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HUMAN GENETICS

Results of Molecular Genetic Testing in Russian Patients with Pendred Syndrome and Allelic Disorders

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Abstract—Pendred syndrome is an autosomal recessive inherited disorder characterized by a combination of sensorineural hearing impairment and euthyroid goiter; its clinical manifestation in children is hardly distinguishable from nonsyndromic hearing loss. Pendred syndrome is one of the most frequent types of syndromic hearing loss. Hearing impairment is accompanied by abnormal development of the bony labyrinth—enlarged vestibular aqueduct (EVA) and occasionally combined with Mondini dysplasia. Mutations in the *SLC26A4* gene, which encodes the pendrin protein, are responsible for both Pendred syndrome and for allelic disorder (nonsyndromic enlarged vestibular aqueduct). The present study for the first time conducted molecular genetic analysis in 20 Russian patients with Pendred syndrome, EVA and/or Mondini dysplasia. As a result, six pathogenic mutations in the *SLC26A4* gene were revealed in four patients. The mutation c.222G>T (p.Trp74Cys) was detected for the first time. Mutations were found in patients with Pendred syndrome and nonsyndromic EVA with or without Mondini dysplasia. Mutations were not detected in patients with isolated Mondini dysplasia. One proband with clinical diagnosis Pendred syndrome was homozygous for the c.35delG mutation in the *GJB2* gene. The absence of frequent mutations, including well-known ones or "hot" exons in the *SLC26A4* gene, was reported. Therefore, the optimal method to search for mutations in the *SLC26A4* gene in Russian patients is Sanger sequencing of all exons and exon-intron boundaries in the *SLC26A4* gene.

Keywords: hearing loss, Pendred syndrome, enlarged vestibular aqueduct, *SLC26A4* gene

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INTRODUCTION

Hearing impairment represents one of the most common sensorineural diseases. This inherited pathology is detected in one of 500 newborns on average. Moreover, inherited hearing impairment comprises about 50% of all the cases of prelingual hearing loss. Frequently, early detection of hearing impairment, as well as the reasons for its manifestation, appears to be a guarantee of successful child rehabilitation. Therefore, many countries, including Russian Federation, conduct universal audiologic screening of newborns for the effective early detection of hearing impairment together with DNA diagnostics for the establishment of inherited pattern of a disease. To date, about a hundred genes which cause hearing loss in the case of mutations in any of them are known [1–3]. More than half of cases diagnosed with medium and severe inherited nonsyndromic hearing loss belong to genetic type DFNB1 caused by recessive mutations in the *GJB2* gene encoding for connexin 26 [4]. About 20–30% of various inherited hearing impairments are accompanied by various syndromes. To date, more than 700 genetic syndromes including hearing loss as one of the parameters have been described [5].

Pendred syndrome represents one of the most frequent types of syndromic hearing loss. Its frequency is 7.5–10 cases per 100000 newborns, which is up to 10% of all cases of inherited hearing loss. Pendred syndrome is characterized by a combination of various type of hearing impairment and euthyroid goiter [6]. The degree of hearing impairment and disease manifestation vary. In a classic case, a severe two-sided preverbal hearing loss is observed; however, some children with Pendred syndrome demonstrate hearing impairment at an older age, which progresses in time. In rare cases, this progress might be related to craniocerebral injury, infection, and other reasons. In 66% of patients with Pendred syndrome, vestibular dysfunction is observed [7, 8]. In most or all cases, hearing impairment affiliated with Pendred syndrome is accompanied by an abnormally developed inner ear,

