Generation of the panel of transgenic human cell lines with stable expression of mutant variants of the *GJB2* gene associated with hearing loss for comparative *in vitro* studies

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Assessment of pathogenicity of novel variants is a primary task for molecular diagnostics of hereditary diseases, and comprehensive evidences, including functional *in vitro* studies, are necessary to support their involving in pathology.

(i) **pathogenic variant**
(ii) likely pathogenic variant
(iii) uncertain significance variant
(iv) likely benign variant
(v) benign variant

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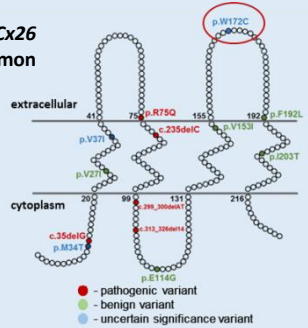
Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology

Criteria for the variant pathogenicity:

- ✓ Obvious segregation of variant with pathology (family analysis);
- ✓ Significant excess of variant frequency in patient sample compared to controls;
- ✓ Rarity or absence of variant in world human genomic databases;
- ✓ Multiple computational predictions of deleterious effects of variant;
- ✓ Confirmation of its pathogenic effects in *in vitro* functional studies

This study aims to generate the panel of transgenic human cell lines with stable expression of mutant variants of the *GJB2* (Cx26) gene associated with hearing loss and to perform the complex assessment of pathogenicity of novel *GJB2* variant c.516G>C (p.Trp172Cys) found in association with deafness in indigenous peoples (Tuvinians and Altaians) of Southern Siberia (Russia).

The structure of the *Cx26* protein with some common variants.



Novel nonsynonymous missence variant c.516G>C (p.Trp172Cys) in the *GJB2* gene which encodes a transmembrane protein connexin 26 (Cx26).

The authors declare that they have no competing interests

Complex assessment of novel *GJB2* missence variant c.516G>C (p.Trp172Cys)

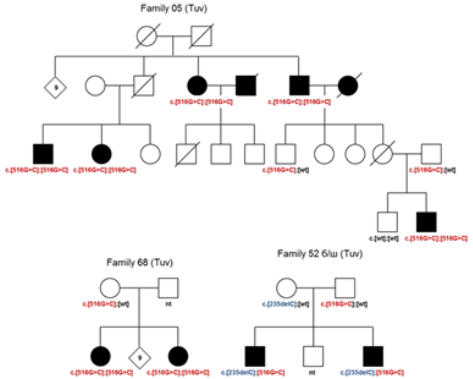
Frequency of c.516G>C (p.Trp172Cys) was significantly higher in the patient's groups than in the controls ($p<0.05$).

Allele frequency of p.W172C (c.516G>C) in patients and control samples.

Tuvinians		Altaians	
Number and frequency of p.Trp172Cys allele		Number and frequency of p.Trp172Cys allele	
Patients (N=182)	Control (N=157)	Patients (N=74)	Control (N=218)
49/364 0.135	> 6/314 0.019	8/148 0.054	> 1/436 0.002
$p= 0.6178 \times 10^{-8}$		$p= 0.1039 \times 10^{-3}$	

Most of *in silico* programs (PolyPhen2, FATHMM, PROVEAN, PANTHER, MutationTaster etc) predicted a likely damaging effect of p.Trp172Cys.

Segregation of a homozygous or compound heterozygous c.516G>C (p.Trp172Cys) with hearing loss was established in analysis of pedigrees of deaf patients.



The c.516G>C (p.Trp172Cys) was not found in the world human genomic databases (ClinVar, dbSNP138, 1000 Genomes Project, Genome Aggregation Database).

Generation of the panel of transgenic human cell lines with stable expression of mutant *GJB2* variants

- To avoid any endogenous *GJB2* expression, we primarily obtained (using CRISPR/Cas9 genome editing system) the *GJB2* knockout HeLa cell line (Hela *GJB2* ko) for subsequent establishment of transgenic cell lines.

- Seven transgenic cell lines carrying different *GJB2* variants were generated:

Novel missense variant c.516G>C (Cx26-p.Trp172Cys) with yet unclear functional significance;

Wild type of the *GJB2* coding region (Cx26-wt);

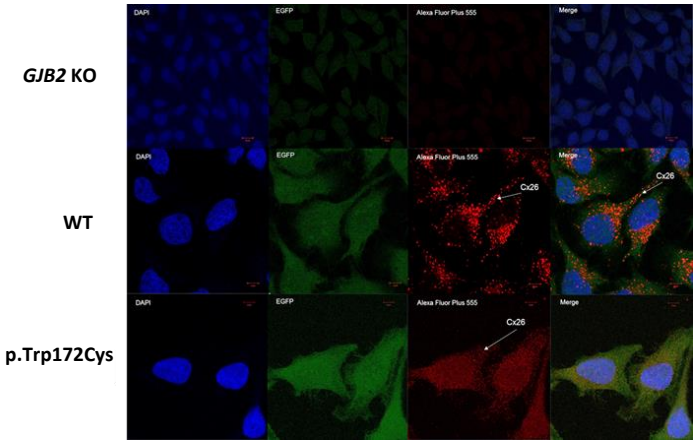
Known dominant mutation c.224G>A (Cx26-p.Arg75Gln);

cis-configuration of two variants c.79G>A (p.Val27Ile) and c.341A>G (p.Val27Ile) of unproven pathogenicity (Cx26-p.[Val27Ile;Glu114Gly]);

Obvious pathogenic deletions c.35delG (Cx26-p.Gly12Valfs), c.235delC (Cx26-p.Leu79Cysfs), c.313_326del14 (Cx26-p.Lys105Glyfs).

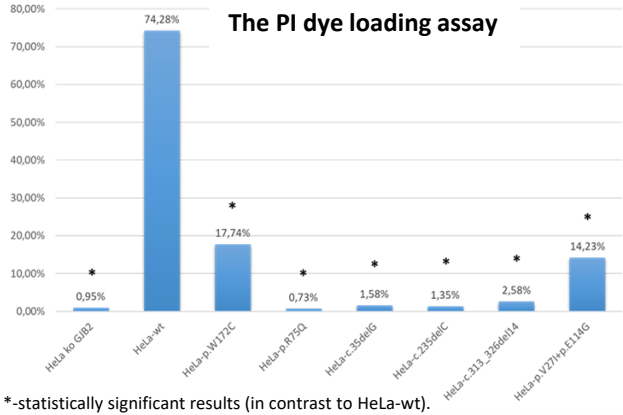
Comparative *in vitro* analysis with using constructed panel of transgenic HeLa cell lines

ICC showed predominantly cytoplasmic localization of the Cx26-p.Trp172Cys protein in contrast to Cx26-wt which represented distinct conglomerates on cell membrane.



Localization of different variants of Cx26 protein in transgenic HeLa cell lines *(confocal microscopy).*

The Dye (PI) loading assay revealed lower PI loading efficiencies in cells expressing mutant variant Cx26-p.Trp172Cys compared to Cx26-wt while the absence of PI accumulation was shown for mutant Cx26-variant with known pathogenic effect.



*-statistically significant results (in contrast to HeLa-wt).

Conclusion:

For the first time, the panel of transgenic HeLa cell lines stably expressing of different *GJB2* variants was generated for subsequent analysis of structure and functioning of Cx26 protein.

The pathogenicity of novel *GJB2* missense variant c.516G>C (p.Trp172Cys) was proven by several lines of evidences including comparative *in vitro* analysis with using constructed panel of transgenic HeLa cell lines.

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