ARTICLE

Clinical evidence of the nonpathogenic nature of the M34T variant in the connexin 26 gene

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Mutations in *GJB2* are the most common cause of congenital nonsyndromic hearing loss. The controversial allele variant M34T has been hypothesized to cause autosomal dominant or recessive nonsyndromic hearing impairment and some *in vitro* data has been consistent with this hypothesis. In this report, we present the clinical and genotypic study of 11 families (seven familial forms of nonsyndromic sensorineural hearing loss (NSSNHL) and four sporadic cases) in which the M34T *GJB2* variant has been identified. The M34T mutation did not segregate with the deafness in six of the seven familial forms of NSSNHL. Eight persons with normal audiogram presented a heterozygous M34T variation and five normal hearing individuals were composite heterozygous for M34T and another *GJB2* mutation. Four normal hearing individuals with a documented audiogram were M34T/35delG and one was M34T/(*GJB6*-D13S1830)del. Screening a French control population of 116 subjects we have found an M34T allele frequency of 1.72%. This percentage was not significantly different from the prevalence of the M34T allele in the deaf population, which was 2.12%. All these data suggest that the M34T variant is not clinically significant in human and is a frequent polymorphism in France.

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Introduction

Hearing loss is the most frequent of the sensory defects. One child in 1000 at birth presents with a severe or a profound deafness and one of 1000 more becomes deaf before adulthood. Recent discoveries in human genetics indicate that more than 80% of congenital deafness is of genetic origin in developed countries.¹ The vast majority,

80–90%, of the nonsyndromic sensorineural hearing loss (NSSNHL) shows autosomal recessive transmission. Since 1994, more than 60 loci of NSSNHL have been identified and 28 different genes cloned.¹ One of them, *GJB2*, encoding connexin 26 is involved in the most frequent form of NSSNHL: DFNB1 (MIM 220290) on chromosome 13p12.² This gene has also been implicated in an autosomal dominant form of NSSNHL and in syndromic deafness.^{3,4}

More than 60 different *GJB2* amino-acid substitutions have been reported to date as recessive (DFNB1) and dominant (DFNA3) (MIM 601154) hearing loss alleles (see The Connexin-Deafness Homepage). One of these, M34T is a missense mutation affecting a methionine in the first transmembrane domain of *GJB2*, conserved in *GJB1* and *GJB2* but not in other β -connexins. To date the effect of the M34T mutation remains controversial. The M34T mutation was first detected in a dominant pedigree of NSSNHL.⁵ This observation was consistent with the results of the functional data using *Xenopus* oocytes, which show a dominant negative effect of the M34T mutant polypeptide.⁶ Further studies on the first family described by Kelsell have shown dermatological signs in deaf patients and isolated another dominant mutation in *GJB2* segregating with the disease.⁷ Several authors described normal hearing in M34T heterozygous carrier.^{8–10} The M34T mutation was also reported to be responsible for a recessive form of NSSNHL.^{11–13} *In vitro* data in mammalian Hela cells seem to demonstrate that M34T could disrupt the normal channel formation or oligomerisation.^{14–16} However, others and we have reported normal hearing subjects carrying a compound heterozygous mutation: M34T/35delG.^{10,17} Furthermore, Cucci *et al*¹⁸ argue that the M34T allele variant may be dependent on the mutations segregating in the opposite allele. The M34T substitution was also implicated in the phenotypic expression of association of a palmoplantar keratoderma (PPK) with deafness⁷ (MIM 124500).

In this study, we have screened affected patients and normal hearing controls from France for the M34T mutation. These analyses were performed in order to determine the pathogenic effect of the M34T variant and also to assess the frequency of this variant in the French population.

Materials and methods

Patients

We enrolled 188 unrelated French families that had members presented with a phenotype compatible with DFNB1 (NSSNHL bilateral, prelingual, with no cochleovestibular malformation, 110 sporadic and 88 autosomal recessive) and 116 normal hearing controls. Every control subject had normal audiometry and was questioned to eliminate familial history of deafness. In each deaf patient,

a complete medical history was obtained to determine the age of onset of the deafness and to exclude the possibility of environmental causes. The deaf subjects underwent an otoscopic examination in an ear nose and throat clinic and a general examination with systematic search for signs suggestive of a syndromic form of deafness in a genetic clinic. They also had an ophthalmologic evaluation, investigation for hematuria and proteinuria and an electrocardiogram. High-resolution temporal bone computed tomography was performed. Controls, deaf children and their parents underwent pure-tone audiometry with a diagnostic audiometer in a soundproof room, with recording of pure-tone air- and bone-conduction thresholds. Air-conduction pure-tone average (ACPTA) thresholds in the conversational frequencies (0.5, 1, 2 and 4 kHz) were calculated for each deaf ear and were used to define the severity of deafness. The protocol was accepted by the Committee for the Protection of Individuals in Biochemical Research as required by French legislation and informed consent was obtained from all patients or their parents.

