High Frequency of *GJB2* Mutation W24X among Slovak Romany (Gypsy) Patients with Non-Syndromic Hearing Loss (NSHL)

G. Minárik[1,2], V. Ferák[1], E. Feráková[1], A. Ficek[1,2], H. Poláková[2] and Ľ. Kádasi[2]

[1] *Department of Molecular Biology, Comenius University Faculty of Natural Sciences, Mlynská dolina B2–210, 842 15 Bratislava 4, Slovakia*
[2] *Institute of Molecular Physiology and Genetics Slovak Academy of Sciences, Bratislava, Slovakia*

Abstract. Mutations in the *GJB2* gene (connexin 26) represent a major cause of autosomal recessive non-syndromic hearing loss (NSHL) worldwide. In most Caucasian populations, the 35delG mutation in this gene was found to account for up to 50 % of cases of the genetic non-syndromic childhood deafness. In populations of non-European ethnic background, other *GJB2* gene mutations are occasionally common, e.g. 167delT in Ashkenazi Jews, R143W in Africaans and 235delC in Koreans.

In this work, DNA samples from 54 unrelated NSHL patients from endogamous and inbred population of Slovak Roms (Gypsies) from Eastern Slovakia were screened for *GJB2* mutations. The coding region of the *GJB2* gene of patients was sequenced and mutations W24X, R127H, V153I, L90P and V37I were found. In Slovak Romany population, mutation W24X accounts for 23.2 %, R127H for 19.4 %, 35delG for 8.3 %, V153I for 3.7 %, L90P for 3.7 % and V37I for 0.9 % of screened chromosomes. As the W24X mutation was previously found in India and Pakistan, were from the European Romanies originate, it was brought by the European Romanies from their Indian homeland.

The carrier frequency of 35delG was estimated for Slovak non-Romany population to be 3.3 %, and for Slovak Romany population to 0.8 %. The carrier frequency of W24X varied in different Slovak Romany subpopulations from 0.0 % up to 26.1 %.

Key words: Genetic deafness — Non-syndromic hearing loss — *GJB2* — Romanies

Correspondence to: MSc. Gabriel Minárik, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava 37, Slovakia
E-mail: minarik@fns.uniba.sk

Introduction

An incidence of congenital hearing loss is estimated to be approximately 1 in 1000 births in most populations. More than a half of cases can be attributed to genetic factors. Non-syndromic forms of hereditary deafness show autosomal dominant, autosomal recessive, X-linked, as well as mitochondrial modes of transmission, respectively. About 80 % of the cases of congenital deafness are autosomal recessive (DFNB). To date, about 39 loci linked to autosomal recessive non-syndromic hearing loss (NSHL) were described, while only 18 genes have been cloned (www.uia.ac.be/dnalab/hhh). From 30 % to 60 % of all DFNB cases are due to mutations in a single gene coding for connexin 26 (Cx26), *GJB2,* as observed in different Caucasian populations (Kelsell et al. 1997; Zelante et al. 1997).

This gene is located in 13q12, and belongs to the connexin gene family. Connexin genes are simple in organization, composed of two exons. The first exon encodes 5′-untranslated region, while the second exon encodes complete open reading frame (678 nucleotides) and the 3′-untranslated region. This makes connexin genes relatively easy target for mutational screening.

Connexins are gap junction proteins forming membrane channels between adjacent cells, which allow the intercellular exchange of small molecules. The single channel is composed of two hemichannels called connexons, which are hexamers of connexin monomers. The *GJB2* encodes a 26 kDa (226 aminoacid) gap junction protein Cx26. In the rat cochlea it is expressed in supporting cells and basal cells of stria vascularis of the Corti organ (Kikuchi et al. 2000). The function of Cx26 gap junctions is proposed to be essential for K^+ recycling pathway in Corti organ. The ion content of cochlea is unique, there is high concentration of K^+ (150 mmol/dm^3), low concentration of Na^+ (1 mmol/dm^3) and low concentration of Ca^{2+} (0,02 mmol/dm^3) outside in endolymph. The influx of K^+ through mechanically gated ion channels of hair cells induce depolarization of hair cell and Ca^{2+} influx through basal membrane and this causes release of neurotransmitter. So the recycling of K^+ is important for accurate function of cochlea. The unique mechanism of signaling in cochlea can be the reason why mutations of *GJB2* gene are associated especially with NSHL; however this gene is widely expressed (Kikuchi et al. 1995).

