Letters to the Editor

J Med Genet 2001;**38**:175–178

Submicroscopic subtelomeric 1qter deletions: a recognisable phenotype?

EDITOR—Since the first report by Mankinen *et al*[1] in 1976, over 30 cases with microscopically visible 1q deletions have been described. Patients with a distal deletion of 1q (q42 or q43→qter) have a recognisable pattern of malformations, albeit rather variable.[2 3] The facial features include microcephaly, a full, round face with prominent forehead (sometimes with a metopic ridge), upward slanting palpebral fissures, epicanthic folds, a short, broad nose with a flat nasal bridge, thin lips with downturned corners of the mouth, micrognathia, apparently low set ears, and an abnormal palate (sometimes cleft). Patients are mentally and growth retarded, and may have variable cardiac, genital, and central nervous system anomalies. As most of these features suggest a chromosomal abnormality, patients with a deletion of distal 1q will usually be diagnosed after routine karyotyping. However, for submicroscopic distal 1q deletions, fluorescence in situ hybridisation with a 1qter specific probe will be required for the cytogenetic diagnosis. This test needs to be specially requested. In recent years, in situ hybridisation has led to the awareness that subtelomeric deletions below the level of the light microscope (<2-3 Mb) are a significant cause of malformation and mental retardation. One study identified previously undetectable abnormalities in 5% of 99 retarded patients.[4] Whereas large deletions of distal 1q have been reported frequently, no submicroscopic distal 1q deletions have been described so far. Here we report the clinical and cytogenetic findings in two unrelated mentally retarded boys and one female fetus, one with a submicroscopic distal 1q deletion and the others with a partial submicroscopic trisomy of distal 13q in addition to a submicroscopic distal 1q deletion.

The first patient is a 7 year old boy who was born at 39 weeks' gestation with a low birth weight of 2300 g (<3rd centile). His 2 year older brother had a ureteric obstruction and his mother had had one miscarriage. There was bleeding in the eighth week of pregnancy for one week. At birth, he had hypospadias, a ventricular septal defect, and subaortic stenosis. His left kidney was non-functioning with vesicoureteric reflux of the right kidney. At the age of 2 months, he underwent a left inguinal herniotomy and a left orchidopexy. Early on, his visual behaviour was virtually absent and he began to use his eyes from 5 months of age. Several non-specific seizures were noticed in his first year for which he was treated with carbamazepine. No EEG abnormalities were seen. At the age of 17 months he underwent a fundoplication to control gastro-oesophageal reflux. The VSD was closed and a subaortic membrane resected at the age of 2½ years. An MRI scan of the brain showed partial agenesis of the corpus callosum with the posterior part of the body and the splenium missing at the age of 2 years 7 months. His development was severely delayed; he sat at 18 months, walked with support at 2 years, and did not develop any speech. At the age of 2½ years his head circumference was 41.5 cm (−5 SD) and his length 76.5 cm (<3rd centile). He had a full, round face, a short, broad nose with a broad base and a flat nasal bridge, and a long, smooth philtrum with a thin vermilion border (fig 1). Periorbital fullness, epicanthic folds, and strabismus were present. His ears had prominent, everted lobes. His fingers were tapering. G banded chromosome study showed a normal male karyotype, 46,XY. Testing for 7-dehydro-cholesterol was normal. Endocrine studies were normal.

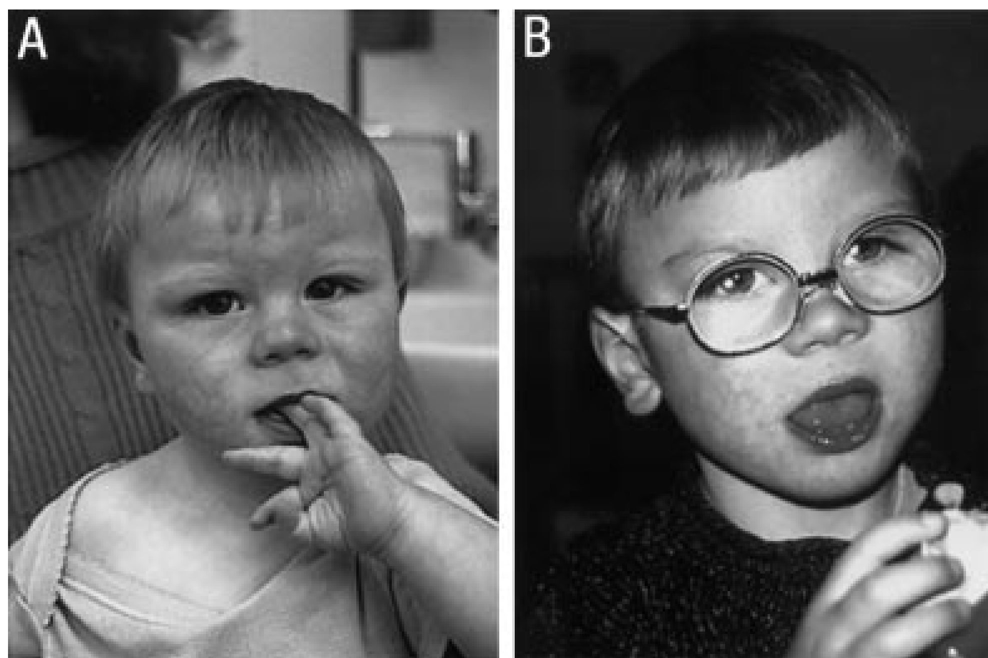

Figure 1 Case 1 at the age of 2 years (A) and 6 years (B). Note the full, round face, periorbital fullness, epicanthic folds, strabismus, a short, broad nose with a broad base and a flat nasal bridge, long, smooth philtrum and thin upper lip, and prominent ears with everted lobes.

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The second boy (case 2) was the first and only child of a non-consanguineous couple. The father had a normal daughter from a previous relationship and the mother was one of monozygotic twins. He was born at term with a birth weight of 2136 g (<3rd centile) and a head circumference on the 3rd centile. Ultrasound scanning at 20 weeks' gestation showed ventriculomegaly, micrognathia, and rocker bottom feet. Fetal blood sampling was performed in view of the above abnormalities and the karyotype was normal. By 28 weeks' gestation, the ventricular size was within normal limits. At birth, he had a cleft palate, small jaw, and hypospadias. Postnatal TORCH screen was negative. From birth, he was hypotonic with failure to thrive and frequent gastro-oesophageal reflux. A small patent foramen ovale was detected which did not require surgical treatment. On examination at the age of 16 months, he had developed severe microcephaly (head circumference 39 cm, −5 SD) with mild trigonocephaly and a metopic ridge. He had a full, round face, sparse, fine hair, periorbital fullness, and upward slanting palpebral fissures with epicanthic folds (fig 2). His nose was short with a long, smooth philtrum. He had a thin upper lip and downturned corners of the mouth with micrognathia and a cleft palate. His neck was short. On the right hand he had a single palmar crease and both hands were small with small nails. The feet were also small with overriding toes and rather oedematous dorsi of both feet. There was severe hypospadias and scoliosis. Testing for plasma amino acids and 7-dehydroxy cholesterol were normal. An EEG was normal. An MRI scan of the brain and spinal cord, at the age of 2 years, showed a rather poorly formed corpus callosum with a generalised paucity of white matter. The skeletal survey showed a hemivertebra at T11 and an anomalous vertebral body at T3. His development was severely retarded; at 22 months he was still not sitting and had no words. At the age of 2 years 5 months, his overall functioning was around the 8 month level.

The mother of case 2 had had a second pregnancy that was terminated at 17 weeks after structural abnormalities were identified in the fetus by ultrasound scan. Necropsy findings of the female fetus showed dysmorphic facies with a very small, receding mandible and a prominent maxilla and philtrum. There was a large midline cleft in the hard and soft palate, thick ear helices, and a flat occiput. The big toes were long and bulbous. A skeletal survey showed a hemivertebra at T7 in addition to the previous findings. A normal female karyotype was found in cultured amniotic fluid cells. The mother's twin sister had a severely retarded daughter with various dysmorphic features. She was macrocephalic (head circumference 57 cm at the age of 16 years, 98th centile) with a small mouth, mild microphthalmia, a high arched palate, small, overfolded ears, and a low posterior hairline. She had short fingers with clinodactyly and very small fifth toes. CT scan of the brain was normal.

FISH analysis using subtelomeric probes (under conditions described previously[5–7]) showed that case 1 had a de novo subtelomeric deletion involving the q arm of one of the chromosome 1 homologues (fig 3A). No other abnormality was detected. For case 2, subtelomeric FISH studies showed monosomy for the 1q subtelomeric region (fig 3B1) and trisomy for the 13q subtelomeric region. The additional copy of the 13q subtelomeric region is translocated onto the q arm of the derivative chromosome 1 (fig 3B2). This rearrangement was also present in the mother's second child, a female fetus aborted at 17 weeks. Subsequent FISH studies showed that both the mother and the mother's twin sister had balanced translocations of the 1q and 13q subtelomeric regions. The severely retarded daughter of the mother's twin sister had the contrasting unbalanced chromosomal rearrangement to her cousin (case 2); FISH showed that she is monosomic for the 13q subtelomeric region and trisomic for the 1q subtelomeric region.

Microscopically visible telomeric deletions sometimes cause specific malformations and mental retardation syndromes such as 4p− (Wolf-Hirschhorn syndrome), 5p− (cri du chat syndrome), 9p−, 13q−, and 18p− syndrome and they can, therefore, be ascertained through specific phenotypes. Deletions of other subtelomere regions often have a less characteristic phenotype particularly for the submicroscopic subtelomere deletions. Recently, two other submicroscopic subtelomere deletions on autosomes have been associated with a distinctive clinical phenotype,

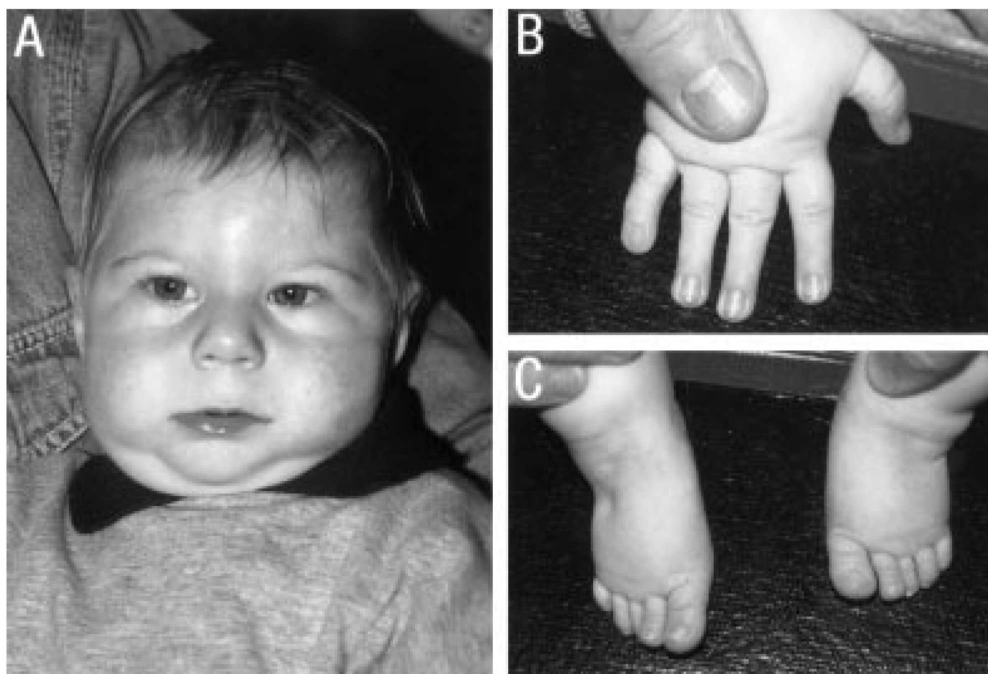

Figure 2 Case 2 at the age of 1½ years. Note (A) full, round face, sparse, fine hair, periorbital fullness, upward slanting palpebral fissures with epicanthic folds, short nose with a long, smooth philtrum, thin upper lip and downturned corners of the mouth. and micrognathia. (B) Tapering fingers with small, narrow nails. (C) Small feet with overriding toes and oedematous dorsi.

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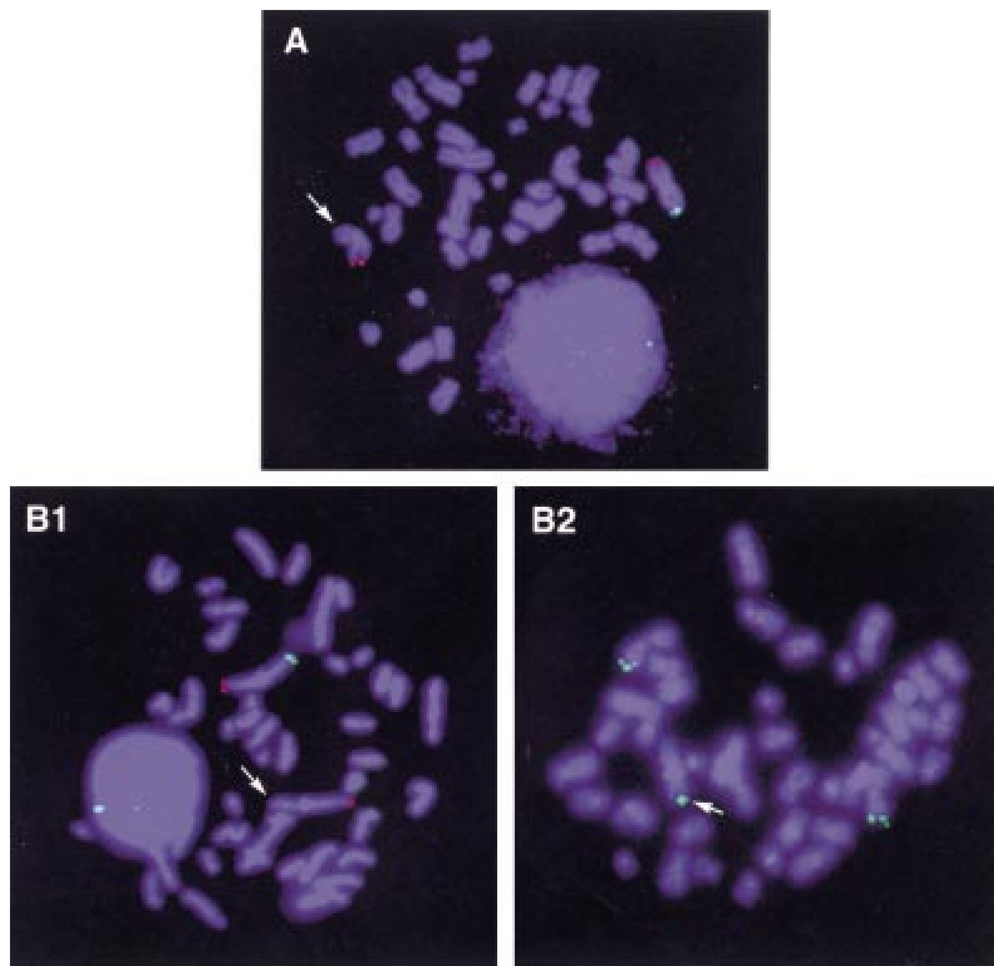

Figure 3 Subtelomeric FISH to metaphase chromosomes from (A) case 1 showing monosomy for 1q, (B1) case 2 showing monosomy for 1q, and (B2) case 2 showing trisomy for 13q. The p arm subtelomeric probe signals fluorescence red, whereas the q arm subtelomeric probe signals fluorescence green.

1p− and 22q−. Children with a distal 1p deletion are growth and mentally retarded with seizures, visual problems, large anterior fontanelle, asymmetrical and low set, dysplastic ears, deep set eyes, a depressed nasal bridge, a pointed chin, and fifth finger clinodactyly.[8 9] 22q telomere deletions are clinically associated with hypotonia, developmental delay, and absence of speech in the child.[10 11]

The two cases in this report support the presence of another recognisable submicroscopic telomere syndrome. Both patients shared a clinical pattern of severe mental retardation, growth retardation (prenatal onset), severe progressive microcephaly, hypospadias, corpus callosum abnormalities, cardiac anomalies, and gastro-oesophageal reflux. Facially, a short nose with a long, smooth philtrum, a thin upper lip, and full, round facies with periorbital fullness were present in both. These features are not restricted to submicroscopic 1q44 deletions as they have also been reported in patients with a microscopically visible distal deletion of 1q43→ter after routine G banding (table 1).[2 3] Other clinical features individually present in the boys have also been reported in visible 1q43→ter deletions (table 1). Some of these features were also present in the 17 week old female fetus with the der(1)t(1;13)(q44;q34), such as micrognathia, cleft palate, prominent philtrum, and abnormal ears. Although the deletions in the present cases were submicroscopic, their phenotype fits into the spectrum of abnormalities observed in large distal deletions of 1q43→ter. This suggests that the "1q− phenotype" is mainly caused by hypoploidy of genes in the subtelomeric region, which is compatible with the fact that the subtelomeric chromosome regions are relatively gene rich.[12] The clinical presentation, however, seems less severe in the

Table 1 Clinical features in cases 1 and 2 compared to microscopic visible 1qter deletions

Manifestations of patients with microscopic visible 1qter deletions[2 3]	*Case 1*	*Case 2*
General		
Mental retardation	+	+
Growth retardation	+	+
Microcephaly	+	+
Hypotonia		+
Seizures	+	
Face		
Sparse, fine hair		+
Prominent forehead/metopic ridge		+
Upward slanting palpebral fissures		+
Epicanthic folds	+	+
Strabismus	+	
Flat nasal bridge	+	
Short, broad nose	+	+
Smooth, long philtrum	+	+
Thin vermilion	+	+
Downturned corners of the mouth		+
Micrognathia		+
Cleft palate		+
Abnormal ears	+	
Other		
Short neck		+
Cardiac anomaly	+	+
Hypospadias	+	+
Abnormal hands		+
Abnormal feet		+
Gastro-oesophageal reflux	+	+
Corpus callosum a/hypoplasia	+	+

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submicroscopic cases, with regard to the motor development and life span, compared to the larger deletions. Obviously, this might be expected for a "contiguous gene syndrome".

Several abnormalities in our three cases were related to the midline. This suggests that gene(s) involved in normal midline development might be located in the deleted region on 1q. The size of the telomeric deletion in two of the cases presented had previously been reported to be between 15.7 and 23.3 cM,[7] which therefore defines the critical region for such a gene(s).

Although several clinical manifestations in the two cases can be observed in other chromosomal disorders, the combination of features seems to be distinctive: severe mental retardation, growth retardation (prenatal onset), severe progressive microcephaly, hypospadias, corpus callosum abnormalities, cardiac anomalies, gastro-oesophageal reflux, and a characteristic facies. Knowledge of the pattern of this "1qter− phenotype" will help clinicians to diagnose this chromosomal abnormality in their patients and to counsel the parents accordingly.

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Pure partial 7p trisomy including the *TWIST*, *HOXA*, and *GLI3* genes

EDITOR—The clinical findings associated with 7p duplication have been well delineated. They comprise large fontanelles and sutures, hypertelorism, large, apparently low set ears, high arched palate, hip joint dislocation or contractures, a high frequency of cardiac septal defect, and mental retardation.[1–5] It usually results from malsegregation of a parental balanced translocation or through abnormal recombination caused by a parental inversion. Some cases, however, result from a partial de novo 7p duplication.[6–15] Because these cases represent pure 7p segmental imbalances, they are of great interest in phenotype-genotype correlation studies.

Here we present a case of pure 7p duplication resulting from an unbalanced inverted insertion of segment 7p13-p21.2 into the short arm of a chromosome 8. A comparative analysis of our case with those published previously suggests that the 7p21.1-p21.2 region might contain a critical region for the 7p duplication syndrome. Moreover, the presence in our patient of some opposite features of Saethre-Chotzen syndrome, which is the result of haploinsufficiency of the *TWIST* gene,[16 17] suggests that these findings may result from a triple dosage of this particular gene.

The patient, a 24 year old man, was referred to us for further investigation because he had dysmorphic features and was mentally retarded. He was the fourth child of healthy, non-consanguineous, Lebanese parents. At birth, the mother was 26 years old and the father 31 years old. The family history was unremarkable. Pregnancy and delivery at term had been uneventful. Birth weight was 3800 g (75th centile) and length 58 cm (97th centile). A right talipes equinovarus was noted at birth. The baby was breast fed and discharged from hospital on the third day of life. A severe delay in developmental milestones was observed as he walked at 5 years of age and said only a few words at 7 years of age. According to the parents, he had a wide open anterior fontanelle that closed only at 4 years of age.

On clinical examination, he was sociable and very affectionate. His height was 170 cm (25th centile), weight 47.5 kg (3rd centile), and head circumference 52.5 cm (60th centile). Physical measurements showed a facial height of 13.5 cm (>97th centile), forehead height 9.5 cm (35th centile), lower facial height 8 cm (>97th centile), arm span 165 cm, total upper limb length 67 cm (35th centile), upper arm length 37 cm (>95th centile), forearm length 27 cm (80th centile), hand length 16.4 cm (3rd centile), and total lower limb length 97 cm (35th centile). The face was long and triangular. There was a long nose with a broad nasal bridge, bushy eyebrows, mild ptosis of the right eyelid, convergent strabismus, and moderate hypertelorism. Ears were low set and protruding, with poorly folded helices. In addition, a deep and short philtrum, a thin upper lip, a small mouth with downturned corners, a high arched and narrow palate, a bifid uvula, and a massive chin were observed. The thorax was narrow with no pectus deformity. A right kyphoscoliosis was present (fig 1). There was a positive thumb sign and mild joint hyperextensibility.

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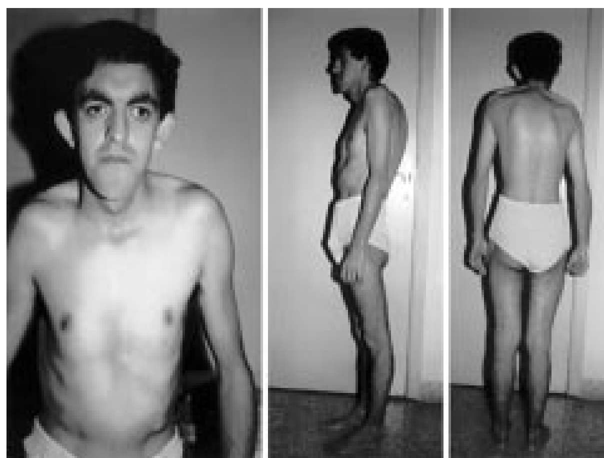

Figure 1 The patient: note long face with hypertelorism and microstomia, large, dysplastic ears, and kyphoscoliosis.

A right single palmar crease was noted. The external genitalia were unremarkable. Heart examination showed a grade 2/6 systolic murmur with maximum intensity in the mitral valve area and a B1 click. Echocardiography showed an ostium secundum atrial septal defect of 27 mm width with probable abnormal pulmonary venous return, a marked dilatation of the right chambers with paradoxical interventricular septum motion, and high pulmonary artery pressure related to pulmonary outflow without any physical obstacle. Full body skeletal radiography was performed and showed a right kyphoscoliosis, thin ribs especially on the right side, and a rectangular form of the vertebrae with broadening of the interpedicular length in L4 and L5. Increased malar angles, long phalanges, and generalised demineralisation were also noted. Magnetic resonance imaging of the brain was unremarkable. Ophthalmological and neurological investigations, abdominal ultrasound, and laboratory tests including liver and thyroid function studies were unremarkable.

High resolution chromosome analysis using RHG, GTG, and replication banding techniques were performed on peripheral blood lymphocyte cultures according to usual procedures. The chromosomes were classified according to the international nomenclature (ISCN, 1995).

Spectral analysis was performed according to the manufacturer's instructions (Applied Spectral Imaging). Briefly, 10 μl of the probe were hybridised to the patient's metaphases. Hybridisation was performed for two days at 37°C. Images were acquired with a SD200 Spectracube (Applied Spectral Imaging) mounted on a Zeiss Axiophot II microscope.

Chromosome 8 painting probe was obtained using *Alu*-PCR from a human-rodent cell line containing chromosome 8 as the sole human material, as previously described.[18]

YAC clone 321d10 and cosmid clones gc550 and gc68 correspond to the *GLI-3* gene locus (7p13).[19] YACs clones 961E5 (7p15) and 933E1 (7p21) encompass the *HOX A* gene complex[20] and the *TWIST* gene locus respectively.[21] The YACs clones 858H6 (D7S2557) and 938A6 (D7S664) (http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map), which map to 7p21.2, and 933A5, which maps to the chromosome 8[22] long arm subtelomeric region, were also used. FISH studies were performed as previously described.[23] Comparative genomic hybridisation (CGH) was carried out as previously described.[24] High molecular weight DNA was extracted from the peripheral blood of the patient and a normal male control. One μg of DNA was labelled by nick translation (Vysis, Downers Grove, IL, USA) using FluorX (FluorX Amido 10dCTP) for patient and cyanine 3 (Cy3-AP3-dUTP) (Amersham Life Science, Arlington Heights, IL, USA) for control DNA. For both patient and control, 200 ng of DNA were coprecipitated with 70 μg of unlabelled Cot-1 DNA (Life Technologies, Pasler, Scotland), resuspended in 12 μl of a hybridisation mixture, and hybridised on normal metaphase spreads for two days at 37°C. After post-hybridisation washing, the slides were analysed using a Leica DMRXA epifluorescence microscope. Images were processed and analysed with the Quips CGH Software (Vysis, Downers Grove, IL).

