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OTOLOGY

Correlation between GJB2 mutations and audiological deficits: personal experience

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Abstract Mutations in GJB2 gene are the most common cause of genetic deafness. More than 100 mutations have been described. The aim of this work is to describe the personal experience in genetic hearing loss, investigating the audiological and genetical characteristics of Cx26 deafness and correlating genotype and phenotype. We performed audiological and genetical evaluation in 154 patients affected by non-syndromic deafness of different degree. All patients showed a bilateral symmetrical sensorineural hearing loss. From the genetical analysis 127 probands resulted as negatives while 27 as positives (51.8% homozygous for 35 delG, 14.8% compound heterozygosis and 33.3% single mutation); 7.5% of patients had a mild deafness, 37% moderate, 33.3% severe and 22.2% profound. The c.35 delG mutation was detected in 66.6% of patients. Three mutations were found in compound heterozygosis with 35 delG, six different single mutations already described, and a new mutation *S138G* were also found. Correlation between genotype and phenotype confirmed the high variability of hearing loss.

Keywords Deafness · Connexin 26 · Genotype · Phenotype · Mutation

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Introduction

Hearing loss is the most common sensory disorder. Approximately 60% of early-onset deafness is genetic, of which 70% is non-syndromic. Of non-syndromic deafness, 75–80% is inherited in an autosomal recessive pattern and 15–20% in an autosomal dominant pattern.

A high number of chromosomal loci and cloned genes have been found to be responsible for hearing loss. Molecular genetic tests are available for many types of syndromic and non-syndromic deafness, although often only on a research basis [1]. Because of this extreme heterogeneity, it could be assumed that genetic hearing loss is caused by a very large number of equally rare genetic types whose specific identification in individual cases would remain an arduous task.

Mutations in GJB2 gene which is located on chromosome 13q and encodes the protein Connexin 26 (Cx26), are responsible for nearly half of genetic cause in many populations [2–5]. The Cx26 is a member of a family of highly related gap-junction proteins that facilitate the exchange of ions, secondary messengers and small molecules. Six connexins assemble to form a half-channel or connexon, which docks with its counterpart in an adjacent cell to form a complete intercellular channel or gap-junction [6]. Connexins have four trans-membrane domains linked by one cytoplasm and two extracellular loops, with cytoplasm carboxy and amino-terminal ends. The presence and integrity of Cx26 gap junctions in the cochlea are believed to be essential for normal auditory function. The Cx26 has been identified along the outer and inner hair cells, the supporting cells, the spiral ligament, the stria vascularis and the basilar membrane [7]. In particular, it provides to the recycling potassium ions that flow into sensory hair cells as part of the transduction current [8].

Mutations of GJB2 can account for up to 50% of all the cases of non-syndromic hearing loss in many populations [9, 10]. In addition, there are a growing number of mutations in GJB2 associated with syndromic forms of deafness and skin manifestations [11]. At present several mutations in the gene (approximately 100) have been identified and these mutations can be inherited recessively or dominantly. However, the degree of hearing loss associated with all reported genotypes has not been clearly demonstrated. The c.35 delG mutation is the most common pathogenic allele in European and American Caucasian people, for whom a single origin has been demonstrated [12]. Its carrier frequency among hearing individuals from different Caucasian populations ranged from 1 to 4% [13]. The c.35 delG is a single-base deletion that results in a frame-shift at position 12 in the coding sequence and premature termination of the protein.

A truncating deletion mutation in the GJB6 gene, which codes for Connexin 30 (Cx30) and lies upstream from GJB2 on the long arm of chromosome 13 has been identified by del Castillo et al. [14]. Deletion of this gene does not directly affect the coding sequence of the GJB2 gene. Both Cx26 and Cx30 proteins are expressed in the inner ear. Compound heterozygosis involving mutations in both GJB2 and GJB6 results in hearing loss and demonstrates a phenotype similar to mutation homozygosis for GJB2 [7].

Previous observations on the audiogram of patients with GJB2 deafness have shown that they exhibit considerable variations of their hearing loss and the full range of phenotypic expression has not been well characterised. However, deafness may range from mild to profound; it can be prelingual or post-lingual, progressive or stable.

