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Linkage Analysis of Progressive Hearing Loss in Five Extended Families Maps the DFNA2 Gene to a 1.25-Mb Region on Chromosome 1p

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Thus far, 13 genes for autosomal dominant hearing loss have been localized to specific chromosomal regions, but none of the genes has been cloned. Only a single family has been linked to each of these loci, with the exception of DFNA2. DFNA2 was originally mapped in two extended families originating from Indonesia and the United States. In this study we report linkage to DFNA2 in three additional large families with autosomal dominant hearing loss from Belgium and The Netherlands. These five DFNA2 families show a similar progressive sensorineural hearing loss, starting in the high frequencies and also affecting the middle and low frequencies later in life. Combining the information from all linked families, the candidate region that is most likely to contain the DFNA2 gene was reduced to a 1.25-Mb region between markers D1S432 and MYCL1. Different haplotypes segregating with the hearing loss were found in all five families, suggesting that different mutations are present in the same gene. These results indicate that DFNA2 is most likely an important gene for autosomal dominant hearing loss.

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INTRODUCTION

Hearing loss is the most frequent condition limiting communication between humans. It has been estimated that approximately 70 million people worldwide have a hearing loss of at least 55 dB (Wilson, 1985). Loss in hearing acuity of at least 25 dB has been reported in approximately 1% of young adults and in-

creases markedly with age to approximately 10% at age 60 and 50% at age 75 or older (Morton, 1991). Progressive hearing loss of the elderly (presbycusis) is probably due to a multifactorial interaction of genetic and environmental factors. In some cases, progressive postlingual hearing loss is monogenic, and many autosomal dominant families with this type of hearing loss have been described (Cohen and Gorlin, 1995). By identifying genes responsible for monogenic hearing loss, more insight may be gained into the molecular process of hearing and the pathology of hearing loss, which may help in understanding the complex multifactorial process of presbycusis.

Initially, localization of genes responsible for nonsyndromic hearing loss has been very slow. To date, however, 26 genes have been localized to specific autosomal regions by linkage analysis. These genes have been classified in two categories: DFNA for autosomal dominant hearing loss and DFNB for autosomal recessive hearing loss. Nearly all DFNA families generally have progressive hearing loss that is often mild at a younger age, while most of the DFNB families have a profound congenital deafness. A list of all published loci, including detailed genetic and clinical information, can be found on-line in the Hereditary Hearing Loss Homepage (<http://www-dnalab.uia.ac.be/dnalab/hhh/>).

Recently, we mapped a gene for autosomal dominant sensorineural hearing loss starting in the high frequencies (DFNA2) to chromosome 1 in an extended family originating from Indonesia, and we found that the same gene is responsible for hearing loss in a family from the United States (Coucke *et al.*, 1994). Here we report three additional large families from Belgium and The Netherlands with a similar type of progressive hearing loss, linked to DFNA2. Combining the informa-

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tion from all five linked families, we were able to reduce the region that most likely contains the DFNA2 gene to a 1.25-Mb region between genetic markers D1S432 and MYCL1.

MATERIALS AND METHODS

Clinical diagnosis. Otologic examination and pure tone audiometry with air and bone conduction were performed on every family member who participated in this study, and blood samples were taken for DNA extraction. The audiometric curves of all family members were compared with age- and sex-dependent percentile curves (International Organization for Standardization, 1984). Family members were considered affected if they had a sensorineural hearing loss with an audiometric curve below the 95th percentile (P95) of the reference curves for most frequencies for both ears. Family members were considered unaffected if hearing thresholds at most frequencies were better than 20-dB hearing level or above the 50th percentile. Normal-hearing individuals below the age of 35 for the Belgian family, below 40 for Dutch family 1, and below 25 for Dutch family 2 were given an unknown disease status in the linkage analysis. Persons with an atypical or doubtful audiogram, or with a history of environmental factors that could be implicated in their hearing loss, were also given an unknown disease status. In a few cases, the family history of married-in individuals revealed a possible hereditary hearing loss. These individuals and their children were also given an unknown affection status.

