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Retinitis Pigmentosa and Progressive Sensorineural Hearing Loss Caused by a C12258A Mutation in the Mitochondrial MTTS2 Gene

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Summary

Family ZMK is a large Irish kindred that segregates progressive sensorineural hearing loss and retinitis pigmentosa. The symptoms in the family are almost identical to those observed in Usher syndrome type III. Unlike that in Usher syndrome type III, the inheritance pattern in this family is compatible with dominant, X-linked dominant, or maternal inheritance. Prior linkage studies had resulted in exclusion of most candidate loci and >90% of the genome. A tentative location for a causative nuclear gene had been established on 9q; however, it is notable that no markers were found at zero recombination with respect to the disease gene. The marked variability in symptoms, together with the observation of subclinical muscle abnormalities in a single muscle biopsy, stimulated sequencing of the entire mtDNA in affected and unaffected individuals. This revealed a number of previously reported polymorphisms and/or silent substitutions. However, a C→A transversion at position 12258 in the gene encoding the second mitochondrial serine tRNA, MTTS2, was heteroplasmic and was found in family members only. This sequence change was not present in 270 normal individuals from the same ethnic background. The consensus C at this position is highly conserved and is present in species as divergent from *Homo sapiens* as vulture and platypus. The mutation probably disrupts the amino acid–acceptor stem of the tRNA molecule, affecting aminoacylation of the tRNA and thereby reducing the efficiency and accuracy of mitochondrial translation. In summary, the data presented provide substantial evidence that the C12258A mtDNA mutation is causative of the disease phenotype in family ZMK.

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Introduction

Retinitis pigmentosa (RP) describes a genetically and clinically heterogeneous group of disorders that are characterized by gradual degeneration of photoreceptor cells, often to the point where the patient is legally blind. Common clinical features include a progressive loss of night vision, leading to night blindness; peripheral-visual-field loss; abnormalities present on electroretinography; tunnel vision; optic-disk pallor; attenuation of the retinal blood vessels; intraretinal pigment deposition; and progressive visual handicap (Humphries et al. 1990, 1992; Wright 1992; Dryja and Li 1995; Kenna et al. 1997). RP can present either alone or as a syndromic disorder. A syndrome incorporating both RP and various degrees of sensorineural hearing loss was first described by Charles Usher in 1914 (Usher 1914). Usher syndrome is inherited in an autosomal recessive fashion, is genetically and clinically heterogeneous, and has been divided into three major categories based on (*a*) whether the hearing loss is congenital or progressive, (*b*) presence or absence of vestibular involvement, (*c*) severity of symptoms, and (*d*) age at onset (Kimberling et al. 1995). Usher syndrome type I is characterized by profound congenital deafness, absent vestibular responses, and onset of RP by age 10 years (Kimberling et al. 1995). Loci for Usher syndrome type I have been localized to chromosomes 14q (USH1A [MIM 276900]; Kaplan et al. 1992), 11q (USH1B [MIM 276903]; Smith et al. 1992), 11p (USH1C [MIM 276904]; Kimberling et al. 1992), 10q (USH1D [MIM 601067]; Wayne et al. 1996), and 21q21 (USH1E [MIM 602097]; Chaib et al. 1997). Usher syndrome type 1B on 11q has been shown to be caused by mutations in the myosin VIIA (MYO7A) gene (Weil et al. 1995). Patients with Usher syndrome type II have normal vestibular responses, onset of RP by the late teens, and moderate to severe congenital deafness (Kimberling et al. 1995). Usher syndrome type II has been linked to chromosomes 1q41 (USH2A [MIM 276901]; Kimberling et al. 1990) and 5q (USH2B [MIM 276905]; W. J. Kimberling, personal communication). Mutations in USH2A have been found in the USH2A gene on 1q41

(Eudy et al. 1998). Usher syndrome type III is characterized by progressive onset of hearing loss and RP at puberty or later and is prevalent in Finland (Karjalainen et al. 1989); it has been localized to chromosome 3q ([MIM 276902] Sankila et al. 1995; Joensuu et al. 1996). With the exception of myosin VIIA and USH2A, the genes causing the remaining forms of Usher syndrome remain to be identified.

Family ZMK, previously referred to as "ZMK-92" (Kumar-Singh et al. 1993*a,* 1993*b*) and "TCD ZMK-92" (Kenna et al. 1997) is an Irish kindred that segregates progressive sensorineural hearing loss and RP. Although the pattern of symptoms observed strongly resembles that noted in Usher syndrome type III (Kenna et al. 1997), family ZMK is unique in that the pattern of inheritance is compatible with autosomal dominant, X-linked dominant, and maternal transmission of the disease locus (fig. 1). Extensive linkage analysis had excluded the majority of loci shown to be involved in various forms of RP, Usher syndrome, and some forms of hearing loss and had resulted in the identification of a putative linkage on the q terminal region of chromosome 9 (Kumar-Singh et al. 1993*a,* 1993*b;* Kenna et al. 1997). LOD scores obtained on 9q marginally exceeded the 3.00 threshold of significance, with markers D9S118, D9S121, and ASS (Kenna et al. 1997). However, the putative disease locus could not be clearly defined, owing to both a lack of mapped markers in this region of the genome at the time and the fact that all three critical markers were unmapped with respect to markers on the Généthon map (Gyapay et al. 1994). We have since mapped the critical markers and have used this map to perform extensive multipoint analyses extending over the entire 9q region. In addition, the clinical status of one family member has altered from unaffected to affected. Results from multipoint analysis and haplotyping indicate that the disease gene in ZMK is now excluded from this region of 9q. Additionally, extensive linkage studies with this family have resulted in exclusion of >90% of the human nuclear genome. The possibility of a large-scale chromosomal abnormality was also ruled out by high-resolution karyotyping (Kenna et al. 1997). Given extensive exclusion of the nuclear genome, a wide variation in disease symptoms experienced by family members, and abnormal findings from a single muscle biopsy, it was decided to screen all 16,569 bp of the mitochondrial genome, for mutations in affected members of this kindred (Kenna et al. 1997). We present evidence that a novel C→A transversion at position 12258 in the second mitochondrial serine tRNA gene (MTTS2) is responsible for the progressive hearing loss and RP observed in members of this family.

Patients and Methods

Patients

The assessment, diagnosis, and clinical attributes of the family have been described elsewhere (Kumar-Singh et al. 1993*a,* 1993*b;* Kenna et al. 1997). Blood samples were obtained from 29 family members, who were clinically assessed, and from 5 spouses of affected persons. Informed consent was obtained from all persons who participated in this study. DNA was extracted from whole blood, according to standard methods. One individual underwent a quadriceps femoris muscle biopsy. One half of this sample was used for electron microscopy as reported elsewhere (Kenna et al. 1997), whereas DNA from the other half was extracted as follows. Muscle tissue was ground, in liquid nitrogen, in a mortar and pestle until reduced to fine powder. The powder was transferred to a tissue homogenizer, with 10 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg of proteinase K/ml [Boehringer Mannheim])/100–200 mg of tissue. After homogenization, the sample was shaken overnight at 37°C. Three phenol extractions were performed, and the sample was precipitated with 1/10 vol of 7.5 M ammonium acetate and 2 vol of 95% ethanol chilled to −20°C. The DNA was spun down at 1,200 *g* and was washed twice with 70% ethanol. The pellet was air-dried and resuspended in sterile dH_2O.

