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ORIGINAL INVESTIGATION

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Genetics of congenital deafness in the Palestinian population: multiple connexin 26 alleles with shared origins in the Middle East

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Abstract In some Palestinian communities, the prevalence of inherited prelingual deafness is among the highest in the world. As an initial step towards understanding the genetic causes of hearing loss in the Palestinian population, 48 independently ascertained probands with non-syndromic hearing loss were evaluated for mutations in the connexin 26 gene. Of the 48 deaf probands, 11 (23%) were homozygous or compound heterozygous for mutations in *GJB2*. Five different mutations were identified: ivs1(+1) G→A, 35delG, 167delT, T229C, 235delC. Nine deaf probands were homozygous and only two compound heterozygous. Among 400 hearing Palestinian controls, one carrier was observed (for 167delT). We show that *GJB2* ivs1(+1) G→A disrupts splicing, yielding no detectable message. Linkage disequilibrium analysis suggests, in the Palestinian and Israeli populations, a common origin of the 35delG mutation, which is worldwide, and of 167delT, which appears specific to Israeli Ashkenazi and Palestinian populations. A high prevalence of deafness, high frequency of homozygosity rather than compound heterozygosity among deaf, and low mutation carrier frequency together reflect the high levels of consanguinity of many extended Palestinian families. Some of the 25 families with multiple cases of inherited prelingual deafness and wildtype *GJB2* sequences may represent as-yet-unknown genes for inherited hearing loss.

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Introduction

Hearing loss is the most prevalent form of sensory impairment in humans, with approximately 1 in 1000 infants being born with a serious hearing deficit (Nadol 1993). In some communities with high levels of consanguinity, the frequency of childhood deafness is especially high (Jaber et al. 1998). Prelingual hereditary hearing impairment occurs in the Palestinian population at a frequency of approximately 1.7 per 1000 (Shahin 2000) and is higher in some villages.

The molecular events leading to deafness are rapidly being elucidated by genetic analysis of human families and mouse model systems. Close to 70 human loci causing non-syndromic hearing loss (NSHL) have been mapped and 22 identified (Van Camp and Smith 2001). In all populations in which the genetic epidemiology of deafness has been evaluated, mutations in the gap-junction protein *GJB2* are the single most frequent cause of inherited deafness (Cohn and Kelley 1999). In some Mediterranean regions, mutations in *GJB2* are responsible for 50% of recessive NSHL (Zelante et al. 1997; Denoyelle et al. 1997; Estivill et al. 1998). Mutations in *GJB2* can also lead to dominant hearing loss (Denoyelle et al. 1998) and to dominantly inherited sensorineural deafness associated with keratoderma (Maestrini et al. 1999; Kelsell et al. 2000). A large number of mutations in *GJB2* exist, more than 60 of which are known to be associated with deafness (Rabionet et al. 2000; GeneDis, http://bioinfo.tau.ac.il/GeneDis). The most common of these are 35delG (Kelley et al. 1998) and 167delT (Morell et al. 1998).

In order to estimate the proportion of inherited deafness attributable to *GJB2* in the Palestinian population, we sequenced this gene in 48 independently ascertained probands with prelingual profound bilateral hearing loss. We determined the effect on transcription of a previously uncharacterized mutation and estimated distances between genes and markers in the region by using new, publicly available, genomic sequence.

Subjects and methods

Subjects

Forty-eight children with profound prelingual bilateral hearing loss were ascertained from records of Palestinian schools for the deaf. Children, their parents, and other informative relatives were interviewed and blood samples were obtained for DNA extraction after informed consent of each child's parents. Control individuals with normal hearing were 510 Palestinians who attended out-patient clinics at the Holy Land Hospital or the Karitas Children's Hospital and 96 Israeli Ashkenazim. Personal identifiers were removed from samples. All research undertaken was performed according to the guidelines of the Palestinian Charter of Patient Rights and the Helsinki Committee at Tel Aviv University.

