Letters to the Editor

Am. J. Hum. Genet. 64:290–292, 1999

The Glu318Gly Substitution in Presenilin 1 Is Not Causally Related to Alzheimer Disease

To the Editor:

With 49 different mutations in the coding region, presenilin 1 (the gene is denoted "PSEN1"; the protein is denoted "psen1") is the most frequently mutated gene in early onset (onset age <65 years) Alzheimer disease (AD [MIM 104300]) (Sherrington et al. 1995; Cruts and Van Broeckhoven 1998). PSEN1 missense mutations are generally considered fully penetrant mutations. Mostly they are found in patients with a positive family history of early-onset AD compatible with autosomal dominant inheritance. Patients carrying the same mutation usually display very similar onset ages (Van Broeckhoven 1995).

An A→G transition at codon 318 in exon 9 of PSEN1, resulting in the nonconserved Glu→Gly substitution, has been reported, by us (Cruts et al. 1998) and others (Sandbrink et al. 1996; Forsell et al. 1997), in familial AD cases with onset ages of 35–64 years (Cruts and Van Broeckhoven1998). However, segregation of Glu318Gly with AD could not be demonstrated, because either no or too few relatives were available for DNA testing. PSEN1 Glu318Gly involves the last codon of exon 9 and is located in the middle part of the sixth hydrophilic loop of psen1. Because of the high variability in onset age of AD and the mutation's location in a psen1 region that is less conserved between psen homologues in human and other species, we previously had hypothesized that the Glu318Gly could be either an incompletely penetrant mutation or a rare polymorphism (Cruts and Van Broeckhoven 1998).

To evaluate the frequency of Glu318Gly and its contribution to AD, we screened incident and prevalent demented cases and age- and sex-matched controls derived from the Rotterdam Study. This is a prospective single-center population-based study of elderly residents ⩾55 years of age who are from a Rotterdam suburb (Hofman et al. 1991). Cognitive functioning was assessed and diagnosis of dementia made on the basis of the DSM-III-R definition (American Psychiatric Association 1987). Possible and probable AD was diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS)–Alzheimer's Disease and Related Disorders Associations criteria (McKhann et al. 1984). Vascular dementia was diagnosed according to NINCDS–Association Internationale pour la Recherche et l'Enseignement en Neurosciences criteria (Roman et al. 1993). At baseline, 474 prevalent demented cases were diagnosed (Ott et al. 1995). During follow-up, another 146 incident cases of dementia were detected (Ott et al. 1998). From 345 prevalent cases, 134 incident cases, and 256 controls, blood samples were available for DNA extraction. Controls were randomly selected among nondemented participants in the Rotterdam Study and were group matched on the basis of age (5-year intervals) and sex. To facilitate rapid screening for Glu318Gly, we developed a mismatch PCR assay that allows detection by *Bst*NI digestion of the mismatch PCR product. The forward mismatched primer was 5′-ATCCAAAAATTCCAAGTATAATCCAG-3′ and the reverse primer was 5′-CTGGGCATTATCATAGTTCTCAAG-3′.

PSEN1 Glu318Gly was observed in 2 (1.5%) incident and 11 (3.4%) prevalent demented cases and in 9 (4.1%) controls. In contrast to previous reports, we detected Glu318Gly in individuals who were elderly. The frequencies in incident and prevalent cases versus those in controls were compared by the Fisher exact test and were found to be not significantly different ($P = .22$ and $P = .65$, respectively). Of 13 demented Glu318Gly carriers, 10 were diagnosed with AD (4 possible AD and 6 probable AD), 1 with dementia associated with Parkinson disease, 1 with vascular dementia, and 1 with dementia associated with multiple sclerosis. Mean age at onset in demented Glu318Gly carriers (83.4 ± 4.7 years, range 72–88 years) was similar to that in demented noncarriers (81.0 ± 7.7 years, range 52–97 years). Cognitive functioning measured by the mini-mental state examination in the control group was similar in Glu318Gly carriers (26.4 ± 2.9) and noncarriers (27.0 ± 2.0). Since the $\epsilon 4$ allele of apolipoprotein E (APOE) is known to increase risk for AD (Pericak-Vance and Haines 1995), we also examined the APOE genotypes in the demented cases. However, the APOE*ϵ4-allele frequency in the demented Glu318Gly cases (19%) was not different from that in the total group of demented cases (21%), excluding a possible interaction between APOE*ϵ4 and Glu318Gly. Together, these find-

ings demonstrate that Glu318Gly is a rare allele (22 carriers/676 individuals, allele frequency 1.6%) in the Dutch population analyzed and that its presence is not associated with AD or dementia in general.

The relatively high frequency of Glu318Gly in the Dutch population analyzed may be explained if all subjects are distantly related. To test this possibility, we genotyped several polymorphic DNA markers located within and near PSEN1 (Cruts et al. 1995, 1998). All Glu318Gly carriers (cases and controls) shared one allele for D14S77 (203 bp; frequency 8%), the PSEN1 promoter (allele T; frequency 12%), and intron 8 polymorphisms (allele A; frequency 54%). Allele sharing was also observed at D14S1028, with 20 of 22 Glu318Gly carriers sharing the same allele (239 bp; frequency 4%). No obvious sharing of alleles was observed at D14S1004. Frequencies of the shared alleles were calculated in 118 control individuals coming from the same Rotterdam suburb (C. M. van Duijn, unpublished data). The probability of detecting this allele combination independently in 22 cases (4×10^{-35}) strongly suggests that all Dutch Glu318Gly carriers have one common founder. Glu318Gly is also frequently observed in populations of different geographic and ethnic origins (Baker et al. 1998; Forsell et al. 1998; Helisalmi et al. 1998; Mattila et al. 1998; Reznik-Wolf et al. 1998; Torres et al. 1998).

The mechanism by which mutations in PSEN1 lead to AD remains largely unknown. However, an increasing amount of in vivo and in vitro evidence suggests that the mutated psen1 expresses its pathogenic effect by processing the amyloid precursor protein (APP) in such a way that increased levels of the 42-amino-acid form of the amyloid β peptide (Aβ42) are secreted (Hardy 1997). Aβ42 is believed to be pathogenic, since it is more prone to aggregation and therefore leads to accelerated amyloid β accumulation in the brain of patients with AD. To assess whether Glu318Gly also influences APP processing, we measured Aβ42 levels in conditioned media of HEK-293 cells stably transfected with the Glu318Gly PSEN1 cDNA, using an Aβ42-specific enzyme-linked immunosorbent assay (De Strooper et al. 1998). No increase in Aβ42 secretion was observed, compared with cell lines stably transfected with wild-type PSEN1 cDNA. The absence of increased APP processing into Aβ42 in vitro is consistent with our findings at the population level, which show no association of Glu318Gly with either AD or dementia.

A few possibilities remain unexplored. First, since we detected the Glu318Gly allele only in the heterozygous state, it cannot be excluded that Glu318Gly is associated with dementia in an autosomal recessive manner. However, there is no evidence supporting autosomal recessive inheritance in familial AD (Rao et al. 1994). The fact that neither none of 479 late-onset patients screened in the present study nor the 100 early-onset cases screened in earlier studies (Cruts et al. 1998) carried two copies of the allele makes it unlikely that homozygosity for Glu318Gly is a frequent cause of AD. Another as yet not excluded possibility is that all Glu318Gly carriers share a (disease) phenotype that is different from either AD or dementia and that has remained undetected in the present study. However, we are not aware of any nondementia phenotypes associated with genetic variations in PSEN1.

In conclusion, we provide evidence that PSEN1 Glu318Gly is not causally related to either AD or other types of dementia. The latter has important implications for genetic counseling, since Glu318Gly carriers are not at increased risk. Our observations also imply that care should be taken in assigning a pathological nature to mutations in PSEN1, when these mutations are reported in isolated cases or in familial cases but in the absence of conclusive evidence for cosegregation with the disease.

Acknowledgments

We are grateful to Hubert Backhovens, Marleen Van den Broeck, and Sally Serneels for their skilled technical assistance in the mutation screening and genotyping. We thank Alewijn Ott, Frans van Harskamp, Inge de Koning, Maarten de Rijk, and Sandra Kalmijn for their contribution in the dementia diagnosis. This work was funded in part by a special research project of the University of Antwerp, the Fund for Scientific Research in Flanders, and the International Alzheimer's disease Research Fund, Belgium; and by the NESTOR stimulation program for scientific research in the Netherlands (Ministry of Health and Ministry of Education), the Netherlands Organisation for Scientific research, the Netherlands Prevention fund, and the municipality of Rotterdam.

Bart Dermaut,[1] Marc Cruts,[1] Arjen J. C. Slooter,[2] Sofie Van Gestel,[1] Chris De Jonghe,[1] Hugo Vanderstichele,[3] Eugeen Vanmechelen,[3] Monique M. Breteler,[2] Albert Hofman,[2] Cornelia M. van Duijn,[2] and Christine Van Broeckhoven[1]

[1]*Laboratory of Neurogenetics, Flanders Interuniversity Institute for Biotechnology, Born-Bunge Foundation, University of Antwerp, Department of Biochemistry, Antwerp;* [2]*Department of Epidemiology and Biostatistics, Erasmus University Medical School, Rotterdam; and* [3]*Innogenetics N.V., Industriepark Zwijnaarde, Zwijnaarde, Belgium*

Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for AD [MIM 104300])

References

American Psychiatric Association (1987) Diagnostic and statistical manual of mental disorders, 3d ed rev. American Psychiatric Association, Washington, DC

Baker M, Perez-Tur J, Eckman C, Younkin L, Younkin N (1998) High frequency of presenilin mutations in first degree relatives of Alzheimer's disease patients with increased plasma Aβ42 levels. Neurobiol Aging Suppl 19:S87

Cruts M, Backhovens H, Wang SY, Gassen GV, Theuns J, De Jonghe CD, Wehnert A, et al (1995) Molecular genetic analysis of familial early-onset Alzheimer's disease linked to chromosome 14q24.3. Hum Mol Genet 4:2363–2371

Cruts M, Van Broeckhoven C (1998) Presenilin mutations in Alzheimer's disease. Hum Mutat 11:183–190

Cruts M, van Duijn CM, Backhovens H, Van den Broeck M, Wehnert A, Serneels S, Sherrington R, et al (1998) Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population based study of presenile Alzheimer disease. Hum Mol Genet 7:43–51

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, et al (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature 391:387–390

Forsell C, Froelich S, Axelman K, Vestling M, Cowburn RF, Lilius L, Johnston JA, et al (1997) A novel pathogenic mutation (Leu262Phe) found in the presenilin 1 gene in early-onset Alzheimer's disease. Neurosci Lett 234:3–6

Forsell C, Mattila KM, Axelman K, Lannfelt L (1998) The Arg269His and Glu318Gly mutations in the presenilin-1 gene found in Swedish early-onset Alzheimer's disease families. Neurobiol Aging Suppl 19:S88

Hardy J (1997) Amyloid, the presenilins and Alzheimer's disease. Trends Neurosci 20:154–159

Helisalmi S, Mannermaa A, Lehtovirta M, Hiltunen M, Ryynänen M, Riekkinen P Sr, Soininen H (1998) Presenilin-1 nucleotide alteration in the coding area of Alzheimer's disease in Finland. Neurobiol Aging Suppl 19:S88

Hofman A, Grobbee DE, de Jong PTVM, van den Ouweland F (1991) Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7: 403–422

Mattila KM, Forsell C, Pirttilä T, Rinne J, Lehtimäki T, Röyttä M, Likius L, et al (1998) The Glu318Gly mutation of the presenilin-1 gene does not necessarily cause Alzheimer's disease. Neurobiol Aging Suppl 19:S88

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34:939–944

Ott A, Breteler MM, van Harskamp F, Claus JJ, van der Cammen T, Grobbee DE, Hofman A (1995) Prevalence of Alzheimer's disease and vascular dementia: association with education: the Rotterdam study. BMJ 310:970–973

Ott A, Breteler MM, van Harskamp F, Stijnen T, Hofman A (1998) Incidence and risk of dementia: the Rotterdam Study. Am J Epidemiol 147:574–580

Pericak-Vance MA, Haines JL (1995) Genetic susceptibility to Alzheimer disease. Trends Genet 11:504–508

Rao VS, van Duijn CM, Connor-Lacke L, Cupples LA, Growdon JH, Farrer LA (1994) Multiple etiologies for Alzheimer disease are revealed by segregation analysis. Am J Hum Genet 55:991–1000

Reznik-Wolf H, Treves TA, Shabtai H, Aharon-Peretz J, Chapman J, Davidson M, Barkai G, et al (1998) Germline mutational analysis of presenilin and APP genes in Jewish-Israeli individuals with familial or early-onset Alzheimer disease using denaturing gradient gel electrophoresis (DGGE). Eur J Hum Genet 6:176–180

Roman GC, Tatemichi TK, Erkinjuntti T, Cummings JL, Masdeu JC, Garcia JH, Amaducci L, et al (1993) Vascular dementia: diagnostic criteria for research studies: report of the NINDS-AIREN International Workshop. Neurology 43: 250–260

Sandbrink R, Zhang D, Schaeffer S, Masters CL, Bauer J, Forstl H, Beyreuther K, et al (1996) Missense mutations of the PS-1/S182 gene in German early-onset Alzheimer's disease patients. Ann Neurol 40:265–266

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, et al (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754–760

Torres O, Cruts M, Backhovens H, Velez P, Arteaga C, Villarea E, Serrano M, et al (1998) Mutation analysis of the presenilin genes (PS1 and PS2) in Colombian familial and sporadic AD patients. Neurobiol Aging Suppl 19:S88

Van Broeckhoven C (1995) Presenilins and Alzheimer disease. Nat Genet 11:230–232

Address for correspondence and reprints: Prof. Dr. Christine Van Broeckhoven, Laboratory of Neurogenetics, University of Antwerp (UIA), Department of Biochemistry, Universiteitsplein 1, B-2610 Antwerpen, Belgium. E-mail: cvbroeck@uia.ua.ac.be

0002-9297/99/6401-0035$02.00

Am. J. Hum. Genet. 64:292–295, 1999

p53 Variants Predisposing to Cancer Are Present in Healthy Centenarians

To the Editor:

Cancer results from the expansion of cell clones that progressively lose control of proliferation, differentiation, and death, owing to accumulation of mutational events in genes that control the cell cycle and apoptosis. Nuclear protein p53 is thought to play a major role in malignancy, since it induces genes that determine apoptosis and cell-cycle arrest, interacts with proteins employed in DNA repair, and binds to DNA strand breaks. As expected, somatic mutations in p53 are found in a variety of human cancers. Mutations are predominantly inactivating, thus eliminating the "guardian of the genome" from the proliferating cells. Germ-line mutations

of p53 also have been described as the molecular basis of a rare familial cancer-prone syndrome, Li-Fraumeni syndrome. At the population level, common variants (polymorphisms) of p53 are present. In particular, a C→G transversion leads to a proline-to-arginine change at p53 codon 72. Several studies have reported data regarding the association of the codon 72 variants with susceptibility to a variety of human cancers, such as breast cancer, lung cancer, and colorectal neoplasia (Birgander et al. 1995; Sjalander et al. 1996). Some reports suggest that Arg/Pro72 alleles should be considered as markers in linkage disequilibrium with other sites able to modulate cancer risk (Sjalander et al. 1995). Recently, a new insight into the role of codon 72 in human cancers has been reported by Storey et al. (1998). In their analysis, the authors found that a majority (76%) of women affected by human papillomavirus (HPV)–induced cervical carcinoma were homozygous for Arg72 alleles, compared with a frequency of 37% among unaffected women. In addition, when functional analysis of p53 variants was performed, the authors found that a p53 protein carrying an arginine at codon 72 binds more effectively to HPV oncoprotein E6 and is degraded and inactivated more rapidly by the proteasome pathway. The result is an estimated sevenfold risk of developing cervical cancer, for people homozygous for Arg72, when infected by HPV that is able to produce E6 protein. Overall, the available data in the literature suggest that p53 variants may be considered as risk factors for some of the major neoplastic diseases in humans, such as lung, colorectal, breast, and cervical cancer, and are expected to affect survival. Hence, an underrepresentation of p53 variants involved in cancer risk would be expected in a group of people who reach very old age in good health and who have escaped any overt cancer disease. Accordingly, healthy centenarians of both sexes were studied, to test this prediction, and the results were compared with those obtained from the study of a group of younger people.

The centenarians and the controls in our study were basically those studied in a previous investigation, in which significant differences in the frequency of apolipoprotein B (ApoB) VNTR alleles were found between the two groups (De Benedictis et al. 1997). The sample of centenarians comprised 176 healthy unrelated subjects (53 males and 123 females) from northern and southern Italy, and the health status of each was assessed as described elsewhere (Capurso et al. 1997). The control group comprised 204 younger unrelated subjects (113 males and 91 females, 20–60 years of age) randomly collected from northern and southern Italy. The ancestry in the specific geographic area of the subjects included in this study was checked as far back as the grandparental generation. In table 1 the frequencies of p53 codon 72 alleles and genotypes in the younger controls and the centenarians are shown. No difference between the two groups was found. Moreover, no difference in allelic and genotypic distributions, between the centenarians and the younger controls, was found when sex and geographic origin were considered in the analysis or when the group of younger controls was split into two subgroups (<40 and ≥40 years of age) (data not shown).

Table 1

Allelic and Genotypic Frequencies of p53 Codon 72 Polymorphism

	No. (%) of Younger Controls [n = 204]	No. (%) of Centenarians [n = 176]
p53 Allele:[a]		
Pro72	128 (31.4)	101 (28.7)
Arg72	280 (68.6)	251 (71.3)
p53 Genotype:[b]		
Pro72/Pro72	18 (8.8)	12 (6.8)
Arg72/Pro72	92 (45.1)	77 (43.8)
Arg72/Arg72	94 (46.1)	87 (49.4)

NOTE.—Hardy-Weinberg equilibrium (HWE) of p53 genotypes was assessed by exact tests. Both groups were in HWE: younger controls, $P = .46$; and centenarians, $P = .61$. χ^2 tests for comparison of allelic and genotypic distributions were performed by use of Monte Carlo algorithms implemented by means of the Statistical Product and Service Solutions package.

[a] $\chi^2 = 0.64$, df 1, $P = .42$.

[b] $\chi^2 = 0.74$, df 2, $P = .68$.

Several explanations can account for the results reported here. First, the most direct explanation is that Arg/Pro72 alleles are neutral and do not exert any censoring, with regard to susceptibility to cancer and life expectancy. This hypothesis is compatible with the cautious conclusions of a recent meta-analysis, which points out that a consensus about the role of p53 variants in human cancer has yet to be reached (Weston and Godbold 1997). Second, we can assume that p53 codon 72 Arg/Pro alleles are not neutral. In view of this hypothesis, our data on healthy centenarians suggest that the long-term consequences of p53 codon 72 Arg/Pro alleles on survival are negligible, even though they are related to increased cancer risk. However, additional data regarding the incidence of and mortality rate for p53-related cancers in the cohort studied and the relative risk of developing these diseases, for different p53 allelic variants, are needed in order to reject the above-mentioned hypothesis. On the other hand, recent data indicate that p53 polymorphisms appear to modulate an individual's risk of developing cancer only when peculiar conditions occur (i.e., a particular viral infection). In this case, only a small proportion of people who carry certain alleles would be selectively lost during aging.

However, these considerations probably are quite simplistic, owing to the possibility that the scenario is much more complex. Indeed, unexpected nonmonotonic age-

related trends of allele frequency can be found for genes related to survival and longevity. This is the case for the ApoB gene (De Benedictis et al. 1998), which we previously showed to be correlated with longevity (De Benedictis et al. 1997). We recently conceptualized these findings and created a model to account for the complex changes, with age, observed for ApoB gene allele frequencies (Yashin et al. 1998). According to this model, complex trajectories can be expected when the existence of "frail" and "robust" alleles of longevity-associated genes are assumed and when the mortality rates of the carriers of the two types of alleles are crossed. A similar conclusion was reached when changes, with age, in the allelic frequencies of genes related to cardiovascular risk factors, such as angiotensin-converting enzyme, were considered (Schachter et al. 1994).

As far as we know, the above-mentioned models (Toupance et al. 1998; Yashin et al. 1998) represent the only theoretical framework available to address the complex problems encountered when biodemographic data and genetic data, concerning longevity genes and genes related to risk factors for major age-related diseases, are merged and compared. A similar model for p53 could be of great interest and could help in (1) testing the hypothesis that the p53 Arg/Pro72 alleles are related to increased cancer risk and (2) answering the question of whether the frequency of the p53 alleles that we found in centenarians is compatible with this hypothesis. We predict that such a model will be quite difficult to develop, given that p53 is involved in a variety of tumors and that there is a relative paucity of data on p53 allele frequencies in people of different age groups and, particularly, in the elderly.

In this regard, if we admit that the data reported here imply no global differential chance of survival between p53 genotypes, our results could be due to compensatory effects. Indeed, opposite effects of genotypes on survival, at different ages, are predicted by the theory of negative, or antagonistic, pleiotropy (Williams 1957). The scenario is particularly interesting and challenging with regard to the healthy centenarians, who are the best models of successful aging and longevity and in whom a variety of other genetic and nongenetic risk factors for cardiovascular diseases was found (Mari et al. 1996; Mannucci et al. 1997; Baggio et al. 1998). Furthermore, healthy centenarians also can rely on other compensatory mechanisms, such as an effective and well-preserved immune system, to cope with internal and external threatening agents (Franceschi et al. 1995).

In conclusion, healthy centenarians may be considered useful models for testing basic theories on aging. Moreover, this selected group of healthy individuals will be useful for evaluating the impact of genetic risk factors on survival and longevity.

Acknowledgments

This study was supported by a grant from AIRC (Italian Association for Cancer Research) (to C.F.) and by the European GENAGE Project, by MURST (Ministry for University and Technological Research) Cofinanziamento 1998, and by POP (Regional Operative Project) 94/99 from regione Calabria, Italy.