The aim of this study is to describe our personal experience in the evaluation of the genotype and the phenotype of subjects affected by different degree of deafness, in order to correlate mutations of GJB2 gene and audiological profiles.

Materials and methods

A total of 154 patients from unrelated families (81 females and 73 males), aged between 2 and 77 years (mean age 31 ± 20.44), affected by non-syndromic deafness, were included in this study. An informed consent was obtained from all participants or from the parents of patients younger than 18 years.

All the probands were initially evaluated for the presence of environmental causes (such as neonatal hyperbilirubinemia, ototoxic medication exposure, meningitis, and acoustic trauma). Syndromic forms of deafness were excluded from the study. Patients affected by middle ear and retrocochlear pathology were also excluded.

From Italy 137 probands came (29 from southern and 118 from central Italy) while 7 were foreign. In 109 patients hearing loss was inherited, in 44 sporadic, and only in 1 little patient was unknown, because the child was foreign and adopted. Deafness was diagnosed in children age (<15 years old) in 93 probands and in adult age (>15 years old) in 61 patients.

Audiological examination

Physical examination of the ears, audiometric tests and tympanograms were performed in all subjects.

Air conduction pure tone average thresholds at frequencies 0.5–1–2–4 kHz (PTA, 0.5–4 kHz) were calculated for each ear and were used to classify the hearing loss severity. Average thresholds ranging between 21 and 40 dB HL were defined as mild hearing loss, 41–70 dB HL as moderate, 71–95 as severe hearing loss, and >95 dB HL as profound hearing loss, as suggested by Liu et al. [15].

Genetic examination

A four-generation family history with attention to other relatives with hearing loss and associated findings was obtained. Documentation of relevant findings in relatives was accomplished through direct examination of those individuals or through review of their medical records, including audiograms.

Mutation analysis on DNA samples was performed by DHPLC screening followed by sequencing analysis to confirm and identify the nature of the mutation. The coding exon was divided into three fragments obtained by polymerase chain reaction with primers BF (5′-TCTTTTCCAG AGCAAACCGCC-3′) and AR (5′-CACGTGCATGGCCA GTAG-3′), 1F (5′GCCAGGCTGCAAGAACGTGT-3′) and 3R (5′-GGAGAAGCCGTCGTACATGA-3′), 2F (5′-TCA AGGGGGAGATAAAGAGT-3′) and BR (5′-TGAGCAC GGGTTGCCTCATC-3′). All PCR amplifications were performed with use of a Applied Biosystems GeneAmp PCR System 9700, according to the following program: 1 cycle of denaturation at 95°C for 10 min; 35 cycles of 95°C denaturation for 1 min, 56°C annealing for 1 min and 72°C extension for 1 min, and a final 1 cycle of extension at 72°C for 5 min. The fragments were analysed by DHPLC (WAVE system, Transgenomic, Crewe, UK); gradient conditions and oven temperatures were determined by the WAVE maker 4.0 software. Possible mutations were subsequently identified by sequencing with Big Dye Terminators as described by the manufacturer (Applera) and analysed on Abi Prism 3100 Avant (Applied Biosystems).

Values of age and PTA were expressed as mean ± standard deviation. The statistical tests used were

paired and unpaired Student's *t* test. Significance was set for $p < 0.05$.

Results

All patients showed a bilateral symmetrical sensorineural hearing loss of different degree. Mean PTA obtained from all patients was 64 ± 29.1 dB HL.

At the genetical evaluation 127 probands resulted negatives (82.47%) while 27 (17.53%) presented modifications of the GJB2 gene.

Patients resulted negatives at the genetic examination were divided in four groups; demographic and audiological data are reported in Table 1.

78.75% of patients presented a mild or moderate deafness. No statistical differences were demonstrated comparing age or sex in four groups. About the origin of patients 77.95% came from central Italy and this difference was present in each group. About the etiology we showed that 74.8% of patients presented a familiar deafness, only in the group of profound hearing loss sporadic and familiar deafness were similar. Finally, age of diagnosis was different among the groups; only in the mild hearing loss group diagnosis was done in adulthood while in the other groups, in childhood.

The data of patients that resulted positive in the molecular genetic analysis are reported in Table 2. The classification of these subjects is the same reported for negative patients, and it is relative to the entity of deafness: mild, moderate, severe and profound [15].

