Letters to the Editor

Am. J. Hum. Genet. 65:247–249, 1999

Mutations of *UFD1L* Are Not Responsible for the Majority of Cases of DiGeorge Syndrome/ Velocardiofacial Syndrome without Deletions within Chromosome 22q11

To the Editor:

Deletions of chromosome 22q11 are associated with a wide spectrum of congenital malformation, encompassed by the acronym "CATCH22" (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, and hypocalcemia on chromosome 22), including velocardiofacial syndrome (VCFS; MIM 192430), DiGeorge syndrome (DGS; MIM 188400), and conotruncal-anomaly face (Emanuel et al. 1998). The major anomalies include outflow-tract congenital heart defects, hypoplasia of the parathyroids and thymus, craniofacial dysmorphism, and learning/behavioral problems (Ryan et al. 1997). Many of these are thought to be due to a defective neural-crest contribution during development. The DiGeorge chromosomal region (DGCR) is entirely cloned (Carlson et al. 1997) and sequenced, and several genes have been reported mapping to the region. Mutation screens of genes mapping to the proximal end of this region, termed the "minimal DiGeorge chromosomal region" (MDGCR; Gong et al. 1996), have been negative (Wadey et al. 1995; Gong et al. 1997; Gottlieb et al. 1997; Lindsay et al. 1998). Attention therefore has turned to the regions adjacent and distal to the MDGCR. Recently, the gene *UFD1L* was proposed as the major gene haploinsufficient in this group of syndromes (Yamagishi et al. 1999). *UFD1L* is downstream of *dHAND,* a gene known to be involved in control of the development of structures affected in DGS, and *Ufd1l* is expressed in the branchial arches, frontonasal mass, and outflow tract. In addition, a single patient has been reported with a de novo deletion affecting *UFD1L* and the neighboring gene, *CDC45L2* (Yamagishi et al. 1999). *CDC45* is required for initiation of DNA replication in yeast, and *CDC45* mutants are nonviable. However, *CDC45L2* expression is not altered in *dHAND* −/− embryos. On the basis of these findings, Yamagishi and colleagues concluded that *UFD1L* haploinsufficiency (perhaps with some contribution from *CDC45L2*) causes DGS.

We conducted mutation screens, in both *UFD1L* and *CDC45L2,* as a three-center collaboration. *UFD1L* was screened by direct sequencing of 12 patients in London, by direct sequencing of all exons and 900 bp of the *5′* UTR in 20 patients in Rome, and by DGGE of 7 patients' DNA in Rotterdam. Local ethical review and consenting procedures were followed. The majority of patients were chosen on the basis of the presence of two or more features of the 22q11 deletion syndromes, but with no detectable deletion of 22q11 or of the DGSII region of 10p13 (Daw et al. 1996). The Rome series contained six patients with an isolated (i.e., nonsyndromic) interrupted aortic arch, a congenital heart defect commonly associated with the deletion. These patients were included because point mutations may be associated with a narrower spectrum of malformation than deletion and—since *UFD1L* was specifically identified as a *dHAND* target—because congenital heart defects might be especially significant. The previously described patient with a balanced 2;22 translocation in association with DGS (patient ADU; Augusseau et al. 1986) was also screened. *UFD1L* primers and conditions are available from the collaborating centers, and the genomic organization of *UFD1L* and the resources for exon PCR amplification have been described elsewhere by Novelli et al. (1998). In London, 24 patients were similarly screened for *CDC45L2* mutations; primers and PCR conditions are available on request, and genomic organization has been published previously (McKie et al. 1998). No mutations of either gene were detected. We did, however, detect a number of sequence variants. Within the *5′*UTR of *UFD1L* we found a single polymorphic sequence, initially detected by SSCP and subsequently shown to involve an A→G transition, located at the −277 position (with respect to the first base of the initiation codon). Screening of 25 unrelated controls generated a heterozygosity value of .40. Within *CDC45L2* we detected an A→G transition 22 bp upstream of exon 17 (at intron 16, with heterozygosity of .3) and a G→T transversion 24 bp into intron 18 (heterozygosity of .5). In addition, Southern analysis of 42 patients was conducted, with four different restriction-enzyme digests (*Hin*dIII, *Eco*RI, *Kpn*I, and *Bam*HI), in an attempt to ascertain rearrangements similar to the

UFD1L/CDC45L2 deletion reported elsewhere. This analysis included all of the London patients screened for point mutations, as well as an additional 18 patients. No rearrangements or deletions were detected, although four RFLPs were observed. Finally, mice with hemizygous targeted mutations of *Ufd1l* were normal (A. Baldini, personal communication).

Where does this leave the molecular genetics of the 22q11 deletion syndromes? Interpretation of current data must consider that, although ⩾10% of deletions are inherited (Ryan et al. 1997), there is no good evidence for inheritance of DGS/VCFS in nondeletion cases. Furthermore, there are a large number of potential phenocopies of the condition (Emanuel et al. 1998). It is therefore possible that only a fraction of nondeleted cases have an etiology related to chromosome 22q11. Therefore, *UFD1L* must still be regarded as a good candidate for contributing to this complex multiple-malformation syndrome. However, it should be kept in mind that a number of genes might be acting to produce a combined haploinsufficiency, especially since other genes within the DGCR are also expressed in affected tissues. In the case of HIRA, for instance, the protein is known to interact with *PAX3,* a gene required for conotruncal septation in the mouse (Magnaghi et al. 1998), and antisense attenuation of HIRA expression in chicks yields an increased incidence of persistent truncus arteriosus (Farrell et al. 1999). However, as with *UFD1L,* mutations within *HIRA* have not been detected. Another consideration is the presence of distinct (i.e., nonoverlapping) rearrangements of 22q11, associated with very similar DGS-like phenotypes (Dallapiccola et al. 1996; Kurahashi et al. 1996; Sutherland et al. 1996; Rauch et al. 1999). Perhaps haploinsufficiency of more than one gene can cause the syndrome, or long-range effects induced by the rearrangements can down-regulate the expression of the relevant gene(s). The role of combinations of genes during development is being tested by chromosome engineering in the mouse (Lindsay and Baldini 1998), although it is conceivable that long-range effects will confuse analysis in the murine system. In agreement with other commentators (Baldini 1999; Hagmann 1999), we think it is too early to call "Closing Time" (Heller 1996) on "CATCH22" (Heller 1955).

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for VCFS [MIM 192430] and DGS [MIM 188400])

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Haploinsufficiency of the *HOXA* Gene Cluster, in a Patient with Hand-Foot-Genital Syndrome, Velopharyngeal Insufficiency, and Persistent Patent Ductus Botalli

To the Editor:

The homeobox-containing *HOX* genes constitute a highly conserved gene family, with a role in specifying the body plan. In humans and in mice, four clusters (A–D) of *HOX* genes are located on different chromosomes. The precise function of the individual *HOX* genes, in humans, can be deduced from their expression pattern during mouse development and from the phenotype of mice with a targeted disruption or overexpression of a specific *HOX* gene. In humans, mutations have only been described in *HOXD-13* and *HOXA-13,* causing synpolydactyly and the hand-foot-genital (HFG) syndrome, respectively (Muragaki et al. 1996; Mortlock and Innis 1997). The mechanisms by which mutations in *HOXA-13* lead to the phenotype—that is, whether through haploinsufficiency or through a dominant negative effect—are currently unknown. Here we report on a patient with HFG syndrome who carries a chromosome 7p14 deletion involving the entire *HOXA* cluster, indicating that haploinsufficiency of *HOXA-13* may cause the phenotype.

The patient is the second child of healthy, unrelated parents. Pregnancy and delivery were uneventful. Facial dysmorphism was evident from birth, with retrognathia, low-set malformed ears, upturned nostrils, large mouth, and upslanted eyes. There were mild anomalies of the hands and feet, with shortened and laterally deviated first toes and clinodactyly of the fifth fingers with short terminal phalanges. Radiographs revealed hand and foot anomalies characteristic of HFG syndrome (fig. 1 and 2) (Stern et al. 1970; Halal 1988). There was left-sided cryptorchidism and a ventral-bowed penis. An intravenous pyelogram was normal. In addition, he presented with severe feeding difficulties during infancy, caused by velopharyngeal insufficiency with a shortened soft palate and very small uvula. On a barium swallow, massive nasal reflux was visible. A persistent patent ductus Botalli was surgically corrected at age 4 years. Growth was normal. Full-scale IQ at age 7 years was 85. Presently,

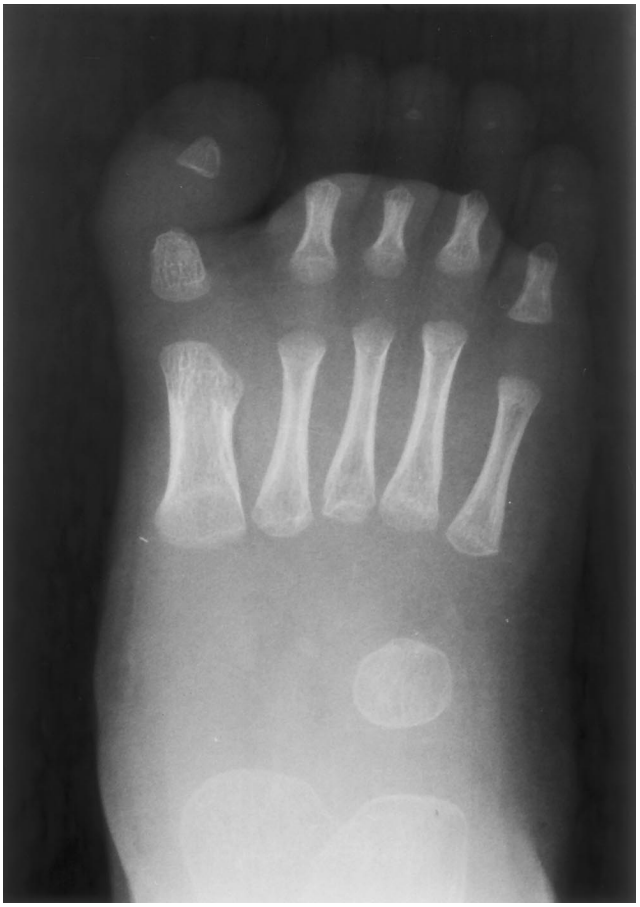

Figure 1 X-ray of the patient's left foot, at age 2 years 10 mo. The first toe is laterally deviated, with a triangular distal phalanx and shortened proximal phalanx. There is absence of calcification of the middle phalanges of toes II–V and distal phalanx of toe II.

at age 21 years, he is healthy and functions at a borderline intelligence level.