Massimiliano Bonafè,[1] Fabiola Olivieri,[2] Daniela Mari,[3] Giovannella Baggio,[4] Rosario Mattace,[5] Paolo Sansoni,[6] Giovanna De Benedictis,[7] Maria De Luca,[2,7] Stefano Bertolini,[8] Cristiana Barbi,[9] Daniela Monti,[10] and Claudio Franceschi[1,2]

[1]*Department of Experimental Pathology, University of Bologna, Bologna, Italy;* [2]*Italian National Research Centers on Aging, Ancona, Italy;* [3]*Institute of Internal Medicine, Scientific Institute of Care and Research, Maggiore Hospital, University of Milano, Milan;* [4]*Department of Internal Medicine, University of Sassari, Sassari, Italy;* [5]*Chair of Geriatrics, University of Reggio Calabria, Catanzaro, Italy;* [6]*Institute of Internal Medicine and Medical Therapy, University of Parma, Parma, Italy;* [7]*Department of Cell Biology, University of Calabria, Rende, Italy;* [8]*Atherosclerosis Prevention Center, Department of Internal Medicine, University of Genoa, Genoa;* [9]*Department of Biomedical Sciences, University of Modena, Modena, Italy; and* [10]*Institute of General Pathology, University of Florence, Florence*

References

Baggio G, Donazzan S, Monti D, Mari D, Martini S, Gabelli C, Dalla Vetra M, et al (1998) Lipoprotein(a) and lipoprotein profile in healthy centenarians: a reappraisal of vascular risk factor. FASEB J 12:433–437

Birgander R, Sjalander A, Rannug A, Alexandrie AK, Sundberg MI, Seidegard J, Tornling G, et al (1995) p53 Polymorphisms and haplotypes in lung cancer. Carcinogenesis 16: 2233–2236

Capurso A, D'Amelio A, Resta F, Gaddi A, D'Addato S, Galletti C, Trabucchi M, et al(1997) Epidemiological and socioeconomic aspects of Italian centenarians. Arch Gerontol Geriatr 25:149–157

De Benedictis G, Carotenuto L, Carrieri G, De Luca M, Falcone E, Rose G, Yashin AI, et al (1998) Age-related changes of the 3′ APOB VNTR genotype pool in aging cohorts. Ann Hum Genet 62:115–122

De Benedictis G, Falcone E, Rose G, Ruffolo R, Spadafora P, Baggio G, Bertolini B, et al (1997) DNA multiallelic systems reveal gene/longevity associations not detected by diallelic systems: the APOB locus. Hum Genet 99:312–318

Franceschi C, Monti D, Sansoni P, Cossarizza A (1995) The immunology of exceptional individuals: the lesson of centenarians. Immunol Today 16:12–16

Mannucci PM, Mari D, Merati G, Peyvandi F, Tagliabue L,

Sacchi E, Taioli E, et al (1997) Gene polymorphisms predicting high plasma levels of coagulation and fibrinolysis proteins. Arterioscler Thromb Vasc Biol 17:755–759

Mari D, Mannucci PM, Duca F, Bertolini S, Franceschi C (1996) Mutant factor V (Arg506Gln) in healthy centenarians. Lancet 347:1044

Schachter F, Faure-Delanef L, Guenot F, Rouger H, Froguel T, Lesueur-Ginot L, Cohen D (1994) Genetic associations with human longevity at the APOE and ACE loci. Nat Genet 6: 29–32

Sjalander A, Birgander R, Hallmans G, Cajander S, Lenner P, Athlin L, Beckman G (1995) p53 Germ line haplotypes associated with increased risk for colorectal cancer. Carcinogenesis 16:1461–1464

——— (1996) p53 Polymorphisms and haplotypes in breast cancer. Carcinogenesis 17:1313–1316

Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, et al (1998) Role of a p53 polymorphism in the development of human papillomavirus–associated cancer. Nature 393:229–234

Toupance B, Godelle B, Gouyon P-H, Schächter F (1998) A model for antagonistic pleiotropic gene action for mortality and advanced age. Am J Hum Genet 62:1525–1534

Weston A, Godbold JH (1997) Polymorphism of H-Ras and p53 in breast cancer and lung cancer: meta-analysis. Environ Health Perspect 105:919–926

Williams GC (1957) Pleiotropy, natural selection, and the evolution of senescence. Evolution 11:398–411

Yashin A, Vaupel JW, Andreev KF, Tan Q, Iachine IA, Carotenuto L, De Benedictis G (1998) Combining genetic and demographic information in population studies of aging and longevity. J Epidemiol Biostat 3:289–294

Address for correspondence and reprints: Dr. Claudio Franceschi, Department of Experimental Pathology, Via S. Giacomo, University of Bologna, 40126 Bologna, Italy. E-mail: c.franceschi@inrca.it

0002-9297/99/6401-0036$02.00

Am. J. Hum. Genet. 64:295–300, 1999

Maternally Inherited Cardiomyopathy: An Atypical Presentation of the mtDNA 12S rRNA Gene A1555G Mutation

To the Editor:

Human mitochondrial disorders comprise a heterogeneous group of multisystem diseases, characterized by morphological, biochemical, or genetic abnormalities of mitochondria. Mutations in mtDNA have been described predominantly in a variety of rare encephalomyopathies but are also emerging in association with more common disorders, such as sensorineural hearing loss (SNHL) and cardiomyopathies (DiMauro and Bonilla 1997). Most of the identified mtDNA mutations are associated with specific clinical phenotypes (DiMauro and Bonilla 1997). In a recent issue of the *Journal*, Estivill et al. (1998) reported that the A1555G mutation in the mitochondrial 12S rRNA is responsible for a significant number of cases of maternally inherited nonsyndromic hearing loss and that its pathogenic role is enhanced by treatment with aminoglycosides.

Idiopathic cardiomyopathies are an important cause of morbidity and mortality throughout the world, both in children and adults, with an annual incidence of 2–8/100,000 in the United States and Europe (Manolio et al. 1992). The application of molecular genetic techniques has started to delineate the molecular bases of these syndromes through the demonstration of alterations of myocardial contractile and structural proteins, such as the cardiac β-myosin heavy-chain (MYH7) gene, which accounts for ~75% of the familial cases of hypertrophic cardiomyopathies (Geisterfer-Lowrance et al. 1990). There is growing evidence that mtDNA mutations can cause cardiac disease, including cardiomyopathies and cardiac conduction block. In addition, cardiomyopathy may result from bioenergetic defects caused by mutations in nuclear-encoded subunits of the respiratory chain or in nuclear genes controlling the integrity, replication, and expression of mtDNA (Cortopassi et al. 1992; Kelly and Strauss 1994; DiMauro and Bonilla 1997).

We report here a 35-year-old woman who was evaluated because of heart failure. At age 23–24 years, during her first pregnancy, the patient noted easy fatigability, shortness of breath, and palpitations. Chest x-ray revealed cardiomegaly with prominent left-atrial enlargement. Cardiological evaluation suggested a restrictive cardiomyopathy. Episodes of atrial fibrillation and flutter required cardioversion on several occasions. Four years later, her clinical condition worsened during a second pregnancy. While in sinus rhythm, she was a New York Heart Association class I-II patient but when in atrial arrhythmia she worsened to class III. A clinical and metabolic work-up for heart transplantation was performed. Family history was remarkable for the mother and maternal grandmother, who had both died of unspecified heart diseases in their late 30s. A 25-year-old brother had a childhood heart murmur but was free of cardiac symptoms. The proposita's two daughters are asymptomatic at ages 11 and 7 years, but the older daughter had had a cardiac murmur in infancy (fig. 1*A*).

At age 35 years, physical examination, including neurological and otolaryngeal evaluations, showed a short and thin woman without other symptoms and signs commonly found in patients with mitochondrial encephalomyopathy, including ptosis, external ophthalmoparesis, pigmentary retinopathy, hearing loss, or diabetes mellitus. A two-dimensional echocardiogram revealed severe restrictive cardiomyopathy, with mod-

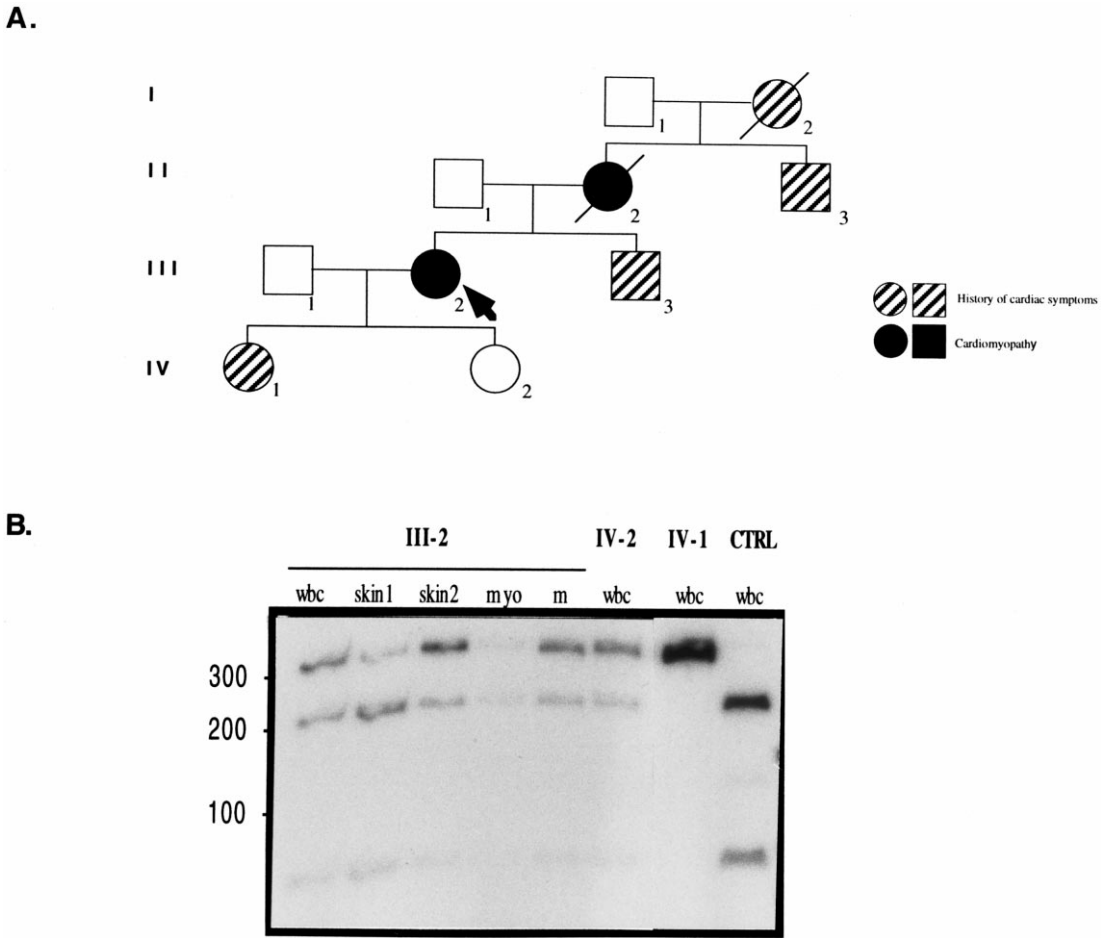

Figure 1 *A*, Pedigree of family harboring the A1555G mutation. Arrowhead indicates proband. *B*, Autoradiogram of the restriction-length–polymorphism analysis used to quantitate mutant mtDNA. The normal 316-bp PCR-amplified fragment is cut by the endonuclease *Alw*26I into two fragments (219 and 97 bp). The A1555G mutation abolishes the *Alw*26I site. Individuals are as shown in figure 1*A*. "CTRL" is a normal control; wbc = white blood cells; skin1 = cultured skin fibroblasts, first passage; skin2 = cultured skin fibroblasts, second passage; myo = cultured myoblasts; m = skeletal muscle.

erate interventricular septum hypertrophy (1.3 mm; normal <1.0 mm). An endomyocardial biopsy showed normal myofibrillar array, minimal hypertrophy of cardiomyocytes, and absence of inflammation. There were no abnormal deposits of glycogen, iron, or amyloid. A diagnosis of idiopathic restrictive cardiomyopathy was made.

After the subjects gave informed consent, we performed our studies under our institutional review board's protocol. Skeletal muscle biopsy of the quadriceps did not reveal typical mitochondrial abnormalities, such as ragged red fibers or cytochrome *c* oxidase (COX)–negative fibers. The most prominent histochemical abnormalities were central or paracentral minicores, which were easily identified, in many muscle fibers, as regions of decreased COX stain. Minicores were also detected by light microscopy in NADH-stained skeletal muscle sections (fig. 2). Electron microscopy revealed foci of myofibers with prominent smearing of Z-lines and absence of mitochondria. Biochemical studies in muscle homogenate and skin fibroblasts showed slightly decreased activities of multiple complexes of the respiratory chain when values were normalized to activity of citrate synthase, a mitochondrial matrix enzyme reflecting total mitochondrial content. Specifically, the residual activities of NADH-dehydrogenase and COX were 50% and 39%, respectively, of the mean values measured in control muscles. PCR followed by SSCP and sequence analyses of our proband's muscle mtDNA identified one possible pathogenic base change, an A→G transition at nt 1555. By PCR, we amplified all 22 mtDNA-encoded tRNA genes and did not identify any additional point mutations by direct sequencing of both strands of the PCR products. The A1555G mutation was hetero-

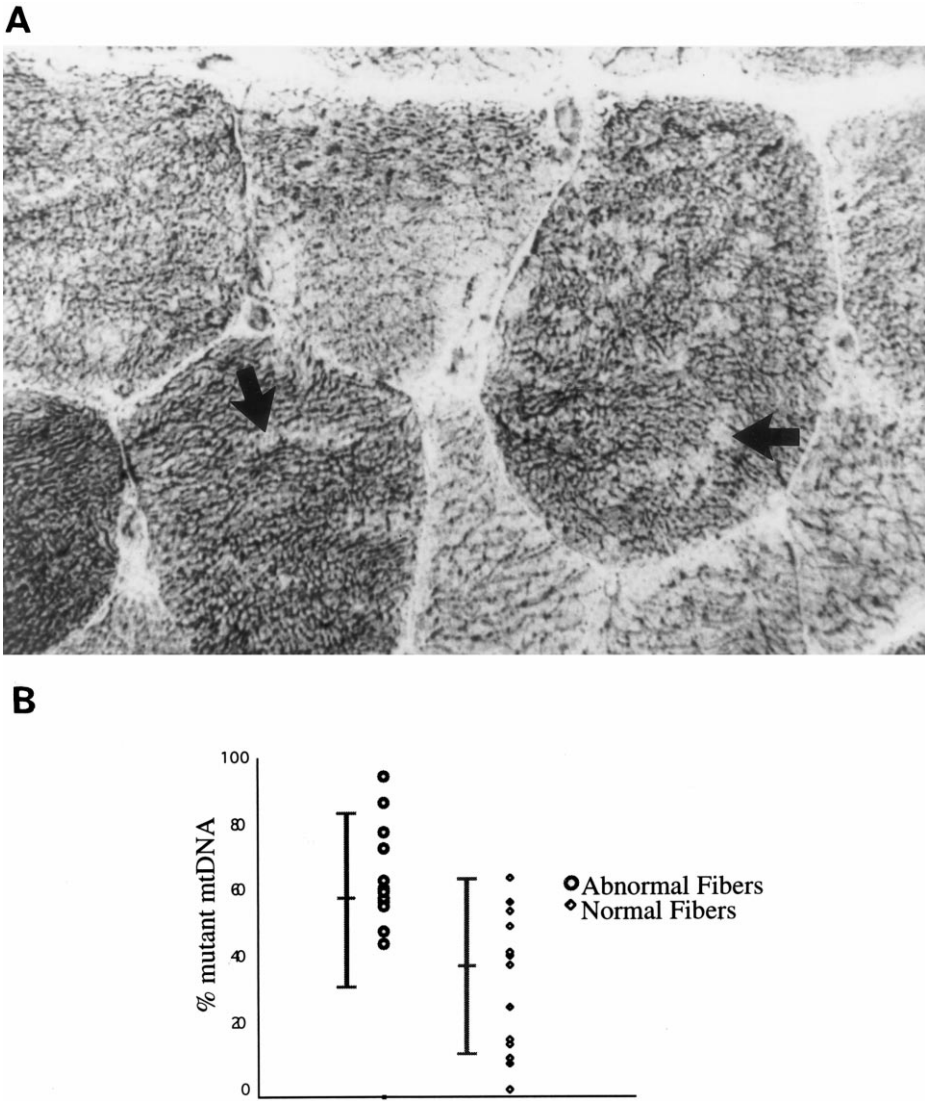

Figure 2 *A*, NADH-TR stain of the skeletal muscle biopsy. Arrows indicate minicores. *B*, Abundance of mutated mitochondrial genomes in normal and abnormal (minicore) fibers, by single-muscle-fiber PCR analysis. The difference is statistically significant ($P < .05$).

plasmic, accounting for 55% of total muscle mtDNA, and similar proportions were detected in blood and primary skin fibroblasts cultures (57% and 60%, respectively). Higher levels of mutated mtDNAs were found in paraffin sections of an endomyocardial biopsy (89%). The mutation was present in high percentages in blood from the two daughters (95% in individual IV-1 and 50% in individual IV-2), the only tissues available for study (fig. 1*B*). No additional maternal members of this family were available for genetic testing.

When we examined the effects of the A1555G mutation on the translational capacity of cultured skin fibroblasts in the presence or absence of aminoglycoside (gentamicin, 0.5 mg/ml), we found moderate protein-synthesis defects under both conditions. The relative labeling ratios and electrophoretic mobility of mitochondrial translation products in cell lines harboring 60% mutated mtDNA did not differ significantly from those observed in controls in three independent measurements. However, there was an overall decrease in the rate of protein labeling, with an average decrease of 35%, which became more apparent (40%) when aminoglycoside was added at concentrations used routinely, in animal cell cultures, to eliminate contaminating microorganisms (fig. 3).

Clinically, our patient suffered from a restrictive cardiomyopathy from early adulthood, with a family history suggesting maternal transmission, whereas her brother and one of her daughters had transient valvular heart disease in early childhood. However, the daughters

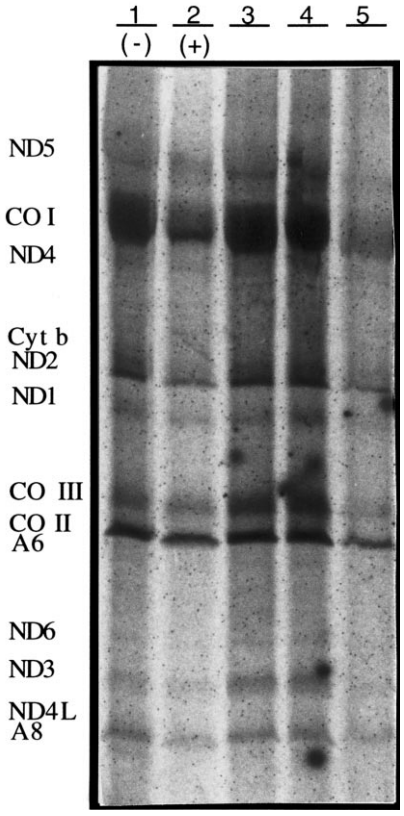

Figure 3 Electrophoretic mobility of the mitochondrial translation products. Lane 1, III-1 skin fibroblasts (aminoglycoside minus). Lane 2, III-2 skin fibroblasts (aminoglycoside plus). Lanes 3 and 4, skin fibroblasts from normal controls. Lane 5, skin fibroblasts from a disease control harboring a high percentage of the G8363A mtDNA mutation (Family A, individual III-4, in Santorelli et al. 1996).

remain at risk for cardiomyopathy, because cardiac symptoms in our proposita did not start until she was in her early 20s and worsened considerably over the course of the next 10 years. Likewise, both her mother and the maternal grandmother died suddenly in their 30s of cardiac failure. We have identified the A1555G mutation, which we deem responsible for her symptoms, on the basis of the following considerations. First, the A1555G mutation was heteroplasmic, both in the patient and in her maternal relatives. Heteroplasmy, the coexistence of wild-type and mutated mtDNA molecules in the same individual, is regarded as an indicator of pathogenicity, and the abundance of mutated genomes usually correlates with the severity of the phenotype. Although this same base change has been associated with either aminoglycoside-induced deafness (AID) or nonsyndromic hearing loss in several Asian, African, and Middle Eastern pedigrees, usually in a homoplasmic state (Prezant et al. 1993), there is evidence of phenotypic heterogeneity. Shoffner et al. (1996) have described a Caucasian family harboring the A1555G mutation, in association with both SNHL and Parkinson disease, with onset in middle age. Complex I activity was decreased in muscle samples of several members of that family, and the degree of the biochemical defect correlated with the severity of the clinical phenotype. It is possible that additional phenotypes will be associated with this mutation. Second, the clinical expression appears consistent across three generations of this family, with variations in age at onset and disease progression, possibly resulting from differences in proportions and tissue distributions of mutant genomes. Because the symptoms do not manifest fully until adulthood, individuals IV-1 and IV-2 are at high risk of developing cardiomyopathy. Third, we noted a statistically significant correlation between the abundance of mutated mtDNAs in single muscle fibers and the presence of "minicores" in our proband ($P < .05$; fig. 2). Whereas minicores and corelike formations have been reported in other myopathies, including nemaline myopathy (Afifi et al. 1965) and limb-girdle dystrophy (Engel et al. 1971), these structural alterations usually are not found in mitochondrial encephalomyopathies. Although the finding of minicores in our proband's skeletal muscle may be nonspecific, the statistically significant association between their presence and the number of mutated genomes in single fibers suggests a causal relationship. To the best of our knowledge, no morphological studies in skeletal muscle of patients harboring the A1555G mutations have been reported. It is also noteworthy that Fananapazir et al. (1993) reported similar morphological changes in soleus muscle biopsies taken from patients with hypertrophic cardiomyopathy as a result of mutations in the MYH7 gene. In those cases, a simultaneous mitochondrial defect was hypothesized but not examined at molecular genetic or biochemical levels. Last, our data suggest that the pathogenetic mechanism of the A1555G mutation involves a primary mitochondrial translation defect, resulting from the base change in the decoding site of the small ribosome. Cells harboring the A1555G mutation showed a decreased rate of mitochondrial protein synthesis when compared with controls, even in the absence of aminoglycoside in the culture medium. Decreased synthesis of the subunits of respiratory complexes is likely to impair ATP production, with deleterious effects on cell functions and ultimately resulting in cell death. If this occurs in cardiomyocytes, it could result in heart failure, especially during periods of higher metabolic demand, such as pregnancy, as in our patient.

An intriguing issue raised by our report regards the phenotypic consequences of the A1555G mutation. Phenotypic heterogeneity is a common feature of diseases associated with mtDNA defects and is thought to result from differential tissue distribution of the mutated genomes. For example, the A3243G base change in the

$tRNA^{Leu(UUR)}$ gene, although primarily associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS), is also responsible for other syndromes, such as diabetes mellitus with deafness, progressive external ophthalmoparesis, and Leigh syndrome (Shoffner and Wallace 1995). The A1555G mutation had been long considered an exception to this rule because it seemed to cause ototoxicity invariably and exclusively, occurring either spontaneously or after exposure to aminoglycosides. However, both this report and the family described by Shoffner et al. (1996) broaden the clinical spectrum for the A1555G mutation. The vulnerability of the auditory system has been attributed to the fact that the mutation affects a 12S rRNA–gene region that is homologous to the aminoglycoside-binding site of the small rRNA in bacteria. Moreover, the mutation lies within a conserved domain that in the *Escherichia coli* 6S rRNA gene forms an essential part of the decoding site of the ribosome. This region is crucial for RNA-protein association, RNA-RNA interaction, or both; therefore, the mutation could enhance sensitivity to aminoglycosides in the hairy cells of Corti's organ, through defective protein synthesis. Deafness is not present in our family; however, restrictive cardiomyopathy is the sole clinical feature, a finding never before reported.

The fact that many patients with the A1555G mutation have been asymptomatic prior to aminoglycoside therapy suggests that the mutation alone is functionally mild (Hutchin et al. 1993). Prezant et al. (1993) hypothesized a "two-hit" model; the 12S rRNA mutation apparently alters the aminoglycoside-binding site, thus causing greater susceptibility to the toxic effects of the drug. In addition, other genetic alterations, perhaps in nDNA, modify the phenotypic expression of this mtDNA mutation. Therefore, we cannot exclude the possibility that, in addition to the abundant mutated mtDNAs detected in the proband's heart biopsy, a second genetic "hit" may have caused the cardiomyopathy in our pedigree with the A1555G mutation. By contrasting the properties of transmitochondrial cybrids harboring different percentages of the A1555G mutation from AID patients and from our cardiomyopathy patient, we may be able to detect biochemical differences, which could be attributed to a second mtDNA alteration. As an alternative, nuclear DNA factors—for example, alterations of a nuclear DNA–encoded mitochondrial ribosomal protein—could modify the phenotypic expression of the A1555G mutation.

Acknowledgments

We thank Dr. Eduardo Bonilla for critical comments. This work was partially supported by National Institute of Child Health and Human Development program project PO1 HD32062, Telethon-Italy grant 844-1996 (to C.C.), Italian Ministry of Health grant 97/02/G/009 (to F.M.S.), and by grants from the Muscular Dystrophy Association. M.H. is supported by National Institutes of Health grant 1RO1 HL59657 and by the Columbia-Presbyterian Medical Center Irving Scholar program.

