Report of five novel and one recurrent *COL2A1* mutations with analysis of genotype-phenotype correlation in patients with a lethal type II collagen disorder

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J Med Genet 2000 37: 263-271
doi: 10.1136/jmg.37.4.263

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Abstract

Achondrogenesis II-hypochondrogenesis and severe spondyloepiphyseal dysplasia congenita (SEDC) are lethal forms of dwarfism caused by dominant mutations in the type II collagen gene (*COL2A1*). To identify the underlying defect in seven cases with this group of conditions, we used the combined strategy of cartilage protein analysis and *COL2A1* mutation analysis. Overmodified type II collagen and the presence of type I collagen was found in the cartilage matrix of all seven cases. Five patients were heterozygous for a nucleotide change that predicted a glycine substitution in the triple helical domain (G313S, G517V, G571A, G910C, G943S). In all five cases, analysis of cartilage type II collagen suggested incorporation of the abnormal α1(II) chain in the extracellular collagen trimers. The G943S mutation has been reported previously in another unrelated patient with a strikingly similar phenotype, illustrating the possible specific effect of the mutation. The radiographically less severely affected patient was heterozygous for a 4 bp deletion in the splice donor site of intron 35, likely to result in aberrant splicing. One case was shown to be heterozygous for a single nucleotide change predicted to result in a T1191N substitution in the carboxypropeptide of the proα1(II) collagen chain. Study of the clinical, radiographic, and morphological features of the seven cases supports evidence for a phenotypic continuum between achondrogenesis II-hypochondrogenesis and lethal SEDC and suggests a relationship between the amount of type I collagen in the cartilage and the severity of the phenotype.

(*J Med Genet* 2000;**37**:263–271)

Keywords: type II collagen disorders; achondrogenesis II-hypochondrogenesis; spondyloepiphyseal dysplasia congenita; *COL2A1*

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Revised version received
12 July 1999
Accepted for publication
5 August 1999

Several heritable osteochondrodysplasias have now been recognised as members of the family of type II collagen disorders, all of which result from dominant mutations in the *COL2A1* gene.[1–4] Phenotypes within this group range from severe lethal dwarfism at birth to relatively mild conditions with precocious osteoarthrosis and little or no skeletal growth abnormality.[2] Achondrogenesis II-hypochondrogenesis and lethal spondyloepiphyseal dysplasia congenita (SEDC) represent the more severe end of the spectrum.[1 5–7] These entities are characterised by severe disproportionate short stature of prenatal onset. The distinction between these phenotypes is mainly based upon clinical, radiographic, and morphological features but considerable phenotypic overlap often hampers proper classification.[1 6–8]

All of the previously reported cases of achondrogenesis II-hypochondrogenesis in which the molecular defect was found were heterozygous for a mutation in the *COL2A1* gene resulting in a glycine substitution in the triple-helical domain of the proα1(II) collagen chain.[9–16] Biochemical analysis of cartilage usually showed the presence of type I collagen and post-translationally overmodified type II collagen. Studies on cultured chondrocytes from one case of hypochondrogenesis with a G913C mutation showed lack of secretion of type II collagen in the medium.[16] Mutations reported in cases of SEDC are more heterogeneous. Single amino acid substitutions, deletions, or duplications in the triple helical domain of the proα1(II) collagen chain have been reported.[17–24]

The aim of this study was to identify the mutation in the *COL2A1* gene in seven cases of achondrogenesis II-hypochondrogenesis or severe SEDC based on the results of the biochemical analysis of cartilage tissue. Instead of performing a "head to tail" mutation analysis of the entire gene, we first started screening those regions of the *COL2A1* gene in which a mutation was most likely to reside, based on the electrophoretic properties of the extracted cartilage collagen. The phenotype of each case was studied in relation to the molecular and biochemical findings and compared with other reported cases of achondrogenesis II-hypochondrogenesis and SEDC in which a *COL2A1* mutation was identified.

Materials and methods

PATIENTS

The seven patients in this study were referred in the period 1982 to 1991 to the International Skeletal Dysplasia Registry at Cedars-Sinai Medical Center, Los Angeles for diagnosis. The clinical features and radiographs were sent to the Registry for evaluation and tissue specimens, taken during necropsy, were collected

Table 1 Clinical, radiographic, and morphological features of seven patients with a lethal type II collagen disorder

	Patient							
	R82-121	*R83-32*	*R85-109*	*R86-6*	*R86-153*	*R87-75*	*R91-68*	
COL2A1 mutation	*T1191N?*	Gly571Ala	Gly910Cys	Gly313Ser	Gly943Ser	Gly517Val	IVS35+4delAGTG	
Clinical								
Sex	Male	Female	Male	Female	Female	Female	Female	
Birth length (gestational age)	39 cm (36 wk)	39 cm (40 wk)	? (37 wk)	? (34 wk)	29 cm (35 wk)	38 cm (42 wk)	34 cm (38 wk)	
Short limbs	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Small thorax	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Cleft palate	No	No	No	Yes	Yes	No	No	
Respiratory insufficiency at birth	No	Yes	Yes	Yes	Yes	Yes	Yes	
Age at death	1 y 1 mth	63 d	1 d	10 h	6 h	2 d	4 d	
Radiographic								
Long bones[a]	++* hands and feet	+++*	++*	+++*	++++*	++++*	+†	
Pelvis[b]	?	Yes	Yes	Yes	Yes	Yes	No	
Spine[c]	Cervical	Cervical	Cervical, sacral	Cervical	Entire spine	Cervical, sacral	Only platyspondyly	
Thorax[d]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Pubic bones[e]	?	No	No	No	No	No	No	
Talus and calcaneus[f]	Yes	No	Only talus	No	No	?	Only talus	
Morphological								
Resting cartilage	Mild hypervascular	?	Very hypervascular	Mild hypervascular	Very hypervascular	Very hypervascular	Slightly increased vascular canals	
Chondrocytes LM	Inclusion bodies	Inclusion bodies	Inclusion bodies, dilated RER‡	Inclusion bodies, dilated RER‡	Inclusion bodies	Inclusion bodies, dilated RER‡	Inclusion bodies, dilated RER‡	
EM								
Growth plate	Abnormal	Abnormal	?	Abnormal	Abnormal	Abnormal	Abnormal	

[a]Degree of shortening of the long tubular bones with* or without† metaphyseal cupping. [b]Presence of flared iliac wings. [c]Platyspondyly with level of incomplete ossification of vertebral bodies. [d]Small thorax with short ribs. [e]Ossification of pubic bones. [f]Ossification of talus and calcaneus. All radiographs were taken in the neonatal period except for patient R82-121 where the radiographs were taken at 1 month of age. ‡Electron micrographs show dilated rough endoplasmic reticulum containing granular material.

for morphological, biochemical, and molecular studies. All patients were sporadic. Parental DNA samples were not available.

PROTEIN ANALYSIS

Cartilage stored at −70°C was used. For analysis of pepsin solubilised collagen, cartilage was extracted, washed, and digested with pepsin as described previously.[11] The resulting collagen type II molecules were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Human epiphyseal cartilage collagen from a 4 day old neonate without signs of a skeletal dysplasia was used as a control. For peptide analysis, cartilage was digested with cyanogen bromide (CB) and the resulting collagen CB peptides were analysed by SDS-PAGE and stained with Coomassie blue as described previously.[11]

NUCLEIC ACID ANALYSIS

Genomic DNA was isolated from cultured skin fibroblasts by standard procedures. PCR amplifications were performed in 25 µl reactions containing 100 ng of genomic DNA, 2 U *Taq* DNA polymerase, 2.5 pmol of each primer, 200 µmol/l of each dNTP, 2 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl, pH 8.3, and 50 mmol/l KCl. The amplification conditions consisted of an initial two minutes at 95°C followed by 36 cycles of 95°C for one minute, 60°C for one minute, and 72°C for one minute, followed by a nine minute incubation at 72°C. Primer sequences were 5'-CTGTTCTCACTCACT GCCTC-3' and 5'-GGATACCATGTGACC TCAG-3' for exon 22; 5'-GGTTGATCACTT CTTGGTG-3' and 5'-CTCAGTGGGACTC CAGGCTAC-3' for exon 31; 5'-GTGCCC GGCTGAGGCGGCTG-3' and 5'-TCCTAA TGCCCAGCAGTCCAG-3' for exon 33; 5'-ACCTTCTTACCCCAGCTCTTC-3' and 5'-GGCCTCGGGCAGAGCCAGGC-3' for exon 35; 5'-CCCTGACCTGACTCAATC GG-3' and 5'-AGGAGGCCTCGGGAAGT CCC-3' for exon 46; and 5'-TCCTCTGA GCTTGCTCCACTC-3' and 5'-TCCTGTC ACTTTAGGACCTG-3' for exon 51. Primer sequences for the other exons are available on request. The PCR generated fragments were examined on non-denaturing 6% polyacrylamide gels (29:1 acrylamide:bis ratio) in 1 × TBE buffer. For single strand conformation polymorphism (SSCP) analysis, 5 µCi of α-[^{32}P] dCTP was added to the PCR reaction and the amount of unlabelled dCTP was reduced to 2.5 µmol/l. Fragments were separated by electrophoresis through MDE gels (AT Biochem) for 11 hours at 3.5 W. The gels were dried and exposed to *x* ray film for 48 hours at −70°C with an intensifying screen. For heteroduplex analysis, PCR products were denatured at 98°C for five minutes, renatured for 60 minutes at 68°C, and loading buffer (50% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol in water) was added. The samples were separated on MDE gels (1 × MDE gel supplemented with 10% glycerol in 0.6 × TBE) overnight at 250 V. The fragments were visualised by ethidium bromide staining. For DNA sequence analysis PCR generated

fragments were purified using a Qiaquick spin PCR purification kit (Qiagen). The purified fragments were either used for direct sequence analysis or ligated into the TA cloning kit (Invitrogen) and sequenced using Sequenase 2.0 (USB). For direct sequencing, single stranded DNA fragments were generated in a second round of amplification using a purified aliquot (2 µl) from the first PCR and only one of the two primers (50 pmol).

