Letters to the Editor

Am. J. Hum. Genet. 64:651–653, 1999

Both Recessive and Dominant Forms of Anhidrotic/Hypohidrotic Ectodermal Dysplasia Map to Chromosome 2q11-q13

To the Editor:

Ectodermal dysplasias (EDs) are a group of conditions characterized by the abnormal development of ectodermal-derived structures including teeth, hair, and eccrine sweat glands. EDs may be either isolated or associated with other clinical manifestations. Hitherto, >100 ED syndromes have been delineated (Freire-Maia and Pinheiro 1988). Whereas the X-linked form of anhidrotic/hypohidrotic ED is well characterized (MIM 305100), the existence of autosomal dominant (MIM 129490) or recessive forms (MIM 224900) has long been discussed. Partial resolution of this controversy was provided when an autosomal dominant form of hypohidrotic ED in a six-generation family was mapped to chromosome 2q11-q13 (Ho et al. 1998). Clinical features included smooth dry skin, hypotrichosis, decreased sweating, and dental anomalies in most affected individuals in that family. The existence of autosomal recessive anhidrotic/hypohidrotic ED is supported by the occurrence of the disease in several families, including a large inbred Moroccan kindred (Munoz et al. 1997; Kabbaj et al. 1998). Here we show that a gene for autosomal recessive ED maps to 2q11-q13, suggesting that dominant and recessive ED may be allelic disorders.

In a large inbred Moroccan kindred, all 14 individuals affected with autosomal recessive ED presented with hypotrichosis, hypodontia, and anhidrosis (Kabbaj et al. 1998). Six of the affected children died in early childhood, probably as a result of dehydration episodes. After obtaining informed consent, we collected blood samples from a total of 32 family members, including 8 affected children. DNA was prepared by standard methods and genotyping was done as reported elsewhere (Belin et al. 1998). After the exclusion of several chromosomal regions encompassing candidate genes, we tested polymorphic markers in the chromosome 2q11-q13 region in which a gene for dominant ED has been mapped (Ho et al. 1998). LOD scores were computed with the LIPED program, version 5.0, under the assumption of autosomal recessive inheritance with complete penetrance and a disease allele frequency of .001 (Ott 1974). Positive two-point LOD scores were obtained with several markers in the region 2q11-q13, with a maximum LOD score of 7.4, at a recombination fraction of zero, with D2S293 (table 1). The allele shared by all the patients at D2S293 was not found on the normal chromosomes of 11 obligatory ED carriers, which suggests the existence of a linkage disequilibrium.

A common haplotype between D2S113 and D2S2269 was identified in six of seven parents of the patients, with an apparent ancestral recombination at the locus D2S135 in individual 21 (fig. 1). Recombination events were observed in patients 24, 25, and 44, at loci D2S373, D2S1895, and D2S121, respectively, which made it possible to locate the disease locus in the 7.47-cM interval between loci D2S135 and D2S121. In all the patients, homozygosity for each of the markers was present in this interval.

During the mapping of a gene for dominant ED, Ho et al. (1998) found several discrepancies in the marker order, throughout the 2q11-q13 region, between the Marshfield genetic map and the Whitehead physical YAC contig sequence-tagged site (STS) content map. In their analyses, which were consistent with the marker order in the Whitehead physical STS content map, they delineated recombinant haplotypes and defined the proximal flanking boundary for the gene for dominant ED (*ED3,* previously known as "EDA3" [Ho et al. 1998]) at D2S1321 and the distal flanking boundary at D2S308. This region entirely contains the interval in which we mapped a gene for recessive ED (fig. 2).

The possibility that affected individuals might be ho-

Table 1

LOD Scores for Linkage of the Locus for the Autosomal Recessive Form of Anhidrotic ED to Chromosome 2q11-q13 Markers

	LOD Score at Recombination Fraction of					
Marker	.00	.05	.10	.20	.30	. 40
D2S373	$-\infty$	−2.82	−.94	−.08	.09	.07
D2S293	7.40	6.86	5.78	4.14	2.53	1.04
D2S1890	5.81	5.39	4.57	3.30	2.04	.89
D2S160	6.33	5.84	4.97	3.59	2.24	.99
D2S121	.34	2.52	2.69	2.03	1.29	.58
D2S1895	$-\infty$	2.89	3.34	2.57	1.61	.68

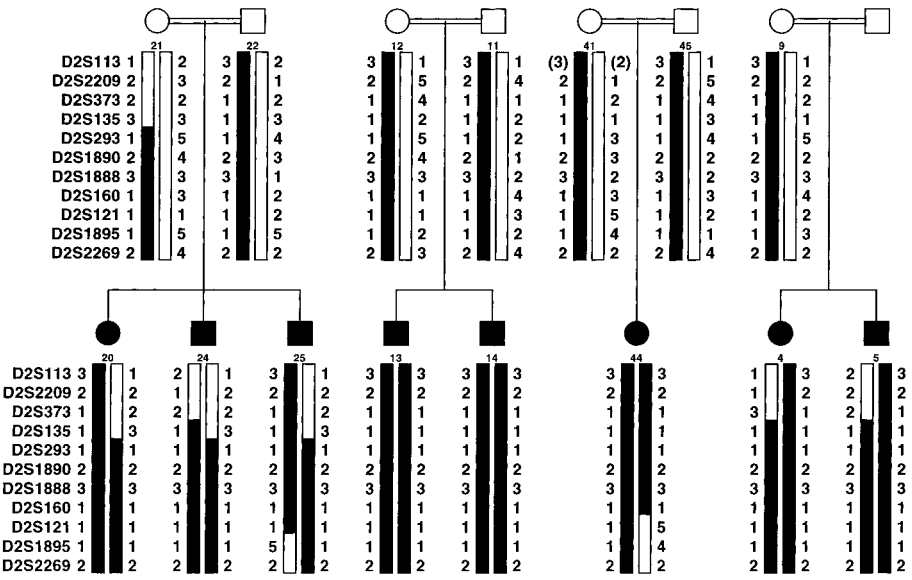

Figure 1 Haplotypes of the chromosome 2q11-q13 region of the patients and their parents. The haplotypes were inferred on the basis of the analyses of additional family members examined but not shown. A complete pedigree of the family appears elsewhere (Kabbaj et al. 1998).

mozygous for a dominant allele with very mild expression in heterozygote carriers was considered. However, the obligate carriers were reexamined and none of them presented any sign of ED. Two main explanations can account for the localization of dominant and recessive ED to the same chromosomal region. First, one can hypothesize that a cluster of genes with related function maps to chromosome 2q11-q13. The existence of gene clusters coding for related proteins such as collagens or keratins is well known, and linked genes may encode various subunits of a single multimeric complex. For instance, mutations in either SUR1 or KIR6.2, which are tightly linked genes on chromosome 11p15.1, result in persistent hyperinsulinemic hypoglycemia of infancy. Alternatively, autosomal recessive and dominant ED might be allelic disorders, as previously shown in several diseases. For instance, collagen gene mutations may cause either dominant or recessive disorders, depending on their nature. Indeed, mutations in COL11A2 cause either Stickler syndrome, a dominant disorder, or OSMED, a recessive bone dysplasia (Vikkula et al. 1995).

As mentioned by Ho et al. (1998), the candidate region contains several genes and anonymous expressed sequences, none which seem to be reasonable candidates by their function or by homology with mouse mutant phenotypes. The study of additional families of both recessive and dominant ED should allow reduction of the interval, toward the cloning of the gene or genes involved in these disorders.

Acknowledgments

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Electronic-Database Information

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Center for Medical Genetics, Marshfield Medical Research Foundation, http://www.marshmed.org/genetics (for marker order in the 2q11-q13 region)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for X-linked anhidrotic/hypohidrotic ED [MIM 305100], autosomal dominant ED (MIM 129490], and autosomal recessive ED [MIM 224900])

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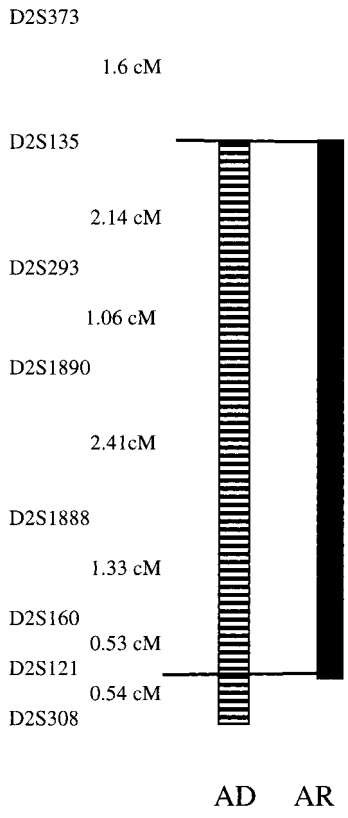

Figure 2 Comparison of the candidate regions on chromosome 2q11-q13 for autosomal dominant (AD) and recessive (AR) ectodermal dysplasia. The order of the principal markers used in the study and the distances are those of the Marshfield sex-averaged chromosome 2 linkage map. Since loci D2S135 and D2S1321 are very close to each other, only D2S135 appears in the figure.