Filippo M. Santorelli,[1,5] Kurenai Tanji,[1] Panagiota Manta,[1,*] Carlo Casali,[4] Sindu Krishna,[1] Arthur P. Hays,[1,2] Donna M. Mancini,[3] Salvatore DiMauro,[1] and Michio Hirano[1]

Departments of [1]Neurology, [2]Pathology, and [3]Medicine, Columbia University College of Physicians and Surgeons, New York; and [4]Istituto di Clinica delle Malattie Nervose e Mentali, La Sapienza University, and [5]Molecular Medicine, Children's Hospital Bambino Gesù, Rome

References

Afifi AK, Smith JW, Zellweger H (1965) Congenital nonprogressive myopathy: central core and nemaline myopathy in one family. Neurology 15:371–381

Cortopassi GA, Shibata D, Soong NW, Arnheim N (1992) A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. Proc Natl Acad Sci USA 89:7370–7374

DiMauro S, Bonilla E (1997) Mitochondrial encephalomyopathies. In: Rosenberg R, Prusiner S, DiMauro S, Barchi R (eds) The molecular and genetic basis of neurological disease. Butterworth-Heinemann, Boston, pp 201–235

Engel AG, Gomez MR, Groover RV (1971) Multicore disease: a recently recognized congenital myopathy associated with multifocal degeneration of muscle fibers. Mayo Clin Proc 46:666–681

Estivill X, Govea N, Barceló A, Perelló E, Badenas C, Romero E, Moral L, et al (1998) Familial progressive sensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment with aminoglycosides. Am J Hum Genet 62:27–35

Fananapazir L, Dalakas MC, Cyran F, Cohn G, Epstein ND (1993) Missense mutations in the β-myosin heavy-chain gene cause central core disease in hypertrophic cardiomyopathy. Proc Natl Acad Sci USA 90:3993–3997

Geisterfer-Lowrance AAT, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG (1990) A molecular basis for familial hypertrophic cardiomyopathy: a β cardiac myosin heavy chain gene missense mutation. Cell 62: 999–1006

Hutchin T, Haworth I, Higashi K, Fischel-Ghodsian N, Stoneking M, Saha N, Arnos C, et al (1993) A molecular basis for human hypersensitivity to aminoglycoside antibiotics. Nucleic Acids Res 21:4174–4179

Kelly DP, Strauss AW (1994) Inherited cardiomyopathies. N Engl J Med 330:913–919

Manolio T, Baughman KL, Rodeheffer R, Pearson T, Bristow J, Michels V, Abelmann W, et al (1992) Prevalence and etiology of idiopathic dilated cardiomyopathy (summary of a

National Heart, Lung, and Blood Institute workshop). Am J Cardiol 69:1458–1466

Prezant TR, Agapian JV, Bohlman MC, Bu X, Oztas S, Qiu W-Q, Arnos KS, et al (1993) Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 4:289–294

Santorelli F, Mak S, El-Schahawi M, Casali C, Shanske S, Baram T, Madrid R, et al (1996) Maternally inherited cardiomyopathy and hearing loss associated with a novel mutation in the mitochondrial tRNA lysine gene (G8363A). Am J Hum Genet 58:933–939

Shoffner JM, Brown MD, Huoponen K, Stugard C, Koontz D, Kaufman A, Graham J, et al (1996) A mitochondrial DNA mutation associated with maternally inherited deafness and Parkinson's disease. Neurology Suppl 46:A331

Shoffner JM, Wallace DC (1995) Oxidative phosphorylation diseases. In: Scriver CR, Beaudet AL, Sly WS, Valle MD (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1535–1609

Address for correspondence and reprints: Dr. Michio Hirano, Department of Neurology, Columbia University College of Physicians and Surgeons, P&S 4-443, 630 West 168th Street, New York, NY 10032. E-mail mh29@columbia.edu

* Present affiliation: Department of Neurology, University of Athens, Athens.

0002-9297/99/6401-0037$02.00

Am. J. Hum. Genet. 64:300–302, 1999

An *Alu*-Mediated 6-kb Duplication in the *BRCA1* Gene: A New Founder Mutation?

To the Editor:

Most mutations in the breast/ovarian cancer–predisposing gene *BRCA1* that have been identified to date are point mutations or small insertions and deletions scattered over the whole coding sequence (5,592 nucleotides long) and over the splice junctions (Breast Cancer Information Core). Although ~65% are unique, because of founder effects several mutations have been found in more than one family, both within specific populations and in more-diverse geographic groups (Neuhausen et al. 1996). The other germ-line mutations published so far are five distinct large deletions (Petrij-Bosch et al. 1997; Puget et al. 1997; Swensen et al. 1997), two of which represent 36% of all *BRCA1* mutations in the Dutch population (Petrij-Bosch et al. 1997). The importance of such large genomic alterations is difficult to estimate, because most PCR-based methods that genetic laboratories use on genomic DNA—such as direct sequencing, single-strand conformation analysis (SSCA), heteroduplex analysis (HDA), denaturing gradient gel electrophoresis (DGGE), and the protein-truncation test (PTT)—will not allow their detection.

Here we report the identification of the first large duplication in the *BRCA1* gene in four apparently unrelated families: it comprises exon 13 and extends over 6 kb of intronic sequences. It was initially identified in one family—F3173—originally ascertained by one of us (H.T.L.) and contained one case of breast cancer and four cases of ovarian cancer. Leukocytes of obligate mutation carriers from F3173 had previously been shown to present a great reduction in the amount of the mutant transcript, but no alteration was identified in the *BRCA1* coding sequence when genomic sequencing and cDNA SSCA were performed (Serova et al. 1996). No genomic rearrangement had been found by Southern blot analysis, and no mutation in the promoter or the *5′* and *3′* UTRs was identified by HDA (Puget et al., in press). To look for splicing defects, we amplified, with 11 primer pairs, cDNA synthesized from leukocyte RNA of two patients from F3173, making sure that each exon was entirely contained within one fragment. Because we knew that the mutant allele was poorly expressed, we considered any abnormal PCR fragment visualized on agarose gels to be potentially interesting, irrespective of its intensity. A faint extra band ~170 bp longer than the expected fragment was visualized in the case of patients from F3173 with primers surrounding exons 12 and 13, which was also seen with primers surrounding exon 13 (fig. 1*a*). Sequencing of this extra band revealed the presence of two consecutive exons 13, leading to a frameshift in the mutant mRNA (ter1460). We then performed long-range PCR on genomic DNA, with overlapping primers in exon 13; although, as expected, no PCR product was obtained with control DNA, an ~6-kb fragment was generated in F3173, indicating that an ~6-kb duplication had occurred in the germ line of the F3173 patient (fig. 1*b*). The ~6-kb fragment was then digested by restriction enzymes, which showed that the duplication junction was contained within an ~800-bp *Xba*I fragment (fig. 1*c*). Duplication-specific primers (dup13F/R) were designed, and a 1.1-kb fragment was PCR amplified and sequenced: it revealed that a 6,081-bp region containing exon 13 (nucleotides 44369–50449 [GenBank accession number L78833]) is duplicated in F3173 (fig. 1*c*). Both breakpoints occurred in a 23-bp region of perfect identity, within two *Sx Alu* sequences in the same orientation (86.7% homology)—one in intron 12 and the other in intron 13—which suggests that the duplication is probably the result of a homologous recombination.

To evaluate whether this mutation, which may have previously escaped detection, is present in other families, we screened, by PCR using primers dup13F/R, 52 additional American families ascertained at Creighton University (of which 29 scored negative for mutations in the coding region and splice sites of the *BRCA1* gene when analyzed by HDA and PTT). This resulted in the

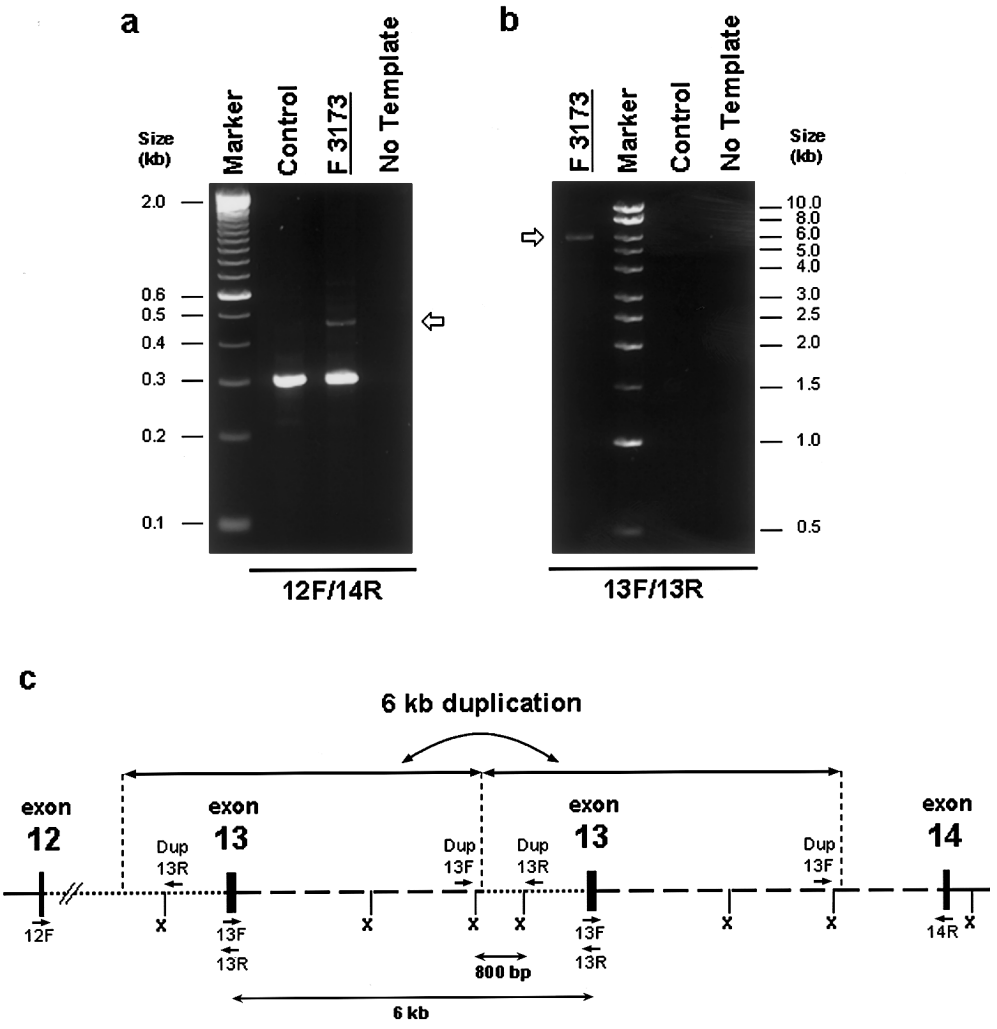

Figure 1 Characterization of the 6-kb germ-line duplication in the *BRCA1* gene in F3173. *a,* Complementary DNA, which was PCR amplified with primers 12F (ACA AGC GTC TCT GAA GAC TGC) and 14R (TGC AGA CAC CTC AAA CTT GTC AGC). Only a 318-bp fragment is generated in the control, whereas in the patient from F3173 a very faint extra band of 490 bp (*unblackened arrow*) containing two consecutive exons 13 as determined by sequencing is also produced. *b,* Genomic DNA, which was PCR amplified with primers 13F (GAT AAA GCT CCA GCA GGA AAT GGC) and 13R (GGC TCC CAT GCT GTT CTA AC). Only the mutant allele in F3173 gives rise to an ~6-kb fragment, shown (*unblackened arrow*), because the wild-type allele cannot be amplified with these primers (see panel *c*). *c,* Duplication of exon 13, schematically represented, with the location and orientation of primers 12F, 14R, 13F, 13R, dup13F (GAT TAT TTC CCC CCA GGC TA), and dup13R (AGA TCA TTA GCA AGG ACC TGT G). The *Xba*I sites (X); introns 12 (*dotted line*) and 13 (*broken line*); and the position and extent of the duplicated region, of the 800-bp *Xba*I fragment generated by the duplication, and of the 6-kb 13F/13R fragment (*two-headed arrows*) are indicated.

identification of two more families bearing this duplication: (1) F3653, which contains seven breast cancer cases, and (2) F2773, which contains seven breast cancer cases and three ovarian cancer cases. The three American families with the duplication are of mixed European (English, Dutch, or Irish) descent. Finally, the 6-kb duplication was also found in one Portuguese family with three cases of breast cancer, when 69 families (scoring negative for mutations in the coding region and splice sites of *BRCA1* when analyzed by DGGE [Stoppa-Lyonnet et al. 1997]) ascertained in Paris by D.S.-L. were screened. Although these families previously had been subjected to quantitative Southern analysis (Puget et al., in press), this rearrangement was missed because, on the one hand, the extra bands generated by digestions with the selected restriction enzymes were identical or very similar in size to the normal fragments, and, on the other hand, the densitometric analysis does not allow duplications to be identified as easily as deletions (1.5-fold signal-strength difference in duplications, compared with a 2-fold difference in deletions).

All four families were found to bear exactly the same

duplication, as revealed by the sequencing of the duplication junction. A founder effect is very likely, since all families could share the same haplotype at nine polymorphic short tandem-repeat markers within or flanking the *BRCA1*locus (D17S776, D17S1185, D17S1320, D17S855, D17S1322, D17S1323, D17S1327, D17S1326, and D17S1329). Of the shared alleles, those at D17S1185 and D17S855 have a population frequency <15%. Given the geographic diversity displayed by these four families' ancestors, the 6-kb duplication might be relatively old and is therefore likely to be found in other families around the world. Apart from the two frequent *BRCA1* mutations 185delAG and 5382insC, which have been found four and five times, respectively, this duplication is the most frequent mutation identified in the set of American families ascertained by H.T.L. (3 of 40 *BRCA1* mutations).

Although this duplication was identified by reverse-transcription PCR, it should be noted that it could be easily missed, since the mutant allele is poorly expressed, presumably because of premature stop codon–mediated mRNA decay. Given the high concentration of *Alu* sequences in the *BRCA1* gene (Smith et al. 1996), founder rearrangements such as the one reported here could explain a substantial fraction of the estimated 37% of breast/ovarian cancer families whose disease is due to *BRCA1* but for whom no mutation has been identified so far in the *BRCA1* coding sequence (Ford et al. 1998).

Acknowledgments

The authors would like to thank the family members. We also thank C. Bonnardel, T. Conway, J. Lynch, S. Slominski, and P. Watson for their expert assistance. This work was supported by program grants from le Comité Départemental de l'Ain et du Rhône de La Ligue contre le Cancer, la Fondation de France, la Fondation Adrienne et Pierre Sommer, Council for Tobacco Research grant 127DR@, U.S. Department of the Army grant DAMD17-94-J-4340, and the Nebraska State Cancer and Smoking-Related Diseases. N.P. is a fellow of the Ligue contre le Cancer de Haute-Savoie.

NADINE PUGET,[1,2] OLGA M. SINILNIKOVA,[1,2] DOMINIQUE STOPPA-LYONNET,[3] CAROLE AUDOYNAUD,[1] SABINE PAGÈS,[3] HENRY T. LYNCH,[4] DAVID GOLDGAR,[1] GILBERT M. LENOIR,[1,2] AND SYLVIE MAZOYER[1,2]

[1]*International Agency for Research on Cancer and* [2]*Laboratoire de Génétique, UMR 5641 CNRS, Lyon;* [3]*Unité de Génétique Oncologique, Institut Curie, Paris; and* [4]*Department of Preventive Medicine and Public Health, Creighton University School of Medicine, Omaha*

Electronic-Database Information

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

Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic (for *BRCA1* mutations)

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for the *BRCA1* gene sequence [L78833])

References

Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, et al (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. Am J Hum Genet 62:676–689

Neuhausen SL, Mazoyer S, Friedman L, Stratton M, Offit K, Caligo A, Tomlinson G, et al (1996) Haplotype and phenotype analysis of six recurrent BRCA1 mutations in 61 families: results of an international study. Am J Hum Genet 58:271–280

Petrij-Bosch A, Peelen T, van Vliet M, van Eijk R, Olmer R, Drusedau M, Hogervorst FBL, et al (1997) *BRCA1* genomic deletions are major founder mutations in Dutch breast cancer patients. Nat Genet 17:341–345

Puget N, Stoppa-Lyonnet D, Sinilnikova OM, Pagès S, Lynch HT, Lenoir GM, Mazoyer S. Screening for germline rearrangements and regulatory mutations in *BRCA1* led to the identification of four new deletions. Cancer Res (in press)

Puget N, Torchard D, Serova-Sinilnikova OM, Lynch HT, Feunteun J, Lenoir GM, Mazoyer S (1997) A 1-kb Alu-mediated germ-line deletion removing *BRCA1* exon 17. Cancer Res 57:828–831

Serova O, Montagna M, Torchard D, Narod SA, Tonin P, Sylla B, Lynch HT, et al (1996) A high incidence of BRCA1 mutations in 20 breast-ovarian cancer families. Am J Hum Genet 58:42–51

Smith TM, Lee MK, Szabo CI, Jerome N, McEuen M, Taylor M, Hood L, et al (1996) Complete genomic sequence and analysis of 117 kb of human DNA containing the gene *BRCA1*. Genome Res 6:1029–1049

Stoppa-Lyonnet D, Laurent-Puig P, Essioux L, Pagès S, Ithier G, Ligot L, Fourquet A, et al (1997) BRCA1 sequence variations in 160 individuals referred to a breast/ovarian family cancer clinic. Am J Hum Genet 60:1021–1030

Swensen J, Hoffman M, Skolnick MH, Neuhausen SL (1997) Identification of a 14 kb deletion involving the promoter region of *BRCA1* in a breast cancer family. Hum Mol Genet 6:1513–1517

Address for correspondence and reprints: Dr. G. M. Lenoir, International Agency for Research on Cancer, 150 Cours A. Thomas, 69372 Lyon Cedex 08, France. E-mail: lenoir@iarc.fr

0002-9297/99/6401-0038$02.00

Am. J. Hum. Genet. 64:303–304, 1999

Cystic Fibrosis Mutations in Heterozygous Newborns with Hypertrypsinemia and Low Sweat Chloride

To the Editor:

Measurement of immunoreactive trypsinogen concentration (IRT) in dried blood spots is the most common technique for cystic fibrosis (CF) neonatal screening. Since a considerable number of newborns show raised IRT levels, the screening specificity is often improved by determining whether infants with hypertrypsinemia have the most common CF mutations: diagnosis is established in neonates carrying two mutations, but a sweat test is required if only one mutation is found, to distinguish between affected individuals—who would have a second, unrecognized mutation—and heterozygotes. Infants with raised IRT, one CF mutation, and normal sweat electrolyte concentrations are usually considered to be carriers only. However, the carrier frequency among nonaffected IRT-positive babies is almost three times higher than that in the general population (Laroche et al. 1991; Castellani et al. 1997); this could be partially explained if some of these babies carry on the other chromosome a mild mutation, associated with scarce symptoms and normal sweat chloride values. A DNA polymorphic sequence of five thymines (TTTTT) in intron 8 of the CF transmembrane conductance regulator (CFTR) gene, which is very common in men with a primarily genital CF form called "congenital bilateral absence of the vas deferens" (CBAVD) (Chillon et al. 1995), has been found to occur more frequently in newborns with raised IRT values than in controls (Castellani et al. 1997; Chin et al. 1997). To look further into the hypothesis that, in at least some babies, raised trypsin levels at birth could be a phenotypic expression of a compound heterozygosity, we investigated a subset of 18 newborns, using the following selection criteria: IRT >99.5 percentile; one identified CFTR mutation among a panel of 15 mutations that are present in 85% of the CF chromosomes in our area; normal sweat chloride, as determined by pilocarpine iontophoresis (mean 16.9 mEq/liter; maximum 32 mEq/liter; minimum 6 mEq/liter). In these neonates and in a control group of 15 healthy subjects (Pignatti et al. 1995), novel and rare mutations of the CFTR gene were sought by use of a complete gene search, with denaturing gradient-gel–electrophoresis analysis of all 27 exons and intronic flanking regions. PCR products that displayed an altered behavior in the gel were sequenced after cloning. Seven CFTR gene mutations were found in eight IRT-positive newborns, compared with one mutation (L997F) in the control group ($P = .02$ by Fisher's exact test; see table 1). Three of these mutations (R117H, Y301C, and E527G) are thought to be disease causing in CF or in CBAVD, since they determine the substitution of an amino acid in evolutionarily conserved residues and therefore are tentatively classified, on the basis of the Cystic Fibrosis Genetic Analysis Consortium (CFGAC) database, as "mutations"; the other four mutations (1716 G/A, 2622+14 G/A, 3041-71 G/C, and 4002 A/G) are not believed to be disease causing in CF and CBAVD, either because they do not determine any amino acid substitutions (in the case of 1716 G/A and 4002 A/G) or because they occur in noncoding regions that, as determined by sequence-analysis software, produce no apparent alteration (in the case of 2622+14 G/A and 3041-71 G/C) and therefore are tentatively classified, on the basis of the CFGAC database, as CF "polymorphisms." Mutations E527G and 2622+14G/A are described here for the first time. It is problematic to understand the clinical significance of the detected "mutations not supposed to cause CF," but, as far as "mutations supposed to cause CF" are concerned, in the 3 (16.6%) of 18 newborns who were compound het-

Table 1

Sweat Chloride Concentration and CFTR Genotypes

Case	Sweat Chloride (mEq/liter)	Mutation	
		Allele 1[a]	Allele 2[b]
1	10	R1162X	3041-71G/C,[c] 4002A/G[c]
2	14	ΔF508	
3	30	R1162X	R117H
4	21	ΔF508	E527G
5	8	ΔF508	
6	12	N1303K, 2622+14G/A[d]	
7	6	ΔF508	
8	20	ΔF508	1716G/A[c]
9	16	ΔF508	
10	10	ΔF508	
11	19	R1162X	
12	19	N1303K	
13	12	G542X	1716G/A[c]
14	32	ΔF508	
15	14	ΔF508	
16	26	N1303K	2622+14G/A[c]
17	18	ΔF508	Y301C
18	18	2183AA→G	

[a] First mutation found, assigned to one gene.

[b] Second mutation found, assigned to the gene other than that to which the first mutation found was assigned.

[c] Mutation located in allele 1 or allele 2 (no segregation analysis was possible, since the parents were not available for testing).

[d] Mutation located in the same gene.

erozygous, the raised IRT probably was not a casual finding but was a biochemical sign of an only partially functioning CFTR protein. Whether these neonates ought to be diagnosed as affected with CF is a moot point. In ~2% of patients with CF, there is an "atypical" phenotype, which consists of chronic sinopulmonary disease, pancreatic sufficiency, and either borderline or normal sweat chloride concentrations (Rosenstein et al. 1998). Unfortunately, it is not possible at present to predict the clinical outcome of our newborns, nor is it possible to provide satisfactory genetic counseling for the family. A close clinical follow-up should help in clarifying the extent of the disease in these subjects.

Acknowledgments

This study was supported by the Italian Ministry of Health, CF Project, law 548/93, and by the Ministry of University and Research. M.G.B. had a fellowship from the Cystic Fibrosis Center of Verona.

C. Castellani[1], M. G. Benetazzo,[2] A. Bonizzato,[1] P. F. Pignatti,[2] and G. Mastella[1]
[1]*Cystic Fibrosis Centre, Ospedale Civile Maggiore, and* [2]*Institute of Biology and Genetics, University of Verona, Verona*

Electronic-Database Information

URL for data in this article is as follows:

Cystic Fibrosis Genetic Analysis Consortium database, http://www.genet.sickkids.on.ca/cftr/ (for mutations in CF and CBAVD)

References

Castellani C, Bonizzato A, Mastella G (1997) CFTR mutations and IVS8-5T variant in newborns with hypertrypsinaemia and normal sweat test. J Med Genet 34:297–301

Chillon M, Casals T, Mercier B, Bassas L, Lissens W, Silber S, Romey M-C, et al (1995) Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. N Engl J Med 332:1475–1480

Chin S, Ranieri E, Gerace RL, Nelson PV, Carey WF (1997) Frequency of intron 8 CFTR polythymidine sequence variant in neonatal blood specimens. Lancet 350:1368–1369

Laroche D, Travert G (1991) Abnormal frequency of ΔF508 mutation in neonatal transitory hypertrypsinaemia. Lancet 337:55

Pignatti PF, Bombieri C, Marigo C, Benetazzo MG, Luisetti M (1995) Increased incidence of cystic fibrosis mutations in adults with disseminated bronchiectasis. Hum Mol Genet 4: 635–639

Rosenstein BJ, Cutting GR, Cystic Fibrosis Foundation Consensus Panel (1998) The diagnosis of cystic fibrosis: a consensus statement. J Pediatr 132:589–595

Address for correspondence and reprints: Dr. Carlo Castellani, Cystic Fibrosis Centre, Ospedale Civile Maggiore, Piazzale Stefani 1, 37126 Verona, Italy. E-mail: cfc@linus.univr.it

0002-9297/99/6401-0039$02.00

Am. J. Hum. Genet. 64:304–308, 1999

A Loss-of-Function Mutation in the Endothelin-Converting Enzyme 1 (ECE-1) Associated with Hirschsprung Disease, Cardiac Defects, and Autonomic Dysfunction

To the Editor:

Hirschsprung disease (HSCR [MIM 142623]) is a congenital disorder characterized by an absence of enteric ganglia over various lengths of the bowel, leading to functional obstruction and resulting in life-threatening bowel distension shortly after birth. The incidence is 1 in 5,000 live births. In ~80% of cases, the rectosigmoid colon is the only part affected, whereas in 15%–20% of cases, the aganglionosis extends to the ileocecal junction. In a small percentage of cases, the entire small bowel and colon are aganglionic, and in some rare cases, so-called skip-lesions occur, in which ganglionic and aganglionic bowel segments alternate.