Karyotype analysis of blood lymphocytes showed a de novo deletion in the short arm of chromosome 7, with karyotype 46,XY,del7(p14). FISH done with probe DO832 did not reveal a microdeletion in chromosome 22q11. FISH and microsatellite analysis were performed for the fine mapping of the deletion on 7p, as described by Devriendt et al. (1997). Informed consent was obtained from the patient and his parents. The physical-map data were from Van Laer et al. (1997). With use of YACs Y915D12 and 920C6 (located telomeric from the *HOXA* cluster) and YAC 961E5 (containing the *HOXA* cluster), no signal was seen on the deleted chromosome 7p. Microsatellites D8S529 and D8S2496 map telomeric and centromeric, respectively, from the *HOXA* cluster (Van Laer et al. 1997). Both markers were informative in this family and their analysis revealed that the patient missed a maternal allele for both markers. These data demonstrated that the entire *HOXA* cluster was deleted on this chromosome.

This patient with multiple congenital malformations carries a de novo interstitial deletion of chromosome 7p14, involving the entire *HOXA* gene cluster. Retrospectively, the hand and foot anomalies present in this patient are typical of HFG syndrome (Stern et al. 1970; Halal 1988). This autosomal dominant disorder is caused by mutations in the *HOXA-13* gene, which is the most centromeric *HOX* gene of the *HOXA* cluster on chromosome 7p (Mortlock and Innis 1997). Mutations in *HOXA-13* have so far been described in three families with HFG syndrome. In two of the families the mutations are predicted to lead to a truncated protein,

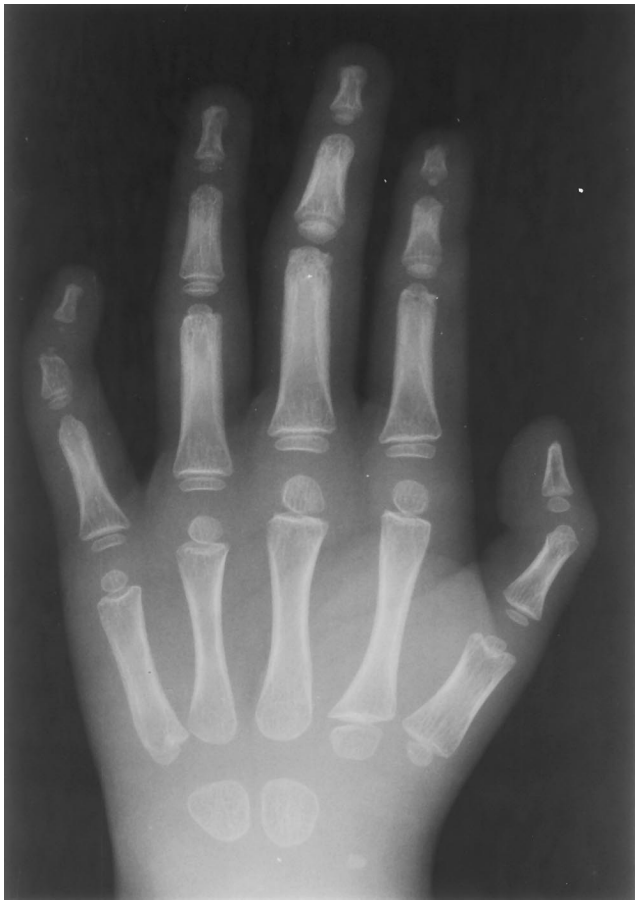

Figure 2 X-ray of the patient's left hand at age 7.5 years. Note the thumb anomalies: shortened metacarpal, pointed distal phalanx, and pseudoepiphysis of the metacarpal. There is a brachymesophalanx V causing clinodactyly and associated with a pseudoepiphysis. There is shortening of the distal phalanx of finger II. Pseudoepiphyses are present at metacarpal II and V. Bone age was 4.1 years.

whereas in one family a polyalanine tract expansion was observed (Mortlock and Innis 1997; Goodman et al. 1998*a*). The different mutations in *HOXA-13* do not result in clear phenotypic differences, although the presence of urinary-tract anomalies in certain male patients seems to be restricted to polyalanine-tract expansion (Goodman et al. 1998*a*).

At the present time, it is unclear whether these mutations result in haploinsufficiency of *HOXA-13* or in a dominant negative effect (Mortlock and Innis 1997; Goodman et al. 1998*a*). The deletion of *HOXA-13* in the present patient leads to haploinsufficiency of this gene and demonstrates that this can result in the HFG phenotype. A polyalanine-tract expansion has also been observed in *HOXD-13* and causes synpolydactyly, probably through a dominant negative effect (Goodman et al. 1997). On the other hand, deletions in this gene that probably result in a null allele also result in a slightly different phenotype (Goodman et al. 1997, 1998*b*).

Interestingly, the patient presented with additional malformations, including persistent patent ductus Botalli, velopharyngeal insufficiency, and a distinct but nonspecific facial dysmorphism. These features have not been reported in HFG syndrome and probably result from the haploinsufficiency associated with one or more of the deleted genes on chromosome 7p14. There is a striking resemblance to the features found in the homozygous *Hoxa-3* knock-out mice (formerly termed *Hox-1.5*) (Chisaka and Capecchi 1991). These mice also display a disorganized musculature in the throat, with a shortened, malfunctioning soft palate. Patent ductus arteriosus was also observed in three *Hoxa-3* knock-out mice. Although we cannot exclude the possibility that the cardiac and velopharyngeal malformations in the present patient are caused by the deletion of another adjacent gene, outside the *HOXA* cluster, the similarity with the *Hoxa-3* knock-out mouse phenotype is very striking and suggests that these anomalies might be related to haploinsufficiency of this gene.

In conclusion, the congenital malformations in the present patient result from the deletion of contiguous developmental genes on chromosome 7p14. The HFG syndrome is caused by haploinsufficiency of *HOXA-13*, whereas the velopharyngeal insufficiency and patent ductus arteriosus are possibly related to haploinsufficiency of *HOXA-3*.

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Highly Skewed X-Chromosome Inactivation Is Associated with Idiopathic Recurrent Spontaneous Abortion

To the Editor:

Recurrent spontaneous abortion (RSA) is a major health concern for women, affecting one in every 100 couples wishing to have children (Stephenson 1996). It has been estimated that 37%–79% of those couples will not receive an explanation for their pregnancy losses, adding to their emotional burden (Hatasaka 1994; Stephenson 1996). Inherited causes of recurrent miscarriage are often assumed, but the presumed high degree of genetic heterogeneity and lack of a carrier phenotype have made genetic studies impossible. Similarly, X-linked recessive lethality has long been proposed for RSA, but the sex of abortuses is generally unknown, and the high population prevalence of pregnancy loss makes the ascertainment of X-linked pedigrees problematic (Motulsky and Vogel 1997, pp. 139–141).

We have recently shown that carriers of X-linked recessive lethal traits may have the "molecular phenotype" of skewed X-chromosome inactivation (Pegoraro et al. 1997). Our model predicts that extreme skewing of X inactivation occurs during embryonic development in the female (asymptomatic) carrier, secondary to cell-autonomous selection against cells in which the abnormal X chromosome is active. All male (XY) conceptions of the carrier that receive the abnormal X chromosome would be spontaneously aborted. The miscarriage rate of female carriers of such traits would be expected to increase from an estimated population rate of ~15% to ~40% (15% + 25% for X-linked recessive recurrence risk). We recently reported a 70-member pedigree that validated this hypothesis: a maternally inherited trait, which caused sole use of the paternally derived X chromosome in female carriers, was associated with a 32% spontaneous abortion rate, whereas noncarriers in the same family all showed the 15% population rate ($P < .05$; Pegoraro et al. 1997).

To test the hypothesis that X-linked lethal traits are a significant cause of RSA, we recruited women who had experienced at least two miscarriages in the absence of any cytogenetic, anatomic, infectious, immunologic, or hormonal abnormalities known to cause RSA (table 1). A priori, "skewed X chromosome inactivation" was defined as preferential use of one allele in ⩾90% of peripheral leukocytes. This value was selected because we proposed that this level of skewing represents negative selection strong enough to be associated with RSA but not such a rare event as to go unobserved in our case group. Genomic DNA extracted from peripheral lymphocytes was assayed for X inactivation at the androgen receptor (HUMARA) locus (Pegoraro et al. 1994).

