Efficiency of SNPs for the Detection of 35DelG Mutation in 50 Cases with Nonsyndromic Hearing Loss

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Congenital sensorineural hearing loss (SNHL) is recognized as a major public health burden. Mutations in the GJB2 gene are among the most frequent encountered etiological factors (approximately 50% of cases of autosomal recessive sensorineural non-syndromic hearing loss in the Caucasian population). Single nucleotide polymorphisms (SNPs) are important markers in studies that correlate the genotype with the phenotype. The main purpose of the study is to develop and validate a molecular-genetic screening algorithm based on the SNP rs80338939 for later use in laboratories in Romania and the Republic of Moldova. A prospective study was conducted on 50 randomly included subjects with profound congenital SNHL. The 35delG mutation was assessed by two methods: a reference method (University Medical Center Freiburg, Germany) and the method to validate: single nucleotide polymorphism (SNP) for the same mutation. We compared the results of the two methods to assess the specificity and sensitivity of the method used in the study. Results obtained indicate a sensitivity of 92% and 98% specificity for the studied method when compared with the reference method. The high sensitivity and specificity of the proposed method confirms that rs80338939 can be used as a biomarker in the assessment of the risk of autosomal recessive SNHL. In fact, we aim to optimize the technique to achieve 100% sensitivity and specificity. At the same time, we acknowledge that the screening of 35delG mutations does not replace the audiological screening tests, because the auditory function involves 1% of the human genes and mutations of any of these may lead to deafness.

Keywords: deafness, molecular analysis, 35delG

Hearing loss is the most common sensory defect, representing a major public health issue [1]. Causes of hearing loss are both genetic (in 50% of cases) and non-genetic [2,3]. Non-syndromic genetic hearing loss occurs in 70-80% of cases, the remaining 20-30% are hearing losses occurring during the various syndromes and are associated with other abnormalities [4-6]. Of the total non-syndromic genetic cases, 75-80% are autosomal recessive (DFNB) transmitted, 18-20% autosomal dominant (DFNA), 1-2% X-linked (DFN) and less than 1% by mitochondrial route [7,8]. In most cases, autosomal non-syndromic SNHL is associated with mutations of the DFNB1 locus from chromosome 13q11-13. In this locus, at a small distance between them, there are two very important genes, GJB2 and GJB6, responsible for the synthesis of connexin 26 (Cx26) and 30 [9] respectively. Cx26 mutations are responsible of various human pathologies ranging from hearing loss to keratitis ichthyosis deafness syndrome. Cx26 contribute also to chemosensory regulation of breathing (10), raising the issue of breathing monitoring during sleep in subjects with these mutations (11).

Mutations in the GJB2 gene account for approximately 50% of the autosomal recessive non-syndromic hearing loss cases in the Caucasian population [5,12]. Worldwide efforts are being made to identify biomarkers: for diagnosis, assessment of etiology, risk, as well as personalization of treatment [13-16].

The main objectives of our study are:

- Development and validation of a molecular-genetic screening algorithm based on the SNP rs80338939 with the potential to be later used in the laboratories in Romania and the Republic of Moldova.

- Creating a multidisciplinary cross-border nucleus of competence in molecular-genetic diagnosis by co-opting and involving experts on the field of interest.

Experimental part

Material and methods

As methodological part, we conducted a prospective molecular-genetic study with the aim to evaluate the prevalence of 35delG mutation in the GJB2 gene in the population with profound non-syndromic SNHL in Romania and the Republic of Moldova, and also to develop and validate an algorithm of molecular-genetic screening based on SNP rs80338939 with the potential to be further used in laboratories in the two countries.

The study group is represented by 50 children with profound autosomal recessive neurosensorineural congenital SNHL, diagnosed and treated at the ENT Clinic within the Clinical Rehabilitation Hospital in Iasi. The study obtained the approval of the Ethics Committee of UMF Grigore T. Popa Iasi. Informed consent was obtained from parents or legal guardians for children before collecting venous blood for molecular analysis.

From each child, 6 mL of venous blood was harvested on the EDTA medium. Two milligrams of blood were sent for analysis to the molecular analysis laboratory of the University Medical Center Freiburg, another 2 mL of blood were sent to the molecular analysis laboratory at the University of Medicine Nicolae Testemitanu from Chisinau and 2mL are kept in reserve.

In order to validate the screening method proposed by the laboratory in Chisinau, the blood was sent for molecular reference analysis at the laboratory in Germany. At the

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molecular lab in Freiburg, DNA was extracted from leukocytes using the standard methods (Qiagen, Hilden Germany). Primer and PCR conditions were selected according to procedures previously optimized for the sequence analysis of exon 1 and the coding exon 2 of the GJB2 gene, including all splice sites [34]. Sequencing of the PCR products was done with standard procedures and analyzed in an automated DNA sequencer Amersham MegaBACETM 500 (American Biosciences, GE Healthcare Europe, Muenchen, Germany).