HSCR is considered to be genetically heterogeneous (Edery et al. 1994; Puffenberger et al. 1994; Romeo et al. 1994; Angrist et al. 1996; Edery et al. 1996; Hofstra et al. 1996; Salomon et al. 1996; Pingault et al. 1998) and even polygenic (Puffenberger et al. 1994; Angrist et al. 1996; Salomon et al. 1996; Bolk et al. 1997). Mutations in five genes, *RET* (Edery et al. 1994; Romeo et al. 1994), *GDNF* (Angrist et al. 1996; Salomon et al. 1996), *EDNRB* (Puffenberger et al. 1994), *EDN3* (Edery et al. 1996, Hofstra et al. 1996), and *SOX10* (Pingault et al. 1998) have been shown to give rise, separately or in combination (Angrist et al. 1996; Salomon et al. 1996), to HSCR. They account for 60%–70% of the familial cases and 10%–30% of the sporadic cases (R. M. W. Hofstra, unpublished data). Conceivably, mutations in other genes that might be part of the signalling pathways to which these proteins belong may also lead to the HSCR phenotype. Here we describe the involvement of one such gene, the gene encoding the endothelin-converting enzyme I. This enzyme, ECE-1, is involved in the proteolytic processing of big endothelin 1, 2, and 3, encoded by genes *EDN1, EDN2,* and *EDN3,* to the biologically active peptides, endothelins ET1, ET2, and ET3, respectively.

For the purpose of the present paper, it is important to summarize the phenotypes of *Edn1, Edn3,* and *Ece1*

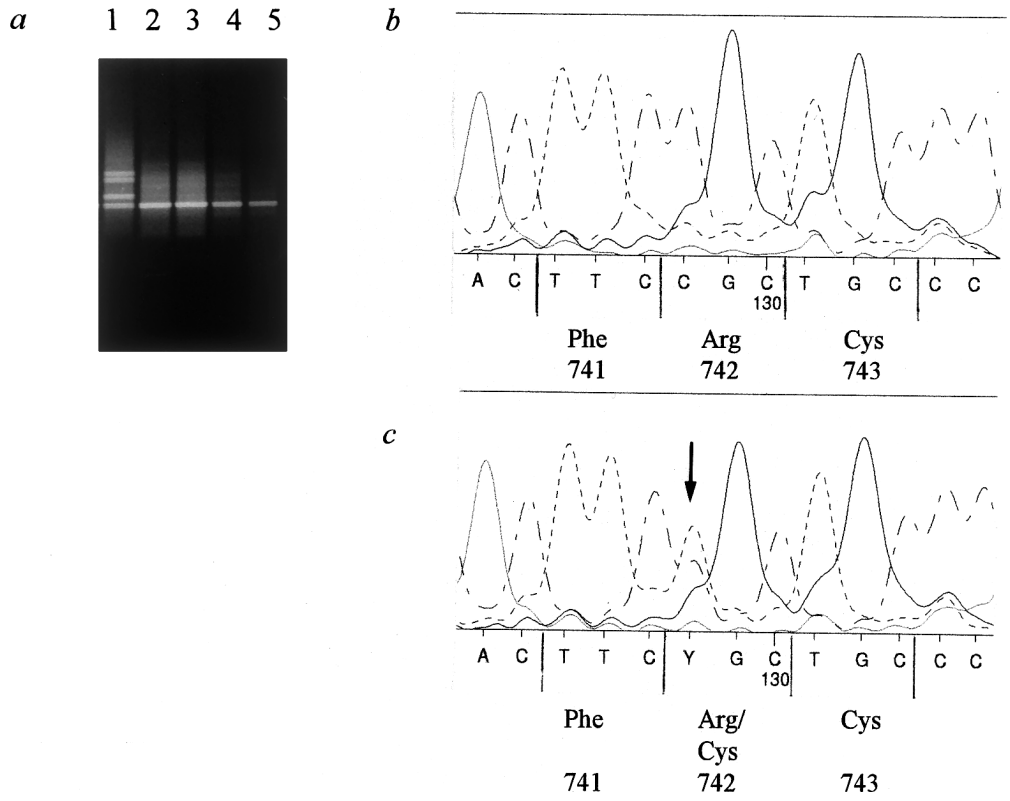

Figure 1 DNA analysis. *a,* DGGE patterns of exon 19 of *ECE-1.* A heterozygous variant can be seen in lane 1. Four normal controls are shown in lanes 2–5. Sequence analysis of *b,* the normal *ECE-1* exon 19 PCR product and *c,* the exon 19 PCR product of the patient described. The PCR primers and conditions and DGGE conditions are available upon request.

knockout mice. In *Edn1*$^{+/-}$ mice, blood pressure is mildly but significantly elevated, whereas *Edn1*$^{-/-}$ mice are characterized by abnormal development of the pharyngeal arches, cleft palate, and small mandibula; abnormalities in the outflow tract of the heart; and abnormal thymus and thyroids (Kurihara et al. 1994, 1995). Similar abnormalities are also seen in the human DiGeorge syndrome (MIM 188400). Genetically, however, these are unrelated, as *EDN1* is located on the short arm of chromosome 6, whereas the locus for DiGeorge syndrome is mapped to the long arm of chromosome 22. *Edn1*$^{-/-}$ mice die shortly after birth (within hours). *Edn3*$^{+/-}$ mice are normal, whereas *Edn3*$^{-/-}$ mice die within a few weeks after birth and have pigment anomalies and aganglionosis in the distal colon (Baynash et al. 1994). Similar abnormalities are seen in the human Shah-Waardenburg syndrome (MIM 277580) (Edery et al. 1996; Hofstra et al. 1996). *Ece1*$^{+/-}$ mice are normal, whereas *Ece1*$^{-/-}$ mice exhibit neonatal lethality due to craniofacial and cardiac defects identical to those seen in *Edn1*$^{-/-}$ mice. In addition, *Ece1*$^{-/-}$ newborns lack enteric ganglia in the terminal colon (Yanagisawa et al. 1998). Thus, *Ece1* knockout mice seem to present a combination of features characteristic for the *Edn1* and *Edn3* knockout mice.

These observations prompted us to scan the human ECE-1 gene (Valdenaire et al. 1995) for mutations in a patient with skip-lesions HSCR, cardiac defects (ductus arteriosus, small subaortic ventricular septal defect, and small atrial-septal defect), craniofacial abnormalities (cupped ears: immature, and posteriorly rotated; and small nose with a high bridge and bulbous tip), and other dysmorphic features (tapered fingers with hyperconvex nails; a single left palmar crease; contractures at the DIP joints of the thumbs; PIP joints of the fingers, bilaterally; and micropenis) and autonomic dysfunction (episodes of severe agitation in association with significant tachycardia, hypertension, and core temperatures as high as 40.5°C; and status epilepticus). The patient had a normal karyotype without a 22q11 deletion.

We screened all 19 exons of the gene, using denaturing gradient-gel electrophoresis (DGGE) (GenBank accession numbers: cDNA sequence, Z35307; exon and intron boundaries, X91922–91939). For DGGE analysis, a 9% PAA gel (acrylamide-to-bisacrylamide, 37.5:1) containing a 40%–80% UF (100% UF = 7 M urea and

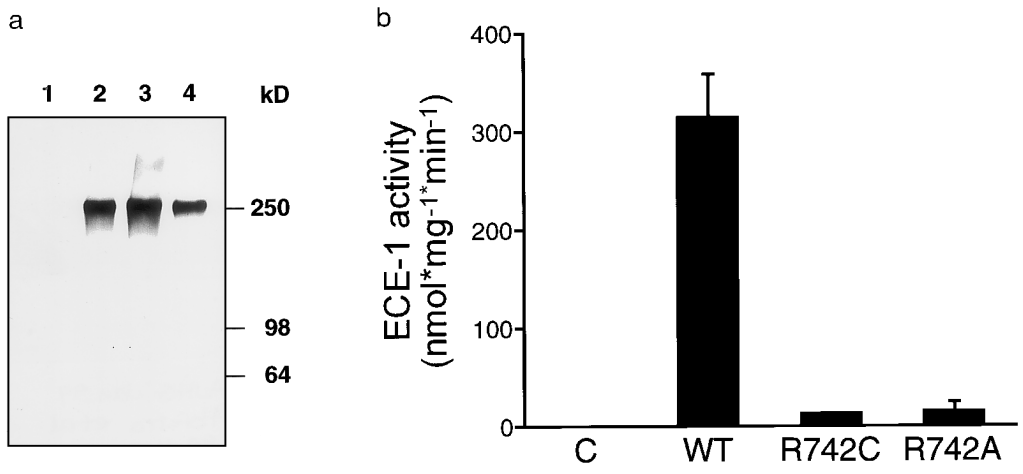

Figure 2 Western blot and activity measurements. *a,* Mutant and native ECE-1b isoforms were transiently transfected into CHO-K1 cells with Lipofectamine (Life Technologies) according to the manufacturer recommendations. Confluent cells in 100 mm plates were harvested 60 h after transfection, and membranes were prepared. Protein levels of expressed ECE-1 were measured on membranes by quantitative immunoblotting as described (Schweizer et al. 1997). Western blot of one set of transfections is shown: lane 1, nontransfected cells; lane 2, wild-type ECE-1b; lane 3, R742C mutant; lane 4, R742A. *b,* ECE-1 activity was assessed by means of a specific radioimmunoassay as described elsewhere (Schweizer et al. 1997). The results shown are the mean ± SD of at least three independent experiments, in nanomoles of produced ET-1 per minute per milligram of ECE-1 protein. C = nontransfected cells; WT = wild-type ECE-1b.

40% deionized formamide) was used. Electrophoresis was performed in 0.5 × TAE (1 × TAE = 40 mM Tris, HAC pH 8.0; 20 mM NaAc; 1 mM Na EDTA) at 11 V/cm and 58°C for 18 h. An aberrant DGGE pattern was detected in exon 19 (fig. 1*a*). For the analysis of exon 19, the following primers were used: ECE-1/19F, 5′-ACAGTGACCCTGGCCTCTCC-3′, and ECE-1/19R, 5′-(40-bp GC clamp)TCTCGTCCTCAGCCCCTTCC-3′. The aberrant PCR products were purified and sequenced. A heterozygous C→T transition, resulting in the substitution of cysteine for arginine at 742, was detected (fig. 1*b*). Unfortunately, the patient's parents were not available for testing. In 100 control individuals, this mutation was never found. Furthermore, no *ECE-1* mutations were found in a further 110 HSCR patients screened. None of them, however, had the phenotype of the described patient.

Amino acid position 742 is in the vicinity of the active site of ECE-1 (Valdenaire et al. 1995). The observed mutation results in the replacement of a basic amino acid by a neutral polar amino acid. Moreover, this might result in the formation of an alternative disulfide bridge. In humans, three ECE-1 isoforms are generated from the same gene (Schweizer et al. 1997). They differ only in their first N-terminal amino acid residues; they share the same extracellular domain (which includes the enzyme active site) and cleave big endothelins with similar efficiencies.

To investigate the functional consequences of the mutation on ECE-1 activity, we introduced it into the human ECE-1b isoform (Valdenaire et al. 1995; Schweizer et al. 1997). A PCR approach was used to construct the mutant (Cys742). Fidelity of the mutants was checked by sequencing. Wild-type and mutant proteins were produced by transient expression of the above-described expression constructs in Chinese hamster ovary cells (CHO-K1). ECE-1 activity was measured on cell membrane preparations by means of a specific radioimmunoassay and quantitative immunoblotting as described elsewhere (Schweizer et al. 1997). The specific ECE-1 activity was calculated as nanomoles of endothelin 1 produced per minute per milligram of expressed ECE-1 ($nM/min^{-1}/mg^{-1}$). A more detailed protocol of this functional assay can be found elsewhere (Löffler and Maire 1994; Schweizer et al. 1997).

An example of a western blot used for quantitative immunoblotting is shown in figure 2*a*. The outcome of the radioimmunoassay is show in figure 2*b*. The specific activity measured in three independent transfections was for the wild-type ECE-1b, 314 ± 44 $nM/min^{-1}/mg^{-1}$ (mean ± SD), and for the Arg742Cys mutant ECE-1b, 14.7 ± 9.8 $nM/min^{-1}/mg^{-1}$ (mean ± SD). Thus, the activity of the mutant ECE-1 is only 4.7% of that of wild-type ECE-1. To determine whether this effect was due to this specific amino acid substitution or more generally to an effect on the catalytic site, we also generated an Arg742Ala mutant. This Arg742Ala mutant ECE-1 had a specific activity of 12.4 ± 0.3 $nM/min^{-1}/mg^{-1}$ (mean ± SD), demonstrating that the position of the mutation is more important than its specific nature.

In addition, from a developmental point of view, there are arguments suggesting that the phenotype described might be caused by reduced activity of the ECE-1 enzyme. The vertebrate enteric nervous system is large and

independent. It develops from cells that migrate to the gut from three regions of the neural crest. The cells from the vagal neural crest colonize the enteric bowel below the rostral foregut, the sacral neural crest cells colonize only the postumbilical bowel, and the cells of the truncal neural crest colonize only the rostral foregut primordia of the esophagus and the cardiac stomach (Gershon 1997). Vagal neural crest cells are also crucial for the development of the outflow tract of the heart, thymus, and parathyroid glands. Neural crest cells from more anterior hindbrain regions play a key role in the patterning of the pharyngeal arches and their derivatives. The phenotypes in spontaneous and induced *Edn3, EdnrB,* and *Ece1* mutant mice are all related to the developmental fate of hindbrain neural crest cells and to the formation of melanocytes, also derived from the neural crest.

Further evidence that reduced levels of ET3 might contribute to the development of HSCR comes from expression studies of this gene in both ganglionic and aganglionic colon segments of HSCR patients and control individuals. Both aganglionic colon and ganglionic colon of HSCR patients show reduced levels of *EDN3* transcripts regardless of the mutation status of genes known to be involved in HSCR (S. E. Kenny, R. M. W. Hofstra, Y. Wu, C. H. C. M. Buys, C. Vaillant, D. A. Lloyd, and D. H. Edgar, unpublished data). This suggests that a low level of ET3 might be a condition for the development of HSCR.

In view of (1) the function of ECE-1 during murine development suggested by the mouse models, (2) the overlap in phenotypic features of these mouse models and our patient, and (3) the functional consequences of the mutation on the enzyme activity, we propose that the Arg742Cys mutation caused or at least contributed to the phenotype of our patient by producing reduced levels of ET1 and ET3.

Robert M. W. Hofstra,[1] Olivier Valdenaire,[2] Ellen Arch,[3] Jan Osinga,[1] Hester Kroes,[1] Bernd-Michael Löffler,[2] Ada Hamosh,[3] Carel Meijers,[4] and Charles H. C. M. Buys[1]

[1]*Department of Medical Genetics, University of Groningen, Groningen, The Netherlands;* [2]*Pharma Division, Preclinical Research, Hoffmann-La Roche, Ltd., Basel;* [3]*Center for Medical Genetics, Johns Hopkins Medical Institutions, Baltimore;* [4]*Institute of Paediatric Surgery/Cell Biology and Genetics, Erasmus University Rotterdam, Rotterdam*

Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for the human cDNA of ECE-1, accession number Z35307; for the nucleotide sequences of all intron-exon boundaries, accession numbers X91922–91939)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Hirschsprung [MIM 142623], for Shah-Waardenburg [MIM 277480], and for DiGeorge [MIM 188400] syndromes)

References

Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A (1996) Germline mutation in glial cell line–derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. Nat Genet 14:341–344

Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE, Yanagisawa M (1994) Interaction of endothelin 3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell 79: 1277–1285

Bolk S, Pelet A, Hofstra RMW, Salomon R, Angrist M, Buys CHCM, Lyonnet S, et al. (1997) Multigenic inheritance of Hirschsprung disease. Am J Hum Genet 61:A41

Edery P, Attie T, Amiel J, Pelet A, Eng C, Hofstra RMW, Martelli H, et al (1996) Mutation of the endothelin-3 gene in the Waardenburg-Hirschsprung disease (Shah-Waardenburg syndrome). Nat Genet 12:442–444

Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, et al (1994) Mutations of the RET proto-oncogene in Hirschsprung's disease. Nature 367:378–380

Gershon MD (1997) Genes and lineages in the formation of the enteric nervous system. Curr Opin Neurobiol 7:101–109

Hofstra RMW, Osinga J, Tan G, Wu Y, Kamsteeg EJ, Stulp RP, van Ravenswaaij-Arts C, et al (1996) A homozygous mutation in the human endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). Nat Genet 12:445–447

Kurihara Y, Kurihara H, Maemura K, Kuwaki T, Kumada M, Yazaki Y (1995) Impaired development of the thyroid and thymus in endothelin-1 knock-out mice. J Cardiovasc Pharmacol 26(suppl 3):S13–S16

Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, et al (1994) Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin 1. Nature 368:703–710

Löffler BM, Maire JP (1994) Radioimmunological determination of endothelin peptides in human plasma: a methodological approach. Endothelium 1:273–286

Pingault V, Bondurand N, Kuhlbrodt K, Goerich DE, Prehu MO, Puliti A, Herbacrth B, et al (1998) SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat Genet 18:171–173

Puffenberger EG, Hosoda K, Washington SS, Nakao K, de Wit D, Yanigisawa M, Chakravarti A (1994) A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. Cell 79:1257–1266

Romeo G, Ronchetto P, Yin L, Barone V, Seri M, Ceccherini I, Pasini B, et al (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 367:377–378

Salomon R, Attie T, Pelet A, Bidaud C, Eng C, Amiel J, Sarnacki S, et al (1996) Germline mutations of the RET ligand

GDNF are not sufficient to cause Hirschsprung disease. Nat Genet 14:345–347

Schweizer A, Valdenaire O, Nelböck P, Deuschle U, Dumas Milne Edwards JB, Stumpf JG, Löffler BM (1997) Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localizations. Biochem J 328:871–877

Valdenaire O, Rohrbacher E, Mattei MG (1995) Organization of the gene encoding the human endothelin-converting enzyme (ECE-1). J Biol Chem 270:29794-29798

Yanagisawa H, Yanagisawa M, Kapur RP, Richardson JA, Williams SC, Clouthier DE, de Wit D, et al (1998) Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. Development 125:825–836

Address for correspondence and reprints: Dr. R. M. W. Hofstra, Department of Medical Genetics, University of Groningen, Ant. Deusinglaan 4, 9713 AW Groningen, The Netherlands. E-mail: R.M.W.Hofstra@med.rug.nl

0002-9297/99/6401-0040$02.00

Am. J. Hum. Genet. 64:308–310, 1999

Variant Manifestation of Cowden Disease in Japan: Hamartomatous Polyposis of the Digestive Tract with Mutation of the *PTEN* Gene

To the Editor:

Because of the clinical heterogeneity and complexity of the group of disorders collectively known as inherited hamartoma syndromes, several attempts have been made to classify them into distinct categories. In the May issue of this *Journal,* Eng and Ji (1998) reviewed recent progress in molecular characterization of these syndromes and classified them into four clinical entities: Cowden disease (CD [MIM 158350]), Bannayan-Ruvalcaba-Riley syndrome (BRR [MIM 153480]), Peutz-Jeghers syndrome (PJS [MIM 175200]), and juvenile polyposis syndrome (JPS [MIM 174900]). Despite some progress in molecular characterization, specific diagnoses of these disorders remain difficult because their phenotypic spectra overlap and because the penetrance of symptoms is age related. Clinical syndromic diagnosis is also dependent on many factors, such as the nature and type of the first clinical symptoms and the medical discipline of the individual(s) diagnosing the syndrome.

PTEN, a gene mapping to 10q23, encodes a dual-specificity phosphatase and is also called *MMAC1* or *TEP1* (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). *PTEN* has been identified as the susceptibility gene for CD and BRR (Liaw et al. 1997; Marsh et al. 1997), and it appears that *PTEN* mutations are detected more frequently in CD and BRR patients when strict clinical criteria are applied to the selection of test subjects (Liaw et al. 1997; Marsh et al. 1997, 1998). *LKB1/STK11,* a serine threonine kinase gene at 19p13.3, has been identified as a susceptibility gene for PJS (Hemminki et al. 1998; Jenne et al. 1998). As for JPS, however, some controversy exists about its molecular basis. Three possibilities have been raised: (1) germ-line mutations of the *SMAD4/DPC4* gene at 18q21.1 are known to be responsible for JPS in some affected families (Howe et al. 1998), (2) *PTEN* mutations appear to be the predisposing elements for some patients diagnosed with JPS (Lynch et al. 1997; Olschwang et al. 1998); and (3) yet another putative locus ("JP1"), centromeric to *PTEN* on chromosome 10q, has been linked to JPS in some affected families (Jacoby et al. 1997). The low penetrance of CD, the sharing of some phenotypic features between CD and JPS, and the possible genetic heterogeneity of JPS make diagnosis complex and confusing.

Pathognomonic hallmarks of CD patients are facial trichilemmomas, acral keratoses, and verucoid or papillomatous papules. This triad of skin lesions occurs in 99% of CD patients (Hanssen and Fryns 1995; Longy and Lacombe 1996). Other, less frequent manifestations of CD include thyroid adenomas or goiters (occurring in 40%–60% of CD patients), breast fibroadenomas (70% of affected females), hamartomatous gastrointestinal polyps (35%–40%), and macrocephaly (38%) (Eng 1998; Marsh et al. 1998). JPS is characterized by gastrointestinal hamartomatous polyps and an increased risk of gastrointestinal cancer (Olschwang et al. 1998).

We examined a 35-year-old Japanese man who had been followed clinically for JPS because of numerous hamartomatous polypoid lesions throughout the entire digestive tract, from esophagus to rectum. Although he had none of the pathognomonic skin lesions of CD, we extended our clinical examination to the patient's whole body and tested him for mutation of the *PTEN* gene, in view of Eng's proposal (1998) that *PTEN* mutation can be a useful diagnostic marker for incompletely expressed CD. After informed consent was obtained, genomic DNAs prepared from blood from the patient and from members of his family were examined by direct sequencing of the entire coding region and exon-intron boundaries of *PTEN,* according to procedures we have described elsewhere (Kurose et al. 1998). The patient's father died of brainstem infarction, a condition unrelated to CD. No other members of his family have been diagnosed as having CD.

The patient was found to be heterozygous for a G→A transition at the second nucleotide of codon 130, which would result in a substitution of Gln for Arg (R130Q). The patient's mother and sister did not carry this mutation (fig. 1), nor was it detected in 192 chromosomes from control Japanese individuals. On closer examination, which included ultrasonography and computed tomography, we found a small thyroid adenoma, a few

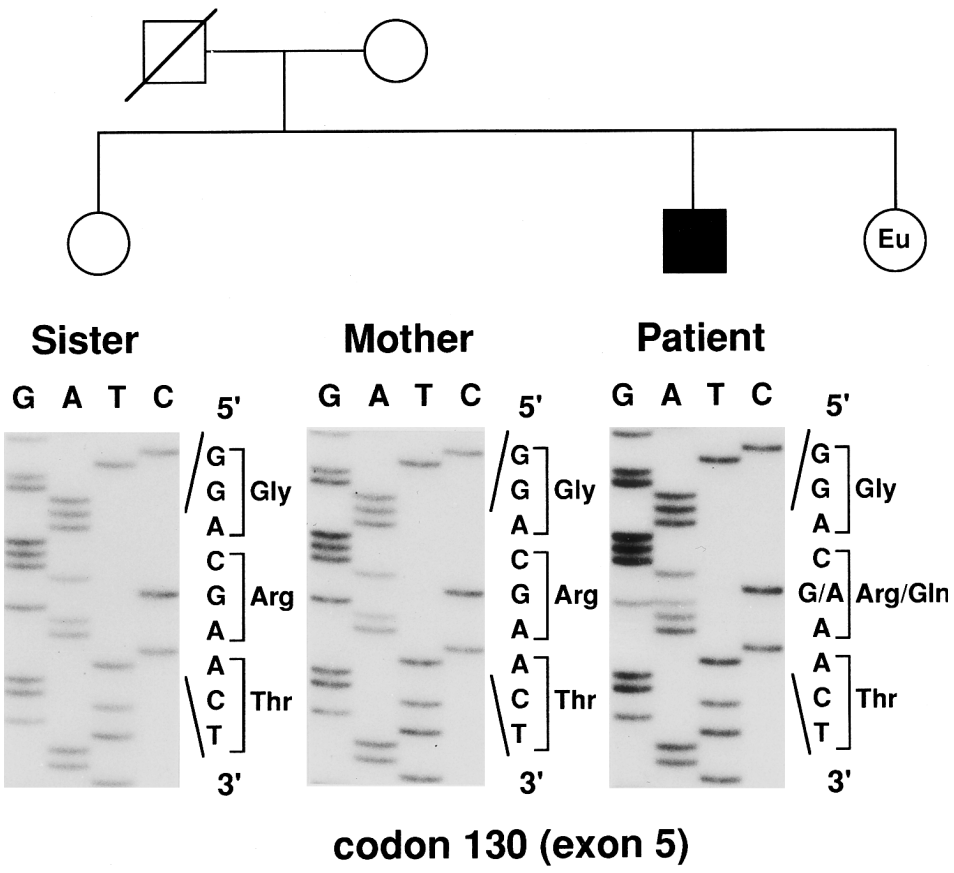

Figure 1 DNA sequencing of the *PTEN* gene in the family of a patient with variant CD. The patient (*blackened square*) carries a G→A transition in exon 5, which is not present in his unaffected mother and sister. Eu: uninformative evaluation.

papillomatous papules in his right hand, and a lung tumor, which is now being examined for possible malignancy. Thus, molecular testing of the *PTEN* gene, as proposed by Eng (1998) in another case of suspected JPS, led us to a diagnosis of CD in a "JPS" patient who manifested atypical symptoms of CD. His germ-line mutation had occurred in the core motif of the phosphatase, at amino acid residue 122–132, encoded by exon 5. Most of the reported germ-line missense mutations of the *PTEN* gene reported in patients with CD have occurred within this core motif (Marsh et al. 1998). Thus, in terms of *PTEN* mutation, our case is typical of a CD entity, although the phenotype is atypical.

