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ORIGINAL PAPER

# Molecular characterization of cystinuria in south-eastern European countries

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**Abstract** Cystinuria is an autosomal recessive disorder caused by defective transport of cystine and dibasic amino acids in the proximal renal tubules and small intestine. So far, more than 128 mutations in *SLC3A1* gene, and 93 in *SLC7A9* gene have been described as a cause of cystinuria. We present a molecular characterization of the cystinuria in 47 unrelated south-east European families. The molecular methodology included direct sequencing, single strand conformational polymorphism, and restriction fragment length polymorphism. A total of 93 (94.9 %) out of 98 unrelated cystinuric chromosomes have been characterized. Mutations in *SLC3A1* gene account for 64.3 % and in *SLC7A9* gene for 30.6 % of the cystinuric chromosomes. Ten different mutations in *SLC3A1* gene were found, and two of them were novel (C242R and L573X), while in *SLC7A9* gene seven mutations were found, of which three were novel (G73R, V375I and c.1048_1051delACTC). The most common mutations in this study were T216M (24.5 %), M467T (16.3 %) and R365L (11.2 %) in *SLC3A1* and G105R (21.4 %) in *SLC7A9* gene. A population specificity of cystinuria mutations was observed; T216M mutation was the only mutation present among

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Gypsies, G105R was the most common mutation among Albanians and Macedonians, and R365L among Serbs. The results of this study allowed introduction of rapid, simple and cost-effective genetic diagnosis of cystinuria that enables an early preventive care of affected patients and a prenatal diagnosis in affected families.

**Keywords** Cystinuria · Cystine · Dibasic amino acids · *SLC3A1* gene · *SLC7A9* gene

## Introduction

Cystinuria is an autosomal recessive disorder characterized by impaired transport of cystine, lysine, ornithine, and arginine in the proximal renal tubule and in epithelial cells of the gastrointestinal tract, resulting in elevated urinary concentrations of these amino acids [1]. Impaired reabsorption of poorly soluble cystine from primary urine leads to a high risk for the formation of cystine calculi in the urinary tract, potentially causing obstruction, infections, and eventually renal failure [2]. The estimated prevalence of the disease is 1/7,000 neonates, ranging from 1/2,500 in Libyan Jews to 1/100,000 in the Swedish patients. Cystinuria counts for 1–2 % of the renal lithiasis in adults and 6–8 % in pediatric patients [3]. Based on the urinary excretion of cystine and dibasic amino acids in obligate heterozygotes, cystinuria is classified into type I and non-type I. Heterozygotes of type I show normal aminoaciduria, whereas those of non-type I show a variable degree of urinary hyper excretion of cystine and dibasic amino acids [4]. Transport of these amino acids is mediated by the rBAT/$b^{0,+}$AT transporter [5], whose subunits are encoded by the genes *SLC3A1*, located on chromosome 2p16.3-21 [6], and *SLC7A9*, located on chromosome 19q12-13.1 [7, 8]. *SLC3A1* gene has 45 kb, and consists of 10 exons with 120–448 bp in length [9]. The product of this gene is the heavy subunit (rBAT) of the heteromeric transporter and this protein consists of 685 amino acids and is approximately 94 kDa in weight [10–12]. *SLC7A9* gene has around 39 kb, and consists of 13 exons with 45–242 bp in length [13]. The product of this gene is the light subunit ($b^{0,+}$AT) of the heteromeric transporter consisting of 487 amino acids with approximately 50 kDa in weight [5, 14]. Mutations in *SLC3A1* gene cause type I, while mutations in *SLC7A9* gene cause non-type I cystinuria [6, 15]. Based on genetic aspects, cystinuria is classified into: type A—caused by mutations in both alleles of *SLC3A1*; the heterozygotes showing a normal aminoaciduria; type B—caused by mutations in both alleles of *SLC7A9*; the heterozygotes usually showing an increase of cystine and dibasic amino acid urinary excretion, but may also have a normal aminoaciduria (in 14 % of the cases); type AB—caused by one mutation in *SLC3A1* and one mutation in *SLC7A9* derived from the two parents, respectively [16]. The type AB is extremely rare [17]. More than 128 mutations in *SLC3A1* gene and 93 in *SLC7A9* gene have been described. There are missense, nonsense, frameshift mutations, small deletions and/or insertions, splicing defects and gross genomic rearrangements [18]. Here, we present a molecular characterization of cystinuria in 47 south-east European families.