The microsatellite marker D7S2564[25] was studied using the following standard PCR conditions: three PCR reactions were performed in a total volume of 50 μl, containing 80 ng of the father's, mother's and patient's genomic DNA, 50 pmol of each primer, 0.125 mmol/l dNTPs, and 1 unit of *Taq* polymerase. Amplification buffer (1×) contained 10 mmol/l Tris base pH 9, 50 mmol/l KCl, and 1.5 mmol/l $MgCl_2$. Amplifications were carried out for 30 cycles of denaturation (94°C for 40 seconds) and annealing (55°C for 40 seconds). An elongation step (72°C for 40 seconds) ended the process after the final annealing.

Analysis of the patient's chromosomes showed, in all metaphases examined, an abnormal short arm of chromosome 8, with the presence of extra material of unknown origin inserted into band 8p23.1 (fig 2). The chromosomes of the parents were normal.

Molecular cytogenetic analysis was performed to characterise this chromosomal abnormality. FISH using a chromosome 8 painting probe excluded the presence of a chromosome 8 duplication. Spectral karyotyping showed that the extra material originated from chromosome 7 and CGH showed a 7p13-p21 duplication (fig 2A, B). Molecular analysis using microsatellite DNA markers mapping to the inserted chromosome 7p13-p21 region showed that this insertion was of paternal origin (data not shown).

To delineate this chromosomal abnormality further, we performed FISH studies using cosmid and YACs clones encompassing different loci mapping along chromosome 7p. This study showed the presence of an unbalanced inverted insertion of segment 7p13-p21.2 including the *GLI-3*, *HOXA*, and *TWIST* genes into the short arm of the chromosome 8 (fig 2C). In particular, we mapped the *TWIST* gene to the telomeric part of chromosome band 7p21.1. Furthermore, as the critical 7p duplication region has been assigned to 7p21-pter,[26] we decided to map the telomeric breakpoint of our patient's insertion in order to define more precisely the 7p duplication region at the molecular level. For this purpose we performed FISH studies using different chromosome 7p21 YAC clones and showed that the insertion telomeric breakpoint mapped in the 7p21.2 band region between YAC 858 H6 (D7S2557) and YAC 938A6 (D7S664) in a 1 Mb region containing the *MOX/GAX* gene locus (NCBI) (table 1).

Numerous patients with complete or partial 7p duplication have been reported.[26] In infants and children, common findings comprise a large anterior fontanelle, hypertelorism, skull anomalies, large, apparently low set ears, high arched palate, joint dislocation or contractures, a high frequency of cardiac septal defect, and mental retardation. The adult phenotype is less well known. Recognition of the clinical spectrum in patients with smaller duplications has suggested restriction of the critical region to 7p15-pter.[5 27] The most recent review, based on the observation of a patient with an unbalanced translocation resulting in 7p21.2-pter duplication and a characteristic clinical phenotype including a large anterior fontanelle, assigned the critical region of the 7p duplication syndrome to 7p21.2-pter.[26] However, the duplicated chromosome

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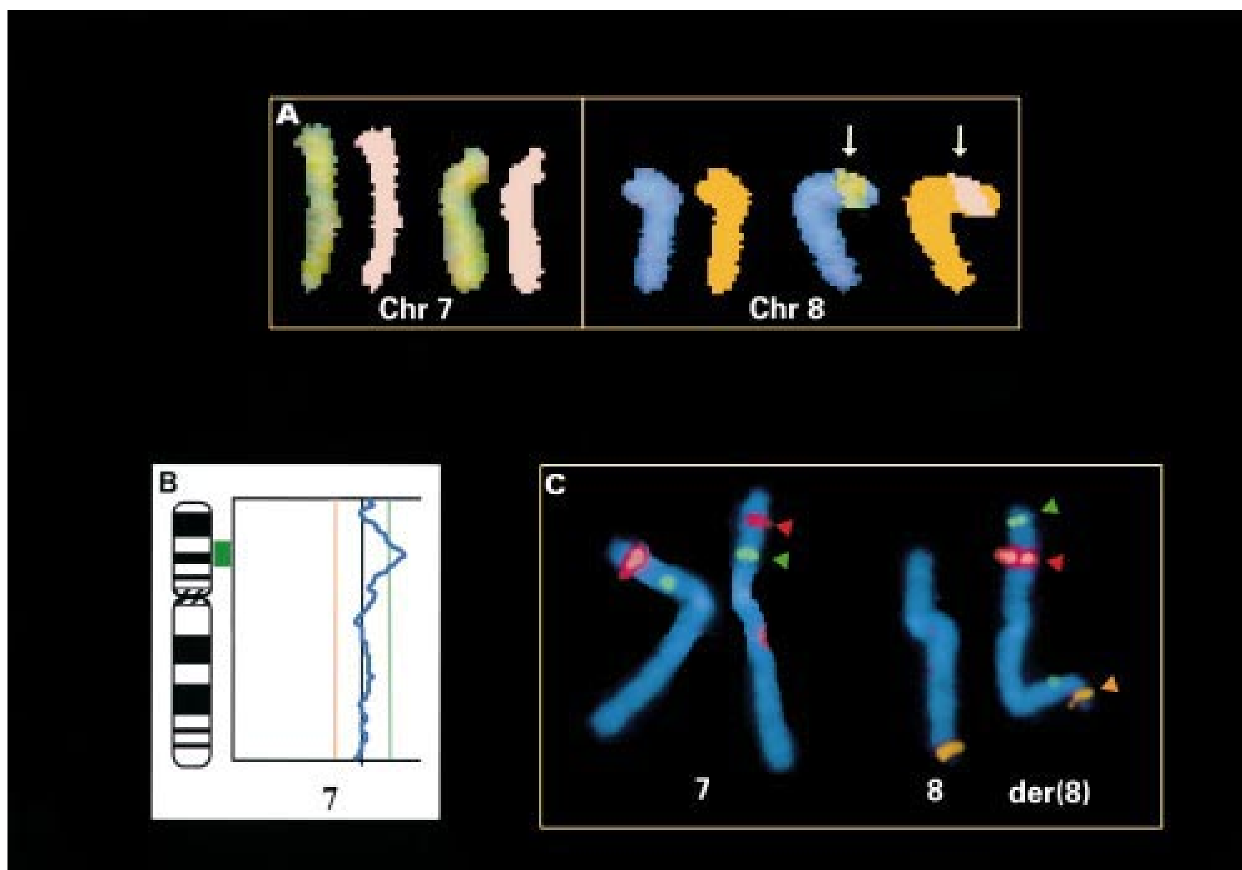

Figure 2 FISH characterisation of the inv ins(8;7). (A) Partial spectral karyotype showing the chromosome 7 insertion into the short arm of chromosome 8. Images were acquired with a SD2000 Spectracube (Applied Spectral Imaging) mounted on a Zeiss Axiophot II microscope. For chromosomes 7 and 8, the two colours correspond to RGB (red, green, and blue) colour and artificial pseudocolour, respectively. The inserted 7p segment is indicated by the arrow. (B) Partial CGH result indicating that the chromosome 7 inserted segment is 7p13-p21. Images were processed and analysed with the Quips CGH Software (Vysis, Downers Grove, IL). (C) FISH with probes corresponding to the GLI-3 (green arrowhead) and the TWIST loci (red arrowhead) as well as the chromosome 8qter region (YAC 933a5, orange arrowhead). The chromosomes were counterstained with DAPI. Note that the order of the GLI-3 and TWIST genes is inverted on the der(8).

segment was not mapped precisely as molecular cytogenetic techniques were not used.

Here we report on a patient with moderate mental retardation and with several clinical features associated with partial 7p duplication, including mild hypertelorism, large, protruding ears, a small mouth with downturned corners, high arched palate, cardiac septal defect, and late closure of a large anterior fontanelle. Detailed molecular cytogenetic analysis showed that the patient carried an unbalanced inverted insertion of the 7p13-p21.2 segment into chromosome 8p23 (fig 3). This observation and previously reported cases suggested that the 7p21.1-p21.2 band region could be critical for the main manifestations of the 7p duplication phenotype.

The 7p21.1-p21.2 band region contains the *TWIST* gene which encodes a transcription factor of the basic helix-loop-helix protein family and plays an important role in mesodermal cell determination. In particular, the *TWIST* gene is involved in membranous ossification occurring during frontal, parietal, and malar bone formation.[28 29] In humans, haploinsufficiency of the *TWIST* gene has been shown to be associated with Saethre-Chotzen syndrome which is characterised by craniosynostosis, a flat face with a thin, long, pointed nose, shallow orbits, plagiocephaly, small, posteriorly rotated ears with long and prominent crus, cleft palate, and often subtle abnormalities of the hands such as mild syndactyly of digits 2 and 3 and bifid terminal phalanges of the hallux, congenital heart defects, and contractures of the elbow and knee.[16 30–32] In addition, mice heterozygous for *TWIST* gene mutations present with craniosynostosis apparently related

Table 1 FISH studies

Clone name	*Origin*	*Localisation*	*Other features*	*Status*
YAC 321d10	CEPH/A Vortkamp	7p13	Gene *GLI3*	T
Cosmid gc550	A Vortkamp	7p13	Gene *GLI3*	T
Cosmid gc68	A Vortkamp	7p13	Gene *GLI3*	T
YAC 961e5	CEPH	7p15	Gene *HOX A*	T
YAC 933e1	CEPH	7p21–22	Gene *TWIST*	T
YAC 858h6	CEPH	7p21	D7S2557	T
YAC 938a6	CEPH	7p21.2	D7S664	N
YAC 933a5	CEPH/T Haaf	8qter	D8S1837	N

The chromosomal map of YACs 961E5, 933E1, 858H6, 938A6, and 933A5 are derived from our own experiments.
T: three signals observed on patient metaphases (two on each chromosome 7 and one on the der(8)).
N: two signals observed on patient metaphases.

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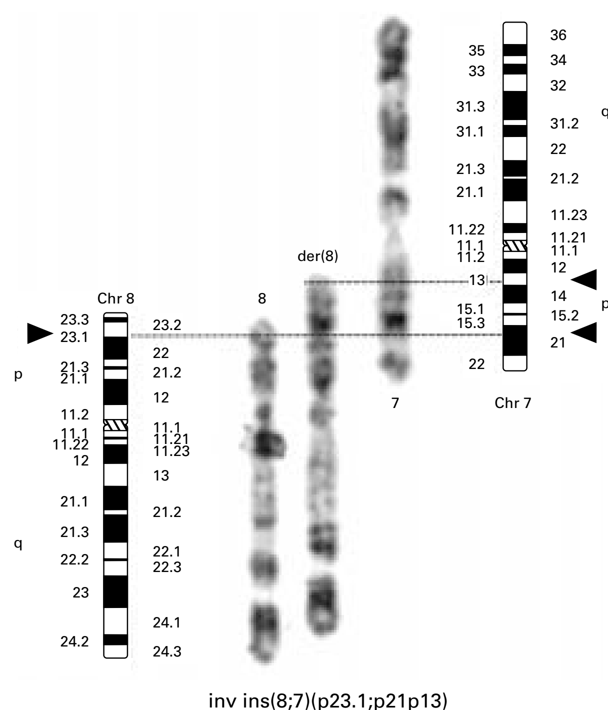

Figure 3 Partial karyotype of the inv ins(8;7)(p23.1;p21.2p13). Chromosome 7 is presented in the inverted position to match the inserted inverted segment into chromosome 8p. Breakpoints are indicated by arrows. Replication R bands were obtained after BrdU incorporation and FPG staining.

to precocious parietal and frontal bone formation as well as abnormal hindlimb development.[29]

Delayed closure of a large anterior fontanelle, a characteristic clinical feature of partial 7p duplication, is the opposite of craniosynostosis, a common clinical finding in the corresponding 7p deletion syndrome,[33] and in the Saethre-Chotzen syndrome.[16] In addition, we mapped the *TWIST* gene precisely in the putative 7p21-1p21.2 duplication syndrome region. Therefore, we would like to suggest that triple dosage of the *TWIST* gene may be responsible for this characteristic clinical feature of the partial 7p duplication syndrome. Indeed, it is not unreasonable to believe that this characteristic may represent a direct reflection of reciprocal gene dosage effects of this particular gene during craniofacial and limb development rather than a mere random event.

Another gene mapping in the putative 7p duplication syndrome region is the *MOX2* gene, which maps in the 7p21.2 band between D75S557 and D7S662 (http://www.ncbi.nlm.nih.gov/Locus.ink/LocRpt.cgi?l=4223) and encodes a homeobox protein implicated in limb muscle and craniofacial development.[34] Interestingly, it has been shown that overexpression of this protein in transgenic mice is associated with decreased cardiomyocyte cell proliferation and abnormal heart morphogenesis.[35] *MOX2* could therefore be a good candidate for heart defects often observed in 7p duplication syndrome. The fact that in our patient the 7p21.2 breakpoint mapped between D75S557 and D7S662 indicates that the *MOX2* gene is likely to be implicated in the duplication.

Finally, in the present observation the duplicated 7p13-p21.1 segment also includes the *GLI3* gene and the homeobox *HOXA* gene complex. Haploinsufficiency of the *GLI3* gene has been associated with Pallister-Hall syndrome,[36] Greig cephalopolysyndactyly syndrome,[37] and postaxial polydactyly type AI,[38] whereas mutations of the *HOXA 13* gene or full deletion of the *HOXA* cluster have been reported in the hand-foot-genital syndrome.[20] No opposite features of the *GLI3* gene or *HOXA* cluster haploinsufficiency were observed in our patient. In particular, the hands, feet, and genitalia are unremarkable. In the present case, the presence of three copies of these genes is not associated with a recognisable impact on the 7p duplication phenotype. It is noteworthy that both of these genes map proximal to the estimated critical segment.

In conclusion, the presence of the *TWIST* gene in triple dosage may be causally related to the presence of a large anterior fontanelle with delayed closure, which is the more characteristic clinical feature of the 7p duplication syndrome. It would be interesting to search for duplication of the *TWIST* gene in patients presenting with a large anterior fontanelle with delayed closure associated or not with mental retardation.

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Suggestive linkage of situs inversus and other left-right axis anomalies to chromosome 6p

EDITOR—Congenital heart disease occurs commonly. One form, heterotaxy, accounts for approximately 3-4% of the total incidence and has a mortality rate approaching 45%.[1] Given that the diagnosis is based on the discordance of the left-right (LR) sidedness between the abdominal viscera and atria,[2] heterotaxy describes a group of malformations arising from the abnormal development of LR asymmetry.[3]

In familial cases one can find subjects with complete, mirror image reversal of normal LR anatomy (situs inversus), and others who manifest the hallmark visceroatrial discordance as well as other laterality malformations (sometimes collectively called situs ambiguus). Moreover other family members with normal LR anatomy (situs solitus) are obligate disease gene carriers by virtue of their pedigree position.

Many genes have been implicated in normal and abnormal LR axis development among non-human vertebrates.[4] Knowledge remains sparse, however, regarding the molecular genetics of human LR malformations. Positional cloning identified a gene, *ZIC3*, on chromosome Xq24-27.1, in which mutations have been found among one sporadic and six familial cases of LR axis malformations.[5] A few mutations have also been found in *LEFTYA* and in the activin receptor type IIB gene (*ACVR2B*), identified on the basis of their homology to the corresponding genes known to cause laterality defects in the mouse.[6 7]

Here we describe a family in which LR malformations segregate across five generations. Although male to male transmission has not occurred, males and females appear to be affected similarly, and linkage analysis has excluded a disease locus on the X chromosome (see below). Both situs inversus and situs ambiguus are found in seven affected subjects and pedigree position implicates four apparently normal subjects as obligate gene carriers. These observations strongly support a model of autosomal dominant inheritance with reduced penetrance. The pedigree comprising 36 subjects is illustrated in fig 1

Seven subjects in five generations manifest laterality defects of multiple organs (fig 1). Of these, four are situs inversus (II.2, III.7, III.10, and IV.6), and three are situs ambiguus (IV.8, V.1, and V.4). There is considerable variability of expression in the situs ambiguus group. IV.8 has mirror image reversal of the heart and of the colon but normal position of the liver, stomach, and spleen, while complex heart malformations were identified in the other two, leading either to prenatal termination (V.1) or surgery (V.4). II.4, III.1, III.6, and IV.4 are obligate disease gene carriers by virtue of their pedigree position but without apparent LR abnormalities. III.9 and V.2 have isolated cardiac defects without any other LR abnormality. The malformation observed in III.9, ventricular inversion in combination with transposition of the great arteries, is usually classified with heterotaxy under the common aetiology of "abnormal looping" defects. Therefore, III.9 was scored as affected in the linkage analysis, while V.2, who showed hypoplastic left heart syndrome (HLHS), which has not been linked embryologically to the cardiac looping defects, was scored as having an unknown disease status. All subjects manifesting laterality defects but III.10, who was unavailable, were included in the linkage analysis and scored as affected. In all subjects, disease status phenotype was assigned before marker genotyping.

Informed consent was obtained from patients participating in this study, which was approved by the Institutional Board at Baylor College of Medicine. Genomic DNA was extracted from whole blood or cell lines (lymphoblast or fibroblast) with the Puregene DNA Isolation Kit (Gentra Systems) according to the manufacturer's protocol. DNA from paraffin embedded tissue was extracted as previously described.[8] Amplifications were performed on HYBAID Omnigene thermocyclers under standard conditions.

The initial screening was performed at the Center for Medical Genetics in Marshfield, WI, using marker screening set 6, consisting of short tandem repeat markers with an

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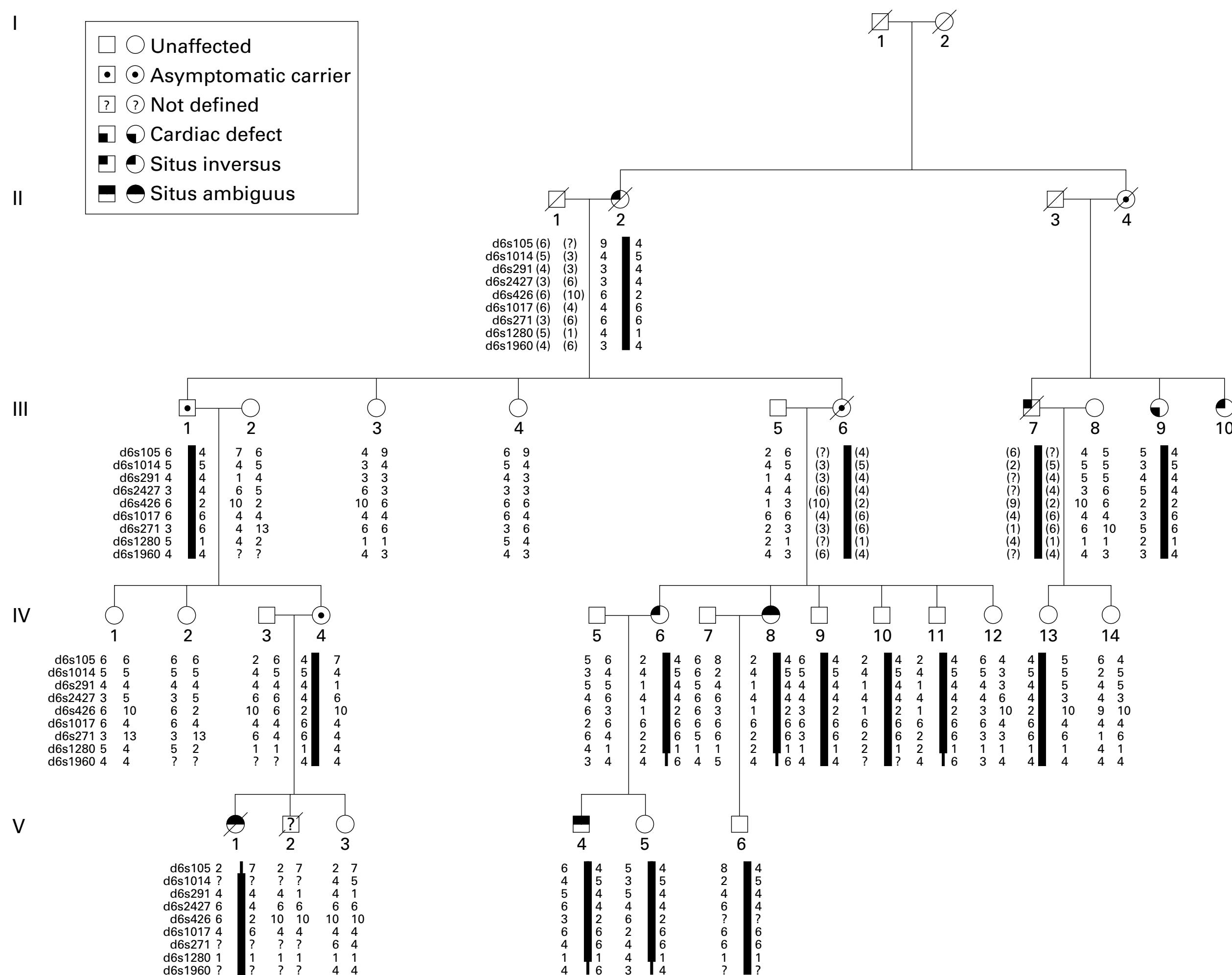

Figure 1 Pedigree of family with laterality defects and 6p21 marker haplotypes. Disease haplotype is boxed. Symbols for situs inversus, situs ambiguus, isolated cardiac defects, and unaffected disease gene carriers are as indicated in symbol definitions. Alleles in parentheses in missing subjects were inferred from their children's marker genotypes.

average heterozygosity of 0.76 and an average sex equal spacing between markers of 10.0 cM.

A total of 346 markers (333 autosomal and 13 X linked) were used for the genome wide screening. Conventional two point and multipoint linkage analysis between the trait locus and the polymorphic markers was carried out using VITESSE version 1.0.[9] For the initial screening, a two point lod score analysis was performed using the affecteds only approach with an autosomal or X linked dominant mode of inheritance. Subjects with confirmed LR abnormalities were scored as affected, married in as unaffected, and the remaining subjects were considered as having an unknown disease phenotype. All marker allele frequencies were equalised and the disease gene frequency was set to 0.00005.

For fine mapping using additional markers, linkage analysis was performed using disease gene penetrance and marker allele frequencies estimated on the basis of our family data using the FASTLINK 3.0 version of ILINK.[10 11] Penetrance was estimated on the basis of disease phenotype status only without including any marker genotype information, and found to be equal to 0.5 for heterozygotes. All unaffected subjects except V.2 were designated as such in the fine mapping linkage analysis. Multipoint linkage analysis was carried out by means of the sliding window technique,[12] using three markers at a time, with genetic distances obtained from the maps developed at the Center for Medical Genetics in Marshfield (WI).

Results of the genome wide screen showed eight markers, one on chromosome 5 and seven on chromosome 6, with a two point lod score between 1 and 2, and two on chromosome 6 with a lod score higher than 2. Three of these 10 markers, two on chromosome 6 and one on chromosome 5, were located in regions that were excluded by negative lod scores obtained from fine mapping after typing additional markers (data not shown). Of the remaining seven markers, five were located on chromosome 6p and two on chromosome 6q (table 1).

As shown in table 1, fine mapping using additional markers in these regions and a reduced penetrance model continued to yield positive lod scores, which increased from 2.33 to 2.67 for D6S426 on 6p21 but decreased from 2.27 to 1.81 for D6S305 on 6q25. Furthermore, multipoint linkage analysis yielded maximum multipoint lod scores of 2.95 at theta=0 from D6S426 on 6p21 (fig 2) and of 2.05 at theta=0 from D6S305 on 6q25 (data not shown). The use of different penetrance values did not significantly modify these results and consistently resulted in increased lod scores for 6p21 and reduced lod scores for 6q25 with respect to the two point analysis.

Among the 6p21 markers, obligate recombination events were observed for D6S105 in V.1 and for D6S1960 in IV.6 and IV.8 (fig 1). These two markers, which are located 32 cM apart, thus define the limits of the critical interval for the disease locus.