Controls

The 270 control DNAs screened for the 12258 mutation were obtained from a panel of unaffected individuals (provided by Dr. L. Mynett-Johnson) from the same ethnic background as the ZMK family.

Linkage Studies

Primer sequences for microsatellite amplification were obtained from the 1994 and 1996 Généthon maps (Gyapay et al. 1994; Dib et al. 1996) and from Genome Database. Markers were amplified by PCR with the incorporation of $\alpha[^{32}P]$-dATP or $[^{32}P]$-dCTP label and then were electrophoresed on 8% denaturing acrylamide gels. Microsatellite data were prepared for analysis using the Linksys version 4.11 data-management package (Attwood and Bryant 1988). Two-point linkage analysis was performed by LIPED. Files for multipoint analysis were generated by LINKSYS and MAKEPED. Multipoint analyses were performed by LINKMAP from the FASTLINK package, version 3.0p (Cottingham et al. 1993; Schäffer et al. 1994). The frequency of the disease phenotype was set at .0001, whereas the frequency of the normal phenotype was set at .9999. One-hundred-percent penetrance was assumed, although some analyses

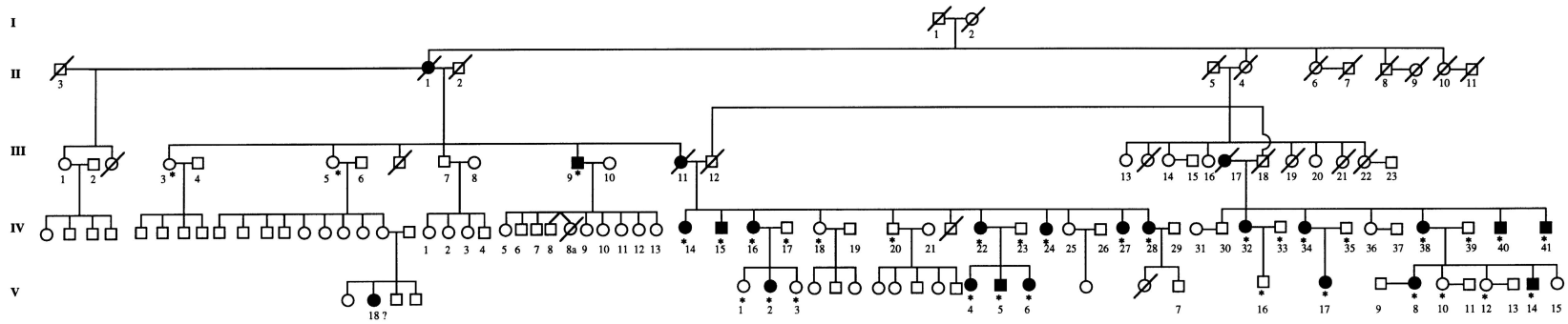

Figure 1 Family ZMK. Individuals denoted by an asterisk (*) have been clinically assessed in detail and have donated DNA samples. The individual denoted by a question mark (?), V-18, is the affected granddaughter of an apparently unaffected individual (III-5). Generation numbers are given on the far left side, and numbers denoting individuals are given below the pedigree symbols.

were completed by use of affected family members only. Frequencies of marker alleles were assumed to be equal. Multipoint analyses were performed under sex-specific conditions. Since there is no confirmed affected-male-to-offspring transmission in this pedigree, it was considered important to take the greater female recombination rates into account. Multipoint analyses were performed by use of marker distances obtained from the LDB maps (Collins et al. 1996*a*), except for chromosome 9, for which the marker distances were obtained from our own map (fig. 2). The chromosome 9 map was generated by CRI-MAP, version 2.4 (Lander and Green 1987). Genotype data used to build the map were, in the case of markers D9S121 and D9S118, generated, by us, from a subset of the CEPH families. The remaining genotype data were downloaded from the CEPH database (Fondation Jean Dausset–CEPH), were converted to CRI-MAP format by MAP+ (Collins et al. 1996*b*), and were combined with our own data by the CRI-MAP merge option. Haplotypes were constructed by hand, from microsatellite data.

Mitochondrial Screening

DNA samples from four ZMK family members were chosen to screen the entire mitochondrial sequence. These family members included two severely affected individuals (IV-22 and V-14), a member of the family who was deemed to be unaffected (IV-18), and an unrelated control (IV-23, spouse of IV-22). The DNA sample from individual IV-22 was extracted from a muscle-biopsy sample, since heteroplasmic mtDNA mutations are occasionally undetectable in blood samples. The other DNA samples were derived from blood. Mitochondrial primers were designed from the consensus Cambridge mtDNA sequence (Anderson et al. 1981; GenBank J01415), which was downloaded from MITOMAP v3.0 (Wallace et al. 1995; Kogelnik et al. 1996), and were synthesized by VHBio and Genosys. The mitochondrial genome was sequenced manually by standard direct-sequencing methodology, since low levels of heteroplasmic banding were thought to be easier to visualize by manual sequence. All family members were screened for the presence of the mutation, by direct sequencing of a PCR product spanning bases 12111–12467. Manual sequencing was performed by standard methods, involving end-labeling of sequencing primers with $\gamma[^{32}P]$-dATP (Amersham), and sequencing using dideoxynucleotides and Sequenase (USB). The 270 normals used as controls were sequenced automatically by an Applied Biosystems DNA sequencing kit and a 373A DNA sequencer (Applied Biosystems). Clones of the MTTS2 gene obtained from PCRs of DNA from the affected individuals discussed above were sequenced in the same manner as were those from the controls.

Southern Blotting

Southern blotting using standard protocols was performed on some family members, to ensure the absence of mitochondrial deletions. A PCR fragment from mtDNA was amplified (positions 9760–11399) by single-stranded PCR in the presence of $\alpha[^{32}P]$-dCTP and then was purified and used as a probe. The probe was subsequently hybridized to total genomic DNA, cut with *Pvu*II, from individuals from family ZMK.

Cloning/Single-Strand Conformation–Polymorphism Electrophoresis (SSCPE)

PCR products were amplified from muscle-biopsy DNA from IV-22 and from blood-derived DNA from IV-22, V-14, and IV-18, by use of forward and reverse primers that incorporated *Hin*dIII and *Xba*I sites, respectively. The primers were as follows (primer tails and enzyme sites are in parentheses): forward, (CTAGAAGCTT)CCGACATAATTACCGGGTTT; reverse, (TTCATCTAGA)TTTGTTAGGGTTAACGAGGG; aside from the primer regions shown in parentheses, these oligonucleotides were identical to those used to amplify control DNA for sequencing. PCR products were cut with *Hin*dIII and *Xba*I and were cloned into pcDNA3 (Invitrogen). Forty clones derived from each individual were sequenced as one method of estimating the percentage of heteroplasmy present in each DNA sample. In addition, PCR fragments (positions 12111–12467) from all members of family ZMK were used for SSCPE (Mansergh et al. 1998), and the wild-type:mutant DNA ratios in different family members were estimated.

