Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

Undescribed *GJB2* c.35dupG homozygous prelingual distinguished from c.35delG homozygous/compound heterozygous deafs, dwelling a German ancestry Venezuelan isolate

Sergio Arias[1], Irene Paradisi[1*], Alba Hernández[1] and Daniela Kanzler[2]

Abstract

Background: Among ten hearing-impaired (HI) families mostly of German descent dwelling the Venezuelan isolate Colonia Tovar, which were initially studied several decades ago to assess the etiology of their profound/prelingual nonsyndromic deafness phenotype, an undescribed genotype/phenotype was found. Forty-eight subjects, including 8 of the still living 143 originally searched with audiograms 4 decades ago, were retested and their DNA collected. A genomic search of 27 loci involved in HI was performed on a randomly chosen prelingual deaf patient. Subsequently, *GJB2* sequencing was performed in all subjects from each pedigree. Haplotypes were constructed with five intragenic *GJB2* SNPs (rs117685390, rs7994748, rs2274084, rs2274083, and rs3751385). Audiograms performed along 5 decades were compared to evaluate age-related hearing loss in the different genotypes found in the population.

Results: Three prelingual deaf siblings, having the highest recorded symmetrical hearing loss of all the known affected in the isolate, carried the very rare mutation c.35dupG (p.V13Cfs*35) at *GJB2* in a homozygous condition. Two additional *GJB2* mutations were identified (p.W77R and c.35delG) in the isolate. Allelic disequilibrium in both c.35dupG and p.W77R carriers (with in-phase haplotype T;T;G;A;C) were found, although not so in the 2 other found c.35delG independent haplotypes. A compound heterozygote in trans (c.35delG/c.35dupG) was audiometrically distinguishable from both the c.35dupG and c.35delG homozygotes.

Conclusions: A relatively higher frequency of mutation of c.35dupG found than elsewhere was retrospectively inferred for the ancient population of the Kaiserstuhl region in Germany, having an opposite epidemiological situation to the one found with the contiguous and very frequent c.35delG. Haplotype analysis suggests founder phenomena and independent occurrence, hundreds of generations back in Caucasoid populations for both mutations.

Keywords: Nonsyndromic deafness, *GJB2* haplotypes, Connexin 26, DFNB1, Diagnostic audiograms

* Correspondence: ireneparadisi@hotmail.com; iparadis@ivic.gob.ve
[1]Laboratory of Human Genetics, Venezuelan Institute for Scientific Research (IVIC), Caracas, Venezuela
Full list of author information is available at the end of the article

Background

A large proportion of human-inherited nonsyndromic profound deafs presenting a DFNB1 phenotype carry mutations at the *GJB2* locus. Those are usually displayed in a recessive prelingual subject either homozygote or compound heterozygote, ordinarily with purported, "normal-hearing" parents. Up to now, published audiometric information of apparently normal-hearing parents of many prelingual deafs is scarce. One of a few exceptions was the situation described in the Colonia Tovar German isolate in northern Venezuela, in which always one or both parents of profound deaf subjects evidenced a high tonal hearing loss emerging with ageing. No examples were initially found of any apparent direct transmission of the deafness phenotype among 9 extended families with hearing losses, when they were studied *in situ* almost 5 decades ago [1]. Originally, they were considered as displaying a single autosomal dominant phenotype; however, at that time the cloning of the *GJB2* (=*CX26*) locus [2]), its chromosomal location [3] and awareness over its consistent etiopathogenic role [4, 5] in most nonsyndromic recessive deafness had not been accomplished. With ageing, some patients, mainly those with dominant phenotypes [6], frequently display a higher hearing threshold. Their heterozygous parents also had revealed a significant degree of presbycusis along the highest pitches [1].

In Caucasoid European/Eurasian populations, almost all mutations (~ 99%) with nonsyndromic recessive phenotypes occurring in codon 12 of exon 2 at *GJB2* are restricted to a deletion of one guanine (c.35delG = p.G12Vfs*2 = rs80338939) in the 13q11 GGGGGG stretch. Only in a mean 0.13% within a large cohort of European Caucasoid samples of profound nonsyndromic deafs from diverse genetic origins [7]; or in 0.04% from another large mainly North American cohort [8]; or in 0.5% in the Volga-Ural region of Russia [9]; the hearing impairment (HI) was due to the compound genotype in trans [deletion G/duplication G] at codons 12/13. No homozygosity for mutation c.35dupG (p.V13Cfs*35 = rs398123814; ClinVar VCV000094392.10) however, has ever been recorded either in Caucasoids nor in a large Mongoloid polyethnic Chinese sample in which it is also present [10]. In the latter, among the mentioned compound heterozygotes in trans, the accompanying mutation occurs mostly outside codons 12/13 in 0.05–1.2% of patients, as also occuring in the Ashanti province of Ghana in only one subject (0.14%) [11] being this a unique instance of African ethnicity recorded. The c.35dupG mutation has been occasionally detected as an eventual single mutant allele among 524 Mongolian recessive nonsyndromic prelingual deafs [12] and either as a single allele or as a compound heterozygote in several instances among the Chinese Han ethnic subgroups [13]. Besides it has never been quoted in comprehensive reviews on connexin 26 hearing deficits [14–18]. Contrarily in 3 prelingual deaf siblings with exclusive German ancestry in the isolate studied anew by us, the etiology for that deafness was homozygosis for the mutation originally described as c.35insG in a compound heterozygous deaf [19]. The low frequency everywhere of such change, with its former name now corrected to c.35dupG [20], explains why up to this day there had been a lack of documented discrimination of their heterozygous parents; but actually, some presbycusis in several obligate heterozygotes can be discussed with this new information, besides that the extreme profound deafness of its homozygotes can be phenotypically contrasted with the one in the compound heterozygote. The partial hearing deficit in some heterozygotes of the surveyed population older than 40 years of age eventually might reveal a late expression of the c.35dupG mutation, added to a founder phenomenon within the isolate which contains different mutations at the same locus. This situation renders an exceptional opportunity for delineating phenotypes of the mutant homozygous and of the simple or compound heterozygous carriers of those mutations. Incidentally the severe/profound compound heterozygous deaf was found to be phenotypically differentiable from both ethnically German polar homozygotes, for the rare c.35dupG and for the usually frequent Caucasoid c.35delG mutation. The ancestral origin of c.35dupG so far has not been explored; despite, it could have been so in geographically specific instances within some populations. Nonsyndromic profound deaf patients in the isolate have shown a wide genetic heterogeneity excluding digenic genotypes with any *GJB2* mutations.

