Original Article

DNA Analysis of Renal Electrolyte Transporter Genes Among Patients Suffering from Bartter and Gitelman Syndromes – Summary of Mutation Screening

(*SLC12A1* / *ClCNKB* / *KCNJ1* / *SLC12A3* / hypokalaemic metabolic alkalosis / Bartter syndrome / Gitelman syndrome

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Abstract. Patients with renal diseases associated with salt-losing tubulopathies categorized as Gitelman and classic form of Bartter syndrome have undergone genetic screening for possible mutation capture in two different genes: *SLC12A3* and *CLCNKB*. Clinical symptoms of these two diseases may overlap. Patients with clinical symptoms of antenatal form of Bartter syndrome were screened for mutations in two different genes: *KCNJ1* and *SLC12A1*. The aim was to establish genetic mutation screening of Bartter/Gitelman syndrome and to confirm the proposed diagnosis. We have identified seven different causative mutations in the *SLC12A3* gene, four in the *CLCNKB* gene, two in the *SLC12A1* gene, and none in the *KCNJ1* gene. Nine of these mutations are novel. In one case, genetic analysis led to re-evaluation of diagnosis between the Gitelman and classic form of Bartter syndrome.

Introduction

Preservation of electrolyte homeostasis and thus water balance is vital to human body functioning. Active re-absorption of sodium generates the driving force for the passive re-absorption of water. At the level of distal convoluted tubule of a nephron about 20 % of H_2O and 7 % of Na^{2+} is reabsorbed. Gitelman syndrome is caused by a defect in transepitelial protein transporter in the distal convoluted tubule. Defects in proteins functioning as transepitelial sodium chloride transporters in thick ascending limb are responsible for Bartter syndromes (types 1, 2, and 3); at this part of nephron about 20 % of Na^{2+} is reabsorbed (Kleta and Bockenhauer, 2006). All of these above-mentioned defects are inherited as autosomal recessive traits.

Gitelman syndrome

Gitelman syndrome (GS), also referred to as familial hypokalaemia-hypomagnesaemia, is characterized by hypokalaemic metabolic alkalosis in combination with significant hypomagnesaemia and low urinary calcium excretion (Gitelman et al., 1966). It is the numerically predominating salt-losing tubulopathy (Simon et al., 1996b). The prevalence is estimated at approximately 1 : 40,000 and accordingly, the prevalence of heterozygotes is approximately 1 % in Caucasian populations, making it one of the most frequent inherited renal tubular disorders.

Clinical symptoms and biochemical markers of GS and classic form of Bartter syndrome (type 3) may overlap (Fukuyama et al., 2003, 2004; Zelikovic et al., 2003) and thus genetic analysis may specify the real cause of symptoms. For diagnostic criteria of Gitelman syndrome (OMIM 263800) see Table 1.

In the majority of GS patients, DNA mutations are found in the *SLC12A3* gene, which encodes the thiazide-sensitive NaCl co-transporter (NCC). At present, more than 180 different NCC mutations throughout the whole protein have been identified (according to online database Human Gene Mutation Database – www.hgmd.org). These mutations include missense, nonsense, frame-

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Abbreviations: GS – Gitelman syndrome, MLPA – Multiplex Ligation-dependent Probe Amplification, NCC – NaCl co-transporter, nsSNP – non-synonymous SNP, SNP – single-nucleotide polymorphism, TAL – thick ascending limb.

Table 1. Features differentiating Bartter and Gitelman syndromes

Features	Neonatal Bartter syndrome	Classic Bartter syndrome	Gitelman syndrome
Age at onset	Neonatal period	Childhood	Childhood or later
Maternal hydramnios	Common	Rare	Absent
Polyuria, polydipsia	Marked	Present	Rare
Dehydration	Present	Often present	Absent
Tetany	Absent	Rare	Present
Growth retardation	Present	Present	Absent
Urinary kalcium	Very high	Normal or high	Low
Nephrocalcinosis	Present	Rare	Absent
Serum magnesium	Normal	Occasionally low	Low
Urine prostaglandins (PGE2)	Very high	High or normal	Normal
Response to indomethacin (improvement of hypokalaemia and renal salt wasting)	Good	Good	Rare

Data taken from Pugh-Clarke, 2007

shift, and splice-site mutations and are distributed throughout the whole protein. Mutations are believed to reduce capability of the affected protein to reabsorb salt in the distal renal tubules, where the co-transporter is specifically expressed (Mastroianni et al., 1996). Altered protein SLC12A3 is usually unglycosylated and remains in endoplasmic reticulum. However, some mutations do not lead to glycosylation disturbance and a partially functioning protein is incorporated into the plasmatic membrane (De Jong, 2002).