A total of 48 women who met the diagnostic criteria for enrollment were assayed for X inactivation, 7 (14.6%) of whom were found to show highly skewed X inactivation (table 1 and fig. 1). In contrast, in the age-matched control group, comprising women from the same demographic region with no known history of

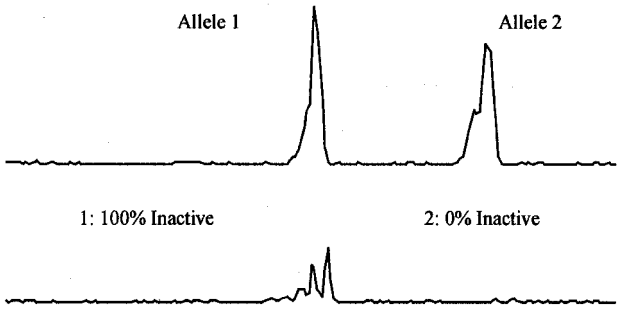

Figure 1 Skewed X inactivation in women with RSA. Genomic DNA samples from women with RSA pregnancy loss were subjected to PCR amplification of the highly polymorphic HUMARA locus,with fluorescent primers. A gravida 5 para 0 (G5P0) woman is heterozygous at this locus (*upper trace*). Digestion of genomic DNA with methylation-sensitive restriction enzymes prior to PCR at the HUMARA locus permits accurate quantitation of X-inactivation patterns (*lower trace*). The G5P0 woman shows complete (100%) skewing. X-inactivation analysis at the HUMARA locus was performed as described elsewhere (Pegoraro et al. 1994). Use of the highly polymorphic HUMARA locus afforded 90.6% (48/53) of individuals informative for the X-inactivation assay.

Table 1

X Inactivation in Women with RSA of Unknown Cause

	No. (%) with X Inactivation		
	Skewed ⩾90%	Random	Total
RSA cases	7 (14.6)	41	48
Controls	1 (1.5)	66	67[a]
Plenge et al. (1997)	4 (3.5)	111	115[b]
Gale et al. (1997)	3 (3.2)	91	94[b]

Note.—Women with RSA of unknown cause have a statistically significant increased frequency of skewed X inactivation, compared with control-group women. RSA cases were women who had undergone an extensive series of diagnostic tests to rule out known causes of recurrent pregnancy loss. The tests performed were as follows: cytogenetic—parental and abortus karyotyping; anatomic—hysterosalpingogram; infectious—cervical cultures for mycoplasma, ureaplasma, gonnococcus, and chlamydia; immunologic—anticardiolipin antibody, antinuclear antibody, and lupus anticoagulant; and hormonal—serum progesterone, late luteal-phase endometrial biopsy, and thyroid-stimulating hormone.

[a] $P < .01$.

[b] $P < .02$.

pregnancy loss, only 1 (1.5%) of 67 exhibited similar X-inactivation skewing (≥90%) with the same assay system (table 1). This finding is statistically significant ($P < .01$, one-tailed Fisher's exact test). The distribution of X-inactivation ratios for both cases and controls is shown in figure 2.

Although the frequency of skewed X inactivation in the control women is lower than that observed by Naumova et al. (1996), this finding remains significant in comparison with the frequency observed in X-inactivation controls in other published reports (table 1). Plenge et al. (1997) found that in 115 unrelated control-group women, 4 (3.5%) showed skewed X inactivation ≥90%. When this result is compared with our case population, the association remains statistically significant ($P < .02$, one-tailed Fisher's exact test). In a study of the effect of aging on patterns of X inactivation, Gale et al. (1997) found that 3 (3.2%) in 94 control-group women in the cohort including the age range of our cases and controls (17–50 years) showed skewed X inactivation ≥90%. Again, our case group shows a statistically significant increase in the frequency of highly skewed X inactivation when compared with this control group ($P < .02$, Fisher's one-tailed exact test).

The excess of women with idiopathic RSA observed in our study who showed highly skewed X inactivation suggests that ~15% of women with RSA may be carriers of X-linked cell-autonomous lethal traits. However, there are two potential confounding variables that merit further discussion: the mechanism of selection in peripheral leukocytes and the effect of aging on X inactivation.

In Belmont's (1996) review of X inactivation and mechanisms of skewing, it was hypothesized that some individuals showing skewed X inactivation in blood samples (peripheral leukocytes) may exemplify somatic selection for a subset of hematopoietic cells. Such selection may or may not be cell-autonomous lethal, since a modest growth disadvantage may result in pronounced skewing over extended time. However, because we show an association between a lethal phenotype (RSA) and highly skewed X inactivation, we believe our hypothesis is also likely, that is, a subset of women with highly skewed X inactivation are carriers of cell-autonomous lethal traits. Such lethal traits could be subcytogenetic deletions, as reported by Pegoraro et al. (1997), or single-gene mutations, either of which would result in RSA.

Since reports of the effect of aging on X inactivation have shown an increased frequency of skewed X inactivation in older women, we need to exclude the possibility that our observed association was caused by an age effect (Busque et al. 1996; Gale et al. 1997). The group of reproductive-age women (17–50 years) studied by Gale et al. (1997) show a statistically significant lower frequency of highly skewed X inactivation, when compared with our case group. The distribution of ages among the controls in the population studied by Gale et al. is not significantly different from the distribution of ages among our cases and controls. Furthermore, the seven women with idiopathic RSA and highly skewed X inactivation in our case group are distributed throughout this range (mean age in case group is 34.8 ± 6.0 years; the ages of case women with highly skewed X inactivation are 28, 37, 39, 40, 41, 42, and 46 years). Thus, we feel it is unlikely that our observed association is caused by an age effect.

Future efforts will be directed at expanding the patient populations studied, with the use of both positive (RSA of known cause) and negative (multiple live-born children in the absence of any spontaneous abortions) control groups. This type of study will enable ascertainment of larger pedigrees cosegregating skewed X inactivation and pregnancy loss, leading to the identification of specific gene loci causing RSA. In such families, the molecular phenotype of skewed X inactivation should permit the genetic mapping of these loci.

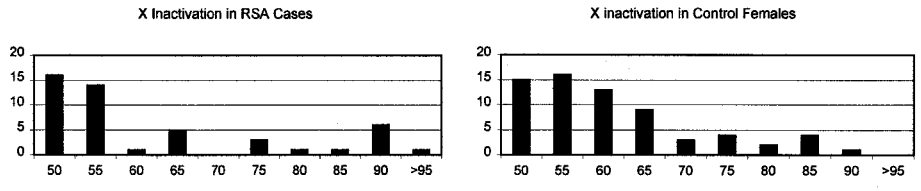

Figure 2 Frequency (*vertical axis*) of X inactivation (*horizontal axis*) in RSA cases ($n = 47$ [*left histogram*]) and controls ($n = 67$ [*right histogram*]). Women with RSA show a statistically significant abundance of highly skewed X-inactivation values, compared with control women. The X-inactivation values, which are reported as the percentage of activity of the more active allele; thus the data range is 50%–100%, inclusive. Although other groups have found the frequency of skewed X inactivation among controls to be closer to 10%, these studies use methodologically different assays, such as digestion with *Hha*I (Naumova et al. 1996). These specific methodological differences appear to yield distributions significantly different from those obtained in the present study and in studies published elsewhere (Busque et al. 1996; Gale et al. 1997; Pegoraro et al. 1997; Plenge et al. 1997).

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No Evidence of Linkage for Chromosome 1q42.2-43 in Prostate Cancer

To the Editor:

On the basis of a genomewide search involving 47 French and German families with multiple cases of prostate cancer, Berthon et al. (1998) reported linkage to chromosomal region 1q42.2-43 (multipoint nonparametric Z score of 3.1, $P = .001$ at marker D1S2785). This finding is interesting because, although D1S2785 is considerably distal to the region 1q24-25—identified by Smith et al. (1996) as containing the putative hereditary prostate cancer locus HPC1—it is only 14 cM away from the marker D1S235, which also produced an elevated Z score in the scan by Smith et al. In an attempt to confirm the finding by Berthon et al., we have evaluated linkage to three markers in the 1q42.2-43 region in 97 unrelated families containing three or more medically verified diagnoses of prostate cancer in first- or second-degree relatives. Eighty-two of these families fulfilled one or more of the proposed criteria for families whose prostate cancer is likely to be hereditary (i.e., three or more affected individuals within one nuclear family, affected individuals in three successive generations, and/or two or more individuals affected at age <55 years). Seven families were African American, four were Japanese American, and three were Chinese American. The families were identified from several sources, described by Hsieh et al. (1997). The mean number, per family, of affected and genotyped individuals was 2.6 (range 2–5), and the mean age at diagnosis of all affected individuals was 66.9 years (67.0 years in white families, 64.1 years in African American families, 69.2 years in Asian American families). The overall number of genotyped affected individuals and the overall mean age at diagnosis are similar to those found for the families reported by Berthon et al. (1998). A total of 382 samples were genotyped for the three markers. Genotyping was performed by the NHLBI (National Heart, Lung, and Blood Institute) Mammalian Genotyping Service at the Marshfield Medical Foundation (Yuan et al. 1997), by use of an ABI 377 sequencer to read fluorescently labeled primers for PCR products. We retyped individuals with ambiguous or missing genotypes and also retyped one or more

Table 1

Multipoint *Z* Values and NPL *Z* Values in 97 Families with Prostate Cancer, for Three Markers in Chromosomal Region 1q42.2–43

Marker	Distance[a] (cM)	Mean Age at Onset <67 Years (48 Families)		Mean Age at Onset >67 Years (49 Families)		All 97 Families	
		Multipoint *Z*	NPL*Z* (*P*)	Multipoint *Z*	NPL *Z* (*P*)	Multipoint *Z*	NPL *Z* (*P*)
D1S235	10.6	−11.46	−1.05 (.85)	−8.82	.40 (.31)	−10.18	.08 (.46)
D1S2785	0	…	…	…	…	…	…
D1S547	2.3	−16.42	−1.52 (.94)	−12.83	−1.01 (.84)	−14.69	−1.04 (.85)
D1S1609	9.3	−18.98	−1.92 (.98)	−10.78	−.36 (.63)	−14.69	−.97 (.83)

[a] From D1S2785, the marker most strongly linked in the data of Berthon et al. (1998).

relatives of each such individual to insure interlaboratory comparability. All samples were typed without knowledge of disease status.