## Materials and methods

### Patients

Patients with cystinuria were selected in collaboration with the Pediatric Clinic at the Clinical Center in Skopje, R Macedonia. In addition to patients from R Macedonia (16) in this study, we included patients from other south-east European countries, i.e. Serbia (13), Montenegro (2), Bulgaria (1), Croatia (2), Kosovo (3), Turkey (9) and Slovenia (1). We examined 47 families with 60 cystinuria patients and 91 family members.

According to the ethnic origin, six families were of Macedonian origin, nine Serbian, nine Albanian, nine Gypsy, nine Turkish, two Croatian, two Montenegrin and one Egyptian. Informed consent was obtained from all patients and their relatives. The study was approved by the Ethics Committee of the Macedonian Academy of Sciences and Arts.

Patients were classified as type I, non-type I, mixed, and untyped on the basis of the urinary excretion profile of their parents for cystine and dibasic amino acids. Parents of type I cystinuria showed normal aminoaciduria, whereas those of non-type I showed a variable degree of urinary hyper excretion of cystine and dibasic amino acids. Mixed cystinuria term was used for the probands, where one parent showed type I and the other non-type I heterozygous cystinuria profile. Untyped patients were those where parents were not available for urine amino acid analysis. When only one parent was available, then the patient was classified as type I (or non-type I)/untyped. In this manner, we had 15 probands type I, 6 probands non-type I, 4 probands were mixed, 7 were type I/untyped, 4 were non-type I/untyped, 11 probands were untyped (Supplement Table 1S).

### Materials

Blood and urine samples were collected from the patients and their family members.

### Methods

Biochemical methods included Brand reaction for determining the presence of cystine in the urine [19], while the

urine amino acid levels of the patients and their relatives were determined using morning or 24 h urine, on Biochrom 30 amino acid analyzer, and corrected per mol creatinine excretion.

Genomic DNA was isolated from peripheral white blood cells using Proteinase K-SDS digestion, followed by phenol–chloroform extraction and ethanol precipitation [20]. Genomic DNA from all patients was amplified by PCR, using intron-derived oligonucleotides for each exon reported in ICC [13] for *SLC7A9* gene and Bisceglia et al. [21] for *SLC3A1* gene. Each PCR was performed in 50 µl total volume using 1× PCR Buffer, 1.5–2.0 mM $MgCl_2$, 200 µM dNTP, 25 pM of each primer (forward and reverse for every exon, respectively) 1 U AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 0.1–0.5 µg DNA. Cycling conditions were established depending on the melting temperature of the primers, ranging from 55 to 62 °C. PCR was performed in Thermal Cycler (Applied Biosystems 2720) under following conditions: initial denaturation 95 °C/10′, followed by 35 cycles of denaturation at 95 °C/1′, annealing temperature at 55–62 °C/1′ and elongation at 72 °C/1–2′. PCR was finished with final elongation at 72 °C/10′.

The mutational screening in all probands was performed by direct sequencing of PCR amplified DNA on ABI PRISM 310 Genetic Analyzer and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and primers used for PCR.

Mutations in the family members were detected by different techniques, including PCR analysis followed by single stranded conformational polymorphism (SSCP), restriction fragment length polymorphism (RFLP), heteroduplex analysis and/or direct sequencing, depending on the mutation in the proband, as detailed below.

The presence of the mutation in the family members of probands with T216M, C242R, c.1136+2T>C, R456C and M467T mutations was confirmed by SSCP analysis. For SSCP, we used 12 % polyacrylamide gel with 39:1 ratio of acrylamide:bisacrylamide. Electrophoresis was performed on BioRad DCode system (Bio-Rad Laboratories, Hercules, CA, USA) under following conditions: preelectrophoresis 15′ and electrophoresis 13–16 h with constant power of 11 W at 4 °C.