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Table 1 Results of two point linkage analysis for chromosome 6 markers

Marker*	Map distance (cM)	Lod score at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4†
Chr 6p21								
d6s105	44.41	−∞	−0.44	0.15	0.31	0.32	0.22	0.10
		−∞	−0.14	0.43	0.58	0.54	0.37	0.17
d6s1014	45.50	1.53	1.50	1.37	1.21	0.88	0.57	0.27
		1.08	1.06	0.98	0.88	0.66	0.42	0.19
d6s291	49.50	1.63	1.59	1.44	1.25	0.87	0.50	0.20
		1.31	1.28	1.18	1.03	0.73	0.43	0.19
d6s2427	53.81	2.11	2.07	1.90	1.70	1.26	0.81	0.38
		2.23	2.19	2.04	1.84	1.40	0.92	0.43
d6s426	60.44	2.33	2.29	2.11	1.89	1.41	0.93	0.45
		2.67	2.62	2.44	2.20	1.68	1.11	0.53
d6s1017	*63.28*	*1.29*	*1.26*	*1.12*	*0.95*	*0.62*	*0.33*	*0.12*
		1.25	*1.22*	*1.10*	*0.95*	*0.64*	*0.37*	*0.15*
d6s271	66.37	1.40	1.37	1.27	1.14	0.88	0.61	0.32
		0.95	0.94	0.88	0.81	0.66	0.48	0.26
d6s1280	*73.13*	*1.29*	*1.26*	*1.15*	*1.02*	*0.76*	*0.51*	*0.26*
		0.82	*0.80*	*0.75*	*0.68*	*0.53*	*0.37*	*0.20*
d6s1960	*76.62*	−∞	*−2.77*	*−1.42*	*−0.89*	*−0.44*	*−0.22*	*−0.10*
		−∞	*−2.73*	*−1.37*	*−0.82*	*−0.36*	*−0.17*	*−0.08*
Chr 6q25								
d6s363	161.59	−∞	−0.75	−0.12	0.10	0.23	0.22	0.14
		−∞	−0.74	−0.10	0.13	0.27	0.25	0.16
d6s1305	164.78	1.79	1.75	1.63	1.46	1.13	0.78	0.41
		1.13	1.11	1.06	0.98	0.80	0.58	0.32
d6s305	*166.39*	*2.27*	*2.23*	*2.06*	*1.84*	*1.38*	*0.91*	*0.44*
		1.81	*1.78*	*1.68*	*1.53*	*1.19*	*0.81*	*0.41*
d6s980	167.78	1.58	1.55	1.45	1.32	1.04	0.74	0.40
		1.05	1.04	1.00	0.94	0.79	0.58	0.33
d6s1599	169.95	0.76	0.75	0.69	0.61	0.47	0.32	0.17
		0.79	0.77	0.72	0.66	0.52	0.36	0.19
d6s1277	*173.31*	−∞	*−0.19*	*0.41*	*0.59*	*0.62*	*0.51*	*0.30*
		−∞	*−0.09*	*0.51*	*0.68*	*0.69*	*0.55*	*0.32*

*In italics are markers that were added after the initial screening. Additional markers typed in the same regions were not informative in our pedigree.
†Results obtained in the affecteds only and reduced penetrance analyses are reported in the first and second lines respectively for each marker.

A genome wide search for linkage in this family has thus tentatively identified a new locus for LR axis malformations on chromosome 6p21. The highest two point lod score obtained was 2.67 at theta=0 with marker D6S426, and a maximum lod score of 2.95 was obtained at the same location by multipoint analysis. The first flanking recombinant markers telomeric and centromeric to this region, respectively, are D6S105 and D6S1960.

Results from simulation analysis carried out with the SLINK program[13] using the same disease model of the actual linkage analysis and one marker with four equally frequent alleles at a genetic distance of 1 cM from the disease locus predicted that a maximum lod score of 3.12 could be obtained in this pedigree. However, this theoretical maximum would be attainable only in the event that none of the unaffected subjects carries the susceptibility haplotype. In contrast, six out of 13 at risk subjects (IV.9, IV.10, IV.11, IV.13, V.5, and V.6) have inherited the disease haplotype and thus appear to be non-penetrant carriers (fig 1). This is in agreement with the penetrance of 50% estimated in our pedigree before marker genotyping on the basis of disease status information only.

Marker D6S305 on chromosome 6q25 gave a lod score of 2.27 in the affecteds only two point analysis. Multipoint analysis of the entire family using a reduced penetrance model consistently decreased the lod scores for this region with a maximum of 2.05. Although a negative lod score of less than −2 is generally deemed necessary formally to exclude a region in linkage analysis, these findings significantly reduced the odds of linkage to 6q25. False positive lod scores of this order of magnitude are not uncommon in genome wide screens. However, while we conclude that there is a high likelihood of one or more genes on chromosome 6p21 influencing LR axis development, we cannot

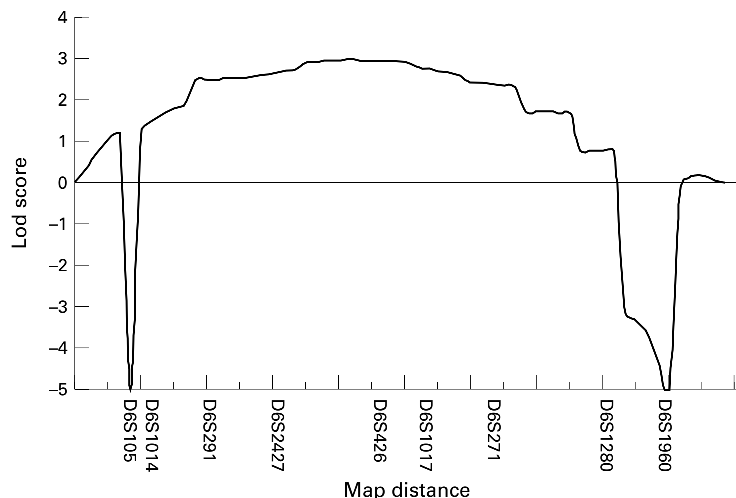

Figure 2 Multipoint linkage analysis for chromosome 6p21 markers.

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formally exclude that a second disease susceptibility gene is located on 6q25. Investigation of these regions in other independent families with the same phenotype will be important in order to provide a definite confirmation of our findings.

The present study cannot determine the precise mechanism by which this locus contributes to the phenotype, particularly given the presence of several non-penetrant carriers. Chance alone may be at work, or the locus may be necessary but not sufficient to cause disease in the absence of modifying loci elsewhere in the genome. It is possible also that there are two or more linked susceptibility loci within this large interval in 6p.

Several genes that affect LR axis development have been identified in the mouse.[4] Where human homologues have been identified, none maps to 6p, and none of the remaining murine genes maps to regions homologous to 6p.[14] A search for candidate genes among those transcripts localised to the critical region shows several that might be involved, but no immediately compelling candidates. Identification of the susceptibility gene (or genes) in this interval awaits further understanding of the genetic pathway in model systems or the future discovery of suitable candidate genes in this region of the human genome.

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Electronic database information: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/ (for asplenia with cardiovascular anomalies (MIM 208530), situs inversus viscerum (MIM 270100), heterotaxy, visceral, X linked (MIM 306955), laterality defects, autosomal dominant (MIM 601086)). Center for Medical Genetics, Screening Sets of Markers, version 6, http://www.marshmed.org/genetics/sets/scrset6.txt

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Founder effect in multiple endocrine neoplasia type 1 (MEN 1) in Finland

EDITOR—Multiple endocrine neoplasia type 1 (MEN 1, OMIM 131100) is transmitted as an autosomal dominant trait with an equal sex distribution and close to full penetrance. Hyperparathyroidism occurs in over 90% of the cases and is invariably associated with multiglandular disease. In addition, the patients may develop tumours of the endocrine pancreas, the anterior pituitary, and the adrenal cortex, as well as lipomas and carcinoids.[1] The *MEN1* tumour suppressor gene at 11q13 was cloned by positional cloning and the protein, menin, has been found to bind specifically to JunD leading to inhibition of JunD activated transcription.[2–5] Following the initial description of the disease gene, over 200 *MEN1* germline mutations scattered throughout the entire gene have been reported. The majority of these mutations are unique and no clear cut genotype-phenotype correlation has been established so far.[3 4 6–13] To date, only a few reports of a founder effect in MEN 1 families have been published.[10 14 15] In all the cases, the evidence for a founder chromosome was based on the presence of a shared disease haplotype and a *MEN1* mutation, in combination with a common geographical origin of the families involved.

To date, more than 30 rare single gene diseases, mostly autosomal recessive, have been identified in the Finnish population.[16 17] The clustering of mutations in the Finnish population can be explained by the mechanisms of isolation, genetic drift, and founder effect. The relatively small founder population, the geographical isolation, and the internal migration movement in the 1500s led to the formation of numerous founder populations. The Finnish population has been widely used in linkage disequilibrium studies for mapping disease gene loci. By combining genetic and clinical investigations with careful genealogical studies, several founder chromosomes for various genetic diseases have been established.[17–19]

A total of 22 MEN 1 families and four isolated MEN 1 cases have been identified in Finland, 20 of them in the northern part of the country. The diagnosis of MEN 1 was based on the findings of tumours in two or more of the

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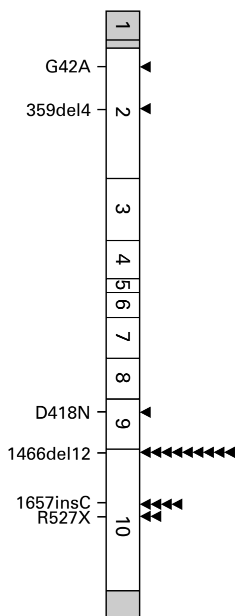

Figure 1 Diagramatic representation of the mutations found in the MEN1 gene in the Finnish population. Each triangle represents one mutation found in one MEN 1 family. Hatched areas are not translated.

principal MEN 1 related glands (that is, the parathyroid glands, the endocrine pancreas-duodenum, and the anterior pituitary). In familial MEN 1, one or more affected relatives also had clinical, radiological, or surgical evidence of MEN 1 related tumours. Isolated MEN 1 was considered in a subject with clinical features of MEN 1 without a known family history of the disease. Informed consent for the mutation screening and genotyping studies was obtained from the participating patients, and the study was approved by the local ethical committee. Some of the families have been published previously,[20] as well as some of the results from *MEN1* mutation screening.[9]

Mutation analysis on 26 subjects representing each of the 22 familial and four isolated cases was performed using single strand conformation analysis (SSCA) and direct DNA sequencing, as previously described.[4 21] For all familial cases where a mutation was detected, its presence in additional family members was tested and confirmed by sequencing or restriction cleavage. Two or more affected members from each of the 22 families were genotyped for five microsatellite markers (D11S1883, D11S4946, D11S4940, D11S4937, and D11S449) spanning the *MEN1* region in 11q13. The pedigrees with the two prevailing mutations were further analysed by genealogical studies. The names, dates, and places of birth of ancestors were traced using the Finnish church records, which frequently cover birth and family information back to the years 1650-1700.

The mutation screening of the *MEN1* gene showed six different germline mutations in 17 familial (77%) and one isolated (25%) cases. The mutations G42A (GGC→GCC)

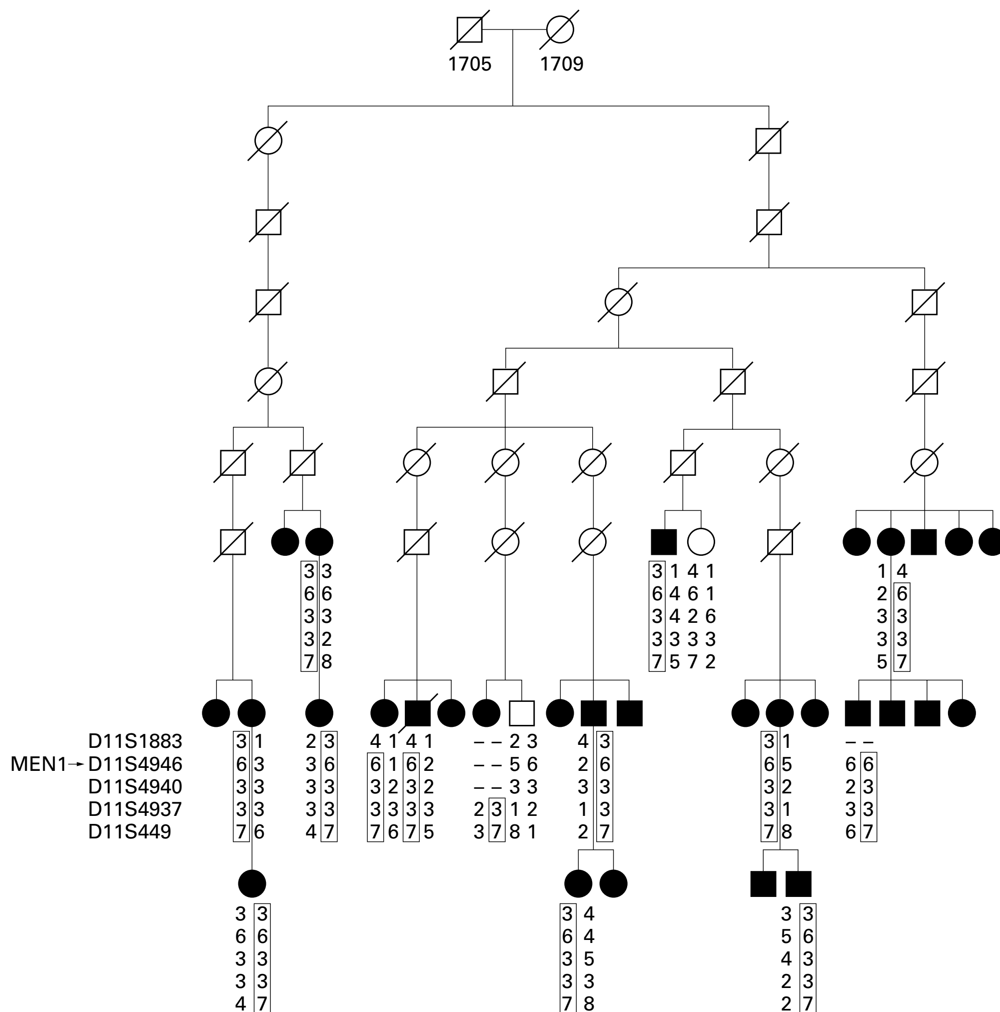

Figure 2 Pedigrees of the families with the 1466del12 mutation. Black symbols indicate clinically affected MEN1 mutation carriers. The affected haplotype is outlined by a box. The common ancestor was found for eight of the nine 1466del12 families.

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and 359del4 (GTCT) were located in exon 2, D418N (GAC→AAC) in exon 9, and 1466del12 (GCAGAAGGT-GCG), 1657insC, and R527X (CGA→TGA) in exon 10 (fig 1). As expected, most of the mutations were frameshift (359del4, 1657insC), nonsense mutations (R527X), and deletions (1466del12), which are likely to cause truncation of the *MEN1* gene product, menin, resulting in the loss of its function. The two missense mutations detected, G42A and D418N, are also expected to be pathogenic since they have not been detected in 100 normal subjects. The mutations 1466del12, 1657insC, and R527X were found recurrently in nine, four, and two probands, respectively (fig 1). Four of the mutations identified in this study (359del4, D418N, 1657insC, and R527X) have previously been reported in familial or isolated MEN 1 patients of non-Finnish origin.[7–12, 14] The regions of the *MEN1* gene where these recurrent mutations have occurred consist of CpG, short DNA repeats, or single nucleotide repeat motifs, which have been reported to be prone to mutations in the *MEN1* gene.[14] The codons 83-84, 210-211, and 514-516 appear to be "warm"spots for *MEN1* mutations.[7, 14] The mutations G42A and 1466del12 have not been reported in other populations.

MEN1 mutations were not detected in five familial and three isolated cases. In these cases, the mutation could be a large deletion located in regulatory or untranscribed regions of the *MEN1* gene, which would be undetectable by the method used. The introns except for the intron/exon boundaries were excluded in the mutation analysis. It has been reported that even 20% of the *MEN1* mutations involve intron sequences.[12] *MEN1* mutations have been found less frequently in isolated than in familial cases, suggesting that some of the isolated cases may have a different genetic aetiology or that two endocrine tumours occur coincidentally in the same patient.[6, 7, 9, 10, 13]

Although the majority of the Finnish population live in the southern part of the country, most of the MEN 1 cases (20/26) were clustered in a limited geographical area in the north of the country. This can be partly explained by clustering of the mutations, a phenomenon, which has been described in many Finnish genetic diseases.[16] Haplotype analysis showed that the nine families with the 1466del12 mutation shared the same haplotype (6-3-3-7) for the region covered by D11S4946 - D11S4940 - D11S4937 - D11S449 (fig 2). By careful genealogical study, eight families were found to be descended from an identifiable founder couple (fig 2). The couple were born in 1705 and 1709 in a small village where most of the descendants still live. The resulting pedigree consists of eight generations, when counted from the present day patients. It is possible that the *MEN1* mutation has occurred beyond the last eight generations, because the connection of the ninth family to this pedigree has not been found even though it shares the same affected haplotype with the other eight families. The mutation analysis showed 1466del12 in the two Finnish families sharing the disease linked haplotype in the previous linkage disequilibrium study.[22] The age of the 1466del12 mutation and the large family sizes, usually seen in northern Finland, suggest that more mutation carriers than presently known could exist in northern Finland.

The families with the 1657insC mutation also shared a common haplotype (3-5-3-3-8) with all the five markers (fig 3). Two patients with the same haplotype on both chromosomes do not indicate homozygosity of the mutation, but a common haplotype in the Finnish population. All the four families could be traced back to a couple born in 1844 and 1846, respectively, four generations from the youngest living patients (fig 3). Two families sharing the common disease linked haplotype in our previous linkage disequilibrium study[22] were found to have the 1657insC mutation.

In the two families with the R527X mutation, the disease was clearly linked to different haplotypes, showing that the mutations had occurred independently.

All the 22 families and four isolated cases with MEN 1 fulfilled the criteria for the MEN 1 syndrome with at least two MEN 1 related endocrine organs affected. The main clinical manifestations of the patients with the identified *MEN1* mutation are detailed in table 1. In total, 65 affected *MEN1* mutation carriers and 10 clinically unaffected *MEN1* mutation carriers were found in these families. Primary hyperparathyroidism (pHPT) was the most common

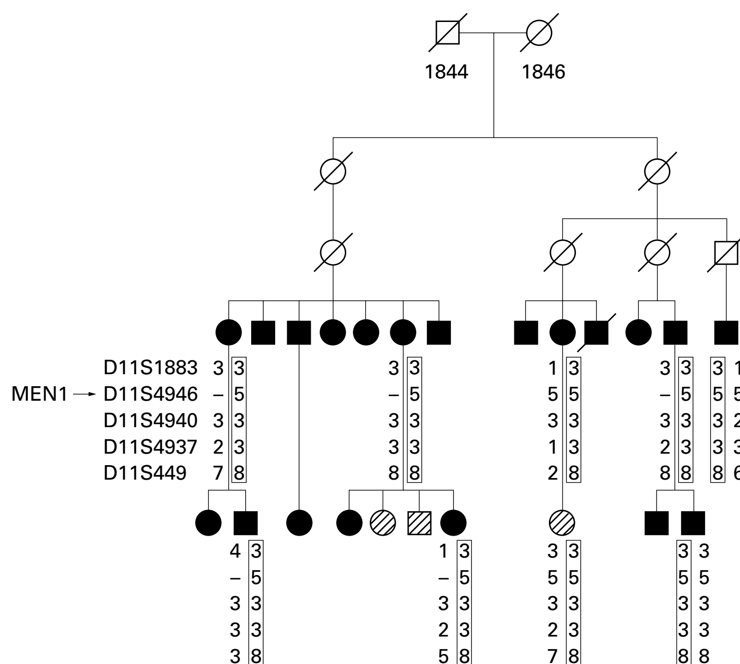

Figure 3 Pedigrees of the families with the 1657insC mutation. Black symbols indicate clinically affected MEN1 mutation carriers and hatched symbols indicate unaffected (or with unknown clinical status) mutation carriers. The affected haplotype is outlined by a box. The common ancestor was found for all the four families with the 1657insC mutation.

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Table 1 Clinical manifestations in 18 Finnish MEN1 families with identified MEN1 gene mutation

	MEN1 mutation						
	1466del12	*1657insC*	*R527X*	*D418N*	*359del4*	*G42A**	*Total*
No of families	9	4	2	1	1	1	18
Affected mutation carriers	31	20	5	5	3	1	65
Main MEN1 lesions							
Primary HPT	30	20	5	4	3	1	63
GEP tumour	21	9	3	3	0	0	36
Pituitary tumour	6	7	2	2	0	1	18
Other associated tumours							
Adrenal hyperplasia	10	4	0	1	0	0	15
Lipoma	8	3	1	0	0	0	12
Carcinoid	1	3	0	0	0	0	4

*Isolated case.

manifestation and was seen in 63 patients (97%). The second most frequent clinical finding was gastroenteropancreatic tumour (55%), followed by pituitary tumours (28%) and benign adrenal hyperplasia (23%), as well as lipomas (18%) and carcinoids (6%).

GEP tumours were more common in the 1466del12 families (68%) than in the 1657insC families (45%), while the occurrence of the pituitary adenomas was the reverse. The differences in the occurrences of the clinical findings might be explained by the varied age and sex of the MEN 1 patients in different families. Families with the prolactinoma variant of MEN 1 families have been reported as a phenotypic variant of MEN 1; however, the mutations were different in these families.[6] Several families with isolated hyperparathyroidism have been reported and in some families with at least three affected subjects a mutation of the *MEN1* gene (E255K, Q260P, L267P, G305D, Y353X, 1350del3) has been detected.[13 21 23–26] pHPT is usually the first symptom of the MEN 1 syndrome and some of the patients in the isolated hyperparathyroidism families may develop MEN 1 syndrome at later ages.

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Familial non-medullary thyroid cancer in Iceland

EDITOR—Incidence of thyroid carcinoma in Iceland is high[1] and papillary carcinoma comprises 80% of all thyroid malignancies in the country.[2] It is well known that medullary thyroid carcinoma aggregates in families and the genetic component in the aetiology has been established.[3] Familial occurrence of non-medullary thyroid carcinoma is rare, but it has been increasingly recognised in recent years.[4–6] There has been increasing awareness that this may constitute a distinct disease subgroup rather than chance occurrence, as reflected by the review of case series by Loh.[7] Familial papillary microcarcinoma has also been suggested as a new clinical entity.[8 9] In an earlier paper, we reported on the occurrence of papillary thyroid carcinoma (373 probands) in first degree relatives of all patients diagnosed between 1955 and 1984 in Iceland.[10] This study showed a non-significant trend to familiality. In the present study, the population includes second and third degree relatives in addition to first degree relatives and we now include all non-medullary thyroid carcinoma patients.

Information on thyroid cancer cases was obtained from the Icelandic Cancer Registry. All cases (n=712) of non-medullary thyroid carcinoma diagnosed in Iceland between 1955 and 1994 form the basis of this study. This includes 147 thyroid cancers found incidentally at necropsy and 565 thyroid cancers diagnosed clinically. The information on first, second, and third degree relatives was obtained from the Genetical Committee of the University of Iceland. This committee has a computerised family tracing resource based on the 1910 census and birth records for 1840-1910. This resource is linked with the national register and death records for later periods. Permission was obtained from the Data Protection Commission to link files from the Cancer Registry with records from the Genetical Committee. The 712 families contributed to the man year calculations on which expected numbers are based. For nine probands of foreign origin, there were no relatives in the study population.

All cases were histologically verified. The histopathology was re-examined by one of the authors (JGJ). Included in the file were all differentiated thyroid cancers. These include papillary and follicular thyroid cancers, as well as anaplastic cancers, as these tumours are derived from the follicular epithelial cells. Thyroid carcinomas showing Hürtle cell differentiation were classified either as follicular or papillary histological type. Medullary cancers and lymphomas of the thyroid were excluded from the study.

The study risk period started in January 1955 or at birth (for persons born after 1 January 1955) and ended on 31 December 1997 or at death if death occurred earlier. Incidence rates of thyroid cancer stratified according to gender, five year age intervals, and five year calendar intervals from 1955 to 1997 were used. Incidence rates of thyroid cancer before 1955 were not available.

Years at risk stratified in the same manner for each degree of relatedness were calculated using the program PERSON-YEARS (PYRS) from the World Health Organization.[11] Expected numbers of cases were calculated as the sums of products of incidence and years at risk. Relative risk (RR) was calculated as the ratio between observed and expected numbers. Pooled relative risk was calculated as the weighted sum of relative risk over the degrees of relatedness 1, 2, and 3 with the weights 1, 1/2, and 1/4, respectively; p values less than 0.05 were considered significant.

Table 1 summarises the study population. Altogether, there were 57 391 relatives in the pedigrees. Of these, 49 949 (25 396 males and 24 553 females) were alive on or after 1 January 1955, thus contributing to the man year calculations. Table 1 describes the distribution of probands and relatives. There were 712 thyroid cancer cases as probands. Among relatives, thyroid cancer occurred 249 times.

Table 2 classifies the probands according to family history, histology, and sex. There were 184 probands with one or more relatives affected. In six pedigrees, three affected relatives were found. There is only a small difference in histology between familial and sporadic cases. Papillary cancer is somewhat more common in the familial group (81%) than in the sporadic group (76%) in males. This difference was not found among females.

