861Cys	22	4
BT103	F	c.1388G>A	p.Gly463Glu	11	29
BT102	F	c.791G>C	p.Gly264Ala	6	31
BT104	F	c.2581C>T	p.Arg861Cys	22	4
BT115	M	c.965C>T	p.Ala322Val	8	This study
BT126	F	c.1956del	p.Asn653ThrfsX19	16	This study
BT131	F	c.791G>C	p.Gly264Ala	6	31
BT136	M	c.887C>T	p.Ser296Phe	7	This study
BT140	M	c.2981G>A	p.Cys994Tyr	26	29
BT141	F	c.2782C>T	p.Arg928Cys	24	4
BT147	M	c.947G>T	p.Gly316Val	7	29
BT164	F	c.3061C>A	p.Gln1021Lys	26	This study
BT167	F	c.2782C>T	p.Arg928Cys	24	4
BT171	M	c.3G>A	p.Met1?	1	This study
BT177	F	c.2576T>C	p.Leu859Pro	22	1
BT181	M	c.938C>T	p.Ala313Val	7	16
BT184	F	c.938C>T	p.Ala313Val	7	16
BT193	F	c.2748-2A>T	Splice defect	23	This study
BT194	F	c.174C>A	p.Tyr58X	1	This study

BT197	F	c.2581C>T	p.Arg861Cys	22	4
BT201	M	c.2221G>A	p.Gly741Arg	18	1
BT209	M	c.2981G>A	p.Cys994Tyr	26	29
BT213	M	c.1046C>T	p.Pro349Leu	8	1
BT215	M	c.1444-4_1460dup	p.Gln481_Gln487dup	12	This study
BT226	F	c.2782C>T	p.Arg928Cys	24	4
BT231	M	c.1195C>T	p.Arg399Cys	10	16
BT234	F	c.1390G>A	p.Ala464Thr	11	29
BT236	M	c.2336G>A	p.Gly779Glu	19	This study
BT243	M	c.1519C>T	p.Arg507Cys	12	This study
BT247	F	c.938C>T	p.Ala313Val	7	16
BT257	M	c.1444-1G>A	Splice defect	11	This study
BT258	F	c.2782C>T	p.Arg928Cys	24	4
GT004	F	c.1664C>T	p.Ser555Leu	13	16
GT007	F	c.938C>T	p.Ala313Val	7	16
GT010	F	c.2581C>T	p.Arg861Cys	22	4
GT034	F	c.965-2_965-1dup	Splice defect	7	This study
GT038	M	c.2883+1G>T	Splice defect	24	1
GT058	F	c.938C>T	p.Ala313Val	7	16
GT059	M	c.2891G>A	p.Arg964Gln	25	1
GT060	F	c.176A>T	p.Asn59Ile	1	This study
GT075	F	c.1390G>A	p.Ala464Thr	11	29
GT078	F	c.2981G>A	p.Cys994Tyr	26	29
GT081	M	c.791G>C	p.Gly264Ala	6	31
GT086	F	c.1180+1G>T	Splice defect	9	17
GT090	F	c.2576T>C	p.Leu859Pro	22	1
GT111	M	c.237_238dup	p.Arg80ProfsX35	1	2
GT115	F	c.2548G>C	p.Gly850Arg	21	37
GT121	M	c.2981G>A	p.Cys994Tyr	26	29
GT122	F	c.2965G>A	p.Gly989Arg	26	19
GT137	M	c.2576T>C	p.Leu859Pro	22	1