Methods

Population and samples

Colonia Tovar is a rural town surrounded by 2 dozen tiny satellite house clusters with specific names each, within an area of 490 km^2, in the coastal mountainous range of northern central Venezuela (67°20' W; 10°10' N), established by German immigrants who travelled there to settle, stimulated by a dual invitation in 1840 from both the Venezuelan national congress and the country government. The enterprise had the intention of developing agriculture in a large extension at 1700–1900 m upon sea level, located among wild forest lands, under a very mild climate, in terrains which had been previously donated for such purpose on behalf of the young republic, by its proprietor, the patrician Manuel Felipe de Tovar. They settled there on April 6th 1843, coming directly from the Baden-Württemberg state in southwestern Germany, after leaving Europe from the harbor of Le Havre in France [21]. A second much smaller group (4%) arrived later between 1852 and 1862, from

more northern states (Hessen, Rheinland, and West Pomerania) plus additional few other independent individuals coming from France, Italy, and Switzerland, who joined the previous immigrants [1, 22]. Most of them stayed in their newly donated lands under considerable geographic and genetic isolation for about 100 years, and have remained there until today, being now however a prosperous, compact population dwelling the Tovar County (= Municipio) which by 2011 had 14,100+ mostly agricultural inhabitants, forming socially and geographically a very well communicated and stable community with a high living standard for any rural population.

Out of a total of 378+ persons who arrived in up to 1862, only 92 contributed to the population with offspring, and their descendants are recognizable today as such by the external phenotype, ancestry assessed back up to 1780 (shown in Fig. 1), and by their persisting German surnames. The ancestors of the presently deaf-prone mutation-carrier families came in decreasing numbers from the towns Wyhl, Endingen, Wasenweiler, Herbolzheim, Erfurtshausen, Oberbergen, and Forchheim and from a few other villages mostly in the Kaiserstuhl region, located to the North of Freiburg im Breisgau, within the limits of the lower left quadrant (III) (shown in Fig. 2) of a geographic focus (i.e., a 55-km diameter circle, with center at Wallburg (48°16'N; 7°51' E)) [1, 23, 24], and further in another concentric one of 22.5-km diameter with its center between Endingen and Forchheim (48°9' N ; 7°43' E). Two thirds of the ancestors of both or at least one of the present HI subjects were born within that quadrant, having as their nearest towns boundaries Ringsheim to the North, Wyhl in the West and Wasenweiler and Hugstetten in the South (shown in Fig. 2). Thus, in less than 398 km^2 eastbound the Rhine river within that ~ 594 km^2 quadrant, there is *a priori* a higher likelihood for the mutation to be present, since 70% of the prelingual deaf ancestors came from that area in the Baden-Württemberg state (Table 1; Fig. 2).

Among 9 extended nuclear families of the isolate (n = 454 persons) containing at least one prelingual deaf subject each first studied in 1970–1972 [1], most of their still-available members were re-examined. Complete genealogies were assessed through direct interview of all families with affected subjects [1] and a further genomic search of 27 loci involved in HI was performed through a public specialized service [25] on a randomly chosen

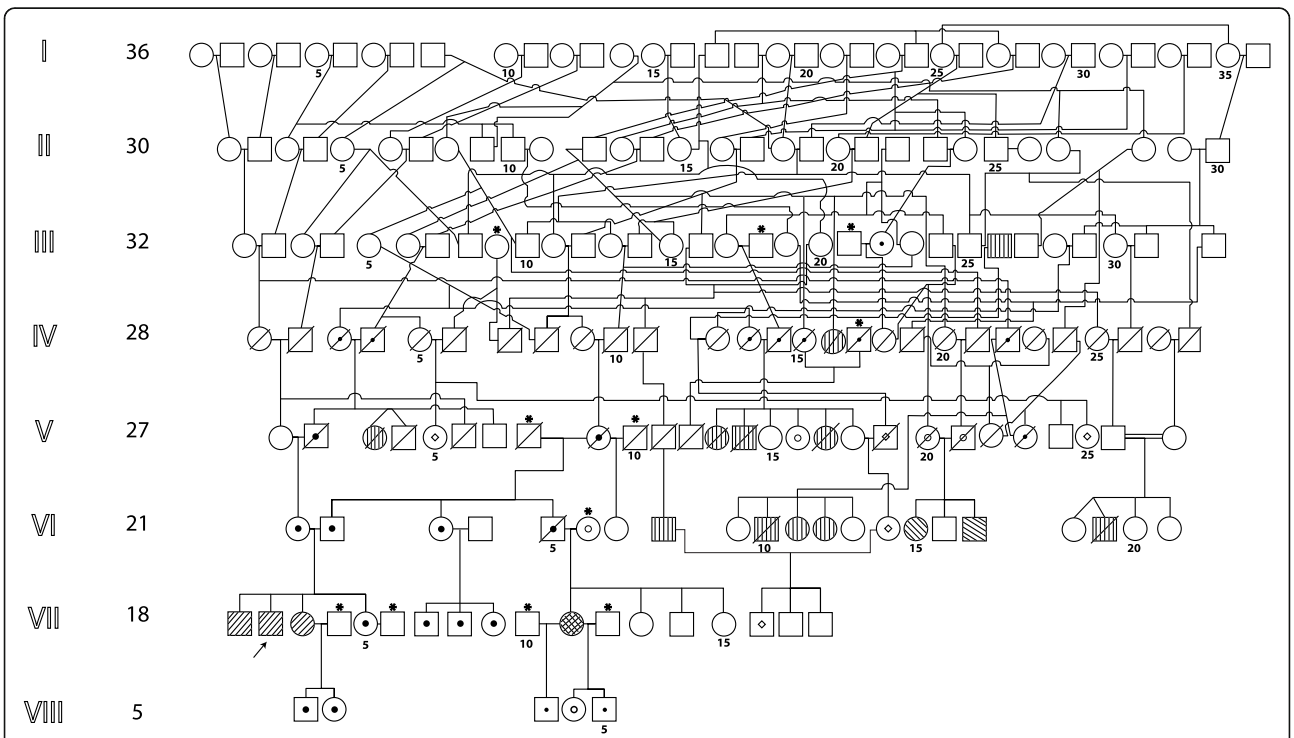

Fig. 1 Extended genealogy of the nuclear families in the isolate, from years 1862 to 2019. Abridged pedigree of 200+ kinship-related (from 9 original nuclear families) prelingual/profound deaf subjects figured as *vertically* hatched = those with any unidentified specific etiology; mutant *GJB2* homozygotes are either, if *left-bent* = c.35dupG; if *right-bent* = c.35delG; if *crisscrossed* = compound heterozygous c.35dupG/c.35delG. *Large* inner central dot = filled: obligate c.35dupG heterozygotes; hollow: suspected/tested c.35delG heterozygotes. *Small* inner central dot = obligate heterozygotes. *Hollow* inner central rhomb = obligate p.W77R (p.Trp77Arg) carriers. *Subject with no German ancestry. All persons from generation IV and upwards were deceased. From generation V on, all living individuals were DNA tested

