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ANALYSIS OF THE C.757A>G P.(ILE253VAL) VARIANT OF THE *SLC26A4* GENE IN *GJB2*-NEGATIVE PATIENTS WITH HEARING LOSS IN YAKUTIA

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In this work, we searched for the missense variant c.757A>G p.(Ile253Val) of the *SLC26A4* gene in *GJB2*-negative patients with hearing loss (n=201) and in the control group of hearing individuals (n=103) in Yakutia. As a result, this variant was detected with a frequency of 2.02% among patients, in the control group - 1.94%. To interpretation the clinical significance, a frequency analysis of this variant and *in silico* evaluation were performed, the results of which are in favor of the likely benign of the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene, as indicated by the high frequency of occurrence in population samples, and the fact that this missense substitution theoretically does not violate the structural stability of the pendrin protein (SLC26A4).

Keywords: variant c.757A>G p.(Ile253Val), *SLC26A4* gene, pendrin (SLC26A4), sensorineural hearing loss.

Introduction. Hearing impairment (HI) is one of the most common congenital pathologies. The prevalence of congenital and childhood hearing loss (HL) and deafness in the world is estimated at 1.33 per 1000 newborns [19]. It is known that up to 50% of cases of congenital deafness have a hereditary etiology [6, 19]. About 70% of genetic causes of HL are thought to be nonsyndromic, with the remaining 30% being syndromic. At the same time, approximately 75% of all cases of nonsyndromic HL and deafness occur in autosomal recessive forms [6, 19].

The contribution of the *SLC26A4* gene to the etiology of autosomal recessive forms of HL is considered one of the most significant, and the proportion of its pathogenic variants among all identified causative variants of other genes in a number of regions of the world is in second place after the *GJB2* gene [5, 7, 9, 10, 16, 31], and in some and in first place (Pakistan) [12]. Pathogenic variants of the *SLC26A4* gene are associated with both autosomal recessive deafness type 4 (DFNB4, OMIM #600791) and Pendred syndrome (PS, OMIM #274600). The *SLC26A4* gene is located on chromosome 7 at the locus 7q22–q31, contains 21 exons, and encodes a transmembrane protein known as pendrin (PDS), which is expressed in the inner ear, thyroid gland, kidneys and airway epithelium [21-24, 28, 30]. Pendrin consists of 780 amino acids forming 12–14 TM segments, including a segment of the intracellular functional STAS domain (Sulfate Transporter and Anti-Sigma factor antagonist) [15, 29].

Earlier in Yakutia, using direct Sanger sequencing of the coding region of the *SLC26A4* gene, an analysis of its mutational contribution was carried out among six patients with HI and inner ear anomalies (IP-1, IP-2, and EVA) [1]. Five variants were identified in the *SLC26A4* gene: c.85G>C p.(Glu29Gln), c.757A>G p.(Ile253Val), c.2027T>A p.(Leu676Gln), c.2089+1G>A (IVS18 +1G>A) and c.441G>A p.(Met147Ile). Pathogenic biallelic variants of the *SLC26A4* gene were detected in four patients (4/6) and monoallelic *SLC26A4*-variants of in two patients (2/6). It should be noted that all monoallelic patients were Yakuts and carried the same heterozygous variant of the *SLC26A4* gene - c.757A>G p.(Ile253Val) [1]. In the ClinVar database, this variant is not annotated (https://www.ncbi.nlm.nih.gov/snp/rs773657545/#publications). However, in the Deafness variation database (DVD), the c.757A>G p.(Ile253Val) variant is classified as likely pathogenic

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(LP), and its allele frequency according to gnomAD is very rare, at only 0.008754% (https: //gnomad.broadinstitute.org/variant/7-107315546-A-G?dataset=gnomad_r2_1). Since the second mutant allele was not found in two patients with the heterozygous variant c.757A>G p.(Ile253Val) of the *SLC26A4* gene, a mutational search was performed in these patients in the genes associated with the hypothesis of digenic inheritance in the *FOXI1* and *KCNJ10* genes, which also did not reveal causative variants in these genes [1]. Due to the "absent" heritability in single-heterozygous patients with c.757A>G p.(Ile253Val) of the *SLC26A4* gene, to assess its pathogenetic role, it is relevant to search for this variant in extended cohorts of patients with hearing impairments and in control groups of hearing individuals.