CARTILAGE MORPHOLOGY

For light microscopy (LM), cartilage specimens were fixed in 10% formalin and embedded in methyl methacrylate. Five µm sections were cut and stained as described previously.[15] For electron microscopy (EM), cartilage specimens were fixed in 2.5% glutaraldehyde, postfixed in osmium, and dehydrated and embedded in Spurr resin. Thin sections were cut and stained as described previously.[15]

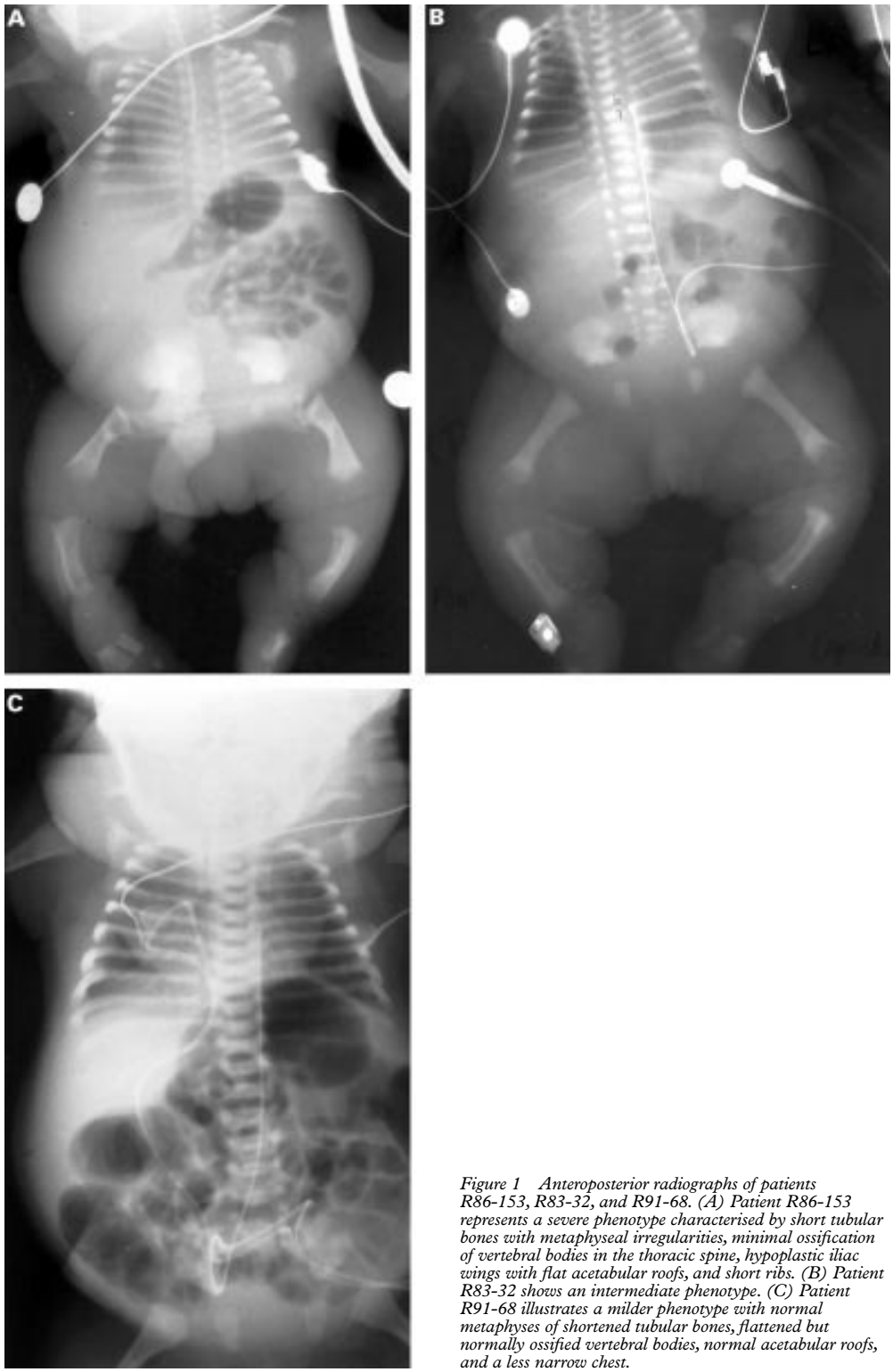

Figure 1 Anteroposterior radiographs of patients R86-153, R83-32, and R91-68. (A) Patient R86-153 represents a severe phenotype characterised by short tubular bones with metaphyseal irregularities, minimal ossification of vertebral bodies in the thoracic spine, hypoplastic iliac wings with flat acetabular roofs, and short ribs. (B) Patient R83-32 shows an intermediate phenotype. (C) Patient R91-68 illustrates a milder phenotype with normal metaphyses of shortened tubular bones, flattened but normally ossified vertebral bodies, normal acetabular roofs, and a less narrow chest.

Results

The diagnosis of a lethal type II collagen disorder was made in each of the patients based on the clinical, radiographic, and morphological features (table 1, fig 1).[1] Significant findings on which the diagnosis was based included the following. (1) Clinical: moderate to severe short limbed dwarfism at birth with respiratory problems owing to a small thorax; cleft palate. (2) Radiographic: incomplete ossification of the vertebral bodies; severe shortening of the long bones and ribs; flared iliac wings; absent ossification of pubic bones, talus, and calcaneus. (3) Morphological: hypervascular resting cartilage; chondrocytes with inclusion bodies (LM) or dilated cisternae of the rough endoplasmic reticulum (RER) containing granular material (EM); disorganised growth plate characterised by extension of hypertrophic cells into the primary trabeculae.

SDS-PAGE of cyanogen bromide (CB) digested cartilage from patient tissues showed slowly migrating, post-translationally overmodified type II collagen CB peptides not seen in the control (fig 2A). The cases varied in their degree of overmodification as best shown by the extent to which α1(II) CB10 was retarded. CB10 was most retarded for R85-109 and R86-153 and less so for R86-6. From R86-6, however, peptide α1(II) CB11 was noticeably retarded compared with control CB11. A doublet for CB10 was visible in R82-121 and an abnormally broad band for CB10 was apparent in R91-68. These observations suggested where along the proα1(II) collagen molecule the mutation may lie, since post-translational overmodification of the procollagen molecules starts at or near the mutation site and proceeds in an amino-terminal direction. For R86-6, a defect in the middle of the triple helix, amino-terminal to CB10 was postulated, whereas for the other patients a defect within or carboxyl-terminal to CB10 was most likely (fig 2C). SDS-PAGE of pepsin extracted collagen from the patient cartilage also showed slowly migrating, overmodified α1(II) chains in most cases compared with the control (fig 2B). The pepsin extract of R85-109 showed a retarded β component not present in the control extract. The band disappeared on reduction with dithiothreitol (not shown) indicating that it was a disulphide bonded β dimer. Type I collagen was also present in many samples as seen from the CB peptide patterns and from the characteristic pattern of three β dimer bands evident in the pepsin digests. However, type I collagen can be present in dissected samples of normal human neonatal cartilage and the amount can