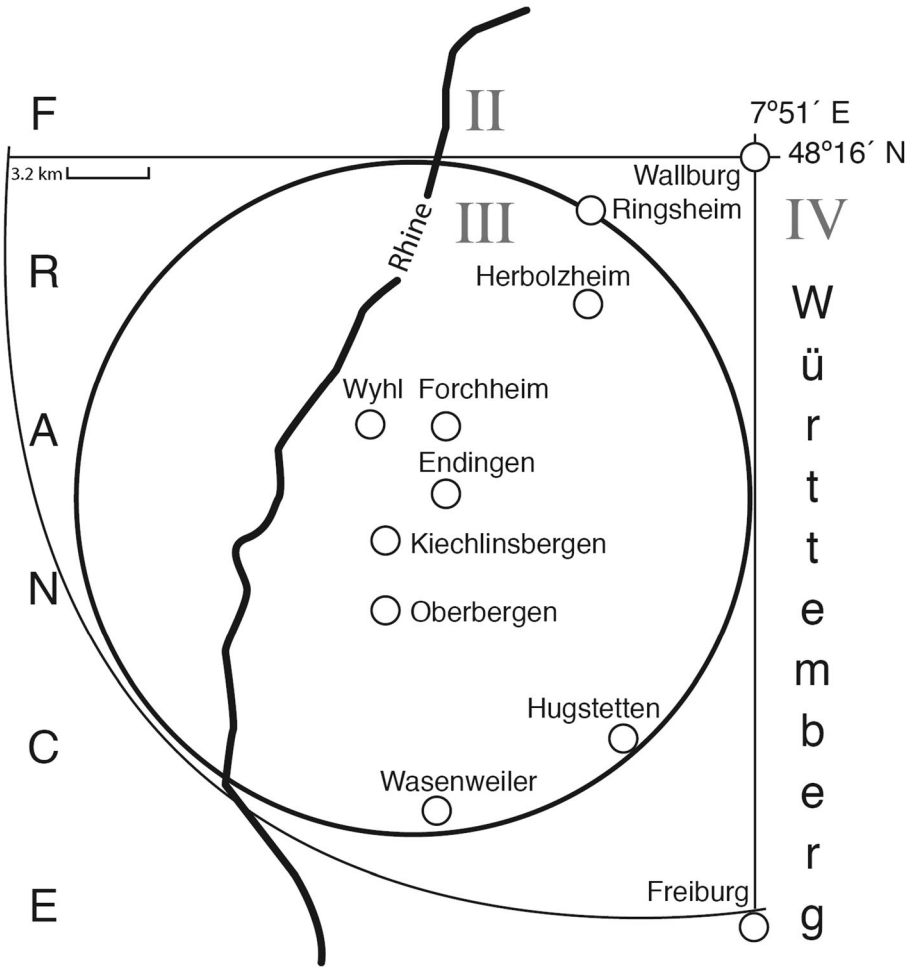

Fig. 2 Kaiserstuhl region eastward the Rhine river in Württemberg state, Germany. Kaiserstuhl region geographic focus III quadrant centered in Wallburg to emphasize the clustering in places of birth within a smaller circular area, for most ancestors of c.35dupG carriers

prelingual deaf patient VII-2 (Fig. 1) in a multiplex sibship whose maternal grandmother V-1 was the consultand for this large pedigree. Written informed consent was voluntarily obtained according to the ethical institutional guidelines and to the ethical standards of the Helsinki Declaration, from all members in studied families with deaf subjects in the isolate, whose blood samples were to be subsequently drawn and new audiometries to be performed; all of whom received audiometric, genetic reports and counseling on deafness.

From the records of unrelated exogamous HI patients and relatives of persons (n = 459) with audiometries formerly performed between 1994 and 1999 at 3 otolaryngologic outpatient clinics from hospitals Domingo Luciani, Carlos J. Arvelo, and Centro Urológico San Román in Caracas, a merged sample was gathered to serve as control.

Families with hearing impairment

One (P-10, in [1]) out of the 10 independently ascertained pedigrees with hearing loss but without any prelingual deafness originally recorded, was used as another control for challenging any eventual phenotypic mutational dosage effect.

All 10 nuclear families proved to be genealogically related, and thus, 190+ subjects belonged to a single largely extended pedigree of 200+ members with both ascertainable kinship and a traceable geographic ancestral origin; of those, only 48 could be contacted anew mostly from year 2014 to 2018 (shown in Fig. 1). Two (P-3, and P-10, in [1]) of the original nuclear families were marginally included in Fig. 1, since several of their ancestors and descendants were common to other ones in different families already included in it. No new relevant genetic information would have been added by showing the complete family data in an overloaded picture, despite it having been previously much abridged for a clearer graphic presentation. None of the now ascertained and proven c.35dupG carriers (V-2, V-9, VI-1, VI-2, VI-3, and VI-5 and their offspring) had been formerly illustrated within P-7 [1] except for the almost-

Table 1 Place of birth in Germany of Colonia Tovar forebears of prelingual deafs

Generation & subject code number	Place of birth of immigrant ancestors 1783 to < 1852										Average year of birth[a]	*N*
	W	**E1**	**W1**	**H**	**E3**	**O**	**F**	**S1**	**Z**	**Others**		
I-1,2								+			**I** 1806 (1792–1828)	36
3,4									+			
6,10,13, 25,26	+											
7,8					+							
9,17,24, 27,28		+										
11,15,16,29,30			+									
12,18,23,35										+		
14,19,20				+								
33,34							+					
36						+						
II-4					+						**II** 1840 (1825–1861)	28
7, 8, 9, 13, 24, 28	+											
14, 23				+								
15, 25, 29										+		
30						+						
Totals												64
n	11	5	5	5	3	2	2	2	2	7		
%	25	11	11	11	7	4.5	4.5	4.5	4.5	16		

Bold = outside of Württemberg state. *Cursive* = outside the Württemberg focus III quadrant in Fig. 2. W = Wyhl, E1 = Endingen, W1 = Wasenweiler, H = Herbolzheim, E3 = Erfurtshausen, O = Oberbergen, F = Forchheim, S1 = Schweich, Z = Zierenberg, Others = *Gräfenhausen*, Ringsheim, *Oberalpfen,* Hugstetten, Munchweier

[a] Range of year of birth of founders, by generation

normal-hearing obligate c.35dupG carrier V-2, since by 1972, when the family was initially studied, the presently prelingual deafs in that pedigree were not yet been born.

Hearing function

After 1970 and lately from year 2000 up to 2002, pure tone audiometry (250 to 8000 Hz or 250 to 4000 Hz for air and bone conduction, respectively) was performed in the field with portable audiometer Amplaid® 200 (Italy) under suitable conditions [1], on all available family members—many of them previously studied in several occasions—and from year 2014, with the audiometer Maico® MA 41 (Germany). The data from the control and the HI family subjects were divided into 5-year-age cohorts of non prelingual individuals, who later were merged for a clearer presentation into 3 age groups (from n = 459), chosen accordingly to their degree of loss (mild, moderate, severe) and compared with 2 age groups from those of the isolate (n = 454) in the years 1970–1972, 2000–2002, and 2014–2016, to assess their progressive hearing loss, if any. A high-pitch hearing loss was defined as any ≥ 25-dB deficit at whatever age starting at 2 kHz and up to 8 kHz, compared with the normal hearing threshold range, presenting any value at that frequency in one or in both ears. The average threshold increment in decibels and its range were estimated for frequencies ≥ 2 kHz if it was ≥ 25 dB at each frequency; in both controls and subjects of the isolate, for comparisons among the latter between those c.35dupG carriers in the one hand and alternate *GJB2* mutant heterozygotes on the other; and for documenting eventual differences in previously estimated regression equations, regarding age at onset of any audiometric loss.

