Functional Analysis of a Novel I71N Mutation in the *GJB2* Gene Among Southern Egyptians Causing Autosomal Recessive Hearing Loss

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Key Words

Gap junction • Connexins • Non-syndromic hearing loss • *Xenopus laevis* oocytes • Functional characterization

Abstract

Mutations in *GJB2*, a gene encoding the gap junction protein connexin 26 (Cx26), are a major cause for inherited and sporadic non-syndromic hearing loss, albeit with highly variable clinical effects. To determine new mutations and their frequencies in a Southern Egyptian population restriction fragment length polymorphism, gene sequencing, and single strand conformational polymorphism revealed only 2 mutations for *GJB2*: c.35delG and p.I71N. The allelic frequency of the c.35delG mutation was 8.7% (found in 27 out of 310 investigated alleles) resulting in a relatively low carrier frequency (1.6%) in Upper Egypt. The new mutation, a substitution of isoleucine (I) (a non-polar amino acid) by the polar amino acid asparagine (N), was localized within the conserved Cx26 structure. The functional significance of p.I71N was tested by injection of cRNA into *Xenopus laevis* oocytes. Cx26 hemi-channel activity was measured

by depolarization activated conductance in non-coupled oocytes. As a result, the p.I71N mutated channel was non-functional. The study discloses a novel, functionally relevant *GJB2* mutation and defines the contribution of Cx26 alterations to the hearing loss in the Southern Egyptian population.

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Introduction

Hearing impairment is the most frequent sensory deficit and the second most frequent chronic disease in man [1]. It is, in most cases, a multi-factorial disorder caused by both genetic and environmental factors. Until now, more than 100 genes involved in non-syndromic and syndromic hearing loss have been mapped and over 40 genes have been cloned [2]. According to country profiles on disability issued by the Japanese international cooperation agency (JICA) (http://www.childinfo.org/mics/Cntry_files/Egyresul.pdf) it is estimated that for

every 100,000 Egyptian children (under 14 years old), there are more than 800 with hearing impairment and more than 600 with speech disability.

In the inner ear, the majority of cells are extensively coupled via gap junctions. These are assumed to form the structural pathways for spatial buffering and circulation of K^+ within the cochlea [3]. Connexin 26 is a member of a family of trans-membrane proteins that is highly expressed in cochlea, and it permits intercellular communication. Hydropathy plots of connexins revealed the presence of two extracellular domains (E1 and E2), four hydrophobic membrane-spanning domains (M1–M4), and three cytoplasmic domains including an intracellular loop, and amino and carboxyl termini (NT and CT, respectively) [4, 5].

Mutations in *GJB2*, the gene encoding the gap-junction protein connexin 26, are the most common cause of autosomal recessive non-syndromic deafness in many populations, being linked to approximately 25% to 50% of all cases in various ethnic populations [6]. More than 90 recessive mutations have been identified in the *GJB2* gene, which can be broadly divided into truncating and non-truncating mutations [7]. Nickel and Forge [3] postulated that truncating mutations are generally associated with a greater degree of hearing loss than non-truncating mutations, and result in defective protein synthesis. It is intriguing to note that all forms of mutations can lead to variable clinical defects suggesting a more complex genetic background of the disease. A deletion of one out of six guanines (G), extending from position 30 to position 35 (c.35delG) which results in a premature stop codon at position 13 and which accounts for the majority of *GJB2* mutant alleles was frequently reported for many populations; and confirmed to be most prevalent in subjects of Mediterranean European ancestry [8].

Here, we examined individuals from Southern Egyptian population aiming at screening for new mutations in *GJB2* and determining the c.35delG frequency in comparison to other ethnic groups. In addition, a functional study of the newly discovered mutation (p.I71N) was performed to assess the impact of this amino acid substitution on cell function.