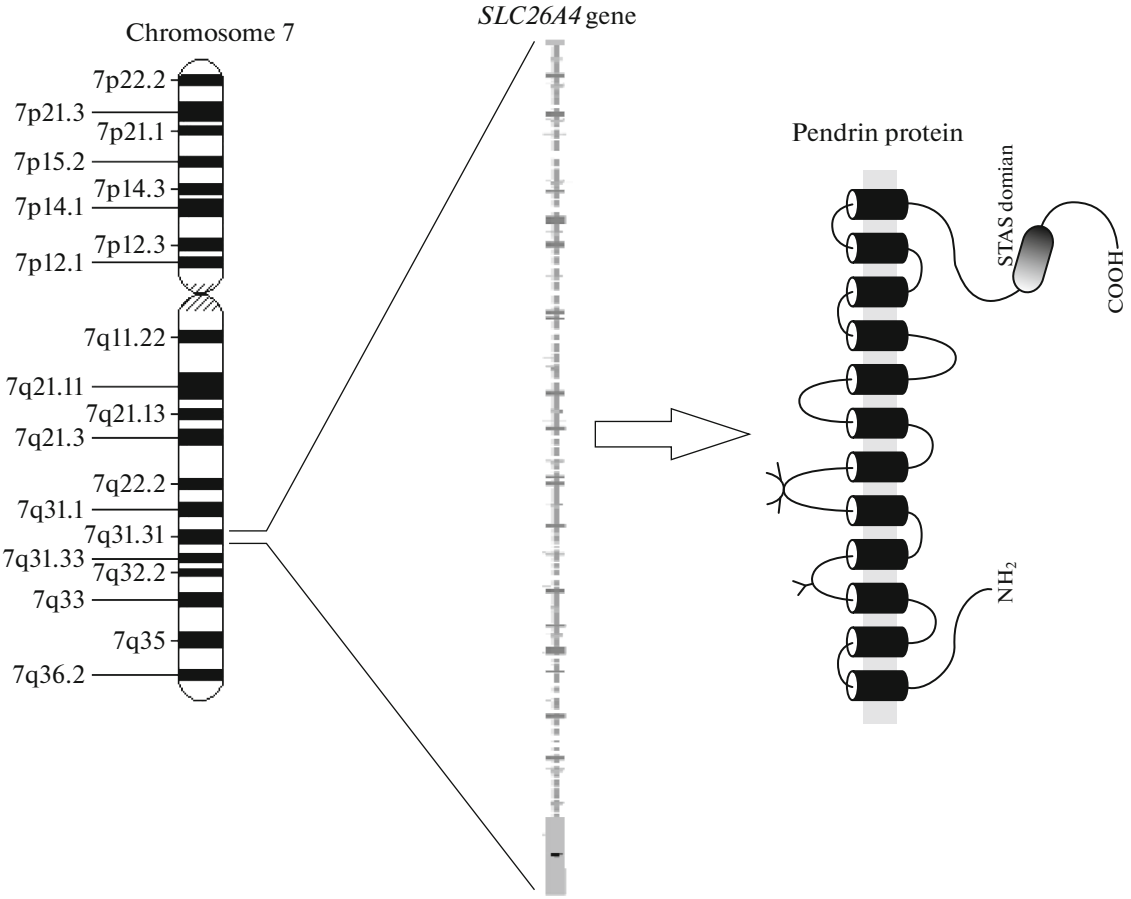

Fig. 1. *SLC26A4* gene and contemporary model of pendrin structure.

including enlarged vestibular aqueduct (EVA) ≥1.5 mm, in some cases in combination with dysplasia Mondini (characterized by incomplete division of cochlea turns, their probable enlargement, and a decreased number of turns) and outer semicircular canal. These abnormalities are rather frequent and are detected via computer and/or magnetic resonance imaging in patients with sensorineural hearing impairment and loss [9].

Mutations in the *SLC26A4* gene result in development of Pendred syndrome. This gene is located at chromosome 7 and consists of 21 exons, which comprise 57173 bp. Initiating codon ATG resides in exon 2. The *SLC26A4* gene encodes pendrin—membrane transport protein, representing a multifunctional anion transporter actively expressed in the thyroid gland, inner ear, and kidneys. Exact transmembrane organization and protein function has not been established. To date, pendrin structure consisting of 13 domains has been suggested (Fig. 1). It is involved in transport of different ions, probably acting as multifunctional anionic transporter. The pathogenetic effect of pendrin mutations in the thyroid gland is probably caused by disturbed flow of iodine anions through the apical membrane into the follicular cavity, which results in disturbed organification of iodine during biosynthesis of thyroid hormones [10]. Moreover, abnormal transport of carbonate ions and abnormal pH of the follicular cavity were also considered in the pathogenesis. Modified pH and acidulation of the inner ear endolymph have also been proposed as the main effect of pendrin mutations [11].

To date, more than 500 different mutations in the *SLC26A4* gene have been identified. They were detected in both homozygote (in children from closely related parents) and compound heterozygote (in familial and sporadic cases), including missense mutations in 70% of cases. Mutations responsible for disturbed splicing and reading frameshift are rare [12]. According to published data, "hot" exons are not found, mutations are even along the whole protein encoding gene sequence, including exon-intron boundaries. Major mutations in the *SLC26A4* gene were described in European, Japanese, Korean, and Chinese populations. The most frequent in several European populations are p.Leu236Pro (26%), p.Thr416Pro (15%), and c.1001+1G>T (14%) mutations. These mutations were detected in 50% of patients with confirmed diagnosis "Pendered syndrome" in Northern European populations [13].

Pathogenic variants c.919-2A>G, p.His723Arg, and p.Val239Asp are frequent in Chinese, Japanese, and Korean populations. Mutation p.His723Arg comprises 53% of mutant alleles in the Japanese population, while in the Korean population it is 40% [14, 15]. Mutation p.Glu384Gly is frequent among Northern Europeans [13], while mutation p.Gln514Lys is frequent among Spaniards [16].

Mutations in the *SLC26A4* gene cause the development of allelic disease, namely, inherited form of enlarged vestibular aqueduct (EVA) (nonsyndromic hearing loss of genetic type DFNB4). Pendred syndrome is considered to clinically differ from nonsyndromic hearing loss with enlarged vestibular aqueduct in the presence of euthyroid goiter, which is formed owing to disturbed iodine organification in the thyroid gland. Usually, goiter is formed in early adolescence in 40% of patients with this disease, but during adulthood in other individuals. Deficit of iodine organification can be detected via the perchlorate test at any age in patients with Pendred syndrome. However, it was considered unreliable because of low specificity and a significant difference in criteria used for interpretation of the results. Recent studies demonstrated that an abnormal result of the loading perchlorate test was also observed in patients with nonsyndromic hearing loss with EVA [17].