In the laboratory in Chisinau, molecular analysis was also made using DNA extracted from leukocytes from the peripheral blood. The difference was that just a single nucleotide polymorphism (SNP) of GJB2 gene, namely rs80338939 - the 35delG mutation - was analyzed. This is known to be the most common mutation in the European population.

Extraction was performed using the GeneJET Genomic DNA Purification Kit (K0722, Thermo Fisher Scientific). Analysis of the extracted DNA quality was performed by spectrophotometric method (NanoDrop 2000c spectrophotometer, Thermo Fisher Scientific). Concentrations of all samples were adjusted to 2 ng/µL.

TaqMan PCR molecular genetic method:

Identification of mononucleotide polymorphism was performed by the TaqMan technique. The Custom TaqMan SNP Genotyping Assay Human Kit (4351379, ThermoFisher Scientific, https://www.thermofisher.com/order/catalog/product/4351379) was used with fluorescence oligonucleotide probes VIC - for Wild Type Allele identification and FAM to identify the allelic form with 35delG deletion (table 1).

The molecular-genetic testing of the SNP was performed on 384-well plates in a total reaction volume of 5 µL. Each plaque included the DNA samples and a negative control to exclude contamination (No-Template Control - NTC).

The PN 4334431 amplification protocol (Thermo Fisher Scientific) was used to ensure appropriate conditions to perform the PCR technique (table 2).

All tests were performed on the QuantStudio 6 flex device (Applied Biosystems, ThermoFisher Scientific). The amplification program, plate design and data collection were performed using QuantStudio Real-TimePCR Software (v.1.3., Applied Biosystems, ThermoFisher Scientific).

Alelic discrimination was performed by the TaqMan Genotyper Software application (v.1.3.1., Applied Biosystems, ThermoFisher Scientific).

The success of genotyping rate was 96%, the undetermined samples were automatically eliminated from alley discrimination.

The results from the two laboratories were analyzed comparatively considering the results obtained in Germany as standard. We statistically determined the sensitivity and specificity of the method proposed for screening.

N/o	Name of gene	NCBI SNP Code	Mutation	Probe [VIC/FAM]
1	*GJB2*	rs80338939	35delG Deletion	GGCACGCTGCAGACGATCCTGGGGG[-/G]TGTGAACAAACACTYCACCAGCATT

Table 1
PROBES USED FOR GENOTYPING

Stage	Parameters		
	Temperature (ºC)	Time	Cycle
DNA denaturation ADN and AmpliTaq Gold® enzyme activation	95	10 min	HOLD
Denaturation	95	15 sec	40
Normalization / Extension	65	1 min	

Table 2
AMPLIFICATION PROTOCOL

	Molecular Lab Chisinau		Molecular Lab Freiburg	
	Alele 1	Alele 2	Alele 1	Alele 2
P1	35delG	35delG	35delG	35delG
P2	35delG	35delG	35delG	35delG
P3	35delG	W	35delG	W
P4	35delG	W	W	W
P5	35delG	35delG	35delG	35delG
P6	35delG	35delG	35delG	35delG
P7	35delG	W	35delG	W
P8	35delG	35delG	35delG	35delG
P9	35delG	W	35delG	W
P10	35delG	W	35delG	W
P11	35delG	35delG	35delG	35delG
P12	35delG	35delG	35delG	35delG
P13	35delG	W	35delG	W
P14	35delG	W	35delG	W

Table 3
COMPARATIVE FREQUENCE OF 35delG MUTATION FOUND IN THE TWO DIFFERENT LABS

P15	35delG	35delG	35delG	35delG
P16	35delG	35delG	35delG	35delG
P17	w	W	W	W
P18	w	W	W	W
P19	w	W	W	W
P20	w	W	W	W
P21	w	W	W	W
P22	w	W	W	W
P23	w	W	W	W
P24	w	W	W	W
P25	w	W	W	W
P26	w	W	35delG	W
P27	w	W	W	W
P28	w	W	W	W
P29	w	W	35delG	W
P30	w	W	W	W
P31	w	W	W	W
P32	w	W	W	W
P33	w	W	W	W
P34	w	W	W	W
P35	w	W	W	W
P36	w	W	W	W
P37	w	W	W	W
P38	w	W	W	W
P39	w	W	W	W
P40	w	W	W	W
P41	w	W	W	W
P42	w	W	W	W
P43	w	W	W	W
P44	w	W	W	W
P45	w	W	W	W
P46	w	W	W	W
P47	w	W	W	W
P48	w	W	W	W
P49	w	W	W	W
P50	w	W	W	W

Table 3
CONTINUATED

Results and discussions

The results obtained after genetic testing in the two laboratories of the 50 subjects with profound congenital SNHL studied are presented in table 3.