Parametric LOD scores, nonparametric Z scores, and one-tailed *P* values were obtained with the software GENEHUNTER (Kruglyak et al. 1996). For the parametric analyses, we assumed an autosomal dominant mode of inheritance of a disease-susceptibility allele with frequency .003 and with penetrances as estimated in the segregation analysis by Carter et al. (1992). For the multipoint analyses, the three markers were assumed to be in the order shown in table 1. We estimated allele frequencies for the three markers in family founders, using the software FASTLINK (Cottingham et al. 1993; Schaffer et al. 1994).

Table 1 shows the three markers analyzed and their estimated positions in relation to D1S2785, the marker most strongly linked in the data of Berthon et al. (1998). Table 1 also shows multipoint LOD scores and nonparametric Z scores among the 48 families with mean age at diagnoses <67 years, among the 49 remaining families, and among all families. The negative values of the LOD scores and Z scores and the nonsignificant *P* values provide no support for linkage. The three markers each had negative two-point Z scores, and either negative or very small positive heterogeneity LOD scores. Berthon et al. found stronger evidence for linkage when analysis was restricted to the nine families in their data for which the age at diagnosis of all affected members in the last generation was <60 years. In contrast, we found negative scores similar to those in table 1 when we analyzed the 14 families in the present data who satisfied this criterion.

Thus, the present data do not support the possibility of a prostate cancer–susceptibility gene in the 1q42.2-43 region. Although the reasons for this lack of confirmation are unclear, several possible explanations come to mind. First, the spikes in this region seen by both Smith et al. and Berthon et al. could be due to chance, since the evidence supporting linkage is somewhat weak. The *P* value of .001 for the Z score of 3.1 for marker D1S2785, reported by Berthon et al., does not reflect the multiple testing involved in their genome scan. As noted by Lander and Kruglyak (1995), a nominal *P* value of .001, such as that reported by Berthon et al., can be expected to occur by chance once in every genome scan. To keep the chance of encountering a false positive $\leq 5\%$, one must impose a threshold of nonparametric Z score >4.1, LOD score >3.6, which corresponds to a significance level of $P = 2 \times 10^{-5}$.

A second possible explanation for the lack of confirmation is differences in ancestry and ethnicity in the two sets of families. Although most of the families in the present analysis were white and of European ancestry, their genetic heritage differs from that of the French and German families analyzed by Berthon et al.

Prostate cancer may be diagnosed at a more advanced stage in France and Germany than in the United States, because of international differences in the prevalence of screening with prostate-specific antigen (PSA). However, such differences are unlikely to explain the discrepant results, because most of the prostate cancers in the present U.S. series were diagnosed before PSA screening became prevalent. Moreover, there is no evidence that PSA screening is less likely to detect inherited cancer than sporadic cancer.

The lack of confirmation for this locus mirrors the difficulties in confirmation of the HPC1 locus. Some data have shown only weak confirmation (Hsieh et al. 1997; Cooney et al. 1997), whereas other data do not support linkage (McIndoe et al. 1997; Eeles 1998). This ambiguity may reflect considerable heterogeneity in hereditary prostate cancer, with any one locus accounting for only a small fraction of such disease. It also may reflect an inability to identify sporadics and to model them correctly.

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A Third Locus Predisposing to Multiple Deletions of mtDNA in Autosomal Dominant Progressive External Ophthalmoplegia

To the Editor:

Autosomal dominant progressive external ophthalmoplegia (adPEO) is a mitochondrial disorder characterized clinically by ptosis and progressive muscle weakness—most severely affecting the external eye muscles—with disease onset in early adulthood. Ataxia, dysphagia, sensorineural hypoacusia, neuropathy, tremor, cataract, and/or depression are present in some families (Zeviani et al. 1989, 1990; Servidei et al. 1991; Suomalainen et al. 1992; Melberg et al. 1996). In a Swedish adPEO family, hypogonadism cosegregated with the disease (Melberg et al. 1996). The typical morphological findings are ragged red fibers in the modified Gomori trichrome staining of muscle samples, and accumulation, enlargement, and abnormal shape of the mitochondria, on electron microscopy. Moderate reduction of the activities of respiratory-chain complexes I and IV is detected in biochemical analysis, and mtDNA analysis shows multiple mtDNA deletions in muscle samples (Zeviani et al. 1990; Servidei et al. 1991; Suomalainen et al. 1992, 1997).

We have shown previously that adPEO is a genetically heterogeneous disorder, by assigning two distinct genomic loci; one, in a Finnish family, on 10q24 (MIM 157640; Suomalainen et al. 1995) and the other, in three Italian families, on 3p14-21 (MIM 601226; Kaukonen et al. 1996). However, several adPEO families studied showed exclusion of both of these loci, thus indicating the existence of one or more additional adPEO loci (MIM 601227; Suomalainen et al. 1995; Kaukonen et al. 1996). Here we report a genomewide search and the assignment of a third adPEO locus.

Figure 1 shows the adPEO pedigree used in the genome scan, and figure 2 shows Southern blot–hybridization analyses of muscle mtDNA of patient 306 and a healthy control. The affected status was determined by observation of marked clinical symptoms in the neurological examination and/or by detection of multiple mtDNA deletions in the analysis of the muscle-biopsy specimen. Muscle samples from patients 306 and

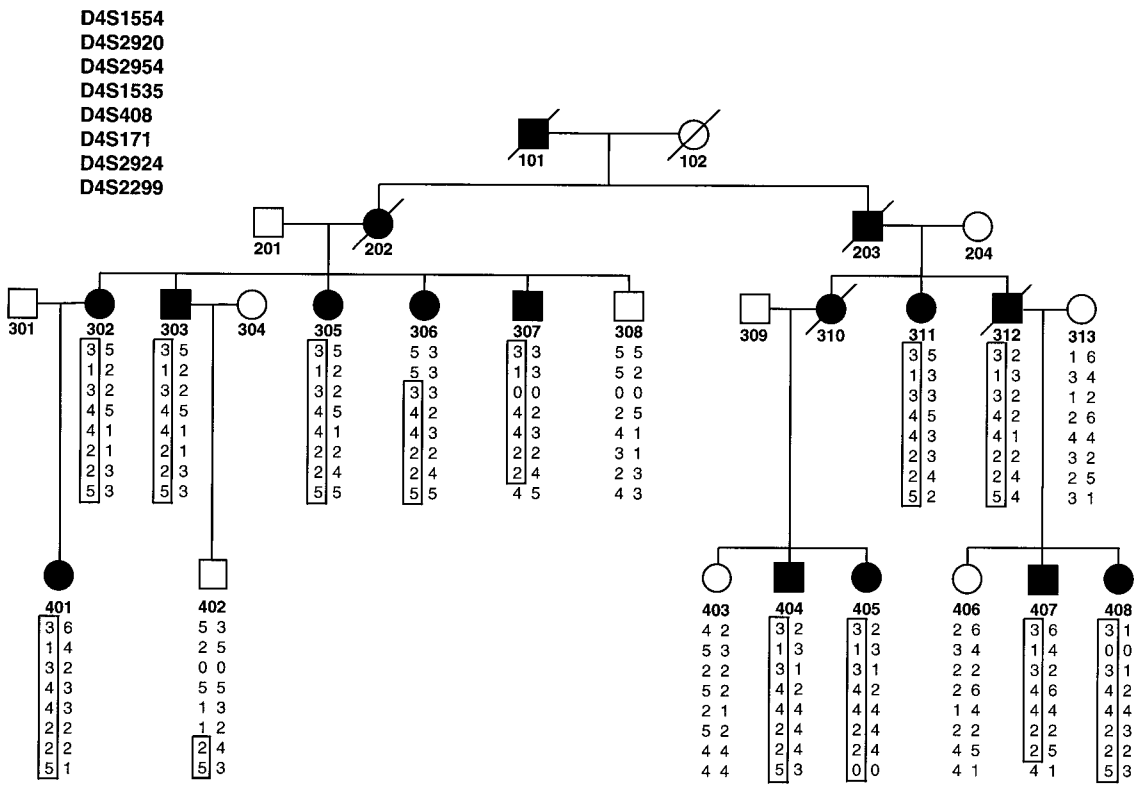

Figure 1 adPEO family with linkage to the markers on chromosome 4q. The individuals with marked clinical symptoms and/or deletions of mtDNA detected by PCR or Southern blot–hybridization analyses are indicated by blackened symbols. The unblackened symbols indicate clinically healthy individuals age >45 years. The markers used in the haplotype construction are shown in the upper-left corner of the figure. The boxes around the haplotypes indicate the shared regions of the affected chromosomes. The recombination events limit the adPEO region, between D4S2924 and D4S2920, to within a distance of 13.5 cM.

408 were examined. The clinical symptoms in this family were milder than those in families with linkage to the 10q and 3p loci (Suomalainen et al 1995; Kaukonen et al. 1996). All the patients had progressive external ophthalmoplegia and ptosis but had no generalized muscle weakness. Age at onset was ~35 years. Several affected family members had sensorineural hypoacusia. Two subjects had goiter associated with hypo- or hyperthyroidism (patients 305 and 306, respectively). Two elderly subjects (patients 310 and 311) suffered from dementia manifesting as impairment of the cognitive functions, with no affective component. An increased serum-lactate level at rest was detected in one patient (patient 408). A typical example of a patient in this family is patient 306, who at age 67 years had ptosis and ophthalmoplegia, bilateral hearing loss, and hyperthyroidism with goiter. Her standard electromyogram was myopathic. Nerve conduction–velocity studies were normal. Multiple mtDNA deletions were detected in an analysis of muscle specimen from the biceps brachialis. Histological analysis of her muscle sample revealed that 3% of the fibers were ragged red and 5% showed partial COX deficiency. No elevation of lactic acid was detected at rest or after standard exercise, and her serum creatine-phosphokinase level was within the normal range. Respiratory-chain analysis showed slightly reduced activities of complexes III and IV (65%–70% of controls' mean), whereas activities of complexes I and II were within the normal range. Informed consent was obtained from all family members, and total DNA was extracted from lymphoblasts or from 10–150 mg of frozen muscle, as described by Zeviani et al. (1988). Southern blot analysis, with *Pvu*II restriction digestion of total DNA, preparation of total human mtDNA as the hybridization probe, and PCR amplifications to detect mtDNA deletions, were conducted as described elsewhere (Zeviani et al 1988; Kaukonen et al. 1996). $\gamma[^{32}P]$-ATP–labeled, PCR-amplified microsatellite markers were separated onto a 5% denaturing polyacrylamide gel and visualized by autoradiography. Fluorescently labeled PCR-amplified microsatellite markers were typed by use of a model 377 Applied Biosystems automatic sequencer (Perkin-Elmer).