Therefore, clinical differential diagnostic in children with Pendred syndrome and isolated hearing loss with EVA is complicated. Moreover, nowadays, different researchers consider nonsyndromic hearing loss with EVA to be a phenotypic variant of Pendred syndrome [18].

Enlarged vestibular aqueduct with/without Mondini dysplasia of the inner ear is also comorbid to other types of syndromic hearing loss and appears to be frequent congenital malformation unrelated to heritability. Sporadic cases might represent a combination between congenital hypothyroidism and autoimmune disorders of the thyroid gland with sensorineural hearing loss, which is clinically similar to Pendred syndrome; however, they are genetically different [19]. The aforementioned factors also complicate clinical differential diagnostics of Pendred syndrome. Therefore, molecular genetic diagnostics of Pendred syndrome and allelic hearing loss with enlarged vestibular aqueduct, which would allow one to confirm the presence of these inherited disorders, is necessary for both effective medical genetic counseling and appropriate estimate of disease development.

A single study in Russia which explored a sample of patients with nonsyndromic inherited deafness reported two mutations in the *SLC26A4* gene in compound heterozygote, namely, c.85G>C (p.Glu29Gln) and c.149T>G (p.Leu50Arg), in one patient from the Republic of Bashkortostan [20]. Targeted study of Pendred syndrome and allelic hearing loss with EVA was not conducted previously; their frequency and etiology in Russian patients remain unknown. Hence, the development of molecular-genetic approaches for the diagnostics of these disorders requires preliminary research, the results of which are reported in the present study.

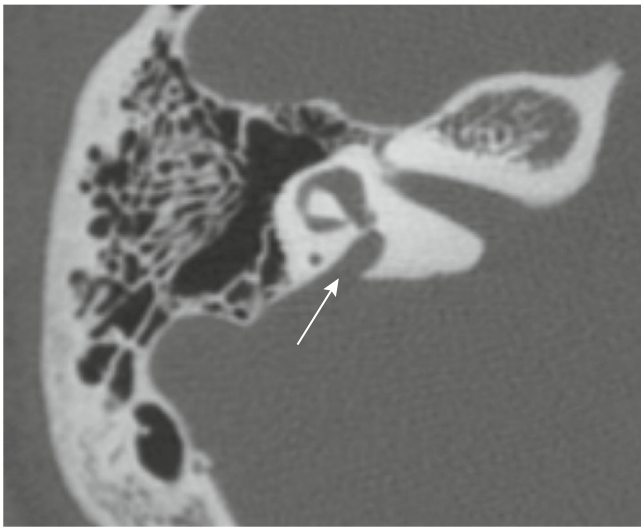

Fig. 2. Enlarged vestibular aqueduct syndrome. Computed tomography scan of the temporal bone, axial view. Enlarged vestibular aqueduct (arrow).

The present study was aimed at estimating the impact of genetic pathology caused by mutations in the pendrin gene in developing Pendred syndrome, enlarged vestibular aqueduct, and Mondini dysplasia in Russian patients, as well as at determining the algorithm of search for mutations in the pendrin gene optimal for Russian patients.

MATERIALS AND METHODS

Patients and population control. Twenty DNA samples obtained from unrelated patients aged from 1 to 16 years reporting hearing loss of various degrees from different regions of Russia served as material for the study. This sample comprised individuals with confirmed diagnosis of "euthyroid goiter" or "hypothyroidism," and also individuals with enlarged vestibular aqueduct and/or Mondini dysplasia detected via computer tomography (CT) (Figs. 2 and 3). The sample of patients was divided into four groups based on clinical characteristics (Table 1).

All the patients were clinically tested at three leading organizations—National Research Centre for Audiology and Hearing Rehabilitation (Moscow), Pokrovsky Medical Center (St. Petersburg), and Perm Regional Children's Clinical Hospital (Perm). DNA obtained from parents of one patient was also studied.

The control sample included 110 DNA samples of unrelated individuals from the genetic bank of the