The mean age of the patients is 23 ± 18 years and ratio between males and females was 10:17. Nineteen patients (70.4%) came from central Italy, six (22.2%) from southern, and two (7.4%) were foreign. The mean PTA was 76 ± 29.2 dB HL. Finally, the etiology was similar between inherited and sporadic deafness whereas most of the diagnosis was established in childhood: 23 patients (85%).

In the mild hearing loss group we observed two patients coming from central Italy, with a genetical pattern of heterozygosis for GJB2 gene. Patient #1 had sporadic deafness diagnosed in childhood and presented a mutation defined as *c.457G > A*; on the contrary the patient # 2 was affected by deafness inherited in autosomal dominant pattern, diagnosed in adulthood and presented a different mutation of GJB2.

In the moderate hearing loss group we observed ten patients (37%), 2 males and 8 females, 8 coming from central Italy; in three patients a sporadic hearing loss was diagnosed in adulthood. Only two subjects had an inherited autosomal recessive deafness and presented at the molecular analysis two different genotypes: a homozygosis for *35 delG* and a compound heterozygosis *35 delG + c.551 C > G*. The remaining patients affected by sporadic deafness presented a single different mutation in heterozygosis, except a patient presenting a double heterozygosis (*35 delG + G167delT*) and another one with a homozygosis for 35 delG. It is interesting to note that mutation c.457G > A was present both in patient #1 (group mild) and #6 (group moderate). Finally, mutation c.412 A > G (#5) is not reported in literature.

In the group of patients affected by severe hearing loss we found nine patients (33.3%), 5 males and 4 females; five patients came from central Italy, three from southern Italy, and one patient from abroad. Deafness was diagnosed in all patients in childhood. Three did not refer any familiarity while five showed a recessive, and only one a dominant pattern of transmission. From the molecular genetic analysis we identified in six patients a homozygo-

Table 1 Results of negative patients at the genetic evaluation

Group	Number of patients (%)	Age	Sex	Origin			Diagnosis		Genetic		PTA
				C	S	FO	Ch	A	F	Sp	
Mild	40 (31.5)	34 ± 17	16/24	31	8	1	13	27	32	8	32 ± 7
Moderate	60 (47.25)	35 ± 22	29/31	47	10	3	35	25	47	13	55 ± 9
Severe	15 (11.8)	29 ± 19	7/8	11	4	0	13	2	11	4	82 ± 7
Profound	12 (9.45)	26 ± 21	11/1	10	1	1	9	3	5[a]	6[a]	>115

Age of patients was expressed as mean ± SD

Sex of patients was expressed as male/female ratio

Origin was expressed as following: *C* centre of Italy, *S* southernern Italy, *FO* foreign

Age of diagnosis was expressed as *Ch* child (<15 years old), or *A* adult

Genetic: patients were divided as *F* familiar deafness and *Sp* sporadic deafness

PTA was expressed as mean ± SD

[a] One patients of this group cannot refer if deafness was familiar or sporadic because was adopted