Results and Discussion

Previous linkage studies resulted in exclusion of 90%–95% of the human nuclear genome, including most previously reported loci for Usher syndrome, RP, sensorineural deafness, and retinopathy (Kumar-Singh et al. 1993*a,* 1993*b;* Kenna et al. 1997; and unpublished results). A putative linkage was identified on the distal end of chromosome 9q, as noted above. Later, however, there were reasons to believe that the identification of this candidate region on 9q was not secure. First, no markers were found to be at zero recombination with the disease locus, and all of the recombinant individuals were affected. Second, D9S118 and D9S121 were analyzed by use of a subset of DNA samples from the CEPH family, and the data were used to build a linkage map of chromosome 9, which incorporated these markers and some of those included in the Généthon maps (fig. 2). The order of markers given by this map indicated that the aforementioned markers were interspersed closely with markers that had more recombinants with

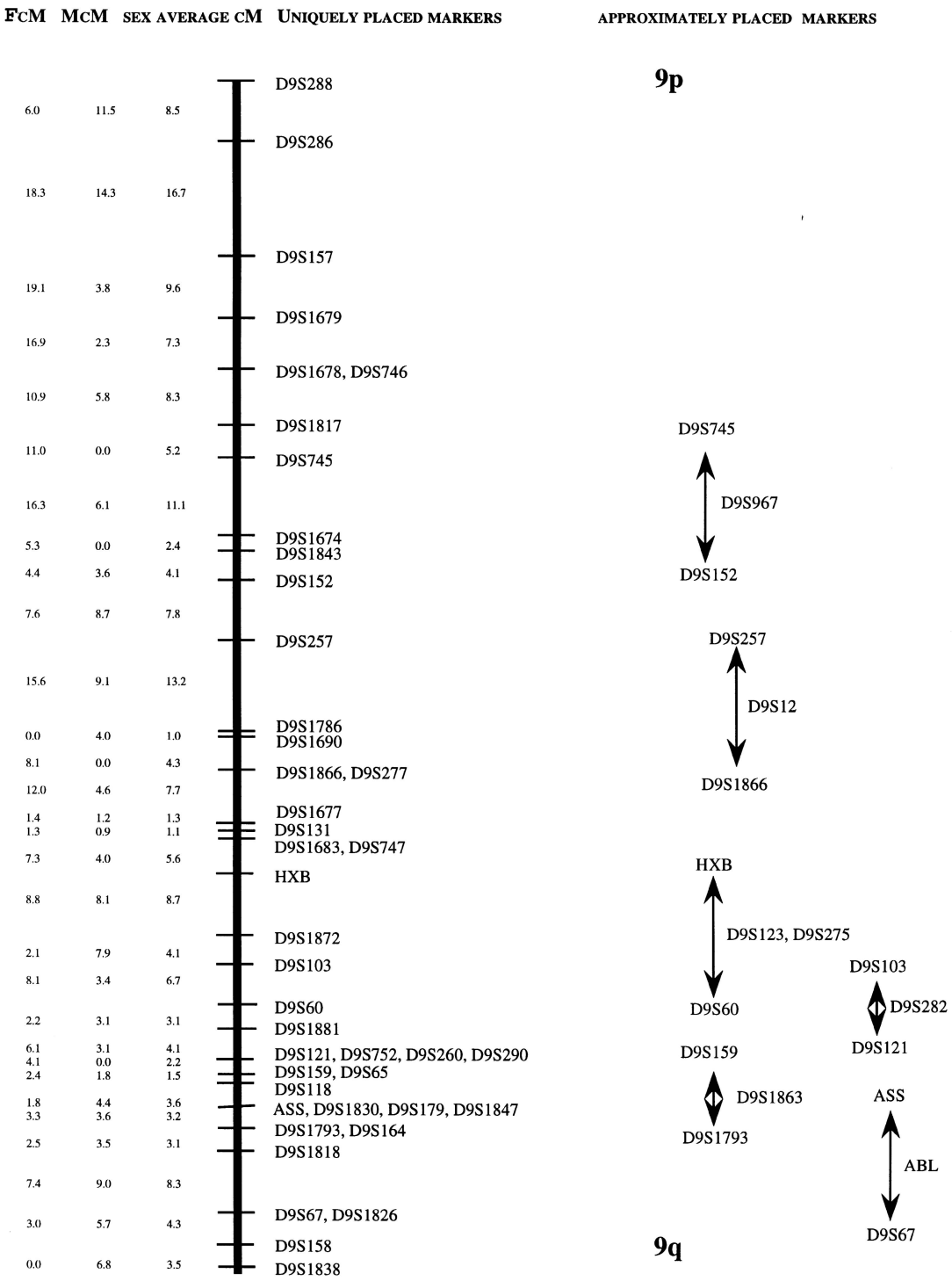

Figure 2 Linkage map of microsatellite markers on chromosome 9. This map was created by CRI-MAP version 2.4 (Lander and Green 1987). Genotype data used to create this map were downloaded from the CEPH database and were merged with CEPH data from markers D9S118 and D9S121 obtained by us. Approximate positions of nonuniquely placed markers are given on the right: markers indistinguishable, by recombination, from just one other locus are placed beside the latter and are assumed to occupy roughly the same position; markers indistinguishable, by recombination, from a number of uniquely placed loci are indicated beside the arrows. Each arrow defines the interval between which the nonuniquely placed marker(s) beside and to the right of the pertinent arrow can be found. Male (McM), female (FcM) and sex-averaged cM values for the genetic distances between the uniquely placed markers are given on the far left side of the diagram.

the disease locus and that gave lower LOD scores. As a result, multipoint analysis of the candidate region excluded it. Multipoint analysis was repeated by use of only affected members of the pedigree, and again convincing exclusions were obtained (fig. 3). Haplotype analysis of this region revealed that no one common haplotype was shared by all affected individuals (fig. 4). Finally, recent diagnostic reassessment resulted in a questionable disease status for one unaffected member of the pedigree. Unaffected individual III-5, although free of symptoms in her 70s, has been found to have a granddaughter with symptoms of RP and hearing loss (R. Carr, personal communication). Although individual III-5 was omitted from all linkage analysis presented, this information raises the possibility of reduced penetrance within the family. Reduced penetrance had not been considered previously, since the majority of family members assessed were affected and no prior evidence for skipped generations had been noted.

