Letters to the Editor

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Genetic Analysis of Families with Parkinson Disease that Carry the Ala53Thr Mutation in the Gene Encoding α-Synuclein

To the Editor:

Linkage of Parkinson disease (PD; MIM 601508) to a highly penetrant genetic locus on chromosome 4q21-q23 (Polymeropoulos et al. 1996) was soon followed by the detection of a missense mutation, in the α-synuclein gene (SNCA; MIM 163890) segregating with the disease (Chen et al. 1995; Polymeropoulos et al. 1997). The mutation was a 209G→A substitution in exon 4 of the gene, resulting in an Ala53Thr mutation in the α-synuclein protein and this change was predicted to revert the whole structure of the protein into beta-pleated sheets, which, in turn, may be involved in the self-aggregation of proteins. This mutation was first identified in a large Italian kindred and three unrelated Greek families, and later it was reported in two more Greek families (Papadimitriou et al. 1999). However, it was absent from several hundred cases of familial PD investigated by groups in the United States (Chan et al. 1998*a*, 1998*b*; Farrer et al. 1998) as well as in Europe (Munoz et al. 1997; Bennett and Nicholl 1998; Vaughan et al. 1998; Zareparsi et al. 1998), indicating that it is indeed a rare cause of PD. A 88G→C nucleotide substitution in exon 3, resulting in an Ala30Pro mutation was subsequently detected in a German patient with autosomal dominant PD (Kruger et al. 1998), giving further support to the hypothesis that α-synuclein could participate in the pathogenesis of the disease. That impaired degradation of abnormal proteins could play a role in PD—and, possibly, in other degenerative disorders—was also suggested by the detection of a mutation in the ubiquitin C-terminal hydrolase-L1 (UCH-L1) in a German family (Leroy et al. 1998).

α-Synuclein is a presynaptic-nerve terminal protein, identified as a precursor protein for the non-β amyloid component of amyloid plaques in Alzheimer Disease (AD) (Ueda et al. 1993; Ueda et al. 1994; Campion et al. 1995; Jensen et al. 1995). The wild type α-synuclein protein is present in the Lewy bodies of familial and sporadic PD patients (Spillantini et al. 1997; Baba et al. 1998).

We present here a molecular-genetic analysis of PD families, with respect to mutations in the α-synuclein gene. Our study was approved by the Ethics Committee of the Medical School of the University of Patras and involved patients with familial PD and as many of their relatives as possible, as well as sporadic PD patients, all voluntary donors of a blood sample. Donors were informed of the content and purpose of the research project and signed an informed-consent form. Ten ml of blood were collected, in presence of EDTA, from each donor. When possible, a second blood sample was drawn from a patient in each family and was used for lymphocyte transformation to provide permanent access to their DNA.

We studied a total of 19 unrelated families, in each of which there were at least two first- or second-degree relatives affected with PD. The three Greek families reported on elsewhere (Polymeropoulos et al. 1997) were included in the study and are analyzed here in an expanded form. Our study involves the recording of pedigrees of at least three successive generations, the recording of available clinical data, and the molecular analysis of the DNA extracted from the blood of patients and their unaffected relatives. Seven of these families had multiple affected members and showed a pattern consistent with autosomal dominant inheritance. The penetrance appeared high, since among family members of age >47 years who were offspring of an affected individual, approximately half were affected (6/13–3/5). All affected individuals had one affected parent, and both males and females transmitted the trait (Mange and Mange 1994). The first clinical data available for the patients of these seven families are given in table 1.

DNA was extracted from peripheral blood and was used for PCR amplification of α-synuclein exon 4, by use of primers 3 and 13 (Polymeropoulos et al. 1997). Exon 3 was amplified by use of forward primer ACTTTGGAGGGTTTCTCATG and reverse primer TGTTATCCTAACCCATCAC. PCRs were prepared in a volume of 100 μl, and 2.5 units of DNA polymerase (GIBCO-BRL) were used. The *Tsp*45I and *Mva*I digestions of PCR products containing exons 4 and 3, respectively, were performed according to the supplier's

Table 1

Clinical and Molecular Analysis of Patients Carrying the Ala53 Thr Mutation

					Status[a]			
Family	Patient	Sex	Age (years)	Age at Onset (years)	Bradykinesia	Muscular Rigidity	Resting Tremor	Ala53Thr Mutation
PDGR1	II3	M	58	52	+ + +	+ + +	−	+
PDGR2	III1	F	54	50	+ + +	+ + +	−	+
PDGR5	III1	F	80	76	+ + +	+ + +	+	−
PDGR5	III2	M	68	58	+ + +	+ + +	−	+
PDGR5	IV5	F	40	36	+ + +	+ + +	−	+
PDGR8	IV1	M	48	43	+ + +	+ + +	+	+
PDGR8	IV6	M	48	47	+ + +	+ + +	−	+
PDGR11	III1	F	57	49	+ +	+ + +	−	+
PDGR11	III9	F	57	51	+ +	+ + +	−	+
PDGR15	III15	M	49	40	+ +	+ + +	−	+
PDGR18	III4	M	61	58	+ + +	+ + +	−	+

+ = Symptom weakly present; ++ = present; +++ = strongly present; − = absent.

directions. The digested material was electrophoresed in 4% Nusieve agarose gel.

Ten microsatellite markers (Gyapay et al. 1994) were used in the haplotype analysis, including two new polymorphisms. The order of the eight previously described genetic markers, from centromere to telomere, is D4S2361-D4S2460-D4S2371-D4S2461-D4S3006-D4S1089-D4S414-D4S2380. The genetic markers were ordered by use of a minimal physical YAC contig. The two additional genetic markers used were (1) TA46, a $(TA)_n$ repeat that was generated from the bacterial artificial chromosome clone 225H6 (Research Genetics) and that contains marker D4S2461 and (2) marker SYN 24,25, which was designed to flank a dinucleotide repeat in the 5′ noncoding region of the α-synuclein gene (GenBank U46895) (table 2).

The Ala53Thr α-synuclein mutation (Polymeropoulos et al. 1997) was detected in 10 patients belonging to the seven autosomal dominant families but was not found in any member of the remaining 12 families (table 1). In patients carrying the mutation the mean age at onset of the disease is 47 ± 11 years, which is considered to be "early onset" PD. Interestingly, one patient from family PDGR5, individual III-1, did not carry the Ala53Thr mutation, although the mutation was detected in two other affected members of that family. This patient had a much later age at onset of the disease, 76 years, and may represent "sporadic" PD. None of 41 sporadic PD patients of local origin, in 13 of whom the age at onset was 35–55 years, carried the mutation. The DNAs from 116 nonaffected members of the seven families were also analyzed. Among them, we found 11 unaffected carriers of the Ala53Thr mutation (not shown), 10 of whom were younger than the mean age at onset. All of these 11 patients had one affected parent carrying the mutation. However, these results are consistent with a high penetrance of the Ala53Thr mutation in the families that we studied, with age at onset >60 years, as was the case in the Contursi family, the original family studied (Polymeropoulos et al. 1997). Finally, we analyzed 100

Table 2

Markers Used for Haplotyping, and the Primers that Generated the Respective Alleles

		PCR Primer (5′→3′)					
Marker	Repeat	Forward	Reverse	PIC	Allele	Size (bp)	Allele Frequency[a]
TA46	$(TA)_{25}$	TGTTTGCTACGACATCTCTC	CTTGAGCCAGAAGGTTGAGG	.76	1	107	.07
					2	109	.3
					3	111	.28
					4	113	.07
					5	115	.13
					6	117	.11
					7	119	.04
SYN24,25	$(TA)_7(CA)_{11}$	AGGATGGATTAGTAGCTATG	CCTATGGAAGACATGAAGAC	.40	1	181	.27
					2	183	.67
					3	185	.04
					4	187	.01

[a]Frequencies based on 56 chromosomes for TA46 and on 92 chromosomes for SYN24,25

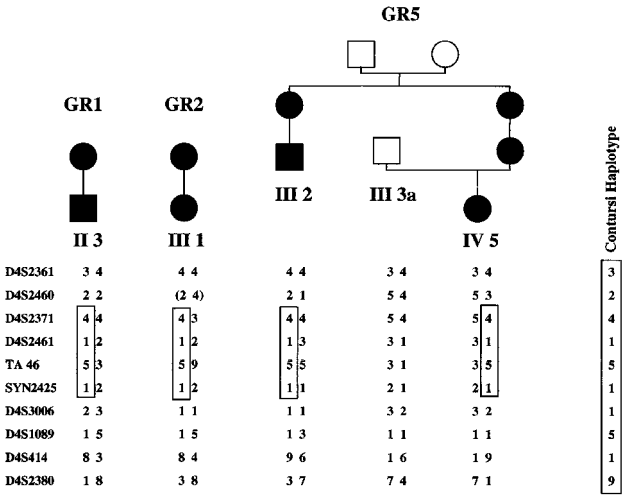

Figure 1 Chromosome 4 haplotype analysis of families carrying the Ala53Thr mutation. The unblackened and blackened symbols represent unaffected and affected individuals, respectively. Roman numerals indicate generations, and arabic numerals indicate individuals. The shared haplotype is boxed.

chromosomes from 50 healthy control individuals, deriving mainly from the Peloponnese and from western Greece, and found none carrying the mutation. Neither any of the patients with familial PD nor any of those with sporadic PD carried the Ala30Pro mutation.

In the Contursi kindred, polymorphic markers established a haplotype shared by all the affected individuals of the kindred, within a region of ~6 cM harboring the α-synuclein gene. To assess the possibility that a founder chromosome is shared by the southern Italian kindred and the seven Greek families that carry the Ala53Thr mutation, we used 10 polymorphic markers to genotype members of three Greek families carrying the mutation (fig. 1). The Greek patients with PD share the portion of chromosome 4 shared by the Contursi kindred, delineated by marker D4S2460 at the centromeric end and by marker D4S3006 at the telomeric end.

On the basis of the information currently available, it appears that these patients with the Ala53Thr α-synuclein mutation have an average age at onset that is at or below the average age at onset in sporadic PD. Clinically, they have prominent bradykinesia and muscular rigidity but rarely have tremor. A more detailed study is currently underway to determine the specific clinical phenotype that may be associated with the α-synuclein Ala53Thr mutation.

All seven Greek families with PD originate from three villages of the northern Peloponnese in Greece, two of which are only 17 km apart and are villages of origin for six of the families. The village of origin of the seventh family was 120 km distant . The Contursi kindred comes from southern Italy, a region geographically and historically linked to Greece. Our data suggest that affected members in these families may all be descendents of a single founder. A study of "early-onset" familial PD in the greater Balkan area would help to establish the contribution of α-synuclein mutations to the PD phenotype.