RFLP was used for the detection of the following mutations: R365L using *Msp*I restriction enzyme (if the mutation is present one of the two restriction sites is lost), M467K using *Alu*I restriction enzyme (if the mutation is present a third *Alu*I restriction site is created), S547W using *Sau*3A1 restriction enzyme (if the mutation is present the restriction site is lost), G105R using *Apa*I restriction enzyme (one of the three restriction sites for *Apa*I is lost) and V375I using *Mse*I restriction enzyme (a new *Mse*I restriction site is formed). RFLP analyses were performed at 37 °C, with the exception of *Apa*I digestion that was conducted at 30 °C.

The presence of the mutations in the parents of the proband homozygous for c.1048_1051delACTC was determined by heteroduplex analysis.

Direct sequencing was used to confirm the mutations in family members of probands with T216M, R365L, and M467T mutations in the *SLC3A1* gene and G105R and c.1265_1266delTG mutations in the *SLC7A9* gene [18].

## Results

In this study, we examined 47 families with 60 cystinuria patients and 91 family members. We analyzed 32 female and 28 male patients. Fifty-nine patients were homozygotes/compound heterozygotes, while one patient had three mutations, two in the *SLC3A1* gene and one in the *SLC7A9* gene (T216M and M467T in the *SLC3A1* gene and G105R in the *SLC7A9* gene). This case is reported elsewhere [22]. The three mutations are the most common cystinuria defects and have been confirmed as deleterious by functional studies using oocyte system [6, 13, 23].

In one family, the cystinuria was present in two generations: the mother and her three children were affected, while the father was a carrier, thus three cystinuria alleles were present in this family. Mother and one child were compound heterozygotes for T216M/M467T, two children were homozygotes for M467T mutation, while father was heterozygote for M467T mutation (type I carrier, having normal values for cystine and dibasic amino acids in the urine).

Two families with probands heterozygotes for non-type I cystinuria were also diagnosed by amino acid analysis of the urine, performed for different purposes. As expected, the DNA sequencing results confirmed the presence of only one *SLC7A9* mutation in both cases. One of the non-type I cystinuria heterozygotes was a male child with mild mental retardation, and he had G105R mutation. The level of cystine and dibasic amino acids in his urine was slightly elevated. He did not have cystine stones formed in the kidneys. The second non-type I cystinuria heterozygote was female that had been prenatally diagnosed with right sided dilatation of the urinary tract. Postnatally, she was found to have reflux grade IV–V. She has failed on conservative treatment and at the age of 4 years underwent anti-reflux surgery that was also unsuccessful. She was also found to have idiopathic hypercalciuria, but on ultrasound she had no evidence for calculi within the urinary tract. The girl had positive Brand reaction and elevated urine cystine and dibasic amino acids. The molecular analysis showed that she is heterozygote for small deletion in *SLC7A9* gene: c.1265_1266delTG.

Among the 98 unrelated cystinuria chromosomes studied, genetic defect was determined in 93 (94.9 %) chromosomes. The distribution of the mutations is given in Table 1; 64.3 % were *SLC3A1* gene mutations, 30.6 % were *SLC7A9* mutations, while 5.1 % were undetermined. All five patients with one undetermined allele have been clinically diagnosed with cystinuria. Three patients have experienced cystine calculi, while in two the amino acid analyses and Brand reaction were consistent with the diagnosis of cystinuria.

In one of these patients, we have indirect evidence that a deletion, involving at least exon 6 of the *SLC3A1* gene is responsible for cystinuria. Namely, the DNA sequencing results showed a homozygous *SLC3A1* R356L mutation. However, the DNA sequencing analysis in his parents showed that the mother was indeed a carrier of R365L, but the father was not. The paternity was confirmed by DNA analysis. DNA sequencing of *SLC3A1* gene showed presence of c.114C/A polymorphism in heterozygote state in exon 1, suggesting that this exon is not deleted. The c.1854A/G polymorphism was also detected, but it was uninformative since it was present in homozygous state in the patient and both parents. Additional analysis should be performed to confirm the size and boundaries of this deletion.