Table 3A shows the relative risk of thyroid cancer for relatives of all thyroid cancer patients. Figures are shown separately for first, second, and third degree relatives and

Table 1 Probands with number of male and female relatives and number of relatives with thyroid cancer

	Study population		*Thyroid cancer*
	No in pedigree	*No alive in 1955*	*No of relatives*
Probands			
Males	206		
Females	506		
Total	712		
Relatives			
Males	29 400	25 396	84
Females	27 991	24 553	165
Total	57 391	49 949	249

Table 2 Number of probands with and without relatives affected with non-medullary thyroid cancer according to histology and sex

	Males	*Females*
Probands with affected relatives		
Papillary	46	102
Follicular	7	19
Anaplastic	4	6
Total	57	127
Probands with no affected relatives		
Papillary	113	303
Follicular	29	47
Anaplastic	7	29
Total	149	379

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Table 3 Relative risk of thyroid cancer for relatives of thyroid cancer patients

		No		Ratio	
Relatedness	Gender	Obs	Exp	O/E	95% CI
(A) All probands (n=712)					
1st degree	Male	28	6.83	4.10	2.74–5.90
1st degree	Female	32	16.56	1.93	1.31–2.70
2nd degree	Male	23	12.17	1.89	1.12–2.66
2nd degree	Female	52	32.90	1.58	1.15–2.01
3rd degree	Male	35	22.55	1.55	1.04–2.06
3rd degree	Female	81	55.96	1.45	1.14–1.77
Pooled	Male			3.83	1.63–5.04
Pooled	Female			2.08	1.58–2.58
(B) Male probands (206)					
1st degree	Male	13	1.95	6.67	3.57–11.29
1st degree	Female	14	4.92	2.84	1.55–4.74
2nd degree	Male	8	3.63	2.20	0.94–4.35
2nd degree	Female	14	9.77	1.43	0.78–2.39
3rd degree	Male	15	7.56	1.98	0.98–2.98
3rd degree	Female	18	17.34	1.04	0.56–1.52
Pooled	Male			6.52	3.56–9.48
Pooled	Female			2.55	1.41–3.70
(C) Female probands (506)					
1st degree	Male	15	4.88	3.07	1.71–5.03
1st degree	Female	18	11.64	1.55	0.92–2.43
2nd degree	Male	15	8.54	1.76	0.87–2.65
2nd degree	Female	38	23.13	1.64	1.12–2.16
3rd degree	Male	20	14.99	1.33	0.75–1.92
3rd degree	Female	63	38.62	1.63	1.23–2.03
Pooled	Male			2.92	1.70–4.14
Pooled	Female			2.02	1.47–2.57

for sex, as well as for all relatives pooled. For all probands, the risk ratio was 3.83 for male relatives and 2.08 for female relatives. For first degree relatives only, the respective risk ratios were 4.10 and 1.93. All these risk ratios are significant. In table 3B and C, the risk ratios for male and female probands are shown separately. For families of male probands, the pooled risk ratio was 6.52 for male relatives and 2.55 for female relatives. For families of female probands, the pooled risk ratio was 2.92 for male relatives and 2.02 for females. All these ratios are higher for male relatives than for female relatives and highest for male relatives of male probands.

Calculations were done separately on the 565 thyroid cancers diagnosed clinically (results not shown). Relatives of these patients (150 males and 415 females) also had a significantly increased risk of developing thyroid cancer, although the risks were somewhat lower than for the total cohort. This shows that relatives of patients with non-medullary thyroid cancer are at significantly increased risk of developing thyroid cancer. The risk is greater in first degree relatives in each sex compared to second and third degree relatives, as shown in table 3. The risk is highest in male relatives of male probands (6.52) and lowest in female relatives of female probands (2.02). As shown in table 1, the disease is more than two times more common in females than males. This finding of greater excess risk of males related to males supports the suggestion that the greater risk in females in the general population in based on either female specific risk factors or greater susceptibility of females to external causes of non-medullary thyroid cancer. Thus, in males, a greater proportion of cancer risk is the result of familial factors than in females.

This study covers the whole Icelandic population, which is relatively small with only around a quarter of a million inhabitants. Because the nation has been rather isolated for centuries, people may be more interrelated than in other countries, which could result in a higher risk of familiality. In a review of case series,[7] the previous study from Iceland[10] showed the highest prevalence among 15 case series. There the prevalence was defined as the number of patients with a family history over the total number of patients with non-medullary thyroid cancers in the respective series.

The aetiology of thyroid cancer is mostly unknown with the exception of radiation. In a previous investigation concerning the effect of radiation used for benign conditions in children, we followed a cohort irradiated between 1930 and 1950. We found three cases of thyroid cancer, none of whom had a relative with thyroid cancer.[12] Therefore, radiation has not contributed to excess familial risk in our study.

Microcarcinomas of the thyroid gland are mostly of papillary histological type and have been thought to be a low risk category of tumour.[13] Recently, reports have been published showing familiality of non-medullary thyroid microcarcinomas.[8 9] Our study shows that when taking into account cases found incidentally at necropsy (which are mainly papillary microcarcinomas), we see a stronger risk for familiality. A block from the thyroid gland is taken routinely at all necropsies in Iceland and microscopically evaluated.

In a study on forensic necropsies specifically looking for incidental carcinoma of the thyroid gland in Iceland, the frequency was 7.5% in males and 5.1% in females.[14] This male to female ratio of incidental tumours is in sharp contrast to the male to female ratio of clinically detected cases. The finding that incidentally found papillary thyroid carcinomas have stronger familiality than clinically diagnosed cases may explain this observation.

Further studies are needed on the molecular genetics of non-medullary thyroid cancer, as well as on the prevalence of neoplasms at other sites and survival of familial cases.

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Sulphate transporter gene mutations in apparently isolated club foot

EDITOR—Diastrophic dysplasia was originally ascribed to sulphate transporter gene (*DTDST*) mutations. The *DTDST* gene is now also known to account for a variety of bone dysplasias including diastrophic dysplasia, atelosteogenesis type II (AO2), and achondrogenesis Ib.[1–3] Abnormally sulphated cartilage proteoglycans with deficient cartilage sulphate content has been reported in these conditions,[4] suggesting that a variable residual *DTDST* activity probably accounts for the broad spectrum of clinical phenotypes at this locus.[5]

While a predominant founder mutation in the splice donor site of a previously undescribed 5' untranslated exon accounts for the disease in Finland, more than 30 mutations have been reported so far world wide[6] and compound heterozygosity for variably deleterious mutations probably explains the broad spectrum of clinical phenotypes at the *DTDST* locus.[2 7 8] The R279W mutation is the most common mutation in non-Finnish patients and, apart from its original report in an AO2 patient, compound heterozygosity for this mutation has been consistently associated with a non-lethal phenotype.

Recently, Superti-Furga *et al*[9] reported homozygosity for the R279W mutation in an adult affected with multiple epiphyseal dysplasia, normal stature, club foot, and double layered patella. Here, we add further support to this view and report on the association of apparently isolated club foot with the homozygous R279W *DTDST* mutation in two unrelated sibships of western French ancestry (Brittany).

In family 1, dizygotic twins, a boy and a girl, aged 14 years, were referred for moderate hand stiffness. They were born after a normal pregnancy (birth weight 2650 g and length 48 cm for the boy and birth weight 2700 g and length 47 cm for the girl). At birth, the girl had a metatarsus varus of the left foot which rapidly recovered after physiotherapy. She developed normally and at 14 years of age she had normal stature (height 158 cm), no facial dysmorphism or abnormal external ears, and did not complain of hip, knee, or ankle pain after physical exercise. At birth, her brother had bilateral club foot which required surgery with persistence of retraction on the left side. His stature was normal (height 160 cm at 14 years). There was no ear swelling, hitch hiker thumb deformity, or limitation of full extension of the elbow and his face and palate were normal. At birth, *x* rays showed a normal spine and lower limbs, but a bone age advanced by one year was observed when the boy was 2 year old. At 14 years, *x* rays showed enlarged metaphyses of the phalanges and shortened metacarpals (fig 1A). When the radiographs were re-examined, mild epiphyseal dysplasia of the upper femoral neck (fig 1B) was noted but no knee abnormality or double layered patella were observed. The parents originated from the same region of Brittany but no consanguinity was known.

In family 2, the first child, a boy (birth weight 4650 g and length 52 cm) had bilateral club foot diagnosed during pregnancy by ultrasound examination. Associated with

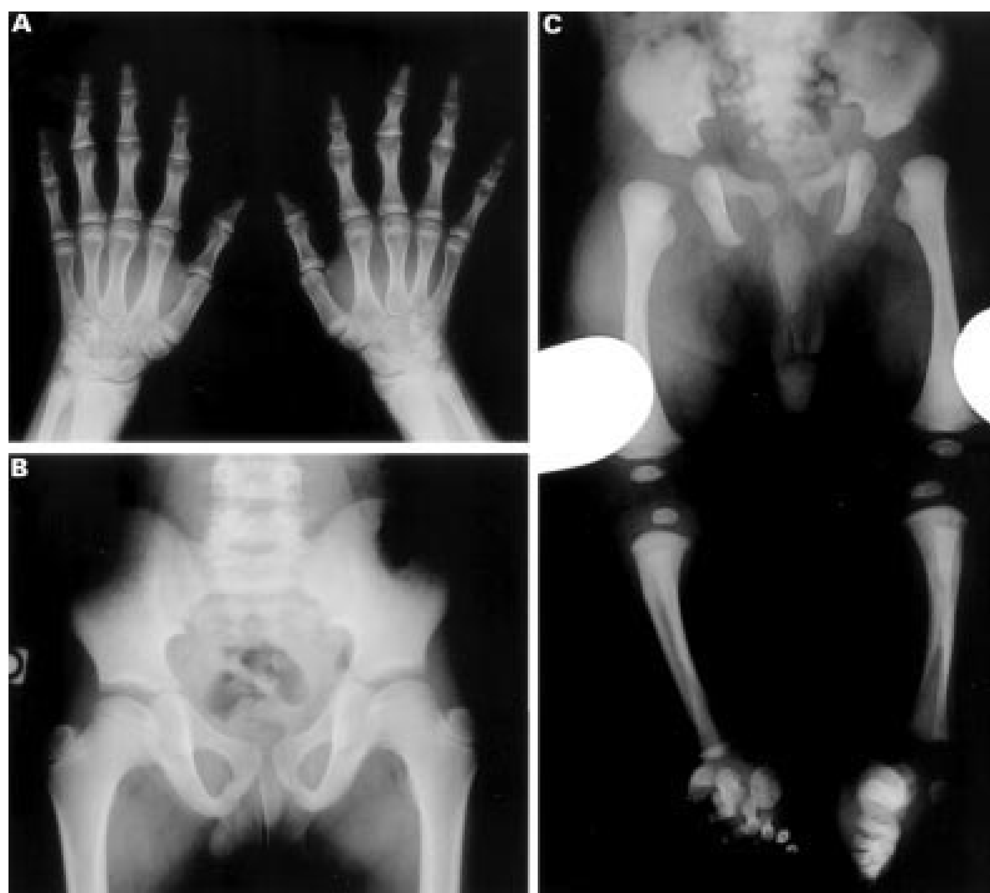

Figure 1 (A) Case 1, aged 14, showing enlarged metaphyses of the phalanges and metacarpals. (B) Case 1, aged 14, showing flattened femoral epiphyses. (C) Case 2, newborn, note the mild enlargement of the femoral metaphysis and the club foot.

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this, mild micromelia with a small chin and mouth was noted at birth. Neither ear swelling nor hand abnormalities were detected, but pronosupination of the elbows was limited. He grew slowly (height 90 cm at 3 years, 25th centile) and *x* rays of the long bones were considered to be normal (fig 1C). Retrospectively, however, a mild flaring of the metaphyses of the long bones with delayed ossification of the epiphyses of the hip were noted at 3 years of age. Vertebral bodies were normal. During the second pregnancy, short femora were detected antenatally. The baby, a girl (birth weight 3250 g, length 46 cm at 37 weeks of pregnancy), had micromelia with brachymesophalangy of the fifth finger and a narrow thorax. The thumbs and feet were normal but a subluxation of the left hip was detected on echography at 2 months and treated by abduction splinting. Radiographs of the upper limb showed two ossified carpal bones and a dislocated elbow at 4 months. Her stature at 9 months was 67 cm (25th centile). There was no functional deficit or pain in the two children. Their parents originated from two villages 10 kilometres apart, but no consanguinity was known.

Because a mild form of diastrophic dysplasia was considered, leucocyte DNA from the probands were screened for mutations in the coding region of the *DTDST* gene. Direct PCR sequencing was performed in both directions using Big Dye terminators (Perkin- Elmer) on an Applied Biosystem 373A apparatus using the two following primers (forward: DTD5: TGC TCT GAT GAT ATG TCT CCA TGC and reverse: DTD8: TAT TCG ATC TAC AGC CAC ACT).[6] Interestingly, the four probands were homozygous for the mutation R279W in the fifth extracellular loop of the sulphate transporter protein.

Club foot is a common malformation, either isolated or associated with various neurological syndromes or chondrodysplasias, including diastrophic dysplasia, spondyloepimetaphyseal dysplasia with joint laxity, Kniest disease, and multiple epiphyseal dysplasia (MED). Isolated club foot (or metatarsus varus with no other clinical abnormality or short stature) is currently regarded as a malformation with a multifactorial mode of inheritance with a recurrence risk of approximately 10%.[10 11] In family 1, club foot was originally regarded as isolated, especially as the two affected children did not complain of functional deficit. They only complained of stiffness of the fingers and the diagnosis of MED was eventually considered after *x* ray discovery of small upper femoral epiphyses. At that time, the association of club foot (metatarsus varus), MED, and stiffness of the hand with short metacarpals and enlarged metaphyseal phalanges prompted us to consider and eventually confirm the diagnosis of a diastrophic dysplasia variant,[12] despite the absence of the double layered patella. The diagnosis of MED has been difficult to reach in family 2 as well, because the two children were too young. However, the delayed ossification of the femoral epiphyses of the hips might have suggested this diagnosis to us earlier.

The association of MED with the homozygous *DTDST* R279W mutation has been previously noted and ascribed to the normal stability of the mutant *DTDST* mRNA.[8] Recently, Superti-Furga *et al*[9] reported that autosomal recessive multiple epiphyseal dysplasia could result from homozygous *DTDST* R279W mutations. The patients reported here add to the view that the clinical spectrum of *DTDST* mutations is much broader than originally believed and includes apparently isolated club foot. However, the molecular mechanisms underlying the intrafamilial or interfamilial clinical variability of the homozygous R279W mutation remain unexplained.[5]

Based on these observations, we suggest giving consideration to *DTDST* mutation analysis in apparently isolated club foot, especially in cases of familial recurrence. Skeletal *x* rays and molecular investigations should help to confirm this diagnosis and determine the actual incidence of *DTDST* mutations in apparently isolated club foot.

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Development and application of linkage analysis in genetic diagnosis of familial hypertrophic cardiomyopathy

EDITOR—Familial hypertrophic cardiomyopathy (FHC) is a prevalent dominantly inherited disease characterised by unexplained hypertrophy of the heart muscle. The clinical manifestations are heterogeneous and the disease is a leading cause of sudden cardiac death among young, otherwise healthy people.[1] More than 120 different mutations have been reported in the following eight genes encoding sarcomeric polypeptides given in parentheses: *TNNT2* (troponin T), *MYL3* (essential myosin light chain), *MYH7* (β myosin heavy chain), *MYBPC3* (myosin binding protein C), *MYL2* (regulatory myosin light chain), *TPM1* (α tropomyosin), *ACTC* (α cardiac actin), and *TNNI3* (troponin I).[2 3] Furthermore, a disease locus on chromosome 7 has been linked to FHC, but the gene has not yet been identified.[4] Additional disease genes probably remain to be discovered since two recent studies found that it was only possible to genotype 50-60% of the FHC population by mutation analyses of seven disease genes.[5 6] In order to optimise risk stratification and management of FHC patients, it is important to identify all disease carriers, which is difficult by physical examination because of the age dependent penetrance of the disease. However, disease carriers may be identified by use of genetic diagnosis, although it is laborious because of the large number of disease genes and the pronounced allelic heterogeneity of the disease loci, with the majority of affected families having their own "private" missense mutation.[7] In addition, genetic diagnosis is complicated by the fact that several amino acid polymorphisms occur in most of the FHC genes[8 9] (unpublished observations). Given this complex genetic background, the use of linkage analysis can be beneficial as it may identify the most likely disease gene and provide evidence for exclusion of some or all of the other candidate disease loci even in small families.[10]

It was the aim of the present study to develop a firm basis for efficient use of linkage analysis in genetic diagnosis of FHC by a well founded selection of polymorphic markers defining nine FHC loci, including a refined genetic mapping of the troponin T gene in a 4 cM interval. For rapid analysis, multiplex PCR panels were developed comprising all markers selected. The feasibility of the method was evaluated by identification of mutations in three families of varying size.

The genetic mapping of *TNNT2* was based on analysis of six informative CEPH pedigrees (obtained from CEPH, France: Nos 102, 884, 1347, 1362, 1413, 1416; 102 subjects) using a previously published intragenic insertion/deletion polymorphism localised in intron 4 (*TNNT2*-Ins/Del).[11] A 340 bp fragment of *TNNT2* including exons 3, 4, and *TNNT2*-Ins/Del (base position 181-520) was amplified using the primers: forward (F) 5'-GTGGCAGGCAGCGTGACTCCAC-3' (the primer sequence was modified in accordance with our own unpublished sequencing results of intron 3 by omitting Gs in position 184, 190, and 198), reverse (R) 5'-CAGGATTTCCACATTGCTGA-3'. PCR was carried out as previously described[3] with primer concentrations given in table 1 and an annealing temperature of 62°C. Multipoint linkage analyses were carried out using chromosome 1 DNA markers in the region previously reported to harbour *TNNT2*[12 13] and the CEPH database version v8.1,[14] essentially as previously described.[15] A 16 point reference map was chosen in accordance with the Généthon genetic map where the marker loci are ordered with odds of at least 1000:1.[16] CMAP analysis was carried out calculating the likelihood for any position of *TNNT2* with respect to the fixed map. The location score curve showed a peak location score of 92.9 between D1S2716 and D1S504, which corresponded to a multipoint lod score of 20.2. The location of *TNNT2* in a 4 cM interval between D1S2716 and D1S504 was favoured

Table 1 Composition of multiplex PCR panels used for linkage analysis in FHC pedigrees

Multiplex name (No PCR cycles)	Marker data					Primer concentration (pmol/μl)	
	Marker name (gene)	Length (bp)	Fluorescent dye	HZ index	No of alleles	Forward	Reverse
MP1 (35 cycles)	D11S1993, (*MYBPC3*)	224–245	HEX	75	8	0.2	0.2
	D7S661, (*FHC locus*)	252–282	TET	76	10	0.4	0.4
MP2 (30 cycles)	D3S3685, (*MYL3*)	195–221	TET	89	13	0.2	0.2
	D3S3564, (*MYL3*)	204–220	HEX	79	7	0.3	0.3
	D3S3582, (*MYL3*)	220–236	FAM	68	7	0.2	0.2
MP3 (30 cycles)	MYO I-CA, (*MYH7*)	90–102	FAM	66	6	0.2	0.2
	MYO II-CA, (*MYH7*)	108–132	HEX	81	13	0.2	0.2
MP4 (30 cycles)	D1S2622, (*TNNT2*)	165–189	TET	79	11	0.2	0.2
	TNNT2 Ins/Del, (*TNNT2*)	335–339	FAM	50	2	0.1	0.1
	D1S2872, (*TNNT2*)	187–207	FAM	76	11	0.4	0.4
MP5 (30 cycles)	D12S1583, (*MYL2*)	219–247	HEX	87	14	0.1	0.1
	D15S153, (*TPM1*)	194–226	TET	86	12	0.1	0.1
	D15S1036, (*TPM1*)	118–140	TET	85	10	0.2	0.2
MP6 (35 cycles)	D12S1343, (*MYL2*)	190–216	FAM	70	9	0.2	0.2
	D12S84, (*MYL2*)	199–219	TET	83	9	0.2	0.2
	HTMα-CA, (*TPM1*)	108–120	TET	60	6	0.1	0.1
MP7 (35 cycles)	D7S505, (*FHC locus*)	262–278	FAM	69	8	0.2	0.2
	D7S483, (*FHC locus*)	166–188	HEX	81	10	0.2	0.2
MP8 (35 cycles)	D3S1578, (*MYL3*)	140–166	FAM	87	14	0.2	0.2
	MYBPC3-CA, (*MYBPC3*)	282–290	HEX	52	5	0.2	0.2
MP9 (35 cycles)	D11S1785, (*MYBPC3*)	268–276	FAM	70	5	0.15	0.15
	D11S903, (*MYBPC3*)	99–109	FAM	74	6	0.2	0.2
	D11S1344, (*MYBPC3*)	273–293	HEX	80	11	0.3	0.3
	D11S4191, (*MYBPC3*)	111–135	TET	87	11	0.2	0.2
MP10 (35 cycles)	ACTC-CA, (*ACTC*)	70–96	TET	86	12	0.2	0.2
	D19S887, (*TNNI3*)	246–262	HEX	72	8	0.3	0.3
	D19S254, (*TNNI3*)	110–150	TET	60	5	0.4	0.4
	D19S210, (*TNNI3*)	165–177	TET	73	6	0.2	0.2

Primer sequences of the polymorphic markers were obtained from the Genome Database (GDB)[37] except for *MYBPC3-CA* where primers were designed in accordance with the published genomic sequence (base position 11 473–11 756 (F-5'-GATTTATTTGGCCAGGGAACC-3'; R-5'-TTTCAAAACTGGGGGAACATC-3')).[26]

by odds of more than 100 000:1 over the second most likely interval, that is, between D1S2716 and D1S2622 (complete data set is available upon request). This refined assignment was in accordance with previous localisations of *TNNT2*.[12 13 17 18]

The remaining FHC loci have all been localised by physical and/or genetic mapping with evidence sufficient to allow a well founded selection of flanking markers. The evidence for the regional localisation of the FHC loci and their relationships to flanking markers were obtained by a thorough integration of mapping information deposited at different databases including the Human Genome Sequencing Project[18] and published reports. The results are summarised in fig 1.

MYL3,[19] *MYBPC3*,[20] and *TNNI3*[21] have all been localised at various radiation hybrid (RH) maps with odds of 1000:1, 1000:1, and a lod >3, respectively. Polymorphic markers flanking the genes were all localised with odds of 1000:1 on the RH maps except the marker flanking *TNNI3* (D19S218), which is a framework marker localised with odds of 300:1 on the Whitehead RH map.[22] The assignment of *MYBPC3* was in accordance with previous localisations.[23–25] *MYBPC3* carries an intragenic polymorphic marker (*MYBPC3-CA*) within intron 20,[26] which was characterised by analyses of 50 chromosomes from unrelated white Danish subjects. Five different alleles were identified with the following frequencies: allele 1 (282 bp) 0.12, allele 2 (284 bp) 0.16, allele 3 (286 bp) 0.34, allele 4

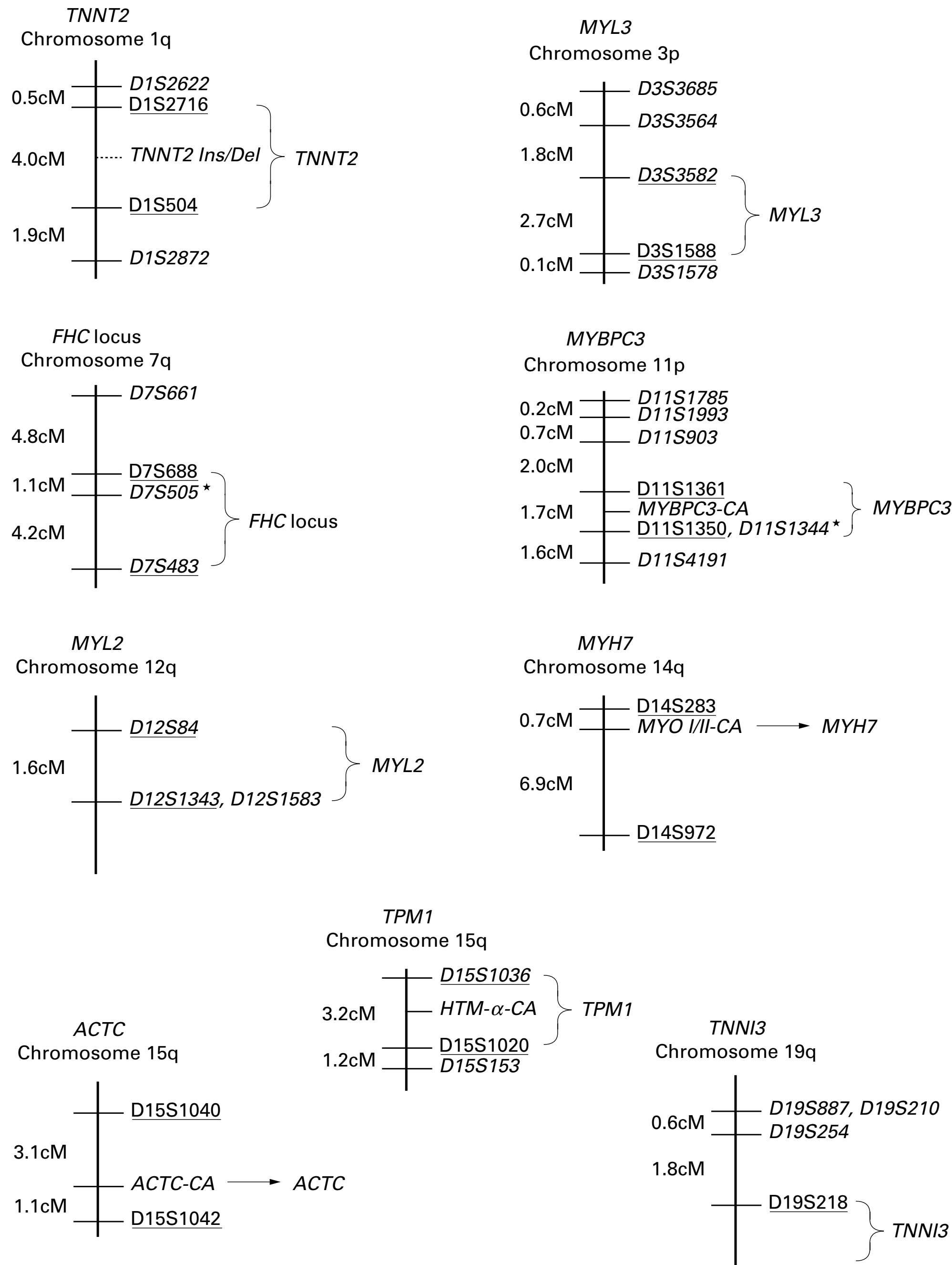

*Figure 1 Localisation of FHC loci in the human genome. The brackets indicate the most likely locations. Polymorphic markers flanking the disease loci are underlined. The markers used for linkage analyses (in italics) were selected on the basis of the highest achievable odds of their localisation, their HZ indices, and their ability to be incorporated in multiplex PCR panels. All markers depicted are localised on the Généthon genetic map with odds of at least 1000:1[16] except D11S1993 and D19S254 which are only part of the Marshfield map.[30] * indicates that the marker could not be localised relative to the disease locus. The distances are in centiMorgans (cM).*

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(288 bp) 0.32, and allele 5 (290 bp) 0.06. The observed HZ index was 52%. The disease loci on chromosome 7 and *TPM1*, which carry an intragenic marker (*HTM-α-CA*), have been localised by linkage analysis with odds of approximately 100:1 and 10 000:1 over the second most likely interval, respectively.[4 15]

MYH7 and *ACTC* carry highly polymorphic markers within intron 1/intron 24 (*MYO I/II-CA*)[27 28] and intron 5 (*ACTC-CA*),[29] respectively. Both genes are part of the Marshfield genetic map.[30] *MYL2* is part of the chromosome 12 sequence released by the Human Genome Sequencing Project.[18] The gene is part of contig NT_000612, sequence AC002351.1. The polymorphic marker D12S1343 is part of the same contig, sequence clone AC002352.1, and localised about 13 kb distally of *MYL2*. No proximal flanking markers within a reasonable distance of *MYL2* are part of the released sequence.[18] However, it was possible to select D12S84 as the proximal flanking marker since the position of *MYL2* has recently been refined by haplotype analysis of a large FHC pedigree carrying a *MYL2* mutation.[5] The position of *MYL2* is in accordance with previous assignments.[18 22]

The markers selected for linkage analysis were incorporated in multiplex PCR panels and the outcome of several optimising procedures with respect to primer, template, and deoxynucleotide concentrations, composition of different markers in each multiplex, and number of amplification cycles are shown in table 1. PCRs were carried out using previously reported conditions.[3] Before the amplified PCR products were analysed on an ABI prism 377 DNA sequencer (Perkin-Elmer Corp), the DNA concentration of the PCR products were estimated by comparing the intensity of the bands with a known concentration of the reference marker λ Dra by 3% agarose gel electrophoresis. In order to achieve reproducible peaks of the markers it was important to dilute the multiplex PCR products to a final concentration of about 0.4 ng/μl before loading the gel.