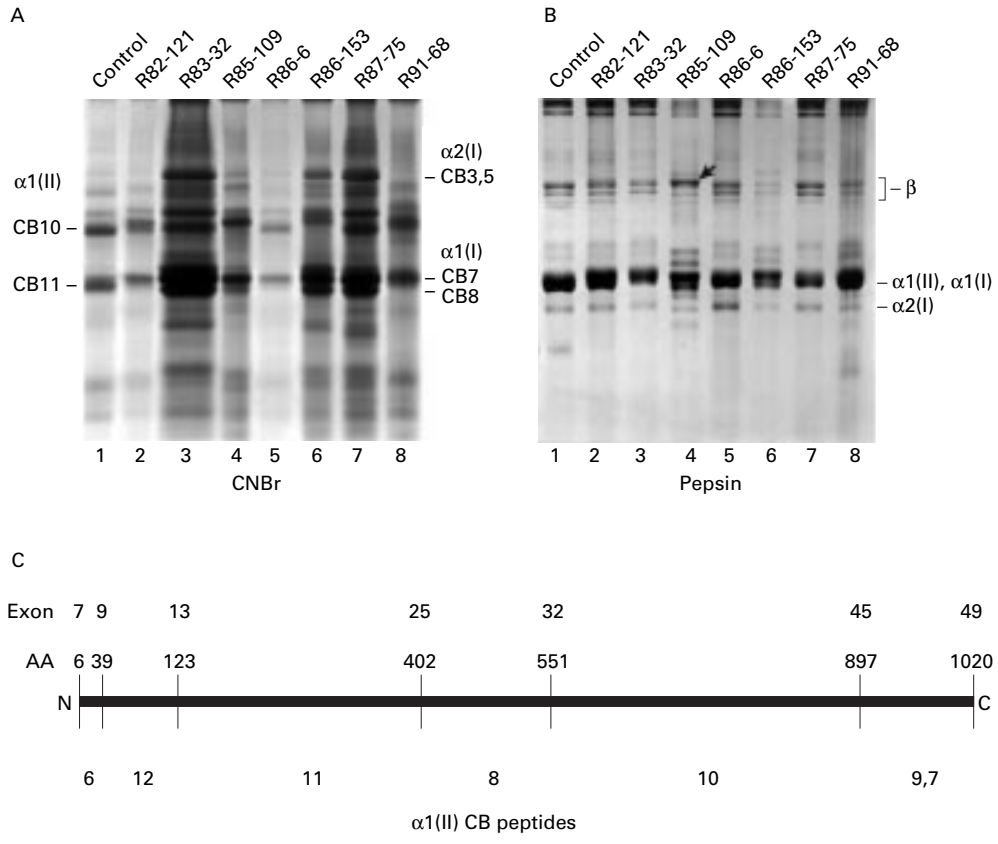

Figure 2 (A) SDS-polyacrylamide gel electrophoresis of cyanogen bromide (CB) peptides extracted from cartilage of each patient and a control (4 day old neonate). The position of the CB peptides derived from control tissue is shown on the left side for the α1(II) collagen chains and on the right side for the α1(I) and α2(I) collagen chains. The major α1(II) CB peptides (CB10 and CB11) derived from patient's tissue exhibit delayed mobility compared to the control. Based on the intensity of the α2(I) CB3-5 peptides, type I collagen seems to be most abundant in patients R83-32, R86-153, and R87-75. (B) SDS-polyacrylamide gel electrophoresis of pepsin extracted collagen from cartilage of each patient and a 4 day old neonate (control) shows slowly migrating, overmodified α1(II) chains in most patients compared with the control. The pepsin extract of R85-109 shows a retarded β component (arrow) not present in control extract. The position of normal α1(II) chains, α1(I) chains, α2(I) chains, and β dimers is indicated on the right side. (C) Cyanogen bromide peptide map of proα1(II) collagen. The corresponding exons and positions of methionine residues are shown at the top.

vary between adjacent samples from the same person. Based on the intensity of the α2(I) CB3-5 peptides, type I collagen seemed to be most abundant in patients R83-32, R86-153, and R87-75 (fig 2A).

Since in the majority of the cases a defect was postulated to reside within or carboxyl-terminal to α1(II) CB10, the region corresponding to exons 32-49 of the *COL2A1* gene was first targeted for mutation analysis (fig 2C). PCR generated genomic DNA fragments for exons 32-49 were examined by SSCP analysis. Primers were designed to include the intron-exon boundaries in each exon amplification. SSCP analysis showed an abnormal pattern for fragments containing exon 46 in patients R85-109 and R86-153 (fig 3), and for fragments containing exon 35 in patient R91-68 (fig 4). Direct sequence analysis of the PCR products of exon 46 showed the mutation in each of the two patients (fig 3). Patient R85-109 was heterozygous for a G to T transversion at the first nucleotide of exon 46. This change implied substitution of glycine[910] by cysteine and abolished a *Bst*NI restriction endonuclease site. Patient R86-153 was heterozygous for a G to A transition, predicted to result in substitution of glycine[943] by serine. The mutation abolished a cleavage site for the restriction endonuclease *Msp*I. Cleavage of the amplified DNA fragments with the corresponding restriction endonucleases was used to confirm that both patients were heterozygous for the mutation (data not shown). DNA sequence analysis of clones containing exon 35 showed a 4 bp deletion at the donor splice site of intron 35 in patient R91-68 (fig 4). Neither cultured cells nor frozen cartilage with which to test the prediction of a splicing mutation were available.

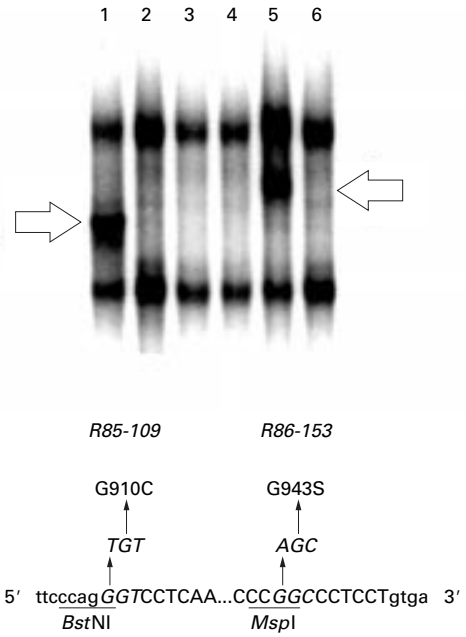

Figure 3 Identification of the COL2A1 mutation in patients R85-109 and R86-153. (Top) SSCP analysis of amplified genomic DNA fragments containing exon 46. The arrows indicate the abnormally migrating fragments from R86-153 (lane 1) and R85-109 (lane 5) compared to unrelated controls (lanes 2-4, 6). (Bottom) Partial nucleotide sequence of exon 46 (upper case) and intron boundaries (lower case) containing both point mutations. For R85-109, the G to T transversion predicts substitution of cysteine for glycine at residue 910. For R86-153, the G to A transition implies substitution of serine for glycine at residue 943. The restriction endonuclease cleavage sites disrupted by the mutation are underlined.

For identification of the underlying defect in the four other patients, the same region (exons 32-49) was first re-evaluated by heteroduplex analysis and thereafter this analysis was extended to the other part of the triple helical domain of the proα1(II) chain, corresponding to exons 6-31. Only for patient R83-32 did polyacrylamide gel electrophoresis of PCR generate products containing exon 33 showing both normal and more slowly migrating fragments in a position which suggested interallelic heteroduplexes. DNA sequence analysis of clones containing exon 33 showed a G to C transversion, implying substitution of glycine[571] by alanine (fig 5). The mutation did not affect a cleavage site of any known restriction endonuclease. Restudy of the earlier performed SSCP analysis of amplified DNA fragments containing exon 33 in patient R83-32 did not show any abnormalities. Taking into account the known lack of 100% sensitivity of SSCP and heteroduplex analysis in mutation detection, direct sequence analysis of PCR amplified genomic DNA fragments spanning the entire triple helical domain was performed in the remaining three patients. This study showed that patient R86-006 was heterozygous for a G to A transversion, predicted to result in substitution of glycine[313] by serine and that patient R87-75 was heterozygous for a G to C transition, predicted to result in substitution of glycine[517] by valine. These mutations did not create or destroy a restriction endonuclease cleavage site. No sequence changes in the triple helical domain were found in patient R82-121. However, in this patient, a C to A transversion at the second nucleotide of codon 1191 was identified. This single nucleotide change implies substitution of threonine[1191] by asparagine (T1191N) in the carboxy-propeptide of the proα1(II) collagen chain.

Discussion

Identification of the molecular defect in patients with type II collagen disorders is usually a challenge because of the relatively large size and complexity of the *COL2A1* gene and its main expression in cartilage. Cartilage is not easily accessible, relatively acellular, and therefore a poor source of mRNA for cDNA synthesis and analysis. Furthermore, sufficient amounts of mRNA can often not be obtained from cultured skin fibroblasts or lymphocytes owing to low basal transcription of the gene. Therefore, mutation analysis of the *COL2A1* gene is often performed in the genomic DNA in a "head to tail" fashion, which is time consuming and laborious. In this study, we used the opportunity of having cartilage tissue for exploring the genetic defect in the *COL2A1* gene in seven patients with a lethal type II

collagen disorder. Those regions of the *COL2A1* gene in which a mutation was likely to reside, based on the overmodification pattern of the type II collagen CB peptides, were screened first with SSCP and heteroduplex analysis. Using these mutation detection methods, the underlying defect was found in four of seven patients. The mutation in two other patients (R86-6 and R87-75) could only be identified with direct sequence analysis of PCR amplified genomic DNA fragments.

The clinical, radiographic, and morphological features of each patient were consistent with the diagnosis of a lethal type II collagen disorder (table 1, fig 1). Significant overlap in phenotype was noticed (table 1). With the exception of patient R91-68, who represents severe SEDC rather than achondrogenesis II-hypochondrogenesis because of the complete ossification of the spine and less severe shortening of the long bones, all cases fell into the achondrogenesis II-hypochondrogenesis spectrum. In comparison to the other cases, the phenotype of patient R82-121 was remarkable, radiographically by more metaphyseal involvement and clinically by absence of respiratory insufficiency in the postnatal period. The baby died at 13 months following seven months' hospitalisation for severe, bilateral bronchopneumonia.