Belgian family. A family pedigree consisting of approximately 150 family members over 4 generations was constructed. Audiometry was performed and blood samples were obtained from 65 family members. Twenty-seven family members were diagnosed as affected. In most affected family members between 10 and 30 years of age, hearing was impaired only at frequencies above 1000 Hz. Above the age of 30, a loss below 1000 Hz generally became apparent, although this was in some cases not below the P95 of the reference curves. The average progression rate of the hearing loss in this family was much higher above 1000 Hz (between 2 and 5 dB per year) than below 1000 Hz (between 0.2 and 0.5 dB per year).

Dutch family 1. A pedigree comprising more than 250 family members in 6 generations was constructed. Eighty-eight family members were tested audiometrically, and blood samples were obtained from all of them. Forty-one family members were diagnosed as affected. In all affected persons, a hearing loss that was most pronounced in the high frequencies and that showed a progress of approximately 1 dB per year could be demonstrated.

Dutch family 2. A pedigree of over 400 family members in 6 generations was reconstructed. Eighty-four of these were tested audiometrically, and blood samples were obtained from them. Twenty-four of them were diagnosed as affected. In this family a progressive hearing loss starting in the high frequencies, which was very similar to Dutch family 1, was found.

Microsatellite analysis. Microsatellites were typed according to standard procedures. Allele sizes were determined by comparison with the known genotype of CEPH individual 134702 (Dib *et al.*, 1996). D1S432 is a microsatellite located 5 cM centromeric from D1S255 and 2 cM telomeric from D1S193 on the Génethon genetic map (Dib *et al.*, 1996). On the physical map of Hellsten *et al.* (1995), the tetranucleotide repeat marker MYCL1 is located close to D1S193, on the telomeric side. Recombinants in several families confirmed the order D1S255–D1S432–MYCL1–D1S193 (data not shown). As D1S432 and MYCL1 are not accurately positioned on the same genetic map, the exact genetic distance between them remains unknown. However, D1S432 and MYCL1 are separated by less than 1.25 Mb from each other, as we identified two CEPH YACS (901_a_1 and 935_e_1) with lengths of 1.25 and 1.8 Mb, respectively, that contain both markers. Detailed information for the genetic markers used in this study can be found in the Genome Database (Fasman *et al.*, 1996). A map showing the relative localization of these four genetic markers flanking DFNA2 is shown in Fig. 1.