Genome Research, http://www-genome.wi.mit.edu (for marker order in the 2q11-q13 region)

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Mosaicism and Sporadic Familial Adenomatous Polyposis

To the Editor:

Familial adenomatous polyposis (FAP [MIM 175100]) is an autosomal dominant heritable disorder caused by germ-line mutations in the APC gene (M74088 [GenBank]). There is a high new-mutation rate, with ~25% of all cases being sporadic (Bisgaard et al. 1994). Parental mosaicism can explain new mutations in genetic disorders (Hall 1988), whereas germ-line and/or somatic mosaicism has been described in genes associated with tumors such as p53 (Kovar et al. 1992), Rb1 (Greger et al. 1990; Lohmann et al. 1997), NF1 (Lázaro et al. 1995), and NF2 (Bourn et al. 1994). We were interested to determine whether APC-gene mutational mosaicism could account for some of the apparentlynew" APC mutations. Systematic studies of our registry identified five in which a germ-line mutation was established (Prosser et al. 1994), parental leukocyte DNA was available, and paternity was assured. These five patients were the subjects of detailed studies to determine the level of parental APC mutational mosaicism (table 1).

During S^{35} sequencing of parental blood-leukocyte DNA samples from the first sporadic FAP case, we noted a faint mutant allele that was reproducible on repeated analyses. The clinical history of this sporadic FAP patient (patient 17) is noteworthy. Dense distal colonic polyposis was diagnosed at age 22 years, with three distinct carcinomas and myriads of smaller polyps, many of which showed carcinomatous change. Mastectomy was required for breast carcinoma when the patient was age 37 years, and disseminated ovarian cancer resulted in death at age 44 years. Her mother (patient 16) had a mastectomy for breast carcinoma at age 46 years, was negative in a screen for colonic polyps when she was age 64 years, and subsequently died from ovarian cancer at age 67 years. There was an extensive family history

Table 1

APC Mutations and Parental-Mosaicism Screening Strategy for Sporadic FAP Patients

Patient	Nucleotide Change	Effect on Coding Sequence	Parental-Mosaicism Screening Assay
17	1354delGT	Frameshift	S^{35} sequencing, color-selective and nonselective PCR, cloning
148	694C→T	Arg→stop at 232	Mutation-specific restriction digestion and PCR enrichment
410	4192G→T	Glu→stop at 1398	PCR cloning, single-cell analysis
893	3926delAAAAG	Frameshift	PCR cloning
1024	4135C→T	Gln→stop at 1379	Single-cell analysis

NOTE.—In all cases, a heterozygous mutation was excluded by standard analysis of parental leukocyte DNA, and paternity was assured. A description of each assay can be obtained from the corresponding author.

of breast cancer due to a BRCA1 mutation (5382insC mutation [Mullen et al. 1997]), with several cases of gastric and colon cancer in the extended family. Patient 17 carried the 5382insC BRCA1 mutation, as well as an APC mutation (table 1).

The APC mutation identified in patient 17 was within a simple repeat region, a GT deletion in a run of four GT dinucleotides in exon 10 (table 1). On observing the faint mutant allele in the maternal leukocyte-DNA sample (patient 16), we were first concerned that it might represent sample contamination by leukocyte DNA of patient 17. However, sequencing of two other samples from patient 16 that had been taken several years apart confirmed the faint mutant allele, and we never saw this sequence in paternal or control leukocyte DNAs. To exclude the possibility that the mutant sequence was due to PCR error, we devised a PCR cloning strategy that reduced the number of PCR cycles and allowed easy identification of bacterial clones carrying the 1354delGT allele. We designed PCR primers for cloning into pBluescript that were out of frame for wild-type sequence and in frame for β-galactosidase in the presence of the patient 17 APC mutation (details are available from the corresponding author, on request). This gave blue colonies for mutant alleles and white colonies for the wild type.

Plating of PCR clones derived from patient 17 gave the expected 50% blue colonies. Ten blue colonies were picked at random and were sequenced, confirming the 1354delGT allele in every clone. All 10 white colonies picked were wild-type sequence, as expected. The blue:white colony ratio was substantially lower in clones derived either from patient 16 or from control DNAs, typically <10% blue colonies in replicate PCR cloning experiments. Approximately 60% of blue clones derived from patient 16 and from control DNA were due to plasmid rearrangements. However, sequencing of 37 blue clones with the correct-size PCR insert from patient 16 DNA identified eight alleles that were identical to the 1354delGT mutation in patient 17 (fig. 1), demonstrating the utility of our PCR cloning–enrichment strategy. Although some blue colonies were due to plasmid rearrangements, mutant sequence was never identified in any clones derived from control DNA, in multiple experiments.

To formally quantify the level of mosaicism in blood leukocytes, PCR amplification products from patients 16 and 17 and from control leukocyte-DNA templates were cloned without selection for color. Mutation screening using heteroduplex analysis of 41 clones that contained the correct-size insert derived from patient 17 DNA templates gave 21 mutant and 20 wild-type alleles. For patient 16's leukocyte DNA, 310 colonies were picked, and 113 contained the correct-size APC fragment, 4 (3.5%) of which were 1354delGT mutant alleles. Screening of 300 colonies derived from control DNA identified 160 clones containing correct APC amplification products, and none were mutant alleles (0/160 vs. 4/113; $P < .03$, by Fisher's exact test). These data establish that patient 16 DNA contained a small proportion of alleles from somatic DNA that are identical to the mutant allele that was responsible for FAP in her daughter. We were unable to determine the tissue distribution of mosaicism, because of the death of patient 16 prior to these studies and because archival paraffin-embedded material had been infected with mold and was destroyed.

Tailored assays were then designed to detect the levels of the respective APC mutation (table 1) in the other four sporadic cases. Preliminary "spiking" experiments demonstrated that an ~0.1% mutant-allele presence was detectable (data not shown). None of the assays detected any other cases of parental mosaicism. Parental blood-leukocyte DNA for patient 148 was analyzed by mutation-specific PCR enrichment; for patient 893, 200 verified colonies for each sample of parental leukocyte DNA were screened by single-strand conformation polymorphism; for patient 1024, PCR products from single-cell preparations of 350 paternal lymphocytes and 120 maternal lymphocytes were analyzed by restriction digestion.

We were surprised to identify a further case of APC mutational mosaicism, in a proband with FAP. Patient 410 has no family history worthy of note but, during the teenage years, developed orbital rhabdomyosarcoma, a tumor very rarely associated with FAP (Lynch et al. 1982; Armstrong et al. 1991). The FAP syndrome

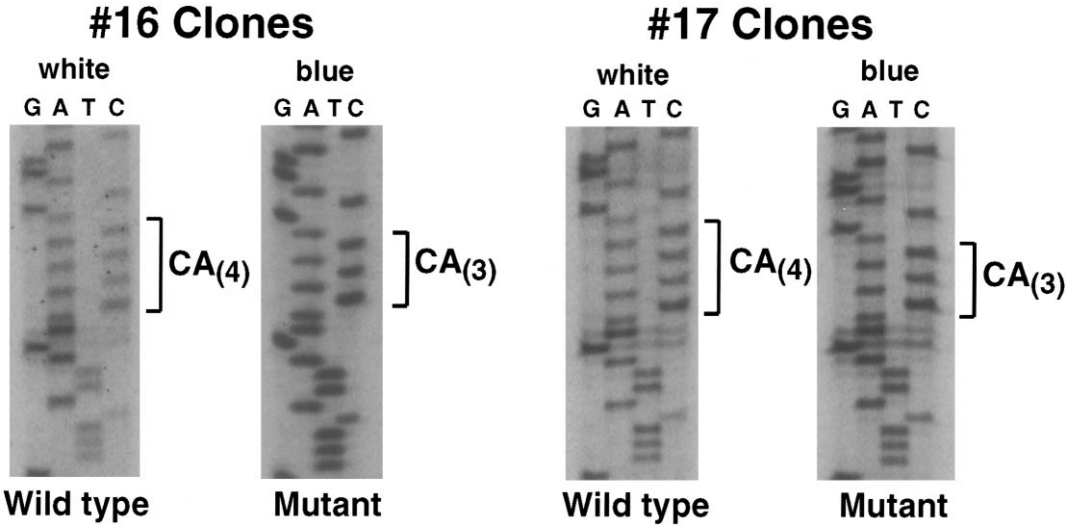

Figure 1 S^{35} sequencing of PCR products from blue and white colonies from patients 16 and 17, showing the presence of mutant (three GT/CA) and wild-type (four GT/CA) alleles, respectively (reverse PCR primer was used to sequence).