Genetic analysis

Blood samples were obtained from one or more affected patient in each family and from the controls. Genomic DNA was isolated from whole blood using Quiamp DNA Blood kits (Quiagen S.A).

Patients were tested for *GJB2* mutations, by denaturing gel gradient electrophoresis analysis (DGGE) and direct sequencing of the coding exon, as described in detail elsewhere.^{2,19} When a monoallelic mutation of *GJB2* was found, a mutation in the first exon of *GJB2* was searched by direct sequencing. A specific PCR assay for the *GJB6*-D13S1830 deletion using the method described by Del Castillo was performed when no mutation or a monoallelic mutation was identified in the *GJB2*.²⁰ When the PCR was positive, the *GJB6*-D13S1830 deletion was confirmed by sequencing.^{2,19}

Results

Detection of M34T variant in deaf patients

A total of 79 (45 of the sporadic group, 34 of the autosomal recessive group) of the 188 unrelated French families, which had members presented with a phenotype compatible with DFNB1, had a *GJB2* mutation. In seven families (Figure 1, families no. 1–no. 7), one or more deaf patients had the M34T variant. In five of them (families no. 1–no. 4 and family no. 6), the M34T mutation was heterozygous. The M34T variation was homozygous in the two deaf brothers (family no. 7). In family no. 5, a sporadic case carried a composite heterozygous mutation: M34T/H100Y. In four families (family nos. 8, 9, 10), the variant M34T was detected only in hearing relatives of the hearing-impaired patients (four parents and one sister).

Figure 1 CX26 sequence variants in the 11 families. Hearing-impaired individuals are indicated by solid symbols, unaffected patients are symbolized with open symbols. The severity of the hearing impairment is indicated.

The M34T mutation did segregate with the deafness in only one (family no. 7) of the seven familial forms of NSSNHL (Figure 1). Eight persons with normal audiogram (seven parents and one brother of a deaf child) had a heterozygous M34T variation (Figure 2). A composite heterozygous mutation (M34T/35delG or M34T/(GJB6-D13S1830)del) was found in five normal hearing individuals (four parents and one sister of a deaf child) (Figures 1 and 2). In three pedigrees (families nos. 1, 3, 6), the genotype was not identical in the hearing-impaired relatives of the same family (Figure 1).

Detection of M34T variant in control patients

A heterozygous M34T mutation was identified in four of the 116 unrelated control patients showing a normal audiogram without any family history of deafness. The frequency of the M34T heterozygous hearing patients was

3.44% (4/116 cases) and the frequency of the mutated allele in the French population was 1.72% (4/232 alleles). These percentages are not significantly different from the frequency of M34T variant in the deaf population (3.72% or 7/188 families *versus* 4/116, $P > 0.05$) or from the frequency of the allele in the hearing-impaired population (2.12% (8/376) alleles *versus* 1.72% (4/232), $P > 0.05$).

Discussion

To date more than 60 GJB2 mutations have been described in deaf patients in the literature. If the pathogenicity of the truncating mutations is highly probable, the pathogenicity of missense mutations is more difficult to determine. It is difficult to differentiate a frequent polymorphism from a pathogenic mutation. We reported here

Figure 2 Audiometric curves of (a) the hearing father of family no. 2 (M34T/WT); (b) the hearing father of family no. 8 (35delG/M34T); (c) the hearing mother in family no. 9 ((35delG/M34T); (d) the hearing father in family no. 10 (M34T/delG/B6). (e) In family no. 11: 1. the hearing father (35delG/M34T) (air-conduction thresholds equal in both ears), 2. the hearing sister (35delG/M34T), 3. The deaf brother (35delG/35delG). The circles represent the air-conduction thresholds of the right ear and the lozenges the air-conduction thresholds of the left ear.

evidence for the nonpathogenic character of the M34T variant and its high frequency in the normal hearing population.