Ten different mutations in *SLC3A1* gene were found, and two of them were novel (C242R and L573X), while in *SLC7A9* gene seven mutations were found, of which three were novel (G73R, V375I and c.1048_1051delACTC). The missense mutation C242R, in exon 3 of the *SLC3A1* gene, involves T to C change at nucleotide 724, leading to substitution of cysteine (TGT) to arginine (CGT). This is a conserved amino acid residue in a part of a subunit that has extracellular localization [23]. The Polyphen bioinformatic program, which assesses the potential for damage based on known protein structure/function analyses and on conservation across a wide range of taxa was used to predict the effect of the novel missense mutations [24]. Polyphen test for C242R mutation in the *SLC3A1* gene has shown that this mutation is probably damaging with a score of 0.999 (sensitivity 0.14; specificity 0.99). The second new mutation in *SLC3A1* gene was the nonsense L573X mutation in exon 10. This mutation leads to a stop codon instead of leucine residue at codon 573, leading to truncated unstable protein.

The G73R mutation in exon 3 of the *SLC7A9* gene includes a substitution of G with A at nucleotide position 402, which leads to replacement of conserved glycine (GGG) by arginine (AGG) in the second transmembrane domain [13]. Polyphen test for novel mutation G73R in the *SLC7A9* gene has shown that this mutation is predicted to be probably damaging with a score of 1.000 (sensitivity 0.00; specificity 1.00). A deletion of four nucleotides (ACTC) at position c.1048_1051 in exon 10 in *SLC7A9* gene was detected in homozygous form in a patient with severe clinical outcome. This mutation was most probably generated by a mechanism of “slipped mispairing” between the two direct repeats (TC), followed by a deletion of one of the direct repeats and the intervening AC sequence [25]. The third novel mutation found in *SLC7A9* gene was V375I in exon 11 that involves a substitution of G with A at position 1123. This mutation involves substitution of valine by isoleucine and is located on the boundary between fifth extracellular loop and tenth transmembrane domain [26]. Polyphen test predicted that V375I mutation in the *SLC7A9* gene is benign with a score of 0.002 (sensitivity 0.99; specificity 0.30). No other mutation has been detected in the coding region and intron/exon boundaries of both *SLC3A1* gene and *SLC7A9* gene. A number of polymorphisms were found in both genes (three in *SLC3A1* and ten in *SLC7A9* gene), thus excluding the presence of deletions involving large portions of these genes. However, other gross genomic rearrangements and intronic mutations involving *SLC3A1* and *SLC7A9* genes are not excluded. Thus, the effect of V375I mutation remains to be clarified in the future.

All mutations were confirmed by a second PCR/sequencing reaction in the patients and in their parents and/or relatives if available. With SSCP, we have detected the following mutations: T216M, C242R, c.1136+2T>C, R456C, and M467T, while with RFLP we detected R365L, M467K, S547W, G105R and V375I. The most common mutation in *SLC3A1* gene was T216M found in 24.5 % of the examined chromosomes, followed by M467T (16.3 %) and R365L (11.2 %). However, the highest prevalence of T216M mutation in the *SLC3A1* gene is due to the fact this mutation is responsible for all cystinuria chromosomes in the Gypsy patients. In the *SLC7A9* gene, the predominant mutation was G105R with 21.4 %. The other mutations determined in both genes were present with a frequency of 1–2 %. A population specificity of cystinuria mutations was observed. Distribution of the cystinuria mutations based on the ethnic origin of the patients is given in Table 1.

### Phenotype–genotype correlation

In 53 cystinuria patients, amino acid analysis was performed to obtain the values for cystine and dibasic amino acids. Mean values of urine cystine and dibasic amino acids of patients with different cystinuria mutations are given in Table 2, while the comparison of the mean values of urine cystine and dibasic amino acids among patients with different cystinuria mutations is shown in Table 3.