followed an aggressive course, with dense polyposis and severe desmoid disease. When testing the primers designed for rapid screening of parental PCR clones, by introduction of a new *Dra*I restriction site into the 4192G→T mutant alleles, we noted a deviation from the expected heterozygote allele ratio. Replicate experiments performed on leukocyte DNA after repeated blood sampling consistently showed that the intensity of the mutant allele was less than expected, even after allowance had been made for the staining intensity of the respective restriction fragments (fig. 2). PCR products from both parents and the patient were cloned, and no mutants were detected in 335 maternal and 210 paternal alleles. However, there were 98 wild-type and only 38 mutant alleles identified on analysis of colonies from patient 410, a result significantly different ($P < .0003$) from the expected 1:1 ratio but in accordance with the dosimetry studies using patient 410's blood-leukocyte DNA templates (table 2). Twenty-eight undigested alleles were sequenced, and all confirmed the presence of a wild-type TTTAAA *Dra*I restriction site, excluding PCR error as the explanation for these findings. Sequencing of three of the clones that did digest with *Dra* I confirmed the presence of the mutant allele, as expected. Further confirmation that a proportion of circulating leukocytes do not carry the mutant APC allele was obtained by sequencing of PCR products from 18 fresh single-cell lymphocyte preparations. Twelve lymphocytes contained exclusively wild-type alleles, whereas the other six showed mutant and wild-type sequence. These studies show that, despite the FAP phenotype, patient 410 is mosaic and does not carry mutant APC in all peripheral blood cells.

We were interested to investigate the tissue distribution of the mutant and wild-type alleles. Determination of the level of 4192G→T mutation in a variety of tissues from patient 410 was possible because surgically resected colon, colonic adenomas, kidney, and orbital rhabdomyosarcoma were available in paraffin-embedded blocks. When the *Dra*I restriction assay was used, substantial mutation-level variation between tissues was noted (fig. 3), with complete absence of the mutant allele in renal tissue. Dosimetry of bands derived from leukocyte DNA agreed well with the results of prior cloning studies (table 2). The mutant:normal volume ratio in rhabdomyosarcoma suggests loss of wild-type allele but with normal-tissue contamination contributing the faint

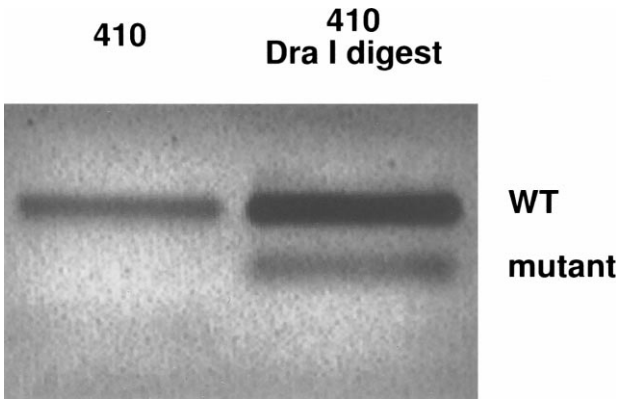

Figure 2 PCR products of patient 410's peripheral blood-leukocyte DNA templates digested with *Dra*I, showing readily visible differences in between wild-type (134-bp) and mutant (112-bp) allele intensities.

Table 2

Quantitation of Mosaicism for the 4192G→T Mutant APC Allele in Patient 410

Tissue	Mutant:Wild-Type Volume Ratio[a]
Peripheral blood leukocyte	.22
Kidney	.08
Normal colonic mucosa	.44
Rhabdomyosarcoma	1.44
Adenoma 1	.42
Adenoma 2	.28

[a] Aggregates of three separate assays for each tissue.

normal allele. The microdissected adenomas have mutant:normal volume ratios that are similar to those for leukocyte DNA, supporting the notion that FAP polyps can be polyclonal in origin (Novelli et al. 1996).

Genetic instability appears to influence the APC mutation spectrum in colorectal tumors (Huang et al. 1996), and so microsatellite analysis (Farrington et al. 1998) was performed on DNA purified from archival paraffin-embedded tissues from patients 17 and 410. Patient 17's colon tumor showed instability at three of the four CA-repeat markers analyzed but at none of three poly-A markers (fig. 4). Although breast cancers rarely show microsatellite instability (Wooster et al. 1994), we are not aware of any report describing microsatellite analysis in colon-tumor tissue from a BRCA1-mutation carrier. Patient 410's tumors showed low-level instability for three CA-repeat markers that gave sufficient data for analysis (different markers in each tumor), but there was no poly-A instability. Genomic sequencing of hMSH2 (U03911/U04045 [GenBank]) and hMLH1 (U07343/U07418 [GenBank]) (Farrington et al. 1998) in patients 16, 17, and 410 did not identify any pathological variants, although hPMS1, hPMS2, and hMSH6 were not analyzed.

In this study, we have established that combined somatic/gonadal mosaicism for an APC mutation can arise rarely and that a clinical phenotype attributable to that mutation is not always apparent. In addition, we have shown germ-line transmission of the mutant allele to offspring. There may be a phenotypic threshold that is likely related to both the overall contribution and the tissue distribution of the mutant allele, as was found in patient 410. Presumably, the patch size in patient 410 was critically distributed, to allow expression of the FAP phenotype. There is one other report of a mosaic APC-gene variant in a patient with FAP (Mandl et al. 1994); however, this variant was not pathogenic, since it was incidental to a frameshift APC mutation segregating with FAP in that family.

Our findings have clinical relevance to counseling regarding recurrence risk to siblings of "sporadic" FAP patients with phenotypically normal parents who do not appear to carry the APC mutation as assessed by conventional methods. We have shown that gonadal mosaicism is a possibility and that therefore the risk to siblings of an index sporadic case is higher than the population risk. Somatic mosaicism has been suggested as a common cause of classic neurofibromatosis 2 (Evans et al. 1998), and isolated gonadal mosaicism has recently been demonstrated in tuberous sclerosis (Yates et al. 1997), emphasizing the broader clinical relevance of our findings. In addition to the implications with regard to germ-line transmission, somatic mutational mosaicism of the APC gene has relevance to cancer risk, since APC plays a pivotal role in neoplastic transformation (Kinzler and Vogelstein 1996). Individuals may carry, in a small proportion of epithelial stem cells, a mutant APC allele that confers an increased cancer risk, even though there is no obvious evidence of FAP.

We hypothesize that APC mutational mosaicism can be due to genetic instability and that passage through the germ line of instability-prone individuals represents a mechanism by which new mutations might arise. In this context, the predominance of CA:GT dinucleotide-repeat instability is noteworthy; and the new germ-line mutation in patient 17 also arose within a short CA:GT repeat in APC. Although we did not identify any germ-line hMSH2 or hMLH1 mutations in patients 16, 17, or 410, the presence of tumor instability associated with mosaicism is intriguing. A constitutional mutator phenotype due to mismatch repair–gene mutations has been reported in association with increased mutation rate in

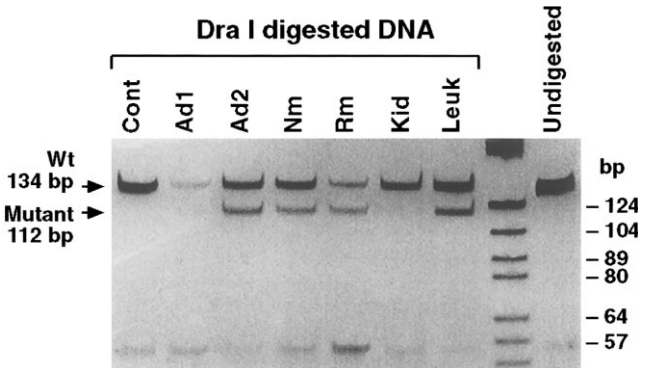

Figure 3 Investigation of dosage of mutant APC allele (4192G→T) in various tissues from patient 410, with *Dra*I digestion of PCR products. Control (i.e., nonmutant) leukocyte DNA lacks the *Dra*I site and so gives a full-length 134-bp fragment on *Dra* I digestion. Results for two adenomatous colonic polyps (lanes Ad1 and Ad2), normal colonic mucosa (lane Nm), rhabdomyosarcoma (lane Rm), renal tissue (lane Kid), and peripheral blood-leukocyte DNA (lane Leuk) are shown.