In a small minority of GS patients, mutations in the *CLCNKB* gene encoding the chloride channel ClC-Kb have been identified. Because *CLCNKB* is expressed not only in the thick ascending limb of Henle's loop, but also in distal convoluted tubules, the features of BS due to a Cl^- channel defect can be shared with GS (Peters et al., 2002). Mutations in the *CLCNKB* gene were previously found to be the cause of classic Bartter syndrome (type 3). It is now evident that the clinical phenotype in patients with *CLCNKB* mutations can be highly variable, from an antenatal onset of Bartter syndrome on the one side of the spectrum, to a phenotype closely resembling Gitelman syndrome on the other (Knoers et al., 2005). Therefore, there is an indication for screening the *CLCNKB* gene in those patients with the Gitelman phenotype who do not have mutations in the *SLC12A3* gene.

NaCl co-transporter NCC is a polypeptide of 1021 amino acids and the 2D-structure is predicted to contain 12 transmembrane domains and long intracellular amino- and carboxy-termini.

Lifelong supplementation of magnesium (magnesium-oxide and magnesium-sulphate) is recommended. Cardiac work-up should be offered to screen for risk factors of cardiac arrhythmias. All GS patients are encouraged to maintain a high-sodium and high-potassium diet (Knoers and Levtchenko, 2008). In general, the long-term prognosis of GS is good.

Heterozygous carriers of inactivating mutations may have moderately decreased renal sodium re-absorption, which protects them against development of hypertension. Abnormal renal handling of magnesium and calcium has been observed in *SLC12A3* knock-out mice (Schultheis et al., 1998). Decreased Na^+ re-absorption in the distal convoluted tubule leads to an increased solute delivery to the collecting tubule, with consequent mild volume contraction and aldosterone-stimulated secretion of K^+ and H^+, finally resulting in mild hypokalaemic metabolic alkalosis (Scheinman et al., 1999).

Bartter syndrome

The thick ascending limb (TAL) is a major part of the nephron urine concentration machinery due to its water impermeability and unique sodium chloride re-absorption abilities. There are several proteins which facilitate the proper ion management and their failure leads inevitably to significant polyuria with all its consequences, especially in infancy and even *in utero* (polyhydramnios). Mutations in genes *SLC12A1* , *KCNJ1*, *CLCNKB*, and *BSND*, encoding bumetanide-sensitive sodium-potassium-chloride co-transporter, potassium inwardly-rectifying channel, chloride channel Kb, and barttin proteins, respectively, cause autosomal-recessive Bartter syndromes types 1, 2, 3 and 4 (Scheinman et al., 1999; Birkenhager, 2001).

Bartter syndrome type 3

On the basolateral side, chloride can exit through at least two chloride channel proteins, predominantly by chloride channel Kb, and, to a lesser degree, by chloride channel Ka. Both of these chloride channels require Barttin as subunit to be present for proper function. Mutations in the *CLCNKB* gene cause Bartter syndrome type 3 (MIM 607364), sometimes classified as classic Bartter syndrome (Simon et al., 1997).

The first symptoms of this disease may be present in the first two years of life, but they are usually diagnosed at school age or later. Diagnostic criteria are summarized in Table 1.

CLCNKB codes for voltage-gated chloride channel with 12 transmembrane domains and intracellular N and C termini. Its functions are regulation of cell volume, membrane potential stabilization, signal transduction, and transepithelial transport.

Mutations listed in the Human Gene Mutation Database (www.hgmd.org) comprise mainly missense and splicing mutations, although deletions/insertions and gross rearrangements were also observed.