The values of cystine and dibasic amino acids in homozygotes with T216M mutation were significantly

**Table 1** Frequency of the mutations in *SLC3A1* and *SLC7A9* genes in 98 unrelated cystinuric chromosomes and distribution of the cystinuria mutation in *SLC3A1* and *SLC7A9* genes in the patients with different ethnic origin

| Mutation | Ethnic origin | | | | | | | | | | | | | | | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Macedonians | | Albanians | | Serbians | | Gipsies | | Turks | | Croatians | | Montenegrins | | Egyptian | | No. of chromosomes | Frequency (%) |
| | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % | | |
| *SLC3A1* | | | | | | | | | | | | | | | | | | |
| T216M | 3 | 20.0 | 2 | 11.1 | | | 18 | 100 | | | | | | | 1 | 33.3 | 24 | 24.5 |
| C242R | | | | | 2 | 11.1 | | | | | | | | | | | 2 | 2.0 |
| R365L | 2 | 13.3 | | | 5 | 27.7 | | | 1 | 5.6 | 2 | 50.0 | 1 | 25.0 | | | 11 | 11.2 |
| c.1136+2T>C | | | | | | | | | 2 | 11.1 | | | | | | | 2 | 2.0 |
| G398R | | | | | | | | | 2 | 11.1 | | | | | | | 2 | 2.0 |
| R456C | | | | | 1 | 5.6 | | | 1 | 5.6 | | | | | | | 2 | 2.0 |
| M467T | 3 | 20.0 | 5 | 27.8 | 3 | 16.7 | | | | | | | 3 | 75.0 | 2 | 66.7 | 16 | 16.3 |
| M467K | | | | | | | | | 2 | 11.1 | | | | | | | 2 | 2.0 |
| S547W | | | | | 1 | 5.6 | | | | | | | | | | | 1 | 1.0 |
| L573X | | | | | 1 | 5.6 | | | | | | | | | | | 1 | 1.0 |
| Total in *SLC3A1* gene | 8 | 53.3 | 7 | 38.9 | 13 | 72.2 | 18 | 100 | 8 | 44.4 | 2 | 50.0 | 4 | 100 | 2 | 100 | 63 | 64.3 |
| *SLC7A9* | | | | | | | | | | | | | | | | | | |
| G73R | | | | | 1 | 5.6 | | | | | | | | | | | 1 | 1.0 |
| G105R | 5 | 33.3 | 9 | 50.0 | 2 | 11.1 | | | 3 | 16.7 | 2 | 50.0 | | | | | 21 | 21.4 |
| A331V | | | | | | | | | 2 | 11.1 | | | | | | | 2 | 2.0 |
| c.1048_1051delACTC | | | | | | | | | 2 | 11.1 | | | | | | | 2 | 2.0 |
| V375I | 1 | 6.7 | | | | | | | | | | | | | | | 1 | 1.0 |
| S379R | | | | | 1 | 5.6 | | | | | | | | | | | 1 | 1.0 |
| c.1265_1266delTG | 1 | 6.7 | | | | | | | 1 | 5.6 | | | | | | | 2 | 2.0 |
| Total in *SLC7A9* gene | 7 | 46.7 | 9 | 50.0 | 4 | 16.7 | | | 8 | 44.4 | 2 | 50.0 | | | | | 30 | 30.6 |
| Undetermined | | | 2 | 11.1 | 1 | 11.1 | | | 2 | 11.1 | | | | | | | 5 | 5.1 |
| Total | 15 | 100 | 18 | 100 | 18 | 100 | 18 | 100 | 18 | 100 | 4 | 100 | 4 | 100 | 3 | 100 | 98 | 100 |

**Table 2** Mean values of cystine and dibasic amino acids in the urine of patients with different cystinuria mutations

| Mutation | Amino acids | | | | |
|---|---|---|---|---|---|
| | Cystine (0.6–20.0 mmol/mol Cr) | Ornithine (0.1–5.0 mmol/mol Cr) | Lysine (0.0–62.0 mmol/mol Cr) | Arginine (0.9–3.6 mmol/mol Cr) | Sum of cystine and dibasic amino acids |
| T216M homozygotes, $N = 9$ | 336.2 ± 69.6 | 439.8 ± 198.4 | 954.2 ± 190.8 | 836.9 ± 326.5 | 2567.1 ± 581.5 |
| M467T homozygotes, $N = 5$ | 148.8 ± 23.6 | 104.9 ± 68.8 | 486.8 ± 264.7 | 116.0 ± 121.3 | 851.0 ± 380.1 |
| G105R homozygotes, $N = 7$ | 222.3 ± 92.2 | 245.8 ± 87.4 | 419.3 ± 200.4 | 515.2 ± 201.8 | 1402.7 ± 459.8 |
| Other homozygotes, $N = 8$ | 189.9 ± 103.7 | 233.3 ± 75.4 | 500.4 ± 335.4 | 582.2 ± 298.9 | 1505.8 ± 588.2 |
| Compound heterozygotes, $N = 22$ | 152.5 ± 100.2 | 164.9 ± 85.0 | 406.3 ± 199.1 | 265.6 ± 192.6 | 989.3 ± 479.3 |