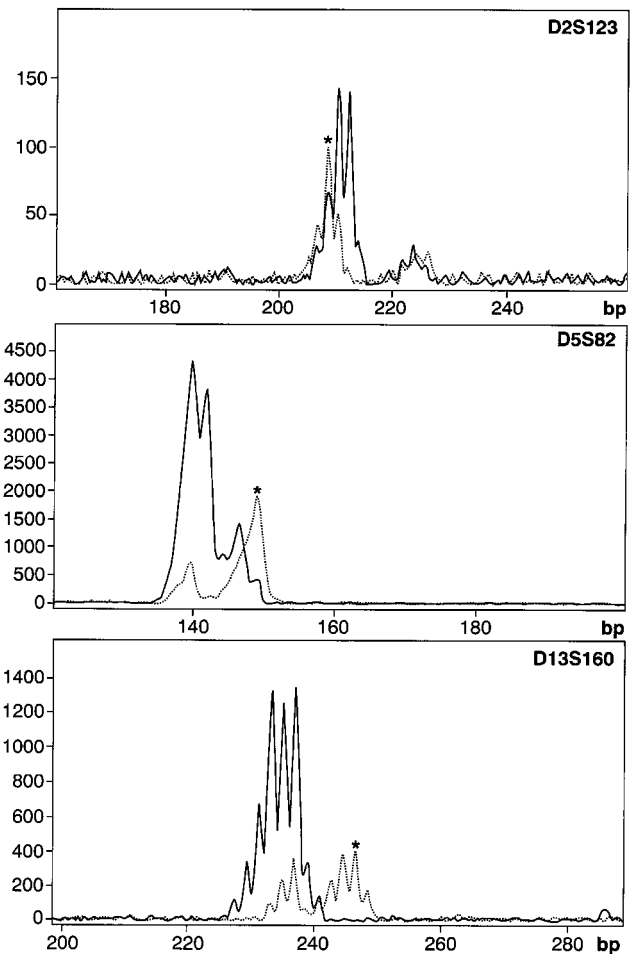

Figure 4 Traces from ABI 310 genetic analyzer with GeneScan software, showing band shifts between patient 17's normal-mucosa DNA template (*solid line*) and colon-cancer DNA templates (*dotted line*), for three CA-repeat markers analyzed. The asterisks (*) indicate the band shifts observed in the tumor tissue.

normal tissues (Parsons et al. 1995; Hackman et al. 1997). Indeed, one of these reported patients had clinical FAP without a detectable heterozygous APC mutation. Of further interest, a defined MLH1 mutation has been reported in association with transmission of instability at the FRAXA locus (Sharrock et al. 1997).

Both the atypical phenotype in these patients and the association with other molecular events—namely, BRCA1 mutation and microsatellite instability—is intriguing and may reflect an underlying process leading to genetic instability. Mutation of BRCA1 would not be expected to induce tumor microsatellite instability, but we estimate that the probability of the association of mutations in BRCA1 and APC is 1/10 million (Bisgaard et al. 1994; Ford et al. 1995), and so a chance occurrence seems remote in the case reported here. It will be of interest to determine whether mosaicism for mutations in other genes is a more general phenomenon than has been previously recognized.

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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 APC cDNA [M74088], hMSH2 cDNA [U03911 and U04045], and hMLH1 cDNA [U07343 and U07418])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for colorectal cancer [MIM 114500] and FAP [MIM 175100])

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HPS Gene Mutations in Hermansky-Pudlak Syndrome

To the Editor:

We recently reported a series of mutations of the *HPS* gene in non–Puerto Rican patients with Hermansky-Pudlak syndrome (MIM 203300) (Oh et al. 1998). One of these mutations was designated incorrectly in some places in the article; a frameshift in codon Q397 was incorrectly designated "E397" in the text (this mutation is now designated "c1189delC," in the new nomenclature; Antonarakis 1998). In addition, subsequent to publication we determined that patient 20 is homozygous for a novel frameshift due to a single-base deletion in codon G96, designated "c288delT," with the first-cousin parents both being heterozygous for this mutation. Thus, *HPS* gene mutations have now been identified in all patients and families who show apparent linkage to 10q23. With our original description of the gene (Oh et al. 1996) and the recent report of Shotelersuk et al. (1998), this brings the number of reported *HPS* gene mutations to 11 and further underscores the lack of missense mutations identified in patients with this disorder.

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A Novel 22q11.2 Microdeletion in DiGeorge Syndrome

To the Editor:

DiGeorge syndrome (DGS; MIM 188400) is a multiple-malformation syndrome characterized by aplasia or hypoplasia of the thymus; immunodeficiency, aplasia, or hypoplasia of the parathyroid glands; conotruncal cardiac defects; and typical facial anomalies(DiGeorge 1965; Conley et al. 1979). Despite causal heterogeneity (Lammer and Opitz 1986), ~90% of patients with DGS have hemizygosity of an ~1.5–3-Mb region within 22q11.2 (Driscoll et al. 1990; Scambler et al. 1991; Driscoll et al. 1992*a*). Because of phenotypic overlap, the same deletion was demonstrated in the majority of patients with velo-cardio-facial syndrome (VCFS; MIM 192430) (Driscoll et al. 1992*b;* Carlson et al. 1997*b*), which was initially characterized by hypernasal speech caused by cleft palate, cardiac anomalies, learning disabilities, and typical facial appearance (Shprintzen et al. 1978, 1981). In addition, 22q11.2 deletions were observed in cases fitting within the spectrum of Cayler syndrome (MIM 125520) (Giannotti et al. 1994), Takao conotruncal anomaly face syndrome (contained in MIM 188400) (Burn et al. 1993), Noonan syndrome (MIM 163915) (Wilson et al. 1993), Kousseff syndrome (MIM 245210) (Nickel et al. 1994), and Opitz GBBB syndrome (MIM 145410) (McDonald-McGinn et al. 1995). Meanwhile, it became evident that deletion 22q11.2 is associated with a phenotypic spectrum that may present as one of the aforementioned syndromes or any condition in between them that has considerable inter- and intrafamilial variability (De Silva et al. 1995; Leana-Cox et al. 1996; Devriendt et al. 1997; Ryan et al. 1997). Therefore, 22q11.2 microdeletion is one of the most common genetic defects, with an estimated incidence of >1 in 5,000 (Wilson et al. 1994; Tezenas Du Montcel et al. 1996). According to Carlson et al. (1997*b*), 90% of patients with deletions have a common 3-Mb deletion, 8.5% have a proximal 1.5-Mb deletion, and 3% have unique nested proximal deletions, which may define a proximal shortest region of overlap within the commonly deleted 3-Mb region. Because of two patients with small deletions in the distal part of the 3-Mb deletion, a distal shortest region of overlap within the deleted region may also be defined (Kurahashi et al. 1997; O'Donnell et al. 1997). However, there is no obvious correlation between the site or size of the deletion and the severity of the clinical manifestations, and position effects have been taken into consideration (Carlson et al. 1997*b;* Kurahashi et al. 1997; O'Donnell et al. 1997). We describe here a novel 22q11.2 microdeletion in a family with mild to severe phenotype. This deletion is adjacent to but does not overlap with the known deletions. Nevertheless, it shows similar clinical characteristics and may therefore give a clue to the mechanisms and genes involved in phenotype determination in 22q11.2 deletions.