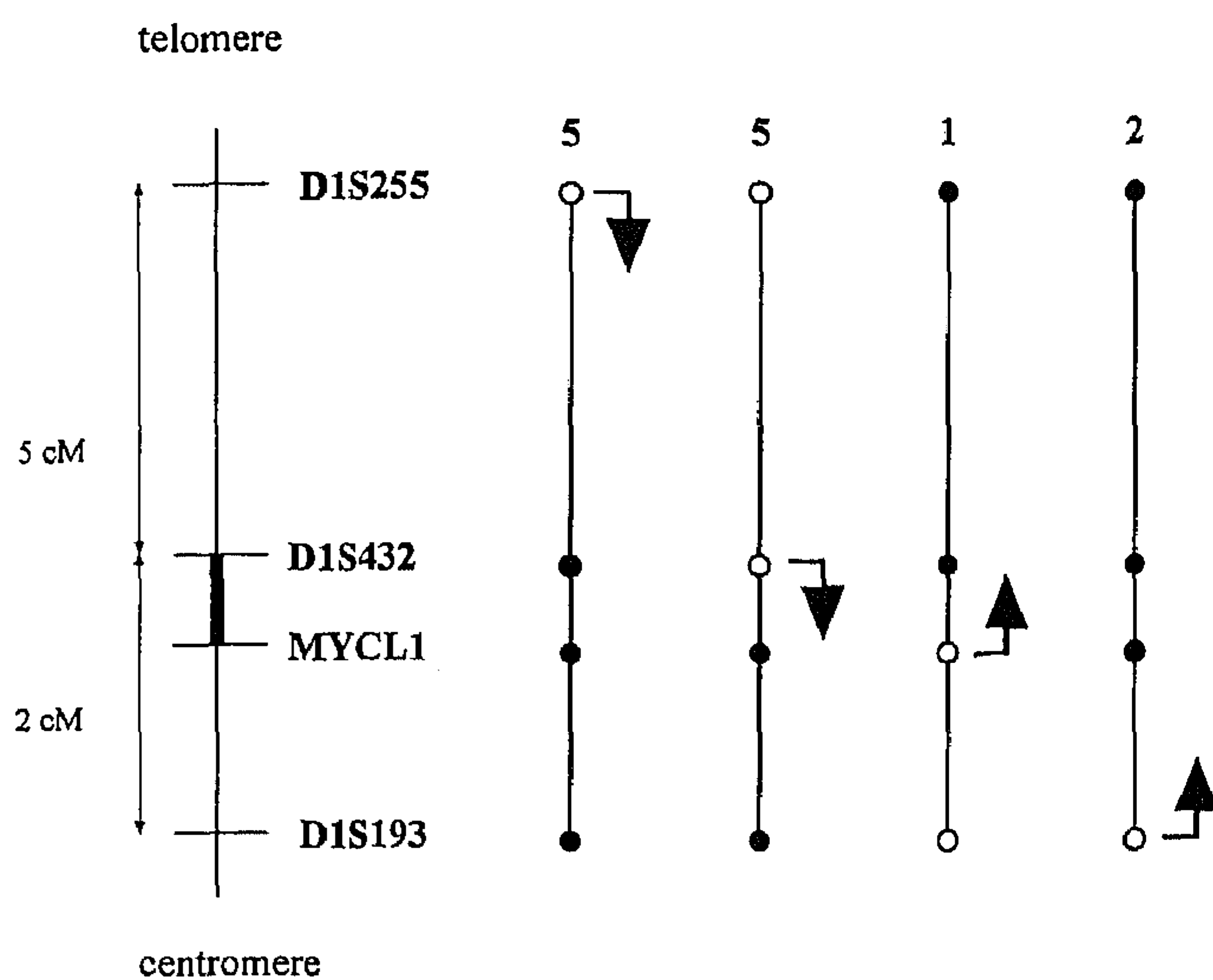


FIG. 1. **Left:** Map of genetic markers flanking DFNA2. The DFNA2 candidate region between D1S432 and MYCL1, as indicated by the key recombinations, is indicated in bold. Both markers are located at a distance less than 1.25 Mb from each other. **Right:** Schematic representation of the chromosomes with key recombinations between DFNA2 and closely linked markers. Informative alleles are represented by circles: the circles are filled in the region containing the DFNA2 gene and open in the regions from which DFNA2 is excluded based on this key recombinant. Arrows indicate the position of DFNA2. The number above the chromosomes indicates how often this type of recombination was observed.

Linkage parameters. Linkage calculations were performed using the Linkage program, Version 5.1 (Lathrop and Lalouel, 1984), and the Fastlink programs (Cottingham *et al.*, 1993). The frequency of hearing loss due to mutations in DFNA2 was set at 1/10,000. Penetrance was set at 98%, and the chance that family members without a mutation in DFNA2 showed hearing loss was set at 2%, to allow for possible phenocopies. Recombination frequencies were assumed to be equal for males and females. For each marker the number of alleles in the lod score calculations was set at the observed number of alleles in the pedigree (N), and allele frequencies were set equal at $1/N$. Changes in the allele frequencies resulted only in minor alterations of the two-point lod scores and did not alter the conclusions of the study.

RESULTS

Linkage Analysis

Positive linkage to the short arm of chromosome 1 had been demonstrated previously in an Indonesian and an American family with autosomal dominant non-syndromic progressive hearing loss (DFNA2) with markers D1S201, D1S255, D1S193, and D1S211 (Coucke *et al.*, 1994).

In this study we identified three additional families with a similar type of hearing loss linked to DFNA2 and analyzed two new markers from the DFNA2 region, D1S432 and MYCL1. The localization of these markers relative to the markers used in the original study is given in Fig. 1. Two-point lod scores for MYCL1 and D1S432 in all five DFNA2 families are given in Table 1. Positive lod scores over +3.0 were obtained for all

TABLE 1

**Two-Point Lod Scores for Linkage between Hearing Loss and Microsatellite Markers
on Chromosome 1p in Five Different Families**