Cytoplasmic inheritance had previously been considered as a possibility in this pedigree but was considered less likely than nuclear inheritance, since information from older family members indicated that individual I-2 had no visual or hearing difficulties well into her 80s, which suggested the possibility that her spouse had transmitted the disease locus in this family. Such an instance of male-to-offspring transmission would rule out mitochondrial inheritance of the disease. However, there is limited information concerning I-1, who died around the turn of the century. Given our recent findings, it is likely that his wife, I-2, may possibly have been an asymptomatic carrier of a germ-line mitochondrial mutation.

Some observations of family ZMK were suggestive of a mitochondrial disorder. There is no male-to-offspring transmission in the pedigree, although, given that few of the affected males have married, this may be merely coincidental. Affected individuals, in addition to having hearing loss, complained of marked recruitment (distortion and physical discomfort as a result of loud noise) and persistent tinnitus (Kenna et al. 1997). Similar symptoms have been noted in persons with hearing loss caused by known mitochondrial mutations (W. J. Kimberling, personal communication). Four affected individuals (IV-22, V-4, V-5, and V-6) were assessed by electrocardiography. All were found to have right-axis deviation, and one individual showed signs of global hypertrophy. Individual IV-22 also underwent electromyography and a muscle biopsy. Results showed electromyographic abnormalities and an excessive number of mitochondria within muscle tissue (Kenna et al. 1997). Such cardiac and muscular abnormalities could be consistent with the presence of a mitochondrial disorder, since respiratory dysfunction in postmitotic tissues such as cardiac and skeletal muscle can result in excessive mitochondrial proliferation and conduction defects (Zeviani et al. 1989; diMauro and Hirano 1998). In addition to the subclinical symptoms noted above, sensorineural hearing loss and pigmentary retinopathy are disorders that have been known to result from mutations within the mitochondrial genome (Zeviani et al. 1989). The aforementioned incidence of reduced penetrance within a pedigree that is otherwise composed largely of affected individuals was also suggestive. Therefore, after excluding most of the nuclear genome, we screened for mutations in the mitochondrial genome of this kindred.

The complete mitochondrial sequence was obtained for four family members, including two severely affected individuals, one mildly affected individual, and an unaffected control. The DNA from one of the severely affected individuals was obtained from a muscle-biopsy sample; the other DNA samples were purified from pelleted blood lymphocytes. A number of variations from the consensus sequence were noted (table 1), most of which were polymorphic variants that have been reported elsewhere (MITOMAP, v3.0). Four previously unrecorded sequence changes were noted. Two of these were within amino acid–encoding regions of the mitochondrial genome and were found to be silent substitutions. The third sequence variant was heteroplasmic and was found within the gene encoding MTTS2 (fig. 5). Noteworthy is that it was found only in family members and was not present in 270 normal individuals from the same ethnic background as family ZMK. Further evidence for the pathogenicity of the C12258A sequence variant was obtained from sequence analysis. A sequence alignment of MTTS2 genes from various species—including those as evolutionarily divergent from *Homo sapiens* as vulture, platypus, and frog—shows that the C at position 12258 is highly conserved (table 2). Therefore, this study provides substantive evidence that the 12258 mtDNA mutation is the cause of the symptoms observed in family ZMK.

Varying degrees of heteroplasmy between family members were also assessed, since levels of heteroplasmy can be correlated with severity of symptoms in mitochondrial disorders (Zeviani et al. 1989; diMauro and Hirano 1998). Given that both muscle- and blood-derived DNA samples from individual IV-22 were available, differences in heteroplasmy between muscle- and blood-derived DNA samples were also noted. Levels of heteroplasmy were assessed by various methodologies. The complete family was sequenced, and differences in relative band intensity were estimated by eye. In addition, an SSCPE analysis of affected family members was undertaken, and relative band intensities were evaluated (fig. 6). Muscle- and blood-derived DNAs from individual IV-22 were amplified by PCR separately and were cloned into pcDNA3. Forty individual clones from each DNA type were sequenced to estimate the proportion of

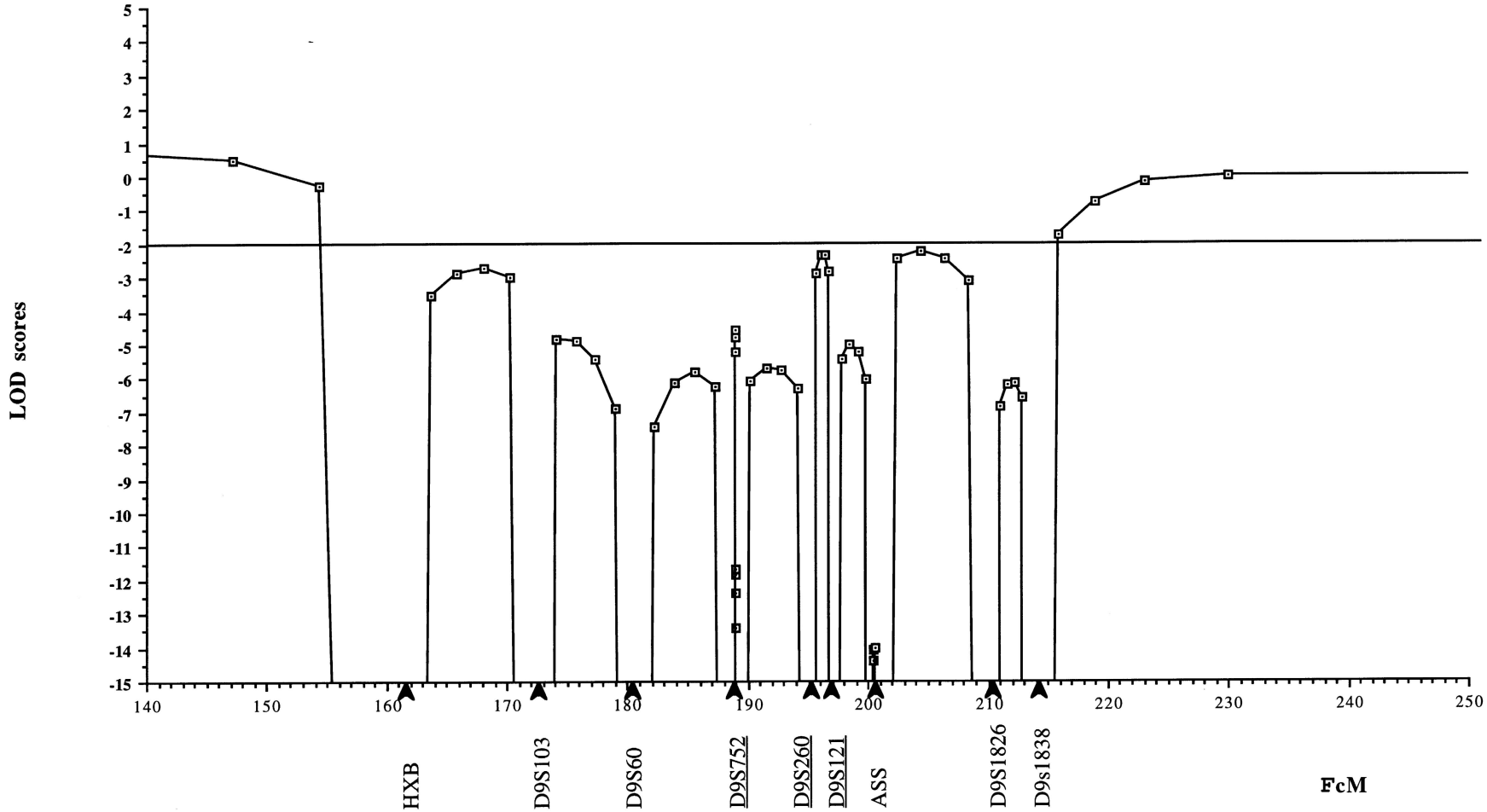

Figure 3 Multipoint analysis of only the affected members of the pedigree, indicating that the disease gene in family ZMK is significantly excluded from the 9q region where there previously had been a suggestion of linkage. Markers that map exactly to the same position are immediately adjacent to one another and underlined.