In this regard, the aim of this work was to analyze the frequency of occurrence of the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene among *GJB2*-negative patients with HL, in comparison with the control group of hearing individuals in Yakutia.

Materials and methods. ***Study sample.*** *Patients.* The study sample consisted of genomic DNA samples of 201 patients (of which 198 were unrelated) with deafness and/or hearing loss from Yakutia, who had earlier, as a result of the analysis of nucleotide changes in the *GJB2* gene, pathogenic variants in the biallelic state, causing autosomal recessive deafness type 1A (DFNB1A, OMIM 220290) was not detected (*GJB2*-negative) [25]. Among patients, men accounted for 42.3% (n=85), women - 57.7% (n=116), mean age 26.63±17.51 years. Ethnic composition: Yakuts - 58.7% (n=118), Russians - 21.9% (n=44), of mixed - 13.9% (n=28) and other ethnicities - 5.5% (n=1). Audiological examination of the state of hearing was carried out using impedancemetry (AA222, Interacoustics, Denmark) and tone audiometry (GSI61, Grason Stadler inc., USA). Air conduction thresholds were obtained at 0.125, 0.25, 0.5, 1, 2, 4 and 8 kHz. The severity of hearing loss was defined as mild (25–40 dB), moderate (41–70 dB), severe (71–90 dB), or profound (above 90 dB).

Control group. In the control group of the studied, the search for the analyzed variant was carried out in 103 individuals with normal hearing from the population sample of Yakuts living in different regions of the republic. Hearing status was assessed using a clinical and audiological examination, including threshold tone audiometry.

Molecular genetic analysis. Genomic DNA samples were isolated by phenol-chloroform extraction. The search for the c.757A>G p.(Ile253Val) variant localized in exon 6 of the *SLC26A4* gene was carried out using PCR-RFLP analysis. For amplification of a 251 bp fragment primers (F) 5'-CAGAGAGTAGGTTTC-TATCTCAGGC-3' and (R) 5'-CCCTG-GAGCAAGAAGCAACA-3' were used. For RFLP analysis, restriction endonuclease *Hpa*I (restriction site GTT↑AAC/CAA↓TTG) was used. RFLP analysis was performed by electrophoresis in 4% agarose gel with registration in the Molecular Imager Gel Doc XR Sistem gel documentation system (Bio-Rad, USA). In the work, reference sequences were used: Gene ID: 5172 (*SLC26A4* - solute carrier family 26 member 4 [Homo sapiens (human)]): NM_000441.2, NP_000432.1, (https://www.ncbi.nlm.nih.gov/gene/5172); Ensembl ID: ENSG00000091137, ENSP00000494017, ENST00000644269 (https://www.proteinatlas.org/ENSG00000091137-SLC26A4).

In-silico analysis. *Database.* To search for genetic information (on the clinical significance and phenotypic relationship of variants, prevalence, publications, evaluation of *in silico* predictive programs for the classification of missense variants), the following databases were used: OMIM® (https://www.omim.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Deafness variation database (DVD, https://deafnessvariationdatabase.org/).

AlphaFold System. AlphaFold was used, which is a computational algorithm that can regularly predict protein structures with atomic accuracy, even in cases in which no similar structure is known [14]. AlphaFold produces a confidence metric for amino acid residues, as a predicted local distance difference test (pLDDT), on a scale of 0 to 100 [14]. An expected value of pLDDT>90 is taken as the high accuracy cut-off (blue), pLDDT>70 indicates low confidence and corresponds to a generally correct backbone prediction (turquoise color, good backbone prediction), pLDDT≤70 indicates that we should also add substantial coverage for sequences without a good template in PDB (yellow color, should be considered with caution), and pLDDT<50 indicates very low confidence (orange, should not be interpreted) [13]. Full details are available at: https://www.ebi.ac.uk/about.

PyMol program. PyMol (PyMOL Molecular Graphics System) - graphics program that provides 3D visualization of proteins, small molecules, molecular surfaces and trajectories. Full details are available at: https://pymol.org/2/#products.