The marker set used for the genomewide gene search

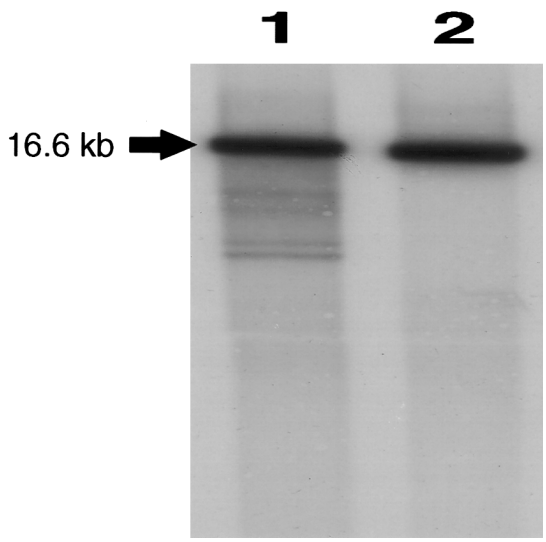

Figure 2 Southern blot–hybridization analysis of total muscle DNA, with full-length mtDNA as a probe. Muscle DNA of patient 306, showing normal-size mtDNA of 16.6 kb (*arrowhead*), and additional bands of lower molecular weight, representing mtDNA populations with multiple large deletions (lane 1) and a muscle sample from a control individual with no mitochondrial disease, with only normal-size mtDNA molecules (lane 2) are shown.

was chosen by use of marker-location information obtained from Généthon (Dib et al. 1996), the Cooperative Human Linkage Center, and the Genetic Location Database (LDB), with an average intermarker spacing of 15 cM. An autosomal dominant model was used in linkage calculations, and the frequency of the disease allele was estimated to be .00001. To avoid the potential danger of considering young, clinically unaffected, and nonmuscle-biopsied patients as healthy, we performed the primary calculations as an affected-only analysis, using even-allele frequencies for each marker allele. Individuals with marked clinical symptoms and/or multiple mtDNA deletions in their muscle were considered to be affected, and all other family members were considered to have an "unknown" affected status. We performed chromosome 4q calculations also by considering the clinically healthy family members age >45 years to be healthy, with .8 penetrance to allow for exceptionally late appearance of the disease.

Data-simulation analyses were performed with the SLINK and MSIM options of the LINKAGE package (Ott 1989; Weeks et al. 1990). We calculated the average expected LOD score from 2,000 replicates with a five-allele marker, using even-allele frequencies, to be 3.34 (SD = 0.62) at recombination fraction (θ) of .0 with the affected-only model, and to be 4.23 (SD = 0.92) when information from clinically healthy family members age >45 years was included. The pairwise and multipoint LOD-score values were calculated with the FASTLINK option (Cottingham et al. 1993; Schaffer et al. 1994) of the MLINK and LINKMAP programs of LINKAGE (Lathrop et al. 1984).

The two known adPEO loci on chromosomes 10q24 and 3p14-21 were first analyzed by genotyping the markers linked to these loci, as described elsewhere (Suomalainen et al. 1995; Kaukonen et al. 1996). These loci were unequivocally excluded from carrying the disease gene in this family, on the basis of haplotype construction and multipoint linkage analyses done across the critical regions. Many recombination events were detected in the disease chromosomes, and the multipoint LOD scores were <-2 across the entire regions of interest (data not shown).

After analysis of 315 markers, primary evidence of linkage was obtained with marker D4S408, which provided two-point LOD scores of 1.89, with the affected-only model, and 2.34, with the inclusion of clinically normal family members age >45 years as healthy, with .8 penetrance in the linkage calculations (table 1). Haplotypes across this chromosomal region were constructed with informative markers D4S1554, D4S2920, D4S2954, D4S1535, D4S408, D4S171, D4S2924, and D4S2299. Recombination events detected in subjects 306 and 402 (fig. 1) limit the third adPEO locus to <13.5

Table 1

Pairwise LOD Scores of Chromosome 4q Markers

Marker and Analysis[a]	Maximum LOD score at θ =				
	.00	.01	.05	.10	.15
D4S1554:					
1	1.61	1.57	1.38	1.15	.92
2	2.28	2.22	1.99	1.70	1.41
D4S2920:					
1	$-\infty$	.44	.93	.96	.85
2	$-\infty$	1.32	1.74	1.69	1.51
D4S2954:					
1	1.29	1.25	1.10	.92	.75
2	1.73	1.68	1.51	1.29	1.08
D4S1535:					
1	2.62	2.56	2.30	1.98	1.64
2	3.51	3.43	3.12	2.72	2.30
D4S408:					
1	1.89	1.85	1.65	1.41	1.18
2	2.34	2.29	2.10	1.85	1.60
D4S171:					
1	1.75	1.72	1.59	1.42	1.23
2	1.86	1.83	1.72	1.55	1.36
D4S2924:					
1	1.97	1.91	1.71	1.46	1.22
2	1.68	1.65	1.53	1.37	1.19
D4S2299:					
1	$-\infty$	−1.80	−.56	−.16	−.02
2	$-\infty$	−.98	.20	.52	.59

[a] "1" denotes affected-only analyses, and "2" denotes analyses done with inclusion of clinically healthy individuals age >45 years, with .8 penetrance. Pedigree of the family is shown in figure 1.

cM, between markers D4S2920 and D4S2924, on 4q34-35. The intermarker distances and cytogenetic localization of this adPEO locus were established by use of the mapping information of the LDB.

The same set of 4q markers was used in pairwise and multipoint linkage calculations. The best two-point LOD scores obtained were 2.62, with marker D4S1535 (affected-only model), and 3.51, when clinically healthy family members age >45 years were considered healthy, with .8 penetrance. The affected-only multipoint calculations across the critical region gave a maximum LOD score of 3.8; 4.7 was obtained when data on the healthy family members were included in the analyses (fig. 3).

Our sample contained four informative Italian families with adPEO (each family alone was informative enough to provide the maximum expected LOD score of >2 at $\theta = .01$, with 2,000 replicates) not previously assigned to known adPEO loci. To study the possible linkage to the 4q locus in these families, haplotypes were constructed across the entire 4q adPEO region. Many recombination events were observed across the region in the disease chromosomes, and the multipoint calculations across the region remained <-2 (data not shown), thus clearly excluding the chromosome 4 locus as the cause of the disease in these families.

adPEO appears to be a genetically heterogeneous disorder, with at least four different nuclear loci causing very similar phenotypes. This heterogeneity could be explained by causative genes that encode different components of related metabolic pathways or by different subunits of an enzyme complex. In databases, we have not found evidence of functionally related proteins previously mapped within the three chromosomal adPEO loci (GeneMap '98). To date, two other autosomally inherited diseases associated with mtDNA deletions have been mapped to distinct nuclear regions. Wolfram syndrome is an autosomal recessive neurodegenerative disorder sometimes associated with single or multiple mtDNA deletions, and it has been shown to be linked to chromosome 4p (Polymeropoulos et al. 1994; Barrientos et al. 1996*a*, 1996*b*). The defective gene (WFS1) was recently identified and it appears to function in the survival of islet β-cells and neurons (Inoue et al. 1998). A recessively inherited mitochondrial neurogastrointestinal encephalomyopathy with multiple mtDNA deletions was recently shown to be caused by mutations in the thymidine-phosphorylase gene on chromosome 22q13.32-qter (Hirano et al. 1998; Nishino et al. 1999).

The clinical symptoms of the patients in the family with linkage to 4q seem to be less severe than those in families with linkage to 10q and 3p. The muscular symptoms are limited to facial muscles: all of the patients presented with ophthalmoplegia and ptosis but with no exercise intolerance or generalized muscle weakness.

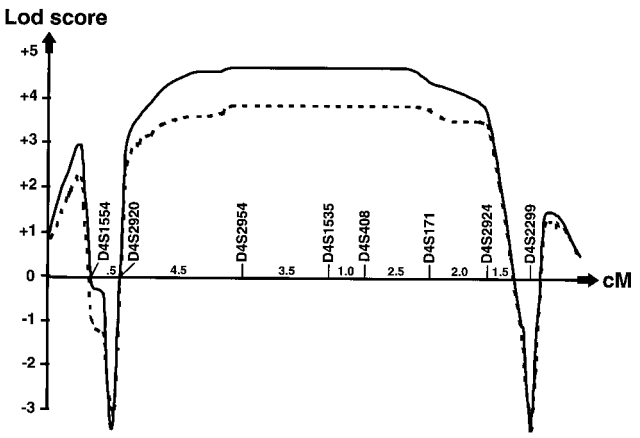

Figure 3 Multipoint LOD-score calculation across the 4q adPEO region. The dotted line represents the analyses done with information from the affected individuals, giving a maximum LOD score of 3.8. The solid line indicates the multipoint calculations done when data from clinically healthy individuals age >45 years were added, giving a maximum LOD score of 4.7. The markers used in the calculations are shown above the X-axis, and the intermarker distances are shown between the markers.