Eight subjects out of 143 members from families with HI audiometrically tested during 1970–1972 [1], plus 40 additional ones from year 2000 to year 2018, were retested for audiometry, and 38 samples of blood were drawn, plus 10 others of saliva, being those whose results conform the core of this study.

DNA analyses and haplotypes

Along years 2000 to 2018, 5 ml of venous blood were drawn, anticoagulated with EDTA and the DNA extracted within 12 h with a hypertonic saline method as published [26]. In 10 cases, saliva was collected instead, immediately frozen at − 20 °C, and processed afterwards within the next 1 to 3 weeks using the proteinase K-extracting procedure. In all subjects from each pedigree, a Sanger sequencing of the *GJB2* locus was done for the codifying region (exon 2) at Macrogen Service, Seoul, Korea, with primers designed with the Primer3 program

(primer3_www.cgi v 0.20), which sequences are available on request. In those deaf subjects not revealing any *GJB2* mutation in exon 2, the *GJB6* locus and the microsatellites [27] were additionally explored.

Chosen intragenic SNPs rs117685390 (NG_008358.1: g.4806T>C), rs7994748 (NM_004004.5:c.-23+792C>T), rs2274084 (NG_008358.1:g.8473G>A; NM_004004.5: c.79G>A; XP_011533351.1:p.Val27Ile), rs2274083 (NG_008358.1:g.8735A>G; NM_004004.5:c.341A>G; XP_011533351.1:p.Glu114Gly), and rs3751385 (NM_004004.5:c.*84T>C, NG_008358.1: g.9159T>C) were searched for assessing kinship, in 3 of them with the appropriate restriction enzymes (New England BioLabs, MA, USA) *BstN* I (g.4806T>C), *Msc* I (c.79G>A), and *Nhe* I (c.*84T>C). After digestion and electrophoresis in 8% polyacrylamide (acrylamide-bisacrylamide 29:1) gels, the fragments were revealed with silver nitrate and the findings confirmed, through Sanger sequencing in both directions.

Results

Vestibular symptoms were neither ascertained, nor referred to by the patients in any of the nonsyndromic HI or deaf examined persons.

Detected mutations and their in-phase haplotypes

c.35dupG

All identified c.35dupG/c.35dupG homozygotes (VII-1, VII-2, VII-3 in Fig. 1) belong to a single sibship which displayed prelingual deafness with a symmetrical ≥ 100-dB threshold at all frequencies. Their parents (VI-1, 2) could eventually reveal a nonzero kinship coefficient (0.004≥ Φij ≥ 0.001), but its actual value was hardly ascertainable. All of their remote and genetically pertinent ancestors along one or both parental lines however, had their origin in the Kaiserstuhl region, located mainly in Wyhl, Endingen, and Wasenweiler (shown in Fig. 2; Table 1). The maternal grandfather (V-2) and the paternal grandmother (V-9) of those homozygotes were the obligate carriers of c.35dupG since V-1 the consultand in this extended pedigree is a still-living and molecularly tested noncarrier with presbycusis, and V-9 was a DNA-tested carrier who had several offspring carriers (VI-2, 3, 5).The nontested father V-8 of the latter was not of German ancestry. VII-5 and VIII-1, 2 were heterozygotes under 30 years of age, all with normal audiograms, and VII-11 is a normal-speaking but c.35dupG/c.35delG compound heterozygote with an asymmetrical 80/120-dB severe/profound binaural threshold phenotype (Fig. 3), whose c.35delG carrier and audiometrically normal mother (VI-6) is not of German ancestry. Either obligate c.35dupG or c.35delG normal-speaking carriers (VIII-3, 5) could not be audiometrically tested. Among 8 c.35dupG carriers older than 40 years of age who showed some audiometric loss, only VI-1 and VII-7 had it higher than 40 dB, at frequencies above 4 kHz; but 9 carriers younger or older than 25 years old (y.o.) did not show any. Therefore, it might be doubtful if the mutation is responsible for the loss in VI-1 and VII-7.

T;T;G;A;C bases in the in-phase (cis) 5-polymorphism haplotypes for SNPs rs117685390 (T>C), rs7994748 (C>T), rs2274084 (G>A), rs2274083 (A>G), and rs3751385 (T>C) were always present in that stated order in all c.35dupG-carrier chromosomes; the remaining out-of-phase chromosome haplotypes in simple heterozygotes as well were T;C;G;A;C and also T;T;

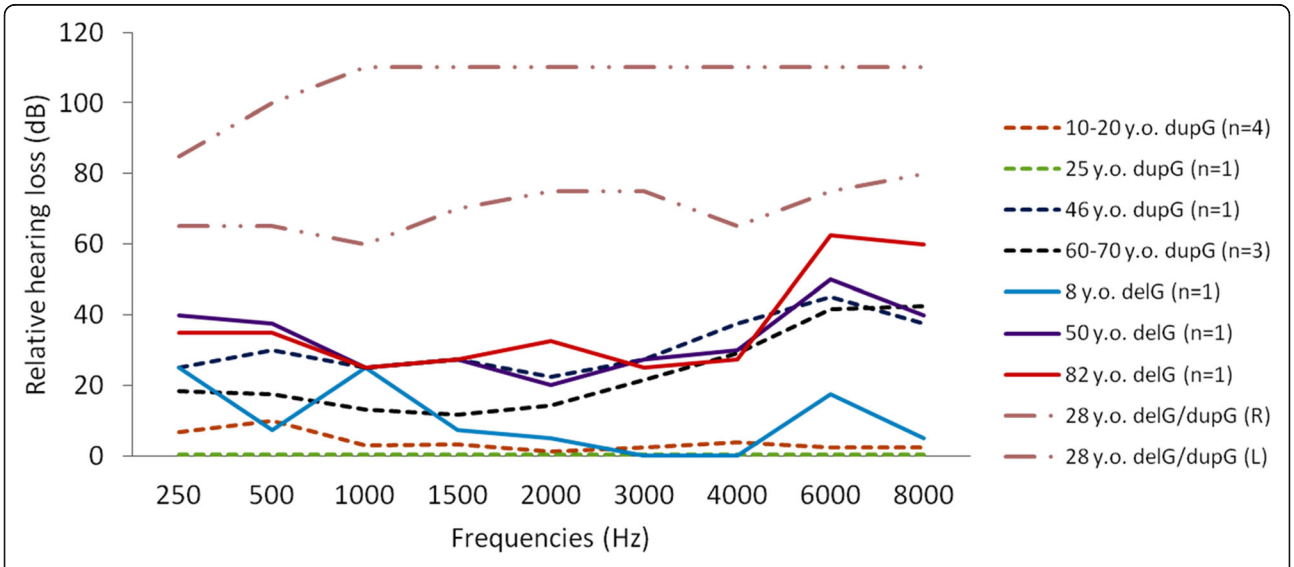

Fig. 3 Air conduction audiograms in heterozygous carriers of codons 12/13 *GJB2* mutations at various ages. Line = interrupted: c.35dupG; continuous: c.35delG. Compound heterozygous c.35dupG/c.35delG: *L*, left ear (upper); *R*, right ear (lower)