Eng and Ji (1998) pointed out that apparent "JPS" patients who carry *PTEN* germ-line mutations (Lynch et al. 1997; Olschwang et al. 1998) may in fact belong to a category of CD patients whose phenotypic features are only partially expressed. Eng and Ji (1998) proposed that the presence of a germ-line *PTEN* mutation could be a good diagnostic sign for CD and BRR. In the future, these inherited hamartoma syndromes should be classified by types of gene mutations, such as the *PTEN* mutation syndrome.

The results described here signal the possibility that a large number of hidden clinical variants of CD may exist among patients who might have escaped correct clinical diagnosis and may have been treated for JPS. Our work underscores the usefulness and importance of molecular methods for achieving differential diagnoses among patients with gastrointestinal polyposis, because JPS and CD predispose to completely different types of cancer.

Acknowledgments

The authors thank the patient and members of his family, for their participation in this study, and Prof. Tetsuro Miki for advice and encouragement. This work was supported by a Grant-in-Aid for priority areas "Cancer Research" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan and by a Research Grant for Cancer Research from the Ministry of Health and Welfare of Japan.

KEISUKE KUROSE,[1,2] TSUTOMU ARAKI,[2] TSUYOSHI MATSUNAKA,[3] YASUHARU TAKADA,[3] AND MITSURU EMI[1]

[1]*Department of Molecular Biology, Institute of Gerontology, and* [2]*Department of Obstetrics and Gynecology, Nippon Medical School, Kawasaki-Tokyo;* [3]*Department of Internal Medicine, Saijo Central Hospital, Saijo, Ehime, Japan*

Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for CD [MIM 158350], BRR [MIM 153480], PJS [MIM 175200], and JPS [MIM 174900])

References

Eng C (1998) Genetics of Cowden syndrome: through the looking glass of oncology. Int J Oncol 12:701–710

Eng C, Ji H (1998) Molecular classification of the inherited hamartoma polyposis syndromes: clearing the muddied waters. Am J Hum Genet 62:1020–1022

Hanssen AM, Fryns JP (1995) Cowden syndrome. J Med Genet 32:117–119

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, et al (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 391: 184–187

Howe JR, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, Tomlinson IP, et al (1998) Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 280: 1086–1088

Jacoby RF, Schlack S, Cole CE, Skarbek M, Harris C, Meisner LF (1997) A juvenile polyposis tumor suppressor locus at 10q22 is deleted from nonepithelial cells in the lamina propria. Gastroenterology 112:1398–1403

Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, Muller O, et al (1998) Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat Genet 18: 38–43

Kurose K, Bando K, Fukino K, Sugisaki Y, Araki T, Emi M (1998) Somatic mutations of the PTEN/MMAC1 gene in fifteen Japanese endometrial cancers: evidence for inactivation of both alleles. Jpn J Cancer Res 89:842–848

Li DM, Sun H (1997) TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. Cancer Res 57: 2124–2129

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, et al (1997) *PTEN,* a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1947

Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, et al (1997) Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 16:64–67

Longy M, Lacombe D (1996) Cowden disease. Report of a family and review. Ann Genet 39:35–42

Lynch ED, Ostermeyer EA, Lee MK, Arena JF, Ji H, Dann J, Swisshelm K, et al (1997) Inherited mutations in PTEN that are associated with breast cancer, Cowden disease, and juvenile polyposis. Am J Hum Genet 61:1254–1260

Marsh DJ, Coulon V, Lunetta KL, Rocca-Serra P, Dahia PL, Zheng Z, Liaw D, et al (1998) Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. Hum Mol Genet 7:507–515

Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C (1997) Germline mutations in *PTEN* are present in Bannayan-Zonana syndrome. Nat Genet 16:333–334

Olschwang S, Serova-Sinilnikova OM, Lenoir GM, Thomas G (1998) PTEN germ-line mutations in juvenile polyposis coli. Nat Genet 18:12–14

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, et al (1997) Identification of a candidate tumor suppressor gene, *MMAC1,* at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15: 356–362

Address for correspondence and reprints: Dr. Mitsuru Emi, Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396, Kosugi-cho, Nakahara-ku, Kawasaki, 211-8533, Japan. E-mail: memi@nms.ac.jp

0002-9297/99/6401-0041$02.00

Am. J. Hum. Genet. 64:310–313, 1999

Failure to Detect Linkage of Preeclampsia to the Region of the *NOS3* Locus on Chromosome 7q

To the Editor:

Preeclampsia is an important inheritable pregnancy-related hypertension syndrome. Since it develops as a result of widespread endothelial dysfunction, the *NOS3* gene responsible for endothelium-derived nitric oxide (NO) production via its gene product, eNOS, has been suggested as an obvious candidate gene for preeclampsia (Morris et al. 1996). We were intrigued, therefore, by the report, by Arngrímsson et al. (1997), of linkage in the region of the *NOS3* gene and have attempted to repeat their findings in our own collection of preeclampsia families.

Evidence for linkage was sought by use of two, separately ascertained, affected sister-pairs (ASPs) collections, from Amsterdam and Cambridge (United Kingdom), that contained 104 sibships. In the Cambridge Centre, a total of 21 extended pedigrees were also iden-

Table 1

Allele Sharing for *NOS3i13* and Flanking Markers

ASP Collection and Marker	No. of Alleles Shared IBD			
	0	1	2	*P*
Amsterdam (n = 46):				
NOS3i13	11.1	20.2	14.6	.250
Cambridge (n = 58):				
D7S483	11.3	32.0	14.7	.388
NOS3i13	18.0	28.0	12.0	.584
D7S505	15.1	30.0	13.8	.584
Combined (n = 104):				
NOS3i13	28.9	47.8	26.3	.547

tified, some on the basis of the ASPs suitable for conventional parametric linkage studies. All affected individuals were Caucasian and met the Redman and Jefferies criteria of preeclampsia: they were proteinuric (>300 mg/24 h) and hypertensive, with a blood pressure of >140/90 occurring after 20-wk gestation and with a ⩾25-mmHg rise in diastolic blood pressure (Redman and Jefferies 1988). These features all resolved by 3 mo postpartum, and none of the subjects had concurrent diabetes, renal disease, or essential hypertension. Some sibships (<5%) contained subjects showing features of pregnancy-induced hypertension (PIH) alone (i.e., they were not proteinuric). These members were not included in subsequent sib-pair analysis, and none of the larger pedigrees used contained subjects with PIH. For the purposes of linkage analysis, all males were assigned to the affection status of "unknown."

Subjects were genotyped for the CA-repeat marker within intron 13 of the *NOS3* gene (referred to here as "*NOS3i13*"). The Cambridge samples were also genotyped for the two flanking markers D7S483 and D7S505. Genotyping was performed by use of PCR amplification of the microsatellite markers by means of primer pairs, in which the forward primer had been 5′ end–labeled with a fluorescent amidite. CEPH primer sequences were used for the flanking markers, and primer sequences published elsewhere were used for *NOS3i13* (Nedaud et al. 1994). The PCR products were multiplexed and were run on an ABI 377, and allele sizes were determined by use of version 2 of GENOTYPER (Applied Biosystems).

The number of alleles shared identical by descent (IBD) was calculated for the ASPs by use of the maximum-likelihood method, as implemented in SPLINK (Holmans 1993; Holmans and Clayton 1995). Parental genotype data were used where available but were incomplete for the Cambridge ASPs and were not available for the Amsterdam ones. Comparison of allele frequencies and marker heterozygosity from the SPLINK output for the two groups showed no significant differences between the two collections. The allele sharing for the *NOS3i13* marker is shown in table 1, for both the Amsterdam and Cambridge ASPs. There is no evidence of excess allele sharing in either group. In fact, the Cambridge group shows a deficit of allele sharing, attributable to chance alone. To confirm this, allele sharing for the two flanking markers for *NOS3*, D7S483 and D7S505, was investigated. These two flanking markers are approximately equidistant from *NOSi13* and span a 4-cM region over 7q36. Again, there was no evidence of a significant positive deviation from a 1:2:1 distribution in the Cambridge group, either for D7S483 or for D7S505.

Parametric analysis of the preeclampsia pedigrees was performed by use of four of the models, employed elsewhere (Arngrímsson et al. 1997), as follows (q is the frequency of disease gene, and f is the penetrance): an autosomal model (AD) of high arbitrary penetrance (q = .02, f = 0.9), a partial-dominance model (AD/LP; q = .10, f_{Aa} = 0.21, f_{AA} = 1.0), a fully recessive model (AR; q = .2), and an affecteds-only model (AO; q = .02, $f_{Aa} = f_{AA}$ = 0.001). The power of the preeclampsia pedigree collection to detect linkage was estimated by means of simulation of each model, by use of SLINK (Weeks et al. 1991; Ott 1989). The results of 500 replications of each model generated by SLINK, if a hypothetical marker with eight equally frequent alleles (PIC = .86) is assumed, are summarized in tables 2 and 3. These results show that such a marker linked tightly to preeclampsia provides an average LOD score sufficient to establish linkage in three of the four models. The power of the simulated marker to detect linkage in the AD/LP model is substantially less than that of the other three models, as has been noted elsewhere (Harrison et al. 1997).

The models were tested by use of all three markers for the 4-cM interval, on 7q, encompassing the *NOS3* locus. Table 4 shows the results of two-point linkage analysis, using allele frequencies provided by SPLINK; the findings were not altered significantly by the assignment of equal allele frequencies throughout. For all the models, linkage to *NOS3i13* could be excluded with LOD < −2. As expected from the SLINK modeling, the

Table 2

Results of Computer Simulations with SLINK, for 500 Replicates

Model	Average LOD Score at θ =				
	.0	.1	.2	.3	.4
AD	6.05	4.89	3.37	1.92	.75
AD/LP	1.70	1.49	.99	.53	.18
AR	4.89	3.81	2.48	1.26	.39
AO	3.88	3.12	2.05	1.08	.37

Table 3

Results of Two-Point Linkage Analysis

Model	% of Replicates with LOD-Score Threshold >		
	1	2	3
AD	100.0	99.4	97.6
AD/LP	78.4	41.4	10.8
AR	100.0	98.4	89.2
AO	98.8	89.6	72.4

low-penetrance AD/LP model provided smaller LOD scores than did the other three models, and linkage to the flanking markers could not be excluded in our preeclampsia pedigrees. However, for neither of the markers under the AD/LP model did the LOD scores approach the required threshold of $3 + \log(4)$ (Kidd and Ott 1984).

These results, therefore, have failed to confirm linkage of preeclampsia to a chromosome 7q 4-cM region containing the *NOS3* gene, as reported by Arngrímsson et al. (1997). This failure occured despite use of a similar combination of ASPs and conventional two-point linkage in pedigrees. Our study used two independently ascertained collections of ASPs, containing a total of 104 ASPs that appeared to be powered adequately. We have estimated the λ_s for preeclampsia at ~10, assuming a local prevalence of preeclampsia (using our strict definition) of some 2%. Since our total ASP collection contains 70 fully informative pairs (estimated by SPLINK) for the *NOSi13* marker, an expected maximized LOD score of 7.6 is given (Risch 1990). Even if λ_s has been overestimated, a figure of 5 would still result in a LOD score substantially >3 (actually 5.7).

Our inability to replicate the earlier linkage report could reflect population differences, although both studies are based on white northern Europeans. It is possible that significant differences in our definition of the preeclampsia phenotype may be important, although the differences in our method of ASP analysis seem unlikely. We have focused in our study only on ASPs with a definite diagnosis of preeclampsia, excluding ones in which the diagnosis either is uncertain or is consistent only with isolated PIH. Nevertheless, Arngrímsson et al. (1997) report that the significance of the excess allele sharing was not substantially altered by removal of the milder phenotypes from the sib-pair analysis. Our method of sib-pair analysis relies on a maximum-likelihood method (SPLINK) to calculate allele sharing that is IBD. The SPLINK program is able to utilize parental genotype data, although this was not available for most of our ASPs. The previous linkage study on 7q also used a likelihood ratio–based method (SIBPAIR), as well as direct testing of increased identity by state (IBS) sharing (APM). A recent comparison of the various methods available to detect linkage in nuclear pedigrees, including sib pairs, showed the superiority of IBD-based programs versus IBS-based programs (Davis and Weeks 1997). In this comparison, SPLINK actually showed comparable power to detect linkage with the SIBPAIR program, except when additional genotypes were available from unaffected members, which was not the case in this study. Our choice of SPLINK, therefore, is unlikely to have increased the possibility of a false-negative result.

The previous positive sib-pair analysis reported by Arngrímsson's group was also supported by two-point linkage results from their pedigree collection (Arngrímsson et al. 1997). They investigated a number of different models, reported elsewhere, from the literature. The LOD score, maximized over the five models, was 4.03, by use of the D7S505 flanking marker rather than the *NOS3i13* marker. This suggests that the preeclampsia locus may be some distance away from the *NOS3* gene itself, although the *NOS3i13* marker and the preeclampsia locus must be in linkage disequilibrium, on the basis of their transmission/disequilibrium-test results. Adopting a similar parametric analysis in our own preeclampsia pedigrees, we have failed to demonstrate a LOD score high enough to confirm linkage in any of the models. In fact, the LOD scores generated actually have enabled us to exclude linkage in all of them, except the AD/LP model. Using the AD/LP model, we did find a small, nonsignificant LOD score, using a flanking marker, but it was by use of D7S483 and not D7S505—that is, at the opposite end of the 7q interval.

A key role for endothelium-derived NO in pregnancy is well supported. An eNOS inhibitor, for example, infused chronically into pregnant animals, produces a preeclampsia-like state, with hypertension, proteinuria, thrombocytopenia, and growth retardation (Molnar and

Table 4

Two-Point Linkage Results for the Four Models Tested

Model and Marker	LOD Score at θ =						
	.00	.01	.05	.10	.20	.30	.40
AD:							
D7S505	−7.22	−5.52	−3.01	−1.59	−.34	.08	.14
NOS3i13	−9.76	−8.40	−5.41	−3.44	−1.40	−.46	−.07
D7483	−2.37	−2.05	−1.27	−.80	−.41	−.26	−.15
AD/LP:							
D7S505	−1.56	−1.40	−.90	−.49	−.07	.07	.07
NOS3i13	−2.08	−1.86	−1.20	−.67	−.12	.07	.08
D7483	.34	.39	.51	.53	.39	.17	.02
AR:							
D7S505	−∞	−7.02	−3.63	−2.08	−.74	−.23	−.04
NOS3i13	−∞	−7.69	−3.98	−2.16	−.58	−.04	.07
D7483	−∞	−3.80	−1.46	−.50	.11	.14	.03
AO:							
D7S505	−3.20	−2.51	−1.03	−.22	.32	.34	.20
NOS3i13	−6.03	−5.05	−2.92	−1.63	−.47	−.06	.04
D7483	−1.91	−1.59	−.86	−.43	−.13	−.09	−.08

Hertelendy 1992). More-recent findings also provide direct evidence for the role of NO production in the fall in peripheral vascular resistance (and blood pressure) seen in normal human pregnancy (Knock and Poston 1996). It is possible that NO forms part of an adaptation pathway, to accommodate the cardiovascular changes of pregnancy and to prevent the development of maternal hypertension and the clinical syndrome of preeclampsia. However, although these data provide a tantalizing circumstantial argument for *NOS3* being a candidate gene for preeclampsia, they do not prove that a *primary* abnormality in eNOS underlies the pathophysiology of preeclampsia. It is equally plausible that the observed changes in NO production during preeclampsia are secondary to free-radical damage of the vascular endothelium.

In summary, then, we have been unable to replicate the previous report of linkage of preeclampsia to the region of the *NOS3* gene. Although abnormalities in NO production have been observed in preeclampsia, we believe that the case for the *NOS3* gene or its product, eNOS, having a *primary* role in the pathophysiology of preeclampsia remains unproved.

Acknowledgments

Generous support for this work was provided by a project grant and Ph.D. studentship (to I.L.) from the British Heart Foundation.

IAN LEWIS,[1] GUUS LACHMEIJER,[4,5] SARAH DOWNING,[1] GUSTAAF DEKKER,[5] CLIVE GLAZEBROOK,[2] DAVID CLAYTON,[3] NICK H. MORRIS,[6] AND KEVIN M. O'SHAUGHNESSY[1]

Departments of [1]Medicine and [2]Anaesthetics, University of Cambridge Clinical School, and [3]MRC Biostatistical Unit, Addenbrooke's Hospital, Cambridge; [4]Division of Clinical Genetics and Prenatal Diagnosis, Department of Obstetrics and Gynecology, University and National Hospital, and deCODE Genetics, Inc., Laboratories, Reykjavik; [5]Academic Department of Obstetrics and Gynaecology, University Hospital, Vrije Universiteit, Amsterdam; and [6]Department of Obstetrics and Gynaecology, Chelsea and Westminster Hospital, London

References

Arngrímsson R, Hayward C, Nadaud S, Baldursdóttir A, Walker JJ, Liston WA, Bjarnadóttir RI, et al (1997) Evidence for a familial pregnancy-induced hypertension locus in the eNOS-gene region. Am J Hum Genet 61:354–362

Davis S, Weeks DE (1997) Comparison of nonparametric statistics for detection of linkage in nuclear families, single-marker evaluation. Am J Hum Genet 61:1431–1444

Harrison GA, Humphrey KE, Jones N, Badenhop R, Guo G, Elakis G, Kaye JA, et al (1997) A genomewide linkage study of preeclampsia/eclampsia reveals evidence for a candidate region on 4q. Am J Hum Genet 60:1158–1167

Holmans P (1993) Asymptotic properties of affected-sib-pair linkage analysis. Am J Hum Genet 52:362–374

Holmans P, Clayton D (1995) Efficiency of typing unaffected relatives in an affected-sib-pair linkage study with single-locus and multiple tightly linked markers. Am J Hum Genet 57:1221–1232

Kidd KK, Ott J (1984) Power and sample size in linkage studies. Hum Gene Mapping 7:510–511

Knock GA, Poston L (1996) Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. Am J Obstet Gynecol 175:1668–1674

Molnar M, Hertelendy F (1992) N-nitro-L-arginine, an inhibitor of nitric oxide synthesis, increases blood pressure in rats and reverses the pregnancy-induced refractoriness to vaspressor agents. Am J Obstet Gynecol 166:1560–1567

Morris N, Eaton BM, Dekker G (1996) Nitric oxide, the endothelium, pregnancy and pre-eclampsia. Br J Obstet Gynaecol 103:4–15

Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F (1994) Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. Biochem Biophys Res Commun 198:1027–1033

Ott J (1989) Computer-simulation methods in human linkage analysis. Proc Natl Acad Sci USA 86:4175–4178

Redman CW, Jefferies M (1988) Revised definition of preeclampsia. Lancet 1:809–812

Risch N (1990) Linkage strategies for genetically complex traits. II. The power of affected relative pairs. Am J Hum Genet 46:229–241

Weeks DE, Ott J, Lathrop GM (1991) SLINK: a general simulation program for linkage analysis. Am J Hum Genet Suppl 47:A204

Address for correspondence and reprints: Dr. Kevin M. O'Shaughnessy, Clinical Pharmacology Unit, Addenbrooke's Clinical Research Centre, Box 110, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom. E-mail: kmo22@medschl.cam.ac.uk

0002-9297/99/6401-0042$02.00

Am. J. Hum. Genet. 64:313–318, 1999

Exclusion of Chromosome 7 for Kartagener Syndrome but Suggestion of Linkage in Families with Other Forms of Primary Ciliary Dyskinesia

To the Editor:

We read with great interest the recent letter by Pan et al. (1998), in which they report a case of uniparental disomy, of chromosome 7, associated with cystic fibrosis (CF), complete situs inversus, and immotile (although ultrastructurally normal) bronchial ciliary apparatus.

Those authors appropriately suggest that linkage studies be conducted in families with Kartagener syndrome (KS), to evaluate chromosome 7 as a candidate location for the gene underlying this disorder.

KS (MIM 244400) is recognized on the basis of a classic triad of symptoms: situs inversus (complete mirror-image reversal of left-right asymmetry of the chest and abdominal organs [MIM 270100]), bronchiectasis, and chronic sinusitis (Afzelius 1976; Schidlow 1994; Afzelius and Mossberg 1995). In families with a KS proband, approximately half of the proband's affected siblings display the full triad of symptoms, whereas the other affected sibs exhibit only bronchiectasis and chronic sinusitis but have normal left-right organ asymmetry. KS is clinically considered a subgroup of primary ciliary dyskinesia (PCD), formerly known as "immotile cilia syndrome" (ICS [MIM 242650]). However, it is unclear whether KS has the same genetic etiology as PCD without situs inversus. These disorders are characterized by dysmotility or immotility of the cilia in airway epithelial cells, spermatozoa, and other ciliated cells of the body. Clinical consequences of PCD cover a wide spectrum of symptoms mainly involving both lower and upper airways and the male reproductive system. Ciliary immotility is caused by various ultrastructural defects of cilia, with major or subtle anomalies detectable, by electron microscopy, in all or nearly all patients (Teknos et al. 1997). The structural defects are predominantly a total or partial absence of dynein arms (70%–80% of cases), defects of radial spokes, nexin links, and general axonemal disorganization with microtubular transposition (Afzelius and Mossberg 1995).

Estimates of the incidence of PCD are in the range 1/16,000–1/60,000 live births, with KS accounting for approximately half (1/32,000–1/120,000 live births) of these (Afzelius and Mossberg 1995). Inheritance in most cases is autosomal recessive, although some examples of dominant or X-linked modes of inheritance have been reported (Narayan et al. 1994*b*). Nearly 200 different polypeptides have been identified within the ciliary axoneme of lower organisms; at least the same number of proteins can be expected in axonemes of humans (Luck et al. 1982). Mutations within many of these 200 genes coding for ciliary proteins might cause the same or similar pathologic consequences of ciliary dysfunction. However, as noted in OMIM (MIM 242650), if this were true, then we might expect that the incidence of PCD would be much higher than that which actually occurs. It is possible that mutations in many of these genes might be lethal—and thus not be found among viable offspring. Alternatively, there may be functional redundancy of some proteins, such that loss of one gene's product may be compensated by other proteins and thus occur without ciliary dysfunction.