Most patients had sensorineural hypoacusia, and some had goiter or dementia. It remains uncertain whether the latter symptoms are a result of the adPEO-gene defect, because of the relatively high prevalence of these disorders in the general population.

To date, ~65 expressed sequence tags representing different genes have been localized to the 4q adPEO region, and eight of these represent known genes (GeneMap '98). The adenine nucleotide translocator is a key metabolic enzyme of the mitochondria, transporting ADP and ATP across the inner mitochondrial membrane. The gene for the heart- and muscle-specific isoform (ANT1) has been localized to 4q35 (Fan et al. 1992). The ANT1 knockout mice showed ragged red muscle fibers and proliferation of mitochondria, lactic acidosis, severe exercise intolerance, and cardiomyopathy (Graham et al. 1997). Our patients lacked the cardiac symptoms, but otherwise the symptoms of the patients resembled those of ANT1 knock out mice, making ANT1 a good candidate gene for adPEO. Whether ANT1 is involved in the pathogenesis of adPEO is being analyzed. In addition, the gene for dominantly inherited facioscapulohumeral muscular dystrophy has been localized to the 4q adPEO region (Wijmenga et al. 1990). adPEO and this dystrophy share sensorineural hearing loss as a symptom of the disease, but our patients had neither generalized muscle weakness nor retinal changes. The eventual characterization of the first adPEO gene will not only reveal one of the pathogenic mechanisms causing the genetically heterogeneous disease but will also enhance the search for the remaining adPEO genes and will improve our funda-

mental understanding of mtDNA stability and maintenance in the cell.

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Electronic-Database Information

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

Cooperative Human Linkage Center, http://www.chlc.org/ (for markers)

GeneMap '98, http://www.ncbi.nlm.nih.gov/genemap/ (for markers)

Genetic Location Database (LDB), http://cedar.genetics.soton.ac.uk/public_html/index.html (for markers)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for adPEO loci in a Finnish family [MIM 157640], in three Italian families [MIM 601226], and from other sources [MIM 601227])

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Possible Interaction between USH1B and USH3 Gene Products as Implied by Apparent Digenic Deafness Inheritance

To the Editor:

The Usher syndromes (USHs; MIM 276900–276904, 601067, 60297, and 602083) are a group of autosomal recessive hereditary disorders characterized by the association of sensorineural hearing impairments and progressive visual loss due to retinitis pigmentosa. Three types of USH are distinguished on the basis of severity and onset of auditory and vestibulary dysfunctions. To date, USHs are mapped to nine different genomic loci: USH1A–F, USH2A, USH2B, and USH3 (Hereditary Hearing Loss home page). USH3 (MIM 276902), assigned to chromosome 3q, is regarded as the rarest form of USH (Sankilla et al. 1995)

The human myosin VIIA gene (MYO7A), located on 11q14, has been shown to be responsible for USH1B (MIM 276903), which is the most common USH1 subtype, accounting for ~75% of all type 1 cases (Weil et al. 1995). More recently, MYO7A has also been shown to be responsible for nonsyndromic recessive and dominant deafness (DFNB2 and DFNA11), both types having been assigned to the same 11q chromosomal region (Liu et al. 1997*a,* 1997*b;* Weil et al. 1997). These findings clearly indicate that the enzymatic activity of MYO7A is critical for normal function in the inner ear and that different mutations may cause different dysfunctions that are manifested by distinct phenotypes. Here we report on two novel MYO7A mutations that may have a synergistic effect on the symptoms of another USH different from USH1B.

Among USH-affected families recruited as part of a study on the genetics of USH, results of which were published in this journal (Adato et al. 1997), was a nonconsanguineous family of Jewish Yemenite origin that included two affected and six healthy siblings. The two affected brothers in this family have different USH phenotypes. One of the affected brothers (1549 in fig. 1) has a typical USH1 phenotype: he has a history of prelingual profound auditory impairment; he uses sign language for communication, since hearing aids are unhelpful in his case; and developmental milestones (Smith et al. 1994) in his childhood are consistent with congenital vestibular dysfunction. The other affected brother (1636 in fig. 1) has a typical USH3 phenotype: he has progressive hearing loss, with postlingual onset; he uses hearing aids and verbal communication; and he receives psychiatric therapy for mental problems. In both affected brothers, the presence of bilateral progressive pigmentary retinopathy has been diagnosed (with onset during early adolescence).

Members of this family were typed for 30 polymorphic markers spanning all nine known USH loci (USH1A-F, USH2A, USH2B, and USH3). Marker alleles were identified and arranged into the most likely haplotypes, as shown in figure 1. Haplotype segregation and linkage analysis resulted in exclusion of all USH1 and USH2 loci (LOD scores range from -1.46 to -3.72) and suggested linkage only to the USH3 locus (with a maximum LOD score of 1.35 for marker D3S1279). Both affected brothers showed homozygosity for alleles of four markers: D3S1315, D3S1279, D3S3625, and D3S1294. Homozygosity of USH3 haplotypes in the affected brothers—and the fact that, although not known to be related, both parents originate from a small Jewish community in Yemen—suggest a possible common origin for both USH3-bearing chromosomes. This "USH3 haplotype" was found to be carried (one copy) by only 2 of 54 Jewish Yemenite control subjects tested for its presence. The homozygote interval in both affected brothers and the position of recombination in the paternal chromosome of one healthy progeny (1643; see fig. 1) suggest that the USH3 gene is located between markers D3S1299 and D3S3625. This result is in agree-

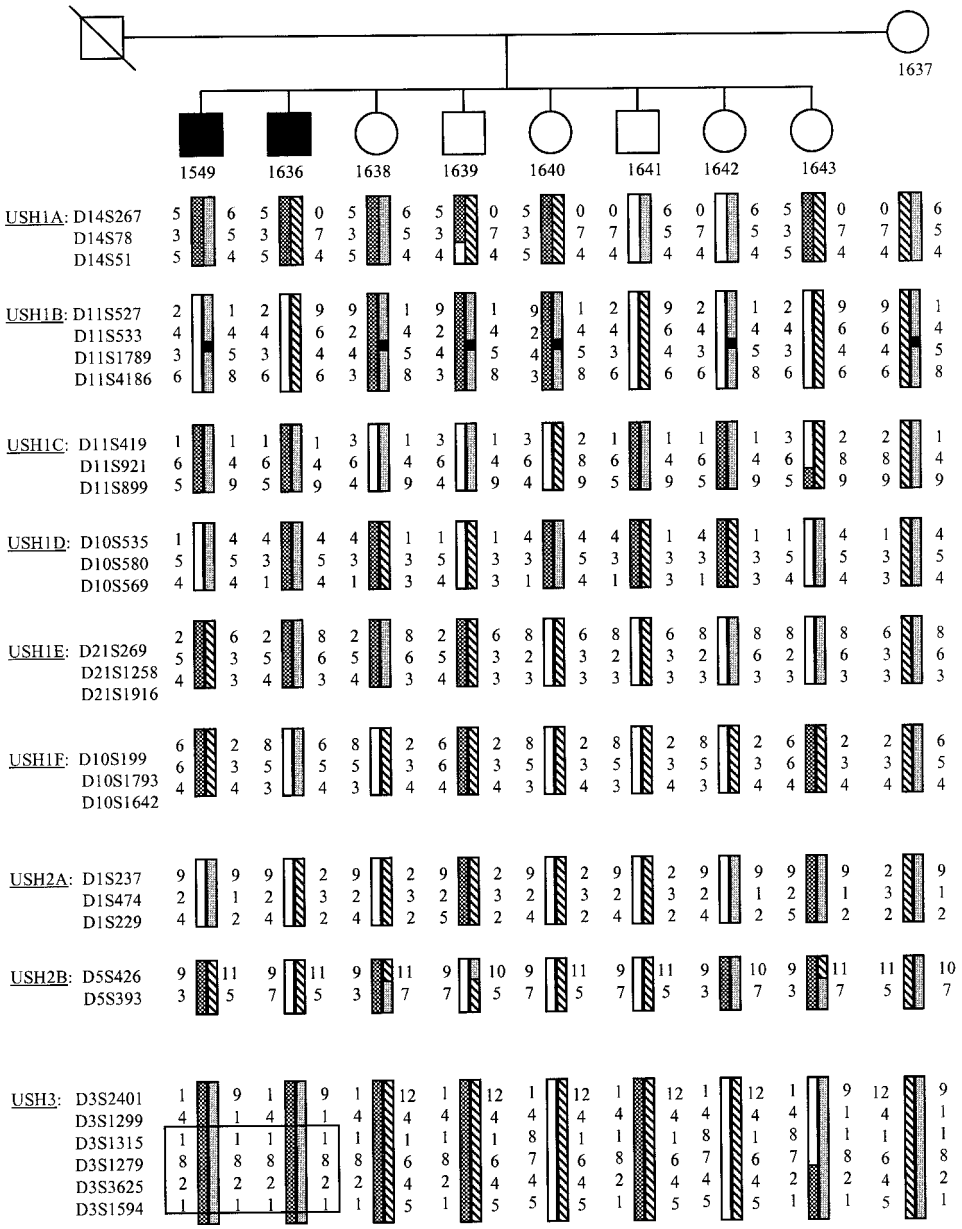

Figure 1 Genomic DNA extracted from blood of family members was used as a template for PCR amplification, which was done with 30 pairs of specific primers of markers spanning all nine USH loci. Marker alleles, identified according to their relative mobility on a denaturing formamide 4% acrylamide gel in all family members, were arranged into the most likely haplotypes. This haplotype arrangement results in exclusion of all USH1 and USH2 loci and suggests linkage only to the USH3 locus. Maternal chromosomes are gray and striped whereas paternal chromosomes are white and dotted. Blackened squares on the gray USH1B maternal chromosomes indicate the presence of the mutated MYO7A. The homozygote interval, of both affected brothers, in the USH3 locus is boxed.

ment with the location suggested by Sankilla et al. (1995) and by Joensuu et al. (1996). The order of markers spanning the USH3 linkage region, as presented in figure 1, is cen, D3S2401, D3S1299, D3S1315, D3S1279, D3S3625, D3S1594, tel, in agreement with the order presented in the Whitehead contig (Whitehead Institute for Biomedical Research). However, in this order, the position of the markers D3S1315 and D3S1279, which our findings indicated were the most closely linked to USH3, differs from the one suggested by Joensuu et al. (1996).