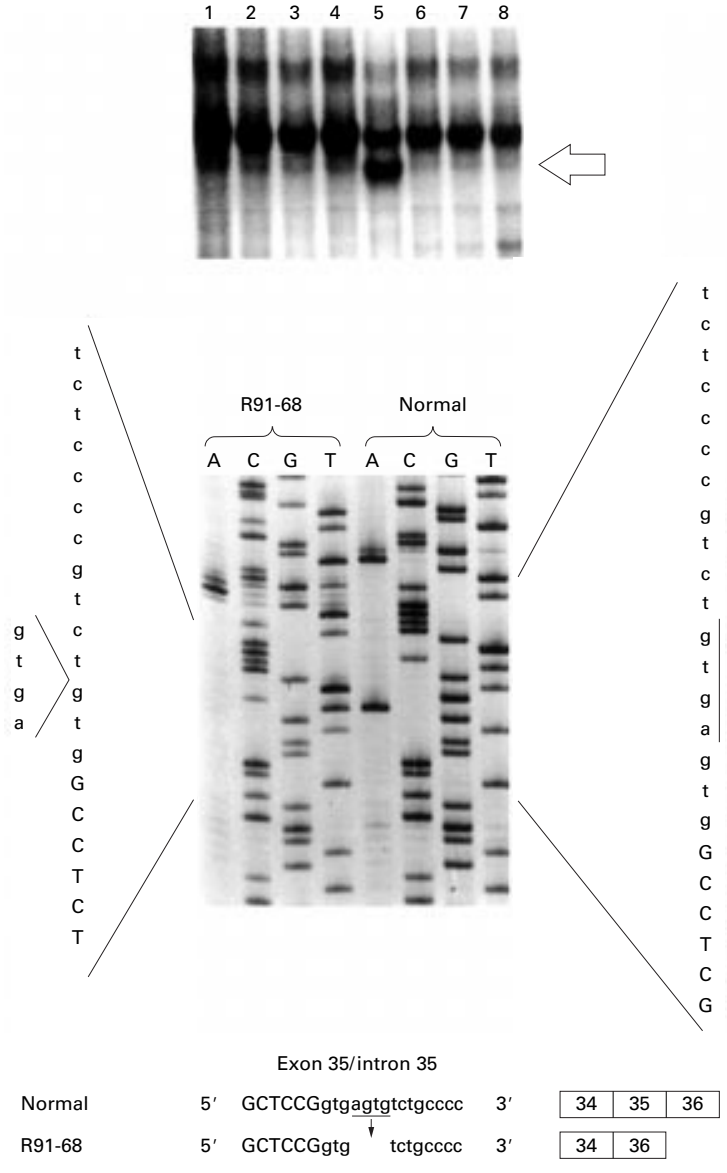

Figure 4 Identification of the COL2A1 mutation in patient R91-68. (Top) SSCP analysis of amplified genomic DNA fragments containing exon 35. The arrow indicates the abnormally migrating fragment from patient R91-68 (lane 5) compared to unrelated controls (lanes 1-4, 6-8). (Middle) Identification of the 4 bp deletion at the splice donor of intron 35. The sequence was determined after cloning of PCR products, containing exon 35 (upper case) and intron (lower case) boundaries, from the mutant and normal alleles. Five of eight clones showed the 4 bp deletion at the splice donor of intron 35. (Bottom) The IVS35+4delAGTG mutation implies skipping of exon 35.

The results of the biochemical analysis showed post-translationally overmodified type II collagen in significant amounts in each patient's cartilage. This is consistent with previous findings that mutant α1(II) chains can be secreted and incorporated into the cartilage matrix in patients with achondrogenesis II-hypochondrogenesis or SEDC.[11 15 18 24] However, in one case of hypochondrogenesis with a G913C mutation, cultured chondrocytes failed to secrete α1(II) chains in the medium.[16] Type I collagen was also present in the cartilage tissue of each patient and appeared to be increased in R83-32, R86-153, and R87-53, who are the most severely affected (table 1). This might suggest a relationship between the amount of type I collagen in the cartilage and the severity of the phenotype. Probably in response to insufficient amounts of type II collagen in the extracellular matrix, the chondrocytes produce and secrete more type I collagen as a substitute. One study has shown that the genes for type I collagen are expressed in chondrocytes of a patient with hypochondrogenesis.[12] This would suggest that hypervascularity of the cartilage is not the main explanation for the presence of type I collagen in cartilage of these patients.

It has been postulated that mutations in the triple helical domain of procollagen α chains delay the folding of the protein into the triple helical conformation, which causes post-translational overmodification of the protein amino-terminal to the mutation site.[25] The availability of cartilage from each case allowed us to screen for an overmodification pattern of the CB peptides and so direct the molecular analysis of the *COL2A1* gene based on the biochemical findings. Because of the slower mobility of peptide CB10 in all but one of the seven cases, it was decided to explore first the domain of *COL2A1* encoding exons 32-49 (fig 2C).

Analysing these exons using SSCP and heteroduplex analysis, three patients (R86-153, R85-109, and R83-32) were found to be heterozygous for a missense mutation that predicted substitution of glycine in the triple helical domain of the α1(II) chain. Their phenotype resided at the more severe end of the achondrogenesis II-hypochondrogenesis and SEDC spectrum. In patient R85-109, with a G910C mutation, the mutant α1(II) chains were clearly expressed in the tissue as seen from the presence of a reducible β dimer in the pepsin extracted collagen (fig 2B). The peptide patterns for R85-109 and R86-153 were remarkably similar for both pepsin and CB

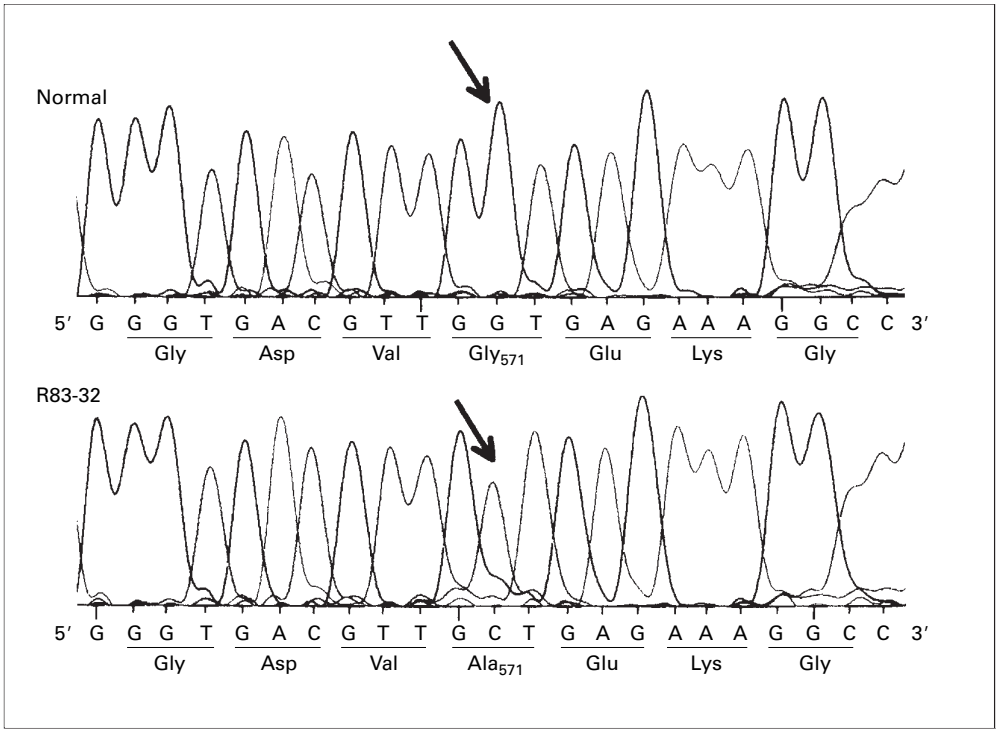

Figure 5 Identification of the guanine to cytosine transversion in exon 33 of the COL2A1 gene for patient R83-32. Partial nucleotide sequence of a clone containing the normal and mutant allele is shown. Three of 10 clones showed the nucleotide change.

digests of tissue and it is notable that the causative single amino acid substitutions (G910C and G943S) were located near the carboxyl-terminus of the triple helix. Interestingly, the G943S mutation, identified in patient R86-153, has been previously identified in another, unrelated case.[9 25 26] The recurrence of the mutation could be explained by its occurrence in the context of a CpG dinucleotide. The clinical, radiographic, and morphological features of both cases were almost identical illustrating the reproducible pathogenetic effects of this mutation (table 2).[9 25 26]

Analysis of the remaining exons 6-31 of the triple helical domain of the proα1(II) collagen chain by heteroduplex analysis did not identify any abnormalities in patients R82-121, R86-6, and R87-75. However, direct sequence analysis of PCR amplified genomic DNA fragments from this region showed a G313S mutation in R86-6 and a G517V mutation in R87-75. As predicted by the overmodification pattern of the CB peptides, the mutation in R86-6 did indeed reside amino-terminal to CB10. Comparable to patients R85-109 and R86-153, the peptide patterns for patients R83-32 and R87-75 were also quite similar. Again, the mutations (G517V and G571A) were located in the same region around the amino-terminal border of CB10. Direct sequence analysis of PCR generated genomic DNA fragments for exons 6-49, spanning the entire triple helical domain, did not show any abnormalities in patient R82-121, despite the observed doublet for CB10. The significance of the single nucleotide change, located in the carboxy-