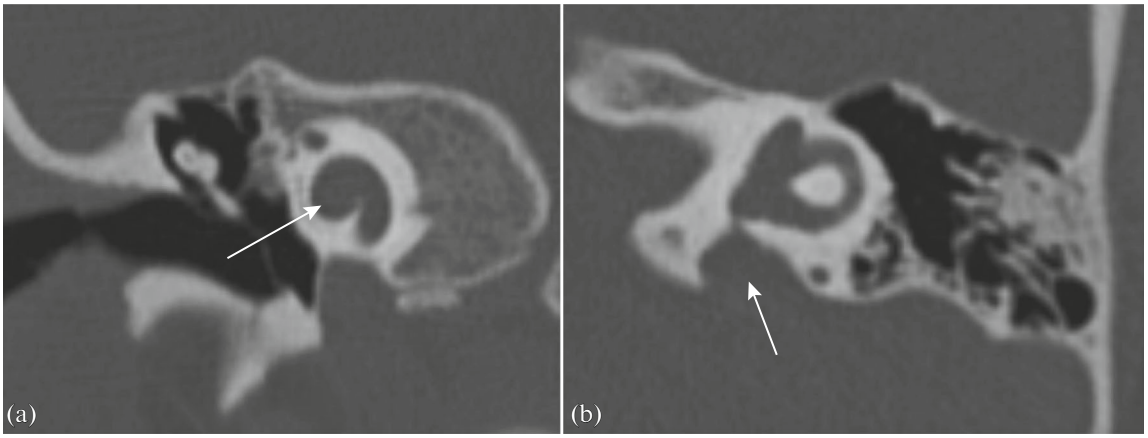

Fig. 3. Mondini Malformation. CT scan, left temporal bone. (a) Coronary projection. "Empty cochlea" (arrow). Incomplete division at the level of apical and medium turn; bone spiral plate is not observed; (b) axial projection. Enlarged vestibular aqueduct (arrow).

DNA diagnostics laboratory at the Research Centre for Medical Genetics.

The studied individuals signed a written informed consent stating the voluntary involvement in the research, including collection of biological material and open access publication of data. Parents signed the informed consent for their juvenile children. The study was approved by Ethics Committee of the Research Centre for Medical Genetics.

Methods. Genomic DNA was extracted from the peripheral blood leukocytes using the DLAtom™ DNA Prep100 kit according to manufacturer's recommendations (Izogen, Russia).

Table 1. Clinical characteristics of screened patients

Patient	Clinical diagnosis	Manifestation age	Sex	Level of hearing loss	EVA	Mondini dysplasia	Euthyroid disorders	Familial anamnesis
1	Pendred syndrome	6 years	f	NA	NA	NA	Goiter	–
2	Pendred syndrome	6 years	m	2	+	–	Goiter	–
3	Pendred syndrome	5 years	m	NA	NA	NA	Hypothyroidism	–
4	Pendred syndrome	1 year	m	2	NA	NA	Goiter	–
5	Pendred syndrome	16 years	m	2	NA	NA	Hypothyroidism	–
6	Pendred syndrome	1 year	f	2	NA	NA	Hypothyroidism	–
7	Pendred syndrome	NA	f	NA	NA	NA	Hypothyroidism	–
8	EVA	2 years	f	4	+	–	NA	–
9	EVA	2 years	m	4	+	–	NA	+
10	EVA	7 years	f	4	+	–	NA	–
11	EVA* + Mondini	8 years	f	4	+	+	Goiter*	–
12	EVA + Mondini	3 years	f	2	+	+	NA	–
13	EVA + Mondini	4 years	f	4	+	+	NA	–
14	Mondini	NA	m	NA	–	+	NA	–
15	Mondini	3 years	m	4	–	+	NA	–
16	Mondini	NA	f	4	–	+	NA	–
17	Mondini	3 years	m	4	–	+	NA	–
18	Mondini	3 years	f	NA	–	+	NA	–
19	Mondini	5 years	f	4	–	+	NA	–
20	Mondini	NA	f	4	–	+	NA	–

"+"—presence, "–"—absence, NA—not available. * Goiter was detected in a child aged 9 years after repeated examination, which followed detection of mutations in pendrin gene.

Amplification of all studied DNA fragments was performed via PCR using an MC2 thermal cycler (DNA Technology, Russia) with Biotaq DNA polymerase (BioMaster, Russia) in a reaction mixture 25 μL in volume consisting of 20–100 ng of genomic DNA, 0.25 μL of each oligoprimer, 200 μM of each dNTP, 1 U of DNA polymerase Biotaq (BioMaster, Russia), PCR buffer (67 mM Tris-HCl; $MgCl_2$ in specific concentration for each primer pair; 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, pH 8.8), and 20–30 μL of mineral oil.

Primers and amplification conditions used in the present study are shown in Table 2. Amplification was performed under the following conditions: preliminary denaturation at 95°C for 5 min; 30–32 cycles consisting of denaturation at 94°C for 45 s, primer annealing at 62°C for 45 s, and elongation at 72°C for 45 s; final elongation at 72°C for 5 min.

Search for p.Leu236Pro, p.Thr416Pro, and p.His723Arg mutations was conducted via RFLP analysis. Restriction was carried out in 20 μL of master mix consisting of 50–200 ng/μL of amplified fragment, 5 U of *Bse*DI restriction endonuclease for p.Leu236Pro and p.Thr416Pro detection, or *Fat*I restriction endonuclease for p.His723Arg detection in 1× buffer. Incubation was performed according to the manufacturer's protocol (Fermentas, Lithuania).