Since one of the affected brothers had an USH1 phenotype, family members were screened for mutations in the human MYO7A gene, which has been shown to be responsible for USH1B. Two new close nucleotide changes were detected in exon 25 of the gene on one

maternal chromosome: a T→C transition and a guanine deletion 5 nt upstream of this transition (fig. 2). None of these changes were found in >200 control chromosomes tested by allele-specific oligonucleotide analysis, as described by Whithney et al. (1993). This mutated MYO7A is carried by the brother with the USH1 phenotype (1549) but not by his affected brother with the USH3 phenotype (1636). The mother (1637) and two unaffected siblings (1638 and 1639), who are all double heterozygotes for the mutated MYO7A and for a single USH3 haplotype, show no evidence of any USH symptoms or nonsyndromic deafness. This suggests a digenic inheritance pattern, with a possible synergistic interaction between MYO7A and the USH3 gene product, where presence of a single defective MYO7A allele seems to increase the severity of deafness as a part of the clinical symptoms associated with USH3.

Evidence for digenic inheritance of nonsyndromic deafness was already presented in the case of a Swedish family (Balciuniene et al. 1998), whose affected members were carriers of DFNA2 and/or DFNA12. Increased severity of deafness was found in family members that were carriers of both alleles. This clear additive effect differs from the situation in our Yemenite family, where mutated MYO7A appears to be phenotypically expressed only on the background of two defective USH3 alleles, suggesting an interaction between the MYO7A and the USH3 gene products. Digenic inheritance was also suggested as one of the possible explanations in the case of DFNB15 (Chen et al. 1997). This is an autosomal recessive nonsyndromic deafness, found in a family of Indian origin, linked to two loci on chromosomes 3q and 19p. Most interestingly in relation to our work, one of these loci, 3q21.3-3q25.2, includes the USH3 locus and the other, 19p13.3p13.1, includes (among others) the MYO1F gene (Hasson et al. 1996), which is another member of the unconventional myosin group.

Human MYO7A is a member of the unconventional myosins group (Weil et al. 1996). All myosins have three different functional domains defined within their heavy chains: an N-terminal motor domain, a regulatory (light-chain–binding) domain, and a tail domain that varies dramatically in length and in sequence among myosins (Mooseker and Cheney 1995). The functions of myosin-tail domains are largely unknown. However, a common assumption is that the tail directs the interaction of a given myosin with its cargo (Mermall et al. 1998). MYO7A is predicted to dimerize, on the basis of the coiled-coil sequence motif at the start of its tail region.

The sequence changes detected in exon 25 of MYO7A in some members of the presented family are expected to result, at the protein level, in a Leu→Pro substitution at codon 1087 and in a frameshift of the reading frame starting at codon 1089. Both these AA codons are located after the coiled-coil domain of the protein. This

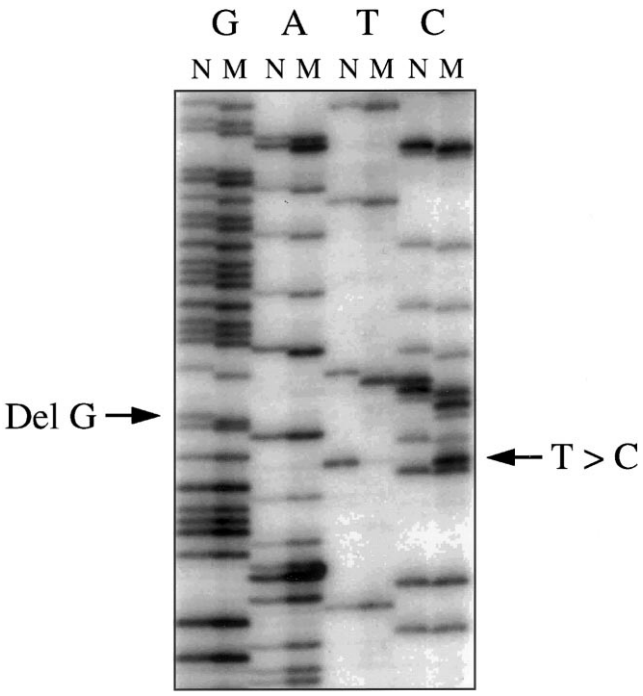

Figure 2 Comparative electrophoresis of normal (N) and mutated (M) exon 25 PCR cycle-sequencing reaction, with SequiTherm EXEL DNA Sequencing Kit (FMC). Products were electrophoresed side by side through Long Ranger Gel (Epicentre Technologies): ddGTP-terminated products of normal exon 25 appear next to ddGTP-terminated products of mutated exon 25; and ddATP-terminated products of normal exon 25 appear next to ddATP-terminated products of mutated exon 25. Arrows indicate the T→C transition and the guanine deletion (5 nt up stream of the transition).

frameshift would result in the formation of a UGA stop codon 18 amino acids downstream from the deletion site and, therefore, in the translation of a truncated protein that lacks >50% of its normal AA sequence, which comprises most of the MYO7A tail domain. Segregation of the mutated MYO7A with healthy members of this family and, on the other hand, with the more severe USH phenotype, suggests a possible biological interaction between MYO7A and the USH3 gene products. This mutated MYO7A appears to be phenotypically expressed only on the background of two USH3 alleles.

Many disease mutations that introduce stop codons were found to lead to mRNA destabilization, as in the mouse MYO6 sv allele (Avraham et al. 1995). In such a case, segregation of the mutated MYO7A, as described above, could indicate that the normal USH3 gene product may affect the stabilization of the MYO7A mRNA or the protein.

If the mutated MYO7A in this Yemenite family was translated, then it would not lack its coiled-coil sequence motif. Unlike the case of DFNB11, where a 9 bp deletion in the coiled-coil region of MYO7A was suggested to

have a dominant negative effect (Liu et al. 1997*b*), segregation of the mutated MYO7A with healthy members of our family clearly determines the recessive nature of its mutation. There are several possible explanations for this difference. The truncated MYO7A produced in our case may be incompetent for dimerization, since it is unknown whether the remainder of the tail is required for self assembly or if the truncated molecule may be destabilized. Another possible reason is the formation of a partially functional heterodimer. These possibilities would yield $\leqslant 50\%$ of the normal protein amounts. In any kind of direct or indirect USH3-MYO7A protein interaction, a reduced-dosage effect of active MYO7A protein is likely to have a synergistic effect on the background of the two impaired alleles of the USH3 gene product.

One possible explanation for the USH3-MYO7A interaction is that the USH3 protein might be involved in targeting or binding MYO7A to the plasma membrane. The tail of MYO7A consists of a direct repeat containing two elements. The distal element, the talinlike domain, shows significant homology to the N-terminus of talin and limited homology to the N-termini of other members of the band-4.1 superfamily of actin-binding proteins (Weil et al. 1996; Chen et al. 1997). In talin and in band 4.1, this region binds to acidic phospholipids and mediates protein-protein interactions. Therefore, it is thought that talinlike motifs serve to bind and/or target the myosin to the plasma membrane (Cheney et al. 1993; Titus et al. 1997).

It is also possible that USH3 is a cytoskeletal component and as such interacts with the MYO7A protein: Actin cytoskeleton is essential for proper the function of the inner ear, and deafness-associated genes such as MYO7A and MYO15 as well as the human diaphanous protein are assumed to be cytoskeletal components (Vasiliki and Petit 1998).

The MYO7A protein may also serve an inner hair-cell–specific role distinct from its role in actin. This role might be complementary to the function of the USH3 gene product. For example, in mice, MYO7A was found to be involved in hair-cell vesicle trafficking of aminoglycosides, which are known to induce ototoxicity (Richardson et al. 1997).

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Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Usher syndrome subtypes [MIM 276900, 276901, 276902, 276903, 276904, 601067, 60297, and 602083])

Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www.genome.wi.mit.edu/ (for Whitehead contig map)

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Anticipation in Familial Chronic Lymphocytic Leukemia

To the Editor:

The term "anticipation" in genetic diseases refers to earlier age at onset and/or increased severity in successive generations. For some neurodegenerative diseases, anticipation results from expansion of unstable trinucleotide repeats in successive generations (La Spada 1997). Epidemiological studies have demonstrated a significant familial effect for leukemia (Goldgar et al. 1994) and chronic lymphocytic leukemia (CLL) in particular (Cartwright et al. 1987; Linet et al. 1989). Families with multiple affected individuals are rare in population studies but may be more common in clinical samples (Cuttner 1992). The mode of inheritance is unknown for leukemia, although it has been hypothesized that in pedigrees with multiple affecteds the disease is due to a single autosomal dominant gene (Horwitz 1997). Evidence for anticipation in familial leukemia has been reported by Horwitz et al. (1996), on the basis of a literature review of published pedigrees with acute myelogenous leukemia (AML) and CLL. The average difference, in age of onset of CLL, between two generations in seven pedigrees (17 individuals), was 15 years, although the mean parent-offspring difference was 21 years. Yuille et al. (1998) have recently confirmed this finding, in 10 families with two generations affected with CLL (mostly parent-offspring pairs) systematically ascertained from a patient registry. They found the age of onset difference between generations to be 22 years. Given that there is a molecular basis for anticipation in some diseases, it is important to determine if there is anticipation in CLL. As with other diseases, anticipation in CLL could be due to a number of well-known sampling biases (reviewed by McInnis 1996), such as the tendency to select early-onset probands, parents with late onset, and families with simultaneous onset of disease in parents and offspring, or other biases that cause a truncation of the sample of families (Hodge and Wickramartne 1995; Fraser 1997). Another bias particular to CLL may arise from the fact that individuals are often diagnosed on the basis of routine blood tests when they are asymptomatic, and they may remain asymptomatic for a number of years. It is conceivable that an anticipation phenomenon could be attributable to changes in medical practice over time, such that, because of the greater routine use of clinical tests, individuals in the younger generations are being diagnosed earlier. Data collected by the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) program during the past 20 years have shown little secular change in the incidence of CLL (SEER). In