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Mutation of the XNP/ATR-X Gene in a Family with Severe Mental Retardation, Spastic Paraplegia and Skewed Pattern of X Inactivation: Demonstration that the Mutation is Involved in the Inactivation Bias

To the Editor:

A family in which severe mental retardation (MR) is segregating with spastic paraplegia (SP) has recently been reported (Martinez et al. 1998). The extended pedigree of this family is presented in figure 1. Obligate-carrier females have a totally skewed pattern of X inactivation, detected by amplification of the $(CAG)_n$ microsatellite repeat in the androgen-receptor gene and previous digestion of genomic DNA with the methylation-sensitive restriction endonuclease *Hpa*II, as described elsewhere (Martinez et al. 1998). Genetic analysis in the family has revealed linkage of the morbid locus to the proximal long arm of the X chromosome, with a maximum LOD score in Xq13.3. Three genes involved in X-linked MR (XLMR) have already been reported in this genomic region. One, encoding oligophrenin-1, is involved in nonsyndromic XLMR (Billuart et al. 1998) and thus does not seem to be a good candidate. The other two are involved in two syndromic XLMR conditions: Menkes syndrome (MIM 309400) and alpha-thalassemia with mental retardation (ATR-X [MIM 301040]) syndrome. The latter is an XLMR condition that associates severe MR, mild alpha-thalassemia, typical facial dysmorphy and a skewed pattern of X inactivation in carrier females (Gibbons et al. 1995*a*). The clinical characteristics of the reported MR+SP family are close to this description, and thus the presence of an allelic mutation at the ATR-X locus could be hypothesized. In addition to the already reported clinical features in the MR+SP family, hematologic analysis revealed that 3% of the patients' erythrocytes showed HbH inclusions after cresyl-brilliant staining, which reinforced the possibility that a mutation in the XNP/ATR-X gene is present in this family. However, ATR-X syndrome has always been reported to be associated with neonatal hypotonia, which can be severe (Gibbons et al. 1995*a*). In the case of the family that we studied, affected

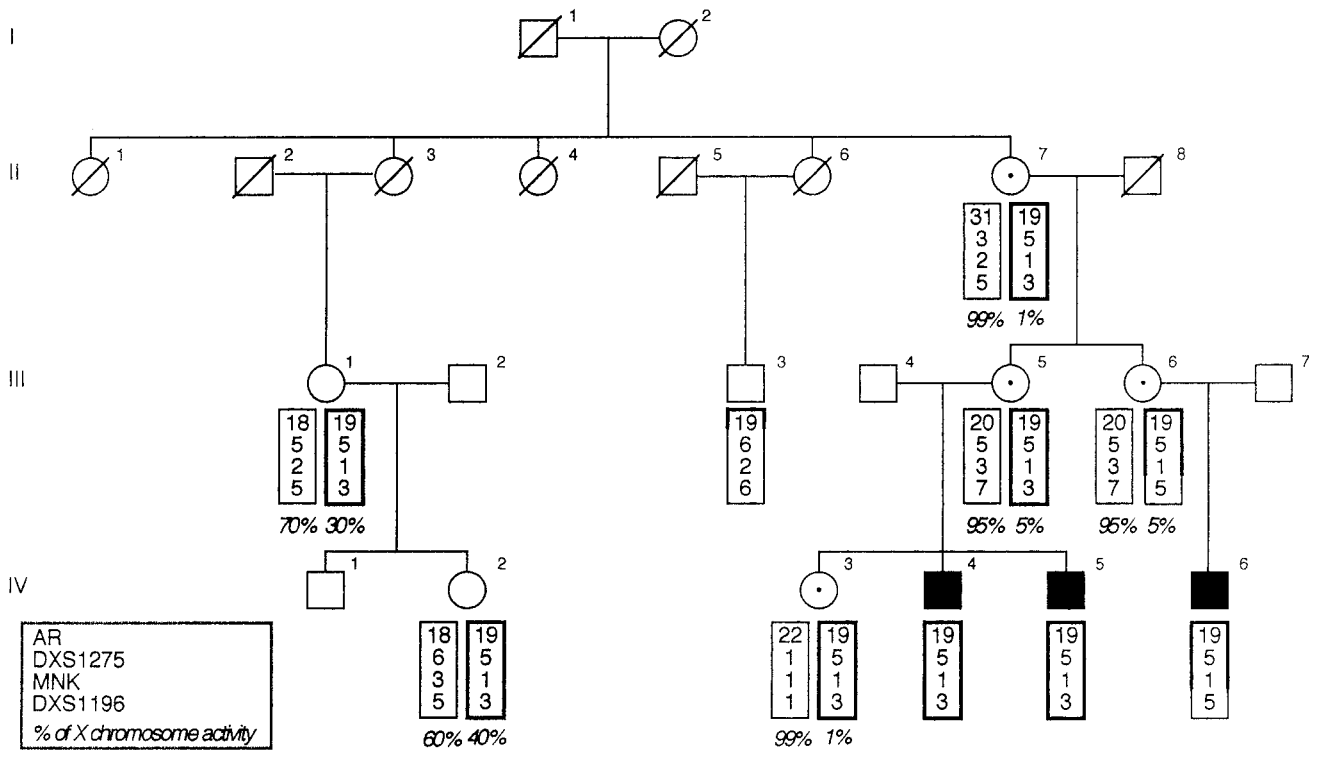

Figure 1 Pedigree of family showing the haplotypes (shown below each analyzed member) of the region linked to the disease. The order of the markers in the region, with respect to the X inactivation–specific transcript (XIST gene) and the XNP/ATR-X gene, is AR-DXS1275-XIST-XNP-MNK-DXS1196. Note that III-1 and IV-2 have inherited the disease-linked haplotype (shown in the thicker frames). The percentages of X inactivation in females are shown (in italics) under the corresponding haplotype (for further details, see text).

patients were affected, from birth, with the opposite sign, hypertonia. It was thus interesting to look for a possible new mutation that might lead to a variant of the disorder.

The gene involved in the ATR-X syndrome was isolated in 1994 (by Gecz et al.) and named "XNP." It has since been shown to be mutated in 13 patients with alpha-thalassemia MR (ATR-X) syndrome (Gibbons et al. 1995*b*). Moreover, mutations in this gene have also been identified in families affected with Juberg-Marsidi syndrome (MIM 309590) and with MR syndromes without alpha-thalassemia (Villard et al. 1996*a*, 1996*b*, 1996*c*). More recently, a new mutation has been found, in Carpenter-Waziri syndrome (Abidi et al. 1999).

The gene is large (probably extending over 300 kb) and consists of 35 exons. It encodes a putative zinc-finger helicase (Villard et al. 1997) that is probably involved, by remodeling the structure of the chromatin, in the control of gene expression. This assumption is supported by the finding of an interaction between the XNP protein and the Ezh2 protein (Cardoso et al. 1998), the human equivalent of *Drosophila* enhancer of zeste, a chromatinian protein of the Polycomb group.

To date, 34 different mutations in 50 pedigrees have been described in the XNP/ATR-X gene, which are located either in the region coding for the three zinc-finger domains of the protein (exons 7 and 8 and the beginning of exon 9) or in the helicase domain, which extends over 3 kb at the COOH terminus of the putative protein.

We thus decided to search for mutations in this gene in a patient from the MR+SP family. This was performed by systematic sequencing of the gene. We found one mutation in the gene, changing an arginine from the conserved helicase domain III into a lysine, R1742K (fig. 2). This mutation perfectly segregates with the disorder in the family (fig. 3). Moreover, the mutation was absent from 100 unrelated chromosomes from the same geographical area as that of the family we studied. We performed reverse-transcription PCR (RT-PCR) on RNA from lymphocytes of the patients, to check for any putative effect of the mutation on the mRNA splicing, which has been previously reported to occur in this gene (Villard et al. 1996*b*) but we failed to find any abnormal-size product (data not shown).

Since this amino acid change (arginine to lysine) is generally considered to be conservative, we used a local structure–prediction program (PEPTIDESTRUCTURE, from the GCG package, which mainly predicts α helices

Figure 2 Position of mutation in the MR+SP family. The top two lines show the normal XNP protein and nucleotide sequence encompassing part of the helicase III domain. The sequences are numbered according to GenBank entry U75653. The R1742 residue is underlined, as is the G5459 nucleotide that is changed into an A and that causes the replacement of R1742 by K (both the normal and mutated codons are shaded).

and β sheets). This program has predicted a change in the local structure of the protein (fig. 4), potentially arising because of steric problems. Arginine 1742 is an amino acid that cannot be strictly considered to be evolutionarily conserved, but it is present at the same position in several members of the helicase family of proteins (e.g., in the yeast Rad54 protein).

Very interestingly, in exploring another branch of the family for the presence of the mutation, we found two females (individuals III-1 and IV-2 in fig. 1) who share the disease-associated haplotype, spanning both the XNP gene and the X-inactivation center, but who lack the R1742K mutation. We thus can deduce that the mutation arose de novo in the germline of one member of the founding couple (I-1 or I-2 in fig. 1). Furthermore, the fact that females in the nonmutated branch of the family do not exhibit a skewed pattern of X-chromosome inactivation demonstrates that the skewing in carrier females is directly linked to the presence of the mutation in this gene. This is the first demonstration of a direct link between a mutation in this gene and the skewed pattern of X inactivation observed in all carrier females, and it further supports the pathogenicity of the R1742K mutation.

We can draw, from these data, two main conclusions. First, the analysis of this family extends the phenotype associated with XNP/ATR-X mutations. All patients reported so far were hypotonic (at least in the neonatal period), which made this trait a major criterion of inclusion. In this family, patients are hypertonic and also present spastic paraplegia. This clinical sign has never been observed either in classic ATR-X patients or in related phenotypes such as Juberg-Marsidi syndrome or Carpenter-Waziri syndrome. Spasticity can develop at later stages in ATR-X patients, despite neonatal hypotonia, but the patients reported in this study were affected with spasticity from birth. We have no explanation for this, except that no mutation affecting the helicase III conserved domain has been identified so far. However, it is difficult to draw conclusions based on the analysis of a single family.

In contrast, a reexamination of the affected males in this family shows that, although one affected male does not exhibit a markedly dysmorphic phenotype, the two other affected individuals present some facial features in common with the ones observed in ATR-X syndrome: everted lower lip with a "carplike" triangular mouth, hypertelorism, small triangular nose, and broad nasal root.

The second point regards the skewed pattern of X inactivation in carrier females. So far, only a close association between a bias in X inactivation and a mutation in the gene has been reported, without any direct proof. In this family, we can observe that the bias in X inactivation occurs only in females carrying the mutation. Other females, who have received the same chromosome but who are not mutated at the XNP locus, do not present a skewed pattern of X inactivation, demonstrating that this phenomenon is directly related to a mutation in the gene. We do not have an explanation for this phenomenon, but we can postulate two hypotheses. First, a mutation in the gene could lead to a selection, during embryogenesis, that favors the cells expressing the normal gene product. The second hypothesis is that the gene, which is involved in chromatin-structure remodeling (Cardoso et al. 1998), could participate directly or indirectly in one of the processes of

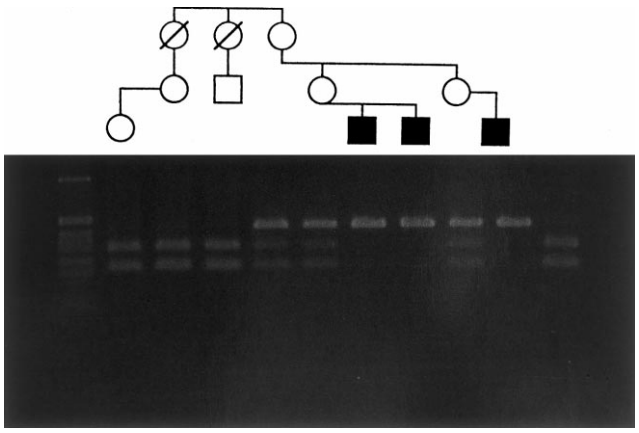

Figure 3 Detection of 5459G→A mutation in the affected branch of the family. A PCR product obtained by use of two oligonucleotides flanking the mutation is digested with *Bse*RI and electrophoresed. The presence of the mutation destroys an internal *Bse*RI site in the amplification product. Affected males have a single undigested amplified allele (one band); carrier-female DNA harboring both a mutated and a nonmutated allele yields both a digested and a nondigested PCR product (three bands); and noncarrier females have two digested alleles (two bands).

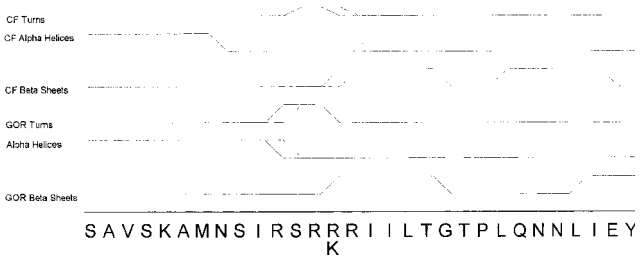

Figure 4 Prediction of secondary structures of the normal (*continuous line*) and the mutated (*dashed line*) proteins. CF = secondary-structure prediction according to the Chou-Fasman algorithm; GOR = the Garnier-Osguthorpe-Robson algorithm. Note the variation for Turns and β-sheet formation (with CF method) and for Turns and α-helix formation (with GOR method), in the region surrounding the R1742K substitution.