Materials and Methods

Mutation analysis of GJB2 gene

The *GJB2* gene was studied in 97 families with nonsyndromic hereditary hearing loss of unknown genesis (155 individuals). The collection, derived from patients seeking au-

diological testing, was consisting of individuals from governorates Minia, Assiut, Suhag, Qena and Aswan. The control samples came from gender-matched healthy individuals of the same ethnicity. The study was performed with the approval of the Assiut University ethics committee, and in compliance with national legislation and international standards (Declaration of Helsinki). Among the subjects, 17 families were analysed by *GJB2* amplification from genomic DNA by using the following primers [1F (forward): 5'-AGA CTC AGA GAA GTC TCC CTG-3'] and [2R (reverse) 5'-CCA GGC TGC AAG AAC GTG TGC-3']. The PCR product was used in a restriction fragment length polymorphism (RFLP) reaction using the restriction enzyme BsiYI. The product was loaded on polyacrylamide gel and electrophoresed. All DNA samples that showed c.35delG in restriction assay were subjected for sequencing of the *GJB2* single coding exon using a capillary sequencer CEQ 8000 (Beckman Coulter) and the following PCR primers [1F (forward): 5'-AGA CTC AGA GAA GTC TCC CTG-3'], [2F (forward): 5'-CCA GGC TGC AAG AAC GTG TGC-3'], [3R (reverse): 5'-GTG TAC CGG AGA CAT GAG-3'], [4F (forward): 5'-GCA GCA TCT TCT TCC GGG TC -3'], [5R (reverse): 5'-GCC AGT TTA ACG CAT TGC CC-3'].

All DNA samples which did not show c.35delG mutations in restriction assays were screened for other eventual mutations in the *GJB2* gene by single strand conformational polymorphism (SSCP). The PCR products were digested by a restriction enzyme (BsmAI) for cutting the products into two pieces (378 bp + 403 bp).

In additional 80 families, one patient from each family, was selected for sequencing the single coding exon of *GJB2* gene according to previous instructions.

Tetra-primer ARMS-PCR

For a novel homozygous missense change (p.I71N) in an affected member from one family, the Tetra-primer ARMS-PCR technique was used for screening 100 subjects from 100 families, to determine the nature of the change, whether it is a polymorphism (SNP) or a real novel mutation in the *GJB2* gene.

The Tetra-primer ARMS-PCR employed two primer pairs (outer and inner primers) to amplify, respectively, the two different alleles of a SNP in a single PCR reaction followed by gel electrophoresis. Special software was used for designing outer and inner primers. The software is available via internet (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). According to this software we designed the outer and inner primers as follows:

[Outer Forward primer: 5' ATG ATC CTC GTT GTG GCT GCAAAG G 3'],

[Outer Reverse Primer: 5' CAT GTC TCC GGT AGG CCA CGT GC 3'],

[Inner Forward primer: 5' AAC GTG TGC TAC GAT CAC TAC TTC CCC AT 3'],

[Inner Reverse primer: 5' GCC CAT AGC CGG ATG TGG GTGA 3'].

A 3' end mismatch was added to the inner primers at position -2 to increase the specificity of the primers.

Functional study of the novel p.I71N mutation in Connexin 26

a) Cloning of PCR products onto Plasmid vector: The *GJB2* coding exon from the patient and a healthy control was amplified by PCR using the following PCR primers: [F (5' AGA CTC AGA GAA GTC TCC CTG 3')], [R (5' GGG CAA TGC GTT AAA CTG GC 3')]. The PCR products were cloned into a pCR®II-TOPO® cloning vector (Invitrogen, California, USA).

b) Transforming competent cells: The cloning reaction is then transformed into chemically competent *E. coli* cells, One Shot DH5α™-T1^R Cells, (Invitrogen, California, USA) according to accompanying protocol. Plasmid DNA was isolated using Qiagen® Plasmid Purification Kit, according to the accompanying protocol. All purified plasmid DNA probes were sequenced, according to routine protocols, using M13 primers [F:5'-GTA AAA CGA CGG CCA G-3'], [R5'CAG GAA ACA GCT ATG AC-3'] to determine the orientation of insertion of the construct in the plasmid. After determining the orientation of the inserts, Plasmid DNA was sequenced again using *GJB2* primers to ensure the right sequence of the *GJB2* gene. The right plasmid DNA was determined (for both, control and mutated samples) according to the results of sequencing. The plasmid DNA was linearized by digestion with the restriction enzyme XbaI (Fermentas Life Sciences, Germany). The enzyme's recognition site is: 5'-TCT AGA-3'.