Table 1

Age at Onset and Year of Diagnosis in 13 Families Investigated for Anticipation

Family and Relationship to Proband	Generation	Age at Diagnosis (years)	Symptomatic at Diagnosis?[a]	Year at Diagnosis
1:				
Father	1	69	yes	1954
Proband (female)	2	47	yes	1972
Brother	2	49	no	1971
Brother	2	47	yes	1968
Sister	2	57[b]	no	1974
2:				
Father	1	75[c]	yes	1967
Uncle	1	63[c]	yes	1955
Proband (male)	2	56	?	1978
3:				
Uncle	1	70	?	1960
Proband (male)	2	50	no	1977
First cousin (female)	2	52	no	1978
4:				
Proband (male)	1	49	?	1984
Nephew	2	53	?	1988
5:				
Mother	1	82	yes	1980
Proband (female)	2	64	?	1985
6:				
Father	0[d]	79	?	1965
Proband (female)	1	74	no	1990
Son	2	55	yes	1991
7:				
Aunt	1	58	?	1961
Proband (male)	2	44	yes	1986
8:				
Mother	1	83	no	1978
Proband (male)	2	55	yes	1983
9:				
Mother	1	32	yes	1961
Proband (female)	2	37	yes	1993
Brother	2	42[b]	yes	1995
10:				
Mother	1	58	?	1965
Proband (female)	2	52	?	1991
11:				
Father	1	76	no	1997
Proband (male)	2	51	no	1997
12:				
Uncle	1	77	?	1984
Proband (male)	2	65	no	1996
Brother	2	50	no	1994
13:				
Mother	1	?	?	?
Proband (female)	2	35	yes	1990

[a] A question mark (?) indicates that data were not available.
[b] Individual sought diagnosis because of family history.
[c] Age at diagnosis is age at death.
[d] Not included in analysis.

fact, recent studies that have reported an increased incidence of CLL find the increase to be limited to *older* individuals (Call et al. 1994; Rozman et al. 1997). We have been studying familial CLL for a number of years and have analyzed age at onset in pedigrees with at least two generations affected. We find evidence for anticipation in these families, even when stage at diagnosis and other potential sampling biases are taken into account.

Since 1974, we have ascertained and collected clinical

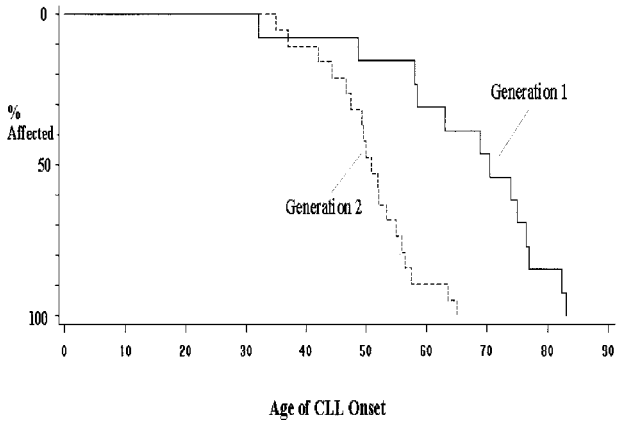

Figure 1 Age at onset of CLL, in two generations

data on 27 families, each of which has two or more confirmed cases of CLL (see Caporaso et al. 1991). Thirteen of these families have cases of CLL in two generations; the remainder have affected siblings and/or cousins. We have complete age-at-onset data for 32 individuals from the 13 two-generation families. One family has missing age-at-onset data on an affected parent. In the majority of cases, age at onset was determined on the basis of medical chart review, but in a few cases we relied on personal reports. Insufficient information was available to determine the disease stage for each individual, by use of either the Rai or Binet staging methods (reviewed in Dighiero and Binet 1996), but, whenever possible, we determined whether the individual was "symptomatic" or "asymptomatic" when diagnosed. We classified as "symptomatic" individuals showing signs or symptoms attributed to CLL, including lymphadenopathy, splenomegaly, anemia, and thrombocytopenia. Individuals were classified as "asymptomatic" if they presented only with a peripheral-blood absolute lymphocyte count (ALC) $\geq 5.0 \times 10^9$/liter (Zwiebel and Cheson 1998). For those individuals diagnosed asymptomatically, we also noted the age at which they became symptomatic, when this information was available in the medical chart. Individuals were classified into two generations, on the basis of their position in the pedigree. Generation 2 was defined as the youngest generation affected; the generation preceding them was considered to be generation 1. All affected individuals in each family were included in the analysis. There was one family with three generations affected; the individual in the oldest generation was not included in the analysis. The age-at-onset data for all individuals are displayed in table 1. In 11 of the 13 families, the proband was in the younger generation.

We found that the average age at onset in generation 1 is 66.7 years (SD 14.6) and that that in generation 2 is 50.7 (SD 7.8). These onset ages are similar to those reported by Horwitz et al. (1996). We used survival analysis to plot the age-at-onset distribution for each generation, using the Kaplan-Meier method as implemented in the SAS Lifetest procedure (Allison 1995). The results are displayed in figure 1. The difference between the two generations is highly significant, whether based on the log-rank test ($P = .0001$) or the Wilcoxon test ($P = .0009$).

We examined the generational differences after taking into account a number of possible biases. In this small sample, there were no significant age-at-onset differences between males and females, and each generation had an approximately equal proportion of males and females. We scored the individuals for whether they were symptomatic, asymptomatic, or unknown at diagnosis. The proportion of symptomatic individuals did not differ by generation (5/13 [.38] in generation 1 and 8/19 [.42] in generation 2; see table 1). In addition—after family 9, in which all members had unusually early ages at onset, was excluded—there was no difference, between symptomatic and asymptomatic individuals. There were two individuals in the sample who sought diagnosis because of their family history. These two individuals, indicated in table 1, had ages at onset of 57 and 42 years, so eliminating them would not change the findings. In order to analyze our data in the most conservative way possible, we increased the age at onset for five individuals (generation 2) who were asymptomatic at diagnosis but whose age at onset of clinical symptoms could be determined. In addition, there were two individuals from generation 1 who were diagnosed on the basis of death certificates and who were assigned onset ages equal to their ages at death. We lowered these individuals' ages at onset by 7 years, since this is the median time between age at onset of symptoms and death (Zwiebel and Cheson 1998). Even under these conservative assumptions, the age at onset in the second generation was significantly lower than that in the first generation (log-rank test $P = .0002$). It should also be noted that, even though we did not analyze all three generations in the three-generation family, the proband's father (generation 0) had a later age at onset than did either the proband or her son. We also considered the possible bias due to preferential ascertainment of families with simultaneous onset in two generations. In 5 of 12 families for which complete data were available, individuals in both generations were diagnosed within a 5-year period (table 1). In the seven other families, the average difference, in calendar year of diagnosis, between generations was 21 years, and the average difference, in age at onset, between generations was 13 years. Thus, our finding of anticipation is not being driven by the presence of families with simultaneous onset in two generations.

We find that, between the two generations in the fam-

ilies we studied, the average decrease in age at onset of CLL was 16 years. One could argue that we have preferentially ascertained early-onset probands. The average age at onset in all 27 of the families that we studied is 54.9 years for probands only and 59.6 years for all affecteds. The average age at onset of CLL has been reported to be ~70 in some population-based samples (Travis et al. 1992; Hjalmar et al. 1996) but ~62–65 in other studies (Radovanic et al. 1994; Rozman et al. 1997). Thus, probands from the multiplex families that we studied have a somewhat decreased age at onset, compared with that in cases from the population. However, for this analysis, we included all affected individuals in a family, which decreases any effect due to earlier onset in the index case. We cannot completely rule out the possibility that anticipation in these families is due to a cohort effect, since, in all of the families that we studied, birth cohort is confounded with generation. As is to be expected from the way in which pedigrees are usually ascertained in genetic studies, the second generation of individuals are from cohorts born more recently than those of the first generation. However, a cohort effect seems unlikely, given the lack of secular trends in CLL, as mentioned above. The age at onset of CLL is also sufficiently late in life that it would not affect fertility, and we are not likely to have missed families with early onset in parents and later onset in offspring. Horwitz et al. (1996) suggested that the very large difference (21 years) that they found between parents and offspring in published families was also consistent with the effect of some common environmental exposure. There is no well-established environmental risk factor for CLL, although susceptibility to some environmental risk factor might exist in subjects with some unidentified genetic susceptibility factor. Although we cannot rule out such an effect, we find a substantial but smaller age-at-onset decrease between generations. We also find the decrease to be present in families that are not correlated for year of diagnosis, arguing against a purely environmental explanation.

In conclusion, we report significant evidence for anticipation in familial CLL and have ruled out various biases that could account for the finding. Therefore, we plan to look for expanded trinucleotide repeats in candidate genes in families showing anticipation.

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SEER, http://www-seer.ims.nci.nih.gov/Publications/CSR7395 (for Cancer Statistics Review, 1973–1995)

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