Hearing was measured by pure-tone audiometry and included bone conduction. Hearing thresholds were obtained between 250 Hz and 8000 Hz in a soundproof room. Probands had bilateral hearing loss of more than 85 dB at all frequencies. The clinical history of each proband was explored to ensure that the hearing loss was not a result of infection, trauma, acoustic trauma or ototoxic drugs. Family histories were evaluated for the presence of hearing loss, diabetes, vision problems, neurological disorders, diabetes and skin disorders.

Genetic analysis

Genomic DNA was prepared by standard techniques from peripheral blood lymphocytes (PureGene, Gentra Systems, Minneapolis, Minn.). *GJB2* was amplified from genomic DNA by using the primers GJB2–1F (5'-TCT TTT CCA GAG CAA ACC GC–3') and GJB2–2R (5'-GGG CAA TGC GTT AAA CTG GC-3') to yield a 724-bp product (Kelsell et al. 1997). Polymerase chain reactions (PCRs) were performed with 100 ng genomic DNA, 20 pmol each primer, 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 1.25 U *Taq* DNA Polymerase (Boehringer Mannheim, USA) in a total volume of 50 μl. PCRs consisted of 35 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 1 min 30 s, following a 5-min denaturation step at 94°C in a PC-100 Thermocycler (MJ Research, San Francisco, Calif.). Prior to cycle sequencing, PCRs were purified on Qiaquick columns following the manufacturer's instructions (Qiagen, Germany). Amplicons were sequenced by using 50 ng purified product, 2 pmol appropriate primer, and 4 μl ABI BigDye Terminator cycle sequencing mix in a 10-μl final volume. Following cycle sequencing, samples were precipitated, resuspended in formamide dye with Dextran blue, denatured and loaded onto an ABI-377XL DNA sequencer. The sequence of each amplicon was confirmed by sequencing in the forward and reverse directions.

To detect the 167delT mutation, we took advantage of a *Mwo*I restriction site gained upon the deletion of T at position 167. Genomic DNA was amplified by using primers 5'-GAT TGG GGC ACG CTG CA-3' and 5'-CCC TTG ATG AAC TTC CTC TTC TTC-3' and cut with *Mwo*I. Wildtype sequence yielded a single uncut 322-bp fragment; the 167delT mutant allele yielded two fragments of 161 bp. The first (noncoding) exon and flanking donor splice site were amplified by using the Advantage-GC Genomic PCR Kit (Clontech, Palo Alto, Calif.) and primers 5'-TCC GTA ACT TTC CCA GTC TCC GAG GGA AGA GG-3' and 5'-CCC AAG GAC GTG TGT TGG TCC AGC CCC-3' (Denoyelle et al. 1999) and then sequenced as described above.

In order to determine the effect of the mutation at ivs1(+1), RNA was isolated from lymphocytes of the proband carrying this mutation by using TRI Reagent (Molecular Research Center, Cincinnati, Ohio). Three separate reverse transcription (RT) reactions were performed with Homo-Oligomeric DNA $d(T)_{12–18}$, Random Hexamer (Amersham Pharmacia Biotech, Piscataway, N.J.) and with primer GJB2–2R. cDNA was amplified from a mixture of the RT reactions by using primers 5'-AGA GAC CCC AAC GCC GAG-3' and 5'-CCT TTG CAG CCA CAA CGA GG-3' and the 203-bp product was sequenced as described above.

In order to examine chromosome 13q11 haplotypes associated with the various *GJB2* mutations, polymorphic markers D13S1316, D13S141, D13S175, D13S250 and D13S1275 were genotyped by published methods (Gasparini et al. 1997). PCR products were labeled by P^{32}-dCTP incorporation during amplification, resolved on a 6% denaturing acrylamide gel and exposed to film.