**Table 3** *p* values obtained from the comparison of the urine cystine and dibasic amino acid values in patients with different cystinuria mutations

| *p* values | Cystine | Ornithine | Lysine | Arginine | Sum of cystine and dibasic amino acids |
|---|---|---|---|---|---|
| T216M vs. M467T | **0.0001** | **0.0036** | **0.0024** | **0.0005** | **0.0001** |
| T216M vs. G105R | **0.0136** | **0.0310** | **0.0001** | **0.0387** | **0.0007** |
| T216M vs. other homozygotes | **0.0036** | **0.0145** | **0.0033** | 0.1157 | **0.0020** |
| T216M vs. compound heterozygotes | **0.0001** | **0.0001** | **0.0001** | **0.0001** | **0.0001** |
| M467T vs. G105R | 0.1156 | **0.0135** | 0.625 | **0.0029** | 0.0531 |
| M467T vs. other homozygotes | 0.4083 | **0.0104** | 0.94 | **0.0073** | 0.0501 |
| M467T vs. compound heterozygotes | 0.9366 | 0.1546 | 0.4489 | 0.1115 | 0.5534 |
| G105R vs. other homozygotes | 0.5359 | 0.7697 | 0.5869 | 0.6249 | 0.7144 |
| G105R vs. compound heterozygotes | 0.1135 | **0.0381** | 0.8817 | **0.0064** | 0.055 |
| Other homozygotes vs. compound heterozygotes | 0.377 | 0.055 | 0.3515 | **0.0019** | **0.0204** |

Bold values indicate statistical significance ($p < 0.05$)

**Table 4** Mean values of cystine and dibasic amino acids in the urine of male and female patients with mutations in the *SLC3A1* and *SLC7A9* genes

| | Amino acids | | | | |
|---|---|---|---|---|---|
| | Cystine (0.6–20.0 mmol/mol Cr) | Ornithine (0.1–5.0 mmol/mol Cr) | Lysine (0.0–62.0 mmol/mol Cr) | Arginine (0.9–3.6 mmol/mol Cr) | Sum of cystine and dibasic amino acids |
| Male *SLC3A1*, $N = 16$ | 162.9 ± 130.4 | 190.9 ± 165.5 | 425.4 ± 291.2 | 364.5 ± 352.7 | 11437 ± 829.7 |
| Female *SLC3A1*, $N = 22$ | 215.2 ± 101.3 | 253.2 ± 169.4 | 639.6 ± 332.1 | 474.8 ± 348.6 | 1582.8 ± 849.9 |
| Male *SLC7A9*, $N = 7$ | 283.0 ± 61.7 | 226.4 ± 100.3 | 483.4 ± 179.0 | 361.5 ± 241.2 | 1354.2 ± 448.3 |
| Female *SLC7A9*, $N = 6$ | 146.2 ± 71.8 | 248.2 ± 75.9 | 439.5 ± 203.2 | 568.6 ± 262.9 | 1402.5 ± 372.2 |

higher compared with other homozygotes and compound heterozygotes, with *p* value <0.05, except for non-significance for arginine values between T216M homozygotes and “other homozygotes”. Higher values of cystine and dibasic amino acids in the urine of the T216M homozygotes might be a result of the substitution of conserved amino acid in the protein. Additionally, certain way of life and environmental influence can lead to hardening the effects of the mutation. This mutation is specific for Gypsies, who represent an isolated group. It is worth mentioning that not all of the Gypsies were from Macedonia, but also from Serbia and/or Kosovo. The mean values of urine cystine and dibasic amino acids in male and female patients with mutations in the *SLC3A1* and *SLC7A9* genes are given in Table 4, while the comparison of theirs mean values is shown in Table 5.