Support for PCD genes potentially located on chromosome 7 is provided by several observations. First, 7q33-q34 is syntenic to a fragment of murine chromosome containing the *hop* mutation (previously named "*hpy*")—mice homozygous for this mutation have a dynein defect in cilia and flagella that is similar to that seen in some cases of PCD (i.e., dynein arms are missing from A-tubules of the outer doublets) (Handel 1985). Second, the gene for the β heavy chain of the outer dynein arm maps to 7p15 region (Kastury et al. 1997), and additional genes containing sequences highly homologous to the dynein-gene family map to 7q21-q22

Table 1

LOD Scores for 23 KS Families

Marker	Map Position (cM)	Summed LOD Score under Homogeneity, for θ = .00001	.01000	.05000	.10000	Maximum LOD Score for Heterogeneity	Alpha at Maximum LOD Score
D7S531	5.28	−12.768	−7.357	−3.647	−2.121	−.01141	.05
D7S517	7.44	−12.722	−7.056	−3.357	−1.885	−.01106	.05
D7S513	17.74	−13.868	−8.068	−3.937	−2.262	−.01309	.05
D7S507	28.74	−8.319	−4.576	−2.133	−1.161	−.00457	.05
D7S493	34.69	−8.053	−4.332	−1.963	−1.056	−.00425	.05
D7S629	37.51	−9.616	−5.046	−2.114	−1.032	.07877	.20
D7S484	53.50	-9.594	−5.027	−1.993	−.850	.06800	1.00
D7S519	69.03	−5.742	−3.035	−1.093	−.331	.15600	1.00
D7S502	78.65	−9.215	−5.308	−2.410	−1.262	−.00372	.05
D7S669	90.42	−4.445	−1.861	−.238	.250	.38000	1.00
D7S657	104.86	−11.184	−6.100	−2.916	−1.630	−.00768	.05
D7S527	108.59	−6.591	−3.671	−1.763	−.989	−.00445	.05
D7S486	124.08	−1.096	−.191	.323	.428	.51927	.55
D7S530	134.55	−7.525	−3.492	−1.162	−.374	.28454	.30
D7S640	137.83	−6.547	−2.833	−.645	.031	.31760	.60
D7S684	147.22	−3.048	−1.115	−.041	.249	.28700	1.00
D7S550	178.41	−6.711	−3.420	−1.198	−.437	.04600	1.00

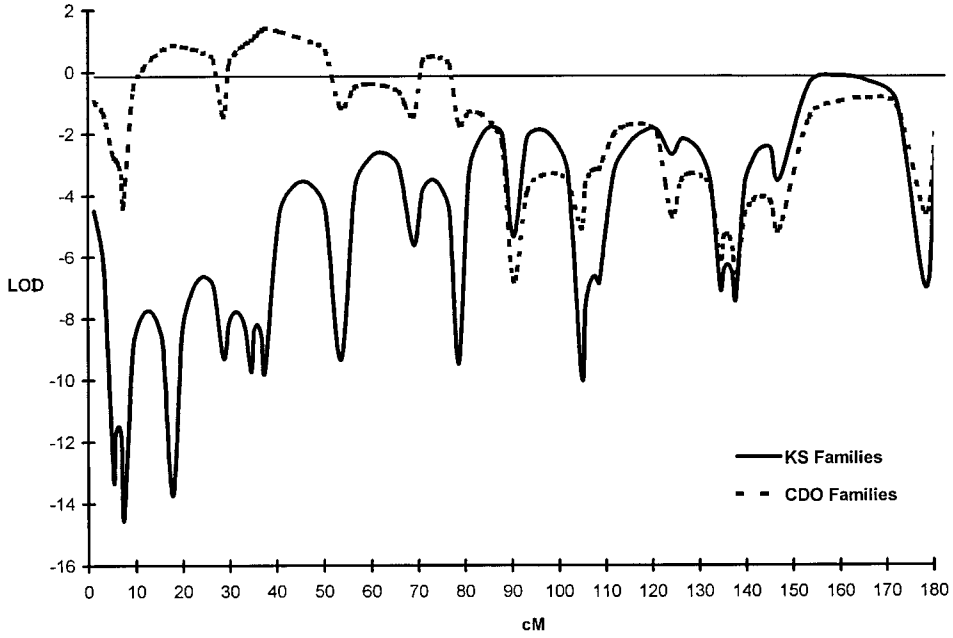

Figure 1 Multipoint LOD scores for KS families and CDO families, for chromosome 7, under the assumptions of recessive inheritance and locus homogeneity.

(GenBank accession number AC002452) and to 7p21 (GenBank accession number AC004002). Third, there is the case of chromosome 7 uniparental disomy and other chromosomal anomalies with KS-like symptoms, summarized in the letter by Pan et al. (1998). PCD candidates on other chromosomes include the following: (1) the HLA region of chromosome 6p, containing the β-tubulin gene (TUBB) (Volz et al. 1994), although limited data reported elsewhere (Gasparini et al. 1994) did not support the motylin gene (MLN), also residing in this region, as being a candidate for involvement in PCD etiology; (2) chromosome 14q32, containing the gene for echinoderm microtubule–associated protein (EMAP), a candidate for Usher syndrome type 1A (the USH1A gene) (these patients exhibit, in the axonemes of their respiratory cilia ultrastructural defects similar to those in PCD) (Bonneau et al. 1993; Eudy et al. 1997); (3) the dynein heavy-chain gene located on 14qter (Narayan et al. 1994*a*); and (4) numerous other dynein, nexin, and other microtubule-related genes rapidly accumulating in the genomics databases. Many genes have been implicated recently in the control of the left-right asymmetry of body development such as that observed in KS (Overbeek 1997; Srivastava 1997; Wood 1997; Levin and Mercola 1998). However, with the exception of the dynein defect associated with the iv mouse mutant (Supp et al. 1997), homologous to the human heavy-chain dynein gene located on chromosome 14 qter, none of these are associated with ciliary dysfunction.

We performed linkage analyses using microsatellite markers spanning chromosome 7 in 30 PCD families recruited in Poland. Each family had at least one member diagnosed with PCD, and no other anomalies or dysmorphologies were present. For linkage analyses, families were further classified either as KS families, if at least one affected member was diagnosed as having KS (i.e., as exhibiting situs inversus), or as ciliary dysfunction only (CDO) families, if none of the affected members had situs inversus. Our sample comprised 23 KS families with 25 KS-affected individuals and 7 CDO-affected individuals and 7 CDO families with 9 CDO-affected individuals. Data from all of these families were consistent with autosomal recessive transmission (i.e., there were no nonsibling affected relatives). Among the KS families, there were four pairs of affected siblings and two trios of affected siblings; among the CDO families, there were two pairs of affected siblings. Two additional KS families were ascertained as having the disease in multiple generations, consistent with a dominant mode of inheritance, but, to date, we have been unable to recruit a sufficient number of members to make these families informative for linkage analysis. We chose to analyze the KS and CDO families separately because of the possibility that different molecular (hereditary) pathologies might underlie these forms of PCD; for example, the *hop* mouse mutation exhibits CDO (without situs inversus).

Seventeen microsatellite markers spanning chromo-

Table 2

LOD Scores for Six CDO Families

Marker	Map Position (cM)	Summed LOD Score under Homogeneity, for θ = .00001	.01000	.05000	.10000	Maximum LOD Score for Heterogeneity	Alpha at Maximum LOD Score
D7S531	5.28	−1.942	−1.008	−.379	−.139	.03100	1.00
D7S517	7.44	−4.170	−2.188	−.934	−.450	−.00035	.05
D7S513	17.74	.833	.812	.723	.607	.83300	1.00
D7S507	28.74	−1.760	−.780	−.192	.005	.09500	1.00
D7S493	34.69	.893	.876	.801	.695	.89200	1.00
D7S629	37.51	.762	.728	.598	.455	.76200	1.00
D7S484	53.50	−1.398	−.496	.021	.152	.15800	1.00
D7S519	69.03	−2.117	−1.119	−.470	−.213	.01030	.85
D7S502	78.65	−1.750	−.745	−.092	.138	.21900	1.00
D7S669	90.42	−6.693	−3.680	−1.790	−1.029	−.00567	.05
D7S657	104.86	−4.870	−2.738	−1.429	−.875	−.00680	.05
D7S527	108.59	−2.540	−1.526	−.819	−.496	−.00308	.05
D7S486	124.08	−4.700	−2.453	−1.149	−.627	−.00311	.05
D7S530	134.55	−4.470	−2.479	−1.192	−.666	−.00335	.05
D7S640	137.83	−7.445	−4.200	−2.217	−1.368	−.01008	.05
D7S684	147.22	−4.761	−2.748	−1.394	−.812	−.00497	.05
D7S550	178.41	−4.643	−2.404	−1.124	−.619	−.00333	.05

some 7, with average interval of 10.8 cM, were analyzed by fluorescence-based, semiautomated DNA-sizing technology (Ziegle et al. 1992) using Applied Biosystems 373 Automated DNA Sequencers and GENESCAN and GENOTYPER software (Applied Biosystems/Perkin-Elmer). Pairwise LOD-score analyses were performed by means of the FASTLINK program (Schäffer 1996). LOD scores allowing for locus heterogeneity (Ott 1991) were calculated by means of a program developed for this purpose (S. R. Diehl, unpublished data). Our unpublished program performs the same simple admixture calculation as is performed by publicly available programs such as HOMOG (and provides identical results in numerous benchmark comparisons), and it is used for data-formatting convenience only. A copy of our program is available on request from the corresponding author. Multipoint LOD scores for all of chromosome 7 were calculated by means of the GENEHUNTER program (Kruglyak et al. 1996) and the sex-average map distances reported by the Marshfield Medical Research Foundation. For all LOD-score analyses, we assumed a recessive mode of inheritance, 50% penetrance for homozygous-mutant genotypes, 0.000013% penetrance for wild-type and heterozygous genotypes (i.e., PCD phenocopies), and PCD disease-allele frequency of .00514. These assumptions yield a population prevalence consistent with that reported for PCD.

Pairwise and multipoint LOD scores for the KS families are shown in table 1 and figure 1, respectively. The last column in the table ("Alpha at Maximum LOD Score") refers to the estimated proportion of families in which there is linkage to the marker under locus heterogeneity (i.e., the maximum LOD score obtained by varying both the recombination fraction and the proportion of families linked). Pairwise LOD scores under locus homogeneity for 17 microsatellite markers of chromosome 7 are all negative and range between −1.096 and −13.868, at a recombination fraction (θ) of .00001, providing no support for linkage. Even if we allow for locus heterogeneity within the KS families (i.e., some KS families have linkage to a gene on chromosome 7, whereas others do not), the maximum pairwise LOD score obtained for any of the 17 markers is only 0.52. Multipoint LOD scores under the assumption of homogeneity exclude (at LOD < -2.0) a KS-susceptibility locus from most of chromosome 7 (fig. 1). The only part of the chromosome not formally excluded is the region between the last two markers on the chromosome, where a gap of >31 cM exists, and even this region is not positive but only lacks power for exclusion. If we allow for locus heterogeneity, the highest multipoint LOD score for the entire chromosome is still only 0.27, which could easily be due to chance. By contrast, pairwise and multipoint LOD scores for the CDO families, shown in table 2 and figure 1, respectively, provide at least a weak suggestion of possible linkage to chromosome 7. We note that, interestingly, the highest multipoint LOD scores for the CDO families, 1.41, occurs at precisely the position on chromosome 7p15 where the gene for the β heavy chain of the outer dynein arm is located (Kastury et al. 1997). The same maximum LOD score is obtained when we allow for locus heterogeneity within the CDO families, since all families provide evidence of linkage to this region (i.e., no evidence of recombination with markers D7S493 or D7S629; see table 2). Analyses of combined KS and CDO families provide no significant evidence of

linkage, with a maximum multipoint LOD score, calculated by the GENEHUNTER program, of only 0.56 for the entire chromosome, occurring at the same location where the highest LOD score (1.41) for the CDO families occurs.

Since our linkage results exclude chromosome 7 as a candidate location for a KS gene, the cytogenetic evidence suggested that this region should be reevaluated. Ciliary structural anomalies are always or almost always found in cases of KS (Teknos et al. 1997); however, despite the absence of normal ciliary motion, no visible ciliary ultrastructural defects were found in the patient with situs inversus and paternal isodisomy of chromosome 7 who was reported by Pan et al. (1998). It is possible that the situs inversus of this patient was caused by a gene solely involved in heterotaxy but without any ciliary ultrastructural anomaly (Overbeek 1997; Srivastava 1997; Wood 1997; Levin and Mercola 1998). Two other studies have reported cytogenetic anomalies of chromosome 7 that are associated with laterality defects (Genuardi et al. 1993; Koiffman et al. 1993). Furthermore, the lack of normal ciliary motion without ciliary structural anomalies, as was observed in Pan et al.'s patient, may represent a secondary effect of cytolysis and cell destruction of bronchial epithelial cells in CF (Cheung and Jahn 1976), although this finding remains controversial (Rutland et al. 1983). Alternative explanations include involvement of genes involving ciliary function (in addition to a gene causing situs inversus). Such a ciliary-function gene might also be located on chromosome 7, or, coincidentally, the patient studied by Pan and colleagues might have a mutation in a gene influencing ciliary function and located elsewhere in the genome. Our suggestive LOD score of 1.41 at the chromosomal location of a dynein gene in the CDO families that we have studied is consistent with the involvement of a ciliary-function gene on chromosome 7, but the ultimate resolution of these issues will require additional data. Although this LOD score is quite small, it may be especially noteworthy in view of the fact that the limited number of CDO families available can produce a maximum LOD score of only 1.49, even under complete linkage and full marker informativeness, as we have determined by means of the SLINK program (Weeks et al. 1990). The KS families, by contrast, can yield LOD scores as high as 3.95, under complete linkage and complete informativeness.

We conclude from our data presented here that the gene(s) responsible for KS is (are) not likely to be located on chromosome 7. Our suggestion of possible linkage for the CDO families should be taken with caution, because of the small size of the sample analyzed; however, especially because the β heavy chain of the outer dynein arm maps to the same location as our positive LOD score of 1.41, we suggest that this region should be considered a high-priority location for follow-up linkage studies in additional CDO families.

Acknowledgments

The authors thank the Polish families participating in this study, for their invaluable cooperation; C. Bock, M. Gregg, D. Smith, and A. Schäffer, for assistance with data management and statistical analyses; D. Freas-Lutz, E. Gillanders, and K. Dziechciowska, for laboratory assistance; A. Miller-Chisholm, for scientific and administrative assistance; and B. Lombard, for editorial assistance. This study was supported by Maria Sklodowska-Curie Fund II grant PAN/NIH 97-310 and by National Institute of Dental and Craniofacial Research, Division of Intramural Research Project Z01 DE-00624.

Michal Witt,[1] Yue-fen Wang,[2] Shengbiao Wang,[2] Cui-e Sun,[2] Jacek Pawlik,[3] Ewa Rutkiewicz,[1] Jerzy Zebrak,[3] and Scott R. Diehl[2]

[1]*Institute of Human Genetics, Poznan, Poland;* [2]*National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda; and* [3]*Institute of Tuberculosis and Lung Diseases, Rabka, Poland*

Electronic-Database Information

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html (for 7q21-q22 [accession number AC002452] and 7p21 [accession number AC004002])

Marshfield Medical Research Foundation (Center for Medical Genetics), http://www.marshmed.org/genetics/ (for sex-average map distances)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for ICS [MIM 242650], KS [MIM 244400], and situs inversus [MIM 270100])

References

Afzelius BA (1976) A human syndrome caused by immotile cilia. Science 193:317–319

Afzelius BA, Mossberg B (1995) Immotile-cilia syndrome (primary ciliary dyskinesia), including Kartagener syndrome. In: Scriver C, Beaudet AL, Sly W, Valle D (eds) The metabolic and molecular bases of inherited diseases, 7th ed. McGraw-Hill, New York, pp 3943–3954

Bonneau D, Raymond F, Kremer C, Klossek JM, Kaplan J, Patte F (1993) Usher syndrome type I associated with bronchiectasis and immotile nasal cilia in two brothers. J Med Genet 30:253–254

Cheung AT, Jahn TL (1976) High speed cinemicrographic studies on rabbit tracheal (ciliated) epithelia: cytolytic effect of cystic fibrosis serum on tracheal epithelial cells. Pediatr Res 10:144–147

Eudy JD, Ma-Edmonds M, Yao SF, Talmadge CB, Kelley PM, Weston MD, Kimberling WJ, et al (1997) Isolation of a novel human homologue of the gene coding for echinoderm mi-

crotubule-associated protein (EMAP) from the Usher syndrome type 1a locus at 14q32. Genomics 43:104–106
Gasparini P, Grifa A, Savasta S, Merlo I, Bisceglia L, Totaro A, Zelante L (1994) The motilin gene: subregional localization, tissue expression, DNA polymorphisms and exclusion as a candidate gene for the HLA-associated immotile cilia syndrome. Hum Genet 94:671–674
Genuardi M, Pomponi M, Sammito V, Bellussi A, Zollino M, Neri G (1993) Split hand/split foot anomaly in a family segregating a balanced translocation with breakpoint on 7q22.1. Am J Med Genet 47:823–831
Handel MA (1985) Allelism of *hop* and *hpy*. Mouse News Lett 72:124
Kastury K, Taylor WE, Gutierrez M, Ramirez L, Coucke PJ, Van Hauwe P, Van Camp G, et al (1997) Chromosomal mapping of two members of the human dynein gene family to chromosome regions 7p15 and 11q13 near the deafness loci DFNA 5 and DFNA 11. Genomics 44:362–364
Koiffmann CP, Wajntal A, de Souza DH, Gonzalez CH, Coates MV (1993) Human situs determination and constitution 46,Xyins (7;8)(q22;q12q24). Am J Med Genet 47:568–569
Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. Am J Hum Genet 58:1347–1363
Levin M, Mercola M (1998) The compulsion of chirality: toward an understanding of left-right asymmetry. Genes Dev 12:763–769
Luck DJ, Huang B, Piperno G (1982) Genetic and biochemical analysis of the eukaryotic flagellum. Symp Soc Exp Biol 35: 399–419
Narayan D, Desai T, Banks A, Patanjali SR, Ravikumar TS, Ward DC (1994*a*) Localization of the human cytoplasmic dynein heavy chain (DNECL) to 14qter by fluorescence in situ hybridization. Genomics 22:660–661
Narayan D, Krishnan SN, Upender M, Ravikumar TS, Mahoney MJ, Dolan TF Jr, Teebi AS, et al (1994*b*) Unusual inheritance of primary ciliary dyskinesia (Kartagener syndrome). J Med Genet 31:493–496
Ott J (1991) Analysis of human genetic linkage, 2d ed. Johns Hopkins University Press, Baltimore
Overbeek PA (1997) Right and left go dHAND and eHAND. Nat Genet 16:119–121
Pan Y, McCaskill CD, Thompson KH, Hicks J, Casey B, Shaffer LG, Craigen WJ (1998) Paternal isodisomy of chromosome 7 associated with complete situs inversus and immotile cilia. Am J Hum Genet 62:1551–1555
Rutland J, Penketh A, Griffin WM, Hodson ME, Batten JC, Cole PJ (1983) Cystic fibrosis serum does not inhibit human ciliary beat frequency. Am Rev Respir Dis 128:1030–1034
Schäffer AA (1996) Faster linkage analysis computations for pedigrees with loops or unused alleles. Hum Hered 46: 226–235
Schidlow DV (1994) Primary ciliary dyskinesia (the immotile cilia syndrome). Ann Allergy 73:457–470
Srivastava D (1997) Left, right…which way to turn? Nat Genet 17:252–254
Supp DM, Witte DP, Potter SS, Brueckner M (1997) Mutation of an axonemal dynein affects left-right asymmetry in inversus viscerum mice. Nature 389:963–966
Teknos TN, Metson R, Chase T, Balercia G, Dickersin GR (1997) New developments in the diagnosis of Kartagener's syndrome. Otolaryngol Head Neck Surg 116:68–74
Volz A, Weiss E, Trowsdale J, Ziegler A (1994) Presence of an expressed β-tubulin gene (TUBB) in the HLA class I region may provide the genetic basis for HLA-linked microtubule dysfunction. J Hum Genet 93:42–46
Weeks DE, Ott J, Lathrop GM (1990) SLINK: a general simulation program for linkage analysis. Am J Hum Genet Suppl 47:A204
Wood WB (1997) Left-right asymmetry in animal development. Annu Rev Cell Dev Biol 13:53–82
Ziegle JS, Su Y, Corcoran KP, Nie L, Mayrand PE, Hoff LB, McBride LJ, et al (1992) Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics 14:1026–1031

Address for correspondence and reprints: Dr. Scott R. Diehl, Molecular Genetic Epidemiology Unit, NIDCR, NIH, 45 Center Drive, Room 4AS-43G, Bethesda, MD 20892-6401. E-mail: scott.diehl@nih.gov

0002-9297/99/6401-0043$02.00

Am. J. Hum. Genet. 64:318–323, 1999

A New Locus for Nonsyndromic Hereditary Hearing Impairment, DFNA17, Maps to Chromosome 22 and Represents a Gene for Cochleosaccular Degeneration

To the Editor:

Over the past several decades, the proportion of the population with hearing impairment attributed to genetic factors has increased as modern medicine has become both more adept at controlling maternal and pediatric infections and better educated about the iatrogenic causes of hearing impairment. At present, as much as one-half of all congenital hearing impairment is considered to have an underlying genetic component (Arnos et al. 1992; Brookhouser 1994; Cohen and Gorlin 1995; Fraser 1995), making hereditary hearing impairment (HHI) one of the most common inherited human deficits.

Cochleosaccular degeneration (CSD) is the most common histopathologic finding in cases of profound congenital HHI. It is estimated to occur in ~70% of cases (Ormerod 1960; Bergstrom 1980; Gulya and Juhlin 1992). CSD was described first by Scheibe in 1892 and is more commonly known as "Scheibe dysplasia." It affects structures that are derived from the pars inferior of the otocyst. Thus, the membranous cochlea and saccule are affected, but the osseous labyrinth, the membranous utricle, and the semicircular canals are normal.

Because there is no clinically available test to diagnose CSD, postmortem histologic examination of the temporal bone is required. The histopathology of CSD is

characterized by a loss of neurosensory hair cells and their supporting cells in the cochleae and sacculae. Cochlear and vestibular nerve atrophy varies and ranges from none to severe. Reissner's membrane and the saccular wall are typically collapsed. The stria vascularis is atrophic with inclusion of abnormal periodic acid-Schiff–positive material. The pathology in the cochlea is typically most severe in the basal turn, with progressive preservation of normal architecture toward the apex. Occasionally, endolymphatic hydrops is present, indicating a disturbance in ionic and osmotic regulation.

Although CSD is relatively common, its molecular pathogenesis remains to be deciphered. Genetic analysis of families with HHI associated with CSD represents a potential route toward identification of genes responsible for intact and functional membranous structures within the cochlea. First, histopathology offers physical evidence of the specific tissues that the disease gene affects. Second, it may provide clues to the functions of the mutant gene. Finally, animals with similar histopathology serve as excellent models for CSD-associated hearing impairment. Previously, no nonsyndromic HHI loci had been associated with CSD, and, with the exception of the DFNA9 locus, there were no nonsyndromic HHI loci that had been both genetically mapped and histologically characterized (Manolis et al. 1996).

Here we present the first reported mapping of a gene responsible for CSD. The family transmitting this mutant gene is a previously described, multigenerational, nonconsanguineous American family with autosomal dominant HHI (Lalwani et al. 1997).

The family studied was identified through the temporal-bone database at the House Ear Institute in Los Angeles. Institutional-review-board approval was obtained for the human-research protocols from the House Ear Institute and the National Institute on Deafness and from Other Communication Disorders at the National Institutes of Health. Eighteen members of the family were enrolled in the study, of whom eight are affected. Extensive medical histories were obtained, and audiological evaluations were performed as described elsewhere (Lalwani et al. 1997). In addition, temporal bones and the brain stem, removed at autopsy from the proband, were analyzed as described elsewhere (Lalwani et al. 1997).

The family has been described in detail previously (Lalwani et al. 1997). In summary, the affected family members exhibit nonsyndromic HHI with an autosomal dominant mode of transmission; there was no pigmentary abnormality in any of the affected individuals. Initially, the hearing impairment would begin at age 10 years and would involve only the high frequencies; by the 3d decade of life, affected family members had moderate to severe deafness. Histologic examination of the proband's temporal bone exhibited classic CSD, with degeneration of the organ of Corti, the saccular epithelium, and the stria vascularis. In addition, there was asymptomatic loss of neurons and gliosis in the inferior olivary nucleus.

Genomic DNA was extracted from whole blood by standard phenol extraction. Samples were quantified by spectrophotometry and were diluted to 25 ng/μl, for amplification by PCR. A 10-cM genome scan was produced with the ABI Prism Linkage Mapping Set, version 1.0 (PE Applied Biosystems), consisting of fluorescently labeled markers detecting microsatellite polymorphisms (Weber and May 1989; Reed et al. 1994). Fine mapping was accomplished with fluorescently labeled MapPairs from Research Genetics.

PCR used 50 ng of genomic DNA in a 10-μl reaction. The final reaction consisted of 1 × PCR Perkin-Elmer buffer; 2 pmol of fluorescently labeled forward primer; 2 pmol of reverse primer; 50 μM each of dCTP, dGTP, dTTP, and dATP; 2.0 mM $MgCl_2$; and 0.25 U of Ampli*Taq* Gold DNA Polymerase (PE Applied Biosystems). Reactions were started, at 95°C for 12 min, to activate the polymerase. Thirty-four cycles of amplification were completed in the following protocol: 94°C for 45 s, 57°C for 45 s, and 72°C for 60 s. Samples were maintained at 72°C for 10 min, for extension. Products were resolved on 4.25% denaturing polyacrylamide gels (6 M urea) and were visualized on a 377 prism (PE Applied Biosystems).