GT142	F	c.2687G>A	p.Arg896Gln	23	30
GT146	M	c.2221G>A	p.Gly741Arg	18	1
GT165	F	c.2576T>C	p.Leu859Pro	22	1
GT184	F	c.111T>A	p.Tyr37X	1	This study
GT185	M	c.1825+1del	Splice defect	14	This study
GT187	M	c.2576T>C	p.Leu859Pro	22	1
GT189	M	c.2581C>T	p.Arg861Cys	22	4
GT196	M	c.473G>A	p.Arg158Gln	3	29
GT210	M	c.727C>T	p.Arg243Trp	5	This study
GT229	F	c.1175C>T	p.Thr392Ile	9	This study
GT235	M	c.514T>C	p.Trp172Arg	4	29
GT243	M	c.2981G>A	p.Cys994Tyr	26	29
GT272	F	c.2576T>C	p.Leu859Pro	22	1
GT278	M	c.1387G>A	p.Gly463Arg	11	This study
GT281	M	c.626G>A	p.Arg209Gln	5	16
GT285	F	c.2929C>T	p.Arg977X	25	1
GT291	M	c.533C>T	p.Ser178Leu	4	16
GT292	F	c.2581C>T	p.Arg861Cys	22	4
B039	M	c.1180+1G>T	Splice defect	9	17
B087	M	c.2782C>T	p.Arg928Cys	24	4
B099	M	c.2883+1G>T	Splice defect	24	1
B104	F	c.1883C>G	p.Ser628Trp	15	This study

*Numbering is according to the cDNA sequence (GenBank : NM_000339.2). The A of the ATG of the initiator Methionine codon is denoted as nucleotide 1.

Table 2. Novel missense changes and *in silico* predictions

Nucleotide (cDNA)	Protein	Exon/ Intron	Conservation between species	Grantham distance[1] (physicochemical difference)	SIFT score[2]	PolyPhen-2[3]	Panther[4]
c.37G>C	p.Ala13Pro	1	Weakly conserved nucleotide Weakly conserved amino acid	-	-	-	NA
c.160C>T	p.Arg54Cys	1	Highly conserved nucleotide Highly conserved amino acid	++	++	++	++
c.176A>T	p.Asn59Ile	1	Highly conserved nucleotide Moderately conserved amino acid	++	+	++	++
c.247C>T	p.Arg83Trp	1	Moderately conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.248G>A	p.Arg83Gln	1	Highly conserved nucleotide Highly conserved amino acid	-	-	++	NA
c.265C>G	p.Leu89Val	1	Highly conserved nucleotide Highly conserved amino acid	-	+	++	NA
c.322C>T	p.Arg108Trp	2	Weakly conserved nucleotide Moderately conserved amino acid	+	+	+	NA
c.403C>T	p.Arg135Cys	2	Highly conserved nucleotide Moderately conserved amino acid	++	++	-	NA
c.443T>C	p.Leu148Pro	3	Highly conserved nucleotide Moderately conserved amino acid	+	++	++	NA
c.602G>T	p.Gly201Val	5	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.647G>A	p.Gly216Glu	5	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.658G>A	p.Gly220Ser	5	Highly conserved nucleotide Highly conserved amino acid	-	++	++	NA
c.679A>G	p.Asn227Asp	5	Highly conserved nucleotide Highly conserved amino acid	-	-	++	++
c.727C>T	p.Arg243Trp	5	Weakly conserved nucleotide Moderately conserved amino acid	+	++	++	++
c.791G>A	p.Gly264Asp	6	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA

c.887C>T	p.Ser296Phe	7	Highly conserved nucleotide Highly conserved amino acid	++	++	++	NA
c.911C>T	p.Thr304Met	7	Highly conserved nucleotide Highly conserved amino acid	+	++	++	++
c.947G>C	p.Gly316Ala	7	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.965C>T	p.Ala322Val	8	Highly conserved nucleotide Moderately conserved amino acid	+	-	-	0.46
c.1001G>C	p.Arg334Pro	8	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.1036A>T	p.Ile346Phe	8	Highly conserved nucleotide Highly conserved amino acid	-	++	++	NA
c.1085G>T	p.Gly362Val	8	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.1124C>A	p.Thr375Asn	9	Highly conserved nucleotide Highly conserved amino acid	-	++	++	NA
c.1175C>T	p.Thr392Ile	9	Highly conserved nucleotide Moderately conserved amino acid	+	-	++	++
c.1196G>C	p.Arg399Pro	10	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.1204T>C	p.Ser402Pro	10	Highly conserved nucleotide Highly conserved amino acid	+	+	++	NA
c.1210G>A	p.Val404Ile	10	Weakly conserved nucleotide Weakly conserved amino acid	-	-	-	-
c.1247G>C	p.Cys416Ser	10	Highly conserved nucleotide Moderately conserved amino acid	+	-	++	NA
c.1262G>A	p.Cys421Tyr	10	Highly conserved nucleotide Moderately conserved amino acid	++	+	++	NA
c.1282A>C	p.Thr428Pro	10	Highly conserved nucleotide Moderately conserved amino acid	-	-	+	NA
c.1289G>A	p.Cys430Tyr	10	Highly conserved nucleotide Highly conserved amino acid	++	+	++	NA
c.1387G>A	p.Gly463Arg	11	Highly conserved nucleotide Moderately conserved amino acid	+	++	++	++
c.1394C>A	p.Thr465Asn	11	Highly conserved nucleotide Highly conserved amino acid	-	++	++	NA
c.1424C>G	p.Ser475Cys	11	Highly conserved nucleotide Highly conserved amino acid	+	++	++	NA