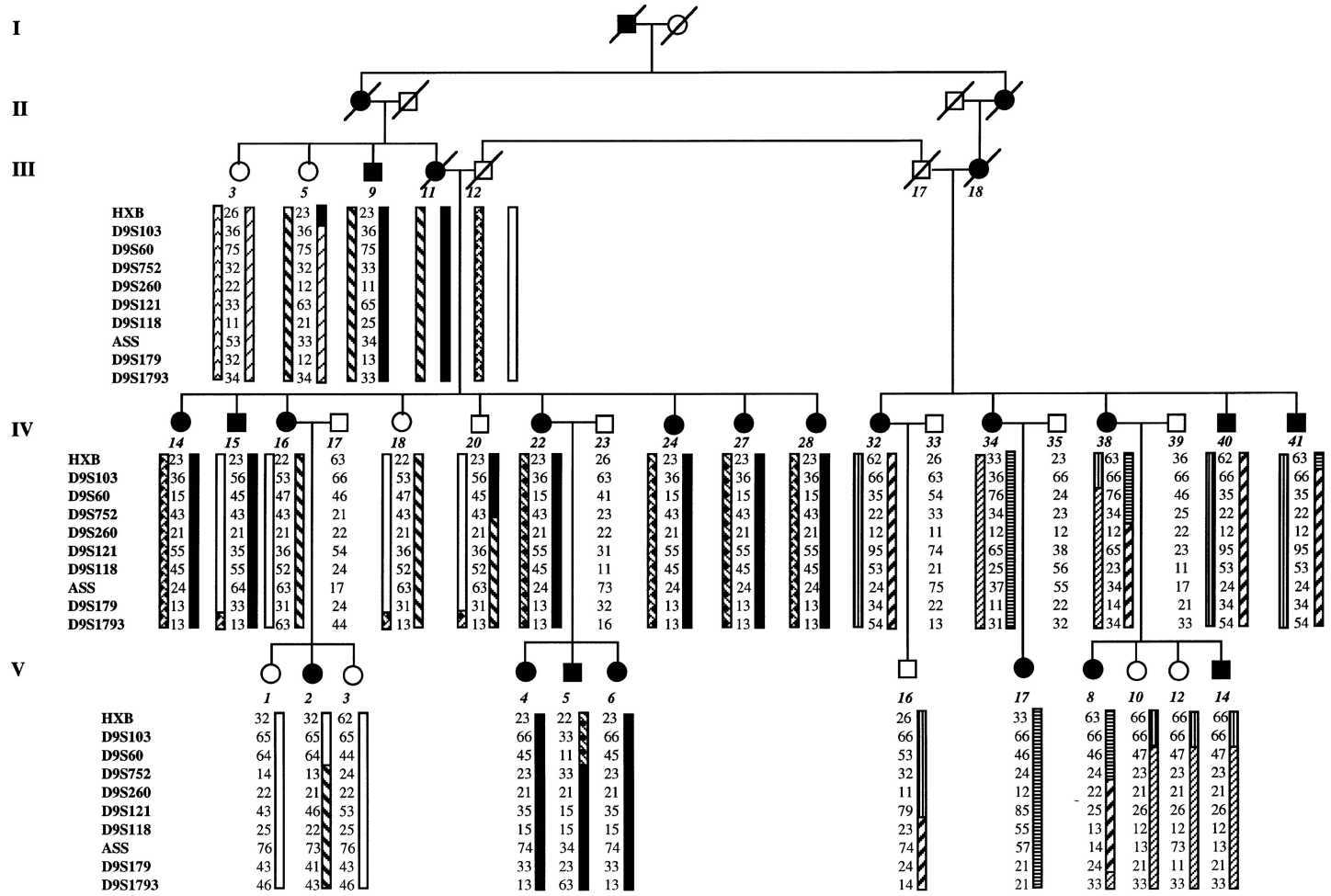

Figure 4 Haplotypes on 9q in family ZMK. Note the transmission of different chromosome types through the pedigree; those shared by related individuals are depicted as shaded bars. No one haplotype is shared by all affected individuals. In addition, the two separate branches of the family do not appear to have in common any 9q portion between HXB and the telomere. The region of tightest linkage is between markers D9S260 and D9S121 inclusive. There are four recombinants with the disease locus in this region—namely, those in IV-16, IV-34, and V-14 and that between the two major branches of the family. Individuals IV-16, IV-34, and V-14 are consistently recombinant with the disease locus, right across the region examined. Two of these individuals have affected offspring, and V-14 is severely affected; he was legally blind at age 21 years. In addition, individual III-5 has an affected granddaughter, increasing to five the number of recombinants with the disease locus. The most likely explanation for these data is that the disease locus is not on 9q.

Table 1

Sequence Variations in Family ZMK

Position	Base in Consensus Sequence	Base in Family ZMK	Gene	Amino Acid
533	A	G	Control	...
6776	T	C	MTCO1	H-H
11953	C	T	MTND4	L-L
12258	C	A	MTTS2	...
13404	T	C	MTND5	I-I
14272	C	G	MTND6	F-L
14569	G	A	MTND6	P-P
14766	A	G	MYCYB	I-T
16239	C	G	Control	...
16519	T	C	Control	...
16569	T	C	Control	...

Note.—The putative disease-causing mutation in the serine tRNA gene is boxed. Additional sequence variations were found both in "married-in" members of family ZMK and across all four individuals sequenced, including the unaffected control individual (data not presented). Many of the variations from the consensus sequence presented above are either previously reported sequence variations (underlined) or do not alter encoded amino acids.

mutant mtDNA present in each tissue. Similarly, blood-derived DNAs from individuals IV-18 (asymptomatic) and V-14 (severely affected) were cloned, and 40 clones per individual were sequenced (table 3).