The linkage approach developed was evaluated in three FHC families shown in fig 2. Informed consent was obtained from each participant in accordance with local ethical committee guidelines. Echocardiography and standard 12 lead electrocardiograms (ECGs) were obtained and evaluated essentially as previously described.[3] In brief, a person was considered to fulfil major diagnostic criteria if the maximal wall thickness (MWT) measured by echocardiography was >13 mm, if ventricular arrhythmia occurred during 48 hours of ambulatory ECG monitoring, or the ECG presented either major Q wave abnormalities, left ventricular hypertrophy, or marked repolarisation alterations. Subjects were considered to fulfil minor diagnostic criteria if their ECG presented minor Q waves in at least two leads. Minor echocardiography criteria were considered to be fulfilled by an MWT=13 mm. The participants in the investigation were classified phenotypically before DNA analysis. All subjects with clinical signs of FHC in families A and B fulfilled major diagnostic criteria while three members of family C fulfilled only minor diagnostic criteria (II.8, III.4, and III.8) and one subject was excluded from all analyses because of hypertension (II.9).

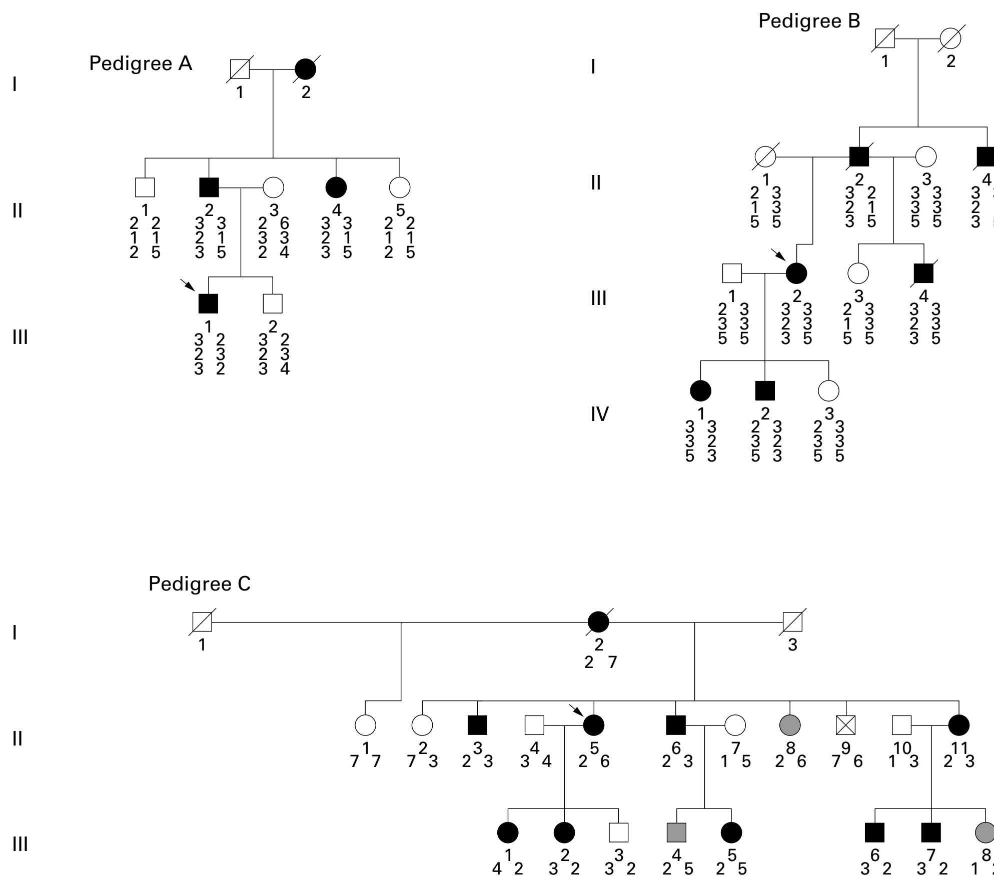

Figure 2 Pedigrees of three FHC families investigated. Index patients are indicated by arrows. Open symbols are unaffected subjects, filled symbols are affected subjects fulfilling major FHC diagnostic criteria, shaded symbols are affected subjects fulfilling minor FHC diagnostic criteria. A cross indicates a subject excluded from linkage analyses because of hypertension. Families A and B carry a TPM1 mutation, while pedigree C carries an ACTC mutation. Alleles defined by markers at the TPM1 and ACTC loci in families A/B and C respectively are shown below symbols. Clinical details of families A and B will be reported elsewhere while details of family C have already been reported.[3]

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Table 2 Maximal lod score values of pedigrees A, B, and C

Pedigree	*Maximal lod score values*									Disease gene identified
	TNNT2	*MYL3*	*FHC locus on chromosome 7*	*MYBPC3*	*MYL2*	*MYH7*	*ACTC*	*TPM1*	*TNNI3**	
A	−1.0	−1.3	−2.6	−3.3	−0.7	−2.7	−3.0	**0.6**	−0.3	*TPM1*
B	−0.9	−3.7	−0.1	−1.1	−1.8	1.2	−1.6	**1.6**	0.03	*TPM1*
C										
Major criteria	−3.0	−2.5	−5.2	−5.4	−6.0	−4.9	**2.7**	−4.2	−2.9	*ACTC*
Minor criteria	−2.6	−5.6	−4.3	−5.9	−3.3	−6.2	**3.6**	−7.4	−2.3	

All values are maximal multipoint lod score values except for *ACTC* (maximal two point lod score value) in which case only one intragenic polymorphic marker was used. Pairwise and multipoint linkage analyses were carried out using the LINKAGE 5.1 package[3 38] and the VITESSE computer program.[39] A mutation was identified in the disease gene indicated for each family.

*The distance from the *TNNI3* locus to the most proximal flanking marker used for linkage analysis (D19S887) was approximately 20 centiRays (cR) corresponding to a genetic distance of approximately 10 cM according to the information given at the Whitehead RH map.[22] This rather conservative assumption was applied in the linkage analysis. Lod score values at positions closer to D19S887 were increasingly negative in all families.

The results of the linkage analysis are summarised in table 2. It should be noted that in all three families the locus showing the highest lod score value was found to be the disease causing gene. Although family A consisted of only three affected subjects, it was possible to exclude four FHC loci showing lod score values $\leqslant -2$. The *TPM1* locus achieved the highest lod score value of 0.6. Subsequently, all exons of *TPM1* were amplified[12] and the PCR products investigated by single strand conformation polymorphism heteroduplex analysis on a precast 12.5% polyacrylamide gel at 4°C or 20°C followed by sequencing of abnormal conformers.[31] These analyses showed a well established mutation in exon 3 resulting in an Asp175Asn amino acid substitution[12 32–35] (data not shown). III.2 was non-penetrant at the age of 18 years. In family B where it was only possible to exclude one FHC locus, the *TPM1* locus obtained the highest lod score value of 1.6. The subsequent mutation analysis of the gene identified the same *TPM1* mutation as in family A. In the large family C, it was possible to exclude eight FHC loci regardless of whether major or minor diagnostic criteria were applied. Using minor diagnostic criteria a significant maximal lod score of 3.6 was obtained at the *ACTC* locus and subsequent mutation analyses showed a mutation in exon 5 resulting in an Ala295Ser amino acid substitution.[3]

The heterogeneous clinical manifestations and the genetic complexity of FHC make the achievement of genetic diagnosis difficult and resource demanding. Linkage analysis is a potentially valuable tool in reducing the number of laborious mutation analyses. However, it is necessary to examine and assess the phenotypes of the entire FHC family in question before linkage analysis can be accomplished. It is important to use stringent phenotypic definitions of the disease in order to identify the correct disease gene within the family, although it is often difficult to define the precise phenotype of FHC. Naturally, the diagnostic criteria change and develop in accordance with the current knowledge of the disease. Even minor clinical abnormalities are more likely to represent FHC within a multiply affected family than they would be outside the context of familial disease. Therefore, it is a sensible strategy to use alternative diagnostic criteria with a subgroup of patients fulfilling conventional disease criteria and, if present, another group of patients fulfilling minor diagnostic criteria. This balanced strategy was used in family C where clinical investigations indicated three subjects with minor signs of FHC (II.8, III.4, III.8). Instead of complete exclusion of the three subjects from the analyses, two lod score calculations were performed using major and minor diagnostic criteria, respectively. As seen from table 2, both lod score values appointed *ACTC* as the most likely disease gene. However, the use of minor diagnostic criteria should be used with caution since there is a greater risk that a subject will be falsely categorised as having the phenotype resulting in no identification of the disease gene. Consequently, minor diagnostic criteria should only be used in context with results obtained by major diagnostic criteria as an additional help in prioritising the order of mutation analyses and certainly not for exclusion of candidate disease genes. The main value of the linkage approach in small families is the ability to prioritise the order of mutation analyses and exclude FHC loci as disease responsible genes, since it is often difficult to achieve a significant lod score because of a limited number of affected subjects. However, once the identification of all FHC genes has been accomplished, significant linkage might be obtained even in small pedigrees as the relative distribution of lod scores among the different FHC loci would determine the significance. For instance, a lod score of 1 would be considered significant if the second most likely candidate locus showed a maximal lod score of –1. Thus, in this future scenario, the linkage approach would play an even greater role in genetic diagnosis of FHC. In the two small pedigrees, A and B, the *TPM1* locus showed the highest lod score value of all FHC loci tested and subsequent mutation analysis identified a previously published *TPM1* mutation. The fact that the pathogenic nature of the *TPM1* mutation identified has been well described,[12 32–35] including expression studies in transgenic mouse models,[36] led us to the conclusion that further mutation analyses of the remaining non-excluded FHC loci were needless. Naturally, the pathogenic impact of any amino acid variation identified needs to be based on substantial evidence since a genetic diagnosis may have profound consequences for disease carriers. It is rarely sufficient to rely only on hypotheses regarding the gene product's functional domains, phylogenetically conserved regions, and three dimensional structure. Similarly, absence of the sequence variation in control chromosomes indicates a possible pathogenic impact, but it is not proof since the sequence variation may still be a rare polymorphism. Linkage analysis may indicate the most likely disease locus, based on quantifiable evidence, and thereby substantiate that a sequence variation identified in this gene is the disease causing agent. Nevertheless, the ultimate proof of disease responsibility is difficult to achieve without studies aiming at expressing the sequence variation in biological test systems. Thus, it is difficult to assess the pathogenic nature of novel missense mutations identified especially in small pedigrees showing insignificant lod score values. Consequently, genetic counselling in small pedigrees associated with novel missense mutations should be cautious and an offer of clinical follow up of all family members without considering their genotype should be considered.

In conclusion, it is apparent that clinical and genetic investigations in FHC are indissolubly connected. The clinical investigations meet the demand of the FHC families in having their disease status clarified and provide the phenotypic assessment necessary for the performance of

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the genetic investigations. Genetic diagnosis is desirable in order to identify as many of the disease carriers as possible and hopefully diminish the number of sudden deaths associated with the disease by improvement of risk stratification and management. The results of the present study provide a firm basis for linkage analysis in FHC pedigrees and thereby a feasible approach, based on quantifiable evidence, for prioritising the order of mutation analyses and for detection of the disease causing agent among nine disease associated loci.

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Genetic heterogeneity and exclusion of a modifying locus at 17p11.2-p11.1 in Finnish families with van der Woude syndrome

EDITOR—The van der Woude syndrome (VWS, OMIM 119300) is the most common form of syndromic cleft lip and palate, affecting 1/30 000 of the general population.[1] The syndrome is inherited in an autosomal dominant fashion with almost complete penetrance.[2] The clinical features of VWS include paramedial pits on the lower lip, cleft lip with or without cleft palate, cleft palate, and hypodontia. However, the presence and severity of the individual features is highly variable within and between families.[3]

A constitutional interstitial deletion of chromosome 1q32-q41 was first described by Bocian and Walker[4] in a patient with lip pits. Linkage of VWS to this locus was then established by Murray *et al*[5] and subsequently Schutte *et al*[6] refined the locus to a 1.6 cM region between D1S491 and D1S205. Recently, a microdeletion test using a novel polymorphic marker D1S3753 derived from the VWS critical region was also used.[7] To date, 1q32-q41 is the only locus described for VWS and the VWS gene has yet to be identified. Recently, a large Brazilian VWS family with multiple cases affected with cleft palate was studied by Sertié *et al.*[8] Linkage analysis was performed to search for a modifying locus for the cleft palate phenotype and suggestive linkage was shown in chromosome 17p11.2-p11.1 with a maximum lod score of 2.05 at D17S1824. The authors proposed the region as a modifying locus for cleft palate in VWS.

The homogeneous nature of the Finnish population is conducive for genetic studies especially for linkage disequilibrium analysis.[9] The clinical features of Finnish VWS patients have previously been described,[1 10] but no genetic studies have been reported. In this study, five Finnish VWS families were tested for linkage to the 1q32-q41 region and to the proposed modifying locus in chromosome 17p11.2-p11.1.

The VWS patients in this study were ascertained through hospital records at the National Cleft Centre in Helsinki, to which all patients with orofacial clefts in Finland are referred. The diagnosis of VWS in the participating patients was based on clinical examination, medical records, telephone interviews, or information from other family members. The pedigrees of the five families are shown in figs 1-3 and the clinical features of all affected cases are detailed in table 1. Within this group of patients the male to female ratio was 3:2 (18:12). Twenty six (87%) affected members had cleft palate or submucous cleft palate, while only three (10%) were affected with cleft lip and palate. Lip pits (including the microform of "wave-like" lower lip) were observed in nine subjects (30%). In family 60, no obvious VWS features were found in the five unaffected subjects who were shown to be disease haplotype carriers (IV.3, IV.5, III.2, III.7, and II.2, fig 2). However, in two of these subjects (II.2 and II.4) speech problems were reported, which might imply the presence of submucous cleft palate.

The five families were analysed for the involvement of the VWS disease predisposition locus in 1q32-q41 as well as for the putative modifying loci in 17p11.2-p11.1 using linkage analysis. Microsatellite markers from the respective regions were amplified by PCR, pooled according to the emission spectra of the fluorescent dyes, and run on an ABI 377 laser fluorescent sequencing machine.

Nine polymorphic microsatellite markers covering the 1q32-q41 VWS region were genotyped and multipoint linkage was calculated as the logarithm of odds (lod) ratio using the GENEHUNTER program.[12] The analysis was performed for individual families as well as the total group of families by considering genetic heterogeneity. VWS was modelled as an autosomal dominant trait with 95% penetrance.[13] The disease allele frequency was taken as 1.5×10^{-51} with a mutation rate of 1.8×10^{-5}.[14] Representative multipoint lod scores for linkage between the VWS phenotype and microsatellite markers at 1q32-q41 are given for each family in table 2. Families 59, 60, and 61 displayed linkage to 1q32-q41 and their cumulative multipoint lod scores were higher than 3.8 across the VWS region. For family 57, haplotype analysis and multipoint lod scores below −10 in 1q32-q41 clearly excluded linkage (table 2, fig 1). Family 62 was uninformative for all nine VWS markers. Therefore, 25 additional microsatellite markers from chromosome 1 were typed. Markers adjacent to the VWS region included D1S1660 - 6 cM - D1S1678 - 8 cM - (nine VWS markers) - 2 cM - D1S2141 - 7 cM - D1S549 - 5 cM - D1S1656 - 2 cM - D1S3462. By observing the extended haplotypes (not shown) and multipoint lod scores of −1.9 along the VWS region (table 2), linkage could be excluded. For the whole group of Finnish VWS families, the maximum multipoint lod scores, under heterogeneity, was 2.53 from D1S245 to D1S205 with an alpha (proportion of linked families) of 53%.

A meiotic recombination between D1S2136 and D1S3753 was observed in an unaffected subject in family 60 (III.4, fig 2), which tentatively placed the VWS locus centromeric to D1S3753 (proposed region 1, fig 4). Furthermore, the affected subjects from family 60 and 61 shared a common haplotype from D1S1663 to D1S3754 (proposed region 2, fig 4). The allele frequencies for each marker making up the haplotype 3 - 5 - 3 - 5 - 3 were 0.53,

Table 1 Clinical characteristics of the VWS patients in the study

Family No	Subject No	(ID)	Genotyped	Sex	VWS phenotype*		
					CL/P	CP	Lip pits
57	II.1	(6387)	Yes	M		+	
	II.2	(6388)	Yes	F	+		
	II.3	(6389)	Yes	M		+	
	II.5	(6381)	Yes	M		+	
	III.3	(6397)	Yes	F		sm	
	III.5	(6396)	Yes	M			+
	III.7	(6383)	Yes	M		+	
	III.9	(6385)	Yes	F		+	
	III.10	(6386)	Yes	M		sm	wl
	IV.2	(6394)	Yes	F		+	
	IV.4	(6399)	Yes	F		+	wl
59	II.5	(5)		M		+	
	III.1	(16)		M		+	
	III.3	(6405)	Yes	F		+	+
	IV.1	(6402)	Yes	M		+	
	IV.5	(6410)	Yes	M		+	+
60	IV.2	(6414)	Yes	F		+	
	IV.6	(6428)	Yes	F		+	
	IV.7	(6426)	Yes	M		sm	+
	IV.10	(34)		M		+	
	V.1	(6411)	Yes	M		sm	
	V.2	(6412)	Yes	F	+		
61	II.1	(5)		M		+	
	III.2	(6379)	Yes	F		+	
	III.3	(7)		M		+	
	IV.1	(6376)	Yes	M		+	
	IV.2	(6377)	Yes	F		+	+
62	II.1	(6432)	Yes	M	+		+
	III.1	(6435)	Yes	F		+	
	III.2	(6433)	Yes	M		sm	+

*+ = present; blank = not observed; CL/P = cleft lip with and without cleft palate; CP = cleft palate; sm = submucous cleft palate; wl = "wave-like" lower lip.

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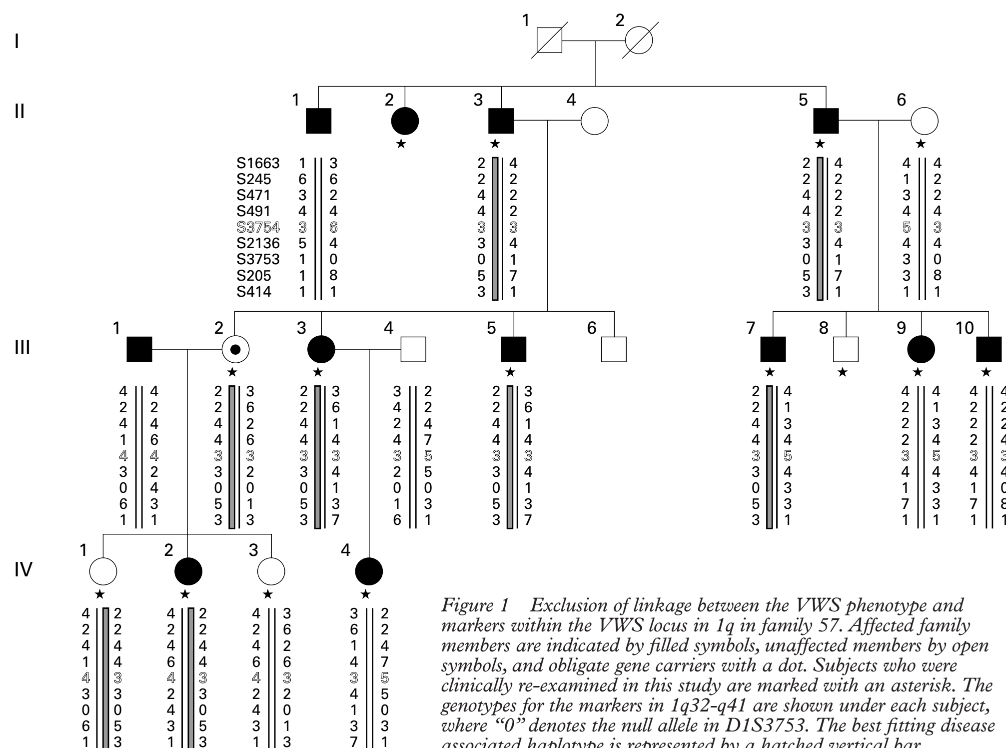

Figure 1 Exclusion of linkage between the VWS phenotype and markers within the VWS locus in 1q in family 57. Affected family members are indicated by filled symbols, unaffected members by open symbols, and obligate gene carriers with a dot. Subjects who were clinically re-examined in this study are marked with an asterisk. The genotypes for the markers in 1q32-q41 are shown under each subject, where "0" denotes the null allele in D1S3753. The best fitting disease associated haplotype is represented by a hatched vertical bar.

0.22, 0.34, 0.34, and 0.59, respectively; therefore, the random population frequency of this haplotype would be 0.8%. Further genealogical studies of the two families may show the contribution of this haplotype to VWS.

During the study, the inheritance of marker D1S3753 was found not to follow a Mendelian inheritance pattern, suggesting the presence of an undetected (null) allele or deletion (figs 1 and 2). The null allele was found in both affected and unaffected subjects, meaning that it may not be related to VWS. To verify the relationship of this allele to orofacial clefts, five control families with no history of cleft lip and palate were typed with this marker. The null allele of D1S3753 was found to be present in these families as well, therefore excluding its role in orofacial clefts.

For the linkage analysis of a modifying locus on chromosome 17, the five most informative microsatellite markers reported by Sertié *et al*[8] were genotyped. The cleft palate phenotype in VWS was modelled as autosomal dominant with a penetrance of 70% and a disease gene frequency of 0.001[8] and only subjects with cleft palate were considered as affected, regardless of the presence of lip pits. Multipoint lod scores at 17p11.2-p11.1 were less than −2 in families 59 and 60 combined (linked to 1q32-q41) as well as in families 57 and 62 combined (unlinked to 1q32-q41) (table 3). Thus, linkage between the cleft palate phenotype and the 17p11.2-p11.1 region could be excluded in these families. Results from family 61 were inconclusive. When all five families were considered together, there was no evidence of heterogeneity and a 20 cM region in 17p11.2-p11.1 could be excluded with lod scores less than –2.

The Finnish population is useful for genetic studies owing to its homogeneous background. As a result of isolation, the Finnish population displays unique clinical and genetic profiles in a number of diseases.[9] In this study, 90% of the Finnish VWS patients in this study had cleft palate in contrast with 30% of VWS patients in other countries.[14] This predominance of cleft palate in our Finnish VWS families implies specific types of VWS gene mutation or the influence of genetic background (modifying genes). The clinical variability of cleft palate including submucous cleft also contributed to the low expressivity of VWS in the Finnish families, especially in family 60, in which five gene carriers did not have overt cleft palate.

To date, chromosome 1q32-q41 is the only reported VWS locus in different populations.[6 8 15 16] In the present study, two Finnish VWS families (57 and 62) were unlinked to this locus, thus representing the first report of genetic heterogeneity in VWS. Both families had classical features of VWS including cleft lip and palate and lower lip pits. For family 57, detailed clinical re-examination was performed on most members to confirm the diagnosis of VWS. In addition, the genotyping was repeated and scored by independent investigators. In family 62, although II.1 was homozygous for all the markers from the VWS region, analysis with additional markers from chromosome 1 clearly excluded linkage.