Locus	Family	Lod score at $\theta =$						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
D1S432	Indonesian	10.92	10.72	9.91	8.85	6.61	4.24	1.89
	American	3.53	3.61	3.59	3.33	2.55	1.62	0.65
	Belgian	6.35	6.23	5.73	5.08	3.71	2.29	0.95
	Dutch 1	0.44	3.27	4.89	5.14	4.39	3.08	1.55
	Dutch 2	4.10	4.17	4.13	3.85	3.03	2.00	0.86
	Total	25.34	28.00	28.25	26.25	20.29	13.23	5.90
MYCL1	Indonesian	6.88	6.90	6.62	6.03	4.53	2.87	1.25
	American	7.16	7.03	6.52	5.86	4.44	2.91	1.33
	Belgian	4.58	4.63	4.50	4.09	3.02	1.82	0.76
	Dutch 1	8.72	8.85	8.70	8.05	6.28	4.26	2.12
	Dutch 2	6.69	6.73	6.54	6.06	4.78	3.23	1.47
	Total	34.03	34.14	32.88	30.09	23.05	15.09	6.93

families, giving conclusive evidence of linkage in each of the families.

Key Recombinants

In our previous study (Coucke *et al.*, 1994), a 6-cM candidate region for DFNA2 was defined between markers D1S255 and D1S211. This candidate region was delineated by a single key recombinations in the American family with D1S255 on its telomeric side and by a single key recombinant in the Indonesian family with D1S211 on its centromeric side. D1S193 located 1 cM distal to D1S211 was uninformative in the Indonesian recombinant. To reduce this candidate region, the new markers D1S432 and MYCL1 were analyzed in the key recombinants from the Indonesian and American families. The Indonesian key recombinant with marker D1S211 also recombines with MYCL1, localizing DFNA2 telomeric of MYCL1. Analysis of the American key recombinant places DFNA2 centromeric of D1S432. Both key recombinants reduce the DFNA2 candidate region to the interval between D1S432 and MYCL1, with a single recombinant on both sides.

To confirm this candidate region, the most likely disease haplotype for D1S255, D1S432, MYCL1, and D1S193 was constructed in the three new DFNA2 families. This revealed five recombinations in the region between D1S255 and D1S432, localizing DFNA2 centromeric of D1S255. Four recombinations in the region between D1S432 and MYCL1 in Dutch family members (three affected individuals in family 1 and one unaffected individual in family 2) localize DFNA2 centromeric of D1S432, confirming the recombinant in the American family. Three recombinations in the region between D1S193 and MYCL1 localize DFNA2 telomeric of D1S193. The Indonesian recombinant localizing DFNA2 telomeric to MYCL1 was not confirmed by other recombinants in the three new families. No key recombinants were present in the Belgian family. Combining all five DFNA2 families, a total of six key

recombinants indicate that the DFNA2 gene most likely lies between D1S432 and MYCL1 (Fig. 1).

Phenocopies

Surprisingly, one individual from the Belgian family and two individuals from Dutch family 1 diagnosed as affected by audiometry did not inherit the disease haplotype from their affected parent but inherited the normal haplotype. A double recombination between DFNA2 and the two informative flanking markers D1S432 and MYCL1 is unlikely as these two markers are very closely linked. Therefore, the hearing loss in these three individuals most likely represents a phenocopy. Their medical history did not disclose any environmental factor that might explain their hearing loss, and reevaluation of the audiograms revealed no misdiagnosis. Most likely, another gene or nongenetic factors are responsible for the hearing loss in these three patients. In the other three families, no phenocopies were found.

Haplotype Comparison

We investigated the possibility that some of the five DFNA2 families are related to each other by comparing the disease haplotypes of the five DFNA2 families. Five different linked haplotypes were found. For the two closest flanking markers D1S432 and MYCL1, respectively four and five different linked alleles are present in the five families. It has been calculated that a region of 1 cM remains identical by descent between two descendants of a common ancestor for more than 15 generations (meiotic count of 30) with a probability of 95% (te Meerman *et al.*, 1994). Therefore, it is unlikely that any pair of the five DFNA2 families is related up to 15 generations ago, with the exception of the American-Dutch 2 pair, who have the same allele for D1S432. However, it is impossible to know whether the allele for D1S432 in these two families is identical by descent

or just by chance. Overall, it is likely that most of the five DFNA2 families carry mutations in the DFNA2 gene that have arisen on separate occasions.

DISCUSSION

In this study we describe very conclusive linkage of three new families with nonsyndromic progressive hearing loss starting in the high frequencies to the DFNA2 locus on chromosome 1p. These three families originate from Belgium and The Netherlands, and together with the Indonesian and American family that we reported earlier, they bring the total of families linked to DFNA2 to five.