c.1484T>C	p.Phe495Ser	12	Highly conserved nucleotide Highly conserved amino acid	++	++	++	++
c.1519C>T	p.Arg507Cys	12	Moderately conserved nucleotide Highly conserved amino acid	++	++	++	NA
c.1619A>G	p.Tyr540Cys	13	Highly conserved nucleotide Highly conserved amino acid	++	++	++	NA
c.1636A>G	p.Ser546Gly	13	Highly conserved nucleotide Moderately conserved amino acid	-	-	-	NA
c.1637G>T	p.Ser546Ile	13	Highly conserved nucleotide Moderately conserved amino acid	++	++	++	NA
c.1751T>C	p.Leu584Pro	14	Highly conserved nucleotide Moderately conserved amino acid	+	++	++	++
c.1836G>C	p.Trp612Cys	15	Highly conserved nucleotide Highly conserved amino acid	++	++	++	NA
c.1840T>C	p.Ser614Pro	15	Highly conserved nucleotide Highly conserved amino acid	+	++	++	++
c.1852G>T	p.Ala618Ser	15	Highly conserved nucleotide Highly conserved amino acid	+	-	+	NA
c.1883C>G	p.Ser628Trp	15	Highly conserved nucleotide Moderately conserved amino acid	++	-	+	++
c.1901T>G	p.Val634Gly	15	Highly conserved nucleotide Highly conserved amino acid	+	+	++	NA
c.1948G>A	p.Gly650Arg	16	Highly conserved nucleotide Highly conserved amino acid	+	++	++	++
c.1967C>T	p.Pro656Leu	16	Highly conserved nucleotide Highly conserved amino acid	+	-	++	-
c.2120C>T	p.Ala707Val	17	Highly conserved nucleotide Highly conserved amino acid	-	++	-	++
c.2186G>C	p.Gly729Ala	18	Weakly conserved nucleotide Highly conserved amino acid	+	++	++	NA
c.2336G>A	p.Gly779Glu	19	Highly conserved nucleotide Highly conserved amino acid	+	-	++	-
c.2498C>T	p.Ser833Leu	21	Weakly conserved nucleotide Moderately conserved amino acid	++	-	-	-
c.2582G>A	p.Arg861His	22	Highly conserved nucleotide Moderately conserved amino acid	-	-	+	++
c.2690T>C	p.Leu897Pro	23	Highly conserved nucleotide Moderately conserved amino acid	+	++	-	++

c.2744A>C	p.Glu915Ala	23	Highly conserved nucleotide Moderately conserved amino acid	+	-	-	-
c.2827C>T	p.Arg943Trp	24	Highly conserved nucleotide Moderately conserved amino acid	+	++	++	++
c.2890C>T	p.Arg964Trp	25	Highly conserved nucleotide Highly conserved amino acid	+	++	-	NA
c.3006G>C	p.Trp1002Cys	26	Highly conserved nucleotide Highly conserved amino acid	++	-	++	NA
c.3053G>A	p.Arg1018Gln	26	Highly conserved nucleotide Highly conserved amino acid	-	++	++	NA
c.3060C>A	p.Asn1020Lys	26	Highly conserved nucleotide Highly conserved amino aci	+	++	-	NA
c.3061C>A	p.Gln1021Lys	26	Highly conserved nucleotide Highly conserved amino acid	-	-	++	NA

[1]For Grantham: ++ indicates a large, + indicates a moderate and - indicates a small physicochemical difference.
[2]For SIFT: ++ indicates the mutation is predicted to be deleterious (score<0.05), + indicates a score <0.05 with low confidence prediction and - indicates the mutation is not predicted to be deleterious
[3]For PolyPhen-2: ++ indicates the mutation is predicted to be probably damaging, + indicates the mutation is predicted to be possibly damaging and - indicates the mutation is predicted to be benign
[4]For Panther: ++ indicates the mutation is predicted to be deleterious ($P_{deleterious}$ >0.50), - indicates the mutation is not predicted to be deleterious ($P_{deleterious}$ < 0.50), NA indicates that predictions are not available.