Table 2 Results of patients postives at the genetic evaluation

Patient	Age	Sex	Origin	Etiology	Genetic	Diagnosis	PTA	Molecular genetic
Mild deafness								
#1	9	M	Centre Italy	Sporadic		Child	25.00	Heterozygosis c.457g > a
#2	50	F	Centre Italy	Familiar	Dominant	Adult	29.3	Heterozygosis c.269 T > C
Moderate deafness								
#3	46	F	Centre Italy	Sporadic		Adult	45.30	Heterozygosis c.249 C > G
#4	36	F	Foreign	Sporadic		Adult	48.12	Heterozygosis c.109 G > A
#5	19	M	Centre Italy	Sporadic		Child	48.10	Heterozygosis c.412 A > G
#6	30	F	Southern Italy	Sporadic		Child	50.00	Heterozygosis c.457G > A
#7	71	F	Centre Italy	Sporadic		Adult	50.40	Heterozygosis c.101 T > C
#8	8	F	Centre Italy	Sporadic		Child	50.31	Heterozygosis c.101 T > C
#9	5	F	Centre Italy	Familiar	Recessive	Child	45.00	Homozygosis 35 delG
#10	18	M	Centre Italy	Sporadic		Child	68.12	Homozygosis 35 delG
#11	9	F	Centre Italy	Sporadic		Child	60.00	Heterozygosis 35 delG + G167delT
#12	23	F	Centre Italy	Familiar	Recessive	Child	53.00	Heterozygosis 35 delG + c.551 C > G
Severe deafness								
#13	4	M	Centre Italy	Familiar	Recessive	Child	80.00	Homozygosis 35 delG
#14	52	M	Southern Italy	Familiar	Recessive	Child	76.25	Homozygosis 35 delG
#15	7	M	Centre Italy	Familiar	Recessive	Child	75.00	Homozygosis 35 delG
#16	4	F	Centre Italy	Familiar	Recessive	Child	74.37	Homozygosis 35 delG
#17	7	F	Southern Italy	Familiar	Recessive	Child	95.00	Homozygosis 35 delG
#18	24	F	Centre Italy	Familiar	Dominant	Child	90.00	Homozygosis 35 delG
#19	6	M	Southern Italy	Sporadic		Child	80.00	Heterozygosis 35 delG + 358 – 360delGAG
#20	38	M	Centre Italy	Sporadic		Child	94.68	Heterozygosis 35 delG + c.551 C > G
#21	10	F	Foreign	Sporadic		Child	93.75	Heterozygosis c.380 G > A
Profound deafness								
#22	34	F	Southern Italy	Familiar	Recessive	Child	120.00	Homozygosis 35 delG
#23	3	F	Southern Italy	Familiar		Child	120.00	Homozygosis 35 delG
#24	22	F	Centre Italy	Sporadic		Child	105.00	Homozygosis 35 delG
#25	40	F	Centre Italy	Familiar	Recessive	Child	120.00	Homozygosis 35 delG
#26	7	M	Centre Italy	Familiar	Recessive	Child	95.00	Homozygosis 35 delG
#27	43	M	Centre Italy	Familiar	Recessive	Child	120.00	Homozygosis 35 delG

sis for *35 delG*, in two with sporadic deafness a compound heterozygosis *35 delG + 358 – 360delGAG* and *35 delG + c.551 C > G*; finally, in one patient with sporadic deafness a single heterozygosis *c.380 G > A*. It can be noted note that the same compound heterozygosis was found in patients #12 and #20, both native from central Italy and in which deafness was diagnosed in childhood. The first one was a female affected by an inherited recessive moderate deafness; the last one was a male with a sporadic profound hearing loss.

Profound hearing loss was detected in six (22.2%) patients, 2 males and 4 females. In this group four patients came from Centre and two from Southern Italy. Two subjects showed a sporadic and four a familiar recessive deafness. Homozygosis for *35 delG* occurred in all patients.

Discussion

The findings of the present study provide evidence that different mutations in GJB2 gene cause deafness, but the degree of hearing loss has a high variability.

In this work we found that 17.53% of patient presents modifications of GJB2 gene (51.8% homozygous for 35 delG, 14.8% compound heterozygosis and 33.3% single mutation). Comparison of our data with those of Liu et al. [15] shows only little differences. As they demonstrated that 19.3% of Chinese patients presented a GJB2 mutation (68.8% homozygous for 35 delG, 3.9% compound heterozygosis and 20% single mutation).

In particular, we identified 11 different mutations of GJB2 gene.

The c.35 delG mutation was detected in 66.6% of patients with mutation in GJB2. As described in literature hearing impairment associated with homozygous c.35 delG mutation can be of different degree from moderate to profound [16–18].

About our four cases presenting compound heterozygosis with 35 delG, in patient #11 we observed a truncating frame-shift mutation *G167delT*, first described by Zelante et al. in 1997 [5], highly common in Ashkenazi Jews with a carrier frequency of 4% [19]. In two subjects (#12, #20) a non-truncating mutation called *c.551 C > G* (namely R184P) occurred in combination with 35 delG. This mutation leads to the loss of an Arginine with a substitution of a Proline in position 184. Gualandi et al. (2004) [20] described the same compound heterozygosis in a patient with a profound familiar dominant deafness. In our experience hearing loss can be inherited by a recessive modality or sporadic, and according to Rikkert et al. (2005) [21] the deafness can be different. Patient #19 presented a heterozygosis *35 delG + 358 – 360delGAG* (also called delE120). It is a non-truncating in-frame deletion, in a short repeated sequence which should lead to the loss of a glutamic acid at position 120 of GJB2 [10, 16].