Four single nucleotide polymorphisms (SNPs) were genotyped in all deaf and hearing individuals. Relative to the ATG of exon 2 (designated +1), the SNPs are at positions –3558 (personal communication, Karen Frederici, Michigan State University), +1946 and +2034. The 5' SNP was identified by using the Advantage-GC Genomic PCR Kit and primers GJB2–5'F (5'-AGA GGA CAA CGA CCA CAG CCA TCC CTG AAC-3') and GJB2–5'R (5'-CCT CTT CCC TCG GAG ACT GGG AAA GTT ACG). The 3' cSNPs were identified by using primers GJB2–3'F (5'-GTG AGC TTG GGA ATT TTA TTG ACA C-3') and GJB2–3'R (5'-GGA GTA CCA TTT TTT GGA AAC CAT G-3'). Linkage disequilibrium values were estimated by using Yule's coefficient, i.e. $\Psi=(p_{11}-p_{12})/(p_{11}+p_{12}-2p_{11}p_{12})$, where p_{11} is the frequency of allele A_1 on chromosomes carrying allele B_1 and p_{12} is the frequency of allele A_1 on chromosomes carrying allele B_2 (Krawczak and Schmidtke 1998).

Results

All 48 probands had bilateral profound sensorineural NSHL. Deafness of all probands was reported by their parents to have been prelingual. Thirty-one probands were from families with more than one child with prelingual hearing loss. Seventeen families reported only one child with prelingual hearing loss.

Eleven of the 48 probands (23%) carried mutations in *GJB2*, including six of 31 probands with deaf siblings and five probands with no deaf siblings. Probands with no deaf siblings were from smaller sibships than familial cases. All deaf probands with *GJB2* mutations were either homozygous or compound heterozygous. All relatives heterozygous for these *GJB*2 mutations had normal hearing.

Five different *GJB2* mutations were identified among the probands (Table 1). Most frequent were 35delG, as had been found in other populations (Denoyelle et al. 1997; Estivill et al. 1998), and 167delT, previously reported only among Ashkenazi Jews (Morell et al. 1998; Kelley et al. 1998; Sobe et al. 1999; Lerer et al. 2000). One proband was heterozygous for *GJB2* 235delC and 167delT. The 235delC mutation is the most common *GJB2* mutation in the Japanese population (Fuse et al. 1999; Abe et al. 2000).

One proband was homozygous for the missense mutation 229 T→C leading to substitution of arginine for tryptophan at codon 77 in the second transmembrane domain of *GJB2*. This allele has been previously reported in an Israeli Arab family (Carrasquillo et al. 1997), but not in any other population. The family of the 229T→C homozygous proband in this series did not report any known relatives with hearing loss but they might be distantly related to the family in which this mutation was previously observed.

Proband J3 carried a mutation in the splice donor of intron 1, ivs1(+1)G→A, corresponding to –3170 G→A relative to the AUG translation-initiating codon (genomic

Table 1 *GJB2* mutations in Palestinian probands with prelingual bilateral hearing loss

Mutation	Effect	Deaf probands with mutation		Allele frequency among deaf
		Homozygotes heterozygotes	Compound	
ivs1(+1) G→A	No transcript detected	–	1 (proband J3)	0.01
35 del G	13 stop	5	1 (proband J3)	0.14
167 del T	56 stop	3	1 (proband D)	0.05
229 T→C	Trp 77 Arg	1	–	0.02
235 del C	81 stop	–	1 (proband D)	0.01
All		9	2	0.23