Table 3. Clinical and biological characteristics of GS patients without mutations in *SLC12A3* and *CLCNKB* genes

Patient	Age at diagnosis	Sex	Clinical presentation	Plasma laboratory findings								Urinary laboratory findings		
				Na	K	Cl	Total CO2	Mg	Renin activity	Renin	Aldosterone	K	Cl	Ca/Creat
				mmol/L	mmol/L	mmol/L	mmol/L	mmol/L				mmol/d	mmol/d	mmol/mmol
				135-145	3.5-4.5	95-107	22-28	0.60-1.05				mmol/L	mmol/L	
BT031*	22	M	Fortuitous diagnosis	143	3.15	102	29	0.67	ND	normal	normal	91	162	ND
BT042	25	F	Fortuitous diagnosis	140	3.3	94	29	0.64	ND	ND	high	83	81	0.1
BT047	29	F	Fortuitous diagnosis	140	2.5	99	31	0.61	ND	high	normal	225 187	307 256	0.04
BT061	42	F	Malaise	137	2.7	103	30	0.63	ND	high	high	86 38	170 76	0.1
BT100	1	M	Growth retardation	140	2.7	96	31	0.67	ND	high	high	129 58	224 100	0.07
BT108	34	F	Fortuitous diagnosis	140	2.6	98	33	0.63	ND	high	high	135 54	345 138	0.15
BT130	27	M	Fortuitous diagnosis	138	2.7	96	35	0.49	ND	high	high	133 78	232 136	0.14
BT164	25	F	Malaise	ND	2.8	ND	ND	0.6	ND	ND	ND	high	ND	ND
BT185	42	F	Malaise, tetany	136	3	103	24	0.49	ND	high	normal	82 65	156 123	0.11
GT003	32	F	Fortuitous diagnosis	ND	3.4	111	28	0.76	ND	normal	normal	50	ND	0.09
GT035*	31	F	ND	ND	3.2	103	32	0.74	ND	ND	ND	74 45	ND	ND
GT043	22	M	Fortuitous diagnosis	ND	3.3	94	37	0.82	ND	47	240	62	ND	1.5 mg/kg/d
GT056	52	F	Malaise with syncope	ND	1.8	105	ND	0.76	ND	109	1470	34	ND	ND
GT126	26	M	ND	139	2.6	99	33	0.49	ND	115	1007	72	218	2.5 mmol/d

GT155	54	M	Fortuitous diagnosis	ND	2.1	ND	30	0.57	ND	ND	ND	66	ND	1.73 mmol/d
GT167	18	F	Fortuitous diagnosis	137	2.3	95	30	0.33	ND	high	normal	102	316	1.5 mmol/d
GT180	29	F	Asthenia, cramps	137	2.4	89	37	0.7	ND	ND	ND	44	65	0.52 mmol/d
GT203	61	F	Chondrocalcinosis	144	2.5	102	27	0.64	ND	ND	ND	60 40	ND	2.9 mmol/d
GT209	15	M	Malaise	133	2.0	89	28	1.11	ND	ND	ND	138 55	183 73	0.03
GT212	42	F	Fortuitous diagnosis	142	3.0	108	28	0.86	ND	normal	normal	89	125	0.6
GT213	41	F	Tetraparesia	140	2.4	97	32	1.2	ND	high	normal	126 36	159 45	0.02 mg/kg/d
GT218	25	F	Asthenia	138	3.1	109	26	0.67	ND	normal	normal	50 39	137 107	1.5 mmol/d
GT223	54	M		144	2.9	103	25	0.65	ND	high	high	ND	ND	ND
GT234	49	F	Asthenia	144	2.5	95	35	1.0	ND	high	normal	55	ND	0.53
GT236	50	M	Fortuitous diagnosis	138.3	3.19	94	31	0.69	ND	high	normal	22	22	0.08
GT240	20	F	Fortuitous diagnosis	136	3.1	90	30	0.66	ND	normal	normal	103	ND	ND
GT245	24	F		138	2.1	93	33	0.67	ND	high	normal	76	290	0.02
GT246	23	F	Arrhythmia	136	2.97	101	25	0.72	ND	high	high	94 82	146 126	0.32
GT247	37	F	Fortuitous diagnosis	135	2.2	83	40	0.69	ND	high	high	41 23	ND	90 mg/d
GT250	34	M	Fortuitous diagnosis	139	2.8	97	27.5	0.52	ND	high	high	104 55	165 87	0.11
GT253	37	F	Weakness	138	3.3	97	30	0.8	ND	normal	normal	169 106	ND	0.24
GT279	44	F	Fortuitous diagnosis	129	2.4	75	39	1.08	ND	high	high	27	45	0.4