c) *In vitro*-Transcription: The linearized purified plasmid DNA from control and mutated samples were used for *in vitro*-transcription process for production of cRNA using MEGAscript® Kit-SP6 Kit (Ambion, USA) according to manufacturer's instructions. DNA template and unincorporated nucleotides were removed using SV Total RNA Isolation System (Promega, Germany) according to the manufacturer's instructions.

d) Expression in *Xenopus* oocytes and electrophysiological recordings: cRNA encoding wild-type and mutant Cx26 was used for expression in *Xenopus laevis* oocytes. Dissection of *Xenopus laevis* ovaries and collection and handling of the oocytes have been conducted according to the protocols described in Wagner et al. [9]. Oocytes were injected with 50 ng cRNA encoding wild type or mutant Cx26. As a control, oocytes were injected with equivalent volumes of solvent. All experiments were performed at room temperature, 2 days after injection of the respective cRNAs.

In two-electrode voltage-clamp experiments, the cell membrane potential was changed from -140 mV to +60 mV in 40 mV increments for 20 seconds. The intermediate holding potential was -60mV. The steady state current at +60 mV was taken as a measure for Cx26 hemi-channel activity. Currents were filtered at 10 Hz and recorded with Digidata AD/DA interface digital to analog converter and pClamp software for data acquisition and analysis (Axon instruments, Foster City, CA, USA). The bath solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM Hepes, pH 7.4. The final solution was titrated to the pH indicated using HCl or NaOH.

Data is provided as mean ± SEM; n represents the number of oocytes investigated. All experiments were repeated with at

least 3 batches of oocytes; in all repetitions, qualitatively similar data were obtained. All data were tested for significance by using ANOVA, and only results with p < 0.05 were considered statistically significant.

Western Blot of Plasma Membrane Proteins

The membrane abundance of Cx26 was analyzed by surface biotinylation. 20 intact healthy oocytes were incubated in Sulfo-NHS-LC-Biotin (1 mg/ml, Pierce, Rockford, IL, USA) for 30 minutes at RT and washed 5 times for 10 min in ND96. The oocytes were homogenized with a pestle in 400 µl buffer H (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% TritonX-100 and Complete Protease Inhibitor [(Roche Diagnostics GmbH, Mannheim, Germany)]). The samples were kept at 4°C for 1 hour on a rotator, and then centrifuged for 2 min at 13000 rpm. The supernatants were supplemented with 25 µl washed immobilized NeutrAvidin Agarose beads (Pierce, Rockford, IL, USA) and incubated at 4°C overnight on a rotator. The beads were then pelleted by a 2 min centrifugation at 13000 rpm, and washed in buffer H (5 times). For total protein western blot, 20 intact healthy oocytes were homogenized with a pestle in 400 µl Buffer-H. The samples were kept at 4°C for 1 hour on a rotator, then centrifuged for 2 min at 13000 rpm. After measurement of the total protein concentration (Bradford assay), 50 µg of protein were solubilized in Roti-Load1 Buffer (Carl Roth GmbH, Karlsruhe, Germany) at 95°C for 10 min. Proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with 5% non-fat dry milk in TBS 0.1% Tween20 for 1 h at RT, the blots were incubated overnight at 4°C with rabbit anti-Cx26 antibody (diluted 1:500, Invitrogen, Carlsbad, CA). After washing (TBST), the blots were incubated with anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling, Danvers, MA, USA) for 1h at RT. For loading control the blot was stripped in stripping buffer (Carl Roth GmbH, Karlsruhe, Germany) at 56°C for 30 min. After washing with TBST the blot was blocked with 5% nonfat milk in TBST for 1 h at RT. The blot was then incubated with a rabbit anti-GAPDH antibody (diluted 1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing with TBST, the blot was incubated with anti-rabbit HRP antibody (diluted 1:1000, Cell Signaling Technology, Danvers, MA, USA) for 1h at RT. Antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). Bands were quantified with Quantity One Software (Bio-Rad, München, Germany).

