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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

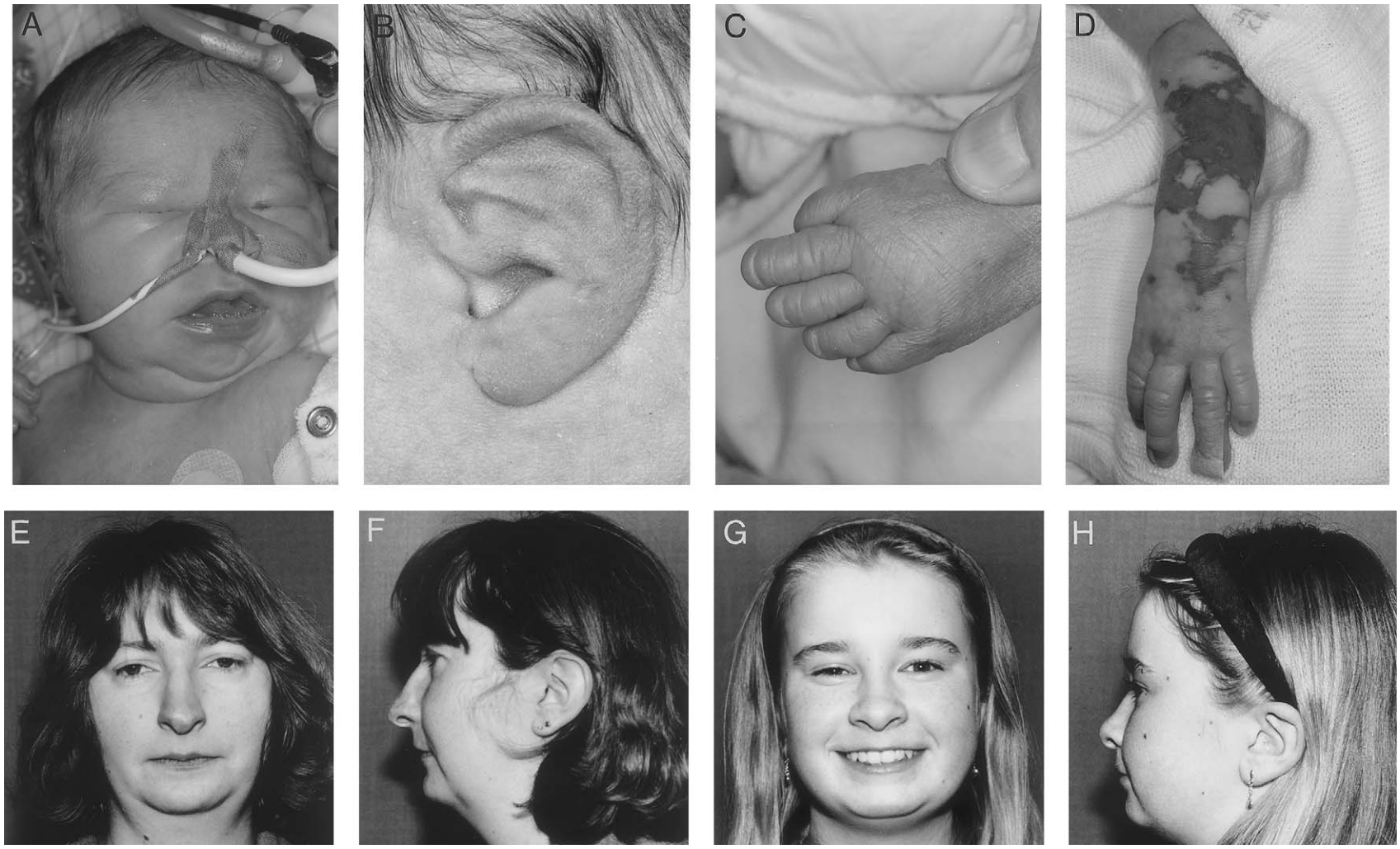

Figure 1 *a–d,* Patient III:3 at age 2 wk. Note retrognathia (*a*), typical short squared-off ears with simple overfolded helix and railway-track sign (*b*), hypoplastic toes and absent halluces (*c*), and purpura fulminans from *P. aeruginosa* sepsis (*d*). *e* and *f,* Facial appearance of the patient's 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.