Regarding single mutations detected in this work, we described in patient #5 a new mutation called *c.412 A > G* (*S138G*). Sequencing of the DNA revealed a heterozygous with a change Alanine > Guanine at nucleotide 412. The patient was an Italian young man who presented a moderate deafness and in which audiological examination of the parents was normal. A similar, non-truncating mutation, called *S138 N*, was described by Rikkert et al. [21] in one patient. We suppose that our new mutation cannot be considered a polymorphism because we have analysed a great number of chromosomes in this study.

Patients #1 and #6 showed the same mutation (*c.457G > A*), on the contrary they presented a different degree of deafness, as described recently by Dalamón et al. [22]. Also patients #7 and #8 had the same mutation but in this case the sporadic hearing loss was similar. *M34T* (also called *c.101 T > C*) is a missense, non-truncating mutation, located in transmembrane domain [10], and its role in non-syndromic hearing loss was largely debated: it could be considered an autosomal dominant mutation [3] (as in our patient) or a polymorphism only [23]. In this regard, Gualandi et al. (2004) [20] described a single heterozygosis in a patient with a profound familiar recessive deafness.

The missense non-truncating mutation *L90P*, also called *c.269 T > C*, showed in subjects #2, was first described by Denoyelle et al. in 1999 [16] and the changes are located in trasmembrane domain. Also Gualandi et al. in 2004 [20] described a heterozygosis in a patient with a severe sporadic deafness; in our experience a mild hearing loss was inherited by a dominant modality.

The *F83L* mutation (or *c.249 C > G*) detected in patient #3 was first described by Scott et al. in 1998 [24]. Later on Rabionet et al. [10] found this single mutation in a patient with a congenital deafness; in contrast to this evidence our patient has a sporadic hearing loss that appeared in adult age.

The *W24X* mutation (or *c.109 G > A*), detected in a foreign patient with a sporadic moderate hearing loss (#4), is a truncating non-sense mutation, already detected in compound heterozygosis and homozygosis, resulting in conversion of a valine to an isoleucine, at codon 24 with a premature stop codon [3, 9]. This mutation, first described by Kelsell et al. in 1997 [3] in a Pakistani family affected by profound deafness, occurs in the first trans-membrane domain of Cx26.

Finally, *R127H* mutationt (*c.380 G > A*) of our little foreign girl #21, affected by a sporadic severe deafness, is a non-truncating mutation first described by Estivill et al. in 1998 [25]. It lies in the cytoplasmatic loop involved in pH gating even if it is not clearly related to hearing loss.

On the basis of our experience we suggest that testing of GJB2 plays a prominent role in diagnosis and genetic counseling; in fact mutations in this gene account for a great number of non-syndromic deafness of various degrees in many world populations. Moreover, patients with unknown causes of severe or profound hearing loss should be routinely tested for GJB2 mutations, but individuals with lesser degrees of hearing loss should not be precluded from testing.

The other finding of this study is that genotype–phenotype correlation in GJB2 deafness should be cautiously interpreted during clinical genetic counseling of patients, due to variability of hearing loss with the same genotypes. The characteristics of audiological profiles of GJB2-related deafness are important for the prevision of prognostic information and genetic counseling of affected and at-risk individuals. On the other hand, future research to uncover the basis of this variation is necessary to provide better counselling to families with GJB2-related hearing loss.

In this context, it can be argued that management of hereditary hearing loss must be performed by a team that includes an otolaryngologist, with expertise in the management of audiological disorders, and a clinical geneticist.

In conclusion, a definitive clinical description of the type, degree, and nature of the hearing associated with abnormal Cx26 expression remains undefined. It could be theorised that the variable clinical presentation may be attributed in part to ethnic diversity in the expression of Cx26 defects, in part to co-existing gene mutations or genetic modifiers. The clinical phenotype is further complicated by the role of environmental factors, which may be important influencing the degree of deafness.

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