Although more than 90 mutations has been described in *GJB2* as NSHL causative (http://www.crg.es/deafness/), mutation 35delG accounts for up to 85 % of causative mutations in Caucasian population (Denoyelle et al. 1997; Zelante et al. 1997; Gasparini et al. 2000). It is a frameshift mutation which creates a stop codon right to the next codon after mutation (13-th codon). In a homozygous state, no functional Cx26 monomers are present in cells. It seems that this mutation is not so frequent in populations of non-Caucasian origin: e.g. mutation 167delT prevails in Ashkenazi Jews (Morell et al. 1998; carrier frequency 4.0 %), R143W in Africans (Brobby et al. 1998), 235delC in Koreans (Park et al. 2000).

The Romany population of Slovakia (about 400,000) represents a genetically isolated population, characteristic with high frequency of consanguinity and in-

breeding, which is about ten-to-hundred times higher than in non-Romany population of the same region (Ferak et al. 1987). Thus, in Slovak Romany population (as well as in other genetically isolated and inbred populations) it is a common place to find relatively high frequency of specific autosomal recessive diseases with one causative mutation prevailing as a consequence of founder effect and genetic drift. This has been shown e.g. for phenylketonuria (Ferakova et al. 1992) or primary congenital glaucoma (Plasilova et al. 1999).

The objective of our study is to asses the spectrum of mutations in the *GJB2* gene in Slovak Romany NSHL patients and to determine the prevailing mutation.

Materials and Methods

Samples

DNA samples from peripheral blood of 54 NSHL-affected patients of Romany ethnic origin from Eastern Slovakia were extracted according to standard protocol (Kunkel et al. 1977). All patients were students of special schools for deaf or hearing-impaired children: 28 from Prešov, 25 from Levoča and 1 from Bratislava.

Sequencing

Preamplification of *GJB2* coding sequence was performed with 50 ng of human DNA in reaction volume 20 μl, containing 2 μl 10×PCR buffer (10 mmol/l Tris-Cl, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.17 mg/ml BSA, 0.1% Triton-X 100); 150 μmol/l dATP, dCTP, dGTP, dTTP; 5 pmol of either the forward (GJB2seqF) and reverse (GJB2seqR) primer and 0.5 U Taq polymerase. Samples were denatured for 4 min at 94℃; followed by 35 cycles of 1 min at 94℃, 1 min at 60℃ and 1 min at 72℃; followed by 7 min extension at 72℃. PCR products from preamplification were cleaned by 1.5% agarose gel electrophoresis and corresponding band was cut-off and extracted using a CONCERT^{TM} Rapid Gel Extraction System (GibcoBRL, UK) according to the manufacturer's instructions. PCR products were sequenced by dye terminator sequencing on an Applied Biosystems (ABI) model 310 Genetic Analyser. For sequencing were used both forward and reverse primer from PCR and another two internal primers GJB2intF and GJB2intR (sequences of all primers are in Table 1).

Table 1. PCR and sequencing primers for sequencing of the *GJB2* coding region

Name	Primer sequence	
GJB2seqF	5′-GGTGAGGTTGTGTAAGAGTTGG-3′	22-mer
GJB2seqR	5′-TAGCGACTGAGCCTTGACAG-3′	20-mer
GJB2intF	5′-GTGGCCTACCGGAGACAT-3′	18-mer
GJB2intR	5′-CACTCTTTATCTCCCCCTTG-3′	20-mer

Population frequency

ARMS PCR method for screening of the two most common mutations found in Slovak Caucasian and Slovak Romany populations, 35delG and W24X, was performed as described Scott et al. (1998). 122 individuals of Slovak Caucasian and 154 individuals of Slovak Romany population were used for estimation of population frequencies.

Results

The coding region of the *GJB2* gene of patients was sequenced and mutations W24X, R127H, 35delG, V153I, L90P and V37I were found. Figure 1 (A to F) show sequences of observed mutations with adjacent sequences, respectively. Frequencies of observed mutations are summarized in Table 2.