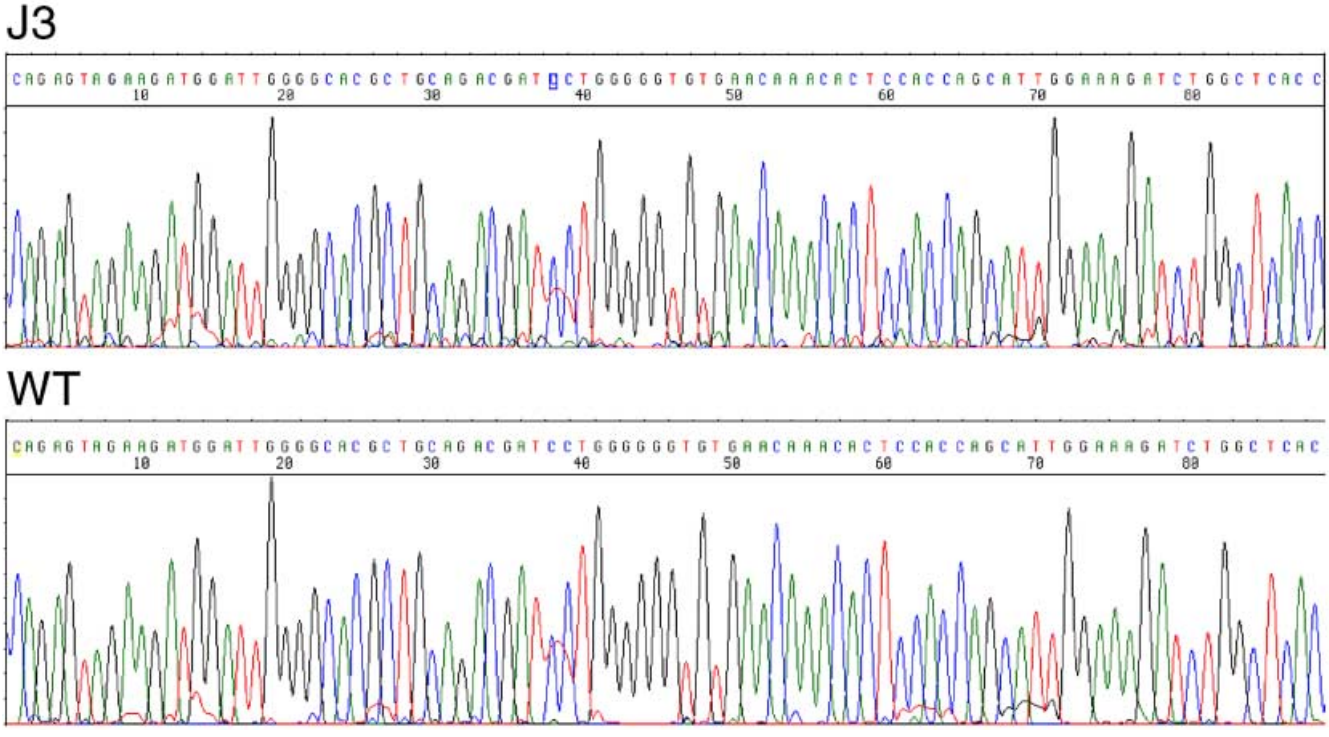

Fig. 1 Loss of transcript attributable to *GJB2* splice mutation ivs1(+1) G→A. cDNA sequence of *GJB2* coding exon 2 from deaf proband J3 (*above*) and from a hearing control (*WT*) individual (*below*). Translation begins at the ATG at position 12 of each electropherogram. The genomic sequence of J3 is compound heterozygous for *GJB2* mutations ivs1(+1) G→A and 35delG (see text). However, the *GJB2* message of J3 includes only the 35delG allele; no transcript from the allele carrying ivs1(+1) G→A was detectable

sequence of this region appears in GenBank U43932). Genotypes of the parents of J3 indicated that ivs1(+1)G→A and 35delG occurred on different parental chromosomes. *GJB2* ivs1(+1)G→A has been identified previously (Denoyelle et al. 1999; Green et al. 1999) but the way in which it contributes to the deafness phenotype is not known. *GJB2* is translated beginning at the first AUG that lies at basepairs 23–26 of exon 2. Hence, exon 1 is entirely in the 5' untranslated region (UTR). The sequence of cDNA from a lymphoblastoid cell line of J3 yielded message only from the 35delG allele, indicating that the ivs1(+1)A→G allele was not transcribed or was extremely unstable (Fig. 1). (The absence of a second transcript was not attributable to any polymorphism in the cDNA primer sequences, because the genomic sequence of J3 was homozygous and wildtype at these sites).