Immunofluorescent staining

After 4% paraformaldehyde fixation for at least 12 h, oocytes were cryoprotected in 30% sucrose, frozen in mounting medium, and placed on a cryostat. Sections were collected at a thickness of 6 µm on coated slides and stored at -20°C. For immunostaining, sections were dehydrated at room temperature, fixed in acetone/methanol (1:1) for 15 min at RT, washed in PBS and pre-incubated for 1 h in 5% bovine serum albumin in PBS. The anti-connexin26 antibody (Abnova) was diluted 1:100 and then applied in a moist chamber overnight at 4°C. Binding of anti-connexin26 antibody was

Family	Case	Age(yrs)	Genotype	Degree of deafness	Ethnic background	Domain	Type of Mutation	Protein substitution
1	I	7	35delG/35delG	Bilateral profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	II	5	35delG/35delG	Bilateral profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	III	71	WT/WT	Bilateral NPH	Southern Egypt(Assiut)	_____	_____	_____
	IV	25	35delG/WT	Bilateral NPH	Southern Egypt(Assiut)	IC1	Frameshift	V13X
2	I	3	35delG/35delG	Bilateral Profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	II	1	35delG/35delG	Bilateral Profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	III	8	35delG/35delG	Bilateral Profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	IV	33	35delG/WT	Bilateral NPH	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	V	48	35delG/35delG	Bilateral Moderate SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
3	I	1.5	35delG/35delG	Bilateral Profound SNHL	Southern Egypt(Minia)	IC1	Frameshift	V13X
	II	40	35delG/WT	Bilateral NPH	Southern Egypt(Minia)	IC1	Frameshift	V13X
	III	35	35delG/WT	Bilateral NPH	Southern Egypt(Minia)	IC1	Frameshift	V13X
4	I	20	35delG/35delG	Bilateral severe SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	II	16	35delG/35delG	Bilateral severe SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	III	10	35delG/35delG	Bilateral Profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
	IV	38	WT/WT	Bilateral NPH	Southern Egypt(Assiut)	_____	_____	_____
	V	40	WT/WT	Bilateral NPH	Southern Egypt(Assiut)	_____	_____	_____
5	I	45	35delG/WT	Bilateral Moderate SNHL	Southern Egypt(Aswan)	IC1	Frameshift	V13X
6	I	1	35delG/35delG	Bilateral profound SNHL	Southern Egypt(Assiut)	IC1	Frameshift	V13X
7	I	6	T212A/T212A	Bilateral Severe to profound SNHL	Southern Egypt(Assiut)	EC1	Missense	I71N
	II	10	T212A /WT	Bilateral NPH	Southern Egypt(Assiut)	EC1	Missense	I71N
	III	33	WT/WT	Bilateral NPH	Southern Egypt(Assiut)	_____	_____	_____

Table 1. Phenotypic and genotypic data of the families studied bearing Connexin 26 mutations. SNHL, sensorineural hearing loss; NPH: normal peripheral hearing; IC, intracellular domain; EC, extracellular domain.

visualised with an anti-rabbit conjugated FITC antibody (1:500, Invitrogen). Slides were analyzed on a confocal laser-scanning microscope (Zeiss LSM 510 Exciter) using Ar laser with 488 nm excitation wavelength and appropriate filter set.

Results

Only 2 mutations were found within the *GJB2* gene in the Upper Egyptian group, the widely occurring

Table 2. % carrier frequencies of 35delG mutation in 12 different populations in addition to Egypt. The accumulated data were transferred from Neocleous et al. [22]. The table reveals that the studied Egyptian population is, in comparison to other European and Mediterranean populations, in the lower range of carrier frequency.