Table 2 Clinical, radiographic, and morphological features of two patients with the G943S mutation in the COL2A1 gene

	R86-153	*Godfrey et al,[25 26] Vissing et al[9]*
Clinical		
Sex	Female	Female
Birth length (gestational age)	29 cm (35 wk)	28 cm (32–33 wk)
Short limbs	Yes	Yes
Small thorax	Yes	Yes
Cleft palate	Yes	Yes
Respiratory insufficiency	Yes	Yes
Age at death	6 h	12 h
Radiographic		
Long bones	Marked shortening with metaphyseal irregularities	Marked shortening with metaphyseal irregularities
Pelvis	Small and flared iliac wings	Small and flared iliac wings
Spine	Only reasonable ossification in the thoracolumbar spine	Only reasonable ossification in the thoracic spine
Thorax	Small with short ribs	Small with short ribs
Ossification of pubic bones	No	No
Ossification of ischia	Yes	No
Ossification of talus and calcaneus	No	No
Morphological		
Resting cartilage	Very hypervascular	Increase in vascularity
Chondrocytes	Inclusion bodies	Dilated RER (EM)
Growth plate	Very irregular with hypertrophic cells extending into the metaphysis	Irregular with hypertrophic cells extending into the metaphysis

propeptide and predicted to result in substitution of threonine[1191] by asparagine (T1191N), remains unclear. Mutations in the carboxy-propeptide of the proα1(II) collagen chain have not been reported before in patients with a lethal type II collagen disorder. However, single amino acid substitutions in the carboxy-propeptide of the proα1(I) collagen chain have been described in osteogenesis imperfecta type II, indicating that mutations in this region can have a dramatic effect on the function of a fibrillar collagen molecule.[27]

In patient R91-68, a 4 bp deletion was found at the donor splice site of intron 35. Although the possibility of exon skipping could not be investigated at the cDNA level, the overmodification of the type II collagen CB peptides (fig 2A) indicates that an abnormal α1(II) chain was expressed and incorporated into the matrix. We concluded that the mutation probably resulted in skipping of exon 35 with shortened chains being incorporated into the tissue fabric. Consistent with the published findings of others, the phenotype of this case was considerably milder than that of the three cases with a glycine substitution.[2] It is notable that most cases of Kniest dysplasia, which is usually a non-lethal type II collagen disorder, are caused by *COL2A1* exon skipping mutations.[28–31] These deletions tend to be concentrated in the amino-terminal half of the molecule. Since the type II procollagen molecule consists of three identical α1(II) chains, heterozygosity for a missense mutation in the *COL2A1* gene should result in seven/eight procollagen trimers containing one, two, or three mutated α1(II) chains. A mutation in one *COL2A1* allele affecting mRNA splicing and resulting in an in frame deletion of several amino acids will give rise to six/eight procollagen trimers containing a mixture of normal and deletion containing chains. Two/eight of the molecules will contain either three normal or three shortened α1(II) chains. This could explain to some extent the difference in phenotypic outcome between glycine substitutions and in frame deletions as a result of aberrant splicing. However, not all glycine substitutions in the α1(II) chain result in a severe phenotype as they can also cause milder cases of SEDC and phenotypically milder spondyloepimetaphyseal dysplasia Strudwick type.[2 32] The phenotype probably depends not only on the mutation type (for example, exon deletion or glycine substitution), but also on the local domain of the triple helix affected.

We thank clinical colleagues M Bridge, N Chaney, A Donnenfield, D Huff, M Lipson, H Stern, and J Waterson for referring the patients described in this study. Excellent organisational assistance was provided by M Priore and S Levin of the International Skeletal Dysplasia Registry. This work was funded by NIH grants AR37318 (to DRE) and HD22657 (to DLR, DHC, and RSL) and by a Belgian National Fund for Scientific Research grant G.0013.97 (to GRM, LN, and ADP). The authors declare that the experiments comply with the current laws of the country in which the experiments were performed and that the study was approved by the Ethical Committee.

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Letters to the Editor

J Med Genet 2000;**37**:785–787

The mutation spectrum in Holt-Oram syndrome

EDITOR—Holt-Oram syndrome (HOS) is a developmental disorder characterised by malformations of the radial ray of the forelimb and by congenital heart disease.[1] The syndrome shows a marked variability in phenotype, with radial ray defects ranging from minor thumb abnormality through to severe reduction defect or phocomelia. The cardiac manifestations of HOS are similarly varied, and patients can present with a variety of structural heart abnormalities, atrial septal defects (ASDs) and ventricular septal defects (VSDs) being the most common, or conduction defects evident on ECG profiles. Previous studies have shown no correlation between the severity of a patient's cardiac and skeletal abnormalities.[2] Intrafamilial variation can be wide.

HOS shows autosomal dominant inheritance and mutations in the T box transcription factor gene (*TBX5*) have been shown previously to be responsible for this disorder.[3 4] There is also evidence for genetic heterogeneity.[5] The mechanism by which mutations in *TBX5* cause a dominant phenotype is not understood at present, and it is anticipated that knowledge of the type of mutations causing HOS may shed light on this. Knowledge of a large number of mutations and the relation of a person's genotype to phenotype is also useful for genetic counselling. In the face of a growing demand for a molecular diagnostic test for HOS, it is also helpful to have a quantitative estimate of the ability of current methods to detect mutations in *TBX5*.

Twenty five cases with a clinical diagnosis of Holt-Oram syndrome have been tested for this study, bringing to 47 the total number of cases studied by us. Minimal diagnostic criteria were as described previously[2]: bilateral radial ray defect, plus either cardiac abnormality or family history of cardiac abnormality. Cases were referred by a variety of clinicians and underwent full clinical assessment including *x* ray, electrocardiography (ECG), and echocardiography. Information regarding the clinical features of these patients came from the referring doctor. The patients represented both sporadic (8) and familial (17) cases of Holt-Oram syndrome.

Mutational analysis was carried out using SSCP methods initially, followed by fluorescence sequencing of exons showing a non-standard SSCP banding pattern. The methods used were described in Li *et al*.[3] Since that study, further analysis of the genomic structure of *TBX5* has recognised that the previously reported exon B is in fact two exons. Extensive resequencing of both genomic and cDNA forms of *TBX5* has also been undertaken, leading to revisions in the previously reported *TBX5* sequence (the new sequence has accession number AF221714). This sequence is the same across the coding region as that produced by others.[6] Of 17 new familial cases tested, eight showed linkage to chromosome 12 and the other families were too small to assess linkage. Table 1 shows that five mutations were identified in familial cases. Only one of these families was sufficiently large to show meaningful linkage to chromosome 12q markers. Of eight new sporadic cases studied, three have yielded mutations. Thus, in the 34 familial cases studied by us, eight mutations have been identified, and six mutations have been identified in 13 sporadic cases. The precise natures of all the new mutations identified are detailed in table 2.

Table 1 Cases studied

	This study	*All studies in our lab*
Familial cases studied	17	34
Sporadic cases studied	8	13
Mutations found in familial cases	5	8
Mutations found in sporadic cases	3	6

The clinical features of HOS in all 47 cases are consistent with the previously described phenotype[2] and show the wide spectrum of cardiac and skeletal abnormalities in this syndrome (see Bruneau *et al*[7] for details of the complexity of cardiac abnormalities in HOS patients). Most patients show at least one defect of cardiac septation (an atrial or ventricular septal defect, or atrioventricular block) and abnormalities of the thumb. Radial hypoplasias and aplasias are present in sporadic cases PpHs, H20s, H22s, and H16s and in the familial cases H6f and Ghf, although not in every affected member.

Twenty five non-translocation mutations have been reported, including those presented here. These mutations are of 19 distinct types, with six mutations being identical to previously described forms identified in unrelated subjects (this paper and Basson *et al*[6]). Of the 19 distinct non-translocation, disease causing mutations in *TBX5* currently known, five are truncations, five amino acid substitutions, three splice site changes, and six reading frame shifts.

We have observed a significant difference in the proportion of cases in which a mutation was detected in our group of sporadic cases as opposed to our group of familial cases. Forty six percent (6/13) of sporadic cases studied in our laboratory have yielded mutations by SSCP screening, whereas only 24% (8/34) of familial cases have done so.

The overall mutation detection rate of 30% may be low for a variety of reasons. An inadequate mutation detection method would explain these results, yet the system in use by us is a standard one used on a variety of projects, all of which produce detection rates nearer the theoretical level for these techniques (approximately 95%). The largest PCR product used in the present analysis is only 326 bp long, well within the size acceptable for this kind of analysis. Mutation detection is also fully repeatable in our hands.

There are four other possible explanations for this low mutation detection rate: (1) clinical misdiagnosis in our patients, (2) genetic heterogeneity of Holt-Oram syndrome,[5] (3) the presence of mutations in the untranslated and promoter regions of *TBX5*, which have not been tested in this analysis, and (4) deletion of whole exons of *TBX5*, which would not be recognised by SSCP.