The FASTLINK program package enabled calculation of two-point and multipoint LOD scores over the entire genome (Cottingham et al. 1993; Schäffer et al. 1994). A dominant mode of inheritance with complete penetrance was assumed. A phenocopy rate of 0.1% was assumed, since this is the incidence of congenital hearing impairment in the United States. The phenotype of individuals <10 years old (V:1 and V:3 are 4 and 8 years old, respectively) was assumed to be unknown, since hearing loss begins at this age in this family.

Previous SIMLINK analysis had shown that the family could generate a maximum LOD score of 4.033, with a mean ± SD of 2.872 ± 0.036 (Boehnke 1986; Lalwani et al. 1997). Genomic scanning at 10-cM intervals identified on chromosome 22 a region with a LOD score >3.0 and exclusion of the remainder of the genome; fine mapping of the region by means of eight additional markers in the linked region was performed. A maximum LOD score of 3.98 was obtained at D22S283 (table 1). Haplotypes were then constructed to determine the critical recombination events (fig. 1). The centromeric recombination occurs in individual IV:6, between markers D22S689 and D22S280. The telomeric recombination occurs between markers D22S282 and D22S444 in several individuals (III-4, IV-7, IV-8, and IV-11). These critical crossovers define a linked region spanning a 16.89–22.97-cM interval, which includes D22S280 near

Table 1

Two-Point LOD Scores Calculated across Linkage Region, with Relative Genetic Distances, According to the Marshfield Medical Research Foundation Genetic Map

Marker	LOD Score at θ = 0	.1	.2	Genetic Distance (cM)
D22S420	−∞	.93	.86	4.06
GCT10C10	−∞	1.86	1.57	18.10
D22S315	−∞	1.90	1.47	21.47
D22S689	−∞	2.04	1.76	28.57
D22S280	3.22	2.55	1.84	31.30
D22S281	3.22	2.93	2.39	31.84
D22S691	2.87	2.26	1.59	32.39
D22S685	2.16	1.72	1.26	32.39
D22S683	3.53	2.81	2.03	36.22
D22S277	3.52	2.89	2.21	36.22
D22S283	3.98	3.26	2.49	38.62
D22S426	3.78	3.06	2.28	41.42
D22S692	1.91	1.48	1.02	41.42
IL2RB	3.30	2.94	2.39	42.81
D22S1045	3.33	2.65	1.92	42.81
D22S445	2.06	1.79	1.44	45.82
D22S423	1.14	.88	.63	46.42
D22S282	2.99	2.27	1.50	48.19
D22S444	−∞	−1.44	−.30	51.54
D22S274	−∞	−1.57	−.51	51.54

the centromere and D22S282 near the telomere. This region corresponds to the cytogenetic bands 22q12.2-22q13.3.

Two individuals, V:1 and V:3, who are <10 years old, were classified as unknown and therefore did not contribute to the LOD score. Individual V:1 does not carry the disease haplotype, and her audiogram is completely normal. Her brother, V:3, is 8 years old and currently has a normal audiogram. However, he carries a portion of the disease haplotype. If he does become affected as he ages, the linked region will be defined by flanking markers D22S689 and D22S423, encompassing a 14.52–17.85-cM region. On the other hand, if he remains unaffected, the linked region will be narrowed to 1.77–5.72 cM, flanked by markers D22S445 and D22S444.

Remarkable progress has been made in the identification of genes responsible for nonsyndromic HHI. To date, the locations of 18 autosomal dominant, 20 autosomal recessive, and 8 X-linked hearing-loss genes have been identified (Hereditary Hearing Loss). Here, we report identification of DFNA17, a new locus for autosomal dominant nonsyndromic HHI, on chromosome 22q12.2-q13.3. Typically, autosomal dominant HHI is characterized by postlingual onset of hearing loss, in contrast to the prelingual onset of deafness observed in autosomal recessive cases. DFNA17 is characterized by high-frequency hearing loss that begins at age 10 years, progresses to severe deafness by the 3d decade, and involves all frequencies. This auditory phenotype is also shared by other previously mapped autosomal dominant nonsyndromic loci, including DFNA2, DFNA5, DFNA7, and DFNA9. High-frequency hearing loss that progresses to involve all frequencies is typical of presbycusis, or hearing loss associated with aging. Considered the most common form of hearing impairment, age-associated hearing impairment is thought to have a multifactorial etiology, with heredity being an important contributing factor. Therefore, the gene responsible for DFNA17, as well as other nonsyndromic HHI genes associated with progressive hearing loss, may provide critical insights into an understanding of the molecular pathophysiology of presbycusis.

The genes responsible for hearing impairment, at seven of the autosomal dominant nonsyndromic HHI loci, have been identified during the past 2 years (Lalwani and Castelein 1999). Mutations in an unconventional myosin gene, myosin VIIA, have been demonstrated to be responsible for DFNA11 (Liu et al. 1997). In the same year, mutations in the diaphanous gene were shown to be the pathogenic cause of DFNA1 (Lynch et al. 1997). In the first 6 mo of 1998, mutations in connexin 26, TECTA, and POU4F3 were found to be responsible for DFNA3, DFNA8/12, and DFNA15, respectively (Denoyelle et al. 1998; Vahava et al. 1998; Verhoeven et al. 1998). These genes have a wide variety of functions, including intercellular communication via gap-junction formation by connexin 26, regulation of actin polymerization by diaphanous-gene, transcription regulation by POU4F3, tectorial membrane constitution by TECTA, and, finally, anchoring of the actin cytoskeleton by myosin VIIA. The wide range of functions subserved by the DFNA genes reflects the heterogeneity of genes involved in nonsyndromic deafness (DFN).

Although the pace of the mapping and identification of mutated genes that cause nonsyndromic HHI has been rapid, their biologic role in the determination of cochlear structure and function is largely unknown. The absence of temporal-bone histologic data from families that have been used for mapping studies has hindered our understanding of the effects of the mutant hearing genes. The DFNA17 family was identified by histologic examination of the temporal bone of the proband, unlike most families with HHI, who are identified initially by clinical symptoms. Hearing impairment in the DFNA17 family is associated with CSD, considered to be the most common cause of profound congenital hearing impairment, accounting for 70% of cases with HHI. DFNA17 represents the first nonsyndromic gene for CSD. However, CSD is likely genetically heterogeneous, because a variety of clinical forms of HHI can lead to the common histopathologic manifestation. DFNA9 is the only other DFN locus for which the human temporal-bone histopathology has been reported. Affected individuals in this

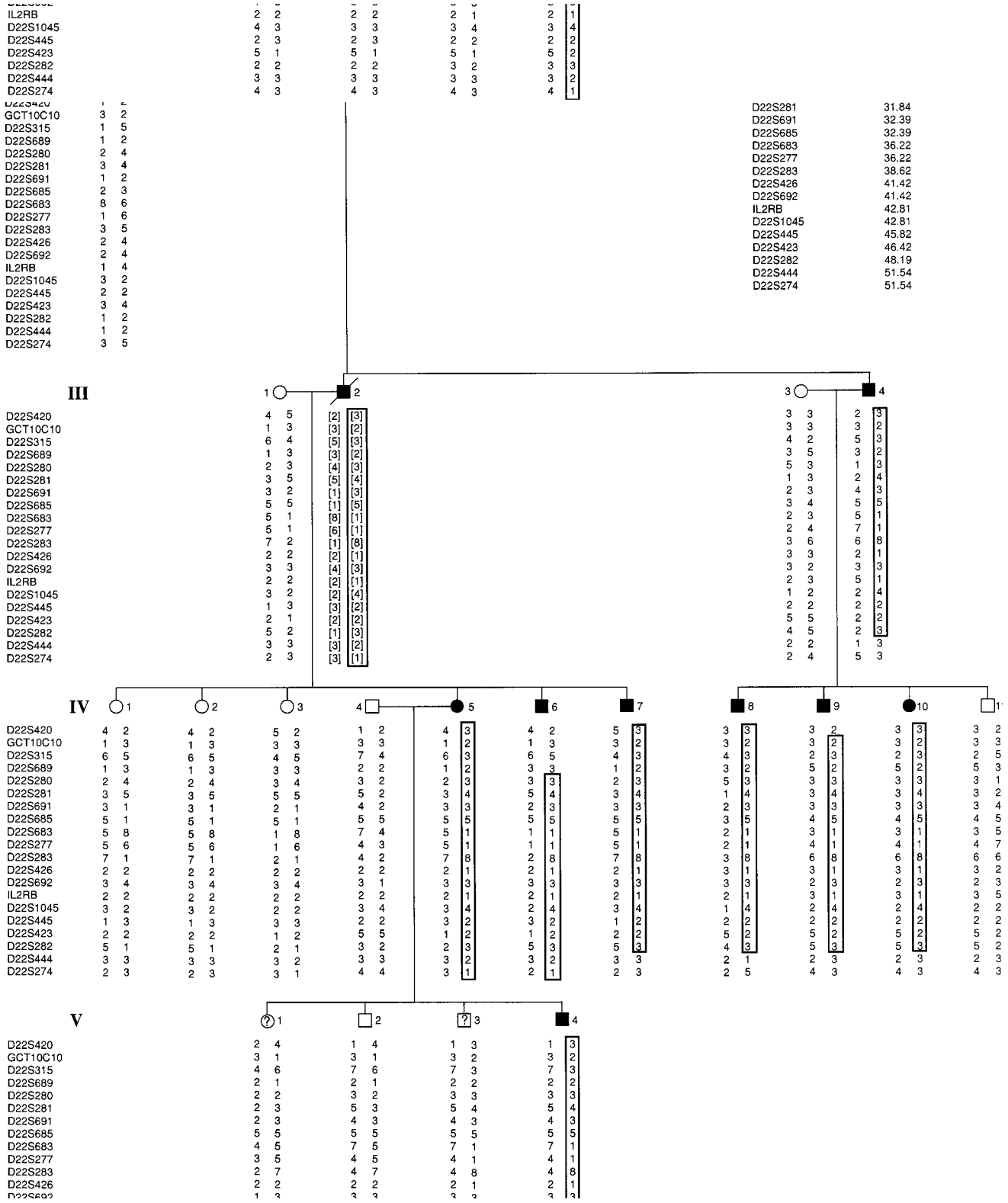

Figure 1 Haplotypes of chromosome 22. Haplotypes for individual III:2, the proband, are inferred from the haplotypes of his children and wife. The disease haplotypes are boxed.

family exhibit mucopolysaccharide depositions in the neural channels of the inner ear (Khetarpal et al. 1991; Khetarpal 1993), and the gene for hearing impairment in this family maps to 14q12-13 (Manolis et al. 1996).

DFNA17 maps to a relatively large genetic region of 16.89–22.97 cM, which is typical for mapping studies that comprise families similar in size to the DFNA17 family. Unfortunately, this region is too large for positional cloning. Alternative approaches to identification of the mutated gene include investigation of cloned genes in the linked region and investigation of mouse models of deafness mapped to syntenic regions. There are many expressed sequence tags and genes that have been mapped to 22q12.2-13.3 and that thus represent potential candidate genes for DFNA17 (Science/The Human Gene Map). The history of the search for hearing-impairment genes has demonstrated that it is difficult to predict a candidate gene on the basis of its known or putative function (e.g., PDS, a putative sulfate-transporter gene, has been found to be associated with hearing impairment). Therefore, it is difficult to select, for mutation analysis, a candidate gene expressed in the DFNA17 region.

A sample of the genes expressed in the linked region includes those for metalloproteinase inhibitor 3 precursors, sodium/glucose cotransporter 1, α-*N*-acetylgalactosaminidase precursor, platelet-derived growth factor, and nonmuscle myosin heavy-chain A (NMMHC-A). Because mutations in two myosin genes are known to cause hearing impairment (Liu et al. 1997; Wang et al. 1998), this class of genes deserves particular attention as potential candidates. Thus the nonmuscle myosin within the linked region represents a strong candidate for DFNA17 (Saez et al. 1990; Simons et al. 1991; Toothaker et al. 1991). Human NMMHC-A is a class II conventional myosin, unlike unconventional myosins VIIA and 15, which have been shown to cause hearing impairment. However, NMMHC-A is not a traditional striated-muscle-cell myosin, since it is expressed in the rat intestine, testis, liver, lung, thymus, kidney, and heart and not in striated muscle (Simons et al. 1991). Recently it has been shown that NMMHC-A is also expressed in the cochlea (authors' unpublished data).

Another approach toward identification of the DFNA17 gene is to use mouse models of deafness that map to the syntenic region in the mouse. Human myosin VIIA and myosin 15 have been identified by initial characterization of the homologous mouse models (Liu et al. 1997; Wang et al. 1998). The mouse syntenic region for DFNA17 includes chromosomes 11 and 15. No mouse deafness models have yet been reported that map to a region syntenic with the human DFNA17. One mouse deafness model—dominant spotting, or kit—displays histology that resembles that of human CSD (Bock and Steel 1983; Steel and Bock 1983), but the gene for this mouse phenotype maps to the homologous region of human chromosome 4. Other animal models for CSD include Dalmatian dogs, Hedlund white mink, and the deaf white cat (Mair 1973; Steel and Bock 1983). However, none of these loci have been mapped, because of the unavailability of genetic markers for these species. Furthermore, unlike the family in the present study, these animal models of CSD are associated with skin-pigment abnormalities due to a lack of melanocytes.

Acknowledgments

We thank the family who made this research possible. We express our deepest appreciation to Drs. Fred Linthicum and Jean Moore, of the House Ear Institute, for their unselfish contributions. Finally, we acknowledge Michael Ng for his efforts in the initial screening of this family. This study was supported in part by National Institute on Deafness and Other Communication Disorders (National Institutes of Health) grants K08 DC 00112 (to A.K.L.) and DC00026 (to E.R.W.); the American Hearing Research Foundation; the National Organization for Hearing Research; the Deafness Research Foundation; Hearing Research, Inc.; and the REAC Jacobsen Fund, School of Medicine, University of California, San Francisco. C.M.C. was supported by grants from the Deafness Research Foundation, the Genentech Foundation, and the School of Medicine, University of California, San Francisco.

Anil K. Lalwani,[1] William M. Luxford,[2] Anand N. Mhatre,[1] Ali Attaie,[1] Edward R. Wilcox,[3] and Caley M. Castelein[1]
[1]*Laboratory of Molecular Otology, Department of Otolaryngology—Head and Neck Surgery, University of California, San Francisco;* [2]*House Ear Clinic, Los Angeles; and* [3]*Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders, Bethesda*

Electronic-Database Information

URLs for data in this article are as follows:

Hereditary Hearing Loss, http://dnalab-www.uia.ac.be/dnalab/hhh
Marshfield Medical Research Foundation, http://www.marshmed.org/genetics (for genetic distances)
Science/The Human Gene Map, http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/chr?22

References

Arnos KS, Israel J, Devlin L, Wilson MP (1992) Genetic counseling for the deaf. Otolaryngol Clin North Am 25:953–971
Bergstrom L (1980) Pathology of congenital deafness: present status and future priorities. Ann Otol Rhinol Laryngol Suppl 89:31–42
Bock GR, Steel KP (1983) Inner ear pathology in the deafness mutant mouse. Acta Otolaryngol (Stockh) 96:39–47

Boehnke M (1986) Estimating the power of a proposed linkage study: a practical computer simulation approach. Am J Hum Genet 39:513–527
Brookhouser PJ (1994) Hereditary hearing loss. In: Gates GA (ed) Current therapy in otolaryngology—head and neck surgery, 5th ed. Mosby, St. Louis, pp 44–52
Cohen MM, Gorlin RJ (1995) Epidemiology, etiology and genetic patterns. In: Gorlin RJ, Toriello HV, Cohen MM (eds) Hereditary hearing loss and its syndromes. Oxford University Press, New York, pp 9–21
Cottingham RW Jr, Idury RM, Schäffer AA (1993) Faster sequential genetic linkage computations. Am J Hum Genet 53: 252–263
Denoyelle F, Lina-Granade G, Plauchu H, Bruzzone R, Chaib H, Levi-Acobas F, Weil D, et al (1998) Connexin 26 gene linked to a dominant deafness. Nature 393:319–320
Fraser GR (1995) The causes of profound deafness in childhood: a study of 3,535 individuals with severe hearing loss present at birth or of childhood onset. Johns Hopkins University Press, Baltimore
Gulya AJ, Juhlin NR (1992) Histopathology of deafness. Ear Nose Throat J 71:494, 499–502
Khetarpal U (1993) Autosomal dominant sensorineural hearing loss: further temporal bone findings. Arch Otolaryngol Head Neck Surg 119:106–108
Khetarpal U, Schuknecht HF, Gacek RR, Holmes LB (1991) Autosomal dominant sensorineural hearing loss: pedigrees, audiologic findings, and temporal bone findings in two kindreds. Arch Otolaryngol Head Neck Surg 117:1032–1042
Lalwani AK, Castelein CM (1999) Inherited hearing loss: cracking the auditory genetic code, part I: nonsyndromic. Am J Otol 20:115–132
Lalwani AK, Linthicum FH, Wilcox ER, Moore JK, Walters FC, San Agustin TB, Mislinski J, et al (1997) A five-generation family with late-onset progressive hereditary hearing impairment due to cochleosaccular degeneration. Audiol Neurootol 2:139–154
Liu XZ, Walsh J, Tamagawa Y, Kitamura K, Nishizawa M, Steel KP, Brown SD (1997) Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene. Nat Genet 17:268–269
Lynch ED, Lee MK, Morrow JE, Welsch PL, Leaon PE, King MC (1997) Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the *Drosophila* gene diaphanous. Science 278:1315–1318
Mair IW (1973) Hereditary deafness in the white cat. Acta Otolaryngol Suppl (Stockh) 314:1–48
Manolis EN, Yandavi N, Nadol JB Jr, Eavey RD, McKenna M, Rosenbaum S, Khetarpal U, et al (1996) A gene for nonsyndromic autosomal dominant progressive postlingual sensorineural hearing loss maps to chromosome 14q12-13. Hum Mol Genet 5:1047–1050
Ormerod FC (1960) The pathology of congenital deafness. J Laryngol Otol 74:919–950
Reed PW, Davies JL, Copeman JB, Bennett ST, Palmer SM, Pritchard LE, Gough SC, et al (1994) Chromosome-specific microsatellite sets for fluorescence-based, semi-automated genome mapping. Nat Genet 7:390–395
Saez CG, Myers JC, Shows TB, Leinwand LA (1990) Human nonmuscle myosin heavy chain mRNA: generation of diversity through alternative polyadenylylation. Proc Natl Acad Sci USA 87:1164–1168
Schäffer AA, Gupta SK, Shriram K, Cottingham RW Jr (1994) Avoiding recomputation in linkage analysis. Hum Hered 44: 225–237
Simons M, Wang M, McBride OW, Kawamoto S, Yamakawa K, Gdula D, Adelstein RS, et al (1991) Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes. Circ Res 69:530–539
Steel KP, Bock GR (1983) Hereditary inner-ear abnormalities in animals: relationships with human abnormalities. Arch Otolaryngol 109:22–29
Toothaker LE, Gonzalez DA, Tung N, Lemons RS, Le Beau MM, Arnaout MA, Clayton LK, et al (1991) Cellular myosin heavy chain in human leukocytes: isolation of *5'* cDNA clones, characterization of the protein, chromosomal localization, and upregulation during myeloid differentiation. Blood 78:1826–1833
Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, et al (1998) Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science 279:1950–1954
Verhoeven K, Van Laer L, Kirschhofer K, Legan PK, Hughes DC, Schatteman I, Verstreken M, et al (1998) Mutations in the human α-tectorin gene cause autosomal dominant nonsyndromic hearing impairment. Nat Genet 19:60–62
Wang A, Liang Y, Fridell RA, Probst FJ, Wilcox ER, Touchman JW, Morton CC, et al (1998) Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. Science 280:1447–1451
Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44:388–396

Address for correspondence and reprints: Dr. Anil K. Lalwani, Department of Otolaryngology—Head and Neck Surgery, University of California, San Francisco, 533 Parnassus Avenue, Room U490A, San Francisco, CA 94143-0526. E-mail: lalwani@itsa.ucsf.edu

0002-9297/99/6401-0044$02.00

Am. J. Hum. Genet. 64:323–326, 1999

Two Novel Single–Base-Pair Substitutions Adjacent to the CAG Repeat in the Huntington Disease Gene (IT15): Implications for Diagnostic Testing

To the Editor:

The CAG-expansion mutation that causes Huntington disease (HD) was first identified in 1993 (Huntington's Disease Collaborative Research Group 1993). The standard PCR assay used by clinical laboratories to determine repeat length amplifies only the CAG repeat (Andrew et al. 1994; The ACMG/ASHG Huntington Disease Genetic Testing Working Group 1998). The ad-

jacent CCG repeat varies in length by 7–12 triplets (Andrew et al. 1994), and the CCT repeat following the CCG repeat can be either two (common) or three (rare) triplets in length (Pecheux et al. 1995). A PCR assay that amplifies across all three repeats (referred to as the "CAG+CCG assay"), taking advantage of the common CCG repeat–length polymorphism, remains valuable for the detection of a second allele in cases in which only a single allele is detected by the CAG-only method (Goldberg et al. 1993; The ACMG/ASHG Huntington Disease Genetic Testing Working Group 1998). By use of a third assay, which determines the combined length of the CCG and CCT repeats (referred to as the "CCG-only assay"; Andrew et al. 1994), CAG-repeat length can be calculated. Previously, an apparently rare mutation was identified, in which the CAA triplet immediately following the CAG repeat is absent, leading to failure of the standard PCR assay for repeat length (Gellera et al. 1996). We now report two additional single-base substitutions that can lead to assay failure or errors in the calculation of CAG-repeat length.

In the first case, a 51-year-old man with a 14-year history of a progressive syndrome typical of HD was referred for testing for the HD expansion mutation. His father had died, at age 56 years, of a myocardial infarction, and an extensive review of the pedigree revealed no affected relatives. After informed consent was obtained, DNA was extracted from blood (Gentra). The CAG-only assay (fig. 1*A*) yielded a single peak, indicating a CAG-repeat length of 19 triplets. The CCG-only assay (fig. 1*A*) generated two peaks, indicating the presence of alleles containing 7 and 10 CCG triplets. The CAG+CCG assay yielded a single peak consistent with a CAG-repeat length of 19 or 20 triplets. A new *5′* primer was synthesized that was identical to HD1, except for the absence of the 3′ terminal C. By use of this primer and primer HD2, the normal length CAG repeat of 20 triplets and an expanded repeat of 41 triplets were detected.

To establish the reason for the failure of the original HD1 primer to amplify the expanded repeat, genomic DNA was reamplified by use of primers HD7-*5′* (*5′*-GGACGGCCGCTAGGTTC-3′) and HD7-3′ (*5′*-CGGCTGAGGAAGCTGAGGAGG-3′) and a PCR protocol similar to the original CAG-only assay. PCR products were cloned into pCRII (Invitrogen), and sequence was obtained from three independent clones containing the expanded allele. Each clone had an expanded CAG repeat of 41 triplets, as predicted by the assay with the shortened HD1 primer, that was adjacent to a CCG repeat of 7 triplets. The sequence also revealed the presence of a C→G substitution of the base immediately preceding the CAG repeat (fig. 1*B*).

In the second case, an unaffected spouse of a patient with HD was tested for HD repeat lengths, after informed consent was obtained, as part of a presymptomatic testing protocol for her child. The CAG repeats determined by the CAG-only assay were 17 and 28 triplets in length. The CCG-only assay yielded a single peak, suggesting two CCG alleles of seven triplets each. The CAG+CCG assay indicated the presence of an allele of (CAG)17, as expected, and a second allele of (CAG)30, two triplets longer than was predicted by the other assays. To account for this discrepancy, genomic DNA was amplified, and the products were cloned into pCRII, as described above. Interpretable sequences were obtained from 11 clones. Eight clones contained a normal allele with, as expected, 7 CCG triplets and either 16 (three clones) or 17 (five clones) CAG triplets. Three clones contained a second allele with either 26 (one clone) or 27 (two clones) CAG triplets. In all three of these clones, the CCG repeat consisted of 12 consecutive CCG triplets without the CCA triplet that normally precedes the CCG repeat (fig. 1*B*).