X-chromosome inactivation: either spreading or maintenance of the inactive state. This would be lethal in females in whom the mutated allele is expressed, because functional disomy of the X chromosome is known to be a lethal condition. Whatever the truth is, it is really striking to observe that a gene defect that leads to such a strong counterselection in female carriers and that is not restricted to certain cell lineages does not impair male viability. It is, to our knowledge, the first instance in which such a negative selection against the cells expressing an abnormal gene product in females does not imply a male-lethal condition (discussed in Pegoraro et al. 1997). Although highly skewed X inactivation has been reported in several other X-linked disorders, it is never systematically present in the obligate-carrier females (Orstavik et al. 1998; Plenge et al. 1999).

A final point is that the bias in X inactivation is, together with severe MR, the only consistent sign in families. This can certainly be important in a first clinical screening of the patients, before a mutation is sought in the gene.

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Electronic-Database Information

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

Human Gene Mutation Database (HGMD), http://www.uwcm.ac.uk/uwcm/mg/search/136052.html (for the mutations reported in the XNP/ATR-X gene)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for ATR-X, Juberg-Marsidi, and Menkes syndromes)

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A 22q11.2 Deletion That Excludes *UFD1L* and *CDC45L* in a Patient with Conotruncal and Craniofacial Defects

To the Editor:

Microdeletions of chromosome 22q11.2 occur with a high frequency in the general population, with an estimated incidence of 1/3,000–1/4,000 (Burn and Goodship 1996). They have been shown to be associated with the malformation phenotypes of velocardiofacial syndrome (VCFS [MIM 192430]), DiGeorge syndrome (DGS [MIM 188400]), and conotruncal anomaly face syndrome (CAFS [MIM 217095]) (Emanuel et al. 1999*a*). Deletions of this region have also been demonstrated in some patients with the autosomal dominant form of Opitz G/BBB syndrome (MIM 145410) (McDonald-McGinn et al. 1995). Significant phenotypic overlap is found among these entities, including conotruncal cardiac defects, craniofacial anomalies, learning disabilities, and cleft palate. The spectrum of clinical findings shows considerable variability, even within families (McLean et al. 1993).

Although the overwhelming majority (>85%) of patients have deletions of the same ~3-Mb region (Emanuel et al. 1999*a*), several reports have described patients with atypical, shorter deleted segments nested within the large typically deleted region (TDR) (Levy et al. 1995; Kurahashi et al. 1996; O'Donnell et al. 1997; McQuade et al. 1999). Recently, a small, 20-kb deletion within the TDR was reported in a patient with a classic VCFS/DGS phenotype. This smaller deletion disrupts the *UFD1L* and *CDC45L* genes, the products of which (in particular, *UFD1L*) have been suggested to play important roles in craniofacial and cardiac development resulting in the phenotype (Shaikh et al. 1999; Yamagishi et al. 1999). However, several of the aforementioned patients (some of whom have cardiac and craniofacial defects) have deletions that do not include the region containing these genes. These observations suggest that additional sequences within the TDR affect early craniofacial and cardiac morphogenesis. Additionally, a patient with features of DGS and with a microdeletion that falls outside the TDR but that does not overlap with any of the known deletions was recently described (Rauch et al. 1999). This patient had craniofacial abnormalities and an interrupted aortic arch (type B) with truncus arteriosus, the same defect seen in the patient described by Yamagishi et al. (1999). The report by Rauch et al. further emphasizes the likelihood that the 22q11.2-related cardiac defects are unlikely to result from defects involving a single gene within the TDR.

We have identified a patient, CH98-18 (Emanuel et al. 1998), with a novel deletion of chromosome 22q11.2. His deletion is distal to the usual 3-Mb deletion found in most patients with VCFS and appears to overlap with a portion of the deleted region described by Rauch et al. (1999). The deletion does not overlap with any of the previously described "minimal critical regions" for VCFS/DGS. The patient was born to a 33-year-old mother at 35 wk gestation. The pregnancy was complicated by a weight gain of 70 lbs and premature rupture of membranes. The baby was delivered by cesarean section, because of breech presentation, with Apgar scores of 7 at 1 min and 8 at 5 min. Physical examination at birth was notable for an appropriate-for-gestational-age infant with hypertelorism, posteriorly rotated ears, micrognathia, a loud cardiac murmur, hypospadias, descended testes, single palmar creases, and 5th-finger clinodactyly bilaterally. Renal and cranial ultrasounds were normal. Echocardiography showed the presence of truncus arteriosus type II and a ventricular septal defect. Borderline hypocalcemia was also present. The patient had surgical repair of his truncus arteriosus at age 3 wk and a replacement graft at age 7 mo.

Motor development was normal. The patient sat at age 6 mo and walked at age 14 mo. However, he had expressive-speech delay, speaking his first words at age 21 mo. At age 26 mo, he had speech appropriate for an 18-month-old. During a recent physical examination at age 26 mo (fig. 1), short stature, microcephaly, a prominent glabella, partially attached earlobes, a broad nasal bridge, a broad nasal tip with a crease, hypoplastic nasal alae, anteverted nares, a featureless philtrum, a downturned mouth, a bifid uvula, and normal hearing and vision were noted. Endocrine evaluation including thyroid-function and growth-hormone panels was unremarkable. Immunologic studies including surface markers for T-cell, B-cell, and NK lineages, myeloid markers, leukocyte adhesion, and Wiskott-Aldrich–associated

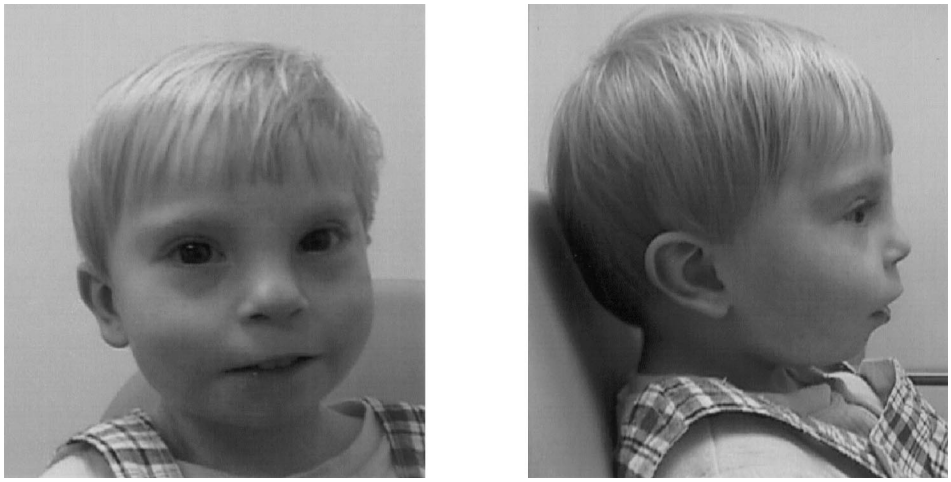

Figure 1 Photographs of patient CH98-18 at age 26 mo. Note hypertelorism, broad nose, and micrognathia.

proteins were all normal. Proliferative responses to mitogen-stimulation tests were also normal, as were functional-antibody responses.

GTG-banded chromosomes prepared directly from peripheral-blood lymphocytes showed a normal 46,XY karyotype, and FISH was negative for a deletion when the N25 (Oncor) probe was used (fig. 2*A*). In addition, CH98-18 did not have a deletion for a number of other cosmid-based FISH markers within the TDR, including D22S788, ZNF74, HCF2, and cHKAD26 (fig. 2*B*) (Emanuel et al. 1998). With the exception of cHKAD26, cosmids used for FISH were isolated by colony hybridization from the chromosome 22–specific cosmid library (LL22NCO3) generated at the Lawrence Livermore Laboratories. The cosmid that contains the cHKAD26 locus was provided by the Japanese Cancer Research Resources Bank. A cosmid, 83C5, containing the portions of the *UFD1L* and *CDC45L* genes deleted in the patient of Yamagishi et al. (Shaikh et al. 1999) was also used for FISH and was not deleted (data not shown). We determined that a cosmid located distally in the TDR (107D7) was not deleted (fig. 2*B*). Because the clinical findings in the patient, including truncus arteriosus, a bifid uvula, hypoplastic alar nasi, and a history of hypocalcemia, were consistent with those seen in VCFS, additional analysis was undertaken. Use of a series of cosmid and bacterial artificial-chromosome–derived probes for FISH demonstrated, initially with a cosmid for locus D22S801, that a deletion adjacent to the TDR was present (fig. 2*A,b* and *B*). Both of CH98-18's parents were analyzed by FISH with the cosmid for D22S801 and were found not to have a deletion, indicating a de novo origin of the deletion.

We and others have implicated blocks of duplicated DNA sequence containing BCRL and GGTL elements in the mechanism etiologic for the 22q11.2 deletions (Emanuel et al. 1998, 1999*b*; Edelmann et al. 1999). We predicted, on the basis of the presence of additional BCRL and GGTL duplicated sequences distal to the TDR, that CH98-18's deletion might involve one of these elements. Thus, the extent of his deletion was investigated on the basis of the map of the region immediately distal to the TDR. This region contains the immunoglobulin-λ light-chain locus (IGLL), within which are located BCRL4 and a copy of GGTL (Kawasaki et al. 1995). The IGLL locus has been completely characterized in a cosmid contig (Kawasaki et al. 1995) and has been sequenced (Kawasaki et al. 1997). Using the cosmid and sequence reagents, we moved distally from D22S801 (LN80) into the IGLL to detect the deletion end point (DEP). To insure that the germline genomic configuration of this region was being investigated, all studies were performed on cultured peripheral-blood lymphocytes. We determined that, although cosmid 61E11 is deleted, 102D1 is the first cosmid not deleted in CH98-18 (fig. 2*A* and *B*). In the sequenced contig, these cosmids are separated by ~40 kb that contain BCRL4 and GGTL. Using DNA sequence information for 102D1, we designed PCR-based 2.0-kb FISH probes and determined that both ends of this cosmid, which is immediately distal to the BCRL4/GGTL duplicated sequence in the IGLL locus, are present (GenBank). These probes are present, then, on both homologues. Results for one of these probes are shown in figure 2*A*. Thus, our patient's telomeric DEP involves a duplicated-sequence block that contains BCRL4 and GGTL sequences. The presence of a DEP in the vicinity of the BCRL4/GGTL duplication suggests unequal crossing-over in the formation of the patient's deletion. Additional cosmids distal to 102D1 were examined by FISH, and all were present on both