Patient III:3 was primarily investigated in the context of a study of incidence and significance of 22q11.2 hemizygosity in patients with interrupted aortic arch (Rauch et al. 1998*b*). Within that study, she was the only patient with symptoms of the DGS/VCFS spectrum who did not have the 22q11.2 deletion and, therefore, prompted further analysis. Phenotype assessment of the patient, her parents, and her sibs was performed before molecular studies and included dysmorphologic analysis of lymphocyte subpopulations by flow cytometry on an Orthoscan, by means of fluorochrome-labeled antibodies against CD3, CD4, CD8, and CD19; and surface immunoglobulin (according to Becton-Dickinson). Diphtheria toxoid and tetanus toxoid were measured after vaccination, by means of a commercially available enzyme-linked immunosorbent assay (ELISA) (ABICAP; Abion). Parathyroid hormone levels were determined by chemoluminescence-ELISA (Nichols) of the patient's sera. Cardiac status was established by echocardiography and angiography in the patient and by echocardiography only in the patient's parents and sibs. Flexible transnasal pharyngoscopy was performed in the patient's sister and mother, to exclude velopharyngeal insufficiency.

In the patient, conventional karyotyping of GTG-banded chromosomes from peripheral T lymphocytes and fibroblasts was performed at an ~550-band level, according to Mitelman 1995, pp 14–21). The patient, both of her sibs, and her mother were investigated by FISH with the DNA probes D22S75 (ONCOR), cHKAD26 (Kurahashi et al. 1994, 1997) (kindly provided by the Japanese Cancer Research Resources Bank)

and bacterial artificial chromosome (BAC) 438P22 (see below) on metaphase chromosomes from peripheral T lymphocytes. In the patient, FISH was performed with the additional probes Tuple1 (VYSIS), M-bcr/abl, m-bcr/abl (ONCOR), and BAC 458J22. The DNA probes D22S75 and cHKAD26 were also analyzed in metaphases from fibroblasts of the patient. The commercially produced probes were used according to the manufacturer's instructions, or two-color FISH was performed with the critical probe biotin-labeled and with a digoxigenin-labeled centromeric 14/22 probe (ONCOR), as described elsewhere (Rauch et al. 1996). The Research Genetics human BAC DNA pools, release IV, were screened with the polymorphic marker D22S425 (see below), according to manufacturer's instructions. Positive BACs were tested and amplified according to Research Genetics guidelines. Two–color fiber-FISH was performed with BAC 438P22 and 458J22, on fixed cultured T lymphocytes from a healthy control, as described elsewhere (Fidlerova et al. 1994).

The patient and her parents had been tested before, for the following 10 short–tandem-repeat polymorphism (STRP) markers from the 22q11.2 region: D22S264 (Marineau et al. 1992); D22S311 and D22S306 (Porter et al. 1993); D22S427 (Gyapay et al. 1994); D22S941 and D22S944 (Morrow et al. 1995); and D22S1638, D22S1648, D22S1623, and D22S308 (Carlson et al. 1997*a,* Genome Database), as described elsewhere (Rauch et al. 1998*b*). Subsequently, the patient, her sibs, her parents, and her maternal grandparents were tested for STRPs at the loci D22S311 (Genome Database 190609), D22S1709 (Genome Database 5865052), D22S306 (Genome Database 190620), D22S308 (Genome Database 190623), D22S425 (Genome Database 199610), D22S303 (Genome Database 190616), D22S257 (Genome Database 180549), D22S301 (Genome Database 190613), D22S156 (Genome Database 177327), TOP1P2 (Genome Database 159908), D22S1144 (Genome Database 606049; SangerCentre bK929C8), and D22S1167 (Genome Database 610902; Sanger Centre bK373H7), by PCR amplification of DNA extracted from fresh peripheral blood and separation on 6% denaturing polyacrylamide gels (41 cm) in a Li-cor (MWG-Biotech) sequencer, as described elsewhere (Rauch et al. 1998*b*). Additional mapping information about the STRP markers was obtained by both a search of the BLAST database by means of the PCR primer sequences and data produced by the Chromosome 22 Mapping Group at the Sanger Centre, which were obtained from the World Wide Web.

In addition to interrupted aortic arch type B, patient III:3 had truncus arteriosus communis type A4, T-cell deficiency, *Pseudomonas aeruginosa* sepsis, hypoplasia of halluces and toenails, choanal stenosis, retrognathia, and ear anomalies (fig. 1*a–d*). After repair of her congenital heart defect, the patient died neonatally, from heart failure and sepsis. Dysmorphologic analysis revealed subtle anomalies in her sister and mother, whereas her brother and father appeared normal. Minor anomalies in the mother included external strabismus, retrognathia, posteriorly angulated ears, broad neck with low posterior hairline, short 5th fingers (Dubois sign), and a high-arched palate with a minimal nick in the uvula (fig. 1*e* and *f*). Her occipitofrontal circumference (OFC) was 52 cm (<3d centile), and her height was 159 cm (10th centile). She had recurrent bronchitis and otitis media, but immunologic investigations revealed normal results. Despite some learning problems, she attended regular school. Her voice was normal. The 12-year-old sister (III:1) also showed mild retrognathia; thin vermillion border of the upper lip; low-set, posteriorly angulated ears with overfolded helices; high-arched palate with a minimal nick in the uvula; and mild muscular hypotonia (fig. 1*g* and *h*). Her OFC was 52.3 cm (25th centile), and her height was 143.8 cm (10th centile). She had a history of recurrent bronchitis, but immunologic investigations revealed normal results. She attends a special school because of minor learning difficulties. Her voice is normal. Echocardiographic, immunologic, endocrine, and pharyngoscopic studies in the parents and sibs of the patient did not show any abnormalities. There were neither attention-deficit/hyperactivity disorders nor behavioral or psychiatric problems in any family members.

Karyotyping and FISH with the probes D22S75, Tuple1, and cHKAD26, of chromosomes from T lymphocytes and fibroblasts of the patient, did not reveal any chromosomal aberration or microdeletion in the commonly deleted 22q11.2 region. FISH with the probes D22S75 and cHKAD26, in the patient's mother and sibs, also showed normal signals on both chromosomes 22. STRP analyses of seven loci within (D22S1638, D22S941, D22S1648, D22S944, D22S1623, D22S264, and D22S311) and two loci flanking (D22S427 and D22S306) the 22q11.2 deletion region in the patient and her parents demonstrated heterozygosity of five markers in the patient. Four markers showed only one allele, but the parental allele constellation was uninformative. At two of the uninformative markers, the patient's mother was heterozygous. At one further distal marker, D22S308, the patient had not inherited the maternal allele. Subsequent STRP analyses with additional distal markers, in the patient and her family, demonstrated a deletion of D22S308, D22S425, D22S303, and D22S257 in the patient, her mother (II:1), and her older sister (III:1), whereas markers D22S301, D22S156, TOP1P2, D22S1144, and D22S1167 were informative for heterozygosity (fig. 2).

Two BAC addresses—438P22 and 458J22—were identified by library screen with D22S425 and were con-