One important goal of linkage analysis is to refine the genetic locus, typically by observing meiotic recombinational events in affected family members. In the three 1q linked Finnish VWS families, one critical recombination was found between D1S2136 and D1S3753 in an unaffected subject (III.4, family 60, fig 2). When combined with previously published linkage data this recombination would place the VWS disease locus between markers D1S491 and D1S3753, in a region of less than 200 kb. However, recombinations in unaffected subjects have to be interpreted with caution, especially when a low expressivity is observed, such as in this family.

Linkage disequilibrium is an efficient approach for mapping disease genes in isolated populations such as the Finnish.[17] In our study, haplotyping of the VWS families showed a common shared region from D1S1663 to D1S3754 between affected members in families 60 and 61. This finding would tentatively refine the VWS locus to the D1S491-D1S2136 interval (proposed region 2, fig 4), the size of which has been estimated to be approximately 130 kb, as determined from genomic sequencing.[18] Genealogical studies are now under way to identify the ancestral relationship between these families.

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Figure 2 Pedigree of family 60 which shows linkage to the 1q VWS region. The disease associated haplotype is represented by a hatched vertical bar. A meiotic recombination was detected between D1S2136 and D1S3753 in III.4 in family 60, placing the disease locus centromeric of D1S3753.

Typing of locus D1S3753 has been suggested as a standard test for microdeletion in VWS and other patients with orofacial clefts.[7] Our results from the five VWS families and from five additional control families showed that this marker contains a commonly occurring null allele, implying that this is a polymorphism without any aetiological role in VWS. Recently, Watanabe *et al*[19] reported a commonly deleted 8 kb segment in the distal part of the VWS critical region in the general population. This, together with previous reports of frequent deletion in 1q32-q41, may represent an unstable region including D1S3753 within the VWS locus.

The Finnish VWS families in our study displayed predominantly cleft palate, which made them suitable for testing the modifying gene on chromosome 17p11.2-p11.1.[8] Linkage was excluded in families either linked or unlinked to 1q32-q41, indicating that 17p11.2-p11.1 does not contain a major modifying gene for cleft palate in VWS.

In conclusion, we have provided the first evidence of genetic heterogeneity in VWS, tentatively refined the VWS locus to a 130 kb interval, and excluded 17p11.2-p11.1 as a major modifying locus for the cleft palate phenotype. The entire VWS critical region has now been sequenced and mutation analysis on both known and new genes within the region is being performed, but no pathogenetic VWS mutations have been reported so far.[18] In the future, the identification of the VWS genes in 1q and elsewhere will shed light on the pathogenesis of VWS.

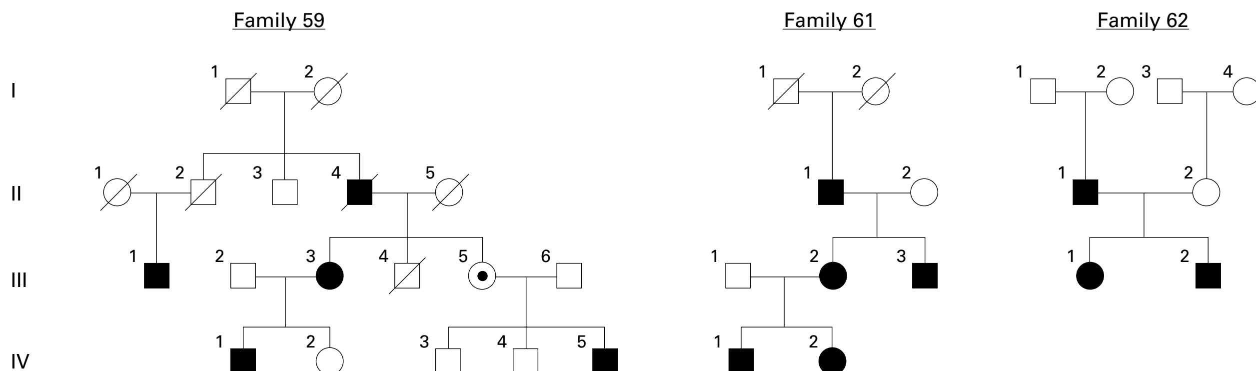

Figure 3 Pedigrees of families 59, 61, and 62.

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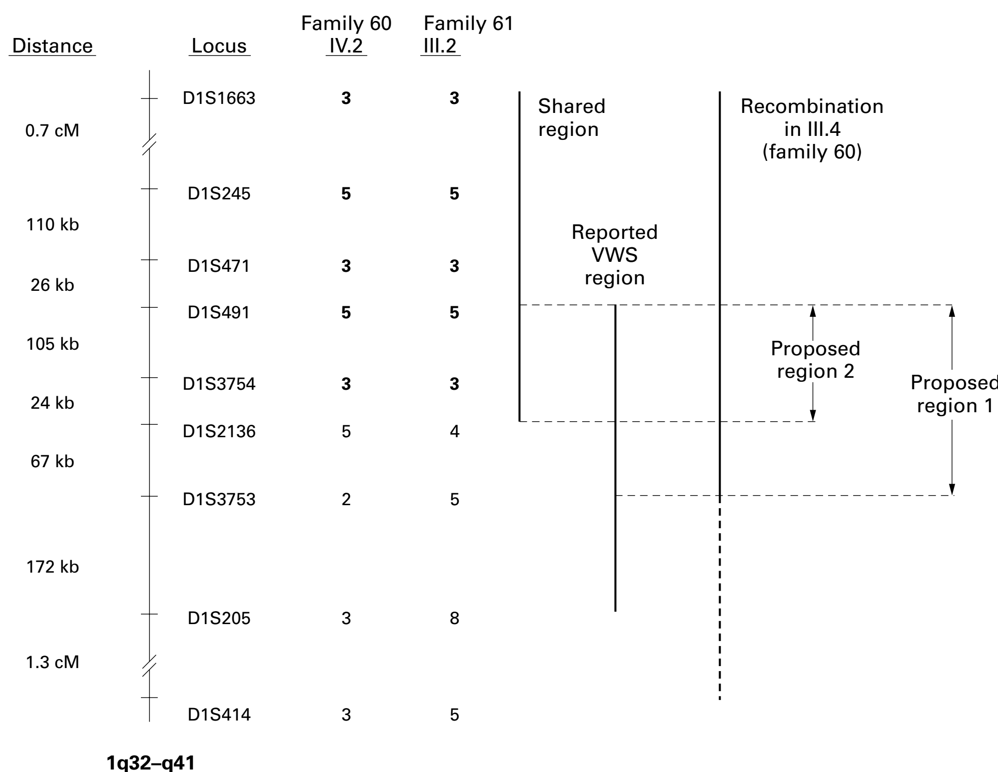

Figure 4 Mapping of the VWS locus within 1q32-q41 to a 130 kb interval, based on the combined results from this study and those previously reported. The physical distance is provided by the Sanger Centre and the VWS region is reported by Schutte et al.[6] The region of haplotype sharing between families 60 and 61 is indicated together with the critical meiotic recombinants. The proposed region 1 is based on the previously reported recombinants as well as on the recombination in III.4 in family 60 and region 2 is based on a common origin of the shared haplotype.

Table 2 Multipoint lod scores for linkage of VWS to 1q32-q41

Locus	Distance (cM)	Family 57	59	60	61	62
D1S1663	0.00	−13.90	1.43	2.07	−5.39	−1.84
D1S245	0.70	−19.47	1.44	2.09	0.30	−1.88
D1S471	0.81	−15.33	1.44	2.09	0.30	−1.89
D1S491	0.84	−14.64	1.44	2.09	0.30	−1.89
D1S3754	0.94	−13.01	1.44	2.08	0.30	−1.90
D1S2136	0.96	−13.12	1.44	2.09	0.30	−1.90
D1S3753	1.03	−13.13	1.44	2.09	0.30	−1.91
D1S205	1.20	−21.17	1.44	2.09	0.30	−1.92
D1S414	2.50	−10.43	1.44	2.06	0.29	−2.08

Table 3 Multipoint lod scores for linkage of the cleft palate phenotype in VWS to microsatellites in the 17p11.2-11.1 region

Locus	Distance (cM)	Family 57	59	60	61	62
D17S1843	0.00	−5.37	0.06	−7.88	0.30	−4.74
D17S953	1.90	−6.28	0.05	−7.88	0.30	−4.74
D17S1824	8.56	−6.36	−3.74	−3.84	0.30	−2.85
D17S1873	9.09	−6.36	−3.74	−7.36	0.30	−4.74
D17S1800	10.52	−6.36	−3.74	−5.65	0.30	−4.74

Electronic database information: Online Inheritance in Man: http://www.ncbi.nlm.nih.gov/omim. Genome DataBase: http://gdbwww.gdb.org. Chromosome 1 Mapping Group at the Sanger Centre: http://www.sanger.ac.uk/HGP/Chr1. Marshfield Medical Research Foundation: http://www.marshmed.org/genetics.

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Analysis of the entire coding region of the cystic fibrosis transmembrane regulator gene in neonatal hypertrypsinaemia with normal sweat test

EDITOR—Measurement of immunoreactive trypsinogen concentration (IRT) in dried blood spots is the most common technique for neonatal screening for cystic fibrosis (CF).[1] Since a considerable number of newborns show raised IRT levels, several laboratories improve the screening specificity by testing infants with hypertrypsinaemia for the most common CF mutations. Diagnosis is established in neonates carrying two mutations, but a sweat test is required if only one mutation is found, in order to detect affected subjects with a second, unrecognised mutation. Infants with raised IRT, one CF mutation, and normal sweat electrolyte concentrations are usually considered to be just carriers.

Unexpectedly, a frequency of CF heterozygotes significantly higher than in the general population has been repeatedly reported among neonates with hypertrypsinaemia and normal sweat chloride levels.[2 3] It is not clear whether having one CF mutation, perhaps together with some unknown pathogenetic factor, is sufficient to predispose to neonatal hypertrypsinaemia. Such a hypothesis is corroborated by the findings of Lecoq *et al*,[4] who showed that the probability of a newborn being a carrier of the major *CFTR* mutation, ΔF508, increases with neonatal IRT concentration, and suggested that heterozygotes may have early subclinical impairment of exocrine pancreatic function. Alternatively, it could be speculated that at least some hypertrypsinaemic newborns who, after testing for a limited number of CF mutations have been found to carry a *CFTR* gene mutation, have on the other chromosome an undetected mild mutation, and possibly suffer from an atypical form of CF, characterised by a negative sweat test. A DNA polymorphic sequence of five thymines in intron 8 of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which is very commonly involved in the pathogenesis of a primarily genital form of CF called congenital bilateral absence of the vas deferens (CBAVD),[5] has actually been found to be more frequent in carrier newborns with raised IRT values than in controls.[3 6] Moreover, a complete scan by denaturing gradient gel electrophoresis of all 27 *CFTR* exons detected nine more mutations in a sample of 18 heterozygous newborns whose IRT was raised and sweat chloride normal.[7]

In order to contribute to the understanding of the correlation between *CFTR* mutations and neonatal hypertrypsinaemia, we analysed the *CFTR* gene in a larger group of IRT positive and sweat chloride negative heterozygous neonates and in a subset of neonates with similar characteristics but negative for a standard *CFTR* mutation panel.

The population under study consisted of 47 screened neonates with IRT at birth above the 99.5th centile and normal sweat test (<40 mEq/kg, Gibson and Cooke method[8]). The screening strategy was as previously described.[3] As non-CF hypertrypsinaemia has been reported in perinatal asphyxia and prematurity,[9 10] no newborn with any of these conditions was included.

The 47 neonates were divided into three groups. Group A consisted of 13 newborns, with one identified *CFTR* mutation among the panel of 15 routinely sought in hypertrypsinaemic babies according to our neonatal screening protocol. In a previous study, all of them had been tested for the (T)5 allele in intron 8 and none of them carried it.[3] Group B consisted of 19 newborns, again carrying one identified *CFTR* mutation out of the panel of 15, but not previously tested for the (T)5 allele. Group C consisted of 15 newborns carrying no *CFTR* mutation out of the panel of 15 and not previously tested for the (T)5 allele. Groups A and B included all the screened carrier newborns, born between July 1993 and January 1998, for whom a blood sample sufficient for genetic analysis was available. These two groups were meant to represent *CFTR* heterozygous hypertrypsinaemic neonates, as opposed to just hypertrypsinaemic neonates (group C); however, as A and B disagreed with regard to the previous 5T analysis, they were processed separately.

In all the neonates and in a control group of 15 healthy subjects whose IRT at birth was unknown but presumed to be normal (the chance of having raised IRT at birth using our cut off is 1 in 200),[11] a complete gene search of all 27 exons and intronic flanking regions with denaturing gradient gel electrophoresis (DGGE) analysis was performed. PCR products that displayed altered behaviour in the gel were

Table 1 IRT, sweat test, and weight Z score values in groups A, B, and C

	Group A median (range)	*Group B median (range)*	*Group C median (range)*	*Intergroup comparison*
IRT at birth (μg/l)	133 (103–285)	110 (97–287)	115 (95–161)	NS
IRT at sweat test (μg/l)	34 (5–73)	40.5 (15–199)	28 (7–60)	NS
Sweat Cl^- (mEq/kg)	14 (6–30)	18 (8–36)	12 (6–26)	B *v* C p<0.01
Sweat Na^+ (mEq/kg)	15 (8–29)	17 (11–31)	13 (9–21)	B *v* C p<0.01
Weight Z score at birth	0.51 (−0.18–2.38)	0.195 (−1.58–1.93)	−0.25 (−1.33–1.23)	
Weight Z score at sweat test	0.06 (−0.74–1.42)	−0.31 (−1.43–1.37)	0.11 (−0.73–2.22)	

NS = not significant.
Weight Z score at birth *v* weight Z score at sweat test: group A p=0.0081, group B p=0.0419, group C NS.

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Table 2 Characteristics of 24 neonates with CFTR mutations other than the 15 routinely sought in IRT positives or the 5T allele. Patients 1A, 2A, 3A, 4A, 5A, 6A, 1B, 8B, and 10B have been partially reported elsewhere[7]

No	IRT at birth (µg/l)	Retested IRT (µg/l)	Sweat Cl⁻ (mEq/kg)	Sweat Na⁺ (mEq/kg)	Weight Z score at birth	Weight Z score at sweat test	CFTR mutation from a panel of 15 mutations	CFTR mutation from DGGE in cis	CFTR mutation from DGGE in trans	CFTR mutation from DGGE, unknown phase	Poly T	TG	M470V
Group A													
1A	144	37	30	29	0.89	−0.74	R1162X		R117H		7/7	10/10	M M
2A	106	34	21	10	0.07	−0.01	ΔF508		E527G		7/9	11/10	M V
3A	123	66	20	15	0.26	−0.10	ΔF508		1716G/A		7/9	11/10	M M
4A	133	65	10	14	0.51	0.96	R1162X			3041-71G/C 4002A/G	7/9	10/11	M M
5A	111	15	12	16	0.31	0.10	G542X			1716G/A	7/9	10/10	M M
6A	107	31	12	16	2.38	1.42	N1303K	2622+14G/A			7/9	11/10	M V
Group B													
1B	105	39	18	19	−0.04	−0.54	ΔF508		Y301C		7/9	11/10	M V
2B	287	199	36	18	−0.71	−0.74	1717-1G→A		D1152H		7/7	10/11	M V
3B	139	—	29	31	—	—	1717-1G→A		1716G/A		7/7	10/10	M M
4B	158	73	22	15	0.16	−0.46	ΔF508		M1137V		9/9	10/10	M M
5B	207	—	23	22	—	—	R553X		L997F		7/9	12/10	M V
6B	150	57	15	15	0.91	−1.31	G542X			L997F	9/9	10/10	M V
7B	100	29	19	15	−0.59	−0.21	ΔF508			L997F	9/9	10/12	M V
8B	103	—	26	26	—	—	N1303K	2622+14G/A			5/9	11/10	M M
9B	113	47	16	15	1.93	1.37	ΔF508				5/9	12/10	M V
10B	108	—	14	11	—	—	ΔF508				5/9	11/10	M M
Group C													
1C	120	36	13	14	−0.71	0.11	—			L997F F1052V	7/7	10/11	M V
2C	161	—	26	21	—	—	—			R117H	7/7	10/11	M V
3C	144	60	14	17	−0.11	−0.04	—			L749L	7/7	11/11	V V
4C	140	15	11	11	−0.07	0.06	—			R75Q	7/7	11/11	V V
5C	96	7	16	15	−1.33	−0.16	—			1716G/A	7/7	10/11	M V
6C	115	59	12	9	−0.26	0.34	—			F1052V	7/9	10/10	M M
7C	161	30	11	11	0.35	−0.30	—			T1424T	7/7	11/12	M V
8C	142	59	6	9	1.11	−0.53	—				5/7	12/10	M V

sequenced after cloning. DGGE primers and conditions in the three groups of newborns under study were as previously described.[12] In addition, the alleles at the polymorphic loci IVS8(TG)m, IVS8(T)n (not in group A), and M470V were studied. M470V was analysed by PCR and *Hph*I restriction enzyme analysis,[13] and IVS8(TG)m and IVS8(T)n with fluorescent primers on an ABI PRISM 377.[4]

The Kruskal-Wallis test was used for statistical analysis of IRT and sweat chloride and sodium values and the Wilcoxon test to compare weight Z scores at birth and at the time of sweat testing. Comparison of mutation and polymorphism frequencies was performed by Fisher's exact test, including Yates's continuity correction.

Preliminary results of DGGE analysis, but not of the polymorphic loci study, of all newborns in group A and five newborns in group B have been previously published.[7]

The median IRT at birth of the three groups is shown in table 1. Sweat chloride and sodium and concomitant IRT values are also included. Trypsinogen levels showed a time related decrease; IRT values were below the cut off at 1 month of age (75 µg/l) in all cases in which data were available, with the only exception of subject 2B in table 2, who was later found to be a compound heterozygote for the mutations 1717-1G→A and D1152H. Table 1 also shows the median weight Z scores calculated at birth and at the time of sweat testing.

Seven more *CFTR* gene mutations were found in group A, eight more in group B, and eight in group C. One mutation (L997F) was found in the control group. The 5T variant was carried by three neonates in group B and one in group C. Table 2 shows genetic and clinical data of the neonates carrying *CFTR* mutations other than the 15 routinely sought in IRT positives or the 5T allele.

Phase was determined in the three babies of group B carrying the 5T allele: in patient 8B, N1303K was in *trans* with (T)5-(TG)11-M470, as was ΔF508 in newborns 9B and 10B with (T)5-(TG)12-M470 and (T)5-(TG)11-M470, respectively.

The incidence of *CFTR* mutations and alleles at (T)n, (TG)m, and M470V polymorphic loci in groups A, B, and C and in controls are shown in table 3.

A complete *CFTR* gene scan of the whole coding sequence has never before, to the best of our knowledge, been performed in unaffected hypertrypsinaemic neonates not carrying any of the commonest mutations, like the ones in group C. Among them, a high incidence of mostly rare *CFTR* mutations, significantly more than in the control group was found.

A high frequency of *CFTR* mutations has been detected in subjects showing some clinical manifestations which can also be found in patients with CF, namely CBAVD,[5] pancreatitis,[15 16] disseminated bronchiectasis,[11] and allergic bronchopulmonary aspergillosis.[17] It is still not completely understood what the correlation between these conditions and carrier status could be. It is generally assumed that carriers of *CFTR* mutations have about 50% of normal function, which is sufficient to remain free from disease. However, most of the studies tested only for a limited number of the more than 850 known CF mutations, and it is therefore possible that more comprehensive genetic analysis might detect additional mutations. Even without postulating a second

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Table 3 Incidence of CFTR mutations and alleles at poly T, (TG)m, and M470V polymorphic loci among the chromosomes of patients in groups A, B, C, and in controls

	Group A incidence (%)	*Group B incidence (%)*	*Group C incidence (%)*	*Controls incidence (%)*	*Intergroup comparison*
CFTR mutations	7/13 (53.8)*	8/19 (42.1)*	8/30 (26.6)	1/30 (3.3)	A *v* controls p=0.0004 B *v* controls p=0.0011 C *v* controls p=0.0257
Poly T: (T)5	—	3/28 (10.7)†	1/30 (3.3)	0/30 (0.0)	NS
Poly T: (T)7	15/19 (78.9)†	19/28 (67.8)†	26/30 (86.6)	26/30 (86.6)	NS
Poly T: (T)9	4/19 (21.0)†	6/28 (21.4)†	3/30 (10.0)	4/30 (13.3)	NS
TG(10)	10/19 (52.6)†	13/28 (46.4)†	11/30 (36.6)	11/30 (36.6)	NS
TG(11)	7/19 (36.8)†	10/28 (35.7)†	17/30 (56.6)	14/30 (46.6)	NS
TG(12)	2/19 (10.5)†	5/28 (17.8)†	2/30 (6.6)	5/30 (16.6)	NS
M470V: M	21/26 (80.7)	25/38 (65.7)	12/30 (40.0)	11/30 (36.6)	A *v* controls p=0.0012 B *v* controls p=0.027 A *v* C p=0.0027 B *v* C p=0.049
M470V: V	5/26 (19.2)	13/38 (34.2)	18/30 (60.0)	19/30 (63.3)	A *v* controls p=0.0012 B *v* controls p=0.027 A *v* C p=0.0027 B *v* C p=0.049

Statistical analysis: Fisher's exact test including Yates's continuity correlation. Two sided p value.
NS = not significant.
*As these newborns were already known to be heterozygotes, only one chromosome per subject was considered.
†As ΔF508 is in linkage with 9T and TG(10) alleles,[19] only non-ΔF508 chromosomes were considered.

CFTR mutation, the contribution of unidentified modifier genes could possibly be sufficient to cause isolated manifestations of CF in heterozygotes.

Similar speculations could also apply to some cases of neonatal hypertrypsinaemia with normal sweat test, and our results seem to confirm that the association of this condition and a *CFTR* mutation may have some phenotypic consequences. Both sweat chloride and sodium in the two groups of carriers, A and B, were higher (the latter with statistical significance) than in group C; similarly, weight Z score in groups A and B was worse at the time of sweat test than at birth.

An alternative, or perhaps complementary speculation to explain the high carrier frequency in hypertrypsinaemic newborns could be that at least some of them carry on the other chromosome an undetected mild mutation, associated with normal sweat chloride values, but able to raise trypsinogen levels. Such a hypothesis has been substantiated by a previous study showing that a few heterozygous newborns with raised IRT and normal sweat chloride do actually carry a second *CFTR* mutation.[7]

The results of the present study confirm such a finding; in 14 out of 32 babies (groups A and B), together with the previously detected mutation, there were either one (in 13 cases) or two (in one case) more *CFTR* gene mutations. The phase could be determined in 10 cases and in eight of them the newly found mutation was in *trans* with the one originally found. As deep intronic mutations remain uncharacterised after screening the coding sequence, we cannot exclude that more infants could be compound heterozygotes.

All missense mutations detected with DGGE analysis (R117H, Y301C, D1152H, M1137V, L997F, F1052V, R75Q) except one (E527G) are located in membrane spanning domains (MSD). It has been reported that some MSD mutations alter the pore properties of *CFTR* by decreasing the amount of current[18]; the residual channel activity may be sufficient to produce a mild phenotype, like the rise in newborn IRT levels.

A role in determining neonatal hypertrypsinaemia could also be played by the 5-thymidine allele in intron 8. The proportion of the correct *CFTR* gene transcript is inversely related to the length of the polythymidine tract in the sequence of the acceptor splice site of intron 8; three alleles ((T)5, (T)7, and (T)9) can be found at this locus, and the (T)5 variant results in a high proportion of abnormal, alternatively spliced *CFTR* mRNA.[19] Subjects with one CF mutation on one chromosome and the (T)5 allele on the other have little normal *CFTR*, and their phenotypes are quite diverse, ranging from good health, to CBAVD, or even to mild CF.[5,20] Former evidence suggests that possibly neonatal hypertrypsinaemia may also be included in this wide clinical spectrum, as the incidence of (T)5 in heterozygous newborns with raised IRT was found to be higher than in other carriers.[3,6] The greater (T)5 frequencies in groups B and C compared to controls, even though not statistically significant, are compatible with such previous findings, and so is the fact that in the three infants in group B carrying the (T)5 allele this is always in *trans* with the originally found mutations.

As for the M470V polymorphism, in vitro studies showed that the V allele yields a lower functional amount of CFTR protein at both transcriptional and translational levels.[21] Although one would have expected to find this allele more frequently among hypertrypsinaemic neonates, it was instead the M polymorphism which was significantly higher in groups A and B compared to group C and controls. This result may be explained by the strong association of the amino acid methionine with mutations located in one of the nucleotide binding folds, mutations which were well represented in both groups A and B.[22]

It is debatable whether the compound heterozygotes detected have or do not have CF. A diagnosis of CF can be made in the presence of a positive neonatal screening test plus the evidence of *CFTR* dysfunction as documented by raised sweat chloride concentrations, or the in vivo demonstration of abnormal ion transport across the nasal epithelium, or identification of two CF causing mutations.[23] None of the mutations found by DGGE were known to be CF causing (R117H would be if in *cis* with (T)5, but in patient 1A, a compound heterozygote for R1162X and R117H, the polyT genotype was (T)7/(T)7).

However, one cannot exclude that neonatal hypertrypsinaemia, in the presence of some degree of genetic abnormality in both CF alleles, could be the first sign of some *CFTR* related disease. In approximately 2% of CF patients, there is an "atypical" phenotype, which often consists of mild chronic sinopulmonary disease, pancreatic sufficiency, and normal to borderline sweat chloride concentrations[23]; compound heterozygous infants could possibly develop a similar phenotype in time, or, if males, CBAVD.