Among 400 hearing controls from the general Palestinian population, one individual was heterozygous for 167delT. No control carried any of the other mutations.

A map of the *GJB2* region of chromosome 13 was developed by using the April 1, 2001 freeze of the UCSC Human Genome Project Working Draft Assembly (http://genome.ucsc.edu/). Distances between *GJB2* and neighbouring genes and polymorphic markers are indicated in Fig. 2. Haplotypes for the region of chromosome 13 flanking *GJB2* were determined for 38 relatives from the three Palestinian families (D, S and L) and for 16 members of Israeli Jewish family 10 by using markers D13S141 and D13S175, which span 125 kb (Fig. 3). For the Israeli and Palestinian families, haplotypes including 167delT are identical at D13S141, which is 38 kb from *GJB2*, and differ at D13S175, which is 70 kb from *GJB2*.

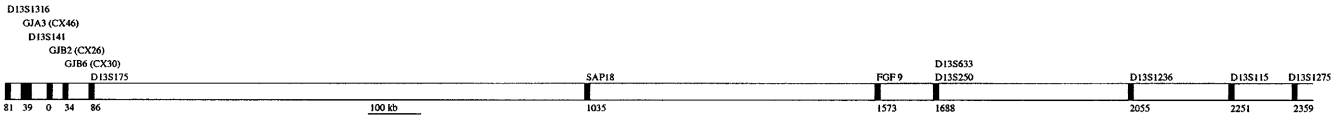

Fig. 2 Map of the chromosome 13 region including *GJB2*. Distances (in kb) between *GJB2* and flanking loci are indicated *below* the line

In order to explore the ancestry of *GJB2* mutations in the Palestinian and Israeli populations, we estimated linkage disequilibrium between 35delG and flanking SNPs, and 167delT and flanking SNPs. Haplotypes spanning 125 kb that could be unambiguously determined for Palestinian and Israeli deaf individuals are indicated in Table 2. The modal haplotype carrying the 35delG mutation is the same in Palestinian and Israeli populations. The three-SNP haplotype that comprises part of this extended 125 kb haplotype is relatively rare among controls in both populations: 23% among Palestinian hearing controls and 14% among Israeli hearing controls. In both Palestinian and Israeli populations, linkage disequilibrium values were significant (P<0.0001) between 35delG and the same alleles at –3558snp, D13S141 and D13S175 (Table 3).

Similarly, all Palestinian chromosomes with the 167delT mutation shared the same six-marker haplotype on chromosome 13q (Table 2). The Palestinian and Israeli 167delT haplotypes were identical at all markers except D13S175. In each population, 167delT mutation was in significant disequilibrium with allele 3 of D13S141, which is relatively uncommon among hearing controls (Table 4).

Fig. 3 Genotypes at markers flanking *GJB2* in Palestinian families S, L and D and Israeli family 10. *Filled symbols* Individuals with prelingual profound sensorineural hearing loss, *boxed haplotypes* chromosomes with the *GJB2* 167delT mutation. *GJB2* lies between D13S141 and D13S175. In families S and D, the shared 167delT haplotype spans more than 2.4 Mb, whereas in family 10, the shared 167delT haplotype spans only 125 kb. The 167delT haplotypes of all four families carry the same allele at D13S141, which is 38 kb from *GJB2*. (The other *GJB2* allele of the proband of family D is 235delC)

Discussion

Connexin 26 mutations are responsible for a substantial proportion (23%) of hereditary hearing impairment in Palestinian families. Diversity of mutations is high even within this small series; five different mutations appeared in eleven probands. However, nine of 11 deaf probands are homozygous for the same allele; the other two probands are compound heterozygotes for one common allele and one rare allele. Carrier frequency of these mutations in the general Palestinian population is low: 1 in 400 individuals. The low carrier frequency is consistent with the high frequency of deafness because the population is highly consanguineous.