G;A;C, as shown in Table 3, without any noticeable audiometric difference between them.

c.35delG

In the isolate, the only ascertained prelingual deaf c.35delG/c.35delG homozygotes were siblings VI-15 and VI-17, descendants of ancestors I-18, I-19, I-23, and I-24 who did not reveal any close/ascertainable genealogical kinship between each other, despite all were born in the Kaiserstuhl region (Table 1, Fig. 2). Audiograms of the former were however clearly distinguishable from those of the c.35dupG homozygotes (Fig. 3) expressing both subjects greater binaural differences and a minor degree of affectation mainly for low frequencies, than the one shown by the c.35dupG homozygotes. Audiometries of c.35delG heterozygote V-16 performed on three occasions along 45 years showed only monaural mild loss above 3 kHz in consecutive tests; but no loss was detected in VI-6 or VIII-4, the only other single heterozygotes examined, whose identity by descent (IBD) mutation had different geographic and eventually partial genetic origin of the one carried by V-16 according to the available haplotype information.

In-phase haplotype C;C;G;A;C was carried by both the homozygous brothers VI-15 and VI-17 and by their heterozygous-half first cousin V-16; contrarily, the haplotype was C;C;G;A;T in VI-6, VII-11, and VIII-4, attesting its not-German origin; but in both cases, allele C is present in rs7994748 polymorphism as found by Parzefall et al. [28], being absent in the cis c.35dupG haplotype. Out-of-phase haplotypes in 2 heterozygotes (V-16, VI-6) were T;T;G;A;T and T;C;G;A;C, respectively. Noncarrier and normal-hearing VI-16 full sib of profound deaf homozygotes VI-15 and VI-17, had T;T;G;A;C and T;T;G;A;T haplotypes, the latter being shared among “Germans” by both controls and carriers.

c.229T>C (Trp77Arg = p.W77R)

Three heterozygous p.W77R subjects (siblings V-5, V-25, and VI-14) were third cousins (Φij ~ 0.004) as assessed through the obligate carrier V-19, since V-18 is a DNA-tested noncarrier. Being I-23 and I-24 their common ancestors, at least one of them should bear an identical-by-descent (IBD) mutation; but only if IV-5 is indeed the c.229T>C (= p.W77R = rs104894397) carrier, whose geographic/ethnic origin then must be exclusively German and eventually not closely related to the inbred eastern Mediterranean first p.W77R-detected carriers [29]. IV-5 was sister of the obligate heterozygote IV-4 for at least one unspecified *GJB2* mutation, who was father of prelingual deaf V-3, eventual compound heterozygous in trans p.W77R/c.35dupG, whose brother V-2 was a c.35dupG obligate heterozygote, as stated previously. At 22 years of age, V-5 (heterozygous p.W77R) showed already a moderate–severe HI (60–70 dB) at all frequencies, and at age 66, she suffers from a bilateral and asymmetrical severe/profound deafness. At ages 8 and 53 years old, her sister V-25 showed an almost invariant with time, bilateral moderate–severe loss (60–70 dB). Audiograms of VI-14 at ages 46 and 62 years displayed only a mild loss (15 dB, above 2 kHz at 62 y.o.); her carrier son VII-16 had at 19 y.o. a HI audiogram with losses from 50 to 65 dB at all frequencies; but her other 2 under 25-y.o. noncarrier sons VII-17, VII-18 had normal audiograms. The latter siblings’ common father VI-8 has a severe HI eventually non Connexin 26-related (molecularly proven) of unknown etiology; he displayed bilateral asymmetrical severe losses above 1 kHz already at 25 y.o., which had progressed to profound ones, at his 54 years of age and further ahead now at his 73 y.o. He is the maternal great-grandchild of II-25 (whose descendant links were not drawn in Fig. 1), whose full sib II-21 was the paternal great-grandfather of V-9. In this family, there were (i) Mendelian segregation of p.W77R and HI (V-5, V-25), (ii) incomplete penetrance (VI-14), or (iii) eventual and still unidentified recessive/semidominant factors at *GJB2* (VI-8, and his son VII-16) as has been proposed in other examples [6] and recently interpreted as gene interaction (epistasis?) over *GJB2* attributed to mutation c.35delG [30], as is argued ahead in the discussion.

In-phase haplotype T;T;G;A;C was the single one in all the p.W77R carriers. Table 3 summarizes the haplotype status of most HI examined subjects.

Non GJB2 mutations

As expected in the population at large, only the profound/prelingual deafs were easily ascertained before any specific examination had to be performed. By the year 1916, very few evident HI subjects were known in the isolate, as was formerly discussed [1]. The first most likely known (prelingual) deaf was IV-16, maternal aunt of 4 siblings—3 prelingual deafs (not displayed in Fig. 1) plus the still-normal-hearing 81-year-old V-12 (recently deceased)—whose father IV-17 was not of German ancestry. His mother IV-15 had close relatives who were certain carriers of both c.35delG (V-20) and of c.35dupG (V-2) mutations. Only 2 sibships containing profound/prelingual deafs illustrated in Fig. 1 did not carry mutations either at *GJB2* or *GJB6* loci. They were the multiplex sibship of VI-10, VI-11, and VI-12, offspring of IV-22 and V-23; and the simplex and closely inbred one (Φij = 0.0156) offspring of V-26 and V-27, respectively. In both instances, their remote ancestors came from northern Germany states, far away from Baden-Württemberg. However, prelingual deaf patient VI-19 had a syndromic phenotype since he bore a congenital cardiac malformation of uncertain etiology, responsible for his premature death at the age of 26 years; but a

recessive etiology cannot then be excluded. His dizygous twin sister was of normal hearing both at ages 7 and 52 years as were currently also at least his other sisters VI-20 and VI-21. Profound deaf VI-8, with a severe HI sister, had parents, one with normal and the other almost normal audiograms; however, its etiology can be excluded as due to any *GJB2*/*GJB6* mutation as was confirmed by Sanger sequencing.

Table 2 shows the audiometric phenotype (air) in obligate heterozygotes and in those with an *a priori* c.35dupG (du), p.W77R (wr), or c.35delG (dl) mutation carrier risk ≥37.5%.