Evaluation of the clinical significance of c.757A>G p.(Ile253Val) of the SLC26A4 gene by ACMG. The classifi-

Table 1

Allele frequency c.757A>G p.(Ile253Val) of the SLC26A4 gene in patients with hearing loss/deafness in comparison with population samples

Country (Region)	Sample of patients*	AF	Population sample**	AF	References
Russia (Yakutia)	8/198	2.02% (8/396)	4/103	1.94% (4/206)	This work
China	1/284	0.17% (1/568)	-	-	[4]
China	1/2352	0.02% (1/4704)	-	-	[18]
China (Tibet)	1/114	0.43% (1/228)	4/106	1.88% (4/212)	[3]
China	1/227	0.22% (1/457)	0/200	0% (400)	[20]
China	1/371	0.13% (1/742)	-	-	[11]
Total	13/3546	0.18% (13/7092)	8/409	0.97% (8/818)	-
χ^2	0.18% (13 из 7095)		0.97% (8 из 818)		$\chi^2 = 17.49$. $p<0.001$

Note: * Number of patients with c.757A>G p.(Ile253Val) of the SLC26A4 gene per total number of patients with hearing loss/deafness, ** - Number of individuals with c.757A>G p.(Ile253Val) of the SLC26A4 gene per total number of individuals from population samples. AF - allelic frequency; "-" - no data.

Table 2

Clinical characteristics of patients with the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene

Country (Region)	*SLC26A4*-genotypes of patients with c.757A>G p.(Ile253Val)	EVA/other anomalies	Hearing	Evaluation	References
China	c.[757A>G];[wt]	-	deafness	VUS	[4]
China	c.[757A>G];[wt]	-	-	-	[18]
China (Tibet)	c.[757A>G];[wt]	norm/ hypoplastic cochleavestibular and semicircular canals	deafness	benign	[3]
China	c.[757A>G];[wt]	EVA	deafness	benign	[20]
China	c.[919-2A>G];[757A>G]	norm	norm	VUS	[11]
Russia (Yakutia)	c.[757A>G];[wt] c.[757A>G];[wt]	EVA+IP-1 IP-1/IP-2	deafness deafness	likely pathogenic	[1]

Note: EVA – enlarged vestibular aqueduct; IP-1 – Incomplete Partition Type 1, IP-2 – Incomplete Partition Type 2; VUS – variant uncertain significance. «-» - no data.

cation of the clinical significance of the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene was carried out according to the standards of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) [26], adjusted for genetic HL [8].

Ethical approval. All examinations provided in this study have been conducted with the informed written consent of all participants. This study was approved by the local Biomedical Ethics Committee of Federal State Budgetary Scientific Institution "Yakut Science Centre of Complex Medical Problems" (Yakutsk, Protocol #7, August 27, 2019).

Results and discussion. ***Search for the c.757A>G p.(Ile253Val) variant of the SLC26A4 gene in GJB2-negative patients and in the control group.*** The search for c.757A>G p.(Ile253Val) of the *SLC26A4* gene in a sample of *GJB2*-negative patients (n=201) with HI in Yakutia revealed this variant in the heterozygous state in 8 Yakut patients. None of the patients had this variant in the homozygous state. In the control group of hearing individuals from the population sample of Yakuts (n=103), this variant was found in 4 individuals, also in the heterozygous state. Thus, the allelic frequency of the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene was practically the same in both study groups, among unrelated patients it was 2.02% (8/396), and in the control group it was 1.94 % (4/206) (Table 1). For a comparative analysis of the allelic frequency c.757A>G p.(Ile253Val) of the *SLC26A4* gene, we analyzed the literature, which described cases of detection of this variant. As a result, the variant we analyzed was previously identified only in isolated cases among