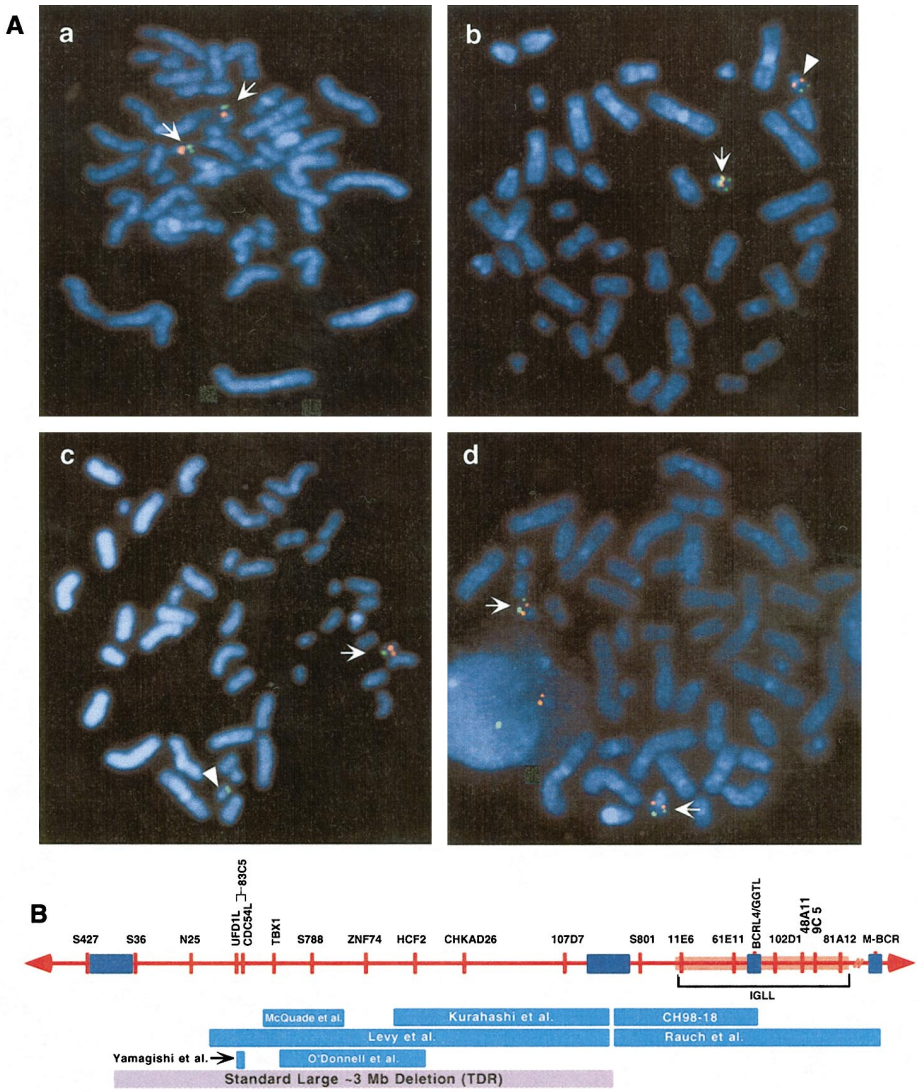

Figure 2 *A,* Dual-color FISH for DEP positioning in patient CH98-18. All metaphase spreads are hybridized with a control cosmid containing the distal marker D22S39. D22S39 was biotinylated and detected with avidin-FITC to identify chromosome 22 (seen at the telomeric end of chromosome 22, in all panels [*green*]). Most test cosmids were labeled and detected with digoxigenin-rhodamine–conjugated probes (*red*), except D22S801, which was biotinylated and then detected with avidin-FITC (*green*). In *a,* N25 (Oncor) is shown (*red*) and is present on both homologues (*arrows*). In *b,* cohybridization with two test cosmids, D22S36 (*red*) and D22S801 (*green*), shows them as overlapping signals on the normal chromosome 22 (*arrow*), with the control signal (D22S39) (*green*). Absence of the FITC test signal on the other chromosome 22 (*arrowhead*) indicates a deletion of D22S801. In *c,* cosmid 11E6 (*red*) is not detected on one of the chromosomes 22 (*arrowhead*) but is present on the normal homologue (*arrow*). In *d,* a PCR-derived probe from the distal end of cosmid 102D1 (*red*) is present on both chromosome 22 homologues (*arrows*). The 102D1 probe was generated by PCR using primers designed from sequence (for nucleotides 34424–36498) available in GenBank (accession number D86994). The PCR product was cloned by ligation into a TA cloning vector (Invitrogen), and the resultant plasmid was labeled by nick translation. *B,* Composite breakpoint map of 22q11.2, depicting the relative locations of several reported atypical deletions (*turquoise bars*). The typical deletion is also shown (*lavender bar*). Within the map of the TDR, genes and markers that identify the cosmids used for FISH experiments are depicted. Addresses of cosmids from the IGLL map (Kawasaki et al. 1995) that were used for FISH experiments are also shown. Individual duplicated-sequence blocks are also shown (*dark blue*). The IGLL locus is indicated by the bracket below the map. The cosmids from within the IGLL locus that are used for FISH are indicated on the top line over the region shaded in light red.

homologues (data not shown). These cosmids included 48A11, 9C5, 75C12, and 81A12 from the previously published IGLL contig (Kawasaki et al. 1995), as well as the commercially available M-BCR probe (Oncor). The region distal to 102D1, extending to the M-BCR probe, was deleted in the family described by Rauch et al. (1999).

Thus, our patient has a novel deletion of chromosome 22q11.2 and features of VCFS, including hypoplastic alar nasi, a bifid uvula, and truncus arteriosus, a cardiac lesion characteristic of the deletion. In a previous study, ~35% of patients with persistent truncus arteriosus were demonstrated to have standard deletions of 22q11.2 (Goldmuntz et al. 1998). To determine whether CH98-18's distal deletion might be related to his particular heart defect, we subsequently analyzed 15 other patients with truncus arteriosus who did not have a deletion when the N25 probe was used. None of these patients had a deletion of D22S801 (B. S. Emanuel, unpublished data). Furthermore, patient CH98-18 has hypertelorism, anteverted nares, a grooved nasal tip, and hypospadias, which, although reported in VCFS, are more commonly described in Opitz G/BBB syndrome (Robin et al. 1996). Opitz G/BBB is a heterogeneous disorder first described in 1969 (Opitz et al. 1969*a,* 1969*b*) and linked to the X chromosome (MIM 300000) and 22q11 (Robin et al. 1995). The X-linked form has subsequently been associated with mutations of the *MID1* gene located on Xp22 (Quaderi et al. 1997; Gaudenz et al. 1998). Patients with features of Opitz G/BBB and an autosomal dominant mode of inheritance have also been reported (Opitz 1987; Robin et al. 1995). Because of phenotypic overlap with the 22q11.2 deletion, several patients were assayed for and were found to have deletions of 22q11.2 (McDonald-McGinn et al. 1995). These patients all had the typical 3-Mb deletion commonly seen in VCFS/DGS. Analysis of several additional Opitz G/BBB cases without TDR deletions has failed to demonstrate a distal deletion similar to the one described in the present report (B. S. Emanuel, unpublished data).

Therefore, the deletion reported here is atypical of that seen with a VCFS/DGS phenotype, and it is also atypical of those deletions that have been seen with autosomal dominant Opitz syndrome. It is notable that CH98-18's deletion does not encompass the region containing the *UFD1L* and *CDC45L* genes. He does, however, like the patient described by Rauch et al., have a cardiac defect similar to that seen in the patient described by Yamagishi et al. These cases with deletions distal to the TDR argue against the hypothesis that *UFD1L* and *CDC45L* alone are sufficient to cause the cardiac defect and craniofacial features typically seen in VCFS/DGS. It is more likely that our patient's phenotype is the result of haploinsufficiency of other genes located in this distal deleted region, which may, perhaps, function with *UFD1L* and *CDC45L* in a common developmental pathway. Alternatively, our patient's deletion raises the possibility of either a "position effect" on genes within the TDR or a more complex mechanism etiologic for features of the disorder, because his deletion is distal to the TDR and appears not to include any of the genes described within the TDR.

Our patient's deletion is contained within the deletion described by Rauch et al. (1999); however, the phenotypes are not concordant. Both patient CH98-18 and the proband from the study by Rauch et al. have persistent truncus arteriosus. Nonetheless, there are no signs of Optiz G/BBB in any members of the family (including the proband) described by Rauch et al. However, since all of the subjects described in their report were female, only the laryngoesophageal anomalies and hypertelorism would be pertinent findings.

Thus, it becomes apparent, when one is studying such "atypical" patients, that delineating a minimal critical region as causal for the VCFS/DGS phenotype spectrum may be of limited applicability. Findings from this patient and the others discussed here imply that disruption of more than one gene most likely contributes to the phenotype. Further definition of the mechanisms that lead to these deletions and of the involvement of the duplicated-sequence blocks found in the region (Emanuel et al. 1998; Edelmann et al. 1999) would enable a greater understanding of (1) why these deletions and their subsequent phenotypes are so frequently encountered and (2) the role that sequences on 22q11.2 have in their etiology.

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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 cosmid 102D1 sequence)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for DGS [MIM 188400], VCFS [192430], CAFS [217095], and Opitz syndrome [145410]).

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De Novo *BRCA1* Mutation in a Patient with Breast Cancer and an Inherited *BRCA2* Mutation

To the Editor:

Individuals with an inherited mutation in both of the breast/ovarian cancer–susceptibility genes, *BRCA1* and *BRCA2,* are rarely described (Ramus et al. 1997; Liede et al. 1998). Despite the large number of variants identified in these genes, there are, in the Breast Cancer Information Core, no published reports of de novo mutations.

During the course of the Australian Breast Cancer Family Study, a population-based study of breast cancer occurring in women at age <40 years (McCredie et al. 1998; Southey et al. 1998, 1999), we identified a proband who developed high-grade breast cancer with axillary nodal metastases. Her father developed prostate carcinoma during his early 50s. Her mother had no history of cancer. The studies outlined below were performed with informed consent from the individuals and were approved by the Peter MacCallum Cancer Institute's review board.

The proband's leukocyte-derived DNA revealed two germline protein-truncating mutations: one in exon 11 of *BRCA2* (6174delT) and a second in exon 11 of *BRCA1* (3888delGA). These mutations were identified by the protein-truncation test and by manual DNA sequencing in our laboratory (fig. 1*A*), and were confirmed, after collection of a further blood sample, by heteroduplex analysis in an outside laboratory (data not shown). The 6174delT *BRCA2* mutation has been reported frequently in individuals of Jewish descent (Neuhausen et al. 1996); however, there was no known Jewish ancestry in this family. The 3888delGA *BRCA1* mutation has not been reported before by the Breast Cancer Information Core. The father of the proband carried only the 6174delT *BRCA2* mutation (fig. 1*A*), and neither parent carried the 3888delGA *BRCA1* mutation. The proband's brother was unavailable for testing. DNA fingerprinting at five informative microsatellite loci supported the genetic relatedness of the parents' and proband's DNA samples (data not shown).

The absence of a germline 3888delGA *BRCA1* mutation in either parent indicated that this variant could represent a de novo alteration that occurred during parental germ-cell gametogenesis. We evaluated this possibility by utilizing a heterozygous polymorphism at nucleotide 1186 (1186 A→G) in the proband's *BRCA1* exon 11. The proband's *BRCA1* exon 11 alleles were separated by cloning, and then were sequenced. The 1186 A→G polymorphism was present on her wild type

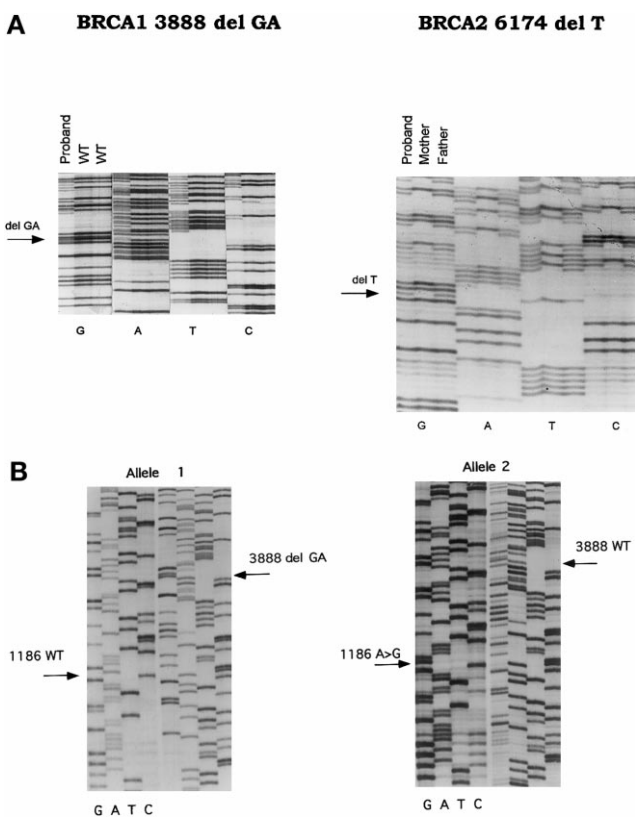

Figure 1 Germline mutations in *BRCA1* and *BRCA2*, and inheritance of *BRCA1* mutation. *A, left panel,* DNA sequence of a segment of exon 11 of *BRCA1* showing 3888delGA mutation in proband, as compared with wild-type sequence (lanes WT). *A, right panel,* DNA sequence of part of exon 11 of *BRCA2* showing 6174delT mutation in the proband and her father, contrasted with the wild-type sequence of the mother. *B, left panel,* DNA sequences of the separate, cloned alleles showing *BRCA1* 3888delGA mutation present on paternal allele (allele 1), with wild-type sequence at 1186. *B, right panel,* wild-type *BRCA1* sequence of the maternal allele (allele 2) at 3888, with the 1186A→G polymorphism. *BRCA1* was sequenced as described elsewhere (Southey et al. 1998), and the primers used to sequence *BRCA2* have been described in the Breast Cancer Information Core. Separation of the proband's *BRCA1* alleles was achieved by cloning a purified PCR-amplified 2.9-kb fragment containing the 1186 polymorphism and the 3888 deletion Bluescript (Stratagene).