M34T variant was first described as an autosomal dominant mutation,⁵ based on its identification in patients affected both with hearing loss and PPK in a family segregating both diseases. Later, Kelsell *et al*⁷ identified in the same family a heterozygote D66H mutation in *GJB2* in trans of the M34T mutation and a heterozygote mutation R32W in *GJB3*. Later, the D66H was demonstrated to be responsible for PPK and hearing defect.²¹ On the other hand, Kelsell *et al*⁷ proposed that the M34T variant of *GJB2* and the R32W mutation of *GJB3* may contribute to the severity of the skin and hearing diseases. However, the R32W variant of *GJB3* has been demonstrated not to be the cause of deafness or skin disease in Spanish patients, even in association with *GJB2* mutations.²² In his report, Kelsell *et al* did not consider that the difference of the severity of the hearing defect could be age dependent as in an other family also carrying D66H²¹ in which the adults members suffered from moderate to severe hearing loss and the children were only mildly affected. The pedigree of Kelsell's family is consistent with this hypothesis because the younger patient (III3) has PPK without deafness. The expression analysis in Hela cells^{14,15} suggested a functional role for M34T as a dominant allele. However, the normal audition of heterozygous M34T carriers, in the general population and in the siblings of deaf patients, like our series and in other reports,^{8–10} indicate that this variation is not sufficient to cause a dominant deafness in these patients. D'Andrea *et al*¹⁵ argue that these clinical observations could be explained by the presence of a second site mutation that compensates the effects of the M34T variant. This hypothesis could not explain the difference of genotypes observed in the patients of two families reported here, in which the hearing loss is transmitted with an autosomal dominant mode (families no. 2 and 6). In these two families, the most probable hypothesis is that *GJB2* is not involved in the phenotype. We cannot exclude a transmission of an unknown mutation of *GJB2* acting with a recessive mode in family no 2 without DFNB1 linkage analysis, but it would imply that two different *GJB2* undetected mutations are present in the deaf child and grandfather.

An autosomal recessive role has also been attributed to the M34T variant, because of its occurrence with other *GJB2* mutations or as homozygous in deaf individuals.^{12,11,18,10,23,13,24} However, as Griffith *et al*¹⁰ we reported here five normal hearing subjects carrying a compound heterozygous mutation in which the M34T substitution is associated in trans with a truncated mutation: the 35delG *GJB2* mutation or the (*GJB6*-D13S1830)del.²⁰ These results indicate that M34T is not a recessive mutation responsible for hearing loss. Cucci *et al*¹⁸

proposed that the effect of the M34T allele may be dependent on the mutation present in trans. However, his hypothesis could not explain the absence of hearing defect in the case of a segregation with frameshift mutations. Indeed, we presented here four normal hearing individuals with M34/35delG and for the first time a normal hearing person with M34T/(*GJB6*-D13S1830)del. Moreover, Cucci report two brothers presenting with the same hearing defect with different genotypes: two siblings carrying the same genotype, but with defects of different severity.¹⁸ All these observations are not consistent with a variant role of M34T according to the mutation segregating on the opposite allele. Two recent reports have found a 10 base pairs deletion in the 5'UTR of *GJB2* that occurs together with the M34T mutation.^{11,24} The last one²⁴ have demonstrated a linkage disequilibrium between these two variants. However, in several families reported in these two studies,^{11,24} neither the M34T nor the –493del10 segregated with the deafness. These observations argue against the pathogenic character of these two variants. The presence of deaf patients homozygous for the M34T variant, as in our report (family no. 7) and in others,^{11,24} should not be considered as an argument for the pathogenicity of this variant because if M34T is a frequent polymorphism, it could be fortuitously present in a homozygous state in some patients.

The similar prevalence of the M34T between the deaf and the hearing population observed in this report and in other¹⁸ is in favor of the absence of pathogenic effect of this variant. The frequency of the M34T allele in the French control population reported here (1.72%) is consistent with prevalence found in other populations:²⁵ 1.5% in the USA,⁸ 2.4% in Belgium,²⁶ 1.98% in the UK,¹¹ 1.33% in the Grampian region.²⁷ In another way, this variant seems to be rare in Asia.^{28,29}

The results of functional studies of M34T mutation in various types of cells are discordant. In *Xenopus* oocytes, the M34T mutant polypeptide appeared to have a dominant negative effect on the intercellular coupling.⁶ Expression analysis in Hela cells showed that the M34T mutant has a strong membranous localization, but it fails to assemble efficiently into hexameric gap junction hemichannels and impaired intercellular coupling.^{14,16} An inhibition of the wild-type *GJB2* channel activity by the M34T mutant was observed by D'Andrea *et al* when the two proteins are coexpressed.¹⁵ However, the very recent experiments performed by Oshima *et al* in Hela cells showed that cells expressing M34T connexins formed normal gap junction plaques and a normal permeability in the dye transfer assays.³⁰ As our data show that the M34T variant is not a cause of deafness in our patients, these more recent *in vitro* results support our conclusion that the M34T mutation is not pathogenic.

Conclusion

The molecular diagnosis of *GJB2* abnormalities has improved genetic counseling for hearing loss. Our data seem to demonstrate that the M34T variant has no phenotypic effect in humans. These results have important implications for genetic counseling. As M34T could not be considered as responsible for the hearing defect, another mutation has to be screened in the first *GJB2* exon or in *GJB3* or in another gene, when this variant is associated with a *GJB2* pathogenic mutation in a deaf patient.

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