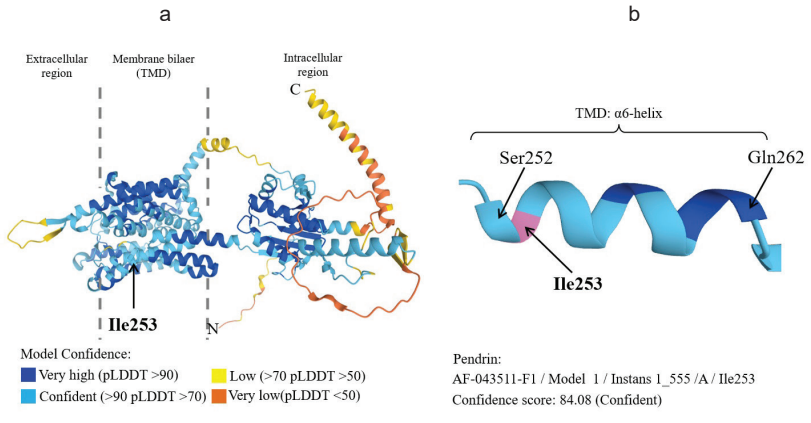

Fig. 1. Three-dimensional (3D) spatial structural model of the human pendrin (SLC26A4) as predicted by Alphafold 2.0. A. General view (https://alphafold.ebi.ac.uk/entry/O43511). Color represents regions of the monomer with varying confidence in conformation prediction (see bottom). Dashed lines indicate extra- and intracellular regions of the monomer. The arrows indicate the analyzed amino acid residues (Ile253). B. Close-up of the location of Ile253 in the α6-helix of the TMD of pendrin (shown in pink), consisting of 10 amino acid residues (from Ser252 to Gln262).

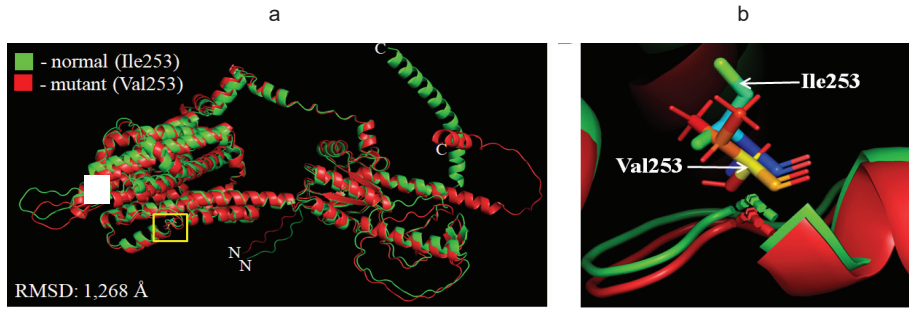

Fig. 2. Alignment of mutant p.(Ile253Val) and normal structures of pendrin (SLC26A4) in PyMOL. A. The yellow square marks the localization site of the amino acid residue at position 253, in the norm Ile (green), in the mutant Val (red). B. Close-up view of the side chains of the amino acid residues Ile253 and Val253, originating from the C-atoms of the main chain.

patients with HI in China [3, 4, 11, 18, 20]. In general, the allelic frequency of c.757A>G p.(Ile253Val) of the *SLC26A4* gene in patients with HL/deafness in the studied regions of the world was 0.18% (13/7095). However, in a comparative analysis of the identified overall frequency of occurrence of the variant among the deaf population, in comparison with the frequency in population samples, it turned out that the c.757A>G variant occurred 5 times more often in popula-

Table 2

Classification of the clinical significance of the missense variant c.757A>G p.(Ile253Val) of the *SLC26A4* gene according to the ACMG recommendation