3888 allele and in her mother's germline but was not present in her father's germline (fig. 1*A*). These data indicate that the *BRCA1* 3888delGA mutation was on the father's allele and further suggest that this mutation arose as a de novo event in a testicular germ cell.

The presence, in the proband, of heterozygous germline mutations in *BRCA1* and *BRCA2* raised the question of whether dysfunction of both these genes was involved in the development of her breast carcinoma.

Loss of heterozygosity (LOH) studies performed on microdissected archival breast-cancer tissue demonstrated consistent LOH of the maternal (wild type) allele at the *BRCA2*-flanking markers D13S260 and D13S290, in multiple independently dissected areas of invasive ductal carcinoma. Loss of the maternal allele was also seen in three adjacent axillary-lymph-node metastases and in multiple apparently separate foci of high-grade ductal carcinoma in situ, a preinvasive lesion (fig. 2). These data are consistent with clonal expansion and dominance of cells that harbor dysfunction of both alleles of *BRCA2*, through multiple phases of malignant progression. In contrast, evaluation of identical microdissected foci at the intragenic *BRCA1* marker D17S855 showed that both alleles were retained, suggesting that LOH-induced inactivation of the second *BRCA1* allele was not selected for during carcinogenesis (fig. 2).

Interestingly, the sequence adjacent to the *BRCA1* mutation—*AGAGGAGAAT* (where GA constitutes the deleted doublet) is similar to that adjacent to the *BRCA2* 6174delT mutation—*AGTGGAAAAT* (where T denotes the deleted nucleotide). Although this similarity could be due to chance, it is also possible that these sequences are problematic for the germ-cell DNA-replication machinery and that they are thus prone to mutation. In support of this, haplotype studies suggest that the 6174delT *BRCA2* mutation has arisen on multiple separate occasions in individuals of varying ethnic origins (Berman et al. 1996). In an attempt to define a possible common mechanism underlying the development of these two mutations, we used the *mfold* software of M. Zuker to evaluate the areas of sequence similarity, together with 100 bp of their respective wild-type *BRCA1*- and *BRCA2*-flanking regions, for their ability to form secondary structures. The segments of both genes preferentially formed consistent hairpin loops arising from unpaired nucleotides at a wide range of bond energies under physiological conditions. Moreover, in each case, the deleted bases lay <2 bp from an unpaired sequence. It is therefore possible that these frameshift mutations occurred because of deletion of nucleotides adjacent to unpaired DNA sequences arising in these quasipalindromic regions. Frameshift mutations occurring in quasipalindromic sequences have been well characterized in *Escherichia coli* (De Boer and Ripley 1984; Rosche et al. 1998) and have been suggested as a basis of the deletion mutations seen in several human genes (Cooper and Krawczak 1993, pp. 185–188).

There is a further potential explanation for the occurrence of the two mutations occurring in this case: *BRCA1* and *BRCA2* have been implicated in the maintenance of genomic integrity (Rajan et al. 1996; Connor et al. 1997; Scully et al. 1997; Bertwistle and Ashworth 1998; Chen et al. 1998; Marmorstein et al. 1998; Patel et al. 1998). Moreover, in the mouse testes, *Brca1* and *Brca2* are expressed in the mitotic spermatogonia as well as in meiotic spermatocytes (Blackshear et al. 1998). Therefore, the presence, in the father, of one mutated *BRCA2* allele in developing germ cells could confer a dosage insufficiency and thus result in a generally increased genomic mutation rate. Such an abnormality could theoretically affect any gene and would thus have a minimal likelihood of being detected, thus explaining the absence of reports of supporting evidence. However, the serendipitous coexistent *BRCA1* variant described here may indicate the need for more-rigorous searches for de novo germline mutations in other genes arising in the background of inherited *BRCA1/2* mutations. Further studies using in vitro methods can address whether sequences such as those described above are indeed more susceptible to mutation, whether *BRCA2* haploinsufficiency results in an increased mutation rate, and whether these two mutation mechanisms could display synergistic effects. This case is, to our knowledge, the first reported example of a de novo mutation arising in *BRCA1*, and it suggests a potential mechanism underlying the development of recurrent germline mutations occurring in association with specific sequences.

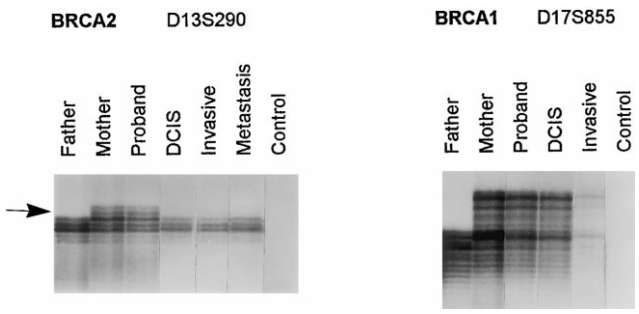

Figure 2 LOH analysis. *Left panel,* Loss of the maternally inherited (wild-type) *BRCA2* allele at the marker D13S290, in ductal carcinoma in situ (lanes DCIS), invasive and metastatic carcinoma from the proband. *Right panel,* Absence of LOH at the intragenic *BRCA1* marker D17S855 in DCIS and in invasive carcinoma. No DNA PCR control lanes are shown. DNA was microdissected from archival tumor samples, was subjected to proteinase K digestion, and was analyzed with standard PCR-based microsatellite-analysis protocols employing published primer sequences for the markers D13S290 and D17S855 (Simard et al. 1994).

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URLs for data in this article are as follows:

Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/

Institute for Biomedical Computing, Washington University, http://www.ibc.wustl.edu/~zuker/seqanal/

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X Chromosome–Inactivation Patterns Confirm the Late Timing of Monoamniotic-MZ Twinning

To the Editor:

We recently reported, in the *Journal*, on the patterns of X-chromosome inactivation in female MZ twin pairs (Monteiro et al. 1998). The data supported the hypothesis that dichorionic (DC) and monochorionic (MC) MZ twin pairs differ in the timing of the twinning event, with respect to the onset of X inactivation. Specifically, DC-MZ twin pairs frequently exhibit dramatic differences, in X-inactivation patterns, between members of the pair, whereas MC-MZ pairs are highly correlated. The most reasonable interpretation of these results is that MC-MZ pairs undergo splitting after the X-inactivation event, whereas DC-MZ pairs must split before or around the time of X inactivation.

We noted in our report that, although MC-MZ twin pairs have X-inactivation patterns that are more highly correlated than those in DC-MZ pairs, they were not as closely correlated as were results of repeated assays of the same individual (Monteiro et al. 1998). We interpreted this as being reflective of heterogeneity in the timing of MC-MZ twinning, with some MC-MZ pairs splitting after—but close in time to—the onset of X inactivation. We further hypothesized that a subgroup of MC-MZ twins—namely monoamniotic-MC twin pairs (MA-MZ)—would have X-inactivation patterns that are even more closely correlated, since MA-MZ twins probably result from much later twinning events that take place many cell divisions after the X-inactivation event. We have now directly addressed this issue experimentally.

MA-MZ twinning is a relatively rare event, with only ~2% of MZ twins falling into this anatomic subgroup (Derom et al. 1995). Nevertheless, we have identified 11 female MA-MZ twin pairs, as part of the East Flanders Prospective Twin Study. The amniotic anatomy of each twin pair was assessed at birth, by examination of the placental structure (Loos et al. 1999). The absence of any septum in a well-preserved fetal sac, when one is able to peel off the amnion from the placental surface, indicates the presence of a single amniotic sac. The X-inactivation patterns in these twins were assessed by a *Hpa*II-PCR assay, which depends on methylation differences, in the androgen-receptor gene, between the inactive and active X chromosomes (Allen et al. 1992). In order to avoid the confounding factor of a shared placental blood supply in MC-MZ twins, the assessment of X inactivation was done by use of DNA isolated from buccal mucosa.

Figure 1 shows a comparison of the absolute difference in X-inactivation patterns, both among the various anatomic subtypes of MZ twin pairs and among patterns in repeated assays performed on the same individual. The data on DC-MZ twin pairs (mean X-inactivation difference 14.3%) and MC-MZ (mean X-inactivation difference 5.9%) are taken from our previous report (Monteiro et al. 1998) and are significantly different, with $P < .05$. (Note that all the "MC-MZ" pairs reported in figure 1 are diamniotic.) Strikingly, MA-MZ twin pairs exhibit identical X-inactivation patterns, at least to the limits of the assay used for this analysis, since the mean difference among MA-MZ pairs (2.1%) does not differ significantly from that observed when the assay is repeated twice on the same individual (3.7%). In contrast, the MA-MZ twin pairs are considerably more similar to one another than are diamniotic MC-MZ pairs, in a pairwise comparison ($P = .02$).

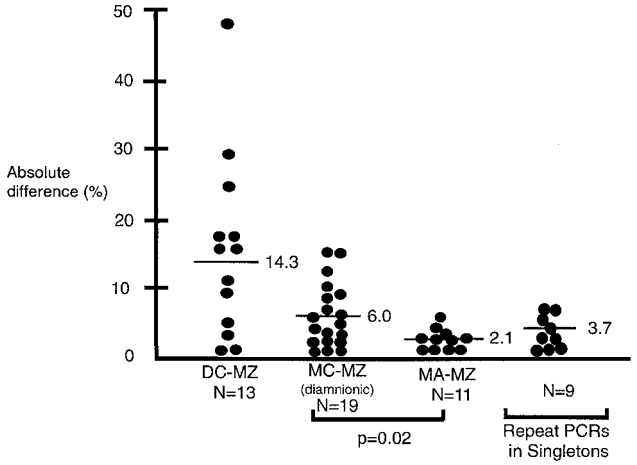

Figure 1 Summary of differences in X-inactivation patterns, among various subgroups of MZ twin pairs and among repeat assays of singletons. An *Hpa*II/PCR methylation assay for the androgen-receptor gene was performed on buccal mucosal DNA, as described elsewhere (Monteiro et al. 1998). The 19 diamniotic MC-MZ twins have been analyzed elsewhere (Monteiro et al. 1998). The repeat assays on singletons were done on buccal mucosal DNA; separate buccal samples were analyzed at different times (Monteiro et al. 1998).