Prevalent mutations

This comprehensive sample of deafs belongs to a large cohort of nonsyndromic high-risk HI members in the isolate; thus, it might be likely biased towards several trends of deafness etiologies. However, most profound/prelingual cases occurred in multiplex sibships, suggesting aggregation of recessive causes in those. From generations I to VII among ethnically mixed couples out of 11 parents with no German ancestors, only IV-14 and VI-6 (18%) may have contributed exclusively with c.35delG to the mutated gene pool; being carriers in cis of either German C;

Table 2 Audiometric phenotype (air) in obligate heterozygotes and in those with an *a priori* c.35dupG (du), p.W77R (wr), c.35delG (dl) mutation carrier risk ≥ 37.5% [(25 + 50%)/2]

Subject code (mutation) (Fig. 1)	Mutation–carrier risk		Audiometric loss ≥ 25 dB		Confirmed DNA status
	100%	≥ 37.5%	At age (years)	For ≥ 2 kHz dB	
III-5 (du)		+	83	35	-
IV-3 (du/wr)	+		57	30	-
IV-4 (du/wr)	+		64	60	-
IV-5 (wr)		+	54	35	-
IV-9 (du)		+	61	50	-
IV-10 (du)		+	63	20	-
IV-13 (du)	+		61	60	-
IV-14 (dl)	+		63	0	-
IV-15 (dl)	+		71	45	-
IV-17 (dl)	+		78	45	-
IV-20 (dl)	+		75	50	-
V-1		+	35, 65, 80	0, 40, 35	+
V-2 (du)	+		40	55	-
V-4		+	30	0	-
V-9 (du)	+		70	50	+
V-16 (dl)	+		37, 65, 81	0, 45, 50	+
V-25 (wr)	+		8, 53	65, 70	+
VI-1 (du)	+		15, 46, 60	0, 40, 45	+
VI-2 (du)	+		65	30	+
VI-3 (du)	+		67	0	+
VI-6 (dl)	+		50	50	+
VI-14 (wr)	+		46, 62	20, 25	+
VII-5 (du)	+		25	0	+
VII-7 (du)	+		47	55	+
VII-8 (du)	+		45	0	+
VII-9 (du)	+		44	0	+
VII-16 (wr)	+		19	70	+
VIII-1 (du)	+		16	0	+
VIII-2 (du)	+		8	0	+
VIII-4 (de)	+			0	+

C;G;A;C or not German C;C;G;A;T haplotypes, respectively.

All c.35dupG and p.W77R mutations were of exclusive German origin as inferred in the latter instance, received from non-tested V-19 through his carrier daughter VI-14 since her mother V-18 did not carry any *GJB2* mutation; although IV-13 had 3 prelingual deaf (V-13, V-14, V-17) siblings, who were either homozygotes, c.35dupG/c.35delG compound heterozygotes, or heterozygous p.W77R. However, she could not be molecularly tested. Both c.35dupG and p.W77R mutations are in cis with haplotype T;T;G;A;C, which is the same one carried by the majority of nonmutated control chromosomes in the isolate (Table 3).

The average minimum prevalence of nonsyndromic prelingual/profound deaf phenotypes within the isolate for generations V–VII was ~ 0.001 = (14/14,100). The present ethnic admixture of not German/German gene sources was estimated as 10–15% within the cohort of mixed couples. Frequency of all *GJB2* mutation carrier subjects (2 pqt) is 1/16; [qt = Σqi] with slightly higher (> 0.01) frequencies for c.35dupG and c.35delG than for p.W77R. Therefore, at least one additionally unidentified mutated locus may be contributing to this genetically very heterogeneous cohort of recessive nonsyndromic HI families.

Audiograms in families carrying different mutations at locus GJB2

Figure 3 illustrates the contrasting but analogous binaural patterns in both c.35dupG and c.35delG heterozygous for those subjects who showed some HI. They had a steep slope after 4 kHz, followed by an opposite inflexion point at 6 kHz, mainly in delG phenotypes. No age- or frequency-related loss pattern was found either in five certain p.W77R carriers, or in the single compound heterozygous c.35dupG/c.35delG (VII-11) who had instead a unique asymmetrical deficit of 40 dB with a higher and steady threshold difference in the right ear, at all frequencies (Fig. 3).

Prelingual deaf homozygotes for both c.35dupG and c.35delG mutations (VII-1, VII-2, VII-3 and VI-15, VI-17, respectively) had audiograms phenotypically differentiable between them, with a ~ 110-dB loss in the former genotype than in the latter (around 100–110 dB) which were entirely different in those cases from the one in the single normal-speaking compound heterozygote (VII-11) who carries also a not-German c.35delG mutation and severe/profound but asymmetrical losses.

Table 3 *GJB2* intragenic haplotypes in hearing impaired and in normal hearing subjects among carriers/noncarriers of mutations c.35dupG, c.35delG, and p.W77R

Haplotype	Chromosomal phase (*n* = 81)				Totals
	c.35dupG	c.35delG	p.W77R	trans	*n* (%)
T;T;G;A;C	17 (100%)		4(100%)	33	54 (66.7%)
C;C;G;A;T*		3		4	7 (8.6%)
C;C;G;A;C		5		3	8 (9.9%)
T;C;G;A;T				5	5 (6.2%)
T;C;G;A;C*				3	3 (3.7%)
T;T;G;A;T				2	2 (2.5%)
C;C;A;A;T*				2	2 (2.5%)
Totals	17 (21.0%)	8 (9.9%)	4 (4.9%)	52 (64.2%)	81 (100.0)

* Carried only by subjects with at least one parent with no German ancestry. SNP haplotype order: rs117685390 (T>C), rs7994748 (C>T), rs2274084 (G>A), rs2274083 (A>G), and rs3751385 (T>C)

Discussion

Ethnic/geographic distribution of mutations

After the original description in one Spanish c.35dupG/c.35delG compound heterozygote, out of 33 *DFNB1* families within a merged sample of 31 Spanish and 51 Italian prelingual deafs [19], the c.35dupG mutation rarely has been detected. In very few populations worldwide, its geographic origins have been suggested, contrarily to those of c.35delG, which display highest frequencies among Mediterraneans, with some populational decaying gradient toward East and North, greatly increased toward China. In contrast, the c.35dupG shows a decreasing gradient westwards, both from Eurasia [9], except as now found in the Kaiserstuhl region, and eastwards toward China, where it is seen mainly among the Han and Hui ethnic subgroups [10, 13, 31–34] and also but very scarcely in Mongolia [35] and in northern Italy [36]. In Africa, it barely appears within the Ghanan population of Ashanti [11]. This universal and still not-explained low prevalence had not allowed so far the assessment of either a single IBS or IBD homozygote among outbred (or even inbred) populations. It has neither been found in former nor in recent surveys within Germany [6, 37], or in northern or southern Europe, the Czech Republic, Morocco, Tunisia, Cyprus, Greece, Turkey, Canada, Siberia, Korea, Japan, Iran, India, southeast Asia, and Australia; neither in the native Amerindian populations in which it could have been already ascertained at least within some enriched subsets of nonsyndromic HI Iberoamerican patients. Available data for the hybrid samples of nonsyndromic deafs revealed its actual absence from Colombia, México, Brazil, Venezuela, Chile [38], and even Guatemala [39], countries all with 50%+ Amerindian genes in their highly admixed general populations, most of which bear easily detectable Caucasoid deleterious recessive phenotypes [23, 40, 41], but none of which have been found among deaf subjects with suspected or evident Amerindian ancestry.