Multiplex ligation-dependent probe amplification (MLPA) was used to search for c.222G>T (p.Trp74-Cys) mutation in the control sample. The ligation of oligonucleotide probes with genomic DNA was performed for 1–2 h at 63°C in a mixture consisting of 0.1–1.0 μg of genomic DNA, 0.16–10 fmol/μL of each oligonucleotide probe (Eurogen, Russia), 0.4 U *Pfu* DNA ligase in a buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP, and 1 mM DTT), and a drop of mineral oil.

The results of PCR and RFLP analysis were assessed via vertical electrophoresis (20 × 20 cm) in polyacrylamide gel. PCR assessment was conducted in 7% PAAG with the acrylamide (AA) to bisacrylamide (BA) ratio of 29 : 1. The results of RFLP analysis were estimated using 8% PAAG with the acrylamide (AA) to bisacrylamide (BA) ratio of 29 : 1.3. The assessment of the results of MLPA analysis was performed using 9% PAAG with the acrylamide (AA) to bisacrylamide (BA) ratio of 19 : 1. Division of fragments was followed by ethidium bromide (0.1 μg/mL in 1× TBE) staining for 20 min and washing. Results were registered using the GelDoc® 2000 gel documentation system.

The search for c.919-2A>G and c.1001+1G>A mutations, as well as the analysis of the total coding sequence, was conducted via direct sequencing of the PCR product from both forward and reverse primers on the basis of the enzymatic Sanger sequencing. Fragments obtained after PCR served as the matrix for sequencing. Sequencing was performed with reagent ABI DyeTerminator, version 1 (Applied Biosystems) using a 3130Xl genetic analyzer (Applied Biosystems, United States).

The protocol, reagents, and PCR conditions used for screening of mutations in the *GJB2* gene were described in detail previously [1].

The reference sequence of genomic DNA from NCBI (http://www.ncbi.nlm.nih.gov/nuccore) (NG_008489.1) was used for the selection of primers for PCR and oligonucleotide probes for MLPA, and also for nucleotide numbering in the *SLC26A4* gene. The numbering of nucleotides was started from nucleotide A initiating ATG codon in exon 2. A selection of respective restriction endonucleases for RFLP analysis was performed using RestrictionMapper (http://www.restrictionmapper.org).

The assessment of pathogenicity of the detected substitutions was primarily based on data obtained from the HGMD database with respect to mutations in the *SLC26A4* gene (http://www.hgmd.cf.ac.uk) and SNP database (http://www.ncbi.nlm.nih.gov/snp). Moreover, bioinformatics resources predicting the effect of mutation, including MutationTaster (http://www.mutationtaster.org), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), and PROVEAN and SIFT (http://provean.jcvi.org/index.php), and predicting possible splicing sites—NetGene2 (http://www.cbs.dtu.dk/services/NetGene2)—were used. Statistical analysis was carried out using STATISTICA 6.0.

RESULTS

Search for Mutations in GJB2 Gene

DNA samples of all patients were screened for the presence of mutations in the *GJB2* gene encoding connexin 26. As a result of the analysis, one proband aged 6 years with clinical diagnosis of Pendred syndrome was homozygous for c.35delG mutation in the *GJB2* gene, while another proband aged 3 years diagnosed with Mondini dysplasia was heterozygous for p.Met34Thr mutation.

Search for Mutations in SLC26A4 Gene

DNA samples of 19 patients were screened for mutations in the *SLC26A4* gene. A proband heterozygous for mutation in the *GJB2* gene was excluded from the following analysis. The first stage included the analysis of the most frequent mutations in the *SLC26A4* gene selected according to published data, including c.919-2A>G, c.1001+1G>A, p.His723Arg, p.Leu236Pro, and p.Thr416Pro. As a result, these mutations were unobserved in the present study.

The second stage included sequencing of the nucleotide sequence of all protein encoding exons, exon 1 noncoding protein, and exon-intron boundaries in the *SLC26A4* gene in DNA samples obtained from 19 patients. As a result, six missense mutations in the *SLC26A4* gene, including five heterozygous com-