Table 2. Frequencies of observed *GJB2* mutations in Slovak Romany patients

Slovak Romany patients (54)			
mutation	No. of chromosomes	frequency of mutation	proportion of mutation
W24X	25	23.2 %	39.1 %
R127H	21	19.4 %	32.7 %
35delG	9	8.3 %	14.1 %
V153I	4	3.7 %	6.3 %
L90P	4	3.7 %	6.3 %
V37I	1	0.9 %	1.5 %
NIM	44	40.7 %	X
all	108	100.0 %	100.0 %

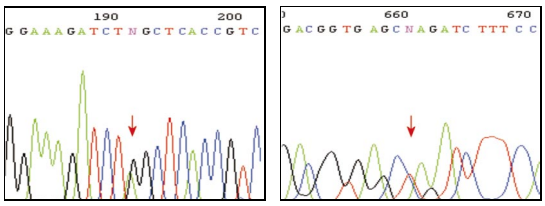
NIM, not-identified mutation in *GJB2* gene.

In 68.5% of patients at least one *GJB2* mutation was identified. The W24X mutation was present in at least one chromosome in 31.5% of patients, R127H in 31.5%, 35delG in 13.0%, V153I in 7.4%, L90P in 5.6% and V37I in 1.9%.

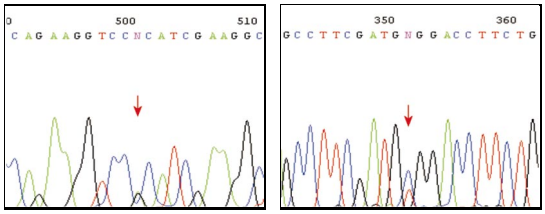
Proportion of the most frequent mutation in Slovak Romanies, W24X, on all observed *GJB2* mutations was estimated to be 39.1%. The carrier frequency was estimated and found to vary in four different subgroups of samples – from 0.0% (0/21), through 3.0% (1/33), 16.7% (9/54) to as much as 26.1% (12/46).

Discussion

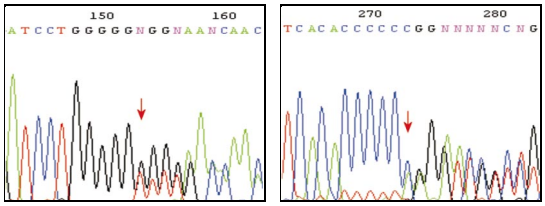
Among *GJB2* mutations described so far in the literature, not all were found as causative, but in some of them decision is not yet definitive. Mutations 35delG,



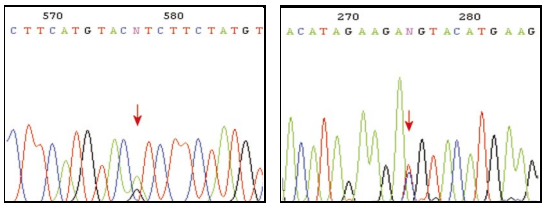
A: W24X heterozygous patient



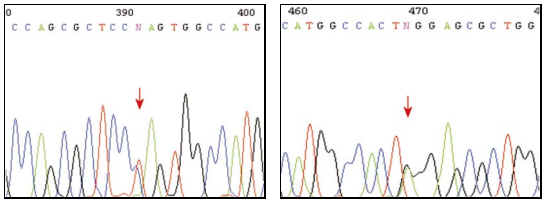
B: R127H heterozygous patient



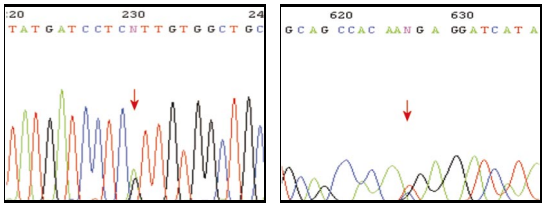
C: 35delG heterozygous patient



D: V153I heterozygous patient



E: L90P heterozygous patient



F: V37I heterozygous patient

Figure 1. Sequences of *GJB2* gene fragments of heterozygous patients with mutations (including flanking sequences). Mutations are highlighted with red arrow. Sequenced with Forward (left) and Reverse (right) primers, respectively (figures A–F).

W24X and L90P were described as NSHL causative, while mutations V37I, R127H and V153I were sometimes described as polymorphisms (http://www.crg.es/deafness/), so additional study is warranted.