The geographic source for this "German" c.35dupG mutation was surprisingly inferred in retrospect within the "general population" of the Kaiserstuhl region, among remote ancestors of people who have dwelled around the towns of Wyhl, Forchheim, and Endingen in the southwestern Baden-Württemberg state (shown in Fig. 2). Since those carrier ancestors in at least 7 ancestral generations did not reveal any close kinship, the mutation prevalence at the time of migration (1842), may be estimated as being higher at least for one order of magnitude than what is currently known for mutated codon 13 at *GJB2*, in all published instances worldwide. An allelic disequilibrium is present with the intragenic T;T;G;A;C haplotype which is common to both c.35dupG and p.W77R carrier chromosomes being also the most frequent (40.7%) among the noncarrier chromosomes in the isolate, a not-shared situation by any of the two other encountered identical-by-state (IBS) c.35delG mutations which are both associated with non-canonical allele C in polymorphic rs117685390, and also with allele C in polymorphic rs7994748 in Brazil [42]. In the former case, this suggests for such remote origins: founder phenomena and independent occurrence, which might explain the association of both the c.35dupG and p.W77R mutations with a haplotype (identical) possibly originated around Kaiserstuhl likelier than elsewhere. In Russia, across the Volga-Ural region, among *GJB2* deafs, the mutation is present in only 0.5% of the total locus allele cohort, without as expected, any identified homozygous subject [9] as in Mongolia [35]. In Linyi (Shandong, eastern China) contrarily, it is seen at 0.1–1.2% [33] in exclusive association with the 2 most prevalent Asiatic mutations in the populations [43]; and recently with the rarer Chinese c.232G>A mutation within one Han family [44], being also the fourth most frequent (at 0.24%) frameshift mutation among Han prelingual deafs [34] supporting thus a plausible old prevalence of the c.35dupG in several areas of China. Those 2 events suggest some concomitant mutation-spreading mechanism, unrelated to the ones producing the allelic disequilibria found for the c.35delG mutation(s). In the former case in Kaiserstuhl, out of 2 genetically "independent" associated haplotypes, it occurred only with T;T;G;A;C of German origin which had a higher frequency (66.7%) rather than with the less frequent not German T;C;G;A;C (3.7%) haplotype within the isolate.

The lack of published information at the present time for the situation described, or with analogous intragenic haplotypes does not allow a stronger support to the conjoint hypotheses of singularity for remote origin [45], and non recurrence [46, 47] of an identical-by-descent c.35delG presence in the isolate, which nevertheless could still be considered as rather likelier *prima facie* the quoted association with allele C as already found in Austria and Spain populations by Ramsebner et al. [48]. These 2 instances of allelic disequilibria do not imply 2 independent c.35delG mutational events, if they did occur quite remotely as has been proposed [45, 46], especially if a single nucleotide change in the fifth polymorphism of the whole haplotype can generate a new identifiable syntenic combination, without necessarily the occurrence of any additional novel codon 12 G deletion, although a second not-German haplotype with 2 different polymorphic specific changes cannot be ruled out as eventually revealing an independent origin; as it has been now proven [35] that western and Eurasian Caucasoid and Mongolian c.35delG carriers share a single unique haplotype despite many generations of genetic separation, different from another haplotype cohort as the one we have found.

The postulated association in 98/252 chromosomes (39%) between the cis rs117685390 allele C genotype of both deaf and hearing Austrian and Spanish dwellers [48] carrying the c.35delG mutation in the intragenic haplotype, is confirmed in our much smaller cohorts, since both ethnically heterogeneous c.35delG carriers who bear slightly different haplotypes, share the same rarer promoter C base, not carried in cis by any of the c.35dupG or p.W77R mutation carriers.

c.35dupG has an original geographic distribution restricted to the Old World including Eurasia up to China, always with a persistent low prevalence. Both circumstances support the hypothesis of a single although not extremely old origin, eventually analogous to one of its contiguous Caucasoid mutation c.35delG, primarily unrelated by origin to it. Together with p.W77R which may probably come originally from the same geographic source [47], it shares an identical haplotype which is different in the first 2 polymorphisms from the former identified c.35delG mutations. The unexpected finding of exceptional homozygous carriers for c.35dupG, who had ancestors without close kinship coming originally way back from southwestern Germany could be the result of a secondary founder effect added to the random drift, rather than being the consequence of any direct and a more recent eastern Mediterranean origin. Absence of negative selective factors eventually concurred for increasing an initial greater frequency in southwestern Germany; or alternatively a random enrichment of carriers by a bottleneck phenomenon among the remaining 92 effective founders of the isolate. Whichever the causes, the explanation for such a known relative frequency difference lower than 50:1 in Kaiserstuhl than elsewhere between those 2 mutations in the contiguous 12/13 codons is still lacking; especially, if both are equally old enough in their origin, likely hundreds of generations back and particularly if as recent findings indicate, there existed an old common Caucasoid/

Mongoloid mixed group [48] out of which one or the two mutations/haplotypes might have arisen. Alternatively, both mutations could be widely separated in their common remote and asynchronous origins, with a much older and exclusive upsurgence in the Caucasoid branch, to explain the present high c.35del G prevalence everywhere; or conversely a still-eventual undisclosed negative allele segregation of c.35dupG, which contrarily shows a similar and common low prevalence in Caucasoid, Mongolian, and Chinese populations as if this mutation might have arisen initially close in time (and previously) to the splitting of the branches [49], thus maintaining its presence and prevalence equally along generations. This evidence supports the hypothesis of the absence of any negative selection (and a low mutation rate), but still leaves unexplained the quantitatively large prevalence difference between the mutations if they appeared synchronously long time ago. Any search for intragenic haplotype markers in Chinese c.35dupG carriers should shed light upon this puzzle.

On the other hand, allele A in the polymorphism rs2274084 (p.Val27Ile) (Table 3) has convincingly an ancestral southern Siberian–Mongolian origin, which is present only in certain populations of Asiatic–Amerindian descent, making it a valuable marker for such ancestry [50], and besides, it is rarely found—if ever—in cis with mutation(s) c.35delG, making thus much less likely any Mongoloid ancestral origin for those 2 c.35delG mutations.