Table 2. Sequence of PCR primers and oligonucleotides for MLPA

SLC26A4 fragment	Oligonucleotide sequence, 5' → 3'
Ex. 1	F: GAGTGAGGATCCGGGTGGC R: CCGCAACCCTCGAGGCCTC
Ex. 2	F: GTCCTCGCTTACCGCGTGTC R: CCCGCTCCGCTTCTCTCTAC
Ex. 3	F: GACTGAGATTGGATTGAAAACCC R: GTGTTAAACTCCTGCTGGAGAC
Ex. 4	F: GTAAGTTGAGGACTTTCTGCATAC R: GAGGTATAATGCACTTAATATAGCC
Ex. 5	F: CCCTATGCAGACACATTGAAC R: GTTTTTAGTAGAATCTTTAAGTCTATATGC
Ex. 6	F: GAGTAGGTTTCTATCTCAGGCAAAC R: CTGGAATGAACAGTGACCCATC
Ex. 7–8	F: GTGCGTGTAGCAGCAGGAAGTA R: GACTGACTTACTGACTTAATGTATTAGTAC
Ex. 9	F: GATGGTGGTCAAATCTTCACAGC R: CCTGTTTCCAGCCCTATAAAACC
Ex. 10	F: CATTCTTAATGTACTTCCTGAAATACTC R: GCCATTCCTCGACTTGTTCTC
Ex. 11–12	F: CACAAGGGAGAAGGACGAATC R: GATATGGCAGGAAGCATATAAGAACC
Ex. 13	F: GTTATCACATGATGGTACCTGATAC R: GTCTGCTTAATGCATAGAAGCACTC
Ex. 14	F: CAAAACACCAGAATGATGGGCTC R: GGCTAGTAAACCCAAGTCCAGC
Ex. 15	F: GACTTGACTCCTTGCTAAGTAGC R: GAAGAGGGTCTAGGGCCTATTC
Ex. 16	F: GCCTTTCCAGATAACAGTTGCC R: GACCCTCTAACTGCTCTCATC
Ex. 17	F: GGTTGAAAGATTTCAAATCTTTGAC R: GCAATACTGGACAACCCACATC
Ex. 18	F: CTGAGCAAGTAACTGAATGCTAC R: CAGACATAATGTGACCACAGTC
Ex. 19	F: CAAAAATTTCTTTTCCTAGGAACTAAC R: GAAATCATGATAGTTTAGAAAAGATACATC
Ex. 20	F: GAGCATCAGGTGGGTTGATGC R: GGGAATTATGTTCCCTGACAGTTC
Ex. 21	F: GGAGAATTCAGTTGTATCAACAC R: GAGTCTGAAGAAATAGTCAATGC
c.222G>T	FG: GTTCGTACGTGAATCGCGGTACCTTGTGCCCATCTTGGAGTGG FT: GTTCGTACGTGAATCGCGGTACTCTCTTGTGCCCATCTTGGAGTGT R: CTCCCCAAATACCGAGTCAAGGTTTTCGATGCGATCCGATGCCTTCATG

Table 3. Substitutions in the *SLC26A4* gene determined in Russian patients

Nucleotide substitution	Location	Effect	Number of patients with mutation	First study publishing this mutation
c.222G>T	Exon 3	Trp74Cys	1	Present study
c.317C>A	Exon 4	Ala106Asp	2	Campbell et al. [23]
c.1001G>T	Exon 8	Splice site mutation	1	Walsh et al. [24]
c.1003T>C	Exon 9	Phe335Leu	1	Campbell et al. [23]
c.1229C>T	Exon 10	Thr410Met	1	Coyle et al. [13]
c.1790T>C	Exon 16	Leu597Ser	1	Campbell et al. [23]
c.2336-33A>C	Intron 20	Neutral polymorphism	18	Present study

pounds and one homozygote, were determined in four patients (Fig. 4). Moreover, all patients were characterized by c.2236-33A>C intronic substitution in the homo- and heterozygous state.

Parents of patient no. 10 with two mutations in the *SLC26A4* gene appeared to be heterozygous carriers of detected mutations; for instance, the c.1003T>C (p.Phe335Leu) mutation was detected in the proband's mother, while the c.1790T>C (p.Leu597Ser) mutation was detected in the proband's father.

Clinical Significance of Detected Substitutions

In total, seven mutations in the *SLC26A4* gene were determined. Five mutations were already described and annotated in HGMD and SNP databases. Two mutations were observed for the first time (Table 3).

An additional analysis of clinical significance of two mutations detected for the first time was conducted. The c.2236-33A>C substitution was detected in all explored probands, including those with two known pathogenic mutations. Bioinformatic analysis aimed at establishing the significance of detected nucleotide substitutions was successfully performed only using the MutationTaster bioinformatics resource, since the substitution is located in the noncoding gene region, which is not available to be tested via other algorithms (PROVEAN, SIFT, PolyPhen2). According to MutationTaster, this substitution is a nonpathogenic one. NetGene2 software predicting splicing sites demonstrated no significant changes in the probability of donor and acceptor splicing sites. The findings make it possible to suggest that c.2236-33A>C substitution is a polymorphism without any clinical significance, i.e., representing the normal neutral variant of a sequence.

The c.222G>T (p.Trp74Cys) substitution was detected in heterozygous compound with known c.317C>A (p.Ala106Asp) mutation (Fig. 4). Assessment of pathogenicity of this mutation was performed using bioinformatic resources MutationTester, PolyPhen2, PROVEAN, and SIFT. According to the results obtained from all the programs, the mutation was predicted to be potentially pathogenic. Multiple alignment conducted with PolyPhen2 revealed that c.222G>T (p.Trp74Cys) substitution was located in the conservative region of the pendrin protein. Moreover, the frequency of this mutation in control sample from the Russian population was estimated. As a result, this substitution was absent among 220 chromosomes. In summary, the results indicate a pathogenic effect of c.222G>T (p.Trp74Cys) mutation, which causes Pendred syndrome in a proband being in compound with c.317C>A (p.Ala106Asp).