In practice, it is not at present possible to predict the clinical outcome of our newborns, nor to provide satisfactory genetic counselling for the family. Close clinical follow up should help in clarifying the extent of the disease, if any, in these subjects.

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Two mutations, D1152H and L997F, deserve further comment. Neonate 2B (table 2) carried, on different alleles, 1717-1G→A and D1152H. His IRT value at birth was unusually high, even for CF, and remained raised at the time of the sweat test as well; also, his sweat chloride level, even though under 40 mEq/kg, was the highest among the neonates under study. D1152H has been reported in association with isolated CBAVD, but also with mild, late onset lung disease and pancreatitis in conjunction with normal sweat values.[24]

Another peculiar result of the study was the finding on four occasions of L997F, a mutation usually rare in CF, but perhaps more common in idiopathic disseminated bronchiectasis,[12] as well as in idiopathic pancreatitis. Unpublished data from our group show that L997H was found in four cases from a subset of 32 subjects suffering from idiopathic pancreatitis, but in none of 100 ΔF508 carriers.

In conclusion, standard mutation panels can detect a high prevalence of *CFTR* mutations among subjects with neonatal hypertrypsinaemia and negative sweat chloride, but even more mutations can be found by a more thorough gene search. Close clinical follow up should help in clarifying the extent of the disease, if any, in compound heterozygous newborns.

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A heterozygous endothelin 3 mutation in Waardenburg-Hirschsprung disease: is there a dosage effect of *EDN3*/*EDNRB* gene mutations on neurocristopathy phenotypes?

EDITOR—Hirschsprung disease and Waardenburg syndrome are congenital malformations involving neural crest derivatives. Several genes are involved in these diseases, defining a complex pattern of inheritance. Hirschsprung disease (HSCR) is characterised by the absence of intramural ganglia in the distal bowel. This lack of enteric innervation results in intestinal obstruction or severe constipation. The incidence of HSCR is 1 per 5000 live births and both genetic and environmental factors are thought to contribute to the phenotype. The mode of inheritance is dominant in some families and recessive or multifactorial in others.[1] In a number of cases, mutations of the *RET* proto-oncogene, a tyrosine kinase receptor, result in a dominant disease with incomplete penetrance.[2–4] Mutations in the *RET* ligand *GDNF* (glial cell line derived neurotrophic factor) may also affect the phenotype.[5–7] A few patients with HSCR were found to have heterozygous mutations in the genes encoding the endothelin B receptor (*EDNRB*)[8–10] or its ligand endothelin 3 (*EDN3*).[11 12]

Waardenburg syndrome (WS) is characterised by a combination of sensorineural deafness and abnormal pigmentation, including a white forelock and eyelashes, heterochromia irides, and areas of skin depigmentation. Four subtypes of WS have been described on the basis of clinical features.[13] Types 1 and 3 and type 2 are associated with mutations in the *PAX3* and microphthalmia associated transcription factor (*MITF*) genes,[14] respectively. Patients with type 4 WS (WS4, Waardenburg-Hirschsprung disease or Shah-Waardenburg syndrome)

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have features of both WS and HSCR.[15] Several WS4 subjects have homozygous *EDNRB* or *EDN3* gene mutations,[12 16–19] whereas other patients have heterozygous mutations in the gene encoding SOX10,[20] a transcription factor expressed in emerging neural crest cells.

Endothelins are a family of three vasoactive peptides recognised by two G protein coupled heptahelical receptors. Endothelin 3 preferentially binds the endothelin B receptor. Endothelin mRNAs are first translated into preproendothelin, which undergoes two step enzymatic cleavage that generates the active endothelin peptide[21 22] (fig 1). This peptide is composed of 21 amino acids, and contains four cysteines involved in two disulphide bonds. Targeted or spontaneous homozygous mutations of the *Ednrb* or *Edn3* gene in mice generate a strikingly similar phenotype, with white coat spotting and aganglionic megacolon,[24 25] suggesting that endothelin 3 is a physiological ligand for the endothelin B receptor. The phenotype is reminiscent of WS4 in humans, in whom homozygous mutations of *EDNRB* and *EDN3* were first described.[16–19] Heterozygous mutations were later reported in patients with isolated HSCR.[8–12] Following these observations, WS4 was described as a recessive condition, and isolated HSCR as a dominant disease with incomplete penetrance when the result of *EDN3* or *EDNRB* mutations.

However, contrary to this simple model, a homozygous *EDNRB* mutation was found in a patient with isolated HSCR[26] and a heterozygous mutation was identified in a patient with WS4, whose affected sibs only had features of WS.[27] *EDNRB* mutations manifest themselves in a more complex manner than previously believed. In their study of a large Mennonite family, Puffenberger *et al*[16] showed that both homozygotes and heterozygotes for a *EDNRB* mutation exhibited the intestinal phenotype, but with very different penetrance (21% in heterozygotes, 74% in homozygotes). They suggested that the *EDNRB* mutation found in this family was dosage sensitive and neither fully dominant nor fully recessive. This explanation for the variable penetrance in homozygotes and heterozygotes might also apply to features of WS. However, it would not predict whether a particular *EDNRB* mutation would be more strongly associated with HSCR or with WS, as modifier genes could also contribute to determining whether a heterozygote develops one syndrome or the other.

Similarly, heterozygous *EDN3* mutations have been identified in patients with HSCR and homozygous *EDN3* mutations in patients with WS4.[12 28] We report a novel *EDN3* mutation carried in the heterozygous state by a girl with WS4, showing that, like *EDNRB*, heterozygous *EDN3* mutations can result in either WS4 or isolated HSCR.

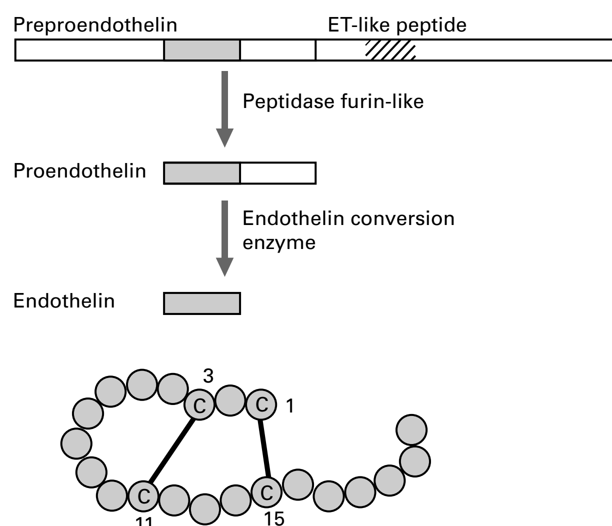

Figure 1 Enzymatic processing of preproendothelin into proendothelin (also termed "big endothelin") and into mature endothelin, according to Yanagisawa et al.[23]

The index case (III.2) was born in a well nourished state after a pregnancy marked by sonographic diagnosis of an intestinal obstruction at 33 weeks. The karyotype was normal (46,XX). Laparotomy on day 3 of life established the diagnosis of HSCR involving the colon and ileum. Multiple biopsy specimens of the distal ileum and colon showed no ganglion cells in the submucosa or intermuscular nerve plexuses and no increase in nerve fibres. An ileostomy in the dilated ileum failed to function and jejunostomy was carried out on day 15, 40 cm from the duodenum. The child required almost total parenteral nutrition. During the neonatal period the baby had a white forelock, which gradually disappeared over a period of months. Mild sensorineural hearing loss was diagnosed when she was 4 months old. Chronic intestinal infection with cholangitis and liver dysfunction occurred, together with several episodes of septicaemia requiring antibiotics (including aminoglycosides). Physical examination at 1 year of age showed areas of hypopigmentation on the hands, and an electrophysiological hearing test showed severe, bilateral, sensorineural hearing loss. Heterochromia of the irises and dystopia canthorum were absent. The child failed to thrive and liver failure necessitated liver and intestinal transplantation at the age of 5 years. She died six weeks later of septic shock.

Pregnancy III.3 (fig 2) was terminated at 29 weeks, in accordance with French law, after an intestinal obstruction was identified sonographically. Necropsy showed the same pattern of HSCR affecting the ileum and colon. There were no other discernible morphogenic defects. The mother (II.4) and father (II.5) are non-consanguineous. I.1, I.2, II.4, and III.1 (9 years old) are healthy. Their physical examination showed no malformations or dysmorphism. Their audiograms were normal. I.1 and I.2 are of Yugoslavian origin. II.2 was born at term in a well nourished state, but died in Yugoslavia in the neonatal period from congenital intestinal obstruction (no medical records are available). The father (II.5) and his family (of French origin) had no relevant history.

The coding sequences of the three genes involved in WS4 (*EDN3*, *EDNRB*, and *SOX10*) were screened by means of single strand conformation polymorphism (SSCP) analysis in the index case, as previously described[12 20] (the sequences of the *EDNRB* primers were kindly provided by J Amiel). A band shift was observed in a fragment corresponding to exon 3 of the *EDN3* gene. Sequencing of the variant fragment showed a heterozygous C→A transition, which introduces a stop codon at position 169 (fig 2B). This mutation, C169X, was inherited from the healthy mother (fig 2A) and was not found in 100 control chromosomes. As WS4 is classically considered to result from homozygous *EDN3* mutations, we investigated this family further.

The coding sequence of the *RET* proto-oncogene was screened for mutations on III.3 fetal DNA by SSCP analysis (exons 1 and 2) and denaturing gradient gel electrophoresis (DGGE) (all other exons) as previously described.[29] No causative substitutions or neutral variants were detected. Haplotypes inherited at the *RET* locus were reconstructed by genotyping the parents' DNA for six known intragenic polymorphisms in exons 2, 7, 11, 13, 14, and 15.[30 31] A single nucleotide polymorphism (SNP) of intron 19 and a microsatellite marker located 80 kb upstream of the *RET* gene (MS, unpublished results) were also analysed. The presence of an interstitial microdeletion

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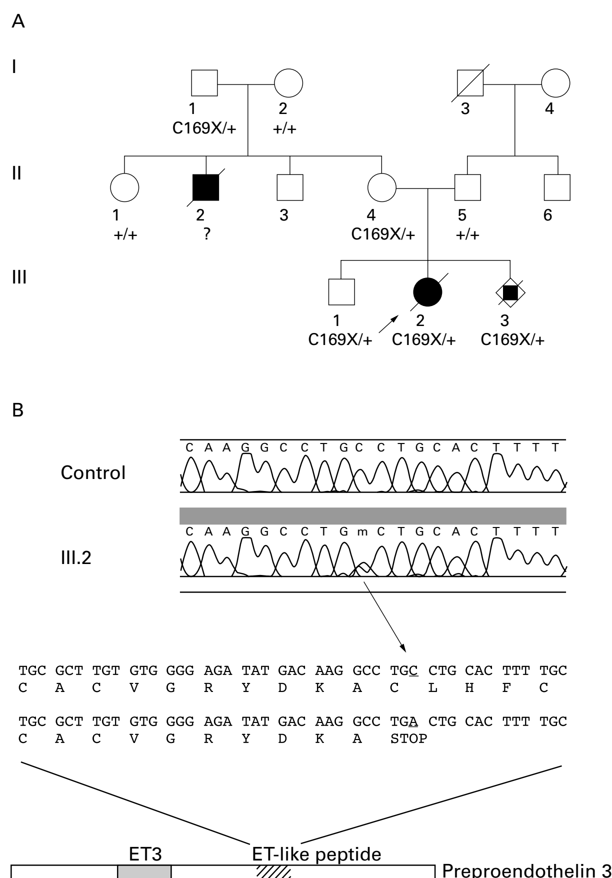

Figure 2 (A) Pedigree of the family. Subjects for whom a DNA sample was available are represented, along with the results of EDN3 genotyping. II.2 is thought to have been affected, as he died from neonatal intestinal obstruction, but no medical records are available. An intestinal obstruction was detected prenatally in the male fetus III.3 and the pregnancy was terminated. (B) The C169X mutation. Results of direct sequencing of EDN3 exon 3 from genomic DNA of a control (above) and the index case III2 (below); consequences of the mutation for preproendothelin 3 structure. The mature endothelin 3 peptide (ET3) is in grey and the ET-like peptide is striped.

was ruled out in the most proximal portion, but loss of heterozygosity (LOH) analysis was not fully informative for the rest of the gene.

Other mutations in the *EDN3*, *EDNRB*, and *SOX10* genes were sought by directly sequencing genomic DNA extracted from the girl's blood cells (III.2) and from a lymphoblastoid cell line established from III.3 fetal cells. No other mutation was found. The absence of partial deletion or rearrangement of the *EDN3* gene was shown by Southern blotting. The girl's (III.2) and control DNA was digested with *BamH*I or *Bcl*I, and human *EDN3* cDNA[32] was used as a probe (ATCC). Finally, to detect possible extinction of the normal *EDN3* allele in the girl, we amplified exon 3 and the junction with exon 4 by reverse transcription from the lymphoblastoid cell line RNA and nested PCR using the following conditions: 20 pmol of each primer, 1.5 mmol/l Mg, annealing temperature 55°C, 35 cycles. First round PCR primers: F 5'CGAACAGACGGTGCCCTATGGAC3', R 5'ATGAGCTTTGGATGGTGGAGGTC3'. Nested primers: F 5'GACTGTCCAACTACAGAGGAAGC3', R 5'CCTGCTTGCTTTGTTGGTCCTTG3'. The PCR products were analysed on 2% agarose gel before sequencing. Several amplification products corresponding to some of the previously described alternatively spliced mRNAs were observed.[32 33] The normal and mutated *EDN3* alleles could be amplified from both the mother and the girl (not shown).

The C169X mutation of the *EDN3* gene lies in a region of the distal preproendothelin called the ET-like peptide (fig 2B). This 15 amino acid peptide shows a very high degree of homology with the mature endothelin peptide, and also with the three preproendothelins from various species. In particular, the four cysteines are conserved. The ET-like peptide might play a role in the first enzymatic cleavage step. The absence of this first cleavage step impairs the final clipping step by endothelin conversion enzyme (ECE-1).[34] As a result, the C169X mutation, by substituting the third cysteine of the ET-like peptide, prevents the disulphide bonds and probably generates an inappropriately cleaved, inactive proendothelin. It is noteworthy that another of the three *EDN3* mutations described to date in WS4 disrupts the disulphide bonds of the ET-like peptide. This defect, C159F, described in the homozygous state,[18] modifies the first cysteine. A functional in vitro assay has been used to show that this mutation results in a virtual absence of the mature endothelin 3 product, supporting the hypothesis of impaired cleavage (Yanagisawa, cited in Hofstra *et al*[28]).

To date, three heterozygous *EDN3* gene mutations have been described in isolated HSCR, and three homozygous mutations have been observed in WS4. Interestingly, in one of the WS4 families, certain members who are heterozygous for the *EDN3* gene C159F mutation have one or more WS features but are free of megacolon.[18] This is incompatible with a recessive mode of WS inheritance and with a dominant mode of HSCR transmission. Another patient, with a congenital central hypoventilation syndrome (CCHS) but free of HSCR and pigmentation defects, carries a heterozygous *EDN3* frameshift mutation involving the carboxy-terminal region of the prepropeptide.[35] However, a functional in vitro test failed to show any effect of this mutation on preproendothelin processing, raising questions as to the deleterious nature of the mutation.

The aborted fetus III.3, which was heterozygous, also had severe intestinal disease, but the presence of WS features could not be assessed. The maternal grandfather and a healthy brother were also heterozygous. It is likely that a maternal uncle, who died at birth from intestinal obstruction, also carried the mutation. As in most other cases described, penetrance was incomplete. Two of the three heterozygous *EDN3* mutations so far identified in isolated HSCR were inherited from an asymptomatic mother,[12] while one was inherited from a mother with a mild intestinal phenotype.[11] Incomplete penetrance and phenotypic variability are frequent in neurocristopathies, particularly in HSCR. This could be explained by environmental factors, multigenic inheritance (see for example Bolk *et al*[36]), or modifier genes, or by stochastic events acting on cell fate or cell differentiation in early embryogenesis. The EDN3/EDNRB ligand/receptor interaction is essential for the development of two different cell lineages, melanocytes and enteric neurones, derived from the neural crest. It is unclear whether this interaction is required by early progenitors of both lineages or only after the lineages diverge. Differences in the chronological order and sites of emergence of distinct subsets of cells derived from the neural crest could partly account for the variable manifestations associated with *EDN3* and *EDNRB* mutations.

This characterisation of the *EDN3* C169X mutation shows that features of both WS and HSCR can result from a heterozygous *EDN3* mutation. One possible explanation for this observation includes multigenic inheritance. Indeed, involvement of an unidentified gene cannot be ruled out in this family. A mutation of the endothelin conversion enzyme gene (*ECE1*) has been described in a patient with a very particular phenotype, including cardiac defects, craniofacial abnormalities, other dysmorphic

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features, and autonomic dysfunction,[37] which were not found in the family investigated here. Another possibility in keeping with our findings is a mode of inheritance which is not fully recessive and not fully dominant, with different penetrance in homozygotes and heterozygotes, as suggested for *EDNRB* mutations. Alternatively, the ET-like peptide mutations could have a particular mode of transmission and phenotypic expression.

This description of a heterozygous *EDN3* mutation in a severe case of Waardenburg-Hirschsprung disease underlines the difficulty in predicting the phenotypic manifestations of *EDN3* mutations. This situation complicates genetic counselling and requires care when assessing the recurrence risk in a family.

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The small patella syndrome: description of five cases from three families and examination of possible allelism with familial patella aplasia-hypoplasia and nail-patella syndrome

EDITOR—The small patella syndrome (SPS, *MIM 14789), also known as ischiopatellar dysplasia, coxopodo patellar syndrome, or Scott-Taor syndrome, is a rare autosomal dominant disorder, characterised by a/hypoplasia of the patellae and various anomalies of the pelvis and feet. This syndrome was first described by Scott and Taor[1] in 1979 in a large family with bilateral small or absent patellae accompanied by anomalies of the pelvic girdle and upper femora in most of the affected subjects. To our knowledge, 42 patients have been reported with this disorder,[1–9] comprising 35 cases from five families and seven sporadic cases. This bone dysplasia is characterised by patellar a/hypoplasia and pelvic anomalies, including bilateral absent or delayed ossification of the ischiopubic junction and infra-acetabular axe cut notches. Other major signs are a wide gap between the first and second toes, short fourth and fifth rays of the feet, and pes planus. Various other skeletal anomalies have been reported, such as elongated femoral necks, flattened and widened proximal femoral epiphyses, hypoplasia of the lesser trochanter, and tarsal anomalies. SPS should be clinically differentiated from disorders with a/hypoplastic patellae, in particular the autosomal dominant disorders isolated familial patella aplasia-hypoplasia (PTLAH) syndrome[10] and the more severe nail-patella syndrome (NPS).[11] The latter is caused by mutations of the *LMX1B* gene on chromosome 9q34. Recently, a locus for PTLAH has been identified on chromosome 17q21-22. As yet, it is unknown whether SPS and PTLAH are allelic disorders. Here we report on five cases from three families with SPS, compare their clinical and radiological anomalies with those of previously reported cases, and propose minimal diagnostic criteria for SPS. Given the clinical overlap between SPS, PTLAH, and NPS, we have studied the possible involvement of candidate regions for these syndromes on chromosome 17q21-22 and 9q34, respectively, by linkage analysis.

Family A, case 1. This male patient, aged 9 years 10 months at the time of examination, was referred because of bilateral absence of the patellae. He was the third child of non-consanguineous Dutch parents. He was born at 37 weeks' gestation after an uneventful pregnancy. Birth weight was 2750 g (10th-25th centile). At birth, talipes equinovarus was noted. Motor milestones were delayed; he sat at 13 months and walked at 24 months. Mental development was normal. At the age of 6 and 8 years, surgery for flat feet was performed, but without success. At the time of examination he complained of unstable knees, muscle weakness of the lower extremities, fatigue on moderate exertion, and inability to run or to stand up from sitting without support. At the age of 9 years 10 months, weight was 30.2 kg (10th-25th centile), height 146.5 (50th-90th centile), and head circumference 51.5 cm (10th-50th centile). The ears were low set and posteriorly angulated. He had a wide gap between the first and second toes bilaterally, short fourth and fifth rays of the feet, and pes planus (fig 1A). The patellae were not palpable. Normal

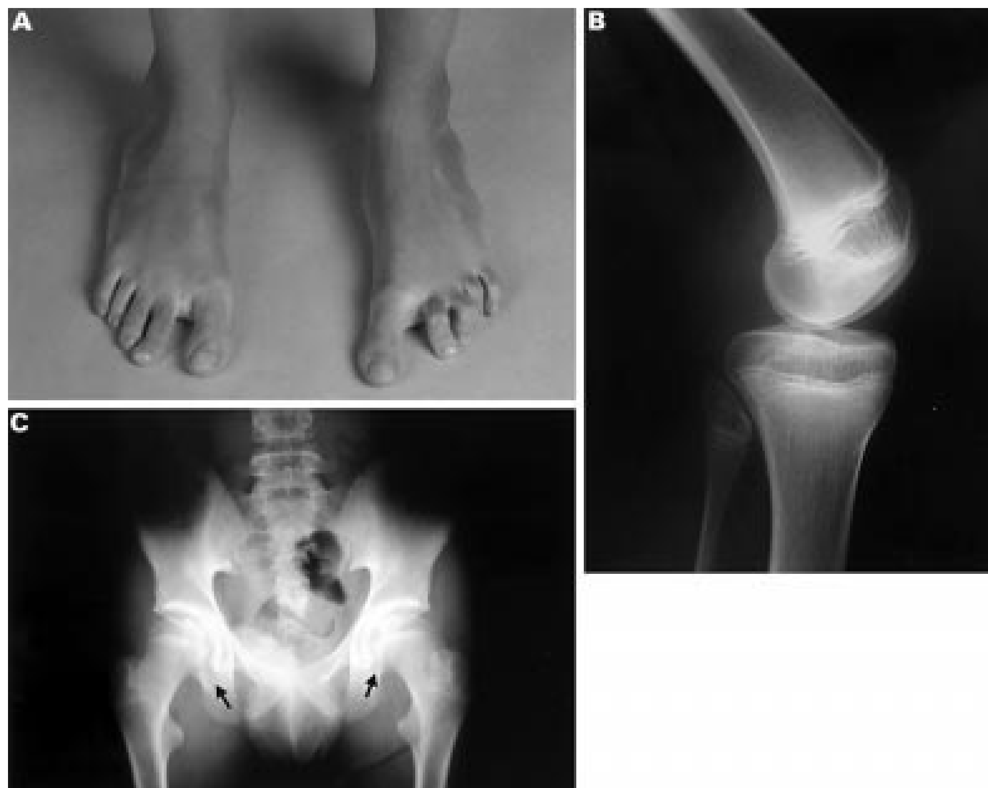

Figure 1 (A) Anterior view of the feet of the proband of family A (case 1) showing an increased space between the first and second toes and short fourth and fifth rays. (B) Radiograph of the knee at the age of 12 years 11 months. Note the absence of the patellae and dysplasia of the epiphyses of the proximal fibula. (C) Radiograph of the pelvis at the age of 12 years 11 months showing the absent ossification of the ischiopubic junction, infra-acetabular axe cut notches (arrows), and elongated femoral necks.

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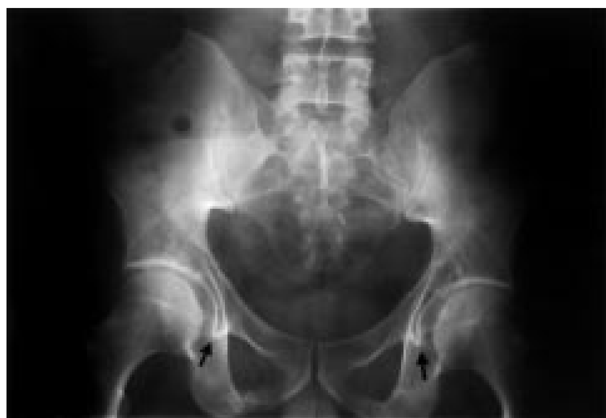

Figure 2 Radiograph of the pelvis of case 2 at the age of 53½ years showing irregular ossification of the ischiopubic junction and infra-acetabular axe cut notches (arrows).

nails and normal joint mobility were found. Radiographs of the knees confirmed the absence of the patellae and showed dysplasia of the epiphyses of the proximal fibulae (fig 1B). Radiographs of the pelvis showed delayed ossification of the ischia and inferior pubic rami, infra-acetabular axe cut notches, and elongated femoral necks (fig 1C). Short fourth and fifth rays of the feet and pes planus were confirmed on radiographs of the feet.

Family A, case 2. This male patient, aged 49 years, the father of case 1, was known to lack both patellae and to have flat feet. In addition to these abnormalities, physical examination showed a wide gap between the first and second toes, and short fourth and fifth rays of the feet. Radiographs showed patellar hypoplasia, irregular ossification of the ischiopubic junction, and infra-acetabular axe cut notches (fig 2). No abnormalities of other joints or nails were found. Chromosome analysis showed a normal male karyotype. His oldest son, daughter, and 10 sibs showed no anomalies of their elbows, knees, or feet on physical examination, except for short fourth rays of the feet in one sib. Radiological examination of this relative showed short fourth metatarsals, but because anomalies of the patellae and pelvis were not seen, he was considered not to have SPS. His parents had died. According to the patient they did not have any complaints or congenital anomalies of the knees and feet.