GT280	23	F	Fortuitous diagnosis	139	3.0	104	ND	ND	0.9 pmol/l/s	ND	high	ND	ND	ND
GT290	27	M	Asthenia	ND	3.1	ND	31	0.91	ND	normal	normal	57 38	ND	0.37 3 mg/kg/d
B001	1.5	F	Fortuitous diagnosis	138	3.1	92	27	0.61	65µg/ml/mn (1.5-5.7)	high	high	115 55	ND	0.08
B002	7	M	Growth retardation	141	2.9	99	21	0.59	28.3 ng/ml/h (1-8.5)	high	normal	84	ND	0.01
B059	0.5	M	Dehydration Growth retardation	129	2.3	72	43	0.78	ND	high	high	158	ND	0.23
B061	43	F	Tachycardia	ND	2.6	93	34	0.84	ND	high	normal	87 23	ND	0.43
B080	22	F	Cramps, muscular pain	ND	3.2	ND	ND	0.4	ND	high	high	109 40.5	ND	0.32

* These two patients were not tested for CLCNKB mutations

Table 4a. Mutations detected in *CLCNKB* gene in patients with GS phenotype

Patient	Sex	Nucleotide*	Protein	Exon/ Intron	Reference	Nucleotide*	Protein	Exon/ Intron	Reference
BT022**	F	c.508G>A	p.Val170Met	6	This study	c.508G>A	p.Val170Met	6	This study
BT099	M	c.1271G>A	p.Gly424Glu	13	This study	c.1271G>A	p.Gly424Glu	13	This study
BT148	M	c.610G>A	p.Ala204Thr	7	38	c.1270G>A	p.Gly424Arg	13	This study
BT153	F	c.610G>A	p.Ala204Thr	7	38	c.1334_135delCT	p.Ser445delfsX450		This study
BT188**	M	c.1340T>C	p.Ile447Thr	14	This study				
BT218**		c.508G>A	p.Val170Met	6	This study	c.508G>A	p.Val170Met	6	This study
GT018	M	c.1173G>A	p.Trp391X	12	This study	c.1173G>A	p.Trp391X	12	This study
GT027	F	c.274C>T	p.Arg92Trp	4	This study	c.274C>T	p.Arg92Trp	4	This study
GT143	F	c.1313G>A	p.Arg438His	14	39	c.1313G>A	p.Arg438His	14	39
GT148	M	Homozygous deletion			38,39	Homozygous deletion			38.39
GT149	F	c.887G>A	p.Gly296Asp	10	This study				
GT160	F	c.1783C>T	p.Arg595X	17	This study	c.1783C>T	p.Arg595X	17	This study
GT207**	F	c.508G>A	p.Val170Met	6	This study	c.508G>A	p.Val170Met	6	This study
GT274**	M	c.1877G>A	p.Cys626Tyr	18	This study				

*Numbering is according to the cDNA sequence (GenBank : NM_000085.3). The A of the ATG of the initiator Methionine codon is denoted as nucleotide 1.
**Neither deletions nor duplications were detected by MLPA

Table 4b. Clinical and biological characteristics of GS patients with mutations in the *CLCNKB* gene.