Bartter syndrome type 3 has the mildest course of all Bartter syndrome types, presumably due to the preserved CLCNKA chloride permeability in the thick ascending limb of Henle's loop. Bartter syndrome is the most important genetic disorder to consider in the differential diagnosis of GS.

Bartter syndrome types 1 and 2

In the thick ascending limb of Henle's loop, sodium chloride is reabsorbed in symport with K^+ via the bumetanide-sensitive Na^+-K^+-$2Cl^-$ co-transporter – NKCC2. As the luminal K^+ concentration is low, a permanent K^+ recycling via potassium inwardly-rectifying channel (ROMK) is essential for proper function of NKCC2 (Boim et al., 1995). Mutations in the *SLC12A1* gene, encoding NKCC2, leads to Bartter syndrome type 1 (MIM 601678) and mutations in the *KCNJ1* gene, encoding ROMK, lead to Bartter syndrome type 2 (MIM 241200), in one group as hyperprostaglandin E syndrome (Simon et al., 1996a).

Prenatal-onset renal salt and water wasting in this autosomal recessive disorder leads to polyhydramnios leading to extreme prematurity. It represents the most severe form of Bartter syndrome and without appropriate treatment patients die shortly after birth; for diagnostic criteria see Table 1. Although most patients have symptoms in prenatal or perinatal period, there are sporadic cases of adolescent age onset (Pressler et al., 2006). Up to date, there are 50 mutations of the *SLC12A1* gene listed in the mutation database (www.hgmd.org), mainly missense.

Mutations in luminal inwardly rectifying potassium channel ROMK (*KCNJ1*) cause Bartter syndrome type 2. This antenatal form is clinically almost indistinguishable from type 1. However, minor clinical and biochemical differences exist. Mutations in the *KCNJ1* gene lead to lower birth weight, the potassium level is normal or slightly increased compared to the lowered potassium level in patients with mutations in the *SLC12A1* gene (Puricelli et al., 2010). And more, the presence of early transitory hyperkalaemia suggests mutations in *KCNJ1* (Brochard et al., 2009).

This channel plays a major role in potassium homeostasis in the kidney. Inward rectifier potassium channels are characterized by a greater tendency to allow potassium to flow into the cell rather than out of it. Their voltage dependence is regulated by the concentration of extracellular potassium. As external potassium is raised, the voltage range of the channel opening shifts to more positive voltages. The inward rectification is mainly due to the blockage of outward current by internal magnesium (Shuck et al., 1994). There are 50 different mutations of *KCNJ1* listed in www.hgmd.org database, mainly missense.

Patients with Bartter syndromes face several difficulties in treatment. Significant fluid and sodium chloride losses need to be replaced, which is more difficult for younger patients. In case prostaglandins are elevated in urine, selective and non-selective cyclooxygenase inhibitors can be used for the treatment, e.g. celecoxib, ibuprofen, or indomethacin (Kleta and Bockenhauer, 2006), but all of these possess possible negative side effects. Some patients with Bartter syndromes show hypomagnesaemia necessitating oral magnesium replacement therapy.

Some clinical characteristics can guide the genetic study (see Table 1). However, these signs might be absent at diagnosis and the genetic classification does not perfectly match the clinical classification. The aim of this study was to find mutations in candidate genes among a set of patients with salt-losing tubulopathies that were distributed according to their clinical manifestation into three categories: presumably Gitelman syndrome (*SLC12A3*), presumably classic Bartter syndrome (*CLCNKB*), and those with presumably antenatal form of Bartter syndrome (*KCNJ1*, *SLC12A1*).

Material and Methods

Patients

We have recruited patients from different regions of the Czech Republic, Slovakia and two siblings from India with clinical manifestation resembling Gitelman and Bartter syndromes. This study was not focused on a specific population mutation spectrum of the four depicted genes, but rather on the possibility to detect mutation in clinically diagnosed Gitelman/Bartter syndrome patients. Affected individuals or their relatives had to sign the informed consent to be included in the study group. All procedures performed throughout the work on the project complied with the ethical guidelines corresponding to relevant laws that were implemented by European Union member states and were included in institutional ethical policies.