Table 2 New mutations identified

Case	*Mutation type*	*Location*	*Mutation form*	*Result*
Nef	Substitution	Intron 4–5	5' splice site(T to C)	Splice site change
FIf	Substitution	Exon 5	nt1170(G to A)	Gly to Arg
FR9f	Substitution	Exon 8	nt1611(G to T)	Stop codon TAG
Jonf	Substitution	Exon 6	nt1233(G to T)	Stop codon TAA
Chas	Substitution	Exon 7	nt1420(G to T)	Ser to Ile
Bers	Deletion	Exon 8	nt1470(del T)	Reading frameshift
PpHs	Substitution	Exon 8	nt1500(C to T)	Stop codon TGA
GHf	Substitution	Exon 8	nt1500(C to T)	Stop codon TGA

Codes ending in f are familial cases, those with s are sporadic.

Table 3 Clinical phenotypes

Family	*Pedigree No*	*Mutation*	*Skeletal abnormality*	*Cardiac abnormality*
H8f	I.1	Exon 8 nt1500 C→T	Bilateral hypoplastic thumbs Syndactyly 1/2	AV block
	II.2		Right triphalangeal thumb Left absent thumb Bilateral radial hypoplasia	ASD
H12f	I.1	Exon 8 nt1500 C→T	Bilateral hypoplastic thumbs Syndactyly 1/2	AV block
	II.1		Bilateral hypoplastic thumbs Syndactyly 1/2	AV block
	II.3		Absent thumbs Bilateral radial hypoplasia	VSD
H22s		Exon 8 nt1500 C→T	Bilateral triphalangeal thumbs Bilateral radial hypoplasia	ASD
PpHs		Exon 8 nt1500 C→T	Bilateral absent thumbs Left radial aplasia Right radial hypoplasia	AV block
Ghf		Exon 8 nt1500 C→T	Bilateral absent thumbs Right radial aplasia Left radial hypoplasia	ASD

Other studies of HOS have not published mutation detection rates. We can only speculate as to why the rate of mutation detection in sporadic cases should be higher than that in familial cases (particularly in those familial cases whose disease loci are known to be present on 12q). An obvious explanation for such a discrepancy is ascertainment bias. A sporadic case must have both detectable heart and limb symptoms to be diagnosed as having HOS, whereas a family need only show both these effects across the pedigree (rather than in one subject) along with an autosomal dominant inheritance pattern.

A group of five of the 25 mutations published so far are the same, a C to T transition at position 1500, generating a stop codon. This mutation is seen in both sporadic and familial HOS cases among patients who do not share common alleles at microsatellite loci closely linked to *TBX5*. This site is likely to represent, therefore, a true "mutation hotspot". The residue is part of a CG duplet, and therefore is likely to be methylated, with a high frequency of mutation to a thymine residue. Studies across a variety of human diseases have found distributions of mutations skewed towards the mutation of CpG sites. The retinoblastoma gene, *RB1*, shows a distribution of mutations severely skewed towards a few C→T transition hotspots (see the *RB1* mutation database[8] for details).

Basson *et al*[6] argue, based on a collection of HOS families of varying symptom severity, that there is a relationship between the phenotype of patients and their specific mutation. It is proposed that truncation mutations in *TBX5* lead to both limb and cardiac malformation, whereas single amino acid changes have different effects depending on their position in the T box. The set of HOS cases we have examined contains no large families of the type studied in this earlier analysis.

The five known cases (two familial and three sporadic) with the same mutation, a C→T transition at nucleotide 1500 in exon 8, present an opportunity to examine more closely the possibility of mutation specific genotype-phenotype correlation in cases with a truncation in *TBX5*. The clinical phenotypes of these cases are presented in table 3. Cardiac defects presented include isolated ASD and isolated VSD but no case with this mutation has a complex cardiac lesion. Syndactyly of the thumb and first finger is common within this group. There is much variation in symptom severity and the group as a whole shows no bias towards a particular severity of either cardiac or skeletal symptoms, in agreement with Basson *et al*.[6]

Along with the truncation forms already described, we have identified two new mutations which each result in a single amino acid substitution. The change in family Fif inserts an arginine in place of a glycine at position 169, which is conserved across T box genes such as *Xbra* (*Xenopus*), *T* (mouse), *TbxT* (chick), and *omb* (*Drosophila*).[9] This introduces a strongly basic residue into a non-polar region in the DNA binding T domain. Family Fif comprises eight affected subjects in two generations, who show significant cardiac involvement compared with very mild skeletal findings. One case has a complex lesion, ASD with VSD, and another case has pulmonary stenosis, a conotruncal malformation which is not typical in HOS. This is consistent with a role for *TBX5* which extends beyond cardiac septation.[10] Only one subject in family Fif has a demonstrable limb abnormality and this is stiffening of the thumbs. The phenotype of this family is therefore consistent with the suggestion that substitution mutations produce predominantly limb or predominantly cardiac features, depending upon their location within *TBX5*.[6]

The change of a serine to an isoleucine in patient Chas is outside the T domain of the protein and its biochemical effects are not known. The phenotype of this patient included a spinal scoliosis, which has not previously been observed in Holt-Oram syndrome, together with bilateral hypoplastic thumbs, syndactyly, and a ventricular septal defect. Currently available details on expression of *TBX5* during development of the mouse and chick give no evidence of expression in the developing spine which would account for such a phenotype being the result of mutation of *TBX5*.[12 13]

In summary, these new data expand our knowledge of the spectrum of mutations that cause Holt-Oram syndrome and also raise interesting questions about the genetic heterogeneity of this disease and its mutations. Clearly there is a need to improve the frequency of mutation detection in HOS and current analysis of untranslated and promoter regions, and screens for whole exon deletions should prove useful. A diagnostic service for *TBX5* mutations is being set up.[13]

The first three authors contributed equally to this work. This work was funded by the British Heart Foundation. Ethical approval for the study was obtained from the Nottingham City Hospital Research Ethics Committee.

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J Med Genet 2000;**37**:787–789

Hemiplegic cerebral palsy and the factor V Leiden mutation

EDITOR—Two mutations have been identified in recent years that predispose a heterozygous carrier to venous thrombosis. One is a mutation localised to the factor V gene, Arg 506 to Gln (factor V Leiden mutation, *FVL*), which has been shown to be the most common cause of familial thrombosis[1 2] through resistance to activated protein C (APC), which is an inhibitor of activated factors V and VIII.[3] The second is in the gene for the coagulation factor prothrombin (factor II), the G20210A mutation.[4]

There are two published estimates of prevalence of the *FVL* mutation in the Australian population, the first in a study of recurrent miscarriage,[5] where 3.5% of the controls had *FVL*. The other is a recent study of blood donors where 3.6% were found to be heterozygous.[6]

Fewer studies have been done on the prothrombin (*PT*) mutation which is not as prevalent, thought to occur in approximately 2% of healthy, normal controls.[1 4]

Of particular relevance to this study are the findings of children with ischaemic strokes[7–9] or thromboembolism,[10 11] who have been reported as having a high prevalence of the *FVL* mutation. A more recent study[12] suggests that neither *FVL* nor *PT* is a risk factor for childhood stroke and that "a large prospective multicentre study is required" to investigate this further. There has also been a report of three babies with hemiplegic cerebral palsy (CP) who were heterozygous for the *FVL* mutation.[13] In the three cases, there was a suggestion that placental infarction/thrombosis or neonatal stroke may have occurred and resulted in the hemiplegia. Other relevant studies have also been done, suggesting that placental infarction and late fetal loss[14 15] may occur more frequently in women who are carriers of *FVL*.

Cerebral palsy (CP) is a common physical disability in childhood occurring in 2.0-2.5 per 1000 live births. In a significant proportion, no cause can be established. Current research suggests that between 6 and 8% of cases are the result of birth asphyxia[16–18] and postnatal problems account for a further 10%.[19 20] Prenatal events are now thought to be responsible for approximately 75% of all cases of cerebral palsy, although it is usually impossible to determine the nature and exact timing of the damaging event. Given the lifetime impact that cerebral palsy has on the person and the family, a search for further causative factors is essential, as new information regarding aetiology may provide the first step to instituting preventive strategies.

In Victoria, there is a CP Register containing demographic and clinical details of 2093 babies born between 1970 and 1995. A letter was sent to the parents of 69 selected patients requesting permission to access the blood spot collected routinely at birth (Guthrie card) for newborn screening purposes. These 69 were chosen because they were private patients of one of the authors (DR) and could therefore be approached directly. The identifiable blood spots are held at The Murdoch Children's Research Institute and are not available for research or other purposes unless permission is obtained from the parents. All had a diagnosis of cerebral palsy with a specific motor diagnosis of spastic hemiplegia.

We estimated that a sample size of 50 would detect a significant increase in prevalence of the *FVL* mutation from a background prevalence of 4% to one of 15%, with a power of 80% and alpha of 0.05.

Two 3 mm^2 sections of Guthrie blood spots were cut out and placed in 50 µl of PCR buffer. They were initially incubated at 95°C for 30 minutes, then kept at 37°C overnight, after which they were pulse centrifuged. The supernatant was then transferred to a sterile 500 µl tube and kept at 4°C until the day of analysis.