Patient	Age at diagnosis	Sex	Clinical presentation	Plasma laboratory findings							Urinary laboratory findings		
				Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	HCO3 (mmol/L)	Mg (mmol/L)	Renin	Aldosterone	K (mmol/L)	Cl (mmol/L)	Ca/Creat mmol/mmol
BT022	43	F	Tetany, cramps	140	2.74	99	34	1.03	high	high	109 40	100 37	0.15
BT99	13	M	Failure to thrive	ND	2.8	91	32	0.78	ND	normal	93	ND	ND
BT148	<1	M	polyuria, failure to thrive	ND	2.5	95	29	0.8	high	high	64.3	ND	0.66
BT153	15	F	Asthenia, paresthesis	ND	2.8	97	32.8	0.83	high	normal	59	ND	0.6
BT188	46	M	cramps, asthenia	ND	3	98	31	0.64	ND	normal	115	157	0.07
BT218	24	F	Malaise, tetany	141	2.8	92	32	0.77	high	high	21	141	0.29
GT18	<1	M	dehydration	ND	2.8	81	31	0.76	high	ND	ND	ND	0.07
GT27	20	F	polyuria, failure to thrive	ND	2	ND	30	0.67	ND	ND	154mmol/d	427.2mmol/d	ND
GT143	30	F	diarrhea, cramps	ND	2.5	97	33	0.73	high	normal	71	40	0.2
GT148	19	M	abdominal pain	ND	2.5	ND	29	0.63	ND	ND	ND	ND	ND
GT149	ND (Infancy)	F	Family history	ND	2.8	ND	ND	ND	ND	ND	ND	ND	ND
GT160	30	F	muscular disability	ND	2.12	ND	ND	ND	high	high	53.8	ND	ND
GT207	60	F	Arrhythmia	ND	2.9	ND	ND	0.78	high	high	80	ND	ND
GT274	52	M	Chondrocalcinosis	135	3.2	90	32	0.49	ND	ND	ND	ND	1.67 mmol/d

Table 5. Primers used for QMPSF method and amplicons' length

	Forward	Reverse	Length
Exon 1	CGTTAGATAGTTAGGCCAACCTCCCTGCCTCC	GATAGGGTTATGCTGCTGTGGGTCAGGTGG	270 bp
Exon 2	CGTTAGATAGGCAGTGGTGCAGGTCAGTGGG	GATAGGGTTACCCTCCACCAGCCCATCAGTCA	182 bp
Exon 3	CGTTAGATAGCGTTGCATGCTCAACATTTGGG	GATAGGGTTAAGAAGGCCTGGCCGTTTGGT	231 bp
Exon 6	CGTTAGATAGTAGAGCCACACTGTCCAGGCCC	GATAGGGTTAGCAGCACAGTGACCGAGACCAC	275 bp
Exon 10	CGTTAGATAGACACAGTGACCCCTGGCTGG	GATAGGGTTAGGGACTGGACAGACCTGGGC	293 bp
Exon 17	CGTTAGATAGACCCCACAAGCAGAGGATGCC	GATAGGGTTATGCAGCAAAAGGGGCTTAAGCAAA	262 bp
Exon 18	CGTTAGATAGGGGGCTCTGGCCAGGTGGAT	GATAGGGTTATTCCACTGTGGCCGGGTGAG	180 bp
Exon 19	CGTTAGATAGGGCGTGGTAGGAAGCAGAGCC	GATAGGGTTATGTACTCACTGTGCGCCTGCAT	221 bp
Exon 20	CGTTAGATAGGAGGCCATGCGCGACTGGAA	GATAGGGTTACTGGCGCTGGCTTCCTTCCC	269 bp
Exon 21	CGTTAGATAGTGCAGTGGACCCCAAGGCCC	GATAGGGTTACCAGGAGCCCCTGTCCCCTT	235 bp
Exon 22	CGTTAGATAGGGATGGACCAGGAGAGAAAGGCGT	GATAGGGTTAACTCCCCTCCCTGTCGTAGCA	307 bp
Exon 23	CGTTAGATAGCCACATCCTCCCTGACATCAACCA	GATAGGGTTACTCAGCCCCAGGGAGGGGAC	251 bp
Exon 26	CGTTAGATAGAAGTGGGGAGGTCATTCTTAGG	GATAGGGTTAAGGATGACTGGAGGTCTGAGGT	260 bp
HMBS	CGTTAGATAGTAGACGGCTCAGATAGCATACAAG	GATAGGGTTAATGCCTACCAACTGTGGGTCA	206 bp