Because of relatively high frequency of the W24X mutation among Slovak Romany patients, and its occurrence in the region where it has been previously identified (India, Pakistan) (Kelsell et al. 1997) we assume, that this mutation was brought by Romanies to Europe from their Indian homeland, and can be present also in other European Romany populations. In order to determine whether the Slovak Romany and the Pakistani W24X mutations are identical by descent, the study of the DNA polymorphic haplotypes in the vicinity of this mutation in patients of Pakistani and of Slovak Romany origin is now in progress. The carrier frequency for W24X mutation in different Slovak Romany population varies from 0.0 % to 26.1 %. The samples for this estimation were drawn from four independent sample groups. Three of them were from three different localities of Slovakia (Plavecký Štvrtok – Western Slovakia, Podskalka – Middle Slovakia and Hostišovce – Eastern Slovakia), while the fourth one, assembled from independent paternity testing, shows the most probable "mean" carrier frequency of 3.0 %. However, this group alone is too small for a reliable estimate (only 33 individuals).

Mutation W24X is a nonsense mutation truncating Cx26 protein (24 AA instead of 226 AA). In homozygous state, no functional Cx26 monomers are present in cells. This has impact on recycling of K^+ to endolymph, and physiological response to sound stimuli is only weak, if any.

Mutation R127H was found to be second most frequent *GJB2* gene mutation in Slovak Romany population with a frequency of 19.4 % of presumed disease alleles. This is a relatively high frequency, which may have two possible reasons. First one is based on the knowledge that Romanies are composed of several subpopulations, genetically highly isolated from each other, and frequency of R127H might have been elevated by drift in only one of them. The second one is based on the assumption that R127H is only polymorphism without any association with NSHL. There are arguments in favor of both these explanations (http://www.crg.es/deafness/; Thönnissen et al. 2002). According to our results we assume R127H being polymorphism rather than causative mutation, as most of our patients carrying R127H had no other *GJB2* mutation on the second chromosome (9 of 17).

Mutation 35delG, which is most common NSHL causative mutation in Caucasian population, was present in only 8.3 % of presumed disease alleles in Romany patients, and it might have been dragged to Romanies from Caucasians, where it is the most frequent one.

V153I was found to account for 3.7 % of presumed disease alleles, but it is most probably just a polymorphism (http://www.crg.es/deafness/). This view is supported by our findings of V153I heterozygous patients without any second NSHL causative mutation in the *GJB2* gene.

Mutation L90P is NSHL causative mutation, accounts for 3.7 % of presumed disease alleles in our patients. This mutation was also found to be relatively frequent

in South-Western Austria (Tyrol) (Löffler et al. 2001) but in contrast it is rather in Eastern Austria (Frei et al. 2002).

Mutation V37I, also generally considered as NSHL causative (http://www.crg.es/deafness/), account for only 0.9% of presumed deafness alleles in Slovak Romany patients, but in our study it was found in compound heterozygous state with R127H in only one patient, so another investigation about its NSHL causativity may be necessary.

These data suggest either that different Romany subpopulations developed as relatively genetically isolated communities with their own different founder mutations, or that the mutation frequency in *GJB2* gene is relatively high. Analysis of polymorphic DNA haplotypes in the vicinity of the *GJB2* mutations is likely to help in solving this problem.

Acknowledgements. The authors thank to authorities of Special Schools for Deaf or Hearing impaired children from Prešov, Levoča and Bratislava for their cooperation. Our thanks go also to the children and their parents, who took part in this study. This study was supported, in part, by research grants: 99/2001/UK from Comenius University, Slovakia, 12/2002 from Faculty of Natural Sciences of Comenius University, Slovakia, 2/1083/21 and 1/7281/20 from Slovak Grant Agency VEGA.