These data strongly support the hypothesis that MC twinning occurs during a relatively broad time frame following X inactivation. Some MC pairs apparently result from splitting quite soon after commitment to X inactivation, leading occasionally to measurable X-inactivation differences between the members of the pair, as much as 15% (fig. 1). In contrast, when twinning occurs late, leading to MA anatomy, members of the twin pair have virtually identical patterns of X inactivation. This conclusion depends on the assumption that roughly equal numbers of cells go to each embryo after splitting (Monteiro et al. 1998). Clearly, highly asymmetric splitting cannot explain MA twinning events,

even though twins in this subgroup are more likely to exhibit birth-weight differences (C. Derom, unpublished data). Of course, asymmetric splitting might occur occasionally but result in one or both fetuses being nonviable. Interestingly, there is marked female predominance in the MA-twin group, with a sex proportion of .23 (Derom et al. 1988). The reasons for this are unclear. It appears that female embryos are relatively delayed in early embryonic development (Pergament et al. 1994). Thus, female embryos could be somewhat less mature at the time of formation of the amnion, and thus splitting of female embryos may be more compatible with survival at this stage. The delay in early female development has been ascribed to the absence of a Y chromosome (Pergament et al. 1994). However, the process of X inactivation, since it may occur when there are $\leqslant$10 cells in the embryo (Puck et al. 1992; Monteiro et al. 1998), might itself contribute to a slight delay in early female-embryo development.

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Refinement of the Locus for Autosomal Recessive Retinitis Pigmentosa (RP25) Linked to Chromosome 6q in a Family of Pakistani Origin

To the Editor:

"Retinitis pigmentosa" (RP) is the term used to define a clinically and genetically heterogeneous group of retinal degenerations primarily affecting the rod photoreceptors. RP is characterized by progressive loss of vision, initially manifesting as night blindness and reduction in the peripheral visual field and later involving loss of central vision (Bird 1995). Ophthalmoscopic examination typically reveals pigmentary disturbances of the mid-peripheral retina. RP may be inherited as an autosomal recessive, autosomal dominant, digenic, or X-linked trait. Autosomal recessive RP (arRP) accounts for ~20% of all cases of RP, whereas sporadic RP, which is presumed to be recessive in most cases, accounts for a further 50% (Jay 1982).

Mutations causing arRP have been found in the genes encoding rhodopsin (Rosenfeld et al. 1992), in the α and β subunits of rod phosphodiesterase (Huang et al. 1995; McLaughlin et al. 1995), in the α subunit of the cyclic-GMP gated–channel protein (Dryja et al. 1995), and in the genes *RPE65* (Gu et al. 1997), *RLBP1* (Maw et al. 1997), *ABCR* (Martinez-Mir et al. 1998) and *TULP1* (Banerjee et al. 1998; Hagstrom et al. 1998). In addition, genetic linkage studies have identified arRP loci at 1q31-q32.1 (van Soest et al. 1994; Leutelt et al. 1995), 2q31-q33 (Bayes et al. 1998), and 16p12.1-p12.3 (Finckh et al. 1998). Recently, linkage of arRP to a region on chromosome 6q has been reported in several Spanish families (Ruiz et al. 1998). All the above are reference at the RetNet website.

We studied 20 members of a three-generation consanguineous Pakistani family in which RP segregated as an autosomal recessive trait (fig. 1). This pedigree contained 12 affected individuals. Examination of all af-

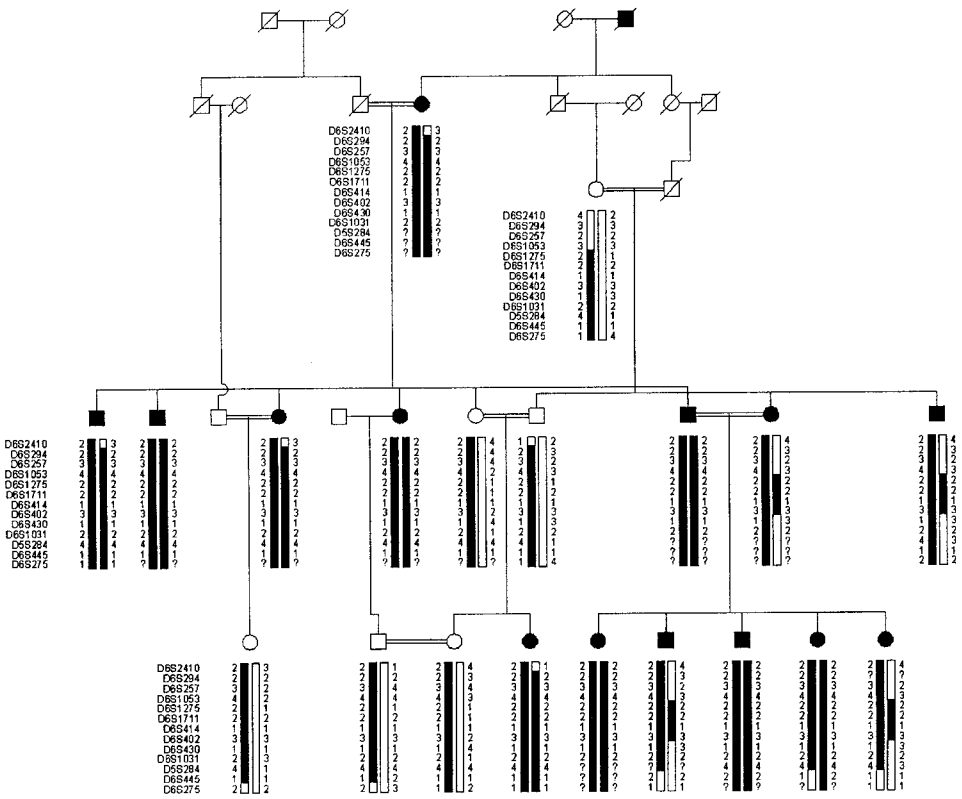

Figure 1 Family pedigree and haplotypes in the disease region of 6q. For each individual, the disease haplotype is shown as a blackened bar.

fected individuals revealed the typical clinical features of RP: pigmentary retinopathy associated with symptoms of night blindness and with the loss of peripheral visual fields. Affected subjects experienced night blindness, beginning at age ~25 years, and deterioration of visual acuity (central vision), beginning at age ~30 years. By age 55–60 years, many affected subjects had no perception of light in either eye.

Genomic DNA for linkage analysis was extracted from the peripheral blood of all 20 individuals in this pedigree (and from 50 unrelated normal individuals of Pakistani origin, who served as controls), by the Nucleon II extraction kit (Scotlab Bioscience). To identify the locus responsible for disease in this family, we performed homozygosity analysis. Genomic DNA from each individual was genotyped for microsatellite markers for all the known arRP loci (*RPE65* [1p31], *ABCR* [1p21-13], 1q31-q32.1, 2q31-q33, *RHO* [3q21-q24], *PDE6B*

Table 1

LOD-Score Calculations for Markers Used to Show Linkage in the Family

	LOD Score at θ =								
Marker	0	.01	.05	.1	.2	.3	.4	Z_{max}	θ_{max}
D6S2410	∞	1.38	1.95	2.03	1.74	1.17	.47	2.03	.1
D6S294	2.83	2.78	2.60	2.33	1.71	1.04	.37	2.83	0
D6S257	3.30	3.25	3.02	2.71	2.02	1.26	.48	3.30	0
D6S1053	3.30	3.25	3.01	2.69	1.98	1.21	.44	3.30	0
D6S1275	2.29	2.25	2.07	1.83	1.34	.82	.32	2.29	0
D6S1711	1.88	1.85	1.72	1.55	1.19	.77	.32	1.88	0
D6S402	1.88	1.85	1.72	1.55	1.19	.77	.32	1.88	0
D6S430	3.13	3.08	2.88	2.60	1.96	1.23	.47	3.13	0
D6S284	∞	−.52	.09	.26	.29	.19	.06	.29	.2
D6S445	∞	−3.06	−1.65	−1.03	−.43	−.14	−.02	−.02	.4
D6S275	∞	−.95	−.34	−.13	0	.01	.01	.01	.3

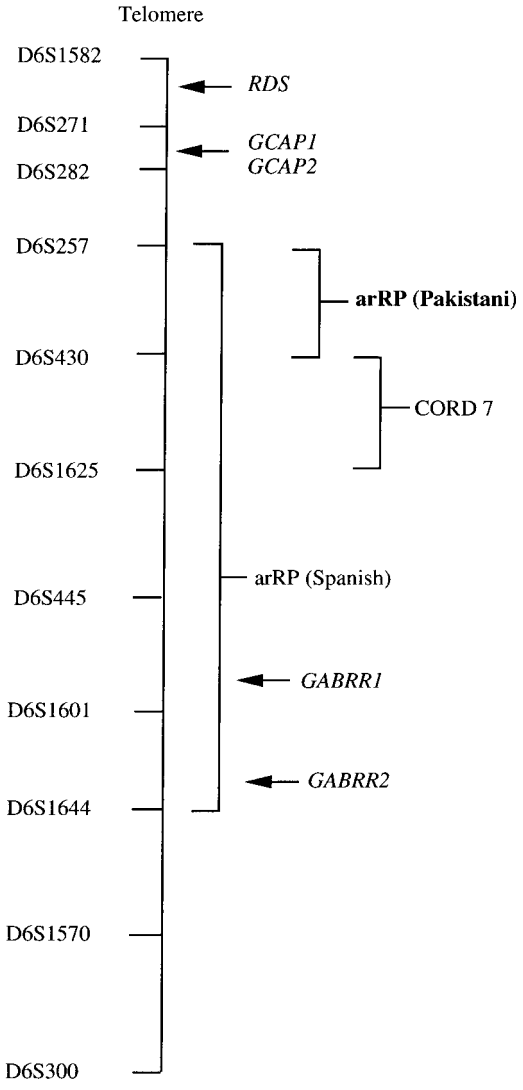

Figure 2 Boundaries of the chromosomal interval containing the 6q arRP gene, in relation to the critical intervals for other eye disorders mapping to this region. Our results indicate either that GABA-receptor candidate genes are no longer candidates for this phenotype or that there are in fact two arRP loci in close proximity on 6q.

[4p16.3], *CNGC* [4p14-q13], *PDE6A* [5q31.2-q34], *TULP-1* [6p21.3], 6cen-q15, *CRALBP* [15q26], and 16p12.1-p12.3). Subsequently, when evidence of linkage was obtained for the 6q locus, further polymorphic markers from this region were analyzed to determine whether analysis of recombinant individuals within the family would permit further refinement of the published disease interval. Marker order was determined from the Généthon sex-averaged genetic map (Dib et al. 1996). Primers were obtained from the MapPairs set (Research Genetics), or were synthesized commercially according to data from Genome Database.

PCR products were separated by nondenaturing PAGE (Protogel; National Diagnostics) and were visualized under UV illumination after being stained with ethidium bromide. Alleles were assigned to individuals, which allowed calculation of two-point LOD scores by the Cyrillic v2.01 (Cherwell Scientific) and MLINK software programs. Allele frequencies were calculated on the basis of data from the spouses in this family and from an ethnically matched control population. The phenotype was analyzed as an autosomal recessive trait, with complete penetrance and a frequency of .0001 for the disease allele.

Significant linkage initially was obtained for three markers on chromosome 6q (table 1). A maximum LOD-score (Z_{max}) value of 3.30 at recombination fraction (θ_{max}) 0 was observed for markers D6S257 and D6S1053 on chromosome 6q (table 1). Recombination events involving the centromeric marker D6S1053 and the telomeric marker D6S430 subsequently permitted refinement of the 6q arRP (RP25)-disease critical region, from the previously reported 16.1 cM (Ruiz et al. 1998) to 2.4 cM (fig. 1).

In their initial linkage report, Ruiz et al. (1998) have suggested, on the basis of a common haplotype for the region surrounding the GABA-receptor candidate genes (*GABRR1* and *GABRR2*) in two families with 6q-linked arRP, that the gene for 6q arRP lies in that region; they therefore have proposed the GABA-receptor candidate genes as candidates for this phenotype. The recombination events seen in the family that we studied exclude both *GABRR1* and *GABRR2* as the disease gene and exclude the area of homozygosity seen by Ruiz et al. (1998) from the critical disease interval.