	ACMG Category			
	Pathogenic (P, LP)		**Benign (B, LB)**	
1	**Population Data**			
	Criteria for classifying pathogenic variants	Evidence	Criteria for classifying benign variants	Evidence
	Strong: very strong – PVS(1) strong – PS(1-4)	-	Strong: stand-alone – BA(1) strong – BS(1-4)	-
	Moderate: PM(1-4)	**PM2_Supporting:** Low MAF in population databases (<0.0007 [0.07%] for autosomal recessive) [8]		
	Supporting - PP(1-5)	-	Supporting - BP(1-6)	-
2	**Functional Data**			
	Strong: very strong – PVS(1) strong – PS(1-4)	-	Strong: stand-alone – BA(1) strong – BS(1-4)	-
	Moderate: PM(1-4)	-		
	Supporting: PP(1-5)	**PP2:** Missense variant in a gene that has a low rate of benign missense variation and in the ClinVar database: out of 389 missense changes, P/LP/VUS-variants account for 94% (https://www.ncbi.nlm.nih.gov/clinvar/?term=SLC26A4%5Bgene%5D&redir=gene) (PP2 - Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease [26])	Supporting: BP(1-6)	-
3	***In silico* Predictions**			
	Strong: very strong – PVS(1) strong – PS(1-4)	-	Strong: stand-alone – BA(1) strong – BS(1-4)	-
	Moderate: PM(1-4)	-		
	Supporting: PP(1-5)	**PP3:** 8 in silico programs (MT, DANN, MetaLR, Polyphen-2, LRT, MutationTaster, PhyloP, GERP++) predict pathogenic effect (https://franklin.genoox.com/clinical-db/variant/snp/chr7-107315546-A-G?app=assessment-tools; https://deafnessvariationdatabase.org/gene/SLC26A4) (PP3 - Multiple lines of computational evidence support a deleterious effect on the gene or gene product [26])	Supporting: BP(1-6)	**BP4:** 1) 4 in silico programs (MUT Assesor, SIFT, GenoCanyon, fitCons) predict benign effect (https://franklin.genoox.com/clinical-db/variant/snp/chr7-107315546-A-G?app=assessment-tools; https://deafnessvariationdatabase.org/gene/SLC26A4) (BP4 - Multiple lines of computational evidence suggest no impact on gene or gene product [26]) 2) Alignment of the mutant (p.Ile253Val) and native protein structures showed that the analyzed substitution does not affect the functional significance of pendrin
Classification	PM2 + PP2 + PP3 = insufficient to classify pathogenic		BP4 = insufficient to classify benign	

tion samples (0.97%) than in people with disabilities hearing (0.18%) ($\chi 2 = 17.49$, $p<0.001$) (Table 1).

When analyzing the clinical characteristics of seven patients with the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene, previously detected in other studies [1, 3, 4, 11, 18, 20], it was found that in most of them (6 out of 7) the variant was found in single-heterozygous condition, the second mutant allele was not detected (Table 2). Only one case is known (1 out of 7), where the c.757A>G p.(Ile253Val) variant was detected in a compound heterozygous state with the *SLC26A4*-variant c.919-2A>G during neonatal screening for deafness in China [11]. However, the authors report that at the time of audiological testing, the child with genotype c.[919-2A>G];[757A>G] had no HL, however, they do not exclude the possibility of progression of HI with age [11].

Due to the absence of certain, strong evidence for unambiguous classification of the clinical significance of c.757A>G (clinical and functional evidence), we performed an *in silico* analysis of this variant using the AlphaFold neural network algorithm.

In-silico analysis of the c.757A>G p.(Ile253Val) variant of the SLC26A4 gene using the AlphaFold neural network algorithm. Since at present, the crystal model of the spatial structure of the human pendrin protein (SLC26A4) has not yet been experimentally established, a native model of the three-dimensional spatial structure was obtained using the AlphaFold system (Fig. 1A). AlphaFold predicted a qualitative model of pendrin with high accuracy (most of the protein regions were predicted with >90pLDDT>70), which allowed us to obtain the necessary information about the architecture of the monomer structure. Thus, in the resulting AlphaFold-model, the analyzed amino acid position Ile253 is localized in segment 6 (α6-helix), which is represented by 10 amino acid residues from 252 to 262 (Fig. 1B).

According to the topology of the pendrin protein (SLC26A4), which is composed of 14 transmembrane segments (α-helices in the form of columns 1-7 and 8-14, forming channel pores), segment 6 (α6-helix) is located in the gate domain, which in turn consists of outer helices, 5-7 and 12-14 segments [2, 15, 17, 27, 29]. It is important to note that the analyzed p.Ile253Val substitution is not located in the critical region of the core domain, the disruption of which can lead to improper substrate transport or the appearance of toxic conformations (misfolded proteins) [2, 17, 29]. It is known that mutant amino acid residues located in the core domain can cause disturbances in the conduction of the SLC26A4 ion channel and cause diseases associated with the pendrin protein. However, it is equally important to note that another pathogenic missense variant is currently annotated in the ClinVar database, which is also located in the 6th protein segment - c.754T>C p.(Ser252Pro) (https://www.ncbi.nlm.nih.gov/clinvar/variation/1065210/). This sequence (Ser252) of the polypeptide chain of helix 6, followed by the region we analyzed (p.Ile253Val), is adjacent to the loop of the extracellular region of pendrin, therefore a mutational change in this region can lead to functional impairment of the protein.