Family B, case 3. This male patient, aged 9 years 2 months, was referred because of bilateral small patellae and knee pain. He is the first child of non-consanguineous Dutch parents. He was born at term after an uneventful pregnancy. Birth weight was 3500 g (50th centile) and length 52 cm (50th-75th centile). Psychomotor development has been

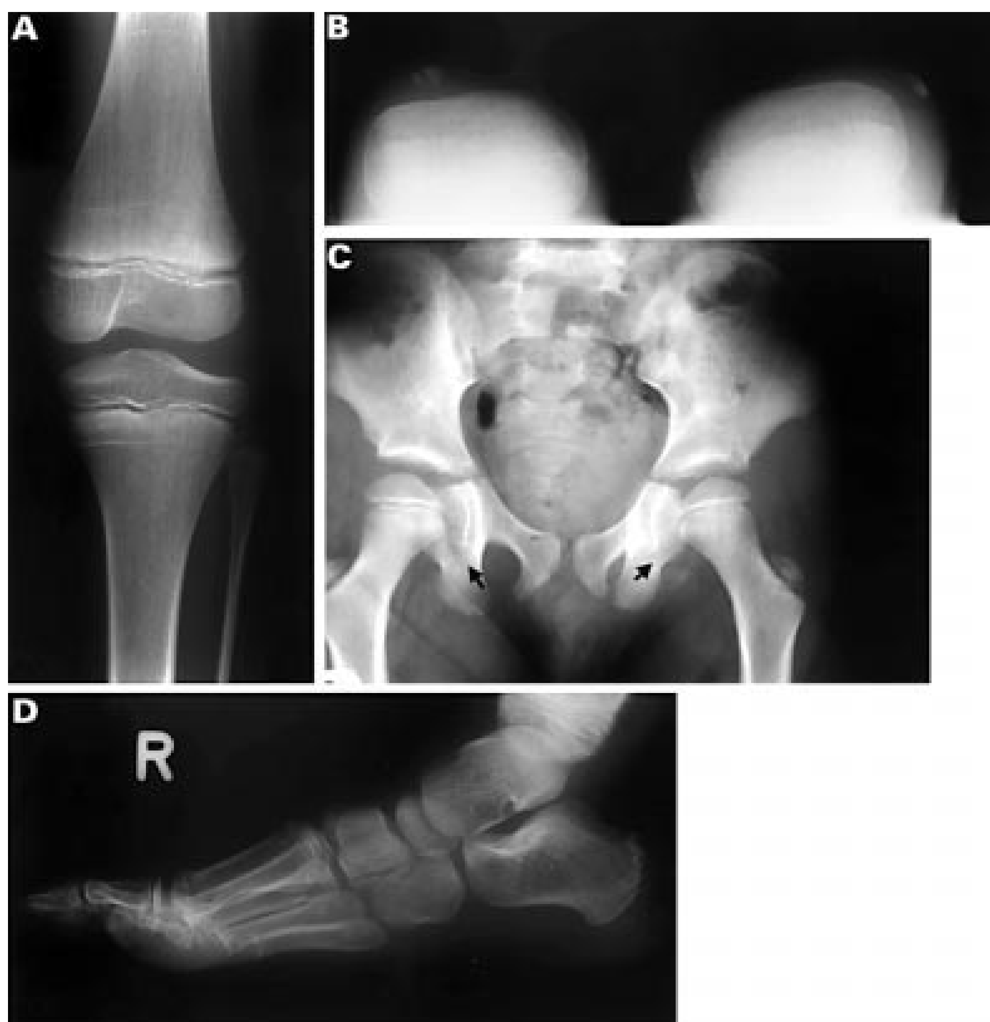

Figure 3 (A) Radiograph of the knees of the proband of family B (case 3) at the age of 9 years 2 months showing smaller medial femoral and tibial condyles compared to lateral femoral and tibial condyles. (B) Note the hypoplastic and dislocated patellae with two ossification centres on the left side, and flattened fossa intercondylares. (C) Radiograph of the pelvis at the age of 7½ years showing bilateral hypoplastic ossification of the ischia and inferior pubic rami for his age and infra-acetabular axe cut notches (arrows). (D) Radiograph of the feet showing hypertrophy of the neck of the talus.

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normal except for an abnormal gait and weakness of the lower extremities. He had surgery for cryptorchidism at the age of 7½ years. At the same age, a traumatic pelvic fracture occurred with subsequent recurrent luxations of the left patella. As a consequence, running was impossible and riding a bicycle very difficult. At the age of 9 years 2 months, weight was 35.1 kg (90th centile), height 148.5 cm (>97th centile), and head circumference 53 cm (2nd centile). The face was unremarkable. The space between the first and second toes was increased, and bilateral short fourth and fifth rays of the feet were noted. There were no contractures, hypermobility of other joints, or nail anomalies. Radiographs of the knees showed small patellae and a dislocated patella on the left side, composed of two ossification centres (fig 3A, B). Radiographs of the pelvis showed bilateral hypoplasia of the ischia and inferior pubic rami, infra-acetabular axe cut notches, flattened and widened capital femoral epiphyses, elongated femoral necks, and hypoplasia of the lesser trochanter (fig 3C). The medial femoral and tibial condyles were smaller than the lateral femoral and tibial condyles. The fossae intercondylares were flattened. Hypertrophy of the neck of the talus and pes planus were seen on radiographs of the feet (fig 3D). Radiographs of the hands were normal. Chromosome analysis showed a 47,XXY karyotype.

Family B, case 4. This female patient, aged 35 years, the mother of case 3, suffered from discomfort of the knees in early childhood. On physical examination, she had bilateral small patellae and an increased space between the first and second toes bilaterally. The other joints and nails were normal. Radiographs showed bilateral small patellae, absent ossification of the ischiopubic junction bilaterally, and infra-acetabular axe cut notches. The medial femoral and

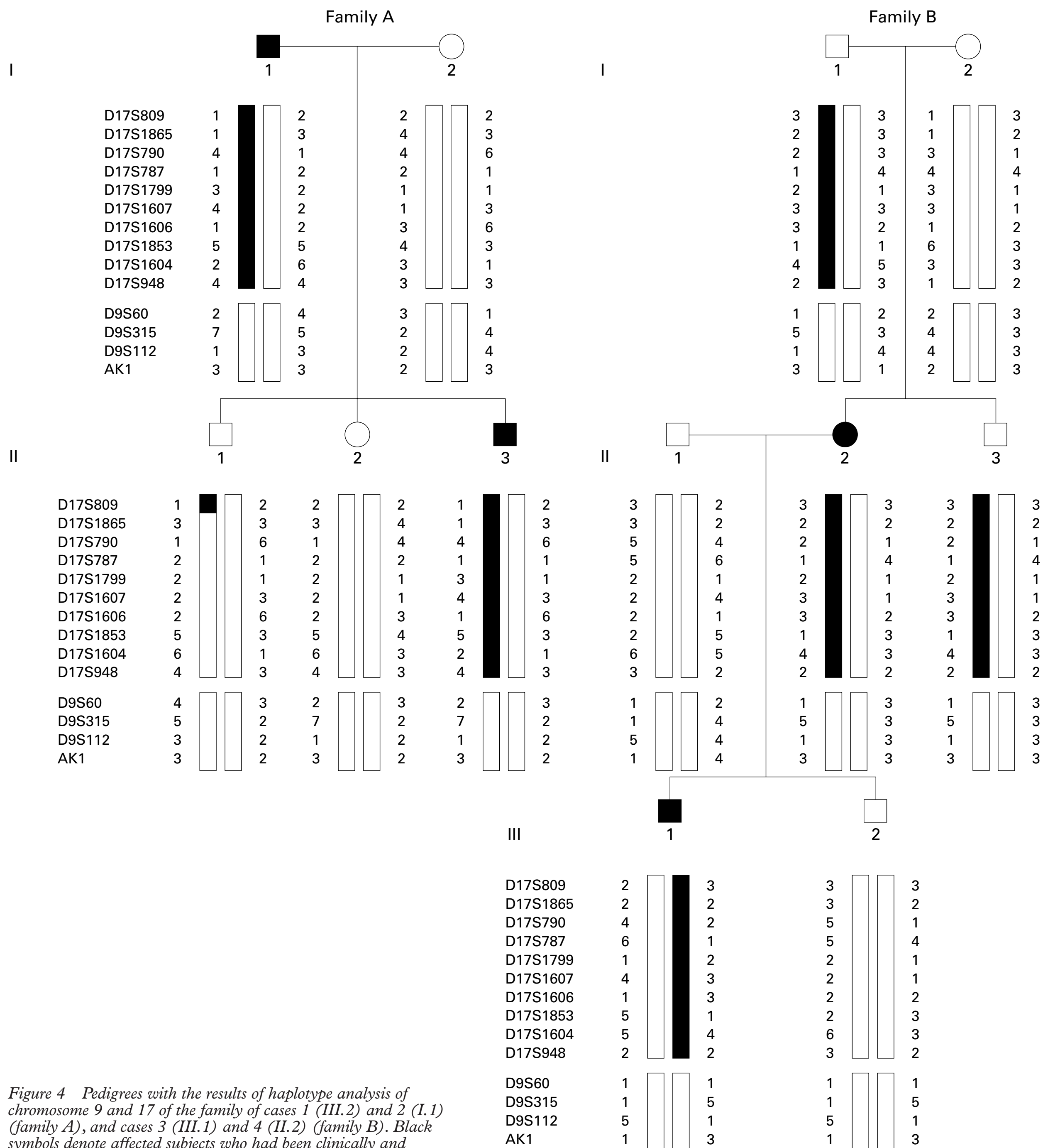

Figure 4 Pedigrees with the results of haplotype analysis of chromosome 9 and 17 of the family of cases 1 (III.2) and 2 (I.1) (family A), and cases 3 (III.1) and 4 (II.2) (family B). Black symbols denote affected subjects who had been clinically and radiographically investigated.

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Table 1 Clinical and radiological features of previously published cases[1–9 12 13] and present cases with small patella syndrome

	Previously reported cases[1–9 12 13]	Frequency (%)	Family A		Family B		Family C
			Case 1	Case 2	Case 3	Case 4	Case 5
Radiological/clinical anomalies of the patella	45/45*	100					
Patellar aplasia	14/45	32	+	−	−	−	−
Patellar hypoplasia	31/45	69	−	+	+	+	+
Radiological anomalies of the pelvis	34/35†	97					
Absence/hypoplasia/irregularity of the ischiopubic junction	24/27‡	89	+	+	+	+	+
Infra-acetabular axe cut notches	8/9	89	+	+	+	+	+
Radiological anomalies of the femora							
Hypoplasia of the lesser trochanter	3/6	50	−	−	+	+	−
Elongated femoral necks/flattened and widened proximal femoral epiphyses	8/15	53	+	−	+	+	−
Radiological/clinical anomalies of the feet							
Pes planus	9/15	60	+	+	+	+	+
Short fourth and fifth rays of feet	10/15	67	+	+	+	+	+
Clinical anomalies of the feet							
Wide gap between first and second toes	13/17	76	+	+	+	+	+

*In 45 of the 46 previously reported cases, patellar a/hypoplasia was mentioned.
†Thirty five of the 46 previously reported cases had radiological examination of the pelvis.
‡Anomalies of the ischiopubic junction were specified in 27 of the 35 previously reported cases with pelvic anomalies.

tibial condyles were smaller than the lateral femoral and tibial condyles and the fossa intercondylares were flattened. Radiographs of the feet showed hypertrophy of the neck of the talus and pes planus bilaterally. Radiographs of the hands were normal. The family history was unremarkable. Physical examination and radiographs of the knees of her father, mother, brother, and youngest son showed no abnormalities. Furthermore, radiographs of the pelvis of her youngest son at the age of 8 years 1 month showed normal ossification of the ischio-pubic junction and absence of infra-acetabular axe cut notches.

Family C, case 5. This female patient, aged 20 years, is the first child of non-consanguineous Belgian parents. She has one healthy brother. She was born at 41 weeks' gestation. Birth weight was 2500 g (<3rd centile) and length 42 cm (<3rd centile). She had surgery on her knees for recurrent luxations of the patellae in infancy. She was referred on suspicion of Marfan syndrome because of general hyperlaxity of the joints and poor wound healing at the age of 20 years. At that age, weight was 105.2 kg (>97th centile), height 174 cm (50th-90th centile), and head circumference 55.1 cm (50th centile). She had a high nasal bridge, high arched palate, and micrognathia. Furthermore, clinodactyly of the fifth fingers, short fifth fingers, bilateral small patellae, flat feet, a wide gap between the first and second toes bilaterally, short fourth and fifth toes, and joint hyperlaxity were noted. The other joints and nails were normal. Radiographs showed hypoplastic patellae, absent ossification of the ischiopubic joint bilaterally, infra-acetabular axe cut notches, and small medial femoral and tibial condyles as compared to the lateral femoral and tibial condyles. Radiographs of the skull, thorax, vertebral column, upper limbs, wrists, hands, and feet showed no abnormalities, except for pes planus. Echocardiography and ophthalmological examination were normal, excluding Marfan syndrome. Chromosome analysis showed a 46,XX,t(1;X)(q12;q27) karyotype which was also found in her mother. Her mother was said to be healthy and was not known to have had knee or foot anomalies. Her father was known to have had scoliosis and pes planus and he had surgery on his knees. Her paternal grandmother was also known to have had knee complaints. Both her father and her mother were unavailable for examination and further clinical or radiological information could not be obtained.

Informed consent for molecular genetic investigation was obtained from two affected and three unaffected members of family A, and two affected and five unaffected members of family B. Genomic DNA was extracted from peripheral blood lymphocytes by a salt extraction procedure.[17] The DNA concentration was measured by optical density (OD_{260}) and purity checked by determining the OD_{260}/OD_{280} ratio. Manual genotyping of microsatellite markers on chromosome 9q34 and 17q21-22 was carried out as described elsewhere.[18] Microsatellite markers were chosen from the final Généthon linkage map.[19] Four patients with SPS from two families were investigated (figs 1-3). Given the overlap of the clinical features of these families with PTLAH[10] and NPS,[11] microsatellite markers from the relevant chromosomal regions were tested for possible linkage in family A and family B. The results of haplotype analysis in these two families are shown in fig 4. Linkage to chromosome region 9q34 was excluded in families A and B. Both families A and B are compatible with linkage to the PTLAH critical region on chromosome 17q21-22,[10] assuming a de novo mutation in case 4 (family B, case II.2, fig 4).

Since the first description of a family with 12 affected cases of SPS by Scott and Taor[1] in 1979, 30 patients have been described with this condition.[2–9] Additionally, several possible cases of SPS have been reported. In 1970, Goeminne and Dujardin[12] described a family with three affected members with bilateral patellar aplasia, bilateral congenital hip dysplasia, hypoplasia of the descending part of the pubic arches, and absent ischiopubic synostosis, accompanied by pes planus, tarsal synostosis, short stature, and oligodactyly of the feet in one subject. Habboub and Thneibat[13] reported a sporadic case with aplastic patellae and bilateral absence of the ischiopubic rami, and suggested the name ischio-pubic-patellar hypoplasia for a possible new syndrome. The clinical and radiological features of the patients described in both reports[12 13] are strikingly similar to those of SPS. Therefore, we believe that these cases do not represent new syndromes but are further examples of SPS. Thus, a total of 51 cases with SPS have currently been described, including the cases of Goeminne and Dujardin,[12] Habboub and Thneibat,[13] and the present cases.

The clinical and radiographic anomalies of the five patients presented here and previously reported cases are summarised in table 1. Patellar a/hypo/dysplasia has been found in all cases, except for one familial case with patellar dislocation in which radiological examination was not mentioned.[8] Complaints and symptoms varied from pain resulting from gonarthrosis in elderly subjects to recurrent luxations from infancy, knee pain, and inability to run and ride a bicycle. In some cases, however, there were no symptoms. In this report, all cases had absent, delayed, or irregular ossification of the ischiopubic joint accompanied by

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infra-acetabular axe cut notches, but no related complaints or additional clinical features were noted. In family B, the medial femoral and tibial condyles were small compared to the lateral femoral and tibial condyles. These features have also been mentioned by Vanek[2] and can also be seen on radiographs from other reports.[3–5] Hypoplasia of the lesser trochanter, elongated femoral necks, and flattened and widened epiphyses of the proximal femora were found in most of the present patients, but have been reported in only a minority of the previously reported cases. The main clinical features of the feet, comprising an increased space between the first and second toes, short fourth and fifth rays, and pes planus, were found in all our patients, and in a majority of the previously reported cases. Furthermore, hypertrophy of the neck of the talus, short fourth metatarsals, tarsal coalition or synostosis, winging or an abnormal shape of the scapulae, short fourth and fifth metacarpals, coxa valga, coxa vara, and genu valgum have been described in a minority of the previously reported cases.

Additional anomalies have only been found in five cases. One of the present cases (case 5) showed a high nasal bridge, micrognathia, and a high arched palate. One of the two sporadic cases described by Kozlowski and Nelson[6] had synophrys, epicanthic folds, a broad nasal bridge, apparently low set, posteriorly angulated ears, anteverted nares, long philtrum, high palate, prominent lower lip, and micrognathia, and the other case had a flattened nose and a prominent forehead. Subsequently, Azouz and Kozlowski[9] described another sporadic case with macrocephaly and cleft palate. As yet, it is not possible to establish whether these anomalies are variable features of SPS. Clinical examination of additional cases and the elucidation of the causative gene defect is required to delineate the phenotypic variability of SPS.

Diagnostic radiographic characteristics of SPS have already been mentioned by Kozlowski and Nelson.[6] Here, a review of the clinical and radiological features of the cases with SPS shows that all the radiologically examined cases have patellar a/hypoplasia as well as pelvic anomalies (table 1). Based on these findings we propose a/hypoplasia of the patellae and absent/delayed/irregular ossification of the ischiopubic junctions or infra-acetabular axe cut notches as minimal criteria for the diagnosis of SPS. In only one child could pelvic anomalies not be excluded[12] because she was too young at the time of radiological pelvic examination. Additional major signs comprise an increased space between the first and second toes and short fourth and fifth rays of the feet accompanied by flat feet. Various other skeletal anomalies have been described but do not contribute to the diagnosis.

This syndrome should be differentiated from disorders with a/hypoplastic patellae, including NPS and isolated familial PTLAH. In NPS, patellar a/hypoplasia is associated with nail anomalies, deformation or luxation of the head of the radius resulting in impaired mobility of the elbow, iliac horns and, frequently, nephropathy. Nail a/hypo/dysplasia and absent or hypoplastic patellae are essential features for the diagnosis. Posterior iliac horns are pathognomonic for NPS, but reported to be present in only 70% of cases. Various other skeletal anomalies, including pes equinovarus, dislocated hips, and contractures of major joints have been described in NPS but do not contribute to the diagnosis. This skeletal dysplasia results from mutations in the *LMX1B* gene.[11] In patients with PTLAH, patellar a/hypoplasia is an isolated anomaly without additional radiological or clinical features. Pelvic anomalies, including anomalies of the ischiopubic joint or infra-acetabular notches have never been mentioned in PTLAH.[10 14–16] Radiological examination of the pelvis and feet should be performed in all patients with a/hypoplasia of the patellae in order to differentiate SPS from PTLAH, and to further evaluate the diagnostic value of pelvic and feet anomalies in SPS.

In conclusion, given the clinical overlap between SPS, PTLAH, and NPS we hypothesised that SPS might be allelic to either of these disorders, which map to the chromosomal regions 17q21-22 and 9q34, respectively. Linkage studies excluded allelism with NPS in two of the present families that were available for molecular analysis. Allelism with PTLAH cannot be excluded in our families at the moment. Further linkage studies in other families with SPS are needed to confirm linkage to chromosome 17q21-22 and to examine whether SPS is genetically homogeneous. In view of the small size of most families, only the elucidation of the genetic defect will provide the final answer for allelism of SPS and PTLAH.

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Uniparental isodisomy for paternal 2p and maternal 2q in a phenotypically normal female with two isochromosomes, i(2p) and i(2q)

EDITOR—Recently, Bernasconi *et al*[1] and Shaffer *et al*[2] described carriers of isochromosomes 2p (i(2p)) and 2q (i(2q)). In both patients maternal uniparental disomy (UPD) (2), the exceptional inheritance of both chromosomes 2 from the mother, was detected. Isochromosome formation of both the short and the long arms of a chromosome in one carrier is a rare event. In addition to isochromosomes 2, there exist single reports on only isochromosomes 4p and 4q,[3] isochromosomes 7p and 7q,[4] and isochromosomes 9p and 9q.[5] In these cases, the parental origin was determined and was mostly maternal.

The phenotypes of the carriers of i(2p) and i(2q) and maternal UPD(2) are rather inconsistent. Bernasconi *et al*[1] reported a healthy woman with a history of five spontaneous abortions. In contrast, the patient of Shaffer *et al*[2] showed features similar to those of three maternal UPD(2) patients ascertained because of confined placental mosaicism (CPM) for trisomy 2.[6–8] In all these four patients, severe intrauterine growth retardation with oligohydramnios or anhydramnios and postnatal growth retardation were observed, with additional findings including hypospadias and pulmonary dysplasia or hypoplasia. Three patients showed good motor and intellectual development[2 6 7] and the fourth patient died of severe pulmonary hypoplasia shortly after birth. The phenotypically normal girl published by Heide *et al*,[9] who shows maternal UPD(2) and a normal chromosomal complement supports the observations that maternal UPD(2) has no clinical effects.

Up to now, partial or complete paternal UPD(2) has never been reported. Here we describe a healthy carrier of i(2p) and i(2q), in whom molecular studies showed a paternal UPD(2p) and a maternal UPD(2q).

The healthy, 36 year old woman was referred for chromosomal analysis because all of her six pregnancies had resulted in spontaneous abortion during the first trimester. She had normal physical and mental development. She went through normal puberty and her final height is 176 cm (+2 SD). She is of normal intelligence and works as a nurse.

GTG banded chromosome analysis on lymphocyte cultures of the proband showed a non-mosaic, 46,XX,i(2)(p10),i(2)(q10) chromosome complement. Cytogenetic analyses of the proband's parents showed normal 46,XX and 46,XY karyotypes.

Table 1 Results of STR typing in the UPD(2) family. Data from markers other than chromosome 2 are not shown; the order and localisation of markers correspond to those published by Gyapay et al[10]

STR	Localisation	Father	Mother	Proband	Informativity
	2pter				
D2S319	2pter-qter	1–2	2–2	1–1	Paternal UPiD
D2S168	2p25	1–4	2–3	4–4	Paternal UPiD
D2S131	2p22-p25	2–2	1–3	2–2	Paternal UPD
D2S160	2p13-q14	1–1	1–1	1–1	—
	Cen				
D2S121	2q12-q13	1–1	2–2	2–2	Maternal UPD
D2S118	2q32	1–2	3–4	3–3	Maternal UPiD
D2S117	2qter-qter	1–1	1–1	1–1	—
D2S116	2q32	2–2	1–1	1–1	Maternal UPD
D2S325	2qter-qter	1–1	2–3	2–2	Maternal UPiD
D2S125	2qter-qter	1–1	2–2	2–2	Maternal UPD
	2qter				

For molecular studies, DNA was isolated from peripheral lymphocytes from the proband and her parents. UPD(2) was determined by short tandem repeat typing (STR). Primers and map location were obtained from the chromosome 2 linkage map published by Gyapay *et al*.[10] Typing of four markers on chromosomes other than chromosome 2 was carried out to confirm normal maternal and paternal contributions.

The results of STR typing are shown in table 1. In three out of four 2p markers, only one paternal allele could be identified; the fourth STR was not informative. On 2q, we detected only one maternal allele in five out of six markers with the sixth not being informative. Therefore, paternal uniparental isodisomy (UPiD)(2p) and a maternal UPiD(2q) is present. A similar condition has been described formerly for chromosome 7[4]; in a postnatally growth retarded girl, a paternal isochromosome 7p and a maternal isochromosome 7q were detected. The following mechanism of formation can be postulated. An incomplete mitotic recombination occurred in a zygote primarily biparental for chromosome 2, followed by the loss of paternal 2q and maternal 2p without the centromere, and centromeric misdivision of the rearranged chromosomes.

To the best of our knowledge, paternal UPD(2) has not previously been described. The finding of a paternal UPD(2p) in a phenotypically normal person indicates that this condition does not seem to have any phenotypic effect. It can be speculated that no paternally imprinted genes are located on the short arm of chromosome 2. Of course, based on only one case, the possibility of the existence of a paternally imprinted gene in 2p cannot be excluded with certainty.

Additionally, maternal UPD(2q) in our proband provides further evidence that there are no imprinted genes on the long arm of chromosome 2, corresponding to the results of Bernasconi *et al*[1] and Heide *et al*.[9] Therefore, our data support the hypothesis that maternal UPD(2) does not influence the phenotype. The clinical findings in the patients with maternal UPD(2) showing abnormalities can probably be attributed to placental dysfunction owing to CPM or to possible mosaicism for trisomy 2. In the case of the phenotypically affected carrier of i(2p) and i(2q),[2] the most probable method is formation of the zygote with biparental disomy 2, subsequent centromeric misdivision resulting in maternal i(2p) and i(2q), and loss of paternal chromosome 2. This mechanism is compatible with CPM for trisomy 2. Thus, this might be the cause of the clinical findings in this patient.

Except for the possibility of homozygosity for recessive mutations, neither paternal UPD(2p) nor maternal UPD(2q) appears to have any adverse effect on the phenotype. There is no evidence for paternally imprinted genes on 2q or maternally imprinted genes on 2p.

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