Table 6. Primers used for LR-PCR and amplicons' length

Exon(s)	**Forward**	**Reverse**	**Length**
2-3	CAATGGCAGAACTGCCCACAACAGA	TGCCATTGGTGGAGATGGCTGAGAT	4579
4-5-6	ATGCTCAACATTTGGGGCGTGATCC	GTTGGCAAAGGAGACCATGATGACA	2434
9	GGACATTTTTGTCCAGAACTTGGTG	GTAATAGTTGATGAGGCCGTAGTGG	6568
14	ATCATTTCCAACTTCTTCCTCTGCT	GTAGTTCTTGATGTGGTCTTCCACC	2941
18	GAAGATCAAGGCCTTCTACTCGGAT	ACACACGCCATAGTTGAAATCAAAG	3281
24-25	ATCATTTCTCTGCTGAGCAAGTTCC	ATCAGGATGACTGGAGGTCTGAGGT	13832
26	ACTCCCGAGACGCTGCTCTCATC	AGCTGGGAAAATGGCAATGACACCC	10191

Table 7. SNPs genotyped in family BT213

dbSNP	Average Heterozygosity	Position on 16q13 (tel- to cen-)
rs34136389	0.500	56909322-56909322
rs11640954	nd	56908884-56908884
rs3829502	0.500	56896730-56896730
rs12921781	0.373	56896189-56896189
rs12599065	0.487	56896036-56896036
rs12920659	0.124	56895873-56895873
rs1436424	0.499	56895034-56895034
rs4784732	0.240	56894519-56894519
rs12932041	0.279	56894505-56894505
rs12443821	0.165	56886203-56886203
rs12445993	0.171	56886109-56886109
rs1347591	0.492	56868700-56868700
rs4784730	0.489	56865249-56865249
rs7199480	0.500	56859595-56859595
rs1529929	0.500	56849496-56849496
rs12444217	0.209	56843443-56843443
rs2043635	0.458	56818987-56818987
rs7202364	0.478	56785390-56785390

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SUPPLEMENTARY FIGURES

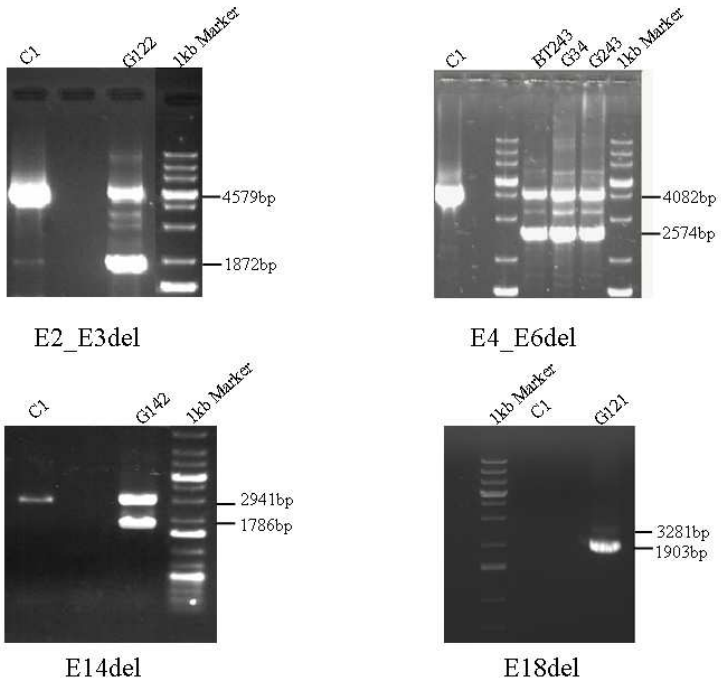

Figure 1. Long Range PCR for patients with 4 heterozygous deletions showing the normal band size in controls (C1) and normal and shortened bands in patients.