Six key recombinants in these five families reduce the DFNA2 candidate region to a small interval between D1S432 and MYCL1. However, it must be kept in mind that only a single proximal recombinant with MYCL1 was found up to now. The possibility that this is not a true recombinational event but a phenocopy cannot be excluded, as this patient has no affected children and phenocopies for this type of deafness are not uncommon. Therefore, a more conservative candidate region for DFNA2, based on at least two recombinants on each side of the DFNA2 gene, would be the 3-cM interval between D1S432 and D1S193. Highly polymorphic markers are present on both sides of this interval, providing a very reliable predictive and diagnostic DNA test that is now available for families in which hearing loss is linked to DFNA2. Early presymptomatic diagnosis based upon DNA analysis can be important in career planning and school choice. Apart from its predictive value in young individuals at risk to develop hearing loss, the DNA test also has a confirmatory value in cases with an uncertain audiometric diagnosis. In addition, the DNA test can uncover incorrect audiometric diagnoses, as illustrated by the presence of three phenocopies in this study.

The presence of three phenocopies in these five DFNA2 families is not unexpected for two reasons. First, a total of 138 patients from these five families were included in our study. Second, hearing impairment of the high tones is very frequent, especially in the older population. To differentiate between hearing loss caused by DFNA2 and age-related hearing loss caused by other factors, we have used age- and sex-dependent reference curves for hearing thresholds. Family members were only considered affected if they had hearing thresholds below the P95 of the curves for several frequencies. However, as 5% of the population has a hearing threshold below the P95 threshold, applying only this criterium could lead to up to 5% phenocopies in the clinical diagnosis. Therefore, anamnestic data, such as noise exposure, and the careful comparison of the audiometric curves for all family members were also used to reduce the number of possible phenocopies. In case of doubt, family members were given an unknown affection status. However, even by applying these strict criteria, phenocopies can never be

eliminated, as some of these phenocopies are phenotypically indistinguishable from hearing loss caused by mutations in the DFNA2 gene.

As the DFNA2 flanking markers, D1S432 and MYCL1, colocalize to the same YAC of 1.25 Mb, this region is amenable to positional cloning of the DFNA2 gene. Mycl1, the mouse homologue of human MYCL1, is located on mouse chromosome 4, and a large region surrounding Mycl1 has syntenic homology to human chromosome 1p. Therefore, the human homologues of genes located close to Mycl1 in the mouse, such as gap junction gene Gja5, colony-stimulating factor granulocyte receptor Csfr, and the collagen gene Col8a2 (Copeland *et al.*, 1993), may be a source of candidate genes for DFNA2. Although several mouse mutations that affect hearing are located on mouse chromosome 4, none of them is located in the vicinity of Mycl1 (Nadeau *et al.*, 1991).

The five families linked to DFNA2 have a similar type of progressive hearing loss, starting in the high tones and progressing with age to include middle and lower frequencies. Nevertheless, statistical analysis of the audiograms shows significant differences in the progression rate of the hearing loss between some of the families from Western Europe (data not shown). This variability suggests allelic heterogeneity, the various families having a different mutation in DFNA2, which is also confirmed by the finding of a different haplotype segregating with hearing loss in each of the five linked families. Many independent recombinations would be necessary to change the haplotypes from all these families into a single haplotype from a hypothetical ancestral founder chromosome. The occurrence of all these hypothetical recombinations, including a number of recombinations in the 1.25-Mb region between D1S432 and MYCL1, would require a large number of meioses. Although a common ancestor for all families can never be formally ruled out, it is more likely that different mutations have occurred in the same gene. This suggests that DFNA2 is an important gene for autosomal dominant hearing loss.

We have tested a total of 21 large families with autosomal dominant hearing loss for linkage with DFNA2 (data not shown), and 5 of them were linked, strongly suggesting that the DFNA2 gene is frequently involved in hereditary progressive hearing loss. The type of hearing loss in the families linked to DFNA2 is similar to that of presbycusis in view of its sensorineural origin and progressive evolution first affecting the high tones. Therefore, DFNA2 might be involved in the pathogenesis of age-related hearing loss, and the DFNA2 families may serve as a model for studying mechanisms leading to presbycusis.

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