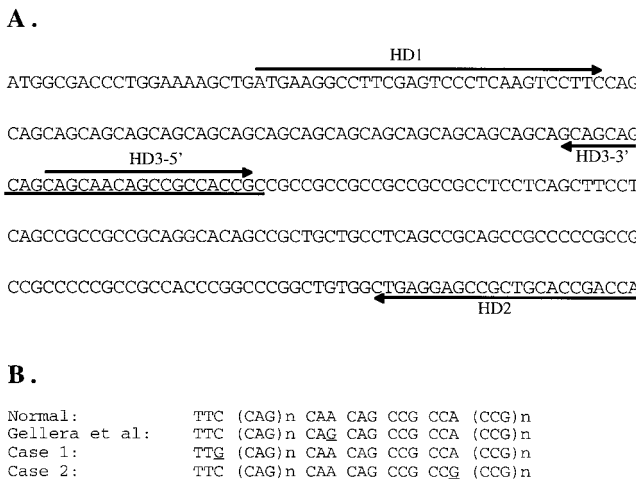

Figure 1 *A,* Repeat region of the HD gene (GenBank accession number L12392, base pairs 316–585) and primers used for amplification of the CAG repeat (HD1 and HD3-3′), the CCG repeat (HD3-5′ and HD2), and the combined CAG/CCG repeat (HD1 and HD2). For the CAG-only protocol, 500 ng genomic DNA was incubated at 99°C for 3 min; 400 nM each of primer HD1 and fluorescently tagged primer HD3-3′, 2.5 U *Taq* polymerase, and buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCL, 200 μM each dNTP, 1.5 mM $MgCl_2$, and 8× MasterAmp PCR enhancer (Epicenter Technologies) were added, followed by denaturation at 95°C for 5 min; 33 cycles of 95°C for 45 s, 67°C for 45 s, and 72°C for 1 min; and an extension at 72°C for 7 min. The CCG-only protocol was as described above, except that annealing was at 60°C and the buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, .01% gelatin, 10% dimethyl sulfoxide, 1.5 U *Taq* polymerase, 100 μM dGTP, 100 μM deaza-dGTP, and 200 μM each of dATP, dTTP, and dCTP. The CAG+CCG protocol was as described elsewhere (Huntington's Disease Collaborative Research Group 1993; Stine et al. 1993), modified for automated fluorescent analysis. All assays are ±1 triplet. *B,* Three substitutions identified in the repeat region of the HD gene.

The sequence of the regions adjacent to the CAG repeat provides an explanation for the PCR results in these two cases. In the first case, the C→G substitution falls precisely at the 3′ terminal base of the HD1 PCR primer, apparently preventing efficient annealing of this primer and, hence, synthesis of a product. The substitution results in a change from phenylalanine to leucine in the encoded protein. The clinical phenotype of the first case is typical of HD, and this substitution of one neutral hydrophobic amino acid for another possibly has no consequences on phenotype. Among the 1,236 subjects that we have tested for HD repeat length, this is the only case for which the HD1 primer has failed consistently, suggesting that this C→G substitution is a rare mutation.

The A→G polymorphism in the second case is silent, because both codons encode proline. The absence of the CCA codon in the second case presumably led to the misannealing of primer HD3-5′, which caused the false finding of a CCG repeat of seven triplets. In 26 other subjects tested with all three assays (CAG-only, CCG-only, and CAG+CCG), we did not detect a repeat-length discrepancy of two triplets, suggesting that absence of the CCA repeat is a relatively uncommon variant. The actual (CCG)12 repeat probably indicates the common (CCG)10 variant coupled with an A→G substitution that converts the adjacent CCACCG sequence into CCG-CCG. However, other combinations of deletions and insertions also could have resulted in this change. The variation in length among the cloned PCR products of the HD region of the second case may reflect either repeat-length instability during plasmid replication in bacteria or somatic variation of the repeat in leukocytes from the subject.

These two variants have implications for the determination of the repeat length in the HD gene. The first case demonstrates that test results indicative of CAG repeat–length homozygosity may be incorrect, particularly if the standard primer, HD1, is used. For cases in which both the CAG-only and the CAG+CCG assays detect a single repeat, a reasonable next step would be to repeat the assays, with the HD1-short primer. If this assay or other PCR assays using alternative primers fail to reveal a second allele, then a search, by Southern blot analysis, for an expanded allele would be prudent. Similarly, the second case suggests the use of an alternative primer in those cases for which CCG-repeat length is important but for which the standard assays of repeat length yield discrepant results. More broadly, these cases illustrate the pitfalls inherent in PCR-based assays of genetic mutations.

Acknowledgments

The authors thank the patients and families of the Baltimore Huntington Disease Project, for their participation in this and other studies. This work was supported by National Institutes of Neurological Disorders and Strokes grant 16375.

Russell L. Margolis, O. Colin Stine,* Colleen Callahan, Adam Rosenblatt, Margaret H. Abbott, Meeia Sherr, and Christopher A. Ross

Division of Neurobiology
Department of Psychiatry
Johns Hopkins University School of Medicine
Baltimore

Electronic-Database Information

Accession number and URL for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/ (HD gene [L12392])

References

American College of Medical Genetics/American Society of Human Genetics (ACMG/ASHG) Huntington Disease Genetic Testing Working Group (1998) Laboratory guidelines for Huntington disease genetic testing. Am J Hum Genet 62:1243–1247

Andrew SE, Goldberg YP, Theilmann J, Zeisler J, Hayden MR (1994) A CCG repeat polymorphism adjacent to the CAG repeat in the Huntington disease gene: implications for diagnostic accuracy and predictive testing. Hum Mol Genet 3:65–67

Gellera C, Meoni C, Castellotti B, Zappacosta B, Girotti F, Taroni F, DiDonato S (1996) Errors in Huntington disease diagnostic test caused by trinucleotide deletion in the *IT15* gene. Am J Hum Genet 59:475–477

Goldberg YP, Andrew SE, Clarke LA, Hayden MR (1993) A PCR method for accurate assessment of trinucleotide repeat expansion in Huntington disease. Hum Mol Genet 2: 635–636

Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983

Pecheux C, Mouret J-F, Durr A, Agid Y, Feingold J, Brice A, Dode C, et al (1995) Sequence analysis of the CCG polymorphic region adjacent to the CAG triplet repeat of the HD gene in normal and HD chromosomes. J Med Genet 32:399–400

Stine OC, Pleasant N, Franz ML, Abbott MH, Folstein SE, Ross CA (1993) Correlation between the onset age of Huntington's disease and length of the trinucleotide repeat in IT-15. Hum Mol Genet 2:1547–1549

Address for correspondence and reprints: Dr. Russell L. Margolis, Department of Psychiatry, Johns Hopkins University School of Medicine, Meyer 2-181, 600 North Wolfe Street, Baltimore, MD 21287. E-mail: rmargoli@jhmi.edu
* Present affiliation: Department of Pediatrics, University of Maryland School of Medicine, Baltimore.

0002-9297/99/6401-0045$02.00

Am. J. Hum. Genet. 64:326–328, 1999

The Interpretation of the Parameters in the Transmission/Disequilibrium Test

To the Editor:

The transmission/disequilibrium test (TDT) proposed by Spielman et al. (1993) is a valid test for linkage in structured populations, irrespective of whether the families are simplex, multiplex, or multigenerational (Spielman and Ewens 1996). Its power to detect linkage of complex traits is potentially greater than that of allele-sharing methods (Risch and Merikangas 1996). The original TDT has been extended by a number of groups—including Sham and Curtis (1995), Schaid (1996), and Spielman and Ewens (1996)—to the study of multiallelic markers. Assuming Hardy-Weinberg equilibrium for the population under study, I present relationships between parameters in the TDT and the disease susceptibility carried by marker alleles. I hope that these relationships make the interpretation of the observed transmission disequilibrium more intuitive and that they yield further insight into the TDT.

Consider a marker H with n alleles, $H_1,\ldots H_n$, having allele frequencies $h_1,\ldots h_n$. Assume that the disease gene D has two alleles, D_1 and D_2, with allele frequencies p_1 and $p_2 = 1 - p_1$, respectively, and that the penetrance for genotype D_uD_v is f_{uv}, where $u, v = 1$ or 2. Furthermore, denote the recombination fraction between H and D as θ and measure the set of linkage-disequilibrium values, between these two loci, in terms of $\delta_{H_iD_u} = P(H_iD_u) - h_ip_u$, where $i = 1, \ldots, n$ and $u = 1$ or 2. First, consider the transmission of a marker allele from *one* parent to the affected offspring. Let $P_{ij} = P$(a parent has genotype H_iH_j and transmits H_i | offspring is affected). Sethuraman (1997) has shown that, if a sample of affected children together with their parents are ascertained at random from a population in Hardy-Weinberg equilibrium, then

$$\begin{aligned} P_{ij} = {} & (p_1f_{11} + p_2f_{12})[h_jP(H_iD_1) \\ & -\theta(h_j\delta_{H_iD_1} - h_i\delta_{H_jD_1})]/K \\ & +(p_1f_{12} + p_2f_{22})[h_jP(H_iD_2) \\ & -\theta(h_j\delta_{H_iD_2} - h_i\delta_{H_jD_2})]/K \ , \end{aligned}$$

$$\begin{aligned} P_{ji} = {} & (p_1f_{11} + p_2f_{12})[h_iP(H_jD_1) \\ & -\theta(h_i\delta_{H_jD_1} - h_j\delta_{H_iD_1})]/K \\ & +(p_1f_{12} + p_2f_{22})[h_iP(H_jD_2) \\ & -\theta(h_i\delta_{H_jD_2} - h_j\delta_{H_iD_2})]/K \ , \end{aligned}$$

where $K = p_1^2f_{11} + 2p_1p_2f_{12} + p_2^2f_{22}$ is the disease prevalence in the population. Sham and Curtis (1995) expressed the P_{ij} in different but equivalent forms. In the following discussion, assume that $\theta = 0$, since, between loci having linkage disequilibrium, θ is generally very close to 0. When $\theta = 0$,

$$\begin{aligned} P_{ij} = {} & h_j[(p_1f_{11} + p_2f_{12})P(H_iD_1) \\ & +(p_1f_{12} + p_2f_{22})P(H_iD_2)]/K \ , \\ P_{ji} = {} & h_i[(p_1f_{11} + p_2f_{12})P(H_jD_1) \\ & +(p_1f_{12} + p_2f_{22})P(H_jD_2)]/K \ . \end{aligned} \qquad (1)$$

Let $P(\text{affected} \mid H_i) = P(\text{an individual is affected} \mid \text{this individual receives allele } H_i \text{ from one parent})$. This conditional probability can be regarded as the genetic risk that allele H_i carries for the disease susceptibility. The value of $P(\text{affected} \mid H_i)$ can be calculated as follows:

$$\begin{aligned} & P(\text{an individual is affected} \mid \text{this individual receives } H_i \text{ from one parent}) \\ = {} & P(\text{an individual receives } H_i \text{ from one parent and is affected})/P(H_i) \\ = {} & \sum_{u=1}^{2}\sum_{v=1}^{2} P(\text{an individual receives } H_iD_u \text{ from one parent, receives } D_v \text{ from the other parent, and is affected})/h_i \\ = {} & \sum_{u=1}^{2}\sum_{v=1}^{2} P(\text{an individual receives } H_iD_u \text{ from one parent and receives } D_v \text{ from the other parent})P(\text{affected} \mid \text{genotype } D_uD_v)/h_i \\ = {} & \sum_{u=1}^{2}\sum_{v=1}^{2} P(H_iD_u)P(D_v)f_{uv}/h_i \ . \end{aligned}$$

The last equation follows from the assumption of Hardy-Weinberg equilibrium. Thus,

$$P(\text{affected}|H_i) = [(p_1f_{11} + p_2f_{12})P(H_iD_1) + (p_1f_{12} + p_2f_{22})P(H_iD_2)]/h_i ,$$
$$P(\text{affected}|H_j) = [(p_1f_{11} + p_2f_{12})P(H_jD_1) + (p_1f_{12} + p_2f_{22})P(H_jD_2)]/h_j . \quad (2)$$

From equations (1) and (2),

$$P_{ij} = P(\text{affected}|H_i)h_ih_j/K ,$$
$$P_{ji} = P(\text{affected}|H_j)h_ih_j/K .$$

Therefore, the following relationship relates P_{ij} to $P(\text{affected} \mid H_i)$:

$$\frac{P_{ij}}{P_{ji}} = \frac{P(\text{affected}|H_i)}{P(\text{affected}|H_j)} . \quad (3)$$

From equation (3), it can be seen that the transmission/disequilibrium ratio P_{ij}/P_{ji} for H_iH_j parents is *independent* of allele frequencies. For n alleles, the $n(n-1)/2$ transmission/disequilibrium ratios P_{ij}/P_{ji} are determined by $n-1$ independent parameters $P(\text{affected}|H_i)$.

Let n_{ij} denote the number of H_iH_j parents who transmit H_i to the affected offspring. Then, conditional on $n_{ij} + n_{ji}$ $(i \neq j)$, n_{ij} follows the binomial distribution

$$B\left[n_{ij} + n_{ji}, \frac{P(\text{affected}|H_i)}{P(\text{affected}|H_i) + P(\text{affected}|H_j)}\right] .$$

This naturally leads to the logistic regression proposed by Sham and Curtis (1995), who did not interpret the parameters as the genetic risks carried by different marker alleles.

Schaid (1996) studied the case when the joint transmission of two parents is considered simultaneously. Let

$P_{ik,jl} = P$(one parent has genotype H_iH_j and transmits H_i and the other parent has genotype H_kH_l and transmits $H_k \mid$ offspring is affected) .

Without the assumption of Hardy-Weinberg equilibrium, it can be shown that

$$\frac{P_{ik,jl}}{P_{jl,ik}} = \frac{P(\text{affected}|H_iH_k)}{P(\text{affected}|H_jH_l)} , \quad (4)$$

where $P(\text{affected} \mid H_iH_k)$ is the conditional probability that a person having genotype H_iH_k is affected—that is, the penetrance for marker genotype H_iH_k (Schaid 1996). Therefore, when two parents are considered jointly, $P_{ik,jl}/P_{jl,ik}$ is determined by the ratio of the penetrances for genotypes H_iH_k and H_jH_l. For n alleles, there are $n(n+1)/2$ possible genotypes; so, a total of $n(n+1)/2 - 1$ parameters are needed to quantify $P_{ik,jl}/P_{jl,ik}$. Schaid (1996) discussed several ways to code the genotypes in the transmission/disequilibrium test.

When each parent is examined separately, the contributions from the two parents are implicitly assumed to be independent. This is true if

$$\frac{P(\text{affected}|H_iH_k)}{P(\text{affected}|H_jH_l)} = \frac{P(\text{affected}|H_i)}{P(\text{affected}|H_j)}\frac{P(\text{affected}|H_k)}{P(\text{affected}|H_l)} ,$$

which holds if and only if $f_{12}^2 = f_{11}f_{22}$ (Knapp et al. 1993).

The relationships in equations (3) and (4) have been used to develop transmission/disequilibrium tests for multiple tightly linked markers (H. Zhao, K. R. Merikangas, and K. K. Kidd, unpublished results). They are also useful in the study of gene-environment interactions. For simplicity, assume that an environmental exposure R—for example, smoking—is classified as being either present $(R = 1)$ or absent $(R = 0)$. Let $P_{ik,jl}^R = P$(one parent has genotype H_iH_j and transmits H_i, and the other parent has genotype H_kH_l and transmits $H_k \mid$ offspring is affected and environmental exposure is R). Denote the penetrance for genotype D_uD_v under environmental exposure R by means of f_{uv}^R. If genotype D_uD_v and environmental exposure R have multiplicative effects on the disease susceptibility—that is, $f_{uv}^1 = \lambda f_{uv}^0$—then it can be shown that

$$\frac{P_{ik,jl}^1}{P_{jl,ik}^1} = \frac{P_{ik,jl}^0}{P_{jl,ik}^0} .$$

Therefore, $P_{ik,jl}^R/P_{jl,ik}^R$ is independent of the environmental exposure variable R when the genotypes and the environmental exposure have multiplicative effects on the disease susceptibility. To test nonmultiplicative gene-environment interactions, standard statistical tests may be performed, to determine whether the transmission/disequilibrium ratios among families having the exposure—that is, $P_{ik,jl}^1/P_{jl,ik}^1$—are the same as the transmission/disequilibrium ratios among families without the exposure—that is, $P_{ik,jl}^0/P_{jl,ik}^0$. However, for nonmultiplicative gene-environmental interactions—for example, additive gene-environment interactions with $f_{uv}^1 = \delta + f_{uv}^0$—there is no simple relationship between $P_{ik,jl}^1/P_{jl,ik}^1$ and $P_{ik,jl}^0/P_{jl,ik}^0$.

Acknowledgments

I thank two referees for their thoughtful and constructive comments. This work was supported in part by National

Institutes of Health grants GM59507, GM57672, and DA09055.

HONGYU ZHAO
Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven

References

Knapp M, Seuchter SA, Baur MP (1993) The haplotype-relative-risk (HRR) method for analysis of association in nuclear families. Am J Hum Genet 52:1085–1093

Risch N, Merikangas K (1996) The future of genetic studies of complex human diseases. Science 273:1516–1517

Schaid DJ (1996) General score tests for associations of genetic markers with disease using cases and their parents. Genet Epidemiol 13:423–449

Sethuraman B (1997) Topics in statistical genetics. PhD diss, University of California at Berkeley, Berkeley

Sham PC, Curtis D (1995) An extended transmission/disequilibrium test (TDT) for multi-allele marker loci. Ann Hum Genet 59:323–336

Spielman RS, Ewens WJ (1996) The TDT and other family-based tests for linkage disequilibrium and association. Am J Hum Genet 59:983–989

Spielman RS, McGinnis RE, Ewens WJ (1993) Transmission test for linkage disequilibrium: the insulin gene and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet 52: 506–516

Address for correspondence and reprints: Dr. Hongyu Zhao, Department of Epidemiology and Public Health, 60 College Street, Yale University School of Medicine, New Haven, CT 06520-8034. E-mail: hongyu.zhao@yale.edu

0002-9297/99/6401-0046$02.00

Am. J. Hum. Genet. 64:328–329, 1999

Cancer Genetics and Insurance

To the Editor:

Rodriguez-Bigas et al. (1998) made a commendable effort to ask 1,000 of a total of 5,178 U.S. health, disability, and life insurance companies about their policies (and conditions) for insuring patients and asymptomatic carriers of the gene for autosomal dominant hereditary nonpolyposis colorectal cancer (HNPCC). The low response rate (7.7%) and the heterogeneity of the insurance companies' attitudes (which ranged from acceptance to rejection of people with these types of risks) do not warrant the authors' optimistic conclusions that "the majority of health, life, and disability insurance providers with an opinion would be willing to sell insurance to both HNPCC gene carriers and at-risk individuals" (Rodriguez-Bigas et al. 1998, p. 737). In The Netherlands (population 15,000,000), the health and life insurance companies expressed the intention to prolong a moratorium on the use of genetic data to control access to life insurance, at the same time that legislative efforts were proposed to reduce the risks of genetic discrimination in access to health insurance and jobs (Committee on Genetic Screening 1994, pp. 86–87). In industrial countries, there is a strong tendency to reduce risk sharing in health insurance and social security systems. This tendency will cause an even greater increase in insurance companies' awareness of risk differentiation based on outcomes of genetic tests (Pokorski 1995; Bodmer 1996).

In view of these nearly global developments, appropriate counseling on the social effects of taking a presymptomatic test for a late-onset genetic disease, such as a cancer syndrome or a neurodegenerative disorder, has become a very delicate matter for clinical geneticists (The Ad Hoc Committee on Genetic Testing/Insurance Issues 1995). The silence of the majority of the insurance companies in the U.S. study by Rodriguez-Bigas et al. (1998) reflects the general neglect of this subject in discussions between the governments of the major economic countries and the regulators of the international insurance and underwriters system.

Families with these genetic risks may become burdened by the unacceptable financial risks of the "wait and see" attitude of the health and life insurance system and the policy makers responsible for these regulations. In Great Britain, life insurers recently started demanding genetic-test results (Wilkie 1998).

Geneticists usually are held responsible for potential adverse socioeconomic effects of genetic testing, by making already foreseeable genetic risks more precise. However, from the onset of presymptomatic testing, society and policy makers have been informed, by the genetics community, of the need to formulate regulations based on fairness and the prevention of genetic discrimination (The Ad Hoc Committee on Genetic Testing/Insurance Issues 1995).

M. F. NIERMEIJER
Department of Clinical Genetics
Erasmus University and University Hospital Dijkzigt
Rotterdam

References

Ad Hoc Committee on Genetic Testing/Insurance Issues, The (1995) Genetic testing and insurance. Am J Hum Genet 56: 327–331

Bodmer W (1996) Genetic testing and insurance. Nature 380: 384–386

Committee on Genetic Screening (1994) Genetic screening. Publ 1994/22 E, Health Council of the Netherlands, The Hague

Pokorski RJ (1995) Genetic information and life insurance. Nature 376:13–14

Rodriguez-Bigas MA, Vasen HFA, O'Malley L, Rosenblatt M-JT, Farrell C, Weber TK, Petrelli NJ (1998) Health, life, and disability insurance and hereditary nonpolyposis colorectal cancer. Am J Hum Genet 62:736–737

Wilkie T (1998) Genetics and insurance in Britain: why more than just the Atlantic divides the English-speaking nations. Nat Genet 20:119–121

Address for correspondence and reprints: Dr. M. F. Niermeijer, Department of Clinical Genetics, Erasmus University and University Hospital Dijkzigt, Westzeedijk 112, 3016 AH Rotterdam, The Netherlands.

0002-9297/99/6401-0047$02.00

Am. J. Hum. Genet. 64:329, 1999

Reply to Niermeijer

To the Editor:

We thank Prof. Niermeijer for his response to and comments on our letter to the editor, regarding health, life, and disability insurance in hereditary nonpolyposis colorectal cancer (HNPCC). As stated in our letter, our survey respondents probably represented <5% of insurance policies sold in the United States. We agree with Prof. Niermeijer that "the silence of the majority of the insurance companies in the U.S. study by Rodriguez-Bigas et al. (1998) reflects the general neglect of this subject in discussions between governments of the major economic countries and the regulators of the insurance and underwriter's system." However, we were encouraged to find, as stated in our earlier letter, that "the majority of health, life, and disability insurance providers with an opinion would be willing to sell insurance to HNPCC gene carriers and at risk-individuals" (Rodriguez-Bigas et al. 1998, p. 737). This apparent dichotomy in our survey reflects the need for legislative bodies, insurance providers, health-care providers (including geneticists and counselors), as well as members of affected kindreds, to enter into dialogues so that further steps can be taken for public and professional education, as well as for prevention of genetic discrimination in our societies.

Similar to what has occurred in the Netherlands, legislative action has been undertaken in the United States, with regard to potential genetic discrimination. The Health Insurance Portability and Accountability Act of 1996, which went into effect on July 1, 1997, includes provisions that deal with genetic discrimination as it relates to the use of genetic information on individuals who have or who are eligible for health insurance under a group health plan offered by an employer. Under this law, genetic information is included in the list of "health status-related factors" that an insurer is prohibited from using to deny, cancel, or refuse to renew insurance coverage. The law also states that genetic information may not be treated as a preexisting condition, without a diagnosis of the condition related to this information. Another provision provides that the health status of an individual or his or her dependents cannot be used to charge premium rates different from those applied to other, similarly situated individuals within the group.

Although this law addresses some potential health insurance–discrimination concerns, it does not apply to individuals ineligible for group health-insurance policies. Furthermore, this law does not restrict the use of individual genetic information for the purpose of setting a particular group premium. These "gaps" in protection demonstrate that, although legislation such as this has been enacted, all issues related to potential health-insurance discrimination regarding individuals affected with and/or at risk for genetic conditions such as HNPCC have yet to be remedied. There is ongoing activity, on both the federal and the state levels, to address these "gaps" and other societal issues associated with genetic status. In the meantime, it is the policy at our institution, as it is in many other centers in the United States and elsewhere, to provide risk assessment and genetic consultation in order to address the exact issues raised by Prof. Niermeijer.

MIGUEL A. RODRIGUEZ-BIGAS,[1], MARY-JO T. ROSENBLATT,[2], AND CAROLYN FARRELL[2]
[1]*Division of Surgical Oncology and* [2]*Clinical Genetic Services, Roswell Park Cancer Institute, Buffalo*

Reference

Rodriguez-Bigas MA, Vasen HFA, O'Malley L, Rosenblatt M-JT, Farrell C, Weber TK, Petrelli NJ (1998) Health, life, and disability insurance and hereditary nonpolyposis colorectal cancer. Am J Hum Genet 62:736–737

Address for correspondence and reprints: Dr. Miguel A. Rodriguez-Bigas, Division of Surgical Oncology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY, 14243. E-mail: mrodriguez@SC3101.med.buffalo.edu

0002-9297/99/6401-0048$02.00