Clinicians monitored levels of specific ions in blood (Na^+, K^+, Ca^{2+}, Cl^-, Mg^{2+}, (P, urea, creatinine)) and urine (Na^+, K^+, Ca^{2+}, Cl, Mg^{2+}), specific symptoms (age of manifestation, muscular weakness, cramps, polyuria/nykturia, nephrocalcinosis, chondrocalcinosis, polyhydramnion, premature delivery, growth retardation, blood pressure, pH of urine, presence of blood, proteins or sugar in urine, levels of specific blood gasses) and distributed patients/families into three groups – presumably Gitelman syndrome (8), presumably classic Bartter syndrome (6) and presumably antenatal Bartter syndrome (3). The majority of patients were in the first two groups. The age of onset spans from neonates to 40 years of age. Informed consent was obtained from all participating

patients or from their parents to take part in this study. It was not possible to screen all adult patients for both genes *SLC12A3* and *CLCNKB* due to the informed consent statement or lack of DNA material.

Candidate genes (*SLC12A3* – reference sequence NM_000339.1, *CLCNKB* – reference sequence NM_00085.2, *SLC12A1* – reference sequence NM_000338.2 and *KCNJ1* – reference sequence NM_000220.2) were tested for the presence of exonic DNA variants. If there was no mutation detected in the *SLC12A3* gene, the sample was tested for mutations in the *CLCNKB* gene, and vice versa. Antenatal Bartter syndrome patients were tested only for the presence of mutation in the *SLC12A1* and *KCNJ1* genes.

We had 100 blood control DNA samples (200 gene loci) of healthy individuals to test the frequencies of certain DNA variants.

DNA analysis

DNA was isolated from peripheral blood by procedures established in our laboratory or sent directly from collaborating institutes. Concentration of the DNA sample was always measured to maintain uniformity among samples. Standard 25-µl PCR reaction consisted of appropriate buffer, Mg^{2+} (1–3 mM), dNTP, primer (10 pmol of forward and reverse primer mix), standard polymerase (Taq DNA polymerase, Fermentas, Glen Burnie, MD), or long PCR polymerase (Long PCR Enzyme Mix, Fermentas), or polymerase for lower quality DNA (AmpliTaq Gold, PE Applied Biosystems, Foster City, CA), and approximately 1 ng of DNA. The PCR method amplified all exons with adjacent splice site junction of all the genes. We used primers described previously (Simon et al., 1997; Fukuyama et al., 2003) or designed by ourselves with the use of Primer3 tool (http://frodo.wi.mit.edu/primer3).

We applied the Multiplex Ligation-dependent Probe Amplification (MLPA) technique to test the *SLC12A3* gene (P136 SLC12A3 kit, MRC Holland, www.mrc-holland.com) for possible gross deletion/insertion of exons which would not be determined by classic direct sequencing strategy. We followed manufacturer's instructions.

Due to the high homology of gene *CLCNKB* (genomic reference sequence NG_013079.1) with *CLCNKA* (genomic reference sequence NG_009359.1) we applied the long PCR strategy with subsequent nested PCR.

Amplified products were directly sequenced with the BigDye terminator kit in ABI PrismTM 310 Genetic Analyzer (PE Applied Biosystems).

To evaluate the pathogenic effect of specific DNA variants, we applied many accessible internet sources, mainly: www.ncbi.nlm.nih.gov (for data regarding gene structure, function, SNP occurrence and frequency), www.biobase-international.com (for data regarding specific mutations), https://genome.ucsc.edu (for data regarding conservativeness of codon position among a great number of species). Missense DNA variants, not listed either in the SNP database or in the mutation database, were tested for position conservativeness with a blat tool of https://genome.ucsc.edu.

One hundred of healthy individuals provided their DNA for laboratory purposes and they served as a control group for testing occurrences of specific DNA variants. DNA variants that were not present in 200 gene loci of the control group were considered as likely pathogenic. We could not, unfortunately, test these variants at the protein level since this procedure is not established in our laboratory.

Results

All mutations are listed in Table 2. Mutation screening has been completed for 10 patients for the *SLC12A3* gene, 10 for the *CLCNKB* gene, three for the *KCNJ1* gene and three for the *SLC12A1* gene, out of total 20 probands (see Table 2). Unfortunately, we could not test all adult patients for both *SLC12A3 – CLCNKB* genes, due to restrictions given in the informed consent or lack of DNA material.