DNA encompassing the *FVL* codon 506 mutation was amplified by PCR. The resulting product was checked on a 1% agarose gel stained with ethidium bromide before restriction enzyme digestion. DNA was digested overnight at 37°C using the enzyme *Mnl*I. Fragments were then

Table 1 Radiological findings

Radiology	*Normal*	*Evidence of ischaemic lesion*	*Hydrocephalus*	*PFVL*	*IVH*	*Non-specific lesion*	*Total*
Ultrasound	3	—	3	1	2	1	10
CT scan	1	10 (2)	2	1	—	3 (2)	17
MRI	—	4 (1)	1	1	—	—	6
Not available/not done							22

Number in brackets refers to number of patients with mutation.
PFVL: periventricular leucomalacia.
IVH: intraventricular haemorrhage.

separated on a 6% polyacrylamide gel and subsequently stained with ethidium bromide. Both normal and heterozygote samples were run as controls in each assay.

The same methodology was applied for detection of the prothrombin 20 210 polymorphism, but with *Hin*dIII restriction enzyme used, and with digest fragments being separated on a 3% agarose gel.

Power calculations were done in SPSS for Windows, version 8.0, SAMPLE POWER, and the binomial comparison of the sample prevalence with the estimated population prevalence was also done in SPSS version 8.0.

Of the 69 parents contacted, 58 responded (84%). All gave permission for use of the blood spot, except for one where the child was in the care of foster parents who did not feel that it was appropriate to give consent. Two Guthrie cards were unable to be found leaving a sample of 55 cases.

Of the 55 subjects recruited, 54 and 52 could be amplified successfully for the *FVL* and *PT* mutations, respectively. Of the 54 samples screened for *FVL*, one was found to be homozygous while three were found to be heterozygous. Therefore, the frequency of cases with at least one mutation of *FVL* was 7.4% which does not differ significantly from the average Australian population heterozygote prevalence of 3.6% (binomial test, one tailed p value=0.13). Only one of the 52 samples analysed for *PT* was found to be heterozygous (1.9%).

The clinical features of the five subjects with positive findings are as follows.

(1) Homozygous for *FVL* mutation. This child was born by caesarean section at 32 weeks following a pregnancy complicated by pre-eclamptic toxaemia. His birth weight was 1815 g. Hemiplegic cerebral palsy was diagnosed at the age of 4 months. A CT brain scan showed appearances consistent with infarction. There was no family history of note but testing showed that his mother is a heterozygote for *FVL*.

(2) Heterozygous for *FVL* mutation. These three children were all normal birth weight, term infants. One child required some resuscitation at birth and meconium had been passed before delivery. Her MRI showed middle and anterior cerebral atrophy infarction. The family history was unremarkable apart from the unexpected death of the paternal grandfather, from a myocardial infarct, at 50 years of age. The other two children had uneventful perinatal periods and hemiplegia became apparent during the first year of life. Both had CT scans which showed loss of cerebral hemisphere substance in both grey and white matter with enlarged ventricles, not necessarily typical of major vessel infarction. While one child had a negative family history, the other child's grandmother had sustained a deep venous thrombosis following a hysterectomy at the age of 50 years.

(3) Heterozygous for the prothrombin mutation. This child had an uneventful perinatal period. A diagnosis of hemiplegic cerebral palsy was made at the age of 8 months. A CT brain scan showed signs of infarction with almost complete absence of the left frontal temporal and parietal lobes, with appearances typical of congenital occlusion of the left anterior and middle cerebral arteries and subsequent porencephaly. There was no family history of note.

All available radiological findings were assessed and are summarised in table 1.

If only cases with radiological evidence of ischaemia (14 cases) are used in the calculations, the frequency of either of the thrombophilia mutations (three cases) is 21%. This is significantly higher than the population prevalence (binomial test, one tailed p value=0.013).

In summary, evidence of the influence of the *FVL* mutation on thrombosis led us to consider whether a significant attributable factor to CP may be the presence of this mutation, which could cause an adverse vascular event such as placental infarcts or stroke in utero or early in postnatal life. To test this hypothesis, we obtained the frequency of the mutation in blood spots from a sample of children, all of whom had hemiplegic CP. This group was chosen because it was thought to be relatively homogeneous and it seemed that if a vascular event were causative, then spastic hemiplegia could be the most likely outcome. However, an examination of their radiological findings, after the mutation detection had been undertaken, showed quite marked heterogeneity. It was among the 14 cases with a known ischaemic event that three mutations were found, giving a significantly higher prevalence of 21%.

From these results, we conclude that there may be a relationship between carrier status for mutations predisposing an infant to thrombophilia and cerebral vascular events in utero (or neonatally) that lead to CP. To confirm such a relationship it will be necessary to study a larger sample of CP patients with a vascular basis, such as venous or arterial occlusion, and compare mutation frequencies with children with CP who do not have a vascular basis for CP.

In addition, investigation of maternal mutation status would be useful, particularly because the frequency of maternal pre-eclampsia in this sample was higher than expected (data not shown), 11% compared with the reported population frequency of approximately 5%[21 22] and it is not known whether these mothers had *FVL* or *PT* mutations.

We report our findings at this stage to illustrate how DNA testing for common polymorphisms, which are of unknown importance in the general population, may be of importance to subjects within subgroups of the population. Primarily this would be for clinical management, but perhaps when potentially interactive aetiological factors, such as smoking in pregnancy, are better defined, this genetic information could be used for preventive measures.

We would like to thank Nick Tzanakos in the newborn screening laboratory of the Victorian Clinical Genetics Service, Murdoch Institute, for his technical assistance and Carole Webley for help in preparation of the manuscript.

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J Med Genet 2000;**37**:789–791

Analysis of the human tumour necrosis factor-alpha (TNFα) gene promoter polymorphisms in children with bone cancer

EDITOR—TNFα (tumour necrosis factor-alpha) is a cytokine produced by macrophages and monocytes with a wide range of activities, and polymorphisms within this gene have been postulated to contribute to MHC associations with autoimmune and infectious diseases.[1] The role of TNFα in cancer is a controversial matter, because while it plays a key role in the "in vitro" killing of tumour cells by macrophages and lymphocytes, it has also been found in high concentrations in patients with cancer, suggesting that it may be an endogenous tumour promoter "in vivo".[2 3] Different results with several tumour types show that TNFα may have both tumour necrotic and tumour promoting activities.

Recently, several genetic polymorphisms have been described in the human *TNFα* promoter.[4–6] Among them, the rare allele at position −308 (TNF308.2) has been proven to be part of a complex haplotype that is involved in higher TNFα levels and has been related to poor prognosis in several diseases.[7] The existence of different *TNFα* alleles, related to different levels of TNFα, raises the possibility that tumour development is somewhat related to the genetic propensity of the person to produce higher levels of TNFα and, therefore, with the presence of genetic variants in this gene. In fact, an increase in the TNF308.1/TNF308.2 genotype has been reported in different tumour types, with a significantly increased frequency of the TNF308.2 allele in patients with malignant tumours.[8] Wilson *et al*[7] have shown that the polymorphism at −308 has a significant effect on the transcriptional activity of the human *TNFα* gene, either because the interaction of the transcription enhancers is increased owing to the different DNA conformations, or because the TNF2 variant is the target for novel binding proteins.

The G to A transition at position –238 (TNF238.2) is also suspected to influence the expression of TNFα, and although its clinical and functional consequences are not clear so far,[9] it has been associated with development and prognosis of different diseases as well.[10 11]

The effects of TNFα on osteoblast differentiation and proliferation are complex but it is generally assumed that it inhibits bone formation and stimulates bone resorption.[12] The activity of TNFα in osteosarcoma, Ewing's sarcoma, and primary human osteoblast cultures has been widely studied showing that it has an antiproliferative and cytotoxic role which usually depends on the type of cell line under analysis.[13 14]

Synergistic cytotoxicity has been described between TNFα and other cytokines, most frequently IFNγ (interferon gamma) and also with certain drugs that are commonly used in the treatment of osteosarcoma and Ewing's sarcoma like the topoisomerase II inhibitors.[13] The data available indicate that TNFα and agents that stimulate its production by host macrophages may have a role in the treatment of osteosarcoma and Ewing's sarcoma.

Based on the role of TNFα in bone biology and the growing evidence of the relationship existing between TNFα genetics and cancer, we tried to test the hypothesis of whether genetic polymorphisms of the *TNFα* promoter contribute to the pathogenesis or prognosis of paediatric bone tumours.

DNA was extracted following standard procedures[15] from peripheral blood lymphocytes of 110 paediatric patients (52 females, 58 males) with bone tumours (63 osteosarcomas and 47 Ewing's sarcomas) and 111 healthy children (53 females, 58 males). All the subjects included in the analysis were white, most of them from the region of Navarre (Spain), and the age distribution was very similar in the tumour and control groups (mean (SD): osteosarcoma 13.5 years (SD 3.3), Ewing's sarcoma 12 years (SD 3.7), controls 11.1 years (SD 5.1)).

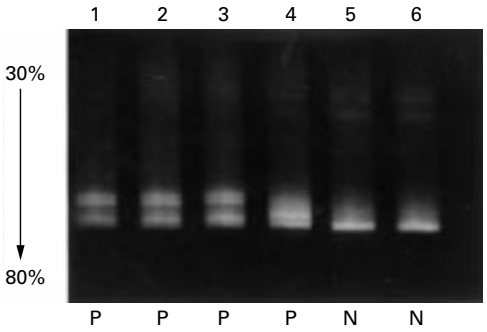

Figure 1 DGGE gel showing polymorphic and normal band patterns. Lanes 1 to 3: genotype normal/−376−238. Lane 4: genotype normal/−308. Lanes 5 and 6: normal, non-polymorphic patterns.