We conducted the comparative statistical analysis of the results obtained by the two laboratories to validate the SNP method proposed to be performed for screening by the laboratory in the Republic of Moldova. We considered the gold standard the technique used in the German laboratory because the results obtained by this laboratory have already been validated and can be considered as reference.

We calculated the sensitivity and specificity of the test we proposed for screening (table 4).

Population genetic studies have shown that the prevalence of GJB2 gene mutations varies according to ethnicity, eg in China 16% of cases of hearing loss [17], in Pakistan the prevalence is 6.1% [18], in the population of Iran - 16 % [19], 9.6% Mexican [20] and may reach up to 50% in the European population [21]. Although several mutations of the GJB2 gene are described, in the European population the 35delG mutation represents 2/3 of the total mutations in the GJB2 gene [22,23]. In contrast, other mutations predominate in other populations, such as: 235delC mutation in Japanese and other Asian populations [24], 167delT in the Ashkenazi Jews [25], W24X in Indians and Roma [26,27].

	35delG present alele 1 + alele 2	35delG absent alele 1 + alele 2
SNP positive	24	1
SNP negative	2	73
	24/ (24+2)	73/ (1+73)
	Sensitivity	Specificity
	0.92 // 92%	0.98 // 98%

Table 4
DETERMINATION OF SNP SENSITIVITY AND SPECIFICITY FOR THE 35delG MUTATION

In the world, based on the studies conducted on population genetics of rare disease, research is conducted into the use of biomarkers to diagnose, evaluate etiology, risk, and to personalize patient treatment [28-30]. One of the expected results of the project was the development of a screening diagnostic algorithm that would allow:

- early diagnosis of children with genetic hearing loss - secondary to the 35delG mutation - by highlighting the presence of this mutation on the 2 alleles of the gene;

-assessing the risk of deafness in brothers and descendants [31] and

-early rehabilitation, thus contributing to the development of preventive medicine and personalized curative medicine [14,32,33].

In order to identify all neonates and infants with permanent hearing loss it is necessary to screen all newborn babies in the country [34]. Universal hearing screening using physiological measures (either OAE or AABR) is already applied almost all over Europe [35-40]. In Romania, as well as in Moldova Republic, in the last 2 years, there has been a struggle to implement universal hearing screening in maternities. Using OAE, as it is planned, it will statistically result in either false negative and also false positive results, as it is recognized that the sensitivity of TEOAE for identifying hearing loss is around 66.7% and its specificity is 98.8% [41]. Some authors have published some cases that show that deafness due to 35delG mutations may have a late onset and consequently the diagnosis may be missed on neonatal screening programs. This may be an argument to consider neonatal screening for GJB2 mutations in order not to miss these late onset cases that cannot be identified at birth [42,43]. The single-nucleotide guanine deletion (35delG) of the GJB2 gene coding for connexin 26 was shown to be the main genetic cause of autosomal recessive deafness among Europeans. The most common GJB2 anomaly is the deletion of one guanine within the six-guanine string at the beginning of the second GJB2 exon (positions 30-35), the so-called 35delG mutation (rs80338939) [44,45].

Our study shows that using SNP detection of 35delG mutation as a neonatal screening of the rate of detection of newborns with potential late onset of hearing loss could be improved. We also found a high specificity (98%) and sensitivity (92%) of our test, much higher than the specificity and sensitivity of the TEOAE test. This means that using SNP rs80338939 screening the rate of false results will be much lower:

- a false positive result in 2% of the cases - so 2 subjects out of 100 will be falsely diagnosed with 35delG mutation and also,

- a false negative result was obtained in 8 subjects out of 100 meaning that 8 patients would be erroneously diagnosed free of 35delG mutation;

However, it should be stressed that 35delG mutation screening does not replace audiological screening tests because it is thought that up to 1% of human genes are necessary for hearing [46], and mutations in any of these may lead to hearing impairment.

Conclusions

In conclusion, the great sensitivity and specificity of the proposed method recommends this technique to be used as a screening method to identify 35delG mutation that in homozygous form is an indicator of deafness and in heterozigous form is a sign of being a carrier of a recessive genetic variant.

Our study confirms that rs80338939 can be used as a biomarker in the assessment of the risk of autosomal recessive SNHL. In fact, we propose that in the next step, we optimize the technique to achieve 100% sensitivity and specificity.

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