Country	% Carrier rate
Greece	3.5
Italy	4
France	2.73
Spain	2.31
Portugal	2.23
Belgium	2.50
Germany	1.6
Bulgaria	0.64
Malta	2.78
Turkey	1.78
Lebanon	2.33
Cyprus	2.47
Egypt	1.6

c.35delG and a new amino acid substitution, p.I71N. Although more than 100 mutations have been detected in *GJB2* worldwide our screen revealed no further sequence deviation in our study group (Table 1).

The c.35delG mutation

The c.35delG mutation was present in 6 out of 97 families with non-syndromic hearing loss representing 6.2% of the families. The allelic frequency for c.35delG was 8.7% (27 out of 310 investigated alleles in 155 individuals). Eleven patients were homozygous and 5 patients were heterozygous. This result indicates a carrier frequency of 1.6% (Table 2). All homozygous patients displayed a phenotype of bilateral severe to profound sensorineural hearing loss while heterozygous subjects were normal carriers except for one subject who was found with bilateral moderate sensorineural hearing loss. When comparing the allelic frequency of c.35delG in the Upper Egypt group with some other ethnicities the Egyptians showed one of the lowest frequencies (Table 2).

The p.I71N mutation

Next to the deletion c.35delG sequencing revealed a novel missense mutation in the *GJB2* gene. It is a substitution (c. T212A) leading to a change from isoleucine (Ile) to asparagine (Asn) at amino acid position 71. This patient, homozygous for p.I71N, was having a phenotype of bilateral severe to profound sensorineural hearing loss. On sequencing another members of the family the

Fig. 1. Functional characterization of the mutated Connexin 26 (p.I71N). *Xenopus* oocytes were injected with wild type or mutated Cx26 or with water. 2-3 days after injection, channel activation was analyzed upon oocyte depolarization from -100mV to +60 mV for 20 seconds. A: Original recordings of wild type Cx26-, mutated Cx26- and water- injected oocytes. B: Mean values ± SEM. *** (p<0.001) indicates statistically significant difference from current in *Xenopus* oocytes injected with H₂O; ### (p<0.001) indicates statistically significant difference from current in *Xenopus* oocytes expressing wild type Cx26. Cx26p.I71N displayed a totally defective phenotype. (H₂O: 0.09 ± 0.01 A (n=15); Cx26: 0.80 ± 0.08 A (n=20); Cx26p.I71N: 0.22 ± 0.03 A (n=22)).

mutation was noted in a heterozygous state in a non-affected brother (with normal hearing) indicating that p.I71N is of recessive nature. No polymorphism has been described at this position so far. In addition, the change was absent in 100 control samples indicating its clinical significance.

Functional analysis of p.I71N

To examine whether the p.I71N mutation affected the function of wild type or mutated *GJB2* sequences were expressed in *Xenopus laevis* oocytes and channel

Fig. 2. Reduced presence of Cx26p.I71N in the oocyte membrane. Binding of anti-connexin26 antibody was visualized with anti-rabbit conjugated FITC antibody. Slides were analyzed on a confocal laser scanning microscope (Zeiss LSM 510 Exciter) using Ar laser with 488 nm excitation wavelength and appropriate filter set. The Figure shows reduced incorporation of the mutated connexin into the cellular membrane in comparison to WT connexin. The left and the right panels depict representative stainings retrieved from two oocytes.

activity was determined by electrophysiological analysis. Depolarization induced a slowly activating current in Cx26 expressing oocytes ($0.80 \pm 0.08 \mu\text{A}$, $n=20$) that was ~ 8 -fold higher than the current measured in water injected control oocytes ($0.1 \pm 0.01 \mu\text{A}$, $n=15$, $p < 0.001$). The p.I71N mutant Cx26 displayed a totally defective phenotype (Fig. 1). A reduced incorporation of the mutant form into the cellular membrane (Fig. 2) supports the observation of diminished activating current. To clarify whether a reduced transcriptional rate of the mutated gene sequence is responsible for this effect, cellular and membrane-bound was compared to the wild type form. Western blots demonstrated an equal synthesis rate (Fig. 3A) but a significantly weaker signal of the Cx26^{p.I71N} membrane fraction (Fig. 3B) in conformity with the results presented in figures 1 and 2.