References

Brobby G. W., Muller-Myhsok B., Horstmann R. D. (1998): Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. N. Engl. J. Med. **338**, 548—550

Denoyelle F., Weil D., Maw M. A., Wilcox S. A., Lench N. J., Allen-Powell D. R., Osborn A. H., Dahl H. H., Middleton A., Houseman M. J., Dode C., Marlin S., Boulila-ElGaied A., Grati M., Ayadi H., BenArab S., Bitoun P., Lina-Granade G., Godet J., Mustapha M., Loiselet J., El-Zir E., Aubois A., Joannard A., Petit C. et al. (1997): Prelingual deafness: high prevalence of a 30delG mutation in the connexin 26 gene. Hum. Mol. Genet. **6**, 2173—2177

Ferak V., Sivakova D., Sieglova Z. (1987): The Slovak Gypsies (Romany): Population with the highest coefficient of inbreeding in Europe. Bratisl. Lek. Listy **87**, 168—175 (in Slovak)

Ferakova E., Ferak V., Kadasi L., Polakova H., Hejcmanova L., Pijackova A. (1992): A unique RFLP haplotype at the phenylalanine hydroxylase locus in Czechoslovak Gypsies with phenylketonuria. Funct. Dev. Morphol. **2**, 139—140

Frei K., Szuhai K., Lucas T., Weipoltshammer K., Schofer C., Ramsebner R., Baumgartner W. D., Raap A. K., Bittner R., Wachtler F. J., Kirschhofer K. (2002) Connexin 26 mutations in cases of sensorineural deafness in eastern Austria. Eur. J. Hum. Genet. **10**, 427—432

Gasparini P., Rabionet R., Barbujani G., Melchionda S., Petersen M., Brondum-Nielsen K., Metspalu A., Oitmaa E., Pisano M., Fortina P., Zelante L., Estivill X. (2000): High carrier frequency of the 35delG deafness mutation in European populations. Genetic Analysis Consortium of GJB2 35delG. Eur. J. Hum. Genet. **8**, 19—23

Kelsell D. P., Dunlop J., Stevens H. P., Lench N. J., Liang J. N., Parry G., Mueller R. F., Leigh I. M. (1997): Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature **387**, 80—83

Kikuchi T., Kimura R. S., Paul D. L., Adams J. C. (1995): Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. Anat. Embryol. **191**, 101—118

Kikuchi T., Kimura R. S., Paul D. L., Takasaka T., Adams J. C. (2000): Gap junction systems in the mammalian cochlea. Brain Res.–Brain. Rev. **32**, 163—166

Kunkel L. M., Smith K. D., Boyer S. H., Borgaonkar D. S., Wachtel S. S., Miller O. J., Bregg W. R., Jones H. V., Rary J. M. (1977): Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc. Natl. Acad. Sci. U.S.A. **74**, 1245—1249

Löffler J., Nekahm D., Hirst-Stadlmann A., Gunther B., Menzel H. J., Utermann G., Janecke A. R. (2001): Sensorineural hearing loss and the incidence of Cx26 mutations in Austria. Eur. J. Hum. Genet. **9**, 226—230

Morell R. J., Kim H. J., Hood L. J., Goforth L., Friderici K., Fisher R., Van Camp G., Berlin C. I., Oddoux C., Ostrer H., Keats B., Friedman T. B. (1998): Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. N. Engl. J. Med. **339**, 1500—1505

Park H. J., Hahn S. H., Chun Y. M., Park K., Kim H. N. (2000): Connexin26 mutations associated with nonsyndromic hearing loss. Laryngoscope **110**, 1535—1538

Plasilova M., Stoilov I., Sarfarazi M., Kadasi L., Ferakova E., Ferak V. (1999): Identification of a single ancestral CYP1B1 mutation in Slovak Gypsies (Roms) affected with primary congenital glaucoma. J. Med. Genet. **36**, 290—294

Scott D. A., Kraft M. L., Carmi R., Ramesh A., Elbedour K., Yairi Y., Srisailapathy C. R., Rosengren S. S., Markham A. F., Mueller R. F., Lench N. J., Van Camp G., Smith R. J., Sheffield V. C. (1998): Identification of mutations in the connexin 26 gene that cause autosomal recessive nonsyndromic hearing loss. Hum. Mutat. **11**, 387—394

Thönnissen E., Rabionet R., Arbones M. L., Estivill X., Willecke K., Ott T. (2002): Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expression. Hum. Genet. **111**, 190—197

Zelante L., Gasparini P., Estivill X., Melchionda S., D'Agruma L., Govea N., Mila M., Monica M. D., Lutfi J., Shohat M., Mansfield E., Delgrosso K., Rappaport E., Surrey S., Fortina P. (1997): Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. Hum. Mol. Genet. **6**, 1605—1609

Final version accepted: July 22, 2003