The genotypes of 20 screened patients with reasonable mutations with consideration of the conducted analysis of clinical significance of detected substitutions are shown in Table 4.

DISCUSSION

The present study demonstrated the results of a pilot molecular-genetic study of Pendred syndrome and inherited enlarged vestibular aqueduct in Russian patients. Since complications of clinical differential diagnostics in children with Pendred syndrome and nonsyndromic hearing loss type DNFB1, which is one of the most frequent, exist, all patients were preliminarily investigated for the presence of mutations in the connexin 26 gene. As a result, one proband diagnosed with Pendred syndrome was homozygous for known c.35delG mutation. This patient was diagnosed with Pendred syndrome on the basis of the presence of sensorineural hearing impairment and euthyroid goiter. No instrumental methods making it possible to diagnose the disorder (computer tomography of temporal bone, perchlorate test) were conducted.

Since only one case of segregation of two recessive genes of hearing loss (*GJB2* and *SLC26A4*) in the world have been described, the insufficiency of clinical data indicating Pendred syndrome results in a low probability of combination of these inherited disorders [21]. Accordingly, this patient probably possesses two widespread disorders—nonsyndromic hearing loss and hypothyroidism of unknown etiology. Accordingly, a search for mutations was not conducted in this proband.

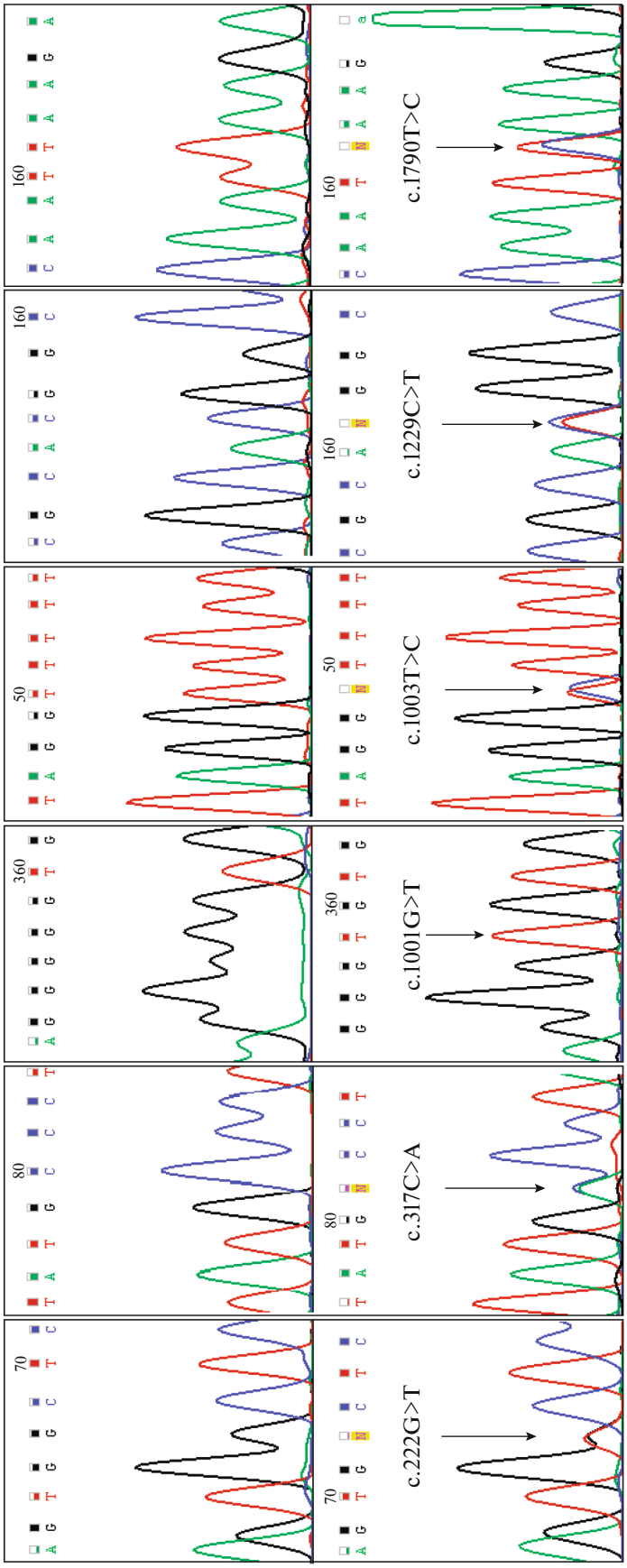

Fig. 4. Fragments of chromatograms of the *SLC26A4* gene sequencing: (top) patients without mutations, (bottom) patients with mutations.