It is noteworthy that sequence, SSCPE, and cloning data demonstrated a large variance in the proportion of mutant mtDNAs in muscle and blood derived from the same individual. Quadriceps femoris–derived skeletal muscle from individual IV-22 appeared to contain ~85%–95% mutant mtDNAs, whereas blood appeared to contain near-equal proportions of mutant and normal mtDNAs (table 2). It is therefore possible that there may also be high proportions of mutant mtDNAs in the retinas and cochleas of affected individuals, thus giving rise to the symptoms observed. This may be the case in affected family members, since it is known that, for some mitochondrial mutations, a low proportion of wild-type mtDNA can be sufficient to protect the cell from the deleterious effects of a pathogenic mtDNA mutation. For example, it has been shown in various mitochondrial disorders that respiratory function is severely compromised only when the proportion of mutated mtDNAs increases to >80%–90% (Zeviani et al. 1989; Chomyn 1998; Schapira 1998).

The mutated A band (fig. 5) was detectable in sequences from all family members, with the exception of individuals III-3 and III-5. Individual III-3 was assessed as unaffected in her 80s and may be free of the mutation. Her affection status is therefore slightly questionable. In contrast, individual III-5, although diagnosed as unaffected herself (in her mid 70s), has a granddaughter (V-1, who is in her 20s) with advanced RP and hearing loss. One possibility may be that individual III-5 inherited a low proportion of mutant mtDNA. Cells carrying a high load of mutated mtDNA molecules may be selected against in rapidly dividing tissues, as a result of their comparatively inefficient energy metabolism (Shoffner and Wallace 1992; Chomyn 1998; diMauro and Hirano 1998; Siregar et al. 1998). Over a lifetime, this can result in a significant reduction in the proportion of abnormal mtDNAs in dividing tissues such as hematopoietic stem cells, thus rendering detection of the mutant mtDNA species more difficult in blood-derived DNA of patients. Although the mutation in individual III-5 is absent in blood, this individual may have carried sufficient levels of the C12258A mutation in germ-line mtDNAs to pass it on to her descendants. There is in mammalian oogenesis a well-documented bottleneck effect that can result in a mother, who herself has a low proportion of a heteroplasmic mtDNA variant, passing on that variant, to her offspring, at high concentrations (Grossman 1990; Shoffner and Wallace 1992; Poulton et al. 1998).

Individuals V-1, V-3, III-18, IV-20, V-16, V-10, and V-12, who were diagnosed as unaffected, show the C12258A band after sequence analysis. Some of these individuals are young and, given the variability, in both age at onset and severity, observed within the family, may yet develop symptoms. Alternatively, there may be a threshold below which lower levels of the mutant do little damage. Marked variability in the severity of symptoms observed for small variations in relative levels of mutant mtDNAs has been noted in other mitochondrial disorders, including mitochondrial encephalomyopathy/lactic acidosis/strokelike episodes (MELAS

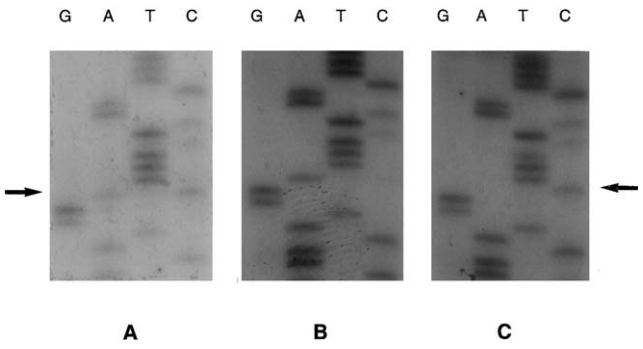

Figure 5 Sequence analysis of mtDNA samples from blood lymphocytes (*A*) and muscle (*B*) from affected individual IV-22 and from blood lymphocytes from an unaffected control (*C*). The C→A mutation at position 12258 (→) is present in both affected individuals and is absent in the unaffected control.

[MIM 54000]), myoclonic epilepsy with ragged red fibers (MERRF [MIM 54500]), and neuropathy/ataxia/retinitis pigmentosa (NARP [MIM 551500]) (Zeviani et al. 1989; Chomyn 1998; Schapira 1998). Finally, the relative proportions of mutated mtDNAs in different tissues may vary widely, both as a result of random genetic drift during development and as a result of selection during aging, as mentioned above (diMauro and Hirano 1998). Levels of a heteroplasmic variant in blood are therefore not a reliable indicator of its frequency in other tissues.

It is noteworthy that a number of syndromes with symptoms overlapping, in part, with those seen in family ZMK have involved large mitochondrial deletions. For example, Kearns-Sayre syndrome (MIM 530000), which is caused by deletions in mtDNA, can include RP, progressive external ophthalmoplegia, and progressive hearing loss among its symptoms (Wallace et al. 1995; Kogelnik et al. 1996), and a familial syndrome incorporating sensorineural deafness and non–insulin-dependent diabetes (MIM 520000) has been linked to a 10.4-kb mitochondrial deletion (Ballinger et al. 1992). Although none of the affected members of family ZMK have diabetes or show the external ocular muscle–movement disorders characteristic of Kearns-Sayre syndrome (Kenna et al. 1997), a Southern blot was undertaken to address the possibility that a large-scale mitochondrial deletion is the cause of disease in family ZMK. It is noteworthy that only the expected linearized mtDNA fragment of 16.5 kb was observed, reducing the likelihood of a mitochondrial deletion segregating through family ZMK. Typically, disorders caused by large-scale mtDNA deletions, duplications, and rearrangements are usually sporadic and often result in premature fatality (Zeviani et al. 1990). There is no evidence to suggest that, as a result of their disorder, affected members of family ZMK suffer significant reductions in life span.

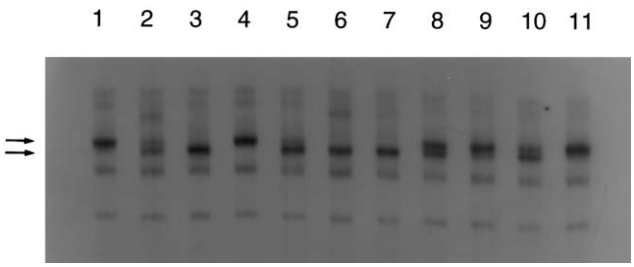

Figure 6 SSCPE analysis using PCR-amplified DNA from members of family ZMK. It is noteworthy that the wild-type (*upper band*) and mutant (*lower band*) mtDNA fragments can be distinguished. Both conformations of mtDNA are present in most affected individuals (e.g., see lanes 2, 5, and 8–10). However, some affected individuals have mtDNA that is mostly mutant. It is noteworthy that the wild-type:mutant mtDNA ratio in blood and muscle tissue varies significantly in individual IV-22 (lanes 2 and 3). The data indicate that there is no clearly evident correlation between the wild-type:mutant mtDNA ratio in blood and disease severity.