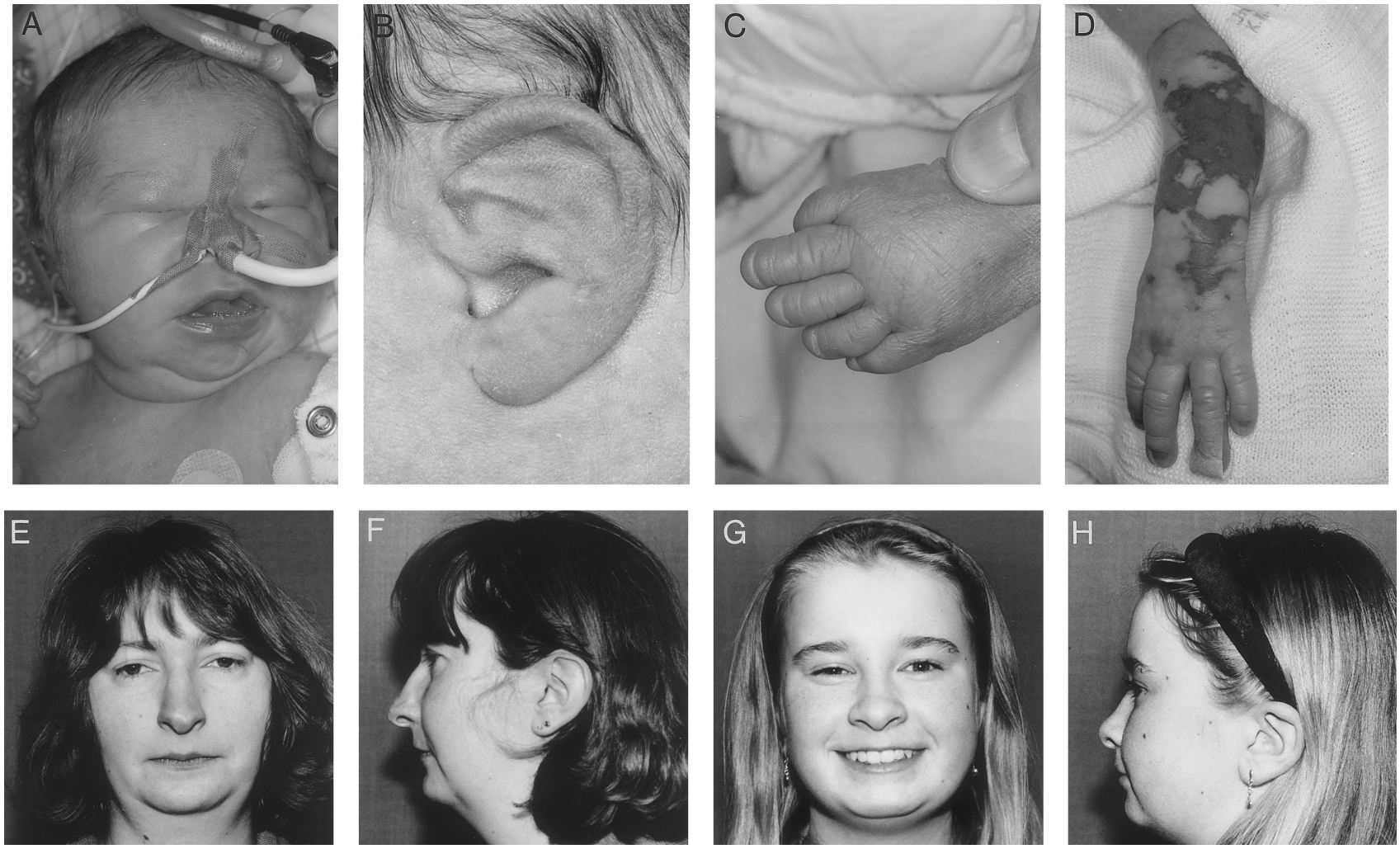

Figure 1 *a–d*, Patient III:3 at age 2 wk. Note retrognathia (*a*), typical short squared-off ears with simple overfolded helix and railway-track sign (*b*), hypoplastic toes and absent halluces (*c*), and purpura fulminans from *P. aeruginosa* sepsis (*d*). *e* and *f*, Facial appearance of the patient's mother (II:1) at age 31 years. Note external strabismus, retrognathia, and posteriorly angulated ears. *g* and *h*, Facial appearance of the patient's sister (III:1) at age 12 years. Note mild retrognathia; thin vermillion border of the upper lip; and low-set, posteriorly angulated ears with overfolded helices.

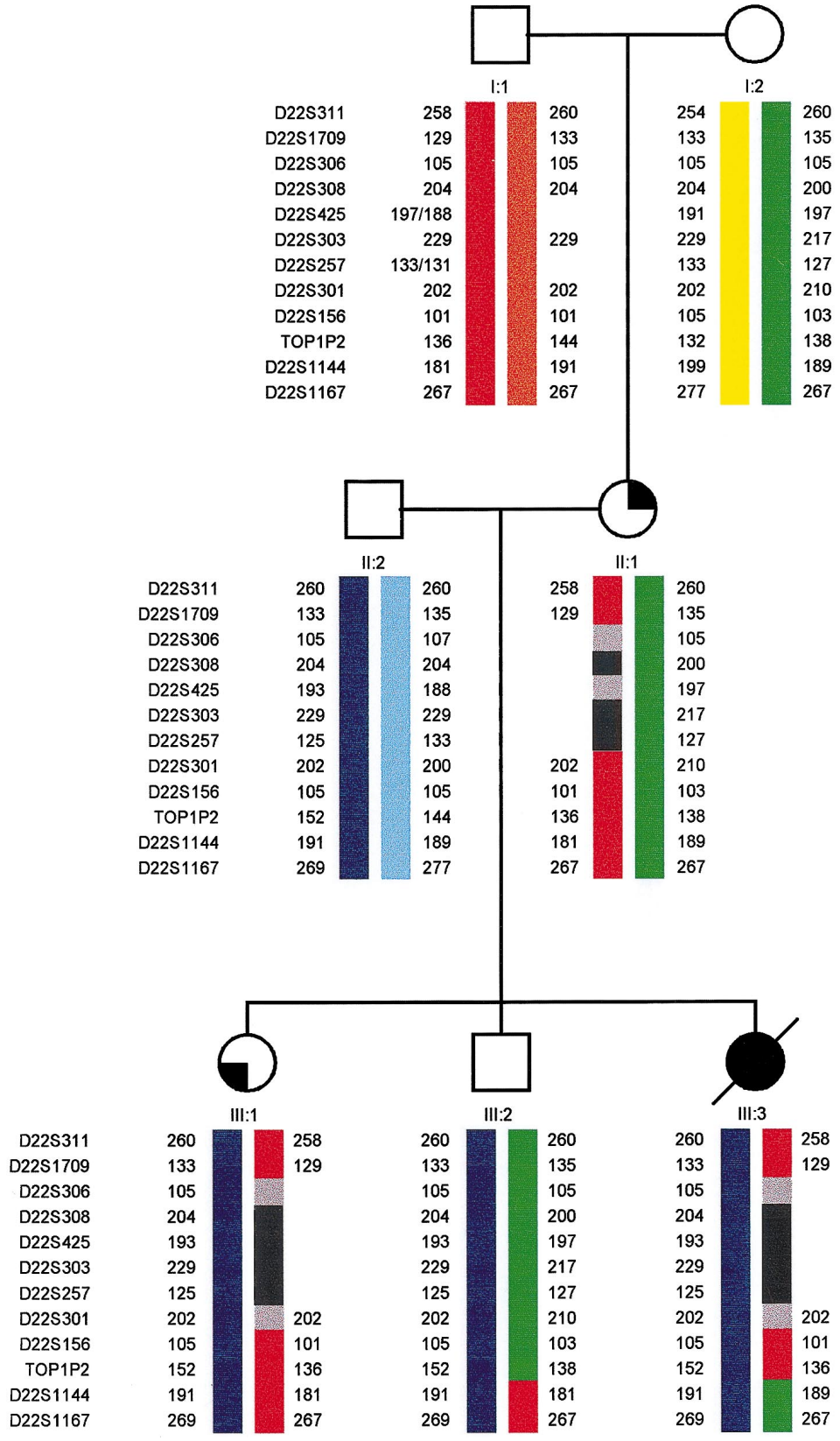

Figure 2 Results of STRP marker analyses in the core pedigree. Black bars denote a deletion; gray bars denote uninformative results either within or flanking the deleted markers. Colors indicate the segregating haplotypes; note the identical paternal wild-type haplotype (*dark blue*) in the mildly and severely affected sisters.

firmed by PCR from single clones. FISH with BAC 438P22 showed only a signal on one chromosome 22 in 50 metaphases from the patient (fig. 3), her sister, and her mother, whereas in her brother, father, and maternal grandparents signals on both chromosomes 22 were seen. In addition, FISH with the probes M-bcr/abl and m-bcr/abl showed a deletion of the BCR (D22S257) signal on one of the chromosomes 22 in the patient. FISH with BAC 458J22 (D22S425) in the patient showed signals on both chromosomes 22, but one signal appeared weaker than the other, in most of the 30 analyzed metaphases. Fiber-FISH with both BACs in a control revealed a relatively short signal by BAC 438P22, which was located at one end of the very long but at least two-times-interrupted signal by BAC 458J22 (fig. 4). Both BACs were negative for markers D22S1709, D22S308, D22S303, D22S257, D22S301, D22S156, TOP1P2, D22S1144, and D22S1167. Therefore, BAC 458J22 does not span either of the deletion breakpoints. On interphase nuclei from a control, BAC 458J22 appeared only rarely as two signals; most of the time it gave split signals. These findings could be explained if BAC 458J22 contained repetitive elements that led to a signal on both chromosomes 22 despite deletion of D22S425.