Fig. 3. Cytoplasmic and membrane-bound presence of Cx26 and Cx26p.I71N A: The upper panel (bar diagram and original blot) depicts one western blot of total Cx26 expression in oocytes injected with water (left lane), with Cx26 cRNA (middle lane) and with Cx26p.I71N cRNA (right lane). The bar diagram depicts the relative Cx26 band intensity of the western blot. GAPDH served as loading control. B: The lower panels (bar diagram and three original blots) depict 5 independent western blots of Cx26 surface expression in oocytes injected with water (left lane), with Cx26 cRNA (middle lane) and with Cx26p.I71N (right lane) cRNA. The bar diagram shows the arithmetic means $\pm$ SEM ($n=5$) of the relative Cx26 band intensity of the western blots. * indicates significant difference from water-injected oocytes ($p < 0.05$, ANOVA), # indicates significant difference from Cx26 ($p < 0.05$, ANOVA).

Discussion

One of the first genes discovered to be contributing to the development of non-syndromic hearing impairment was *GJB2*, coding for connexin 26 (MIM 121011) with a plethora of mutations found until present [2]. The most frequent one in all populations investigated so far is a deletion of one of 6 guanine bases at the position 30-35 of the single coding exon of the gene (c.35delG) [7, 10]. In our pool of 151 individuals from Upper Egypt encompassing 97 families with recessive hearing impairment the allele frequency for c.35delG was 8.7% with a carrier frequency of 1.6%. While numerous studies of different Mediterranean populations and in central-European countries indicated a rather high variability of the deletion's prevalence (see table) the carrier frequency found in this study appears to present a value at the lower end of the list but is similar to the frequency reported by Megiud et al. [11] for 59 Egyptian patients with non-syndromic hearing impairment.

All our patients homozygous for the c.35delG mutation showed a bilateral severe to profound sensorineural hearing loss dating back to their birth. This result agrees with Salvinelli et al. [12] who reported profound hearing loss in 92.3% of such patients. Cryns et al. [13] also noted a significantly stronger phenotype in c.35delG homozygotes in comparison to c.35delG/non-35delG compound heterozygotes.

In contrast to the rather frequent c.35delG mutation the sample collection did not display any other known mutation with the exception of one family where a novel amino acid substitution (p.I71N) was found. This mutation (replacing non-polar Ile vs. polar Asn) is positioned in the first extracellular loop of connexin 26. Such extracellular loops of gap junctions have been discussed to be crucial

for the docking of two hemi-channels to generate a gap junction unit [14, 15]. In other studies, however, Cx26 mutations causing deafness were shown not to be restricted to any functional domain but to be distributed throughout the length of the protein [7, 16]. Therefore, a functional investigation was conducted for our mutation p.I71N showing a defective phenotype with a markedly reduced current in *Xenopus* oocytes after depolarization pointing to an impaired performance by the mutated protein. Reduced incorporation of the mutated Cx26 form into the cellular membrane is confirming this finding. Interestingly, most of the mutants (about 80%) analyzed by biochemical and electrophysiological methods were less active than the wild type Cx26 or completely inactive [17-21].

Further studies may reveal whether this new Cx26 mutation exerts an inhibitory effect on other connexins when heterohexamer connexons are formed, as was shown e.g. for wt Cx30 and Cx26 p.V37I or wtCx31 and Cx26 p.L90P [14]. Such data should aid in genetic diagnosis and counseling of patients providing evidence-based risk assessment. This approach is important since early diagnosis of recessive non-syndromic hearing impairment enhances the timely detection of affected newborns which, in turn, will allow a directed treatment to limit delayed development of speech and communication.

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