Given that this study provides substantive evidence that a mitochondrial mutation, C12258A in the gene encoding MTTS2, is the cause of progressive sensorineural deafness and RP in family ZMK, it is of interest

Table 2

CLUSTAL W(1.7) Multiple Sequence Alignment of MTTS2 in Various Species

Source/Species	Sequence[a]
	* ******* * ** * * *
MTTS2 (mutation)[b]	GAGAAAGCTCA----CAAGAACTGC-T-AACT-CATGCCCCCATGTCTAAC-AA-CATGGATTT-CTC
Human	GAGAAAGCTCA----CAAGAACTGC-T-AACT-CATGCCCCCATGTCTAAC-AA-CATGGCTTT-CTC
Chimpanzee	GAGAAAGCTTA----TAAGAACTGC-T-AATT-CATATCCCCATGCCTAAC-AA-CATGGCTTT-CTC
Gibbon	GAGAAAGCCCA----CAAGAACTGC-T-AACT-CACTATCCCATGTATAAC-AA-CATGGCTTT-CTC
Mouse	AAGAAAGATTG----CAAGAACTGC-T-AATT-CATGCTTCCATGTTTAAA-AA-CATGGCTTT-CTT
Rhinoceros	GAGAAAGCACT----CAAGAACTGC-T-AACT-CATGCCCCCATATTTAAC-AA-TATGGCTTT-CTC
Cat	GAAAAAGTATG----CAAGAACTGC-T-AATT-CATGCCTCCACGTATAAA-AA-CGTGGCTTT-TTC
Seal	GAAAAAGAATG----CAAGAACTGC-T-AACT-CATGCCCCCACGTATAAA-AA-CGTGGCTTT-TTC
Horse	GAGAAAGTATG----CAAGAACTGC-T-AATT-CATGCCCCCATGTCCAAC-AAACATGGCTCT-CTC
Donkey	GAGAA-GTATG----CAAGAACTGCCT-AATT-CATGCTTCCGCGTCTGAC-AAACACGGCTCT-CTC
Opossum	GAGAATGCA-T----CAAGAACTGC-T-AATT-CATGAACCCATATTTAAC-AA-TATGGCTTT-CTC
Platypus	GAGAGAGAA-A----TAAGAACTGC-T-AATC-CTTAACTTCATGCCTAAC-CA-CATGACTCTACTT
Vulture	GTTACAACCAG----CAGGAACTGC-T-AACT-CTTGCATCTGAGTCTAAA-AC-CTCAGCCC-CCTT
Frog	GAACTTGACTGGACCTAAGAACTGC-T-AATTACTTACG-CTGTG-TTCATTC--CACGGCTTG-TTC

[a] An asterisk (*) denotes a position that is conserved in all species listed as well as in MTTS2 in family ZMK. Note the conservation, at position 12258 (underlined), of the normal C base in all species included in the alignment; *Drosophila, Saccharomyces crevisiae,* and *Asterinia pectinifera* sequences were also examined but showed no consensus in the sequence.

[b] Variant found in family ZMK.

Table 3

Heteroplasmy Estimated by Sequencing and by Cloning

Heteroplasmy-Estimation and Individual	A:C Ratio[a]	Tissue	Status[b]
Sequencing:[c]			
III-3	0:100	Blood	Asymptomatic
III-5	0:100	Blood	Asymptomatic
III-9	5:95	Blood	Unclear
IV-14	50:50	Blood	Affected
IV-15	30:70	Blood	Affected
IV-16	40:60	Blood	Affected
IV-17	0:100	Blood	Married-in
V-1	80:20	Blood	Asymptomatic
V-2	95:5	Blood	Affected
V-3	40:60	Blood	Asymptomatic
IV-18	60:40	Blood	Asymptomatic
IV-20	40:60	Blood	Asymptomatic
IV-22	50:50	Blood	Affected
IV-22	95:5	Muscle	Affected
IV-23	0:100	Blood	Married-in
V-4	80:20	Blood	Affected
V-5	80:20	Blood	Affected
V-6	80:20	Blood	Affected
IV-24	50:50	Blood	Affected
IV-27	30:70	Blood	Affected
IV-28	50:50	Blood	Affected
IV-32	10:90	Blood	Affected
IV-33	0:100	Blood	Married-in
V-16	20:80	Blood	Asymptomatic
IV-34	50:50	Blood	Affected
IV-35	0:100	Blood	Married-in
V-17	80:20	Blood	Affected
IV-38	40:60	Blood	Affected
IV-39	0:100	Blood	Married-in
V-8	50:50	Blood	Affected
V-10	40:60	Blood	Asymptomatic
V-12	40:60	Blood	Asymptomatic
V-14	60:40	Blood	Affected
IV-40	20:80	Blood	Affected
IV-41	50:50	Blood	Affected
Cloning:			
IV-22	37:3	Muscle	Affected
IV-22	25:15	Blood	Affected
V-14	24:16	Blood	Affected
IV-18	16:24	Blood	Asymptomatic

[a] A = mutated variant; C = normal base.

[b] "Asymptomatic" has been used to denote unaffected individuals, since some of them are still young and there is evidence for a widely variable age at onset and penetrance in the pedigree. Similar results were obtained with SSCPE analysis (see fig. 6).

[c] Heteroplasmy estimated by eye.

to speculate as to possible mechanisms by which this mutation has a deleterious effect. A cursory review of tRNA structure indicates that the C12258A mutation is situated within the amino acid–acceptor arm of the molecule. The presence of the mutant A at this position would be expected to disrupt base pairing of the acceptor arm, resulting in a change or destabilization of the tRNA secondary structure. Such a change in the tRNA secondary structure could result in inefficient aminoacylation of the tRNA. Alternatively, the variant tRNAs may be degraded more rapidly. Either or both mechanisms would probably result in a reduction in the pool of charged serine (i.e., AGY) tRNAs available for translation. An increase in the uncharged:charged tRNA ratio may lead to incorporation of uncharged tRNAs into the ribosomal active site, resulting in premature termination of transcripts. Prior studies of the MERRF 8344 tRNA Lys mutation (MIM 590060.0001) show precedents for both mechanisms. In this condition, levels of the tRNA have been thought to decline by 18%–35% while charging of the variant tRNA with lysine is reduced by 30%–40%. Prematurely terminated transcripts have also been observed (Chomyn 1998). Another possibility is that the mutation could interfere in some way with processing of the polycistronic mRNA produced from transcription of the mitochondrial heavy strand. The previously described 7445 tRNA Ser (UCN) deafness (MIM 590080) mutation is thought to act in this way, at least in part (Fischel-Ghodsian 1998*a,* 1998*b*).

Regardless of the precise mechanism, if the pool of charged serine tRNAs is reduced, one would expect a resulting reduction in the efficiency of mitochondrial translation, which would impair mitochondrial ATP production, thereby reducing the energy available to the cell. Neural tissues (including retina and cochlear hair cells), have the highest energy demands of any tissue in the body, followed by muscle tissue (Zeviani et al. 1993; Fischel-Ghodsian 1998*a,* 1998*b;* Schapira et al. 1998). This provides a coherent reason why symptoms involving various progressive visual, auditory, muscular, cardiac, and neuropathic abnormalities almost always accompany mitochondrial disorders. Another factor that increases the vulnerability of neural and muscular tissues to mitochondrial disorders is that cells containing high loads of dysfunctional mitochondria are less efficiently selected against in nondividing cells, leading to the presence of a higher proportion of such cells in these tissues.