**Table 5** *p* values obtained from the comparison of the urine cystine and dibasic amino acid values between male and female patients with mutations in the *SLC3A1* and *SLC7A9* genes

| *p* values | Cystine | Ornithine | Lysine | Arginine | Sum of cystine and dibasic amino acids |
|---|---|---|---|---|---|
| M vs. F (*SLC3A1*) | 0.172 | 0.2663 | **0.0462** | 0.3442 | 0.121 |
| M vs. F (*SLC7A9*) | **0.0035** | 0.6711 | 0.6865 | 0.1666 | 0.8384 |
| M (*SLC3A1*) vs. M (*SLC7A9*) | **0.0315** | 0.6072 | 0.6331 | 0.984 | 0.5374 |
| F (*SLC3A1*) vs. F (*SLC7A9*) | 0.132 | 0.9455 | 0.1749 | 0.5473 | 0.6204 |

Bold values indicate statistical significance ($p < 0.05$)

Clinical data of the patients are shown in the supplemental material (Table 1S). The data regarding the age of the appearance of the first stone were available for 34 patients and data on the number of stones in 31. Twenty-eight patients had the first stone in the first decade of life and 19 of them were under 3 years old. There was no difference in the age of appearance of the first stone and the number of stones between male and female patients. Comparison of the cystine and dibasic amino acids in the urine among male and female patients with *SLC7A9* mutation showed that male patients have significantly higher values for cystine compared to the female patients (Table 5). This is opposite to the study of Dello Strologo et al. [16] in which it was observed that stones occur earlier in male than in female patients. Also, female patients excreted higher amount of cystine than the male patients. This difference might be due to the small number of patients included in our study, the difference in the age of male and female patients in both studies or the influence of some environmental factors.

In addition, we have compared the cystine and dibasic amino acids levels in the urine among male and female patients with *SLC3A1* gene mutation, showing that the female patients have significantly higher lysine values in comparison to the male patients. Significant difference was also found for cystine values between male patients with mutations in the *SLC3A1* gene when compared to male patients with *SLC7A9* gene (Tables 4, 5).

## Discussion

This study presents the molecular characterization of cystinuria patients from R Macedonia and several other SEE countries (R Serbia, Kosovo, Montenegro, Croatia, Turkey and Slovenia). Among 98 unrelated cystinuric chromosomes, the genetic defect was determined in 93 (94.9 %) chromosomes. The distribution of the mutation in *SLC3A1* gene and *SLC7A9* gene is 64.3 and 30.6 %, respectively, while 5.1 % are undetermined. Similar to our findings, mutations in *SLC3A1* gene were predominant in Italian [21], Slovak and Czech [27], and German patients [28], while in the studies of Font-Llitjos et al. [17] and Gitomer et al. [29] that investigated cystinuria patients of different origin and American population, respectively, an equal distribution was found. Predominance of *SLC7A9* mutations was found in the Spanish population [30].

The most common mutations in our study are T216M and M467T in *SLC3A1* gene, and G105R in *SLC7A9* gene. These results are in agreement with the results of other studies [31–33]. Macedonians, Albanians and Turks had similar percentage of mutations in both cystinuria genes, while among Serbian patients mutations in *SLC3A1* gene were prevalent.

The T216M mutation in *SLC3A1* gene was predominant among Gypsies, while G105R in *SLC7A9* gene was present with highest frequencies among Albanians and Macedonians. Mutation M467T in *SLC3A1* gene was found in patients from different ethnic origin, while R365L in *SLC3A1* gene was found mainly in Serbs.

T216M mutation was the only mutation responsible for the cystinuria among Gypsy patients. This mutation has been previously described as a most common mutation in south-eastern Europe [23, 31]. This mutation was found to be prevalent among cystinuria patients of different ethnic origin in Europe with prevalence of around 4 % in middle and south Europe and 54 % in south-eastern Europe [28]. According to Albers et al. [34], this mutation is the most common in the Greek population. In studies of Bisceglia et al. [21] and Skopkova et al. [27], this mutation was also detected in patients with Gypsies origin. In Chinese patients, this mutation has not been found [35]. In our study, this mutation was also present among Albanian, Macedonian, and Egyptian patients (Table 1).