Mutations found in SLC12A3

Seven different causative mutations were identified in the *SLC12A3* gene in five patients (Nos. 33, 60, 64, 65, 78). Among those, we recorded five missense, one nonsense and one homozygous frame-shift DNA variants. The MLPA technique was applied to all adult patients from the presumably Gitelman/classic Barrter syndrome group (17 individuals). In none of the DNA tested we recorded a misbalance in the amount of specific exons in the *SLC12A3* gene that would be diagnosed by the MLPA technique.

Patient No. 33 was first examined for *CLCNKB* mutations and then consequently for *SLC12A3*. Variant p.Gly741Arg was reported as pathogenic previously (Simon et al., 1996b). The second mutation detected is surely pathogenic, since c. 2245 C>T transition leads to formation of a stop codon at the position 749. The final protein is only 72 % length of the proper co-transporter, and thus not functional.

Patient No. 60 is a compound heterozygote, p. Arg135His and p. Arg896Gln (Kurschat et al., 2003). Both mutations are caused by transition of G>A at the DNA level. The first mutation is novel. It changes arginine to histidine; both amino acids are polar and basic. This substitution is not in the SNP database, its position is highly conservative among selected species (rhesus, mouse, dog, elephant, opossum, chicken, zebrafish). It was not observed among 200 chromosomes of the control group. We decided to consider this variant as highly likely pathogenic.

Patient No. 64 is a carrier of a single mutation c. 1315 G>A, p. Gly439Ser (Mastroianni et al., 1996), which is common among patients with Gitelman syndrome in the Czech Republic and Slovakia (Urbanova et al., 2006). The second DNA mutation was not, unfortunately, detected.

Table 2. DNA variants found in SLC12A3, CLCNKB, SLC12A1 and KCNJ1 genes

***SLC12A3*, ref seq NM_000339**		
proband	**mutation**	**polymorphism**
33*	c.2221G>A,p.Gly741Arg, Simon et al., 1996 **c.2245C>T, p.Gln749X****	
57	-	c.2142C>T,p.Ala714Ala, SNP c.2625 C>T, p.Gly875Gly, SNP
59	-	c.366 A>G, p.Ala122Ala, homozygous, SNP c.1860C>G, p.Ser620Ser c.2142C>T,p.Ala714Ala, SNP c.2625 C>T, p.Gly875Gly, SNP
60	**c. 404 G>A, p. Arg135His** c. 2687 G>A, p. Arg896Gln, Kurschat et al., 2003	c.366 A>G, p.Ala122Ala, homozygous, SNP c. 1023 C>T, p. Phe341Phe, SNP
62	-	c. 2782 C>T, p.Arg928Cys, SNP
63	-	c.366 A>G, p.Ala122Ala, SNP c.2142C>T,p.Ala714Ala, SNP
64	c. 1315 G>A, p. Gly439Ser, Mastroianni et al., 1996	-
65	**c. 854 C>G, p. Ala285Gly**	-
78A, 78B***	**c.2878_2879ins.AGGGGTGCACCCTC, p.V960EfsX12, homozygous**	c.366 A>G, p.Ala122Ala, homozygous, SNP c.790G>C, p.Arg264Gly, homozygous, SNP c. 2952 C>T, p. Ile984Ile, homozygous
***CLCNKB*, ref seq NM_000085.2**		
proband	**mutation**	**polymorphism**
31	**c.226 C>T,p. Arg76X, homozygous**	-
32	c.1312 C>T, p.Arg438Cys, Simon et al., 1997	c.1441 A>T, p.Thr481Ser, SNP c.80 T>G, p.Leu27Arg, homozygous, SNP c.860 T>C, p.Val287Ala, SNP
33	-	c.80 T>G, p.Leu27Arg, SNP c.860 T>C, p.Val287Ala, SNP c.1455 G>A, p.Ala485Ala, SNP c.1732 G>A, p. Glu578Lys, homozygous, SNP
36	**c.908 A>C, p.Gln303Pro** **c.1269delC,p.Ala423AlafsX56**	c.1732 G>A, p. Glu578Lys, SNP
49A,49B,49C	-	c.80 T>G, p.Leu27Arg, SNP
52	-	c.80 T>G, p.Leu27Arg, SNP c.324 G>A, p.Ser108Ser, SNP c.860 T>C, p.Val287Ala, SNP c.1254 C>T,p.Thr418Thr, SNP c.1255 A>G, p.Ile419Val, SNP c.1308 C>T, p.Ile436Ile, SNP c.1441 A>T, p.Thr481Ser, SNP c.1732 G>A, p. Glu578Lys, SNP
62	**-**	c.80 T>G, p.Leu27Arg, SNP c.2055C>T, p.Ala685Ala, SNP
63	-	-
***SLC12A1*, ref seq NM_000338.2**		
proband	**mutation**	**polymorphism**
48	-	-
58	**c. 2095 G>A, p.Asp699Asn**	-
90	**c. 1411 C>T, p.Arg471X**	c.1614T>C, p.Tyr538Tyr, SNP
***KCNJ1*, ref seq NM_000220.2**		
proband	**mutation**	**polymorphism**
48	-	-
58	-	-
90	-	-