Table 4. Pathological genotypes of the *GJB2* and *SLC26A4* genes in patients

Patient	Clinical diagnosis	*GJB2*	*SLC26A4*
1	Pendred syndrome	N	c.[1001G>T]+[1001G>T]
2	Pendred syndrome	N	c.[222G>T]+[317C>A]
3	Pendred syndrome	N	N
4	Pendred syndrome	N	N
5	Pendred syndrome	N	N
6	Pendred syndrome*	c.[35delG]+[35delG]	N
7	Pendred syndrome	N	N
8	EVA	N	N
9	EVA	N	N
10	EVA	N	c.[1003T>C]+[1790T>C]
11	EVA+Mondini**	N	c.[317C>A]+[1129C>T]
12	EVA+Mondini	N	N
13	EVA+Mondini	N	N
14	Mondini	N	N
15	Mondini	N	N
16	Mondini	N	N
17	Mondini	N	N
18	Mondini	N	N
19	Mondini	N	N
20	Mondini	N	N

N—"normal" genotype.

* Diagnosis was defined more precisely as nonsyndromic sensorineural hearing impairment of genetic type DFNB1 on the basis of molecular analysis.

** Goiter was observed in a child 9 years old after repeated examination following detection of mutations in pendrin gene, which made it possible to define his diagnosis more precisely as Pendred syndrome.

Moreover, one patient with Mondini dysplasia was characterized by heterozygous p.Met34Thr mutation. A patient with mutation in a one of the *GJB2* alleles probably possesses a random combination of hearing loss and population heterozygous carrier state of mutation, since the population frequency of heterozygous carrier state of mutations in the connexin 26 gene is rather high, being 1/20 [1]. Since the connexin 26 gene is short, consisting of two exons, and the search for mutations in this gene is less intensive and expensive, a preliminary analysis of patients with Pendred syndrome for mutations in the *GJB2* gene encoding for connexin 26 appears to be expedient.

The screening of DNA samples obtained from 19 patients for any of five frequent mutations, c.919-2A>G, c.1001+1G>A, p.His723Arg, p.Leu236Pro, and p.Thr416Pro, was performed. Interestingly, no mutation from the ones mentioned was detected in Russian patients. Therefore, the detection systems of the most frequent mutations in the world in the *SLC26A4* gene are not informative for Russian individuals.

Since no "hot" mutation regions in the gene are known on the basis of published data, the following step included Sanger sequencing of all protein encoding exons, noncoding protein exon 1, and exon-intron boundaries in the *SLC26A4* gene. As a result, six pathologic mutations in different genotypes were determined in four patients. One of the mutations, namely c.222G>T (p.Trp74Cys), was detected for the first time. The majority of mutations detected in Russian patients belong to missense mutations resulting in amino acid change, which is congruent with published findings. One of the substitutions appeared to be a splicing site mutation.

It should be noted that c.317C>A mutation was observed in two probands. The following analysis demonstrated that both probands originated from Nizhny Novgorod oblast, which does not exclude that they are distant relatives; hence, we do not consider this mutation as repeated or frequent.

One proband was characterized by homozygous c.1001G>T mutation. We succeeded in establishing that this proband originated from the Caucasus; no other data concerning familial anamnesis were available. Interestingly, published findings reported that c.1001G>T mutation was detected in several Turkish families, and in the homozygous state, in children of blood-related parents. Since it was not possible to

exclude that the parents of this proband were relatives, this mutation could not be considered as frequent in Russian patients.

Accordingly, the findings, together with the results published by Lobov et al. [20], who detected two mutations in the *SLC26A4* gene in a patient, indicate the absence of "hot" exons and frequent mutations in the pendrin gene in Russian patients.

A significant impact of genetic pathology caused by mutations in the pendrin gene in both Pendred syndrome and nonsyndromic EVA in Russian patients was established. Biallelic mutations were detected in 4 of 19 patients, which is 21% (95% CI: 7–46%): 33% had Pendred syndrome (2 of 6 patients; 95% CI: 6–76%) and 33% had hearing loss with enlarged vestibular aqueduct with or without Mondini dysplasia (2 of 6 patients; 95% CI: 6–76%). No mutations were detected in a sample of patients with isolated Mondini dysplasia.

All patients with mutations in pendrin gene were characterized by enlarged vestibular aqueduct, which makes it possible to consider EVA as one of the major diagnostic criteria for patient screening for mutations in the *SLC26A4* gene. The findings are congruent with published data [22]. However, this suggestion should be confirmed by future research. One patient with diagnosis of "enlarged vestibular aqueduct with Mondini dysplasia" was examined by an endocrinologist after the results of molecular-genetic analysis. As a result, this patient at the age of 8 years was also diagnosed with stage I goiter and the appropriate treatment was prescribed. This case demonstrates a significant role of correct and timely diagnostics, including molecular-genetic diagnostics.

The findings indicate that Sanger sequencing of all exons and exon-intron boundaries in the *SLC26A4* gene is the only informative method for screening of mutations in the pendrin gene in Russian patients. Moreover, the search for mutations in the pendrin gene is not informative in patients with isolated Mondini dysplasia.

In summary, molecular-genetic investigations make it possible to establish an inherited etiology of hearing loss and to obtain novel data of clinical manifestation of inherited forms of hearing loss.

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