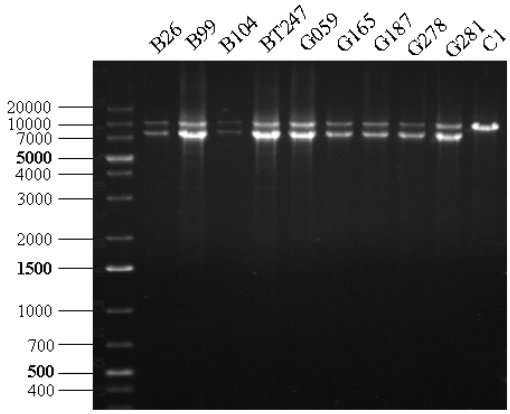

Figure 2. Long Range PCR for patients with heterozygous E26del showing the normal band size in control (C1) and normal and shortened bands in 9 patients.

SUPPLEMENTARY MATERIALS

Multiplex ligation-dependent probe amplification (MLPA) analysis

The procedure can be divided into 5 important steps. Briefly, denaturation of genomic DNA (20ng/µl in TE buffer) at 98°C, hybridization of oligonucleotides contained in the MLPA® probe mix to the target sequence, probe ligation with a thermostable ligase, amplification using primer pair, one unlabeled and one labeled, complementary to the universal primer sequences of the MLPA® probes, and finally fluorescent amplicon separation by capillary electrophoresis on an ABI Prism 3730XL DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA). Data were analyzed using GeneMarker Software version 1.85 (Applied Biosystems, Foster City, CA). For each patient, the mean value of each probe was obtained by comparing the peaks between the patient and a reference sample. If this mean value was below 0.7, the respective exon was defined as deleted; a value between 0.7 and 1.3 was defined as normal, and the exon was defined as duplicated for values between 1.3 and 2.0.

Quantitative multiplex PCR of short fluorescent fragments (QMPSF)

QMPSF consists of a fluorescent multiplex PCR that permits simultaneous amplification of multiple short exonic fragments under semi-quantitative conditions. In each QMPSF, a fragment from the hydroxymethylbilane synthase (*HMBS*) gene was amplified as an internal control. After the PCR, the 6FAM-labeled amplicons are separated by capillary electrophoresis on an ABI Prism 3730XL DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA). Data were analyzed using GeneMarker Software version 1.85 (Applied Biosystems, Foster City, CA).

Mapping the deletion breakpoints by Long Range PCR

Gene-specific 25bp primers located in proximal and distal non-deleted exons were designed for each type of deletion (Supplementary table 2). Genomic DNA (25ng/µl) was used in each PCR assay in a final reaction volume of 50µl using the Expand Long Template PCR system (Roche Applied Science). Amplification was performed on an AB Perkin thermal cycler GeneAmp PCR System 9700 as follows: initial denaturation at 94°C for 2min; 10 cycles of 94°C for 10s, 60°C for 30s and 68°C for 8min; 20 cycles of 94°C for 10s, 60°C for 30s, and 68°C for 8min with a 20s cycle of temperature increments and a final elongation at 68°C for 7min. Following electrophoresis, the DNA bands of interest were excised. The gel slices containing the PCR products were weighed and DNA extraction was performed using Qiagen QIAkit gel extraction kit protocol, according to manufacturer's instructions. To increase DNA concentration, a second PCR was performed with the same primers. Direct sequencing with specific internal primers was performed using an AB Prism 3730XL DNA Analyzer Sequencer (Applied Biosystem) to map the deletional breakpoints.

Comparative genomic hybridization (CGH)

Whole genome array CGH analysis was performed using an Agilent 244k oligonucleotide array (Agilent, Santa Clara, CA, USA), according to the manufacturer's recommendations following protocol version 4.0. Briefly, 1µg of patient and reference DNA (pool of seven male DNA; Promega, Madison, WI, USA) were digested with *Alu*I and *Rsa*I (5U each) and subsequently labelled with Cyanine-5 dUTP and Cyanine-3 dUTP, respectively, using the BioArray Genomic DNA Labelling System (Enzo, Farmingdale, NY, USA) (Grisart B et al, J. Med Genet 2009.46:524-30). Data analysis was performed with the CGH Analytics 3.4 software platform (Agilent, Santa Clara, CA, USA).