We have demonstrated a novel microdeletion at 22q11.2 in a patient with DGS who had neither a deletion in the known 3-Mb 22q11.2 deletion region nor any other detectable chromosomal aberration such as deletion 10p. The deletion most probably comprises the loci D22S306, D22S308, D22S425, D22S303, and D22S257 in all affected family members. According to the physical map provided by Morrow et al. (1995), the size of the deletion should be ~2 Mb; however, the exact physical distance to the distal breakpoint is not known. The presented deletion is distal not only to the commonly deleted 22q11.2 region but also to the small distal deletions described by Kurahashi et al. (1996, 1997) and O'Donnell et al. (1997) (fig. 5). Since this novel deletion is adjacent to the commonly deleted region, a position effect on genes located in the commonly deleted region, or vice versa, may explain the DGS/VCFS phenotype in both the patient and those with the common or published small distal 22q11.2 deletions. Since four of the markers (i.e., D22S306, D22S308, D22S425, and D22S303) from our novel deletion region are within the immunoglobulin light-chain (IGLC) region, immunodeficiency in the affected family members may partly be explained by a reduced number of possible combinations during differentiation of antibody-forming cells. However, the cause of T-cell deficiency, which is the primary immunologic finding in DGS, remains unclear. One should also be aware that deletions of the IGLC region in differentiated B-lymphocytes is not a pathological finding and could lead to misdiagnosis of a germ-line deletion.

Since it has been shown that 22q11 contains several

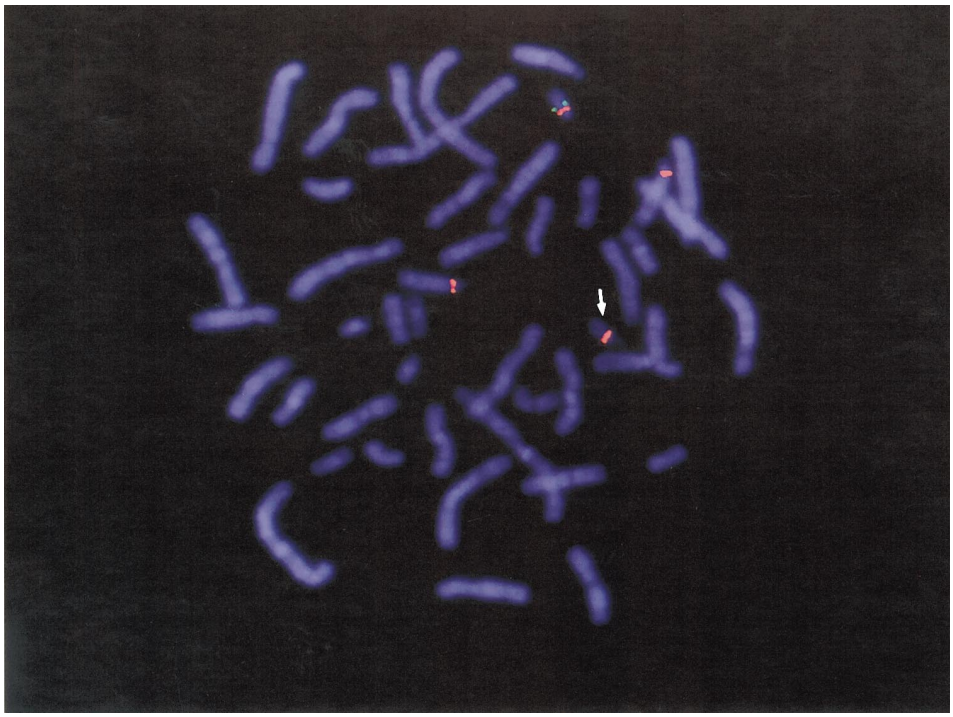

Figure 3 FISH with BAC 438P22, confirming a deletion of D22S425, on the basis of a lack of the green fluorescein signal at one of the chromosomes 22 (*arrow*) detected by red rhodamine signals from a centromeric 14/22 probe.

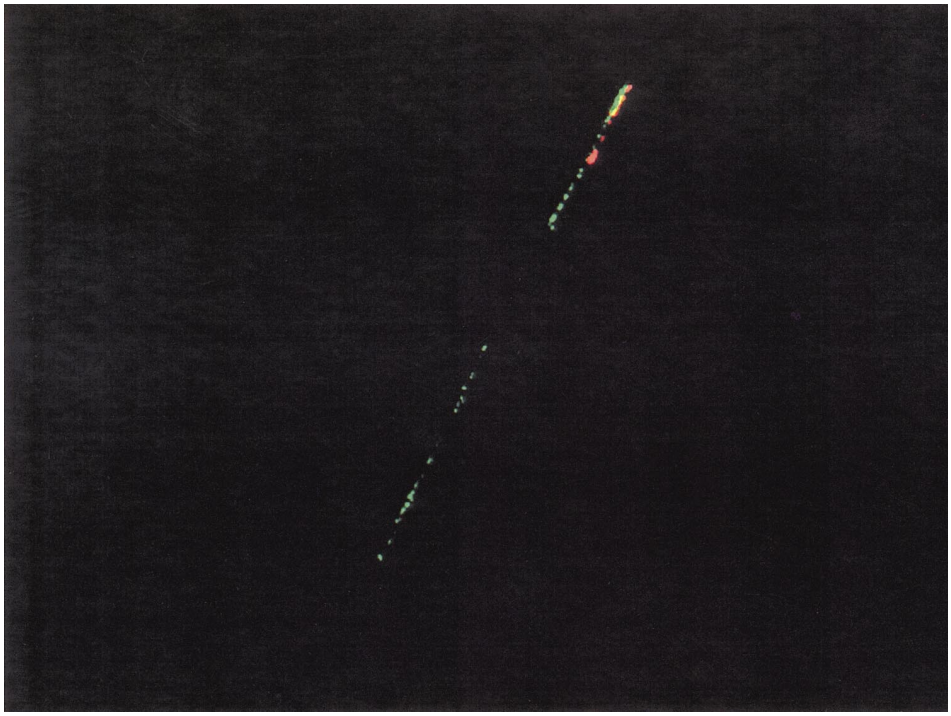

Figure 4 Fiber-FISH with the D22S425-positive BAC clones 438P22 (*red*) and 458J22 (*green*), suggesting the existence of repetitive elements within BAC 458J22.

low-copy repeats (Collins et al. 1997*a*), one might also consider that there are either similar genes or several copies of critical genes within the common and the presented deletion regions. Therefore, the search for such similar genes might give a clue to the answer to the question of whether any of the many genes already known in the commonly deleted region might have a major impact on the pathogenesis in 22q11.2 microdeletion syndromes—and, if so, which ones.

Recently, Chen et al. (1997) identified flanking repeat sequences within the Smith-Magenis syndrome critical region, which may lead to this common microdeletion via chromosomal recombinations. Accordingly, Morrow et al. (1997) mentioned a duplicated element within the breakpoints of the common 3-Mb deletion in VCFS/DGS patients. The proximal breakpoint of the 22q11.2 deletion in the family that we studied is in the same region as the distal breakpoint of the known deletion (Carlson et al. 1997*b*) and, therefore, may lie within this repeat sequence. According to the corrected low–copy-repeat map provided by Collins et al. (1997*b*), this repeat sequence also occurs between the genomic markers

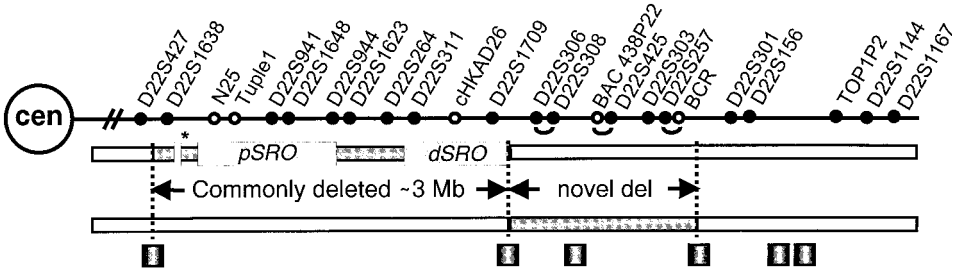

Figure 5 Scheme of the known DGS/VCFS deletion region (Morrow et al. 1995; Carlson et al. 1997b) and the novel microdeletion in the presented family. pSRO: proximal shortest region of deletion overlap defined by Carlson et al. (1997b); dSRO: distal shortest region of deletion overlap defined by Kurahashi et al. (1996; 1997) and O'Donnell et al. (1997). Asterisk: ADU translocation breakpoint according to Carlson et al. (1997b). del: deletion. Empty circles represent FISH probes. Shaded boxes below the scheme: location of low–copy-repeat elements (Collins et al. 1997b).