Compromised respiratory function has a number of deleterious effects on cells. It is known to result in the excessive generation of free radicals (Zeviani et al. 1989; Schapira 1998), which are mutagenic to any remaining wild-type mtDNAs. A further increase in the number of mutant mtDNAs can only impair the respiratory processes further, thus amplifying the problem. Impaired oxidative phosphorylation may lead to decreases in mitochondrial transmembrane potential. Such decreases are known to play an early part in apoptosis, or programmed cell death (Schapira 1998). In this regard, it is of interest that some photoreceptor degenerations have been shown to involve apoptotic mechanisms (Travis 1998). Hence, one possible future approach to therapy

may be to modulate key proteins involved in the apoptotic pathway (Hafezi et al. 1997).

There are a number of possibilities as to why the retina and cochlea in family ZMK are more vulnerable to the deleterious effects of the 12258 mutation than are other neural or muscle tissues. First, these tissues may have energy demands that are exceptionally high even in comparison with those of other neurons. Photoreceptors are thought to be exceptionally sensitive to reductions in respiratory capacity (Travis 1998). In addition, mild mitochondrial mutations that can appear in homoplasmic form, such as those causing Leber hereditary optic neuropathy (LHON [MIM 535000]) and deafness, cause ocular or auditory symptoms, typically without involving other tissue types. Further support for this hypothesis is provided by the observation that two of the most severely affected family members are also mentally subnormal, suggesting the possible involvement of other neural tissues as the mutation load increases. Other explanations have been put forward for the observed tissue-specific effects of various mitochondrial mutations. Mitochondrial gene products may have tissue-specific functions unrelated to their mitochondrial function. Alternatively, gene products that help to regulate mitochondrial function may vary in a tissue-specific manner, rendering some tissues more sensitive to particular mutations (Fischel-Ghodsian 1998*a,* 1998*b*). Varying levels of mutant mtDNAs between individuals could explain the differences in both severity and age at onset that have been observed in family ZMK. Another explanation for variations in severity and age at onset would be interindividual differences in the nuclear-DNA background. However, linkage studies thus far have shown little evidence for susceptibility loci of large effect. Multipoint analyses in the area of the putative disease locus on chromosome 9 resulted in convincing exclusion of the region. Moreover, two-point linkage analyses have been undertaken that have used affected members only and the 400-plus markers that have been typed through the family. It is noteworthy that no significant areas of linkage were established (data not shown).

Although there are no precedents for the precise combination of symptoms observed in this family, there are many examples of mitochondrial disorders that manifest either progressive hearing loss or a retinopathy. RP or similar retinal degenerations are a feature of NARP (MIM 551500) (Holt et al. 1990) and Kearns-Sayre syndrome (MIM 530000) and are sometimes associated with the MELAS (MIM 54000) 3243 mutation (Sue et al. 1997). Mutations at positions 1555 (MIM 561000.0001) and 7445 (MIM 590080.0002) have been shown to cause both aminoglycoside-induced or spontaneous hearing loss (Prezant et al. 1993, Reid et al. 1994), whereas other mtDNA anomalies can result in hearing loss associated with diabetes or with ataxia and myoclonus (Ballinger et al. 1992; van den Ouweland et al. 1992; Tiranti et al. 1995). Cardiac and musculoskeletal abnormalities are also associated with a large number of mitochondrial disorders (Zeviani et al. 1989) and usually manifest much more severely than they do in family ZMK.

The results from this study have significant implications for the etiologies of various retinopathies and forms of hearing loss, especially the various forms of Usher syndrome. To date, eight loci for Usher syndrome have been mapped to the nuclear genome; two of these genes, those causing USH1B and USH2A, have been identified (Weil et al. 1995; Eudy et al. 1998). Additionally, families with some Usher syndrome do not show linkage to any previously mapped loci, suggesting further genetic heterogeneity. It is possible that some unmapped families with Usher syndrome may carry the C12258A mutation. Seemingly dominant or partially dominant inheritance of Usher III has been noted in some patients (W. J. Kimberling, personal communication). Hence, an extensive screening for mitochondrial mutations in patients with Usher syndrome should be undertaken. Such a study may subsequently be extended to patients suffering from disorders involving visual or hearing loss alone. The observation that a mitochondrial mutation causes a disease akin to Usher syndrome raises the previously unconsidered possibility that the mapped but unidentified genes causing some forms of Usher syndrome may be involved in mitochondrial metabolism. Such a situation would not be without precedent; syndromes resembling those caused by mitochondrial mutations have been mapped to the nuclear genome; these include mtDNA deletion syndromes inherited in an autosomal dominant fashion ([MIM 550000] Zeviani et al. 1989, 1990; [MIM 601226] Kaukonen et al. 1996), a form of Leigh syndrome ([MIM 256000] Bourgeron et al. 1995), and Friedreich ataxia ([MIM 229300] Schapira 1998), some cases of which are known to be caused by mutations in genes involved in mitochondrial metabolism. The mammalian mitochondrial genome encodes only 13 of the $\geqslant$50 proteins that constitute respiratory complexes and encodes none of the proteins involved in mitochondrial replication, transcription, and translation. To date, few of the nuclear genes that play a role in healthy mitochondrial function have been cloned. The involvement of such genes in the etiology of diverse genetic degenerative disorders may have been underestimated thus far. We would therefore hope that discovery of the C12258A mutation will aid in both the further understanding of the molecular pathogenesis of Usher syndrome and our knowledge of mitochondrial disorders in general. Undoubtedly, such knowledge will aid in the future development of therapies for this group of debilitating disorders.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Fondation Jean Dausset–CEPH, http://ceph-genethon-map.cephb.fr/ (also see CEPH version 8.0 database, ftp://ftp.cephb.fr/pub/)

Genome Database, http://gdbwww.gdb.org

LDB (The Location Database), http://cedar.genetics.soton.ac.uk

MITOMAP, version 3.1, http://www.gen.emory.edu/mitomap.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for USH1A [MIM 276900], USH1B [MIM 276903], USH1C [MIM 276904], USH1D [MIM 601067], USH1E [MIM 602097], USH2A [MIM 276901], USH2B [MIM 276905], and Usher syndrome type III [MIM 276902]; also Friedreich ataxia [MIM 229300], a form of Leigh syndrome [MIM 256000], LHON [MIM 535000], MELAS [MIM 54000], MERRF [MIM 54500], MERRF 8344 tRNA Lys mutation [MIM 590060.0001], NARP [MIM 551500], Kearns-Sayre syndrome [MIM 530000], autosomal dominant mtDNA deletion syndromes [MIM 550000 and MIM 601226], sensorineural deafness and non-insulin dependent diabetes [MIM 520000], 7445 tRNA Ser [UCN] deafness [MIM 590080], and mutations at 1555 [MIM 561000.0001], and 7445 [MIM 590080.0002])

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