This table shows DNA variants found in the four examined genes. Expression "ref seq" stands for the reference sequence listed in the sequence database (www.ncbi.nih.gov); DNA variant nomenclature according to recommendations of the Human Gene Mutation Database (www.hgmd.org).
*bold numbers of probands represent those that were first tested for a different gene; **mutation in bold represents a novel DNA variant; ***probands with letters represent affected siblings; symbol " –„ (dash) represents that no DNA variant was observed

Patient No. 65 is a carrier of a single variant c. 854 C>G, p. Ala285Gly. This transversion causes missense substitution at highly conservative position Ala285 (9/9 selected species also had alanine at position 285 of the *SLC12A3* gene). Alanine is a non-polar neutral amino acid, similarly as glycine, but this specific DNA variant was not observed among our control group of 200 gene loci. We consider this variant as highly likely pathogenic.

Patients Nos. 78A and 78B are siblings, Indian Bedouins, with very unusual mutation – insertion of 14 nucleotides in exon 24. Its homozygous state was confirmed by analysis of probands' parents, who were consanguineous. This mutation leads to protein shortening to 94 % of its previous length.

Regarding SNP polymorphisms of *SLC12A3*, we observed mainly heterozygotes with average occurrence of about 0.2 (20 %) in standard Caucasian population (data taken from www.ncbi.nlm.nih.gov database). Patient No. 60 is a carrier of SNP: p.Ala122Ala in homozygous constitution, which occurs in the population only in approximately 0.06 %. Samesense substitution p. Ser620Ser is not listed in the SNP database. The position of this variant is in the middle of exon 15, substitution c. 1860 C>G does not interfere with splicing. However, this variant was not observed among our control group sample (200 gene loci), and we consider this variant as not likely pathogenic. Some SNP variants with very low occurrence might have been missed when only 200 loci were analysed. Patient No. 59 (carrier of this DNA variant) has, unfortunately, no causative mutation detected to analyse the parental pattern of inheritance which could provide additional information. SNPs p.Phe341Phe and Arg928Cys are not very commonly observed, the occurrence of heterozygotes is approximately 0.07 and 0.067, respectively.

Mutations found in CLCNKB

Four different causative mutations were identified in the *CLCNKB* gene in three patients. We recorded two missense, one homozygous nonsense and one frameshift mutations.

In patient No. 32, we found only one pathogenic variant. This p.Arg438Cys has been previously described as pathogenic (Simon et al., 1996a). Diagnosis of this proband was not proved, but is highly likely.

In patient No. 31, we found nonsense mutation p.Arg76X. According to our finding, it looked like a homozygous mutation, but since we could not prove it by examination of the probands' parents, we must also consider the possibility of second allele deletion.

In patient No. 36, we found p. Gln303Pro and p. Ala423AlafsX56. Both of these mutations were not detected previously. The first variant changes amino acid glutamine (polar, neutral) to proline (non-polar, neutral). The codon position is conservative among 7/9 species (human, rhesus, mouse, dog, elephant, chicken, x-tropicalis). It was not detected among 200 chromosomes of the control group. We consider this variant as likely pathogenic. The second DNA variant is a deletion of C, leading to a frame-shift defect at the protein level. Nonfunctional protein has only 46% length.