Age-related phenotypic differences in hearing thresholds of homozygous and heterozygous HI mutants

Now, we can rely on informative audiograms for comparison between novel c.35dupG/c.35dupG homozygotes, c.35delG/c.35delG homozygotes, the c.35dupG/c.35delG compound heterozygote, and simple c.35dupG and c.35delG heterozygotes. In several instances, they were obtained along wide life-time intervals, allowing us to perform a survey by searching for aging effects, which were indeed found, although inconstant. The results have indicated however that polar homozygotes are clearly and consistently different in degree of HI between 3 mutations, and also phenotypically distinct from those of the single compound heterozygotes, when it is accompanied in trans at least by one of the two c.35delG mutations found, which had different cis haplotypes as discussed. Some heterozygous subjects in both single mutations at codons 12 and13 can also be distinguishable after age 35–40 years. Most heterozygotes did not show any loss in high frequencies, supporting similar findings at the same locus in other nonsyndromic mutation carriers. Therefore, their phenotypes cannot always allow with confidence to infer through the audiogram the specific genotype in the heterozygote as has been also found by some [51], although not by others [52] at least with one of those 2 frameshift > nonsense mutations. The mutant protein differs in 30 out of 34 amino acids along the consensual sequence of the (relatively) longer (although truncated) one codified by c.35dupG, apparently with a stronger functional effect on hearing. In addition, recently, presbycusis has been demonstrated in obligate c.35delG carriers (thus far in a single explored mutant allele) and attributed to the partial loss of function for a truncated protein, plus the critical participation of the gene complex system *NFR2*/ARE (NFE2-related factor 2/antioxidant response element) exhibiting epistasis [30, 52]. A similar *in vitro* pathogenic (hypomorphic) result had been documented with the non-truncating mutation p.W77R [53] regarding its function. Therefore, any heterozygous presence of a somewhat hypomorphic allele seems unlikelier as the main or single predisposing etiology for *GJB2*-associated presbycusis, especially with so many known unaffected heterozygotes who happen not to be known as carriers of *NFR2* epistatic mutations. This lent a stronger support to the hypothesis of a redox *NFR2*-dependent phenomenon partially revealed so far with mutated alleles c.35dupG, c.35delG, and p.W77R as described, which perhaps could be found with other mutations as well if they were explored.

On the other hand, the apparent trend toward the hearing deficit we observed in several p.W77R heterozygotes in a single family, found previously in other experiences [18], and the exceptional dominant phenotype of the also missense and almost contiguous Ala74Thr = A74T [44] suggests that a close surveillance upon future eventual heterozygotes may be justified, because the in vitro demonstrated functional burden [18] as mentioned could express a milder recessivity in most carriers' audiograms. That seems to be the case with an apparently more strict Mendelian recessive c.35dupG mutation. In relation to both analogous c.35dupG and c.35delG homozygosis, being a frameshift > truncating dual case with an etiopathogenic situation different of the one found with the missense mutation p.W77R (or the A74T), the loss of function of the latter comparatively was not milder, a finding which for this locus and those 2 other mutations does not support the hypothesis of a universal and regular higher deleterious effect (albeit certain information loss is indeed present) attributed either to nonsense or to frameshift mutations [54]. The unique genotypic find of 2 indels truncating homozygous allelic and contiguous mutations, in only one of which there is also a frameshift for 34 codons, has granted the opportunity to relate the phenotypic changes if any, that may be attributed to a single variable (the 30 nonconsensual protein amino acids) from the newly misread sequence, to another equally truncated but smaller

molecule. The phenotypes differ qualitative and quantitatively, a difference which otherwise is functionally marked in the compound heterozygote of two frameshift mutations, from different origins. The single-out published instance of any molecularly diagnosed deaf phenotypically graded as a severe compound heterozygous c.35dupG/c.35delG [36] behaved similarly to our normal-speaking compound. This find expresses a phenotypic contrast with the identical profound HI in such 3 novel homozygous c.35dupG siblings; thus empirically grounding an objective eventual difference between homozygous c.35dupG/c.35dupG in the one hand always-profound prelingual deafs, and mostly severe/profound c.35dupG/c.35delG compound heterozygous ones on the other.

Conclusion

Caucasoid c.35dupG and c.35delG homozygotes plus both their alternate compound and single heterozygotes, can all eventually be reliably identified with an audiogram, which does not show any noticeable or consistent aging influence before 35 years of age in the latter ones. However, there is a slightly earlier threshold effect in c.35dupG carriers above 2 kHz. Within the Old World populations, a wider geographic distribution of mutation c.35dupG is evident, and everywhere, its prevalence is always considerably lower than that of c.35delG, for still unclear reasons, since its segregation frequency does not seem to be lower than expected and therefore, no negative selection factors seem to be acting. The mutation is steadily absent from most non-Caucasoid groups, except Chinese Han and Hui ethnic subgroups, being clearly independent of c.35delG despite its genomically extreme physical proximity. A center for its dispersion has now been located in southwestern Germany, most probably within an allele-enriched topodeme as is exemplified by many other recessive phenotypes in isolated populations (i.e., [55]); its likely IBD source was presently discovered among nonsyndromic deafs in northern Venezuela, distant ahead several generations from their original geographic/ethnic source, showing so far from western Europe, a novel quite elusive genotype, among the offspring of parents with very low close ancestral but likely certain, remote kinship.

Limitations

Regardless of the missing data on the genetic constitution of some critical-alive and defunct-pedigree members that might lessen the whole informational content of the cohort; the relatively low close kinship and the high mutational heterogeneity of the latter are fortunate and robust counterweights, which within an isolate, allow a proper assessment of both the frequency and original geographic distribution of the genetic variables producing hearing impairment phenotypes in a specific topodeme, unlikely to be obtained by surveying largely outbred populations.

Abbreviations

dB: Decibels; HI: Hearing impairment; IBD: Identical by descent; IBS: Identical by state; kHz: Kilohertz; SNP: Single nucleotide polymorphism; y.o.: Years old

Acknowledgments

To all the families who generously participated in the study through several decades, and especially to MTGF within family GF for her efficient cooperation in bringing together most of its members. To Dr. Oscar Ferrer Roo for his kind offer of a large control sample of deaf subjects from the general Venezuelan populations. To Vassiliki Ikonomu for efficiently extracting DNA samples, to Gilberto Gómez for his contribution in some samples collecting activities, and to Juan Carlos Granadillo from the IVIC Graphic Design Department for his assistance with the skillful drawing of the figures.

Authors' contributions

SA: conceptualization, validation, formal analysis, writing—original draft, review & editing, supervision. IP: conceptualization, methodology, investigation, validation, formal analysis, review & editing, project administration, supervision. AH: investigation, formal analysis, review of the final version of the manuscript. DK: investigation, review of the final version of the manuscript. All authors have read and approved the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the ethical standards of the Helsinki Declaration. Informed written consent was obtained from each participating subject, in accordance with the guidelines of the Bioethics Committee of the Venezuelan Institute of Scientific Research (IVIC); reference number is not available.

Consent for publication

Not applicable

Competing interests

The authors have no conflicts of interest to disclose.

Author details

[1]Laboratory of Human Genetics, Venezuelan Institute for Scientific Research (IVIC), Caracas, Venezuela. [2]Center of Experimental Medicine, Venezuelan Institute for Scientific Research (IVIC), Caracas, Venezuela.

Received: 23 February 2021 Accepted: 24 March 2021
Published online: 07 May 2021

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