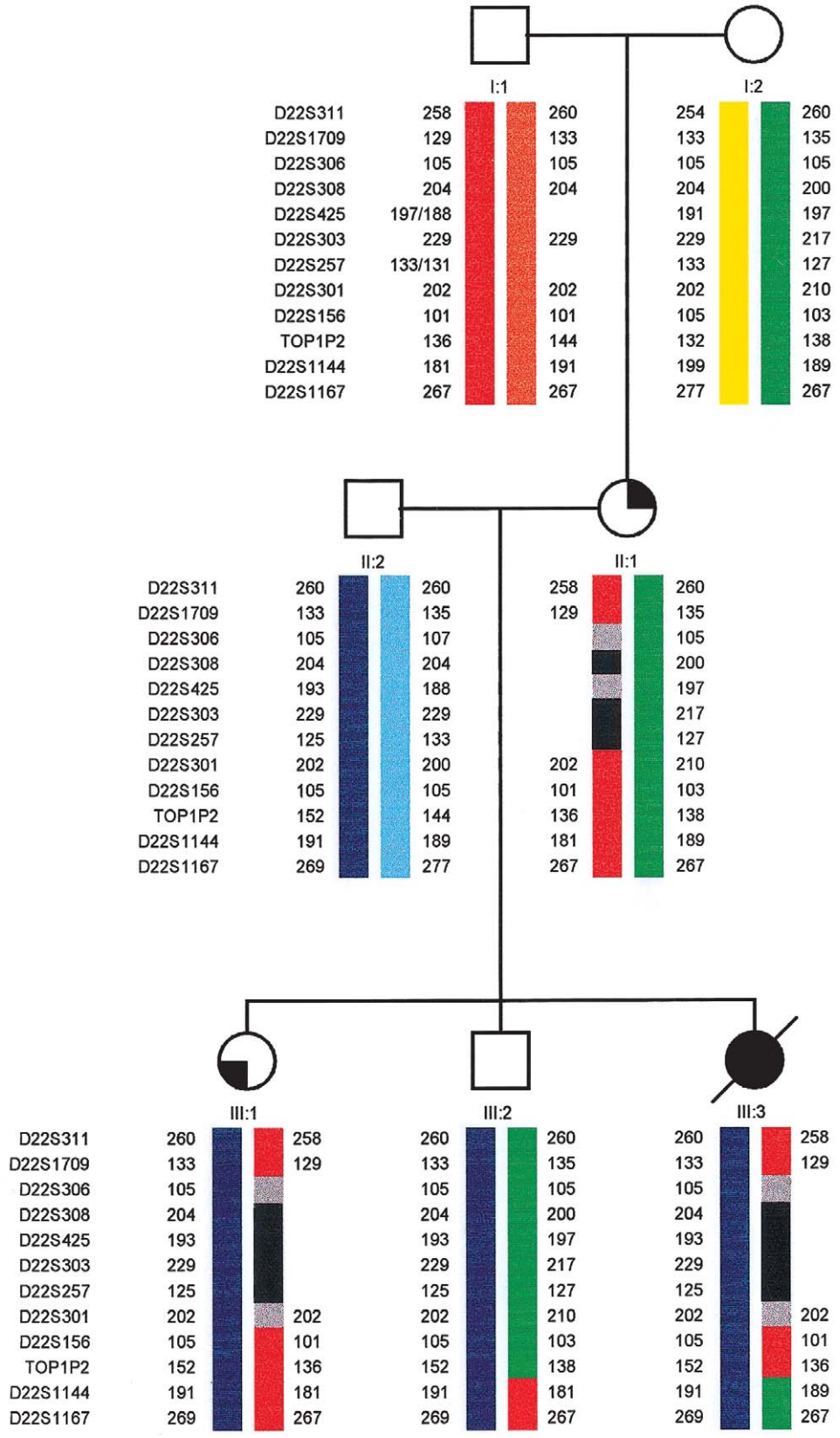

Figure 2 Results of STRP marker analyses in the core pedigree. Black bars denote a deletion; gray bars denote uninformative results either within or flanking the deleted markers. Colors indicate the segregating haplotypes; note the identical paternal wild-type haplotype (*dark blue*) in the mildly and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

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 confirmed 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, immuno-