Further, using the PyMOL program tool based on the native structure of the SLC26A4 protein, modeled by the AlphaFold 2.0 program, we aligned the three-dimensional folding of the mutant and normal pendrin chain (Fig. 2). As a result of alignment of the three-dimensional folding of the mutant and normal chains using the PyMOL program, the obtained RMSD value: 1,268 Å is within the full similarity criterion (<2 Å) and indicates that the studied missense variant does not lead to a change in the spatial structure of the synthesized protein. This similarity of the two compared structures, with a slight difference, is probably due to the physicochemical properties of the considered amino acid residues in the polypeptide chain. It is known that the amino acids isoleucine and valine belong to the same functional group of amino acids with hydrophobic uncharged side radicals and their isoelectric point is approximately the same (Ile: p*I* = 6,1, -COOH = 2,4, $-NH_3$ = 9,7; Val: p*I* = 6,0, -COOH = 2,3, $-NH_3$ = 9,6).

Evaluation of the clinical significance of c.757A>G p.(Ile253Val) of the SLC26A4 gene. In total, the data obtained made it possible to collate the traits (allelic frequency of occurrence, *in silico* assessment) to classification the clinical significance of the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene according to the ACMG criteria [8, 26], which are presented in table 3. The resulting combining of criteria does not allow it to be considered as a pathogenic or benign variant, thus, it is interpretation as a variant of uncertain significance - VUS.

Conclusions. The results of this study indicate that the c.757A>G p.(Ile253Val) variant of the *SLC26A4* gene is to be likely benign, since this is indicated by a high frequency of occurrence in population samples, and the fact that this missense substitution theoretically does not violate the structural stability of the pendrin protein (SLC26A4). However, given the rare occurrence in the world, the lack of genotype-phenotypic and functional data, currently the c.757A>G p.(Ile253Val) variant is classification as a variant of uncertain significance according to the ACMG criteria.

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ENDOTHELIAL DYSFUNCTION IN THE PATHOGENESIS OF INFLAMMATORY PERIODONTAL DISEASES

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Manifestations of endothelial dysfunction that occur in response to microbial invasion in inflammatory periodontal diseases may underlie the occurrence and progression of these diseases. The aim of the study was to determine the level of secretion of adhesive molecules of the selectin family and the superfamily of immunoglobulins in the gingival/periodontal pocket and their relationship with marker periodontal pathogens.

For the study, flushes of the gingival pocket (a total of 88 samples) of patients with chronic generalized periodontitis and intact periodontitis were obtained. The content of soluble forms of the adhesion molecules sICAM-1, sVCAM, sE-selectin, and sL-selectin was determined by ELISA. Marker periodontal pathogens were isolated by real-time PCR. The study revealed changes in the adhesiveness of molecules in individuals with chronic generalized periodontitis (CGP): the concentrations of sL-and sE-selectin molecules in the gingival/periodontal pocket discharge in patients with CGP increased by an average of 80,4% (p=0.045) and 63,6% (p=0,038), respectively. While the concentrations of adhesive proteins of the superfamily of immunoglobulins sICAM-1 and sVCAM in individuals with CGP exceeded the corresponding concentrations of the control group to a greater extent: 9,7 (p=0,022) and 18,1 (p=0,023) times, respectively. The frequency of detection of periodontal pathogenic bacteria genes was 96,4% in patients with CGP and 28,6% in the group with intact periodontitis. Statistically significant correlations of moderate and high degree were found between the content of sVCAM and T. forsythia (r=0,683, p=0,02) and A. actinomycetemcomitans (r=0,621, p=0,04), as well as sICAM-1 and P. gingivalis (r=0,628, p <0,001) and A. actinomycetemcomitans (r=0,821, p=0,04) in the group of patients with CGP. In the examined patients with intact periodontitis, weak negative correlation between sL-selectin and T. denticola was found (r=-0,482, p=0,03). Thus, elevated concentrations of the soluble adhesive molecules sICAM-1, sVCAM, sE-and sL-selectin may indicate endothelial cell alteration due to persistent inflammatory process caused by virulence factors of specific subgingival bacterial flora.

Keywords: chronic generalized periodontitis, periodontal pathogenic bacteria, inflammation, adhesion molecules, endothelial dysfunction.