Figure 2 shows the boundaries of the chromosomal interval containing the 6q arRP gene (RP25), in relation to the critical intervals for other eye disorders mapping to this region. If a single gene underlies arRP in both the Spanish families and the Pakistani family that we studied, then our results indicate that GABA-receptor candidate genes are no longer candidates for this phenotype. However, we cannot exclude the possibility that there are in fact two arRP loci located in close proximity on 6q, one in the Spanish population and one in the Pakistani population. There is no overlap between the locus that we report here and that of the dominant cone-rod dystrophy (CORD 7) (fig. 2).

The 2.4-cM critical interval defined by recombination events in the family that we studied contains no well-characterized candidate genes; however, four expressed sequence tags (ESTs) were identified from the human genome transcript map that are expressed in the retina, as were a further 29 ESTs that were of brain origin. These represent the best candidates available at this time. Further analysis of these cDNA clones will be needed before mutation screening in this family can be undertaken.

In the past, linkage to many of the loci identified as associated with RP (dominant or recessive) have been reported in single families. The identification of a family of Pakistani origin, in addition to the five Spanish families in which linkage to this locus has been reported by Ruiz et al. (1998), suggests that this may be an important gene in arRP, since the disease occurs in two different ethnic populations and in many different families that, according to haplotype analysis, are not ancestrally related.

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Electronic-Database Information

URLs for data in this article are as follows:

Genome Database, http://gdbwww.gdb.org/ (for primers)
Human Transcript Map, http://www.ncbi.nlm.nih.gov/SCIENCE96 (for retinal ESTs)
RetNet, http://www.sph.uth.tmc.edu (for linkage of arRP to chromosome 6q)

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Evidence for a BRCA1 Founder Mutation in Families of West African Ancestry

To the Editor:

Inherited mutations in the BRCA1 gene (MIM 113705; GenBank U14680) (Miki et al. 1994) are less common among breast cancer patients of African American ancestry than among those of white ancestry. For example, in a population-based series of breast cancer patients from North Carolina, the prevalence of BRCA1 mutations was 3.3% among white women and 0% among African American women (Newman et al. 1998). Nonetheless, inherited BRCA1 mutations have been identified in families of African and African American ancestry at high risk of breast cancer (Gao et al. 1997; Stoppa-Lyonnet et al. 1997; Panguluri et al. 1999 [in press]). To provide effective genetic testing for African American families at high risk for breast and ovarian cancer, it would be helpful to identify ancient BRCA1 mutations of African origin analogous to ancient mutations in other populations (Simard et al. 1994; Peelen et al. 1997; Petrij-Bosch et al. 1997). Here we have described one apparently ancient, African BRCA1 mutation.

BRCA1 mutation 943ins10 was detected in breast cancer patients from the Ivory Coast (Stoppa-Lyonnet et al. 1997), the Bahamas, and the United States (Arena et al. 1997; Panguluri et al. 1999 [in press]) (fig. 1). To confirm the identity of the mutation for the five probands and their relatives, the critical region of BRCA1 was genotyped by fluorescent sequencing with dRhodamine-dye terminators (Applied Biosystems). Primers 5′-GGAATTAAATGAAAGAGTATG-AGC-3′ and 5′-CTTCCAGCCCATCTGTTATGTTG-3′ revealed the heterozygous frameshift mutation 943ins10, a 10-bp insertion in exon 11, leading to a stop at codon 289. The mutation is a tandem duplication, in a repeated-sequence motif, that could have occurred at any site between BRCA1 nucleotides 926 and 943 (fig. 2). The notation "943ins10" designates the most-3′ site of insertion possible (Antonarakis et al. 1998). The 943ins10 variant can be easily detected on agarose gel by amplification of genomic DNA or cDNA with BRCA1 primers 5′-CTGCTTGTGAAT-TTTCTGAGACGG-3′ and 5′-TGCTGTAATGAGC-TGGCATGAG-3′ under standard conditions. Wild-type BRCA1 sequence yields a product of 184 bp, and 943ins10 yields a product of 194 bp.

Genotypes of relatives in these five families were consistent with BRCA1 943ins10 being a founder mutation of African origin. Nine markers within and flanking BRCA1 were genotyped (Genome Database): D17S1325, D17S1326, and D17S1327 (5′ of BRCA1); D17S1323 (intron 12), D17S1322 (intron 19), and D17S855 (intron 20); and D17S1321, D17S1320, and D17S1185 (3′ of BRCA1) (Neuhausen et al. 1996; Smith et al. 1996). The 943ins10 mutation occurred on a single haplotype spanning D17S1320–D17S1326 (fig. 1), a distance of ~700 kb.

The families inheriting BRCA1 943ins10 were from widespread locales of Africa and the African diaspora: the Ivory Coast, the Bahamas, the southeastern United States, and Washington, DC. The families are not recently related, and the four families in North America can trace their history in this hemisphere to the slavery period. The length of the 943ins10 nonrecombinant BRCA1 region is similar to the length of the shared region flanking the BRCA1 mutation 185delAG. Hence, the ages of these mutations may be comparable (Bar-Sade et al. 1998). The shared BRCA1 region flanking 943ins10 is shorter than the BRCA1 regions flanking 5382insC or 2800delAA, so the African mutation is probably older than these European mutations (Neuhausen et al. 1996; Friedman et al. 1995). West Africans were brought to North America as slaves between 1619 and 1808. Hence, the social history of the families studied indicates that the mutation is >200 years old and could be much more ancient.

Figure 1 indicates additional, known cases of breast and ovarian cancer in each family. In families UM94003 and UM95027, mothers of probands were affected. In families UM96034 and HU003, in which mothers were not affected, the 943ins10 allele was inherited from the father. Age at breast and ovarian cancer diagnosis was $\leq$50 years for all probands and affected relatives. Family IC564 includes four women with breast or ovarian cancer, all of whom live in the Ivory Coast, where breast and ovarian cancer are rare (Parkin et al. 1997). In this family, the mother of a patient with ovarian cancer remains unaffected at age 83 years, though she is likely to carry the mutation. That there are elderly carriers without cancer suggests that nongenetic factors may influence the penetrance of BRCA1 alleles in geographic regions with a low background risk for breast cancer.

The geographic distribution of 943ins10 in North America is intriguing and is not completely known. BRCA1 943ins10 occurred in 3 of 96 African American patients seen at the University of Miami, who had breast cancer diagnosed at an early age, and in 1 of 55 African American patients seen at Howard University in Washington, DC, who had breast cancer diagnosed at an early age or who had families with a high incidence of breast cancer. However, in the population-based Carolina Breast Cancer Study, the mutation did not appear among 263 African American breast cancer patients, 50% of whom were aged <50 years and 50% of whom were aged $\geq$50 years at diagnosis (Newman et al. 1998). The 943ins10 allele has not been observed in any patients

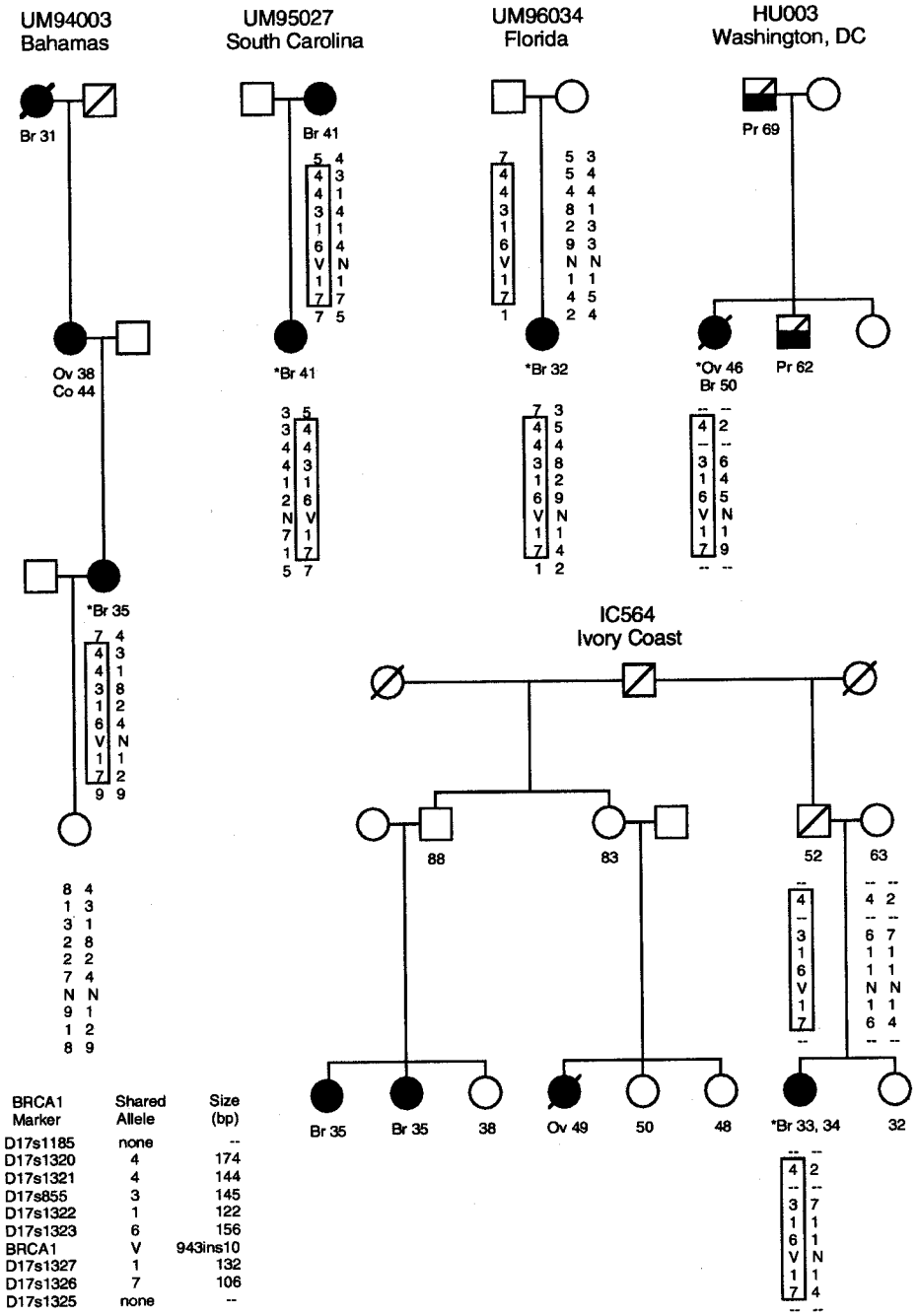

Figure 1 Pedigrees of families carrying the BRCA1 943ins10 mutation. Affected individuals are indicated by a blackened symbol, and probands are denoted by an asterisk (*). The shared haplotype segregating with 943ins10 is boxed. Haplotypes of the fathers of probands in families UM96034 and IC564 have been reconstructed.

with breast cancer who identify their ancestry as solely European.

The migration patterns of African Americans and, hence, the current areas of residence of African American families, may explain the difference, among clinical centers, in the prevalence of the mutation. To determine, among African American women, the proportion of inherited breast or ovarian cancer attributable to BRCA1 943ins10, we would like to encourage testing for this mutation among African American breast and

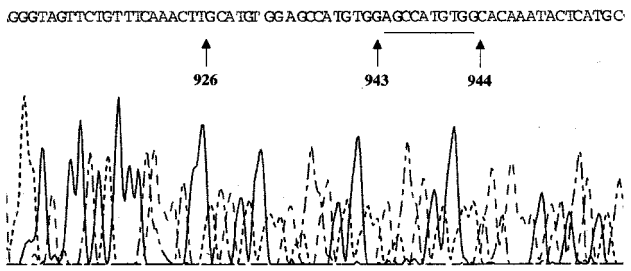

Figure 2 Sequence of the BRCA1 943ins10 mutation. A duplication and insertion of 10 bp causes a frameshift and premature truncation at amino acid 289.

ovarian cancer patients from various regions of the United States. Given the increasing incidence of and higher mortality from breast cancer among African American women, it would be useful to obtain as much information as possible about the roles of BRCA1 and BRCA2 in this population.