We recorded a high number of SNP polymorphism variants, among which p.Leu27Arg was the most frequent. Patient No. 52 is a carrier of a large number of different SNPs.

Two patients (Nos. 32 and 52) have SNP c. 1441 A>T (p. Thr481Ser), which was tested by Jeck (Jeck et al., 2004) and described, thereafter, as causative for chloride channel activity increase. Four patients from a cohort do not have any mutation in all examined exons and adjacent splice junctions. Two of these patients (Nos. 33 and 62) with no mutation identified were further analysed for possible alterations in the *SLC12A3* gene. Unfortunately, we could not test other two patients (Nos. 49 and 52) due to the lack of DNA material. Analysis showed that only patient No. 33 was misdiagnosed as having the classic form of Bartter syndrome, because we could identify two pathogenic variants in the *SLC12A3* gene.

Mutations found in SLC12A1 and KCNJ1

Among the group of three antenatal Bartter syndrome patients, we could identify only two causative DNA mutations in the *SLC12A1* gene, but none in the *KCNJ1* gene.

Patient No. 58 is a carrier of a single variant p. Asp699Asn. This substitution is not listed in the SNP database. Position Asp-699 is strictly conservative among different species (human, rhesus, mouse, dog, elephant, opossum, chicken, x-tropicalis, zebrafish). This variant was not observed among 200 chromosomes of the control group. We consider this missense variant highly likely pathogenic. Unfortunately, we were not successful in finding the second causative mutation.

Patient No. 90 is a carrier of a nonsense variant p. Arg471X (novel) (see Table 2), which leads to formation of a non-functional shorter form of protein. SNP p. Tyr538Tyr is very common in Caucasian population (T/C 0.49).

Discussion

Gitelman and Bartter syndromes are genotypic and phenotypic heterogeneous diseases. Clinical manifestation and laboratory findings might be misleading in proposing diagnosis and managing appropriate treatment.

In the present study, we searched for mutations in four genes causative for development of Gitelman/Bartter syndromes to prove diagnosis in order to determine proper medical treatment. We were not successful in finding mutations for all patients that were recruited in the study group. However, several causative sequence variations were observed. It was possible to prove clinical diagnosis in 5/17 cases by finding two causative mutations.

In five patients we could find only one causative mutation. Since the prevalence of heterozygotes of the Gitelman syndrome, the most frequent renal tubulopa-

thy, in Caucasian population is approximately 1 %, we supposed that all the patients with only one detected causative DNA mutation would be affected by a specific syndrome, but their second causative mutation remained undetected.

There are even reports from the past that different kinds of mutations in one gene can cause both dominant and recessive phenotypes (Deen et al., 2000; Hendy et al., 2000). Our one-allelic mutation detection was rather a result of incomplete gene screening than dominance since the phenotype did not occur more often in different generations in the families.

In 12 patients/families it was not possible to give final confirmation of the proposed diagnosis. Our applied laboratory procedure could, unfortunately, miss some DNA variants in the regulatory or intron regions which were not analysed. At the same time, gross deletions/insertions could be missed. The possibility of large deletions/insertions was excluded by application of MLPA, because no difference in the copy number of all 26 exons of *SLC12A3* was detected.

Several former studies tried to focus on the correlation between specific DNA mutation and phenotypic clinical outcome. In a study by Coto (Coto et al., 2004), many individuals carrying exactly the same mutation coming from unrelated families did not correlate in values of ionic composition in blood and urine. Their clinical symptoms also differed. It seems to be clear that the genetic background of unrelated individuals plays the most important role. It showed that even persons with the same DNA variants, and even siblings, could present differences in clinical symptoms, and even mimic a different syndrome. This was the case in a study by Zelikovic (Zelikovic et al., 2003), where a large Bedouin family sharing *CLCNKB* mutation presented clinical characteristics specific for Gitelman syndrome, on the one side of the spectrum, to classic Bartter syndrome, on the other. There are reports of males having more severe phenotype when compared to women with the same mutation in the *SLC12A3* gene, which could be explained by a positive effect of oestrogens (Lin et al., 2005; Riveira-Munoz et al., 2007). Our collection of patients is too small to correlate the obtained mutation data and phenotypic severity.