Analysis of chromosome 13q haplotypes spanning the 125 kb *GJB2* region suggests a shared origin for a 35delG allele in Palestinian and Israeli populations. The modal haplotype surrounding *GJB2* 35delG is the same and, at three markers of this haplotype, the same alleles are in significant disequilibrium with the mutation in both populations. Analysis of haplotypes including 35delG from deaf individuals of other Mediterranean populations also suggests a single origin of this mutation (Rothrock et al. 2001). Furthermore, analysis of 35delG and SNPs (other than the ones used in our project) among deaf individuals from Belgium, Britain and America supports a common founder for this mutation (Van Laer et al. 2001).

The ancestry of *GJB2* 167delT is more difficult to determine, because the shared haplotype carrying 167delT is common in the general population. This mutation has only been reported previously among the Ashkenazi Jews (Morell et al. 1998; Sobe et al. 1999, 2000; Lerer et al. 2000). The hypothesis for a shared origin for 167delT among deaf Palestinians and Israelis is supported by significant disequilibrium of D13S141 allele 3 with 167delT in both Palestinian and Israeli populations. Because the SNPs comprising the 167delT haplotype shared by all the

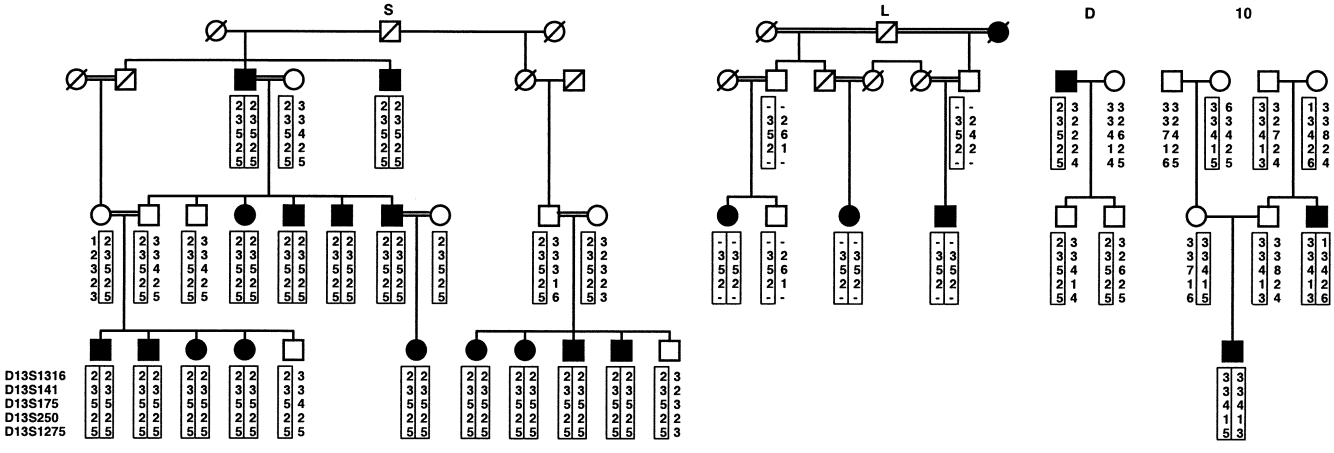

Table 2 Haplotypes of mutant *GJB2* alleles in Palestinian and Israeli deaf probands

D13S141	–3558	*GJB2*	+1946	+2034	D13S175	Chromosomes of deaf individuals	
						Palestinian	Israeli
3	C	35 del G	T	A	4	9	18
3	C	35 del G	T	A	2	1	0
3	T	35 del G	T	A	4	0	6
3	T	167 del T	T	A	5	7	0
3	T	167 del T	T	A	4	0	9
3	T	167 del T	T	A	8	0	1