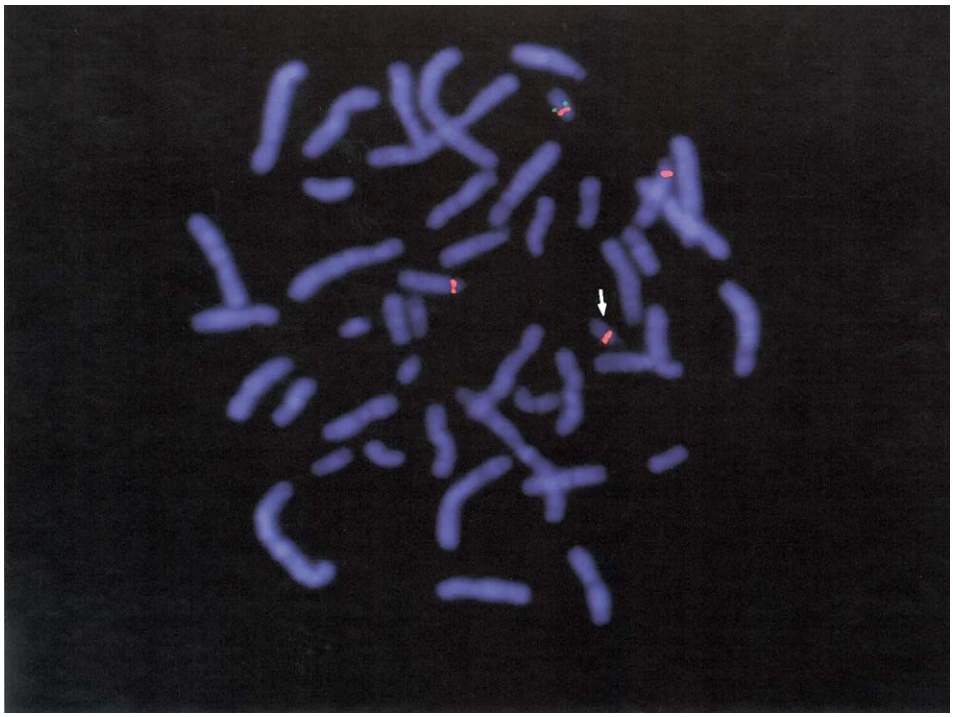

Figure 3 FISH with BAC 438P22, confirming a deletion of D22S425, on the basis of a lack of the green fluorescein signal at one of the chromosomes 22 (*arrow*) detected by red rhodamine signals from a centromeric 14/22 probe.

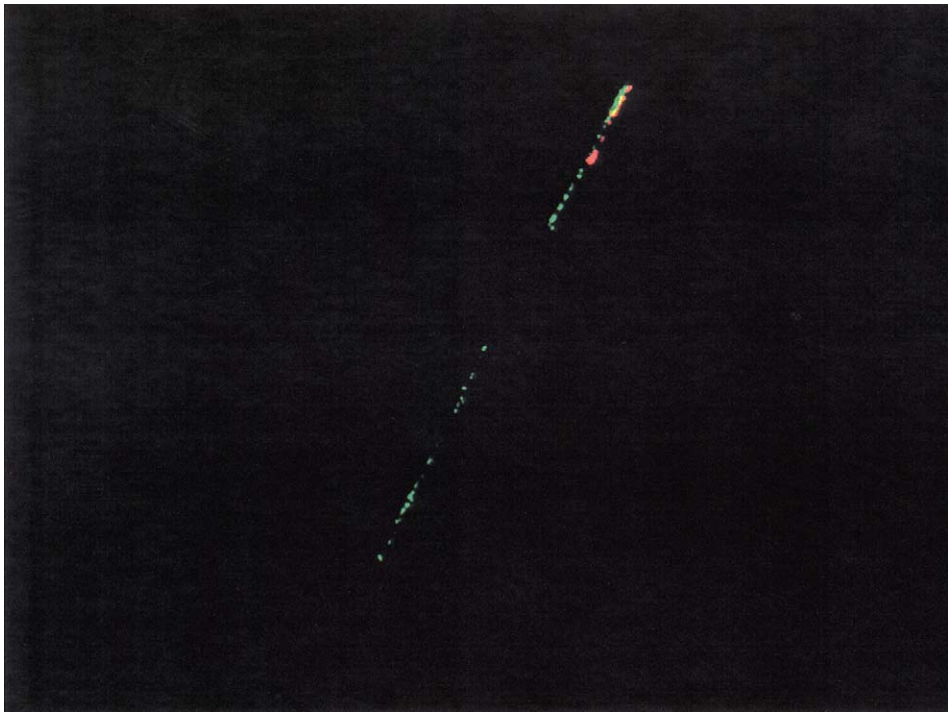

Figure 4 Fiber-FISH with the D22S425-positive BAC clones 438P22 (*red*) and 458J22 (*green*), suggesting the existence of repetitive elements within BAC 458J22.

deficiency 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 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

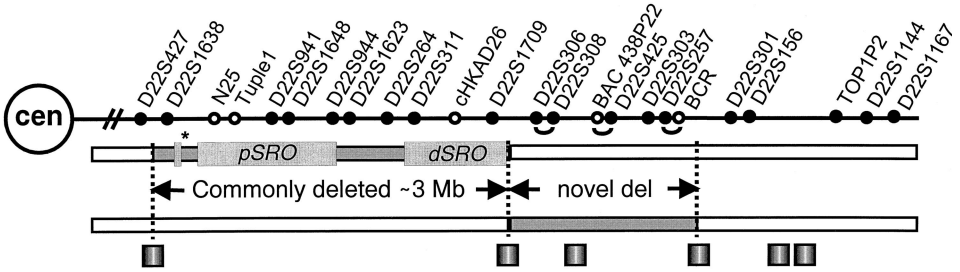

Figure 5 Scheme of the known DGS/VCFS deletion region (Morrow et al. 1995; Carlson et al. 1997b) and the novel microdeletion in the presented family. pSRO: proximal shortest region of deletion overlap defined by Carlson et al. (1997b); dSRO: distal shortest region of deletion overlap defined by Kurahashi 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).

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 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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Electronic-Database Information

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

BLAST, http://www.ncbi.nlm.gov
Genome Database, http://gdbwww.gdb.org
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/Omim (for DGS [MIM 188400], velo-cardio-facial syndrome [MIM 192430], Cayler syndrome [MIM 125520], Takao conotruncal anomaly face syndrome [contained MIM 188400], Noonan syndrome [MIM 163915], Kousseff syndrome [MIM 245210], and Opitz GBBB syndrome [MIM 145410])
Sanger Centre, http.//www.sanger.ac.uk/HGP/Chr22

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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