In our study we found several different SNP variants and the occurrence of their majority approximately matched their occurrence in a standard population (data not shown). However, non-synonymous SNP (nsSNP) polymorphisms may interfere with protein function (Ng and Henikoff, 2002). Patient No. 52 is a carrier of four non-synonymous *CLCNKB* SNP variants (one is rare: p.Ile419Val – 0.023), and we can speculate that the function of the chloride channel protein might be affected. Computational prediction tests applied to nsSNPs estimated that 20–30 % of these nsSNP damage protein function (Chasman and Adams, 2001).

Some authors suggest that Bartter and Gitelman syndromes might be caused by several one-allelic mutations among a set of causative transporter genes (Fukuyama et al., 2003). In our study group there is no example of such theory. None of the probands displayed occurrence of a single causative mutation in doublet *SLC12A3-CLCNKB* or *SLC12A1-KCNJ1*. We must mention here that we could not, unfortunately, test all probands for the *SLC12A3-CLCNKB* doublet due to restrictions in the informed consent of a patient or due to the lack of DNA material sent to our laboratory for screening.

Our study group is very small for evaluation of specific hot-spots of the four examined genes. In our previous paper (Urbanova et al., 2006) we found mutation p. Gly439Ser (*SLC12A3*) to be very frequent among Gitelman syndrome patients from the Czech Republic and Slovakia. This variant was detected only in one patient (No. 64) in this new study group, and we broadened the spectrum by finding five novel mutations. All missense mutations of *SLC12A3* reported to date are located throughout the gene, but most of these mutations are most frequently found in the intracellular domains of the protein (Simon et al., 1996b; Reissinger et al., 2002).

DNA sequence homology and close distance on chromosome 1 between *CLCNKA* and *CLCNKB* genes may lead to genetic conversion during the crossing over process. We have identified DNA variants specific for the *CLCNKA* gene in nested PCR products of the *CLCNKB* gene. This result led to us to the conclusion that the genetic conversion process occurred in these patients. However, after close inspection (new long PCR primer design), we have to re-evaluate our result as a consequence of erroneous interpretation, where a *CLCNKA* fragment was amplified during long PCR together with a *CLCNKB* fragment. Primers for the *CLCNKB* fragment were specific only for the *CLCNKB* gene, but this sequence also matched the *CLCNKA* fragment, where two SNPs (one at 3' site) were present. These facts were not previously considered during the primer design. After final re-evaluation, we have not recorded the genetic conversion process among our patients.

Some patients having mutations in the *CLCNKB* gene may also present polyhydramnios leading to premature delivery that may mislead in genetic screening of the *KCNJ1* or *SLC12A1* genes. However, in the case of Bartter syndrome of types 1 and 2, the urine level of potassium is remarkably lower when compared to the classic form of type 3 (Brochard et al., 2009).

Only three patients had clinical symptoms resembling antenatal form of Bartter syndrome (types 1 and 2), the most severe salt-losing tubulopathy examined. We were successful in finding DNA variants only in the *SLC12A1* gene, which codes for bumetanide-sensitive Na^+-K^+-$2Cl^-$ co-transporter NKCC2. This gene is larger than *KCNJ1*, but its transcription is less complex. Therefore, *KCNJ1* is expected to be more prone to mutation occurrence. According to Brochard et al. (2009), the *KCNJ1* gene was observed to be predominating in frequency of gene mutations among Bartter syndrome patients. We found two causative mutations in *SLC12A1* in only two

patients. The pathological effect of p.Arg471X is evident, but the effect of p. Asp699Asn is only highly likely and should be further tested.

In conclusion, our study applied genetic techniques to screen DNA variants in the exonic regions of four selected genes among a group of patients suffering from salt-losing tubulopathy. It was possible to confirm the proposed diagnosis in four patients/families and to re-evaluate the proposed diagnosis in one patient by finding two causative mutations. This study shows how genetic screening may be helpful in confirmation of diagnosis based on clinical findings.

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