In vitro expression analysis of this mutation shows protein with very low transport activity [23]. We have observed that T216M homozygous patients have significantly higher values of cystine and dibasic amino acids in comparison to the patients with other mutations.

The second most common mutation in *SLC3A1* gene, with frequency of 16.2 % of all alleles, and 25.4 % of the alleles in *SLC3A1* gene, was M467T mutation found among different ethnic groups with different frequency. It has been shown that this mutation is the most common in

north, middle and south-western Europe [21, 36]. Similar frequencies for this mutation were described by other authors [17, 21, 37]. In American population the frequency was 17 % [38], and among Slovak and Czech [27] and Swedish patients [39] M467T mutation was detected with a rate of 36 and 41 %, respectively. In Spanish population, this mutation was found with a low frequency of 5.2 % [30]. In Chinese patients, this mutation has not been found [35]. In the studies analyzing patients from Turkey, this mutation has also been found with the high frequencies [40, 41]. In our study, M467T mutation was not observed among the nine patients that originated from Turkey.

The third most common mutation in *SLC3A1* gene with a frequency of 11.2 % of all affected alleles, and 17.5 % of the alleles in the *SLC3A1* gene was R365L. This mutation was determined in the majority of patients with Serbian ethnic origin and we assume that this mutation is specific for this ethnic group. This mutation was described for the first time by Albers et al. [34], and was found only in population from south-eastern Europe with a frequency of 9.1 % [28]. The R365 position in the rBAT protein is a highly mutable position since there are several other mutations at this site (R365W [37], R365Q [31] and R365P [27]). In our research, we confirmed a connection between the R365L mutation and the polymorphism c.1136+3delT. This polymorphism was found in all patients that had R365L mutation, and in only one patient that is compound heterozygote for mutations in the *SLC7A9* gene (G105R/c.1265_1266delTG) out of 19 with different mutation.

The other mutations in the *SLC3A1* gene (C242R, c.1136+2T>C, G398R, R456C, M467K, S547W, and L573X) were found with a frequency of 1–2 %.

A total of seven mutations were found in *SLC7A9* gene, accounting for 30.6 % of all cystinuria alleles studied. The most common mutation in this gene was G105R with a frequency of 21.6 % of all cystinuria alleles and 70 % of the alleles in the *SLC7A9* gene. This mutation was described for the first time by the ICC [15], and it was the most common mutation in the *SLC7A9* gene with a frequency of 25 % in the study that considered 61 non-type I cystinuria probands from Italy (38), Spain (11), North America (3), Libian Jews (9) [13]. In our study, we found this mutation among patients with different ethnic origin and it was the most common among Albanians. Although Albanian patients had mutations in both cystinuria genes, G105R mutation was the only *SLC7A9* mutation.

The other six mutations in *SLC7A9* gene (G73R, A331V, c.1048_1051delACTC, V375I, S379R, c.1265_1266delTG) were found with a frequency of 1–2 %.

Despite the high mutation detection rate in this study, 5 (5.1 %) of the chromosomes remained uncharacterized. Since the methodology used in our study allowed for determination of mutations in the exon sequences and intron/exon boundaries, most probably the undetermined defects include large genomic rearrangements and mutations involving introns. Large genomic rearrangements were shown to represent 11 % of the defects in both *SLC3A1* and *SLC7A9* genes [42]. According to the HGMD [18], there are 18 large rearrangements in the *SLC3A1* gene and 5 in the *SLC7A9* gene representing 14 and 5.4 % of the mutations in these genes respectfully. We have indirect evidence that a deletion, involving at least exon 6 of the *SLC3A1* gene is responsible for cystinuria in one family from Serbia. The other mutation in this family is R365L. Additional testing should be performed to confirm the size and boundaries of this deletion.

The results of this study allowed for determination of a protocol for rapid, simple and cost-effective genetic diagnosis in cystinuria patients from Macedonia and from the region. The knowledge of the molecular defects allows for early preventive care and prenatal diagnosis in affected families.

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