D22S257 and D22S301, which flank the distal deletion breakpoint in the atypical deletion presented here. Therefore, it is conceivable that an intra- or interchromosomal rearrangement because of repeated elements at the deletion breakpoints has led to the presented deletion as it is postulated in the common deletion. The repetitive nature of the 22q11 region may also give rise to misinterpretation of results, as could have easily happened with the FISH results of BAC 458J22, which appeared to be not deleted but which, on the basis of Fiber-FISH, seems to contain a multiple repeated element in addition to the specific deleted sequence.

Affected family members with the presented microdeletion show several symptoms that are typical of the common 22q11.2 microdeletion: interrupted aortic arch type B; immunodeficiency; hypotonia; mild short stature; microcephaly; short neck; learning difficulties; small, squared-off ears with overfolded helices; retrognathia; high-arched palate; choanal stenosis; and limb anomalies (Ryan et al. 1997; Rauch et al. 1998*b*). However, the typical facial gestalt, nearly always seen in the common 22q11.2 deletion, was not evident in this family. Therefore, the characteristic facial gestalt of the common deletion should occur because of unique and probably contiguous genes from that region. The search for this novel 22q11.2 microdeletion in patients with the 22q11.2 deletion phenotype but without the common deletion will further delineate differences and similarities in both phenotype and genotype and could lead to a better understanding of mechanisms in the pathogenesis of DGS/VCFS.

Because the patient's mother and sister have only some minor anomalies, the novel microdeletion shows a clinical variability similar to that of the known 22q11.2 deletion. To explain the inter- and even intrafamilial variability (De Silva et al. 1995; Leana-Cox et al. 1996; Devriendt et al. 1997), additional factors have been taken into consideration, such as imprinting, recessive mutations or polymorphisms unmasked by hemizygosity, unbalanced regulatory effects, a second-hit theory, and environmental factors (Hall 1993; Dallapiccola et al. 1996; Hatchwell 1996). However, the observation of MZ twins with a concordant phenotype and 22q11.2 deletion strongly argues in favor of a predominant genetic determination of the 22q11.2 deletion phenotype (Rauch et al. 1998*a*). Since both affected sisters have inherited the deletion from their mother, the considerable clinical variation cannot be explained by imprinting. Moreover, both sisters share the same paternal haplotype at the remaining wild-type chromosome 22, which makes the unmasking of different recessive mutations or polymorphisms by hemizygosity unlikely. Therefore, several types of 22q11.2 hemizygosity might result in a susceptibility to certain syndromes, the expression of which might be dependent on other factors, unlinked to this region.

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RB1 Gene Mutations in Peripheral Blood DNA of Patients with Isolated Unilateral Retinoblastoma

To the Editor:

Two recent reports in this *Journal* (Lohmann et al. 1997; Sippel et al. 1998) indicated that the proportion of patients with isolated unilateral retinoblastoma who carry RB1 gene mutations in constitutional cells is higher than estimated previously (Vogel 1979; Draper et al. 1992). Mutation analysis in patients with unilateral tumors is important because it helps to significantly reduce the number of infant relatives who require clinical surveillance for retinoblastoma (Gallie 1997). Moreover, molecular investigation of these patients can identify carriers of mutations associated with incomplete penetrance and reduced expressivity and thus can extend our knowledge of the genotype-phenotype correlation (Gallie 1997; Lohmann et al. 1997). We have now analyzed additional tumors and have found that the frequency of constitutional mutations in patients with isolated unilateral retinoblastoma is not as high as indicated by our previous study (Lohmann et al. 1997).

Forty-two retinoblastomas that showed loss of constitutional heterozygosity (LOH) at the intragenic loci RBi2 (Toguchida et al. 1993) or RB1.20 (Yandell et al. 1989) were available for mutation analysis. Twenty-one of these tumors had been part of a previous study but were not analyzed for small mutations at that time (Lohmann et al. 1997). We analyzed the methylation status at the *5′* end of the RB1 gene by Southern blot analysis, using the methylation-sensitive enzymes *Bss*HII and *Sac*II as described elsewhere (Greger et al. 1994). Hypermethylation was identified in tumors from six patients. We performed SSCP to screen for small mutations, using a method reported elsewhere (Lohmann et al. 1996). Single base substitutions, including 17 transitions at CpG-dinucleotides, and small length alterations were identified in 24 and 3 tumors, respectively. To identify mutations in the remaining 10 tumors, we sequenced all 27 exons and the promoter region of the RB1 gene. However, no small mutation was identified.

In all, mutations were identified in tumors from 32 (76%) of 42 patients (RB1 gene mutation database). In the tumor of one patient (M6485), a missense base change (c.929G→A, E310G) in exon 9 and a nonsense mutation in exon 15 (c.1399C→T, R467X) were identified in addition to LOH. The missense base change was also present in peripheral blood DNA of this patient. Further investigation showed that this variant RB1 allele was inherited from the father and is, at least, carried by four adult relatives who are unaffected by retinoblastoma. The sequence variant, which, to our knowledge, has not been reported before, is expected to alter an amino acid located N-terminal of the pocket domains A and B (Hu et al. 1990). Only a few reported missense mutations with putative oncogenic effect are located outside the regions that code for these domains (RB1 gene mutation database). Considering that the tumor of patient M6485 also shows a somatic nonsense mutation and LOH, the missense base change is probably a neutral polymorphism and has not contributed to tumorigenesis. However, detailed analyses are required, to demonstrate that this sequence variant does not change the functional properties of the Rb protein (Bremner et al. 1997; Otterson et al. 1997).

In our previous study, we detected small RB1 gene mutations in leukocyte DNA from 6 (17%) of 36 patients with isolated unilateral tumors (Lohmann et al. 1997). In the present study, none of the bona fide oncogenic mutations identified in tumors was also detected in corresponding peripheral blood DNA by direct sequencing of PCR products. Therefore, when the data presented here are included, the proportion of patients with mutations in leukocyte DNA drops to 6 (9%) of 68. Because of mutational mosaicism (Lohmann et al. 1997; Sippel et al. 1998), this figure underestimates the true prevalence of constitutional RB1 gene mutations in patients with isolated unilateral retinoblastoma. However, in almost all patients with isolated unilateral retinoblastoma who have affected children, the mutation is readily detectable in peripheral blood DNA (Sippel et al. 1998; authors' unpublished data). It is reasonable to assume, therefore, that the proportion of unilaterally affected patients with mutations in leukocyte DNA approximates the prevalence of hereditary retinoblastoma among patients with isolated unilateral tumors. The percentage obtained from our conjoint studies is now in accord with a previous estimate by Vogel (1979) that was based on epidemiological data.

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Electronic-Database Information

URL for data in this article is as follows:

RB1 gene mutation database, http://home.kamp.net/home/dr.lohmann

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TDT Clarification

To the Editor:

A potentially misleading statement occurs in the invited editorial "The TDT and other family-based tests for linkage disequilibrium and association" by Spielman and Ewens (59:983–989), published in the November 1996 issue of the *Journal*. We wish to make the following clarification.

In discussing some issues that arise when the transmission/disequilibrium test (TDT) is used in families where genotype data are unavailable for one parent, we noted the finding of Curtis and Sham (1995) that, when there is a single affected offspring who is homozygous for an allele present in the available (heterozygous) parent, the TDT gives a biased result and should not be used. We then stated that "[w]hen there is more than one offspring in the sibship, it sometimes will be possible to deduce that the unavailable parent [is also heterozygous], and, in these cases, we may proceed as though this [reconstructed] genotype were known directly" (Spielman and Ewens 1996, p. 987).

We should have emphasized that this claim assumes that the reconstruction is done from the genotypes of unaffected offspring. A bias will usually arise in the TDT statistic if the reconstruction uses, in whole or in part, genotype data from affected offspring whose genotypes are then used in the TDT. A bias can also arise when both parental genotypes are reconstructed from the genotypes of the offspring.

The bias resulting from reconstruction occurs for a reason different from that noted by Curtis and Sham (1995). Since reconstruction is possible only with certain offspring genotype combinations, families in which parental genotypes can be reconstructed are not random as far as offspring genotypes are concerned. This restriction leads to a bias if the offspring genotype data that were used for reconstruction are also used in the TDT.

Knapp (in press) has calculated the size of this bias in a number of important cases and has thus been able to establish more general TDT tests where this bias is allowed for.

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