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Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Entrez (for BRCA1 [U14680])

Genome Database, http://gdbwww.gdb.org (for D17S1325, D17S1326, D17S1327, D17S1323, D17S1322, D17S855, D17S1321, D17S1320, and D17S1185)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for breast cancer, BRCA1 [MIM 113705])

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Power Comparisons of the Transmission/ Disequilibrium Test and Sib–Transmission/ Disequilibrium-Test Statistics

To the Editor:

Several recent papers have considered the extension of the transmission/disequilibrium test (TDT) to families in which parental DNA is not available but in which unaffected siblings can be sampled. Each of these tests compares the alleles in the affected offspring with those in the unaffected offspring. The tests differ both in the precise statistics used and in the numbers of affected and unaffected offspring included. Spielman and Ewens (1998) have developed the sib TDT (S-TDT) for families with an arbitrary number of affected and unaffected members (including at least one of each). Curtis (1997) has used families with a single affected offspring and an arbitrary number of unaffected offspring but has analyzed only that unaffected offspring who has the genotype most different from that of the affected offspring. Boehnke and Langefeld (1998) have used a discordant-sib-pair approach. The S-TDT is a test of linkage, but it is also valid as a test of allelic association in which precisely one affected sibling and one unaffected sibling are used, as is the case in the tests that have been described by Curtis (1997) and Boehnke and Langefeld (1998).

These authors have considered power in different contexts—for example, across offspring genotype configurations (Spielman and Ewens 1998) and across genetic models (Boehnke and Langefeld 1998)—but none of the approaches used was intended to provide an overall assessment of the power of a sibling-based TDT statistic compared with that of the original formulation of the TDT. Here we derive a relationship between power for the S-TDT and the TDT, which shows that, to achieve similar power, considerably more genotyping is required for the S-TDT than for the TDT. This is intuitively clear, for the following reason. For both tests, a family is informative only if at least one parent is heterozygous. The S-TDT requires an additional condition to be true: both alleles from the heterozygous parent must be present in the offspring. This implies that the informativeness of the S-TDT statistic increases with the number of siblings genotyped. Because of the variation associated with the alleles inherited by the n unaffected siblings, we expect that, for finite n, the S-TDT will be less powerful than the TDT, with the power of the S-TDT tending toward that of the TDT as $n \to \infty$. Below we formalize this argument. Our results extend the power calculations of Spielman and Ewens (1998): in table 5 of their paper, they give the power of both the S-TDT and the TDT, for families with one heterozygous and one homozygous parent, a single affected child, and two to four unaffected children. Their power calculations are conditional on both alleles from the heterozygous parent being present in the offspring, which, as the authors acknowledge, covers only a small proportion of possible family genotype configurations. This conditioning on the offspring genotypes implies that all families are informative for the S-TDT, and therefore it crucially affects the power of the S-TDT. With this conditioning, the power of the S-TDT is almost as great as that of the TDT; without it, the power of the S-TDT may be considerably reduced.

For the sake of simplicity, we consider a sample of k families, assuming that in each family there are a single affected offspring and n unaffected offspring. All individuals have been genotyped at a diallelic marker locus with alleles M and m; let the numbers of M alleles in the offspring in the ith family be X_i for the affected sib and Y_{ij}, $j \in \{1,2,\ldots,n\}$, for the unaffected sibs. We condition on the parental genotypes in the sample and compare the TDT and S-TDT for this sample. The difference between the two statistics can be summarized as follows. The TDT compares $X_\cdot = \Sigma_{i=1}^{k} X_i$, with $\mathbf{E}(X_\cdot \mid H_0)$, where this expected value is calculated from the parental marker information, under the assumption that the null hypothesis is true—that is, either of the two alleles in a heterozygous parent is equally likely to be transmitted to an affected child. The S-TDT, however, is designed for use when this parental information is unavailable; instead, $X_\cdot$ is compared with $Y_\cdot/n$, where $Y_\cdot = \Sigma_{i=1}^{k}\Sigma_{j=1}^{n} Y_{ij}$ is the total number of M alleles in the unaffected offspring.

Our test statistics for the TDT and the S-TDT (T_{TDT} and $T_{\text{S-TDT}}$, respectively) are obtained by the method described, by Spielman and Ewens (1998), as the Z-score procedure: test statistics are standardized to mean 0 and variance 1 and are assumed to follow a standard normal distribution. This gives $T_{\text{TDT}} = (X_\cdot - \mu_0)/\sigma_0$, where μ_0 and σ_0^2 are, respectively, the mean and variance of $X_\cdot$, under the null hypothesis of no linkage.

The S-TDT permutation statistic compares X_i with a permutation of genotypes from the affected and unaffected individuals and then sums the resulting statistic over families. This is equivalent to a comparison of $X.$ with a pool of $X.$ and $Y..$, giving

$$\begin{aligned} T_{\text{S-TDT}} &= \frac{X. - \frac{X.+Y..}{n+1}}{\sqrt{\text{Var}\left(X. - \frac{X.+Y..}{n+1} \mid H_0\right)}} \\ &= \frac{nX. - Y..}{\sqrt{\text{Var}(nX. - Y.. \mid H_0)}} \\ &= \frac{nX. - Y..}{\sqrt{n(n+1)}\sigma_0} , \end{aligned}$$

since, under the null hypothesis, the random variables X_i and Y_{ij} are independent and identically distributed for each i, so $\text{Var}(Y.. \mid H_0) = n\text{Var}(X. \mid H_0)$ and

$$\begin{aligned} \text{Var}(nX. - Y.. \mid H_0) &= n^2\text{Var}(X. \mid H_0) + n\text{Var}(X. \mid H_0) \\ &= n(n+1)\sigma_0^2 . \end{aligned}$$

Note that, whereas Spielman and Ewens (1998) exclude from the S-TDT sibships when all sibs have the same genotype, we include them. This does not affect the value of the test statistic, because such families have 0 mean and 0 variance, but it does facilitate comparisons of the TDT and S-TDT, because both test statistics now use the same set of families.

We can define a second TDT statistic in these families, looking at inheritance of M alleles from heterozygous parents to unaffected children, giving $T'_{\text{TDT}} = (Y.. - n\mu_0)/\sqrt{n}\sigma_0$. Then, using the expression for T_{TDT} above, we can write $T_{\text{S-TDT}}$ as

$$T_{\text{S-TDT}} = \frac{1}{\sqrt{n+1}}(\sqrt{n}T_{\text{TDT}} - T'_{\text{TDT}}) .$$

Power comparisons of T_{TDT} and $T_{\text{S-TDT}}$ can be obtained through the expected values and variances of these statistics. For the models appropriate for many complex diseases, the probability that an M allele is transmitted from a heterozygous parent to an unaffected sib is very close to .5 (Spielman and Ewens 1998), so the genotypes of unaffected offspring can be treated as random observations from the parental genotypes. Then $\mathbf{E}(Y..) \approx n\mu_0$, and $\mathbf{E}(T'_{\text{TDT}}) \approx 0$, giving $\mathbf{E}(T_{\text{S-TDT}}) \approx \sqrt{n/(n+1)}\,\mathbf{E}(T_{\text{TDT}})$.

We now show that the variances of T_{TDT} and $T_{\text{S-TDT}}$ are approximately equal. We define γ_{A} and γ_{N} as the probabilities that an M allele is transmitted from a heterozygous parent to, respectively, an affected or unaffected sibling. Good approximations to the sampling distributions of $Y..$ and $X.$ are $Y.. - c_{\text{N}} \sim \text{Bi}(nh,\gamma_{\text{N}})$ and $X. - c_{\text{A}} \sim \text{Bi}(h,\gamma_{\text{A}})$, respectively, where h is the number of heterozygous parents in the sample and c_{N} and c_{A} are constants determined by the number of MM parents in the sample. The approximation arises because the alleles transmitted from parents to a particular child are not independent conditional on the disease status of the child (Bickeböller and Clerget-Darpoux 1995), but it is adequate for most complex diseases and is exactly true for multiplicative disease models (e.g., see Whittaker et al. 1998). Thus,

$$\text{Var}(X.) = h\gamma_{\text{A}}(1 - \gamma_{\text{A}}) = h\left[\frac{1}{4} - \left(\gamma_{\text{A}} - \frac{1}{2}\right)^2\right]$$

and

$$\text{Var}(Y..) = nh\gamma_{\text{N}}(1 - \gamma_{\text{N}}) = nh\left[\frac{1}{4} - \left(\gamma_{\text{N}} - \frac{1}{2}\right)^2\right] .$$

For complex disease models, γ_{A} and γ_{N} will be sufficiently close to $\frac{1}{2}$ that $\text{Var}(Y..) \approx n\text{Var}(X.)$ and

$$\begin{aligned} \text{Var}(T_{\text{TDT}}) &= \frac{1}{\sigma^2}\text{Var}(X.) \\ &\approx \frac{1}{n\sigma^2}\text{Var}(Y..) = \text{Var}(T'_{\text{TDT}}) . \end{aligned}$$

Conditional on parental genotypes, $X.$ and $Y..$ are independent—and, hence, T_{TDT} and T'_{TDT} are independent—and

$$\begin{aligned} \text{Var}(T_{\text{S-TDT}}) &= \frac{1}{n+1}[n\text{Var}(T_{\text{TDT}}) + \text{Var}(T'_{\text{TDT}})] \\ &\approx \text{Var}(T_{\text{TDT}}) , \end{aligned}$$

as required.

We have shown that $\mathbf{E}(T_{\text{S-TDT}}) \approx \sqrt{n/(n+1)}\,\mathbf{E}(T_{\text{TDT}})$ and that T_{TDT} and $T_{\text{S-TDT}}$ have approximately equal variances. When the standard formula for power (e.g., see Risch and Merikangas 1996) is used, it follows that, if the two tests are to have the same power, then, for the TDT, we require $n/(n+1)$ as many families with a single affected and n unaffected offspring as are required for the S-TDT.

The S-TDT is required only when parental genotypes are missing—and, hence, when σ_0 is unknown and must be estimated from the sib data. Spielman and Ewens (1998) have provided an estimator based on their permutation procedure; an alternative would be to use the

sample SD of $X_i - (X_i + Y_{i\cdot})/(n + 1)$. For large sample sizes the distribution of $T_{\text{S-TDT}}$ is well approximated by the standard normal, whereas for small sample sizes exact tests should be used. The results given above depend on the assumption that the probability that an *M* allele is transmitted from a heterozygous parent to an unaffected sib is .5. This probability is actually slightly <.5, so that $\mathbf{E}(Y_{\cdot\cdot})$ is slightly less than $n\mu_0$, and the formula above slightly understates the power of the S-TDT; but, for complex diseases, the discrepancy is insufficient to be of practical importance.

These results allow us to evaluate the optimum number of unaffected sibs to genotype if multiple unaffected sibs are available. Using only one unaffected sibling ($n = 1$) will require twice the number of families as is required for the TDT. Two unaffected siblings ($n = 2$) give the same genotyping load per family as is given for the TDT but require 50% more families to achieve the same power. These results can also be used to consider the trade-off between genotyping costs and power. For example, for a specific number of genotypes, maximum power is obtained for the S-TDT by inclusion of only one unaffected sibling per family.

The loss of power in the S-TDT may be severe, particularly if only a single unaffected sib is available. Also, of course, families with no unaffected sibs cannot be used in the S-TDT. However, the extension of TDT to sibling-based sampling will allow family-based association testing to be performed for late-onset diseases when parental samples are not available. In this case, the loss of power will be outweighed by the utility of the study design. An overall assessment of design of a study can be made, allowing for the availability of different family members and for costs of family ascertainment, phenotype testing and genotyping.

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