Table 3 Linkage disequilibrium between *GJB2* mutation 35delG and flanking markers in Palestinian and Israeli populations (*ns* not significant)

Marker	Allele	Palestinians *GJB2*				Israelis *GJB2*			
		35 del G	Wildtype	Ψ	*P*	35 del G	Wildtype	Ψ	*P*
D13S141	3	10	20	1.0	<0.0001	24	32	1.0	<0.0001
	Other	0	172			0	156		
–3558	C	10	49	1.0	<0.0001	18	27	0.90	<0.0001
	T	0	143			6	165		
1946	T	10	169	1.0	ns	24	169	1.0	ns
	C	0	23			0	23		
2034	A	10	192	na[a]		24	183	1.0	ns
	G	0	0			0	9		
D13S175	4	9	16	0.98	<0.0001	24	54	1.0	<0.0001
	Other	1	172			0	130		

[a]Not applicable (*na*), because allele A at this SNP is monomorphic in the Palestinian population

Table 4 Linkage disequilibrium between *GJB2* mutation 167delT and flanking markers in Palestinian and Israeli populations (*ns* not significant)

Marker	Allele	Palestinians *GJB2*				Israelis *GJB2*			
		167delT	Wildtype	Ψ	*P*	167delT	Wildtype	Ψ	*P*
D13S141	3	7	20	1.0	<0.0001	10	32	1.0	<0.0001
	Other	0	172			0	156		
–3558	T	7	49	1.0	ns	10	165	1.0	ns
	C	0	143			0	27		
1946	T	7	169	1.0	ns	10	169	1.0	ns
	C	0	23			0	23		
2034	A	7	192	na[a]		10	183	1.0	ns
	G	0	0			0	9		
D13S175	4	0	16	1.0	<0.0001	9	54	0.91	<0.0001
	5	7	21			0	22		
	Other	0	153			1	108		

[a]Not applicable (*na*), because allele A at this SNP is monomorphic in the Palestinian population

deaf Palestinians and Israelis are the most common SNPs in both populations, they provide little additional information for assessing linkage disequilibrium. Alleles of D13S175 on 167delT haplotypes differ both between Israeli and Palestinian populations and within the Israeli population. Taken together, these observations seem to us most consistent with a single occurrence of 167delT in Middle Eastern antiquity, with subsequent mutation at D13S175 and/or recombination in the 86-kb region between *GJB2* and D13S175.

Connexin 26 is a member of a large family of gap-junction membrane proteins that mediate electrical and metabolic coupling between adjacent cells. Several findings support the importance of connexin 26 in auditory transduction. Immunohistochemical and structural analysis of the connexin 26 protein in the rat cochlea suggest that gap junctions in epithelial and connective tissue cells are involved in recycling endolymphatic potassium ions through nonsensory cells during the transduction of voltage gating in these channels (Kikuchi et al. 1995; Lautermann et al. 1999). In human families, the identification of mutations in *GJB2* in patients with autosomal recessive congenital deafness further supports the involvement of *GJB2* in normal auditory hearing processes (Kelsell et al. 1997).

Given the high frequency of *GJB2* mutations among Palestinian deaf children, the testing of *GJB2* among deaf children in the Middle East is worthwhile in order to pro-

vide special services at an early age. Furthermore, unnecessary invasive follow-up could be avoided, since *GJB2* mutations are not associated with syndromic hearing loss or with inner ear malformations (Cohn and Kelly 1999; Denoyelle et al. 1999).

Given the allelic diversity of *GJB2* mutations and the small size of the *GJB2* gene, this is best accomplished by amplifying both *GJB2* exons separately from genomic DNA and directly sequencing the entire 5' UTR and coding region of the gene. Mutations in *GJB2* account for a large percentage of hearing loss in the Palestinian population, making *GJB2* of primary importance in any program for understanding the genetics of deafness in the Middle East.

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