Table 1 Genotypes for the TNFα gene promoter in the control subjects and the bone tumour groups

	*Controls (n=111)**		*Osteosarcoma (n=63)*		*ES† (n=47)*	
Genotypes	*No*	*%*	*No*	*%*	*No*	*%*
Normal/normal	67	60.4	47	74.6	35	74.5
Normal/−308	26	23.4	13	20.6	6	12.8
Normal/−238−376	11	9.9	2	3.2	4	8.5
−308/−238−376	2	1.8	0	0	0	0
Normal/−238	5	4.5	0	0	2	4.3
−308/−308	0	0	1	1.6	0	0

*Number of subjects analysed.
†ES, Ewing's sarcoma.

The human *TNFα* promoter region between nucleotides −398 and −103 was analysed by PCR-DGGE (polymerase chain reaction coupled to denaturing gradient gel electrophoresis) as previously published[16 17] (fig 1).

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 9.0). The chi-square contingency test, with Yates's modification for small numbers if needed, was used to test for a significant association between disease and *TNFα* genotypes or haplotypes. Crude odds ratios (ORs) were calculated and given with 95% confidence intervals (CI). To test for association between clinical parameters and genotypes or haplotypes, *t* tests or one way analysis of variance (ANOVA) were performed. Statistical significance was assumed if $p \leq 0.05$ and highly statistical difference if $p < 0.01$; differences between variables with $p > 0.05$ were considered not statistically different. Osteosarcomas and Ewing's sarcomas were treated independently in the statistical analyses given that they are different clinical entities, both in genetic background and in the clinical and histological aspects.

We have analysed the distribution of the *TNFα* gene promoter polymorphisms at positions –376, −308, −238, and –163 in 110 bone sarcoma paediatric patients and in 111 paired healthy children, to search for the putative association between the *TNFα* gene and tumour development or prognosis.

The overall distribution of *TNFα* polymorphic alleles in bone cancer paediatric patients and control subjects is shown in table 1. As in previous reports, polymorphisms at −376 and −238 were found to be in linkage disequilibrium.[5] To date, there are no consistent data on the frequency of the polymorphic alleles at –376 and –163 in white populations, and larger series should be screened to establish the frequencies for these alleles in both normal and disease related populations.

The frequencies of the alleles at −308 and –238 in the Spanish population (0.87 and 0.92, respectively, deduced from table 2) were found to be very similar to those described for European white populations.[10 18]

Tables 2 and 3 show the allele and genotype frequencies of the genetic variants of the *TNFα* gene promoter at –308 and –238 in both cancer patients and healthy subjects. The frequency of the TNF238.2 allele (adenine at position –238) and TNF238.1/TNF238.2 heterozygote genotype was significantly lower in the osteosarcoma group, but not among Ewing's sarcomas. We did not find any outstanding difference in the distribution of the TNF308.2 allele (adenine at position –308) between bone sarcomas and controls.

No relationship was found between the presence of genotypes for the *TNFα* gene promoter and any of the clinical parameters tested: tumour stage, tumour location or size, development of metastasis or relapse, and age at diagnosis (data not shown).

Surprisingly, the percentage of males carrying polymorphisms of the *TNFα* promoter was statistically higher than in females (p=0.025, OR=4.05, CI=1.13-14.43). We did not detect this difference in the group of healthy controls, in which the distribution of polymorphic patterns was similar in both sexes.

Several reports have indicated that different HLA (human leucocyte antigen) products and related genes may be risk factors for and also protective factors against cancer. The *TNFα* gene is of particular interest because of its involvement in tumour immunity and cancer pathogenesis and the relationship existing between certain *TNFα* genetic variants and human tumours.

Although the exact regulatory mechanisms altered by the *TNFα* promoter polymorphisms are not completely delineated, studies on these polymorphisms have shown that those at −308 and −238 are associated with the development and even prognosis of certain types of cancer. Chouchane *et al*[8] detected a marked decrease of the TNF308.1 homozygous genotype in patients with non-Hodgkin's lymphoma, breast carcinoma, and in a group of different malignant tumours. Nevertheless, it must be taken into account that the results of this study were obtained with

Table 2 Frequencies of the TNFα promoter alleles –238 and –308 in the paediatric bone sarcoma groups compared to control children

	*Controls (n=222)**		*Osteosarcoma (n=126)*		*ES† (n=94)*	
Allele	*No*	*%*	*No*	*%*	*No*	*%*
Locus TNFα -308						
G (TNF308.1)	194	87.4	111	88.1	88	93.6
A (TNF308.2)	28	12.6	15	11.9	6	6.4
			p=0.847, NS, OR=0.94 (CI 0.48, 1.83)		p=0.102, NS, OR=0.47 (CI 0.19, 1.18)	
Locus TNFα -238						
G (TNF238.1)	204	91.9	124	98.4	88	93.6
A (TNF238.2)	18	8.1	2	1.6	6	6.4
			p=0.012, ‡, OR=0.18 (CI 0.042, 0.8)		p=0.6, NS, OR=0.77 (CI 0.29, 2.01)	

*Number of alleles analysed.
†ES, Ewing's sarcoma.
‡Statistically significant.

Table 3 Genotypes for the TNFα promoter variants at –238 and –308 in bone sarcoma patients and in healthy controls

	*Controls (n=111)**		*Osteosarcoma (n=63)*		*ES† (n=47)*	
Genotype	*No*	*%*	*No*	*%*	*No*	*%*
Locus TNFα -308‡						
GG	83	74.8	49	77.8	41	87.2
GA	28	25.2	14§	22.2	6	12.8
			p=0.65, NS, OR=0.85 (CI 0.41, 1.76)		p=0.08, NS, OR=0.43 (CI 0.17, 1.13)	
Locus TNFα -238						
GG	93	83.8	61	96.8	41	87.2
GA	18	16.2	2	3.2	6	12.8
			p=0.0095, ¶, OR=0.17 (CI 0.04, 0.76)		p=0.58, NS, OR=0.76 (CI 0.28, 2.04)	

*Number of subjects analysed.
†ES, Ewing's sarcoma.
‡In each polymorphism G is the normal allele and A is the polymorphic one.
§13 patients are GA and one is AA.
¶Statistically significant.

adult patients in the Tunisian population and the polymorphisms of *TNFα* are dependent on ethnicity.

In the present study, we report a decrease in the TNF238.2 rare allele among osteosarcoma paediatric patients and no difference from the control population in the distribution of the TNF308.2 variant in either of the tumour groups analysed, while other authors have described a decreased representation of the TNF308.2 allele in cancer populations (for example, in chronic lymphocytic leukaemia).[19]

Although the exact influence of the TNF238.2 variant on TNFα function and expression is not fully understood to date, the localisation of this polymorphism in the regulatory Y box[20] suggests that it may contribute to the optimal function and regulation of the *TNFα* promoter. However, it has been proven, in transfection assays, that there are no differences in TNFα production after stimulation of TNF238.2 heterozygous or normal homozygous cells; therefore TNF238.2 is not likely to be of functional relevance for transcriptional activation, and the actual meaning of the –238 promoter polymorphism remains a controversial matter.

There is evidence that the −308 TNF2 allele is overexpressed in diseases where TNFα levels are associated with poor prognosis. In our bone sarcoma series, we did not find a relationship between this or any other polymorphic variant of the *TNFα* promoter and tumour prognosis, disease free survival or development of metastasis or relapse (data not shown). One hypothesis is that these polymorphisms may serve as markers for additional polymorphisms or mutations in neighbouring genes that may be involved in the disease.

With regard to the increased number of *TNFα* polymorphic alleles in male osteosarcoma patients, several other authors have reported gender differences and increased TNFα levels in male patients affected by type II diabetes mellitus.[21] A possible explanation for this finding is that the increased plasma levels of TNFα in males are the consequence of the presence of an increased number of the TNF308.2 allele, which has been associated with higher expression of the *TNFα* gene. In fact, in our series of osteosarcoma, there is a tendency for a higher number of TNF308.2 alleles in male patients, without reaching, however, statistical significance (p=0.079).

Computer DGGE programs Melt87 and SQHTX were kindly provided by Dr L Lerman. We are also grateful to Dr H Holden from the Laboratory of Molecular Medicine of the University of Sheffield for providing us with some polymorphic samples and to Dr Reyes López de Mesa for statistical assistance. This work was supported by a PIUNA grant from the University of Navarra.

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J Med Genet 2000;**37**:792–794

Low prevalence of germline *BRCA1* mutations in early onset breast cancer without a family history

EDITOR— Germline mutations in the *BRCA1* and *BRCA2* genes cause predisposition to breast and ovarian cancer.[1] Epidemiological evidence and linkage studies suggested that the likelihood that a woman with breast cancer has a genetic susceptibility to the condition is greater the younger she was at diagnosis and with increasing extent of family history of the disease. Studies of the prevalence of germline mutations in *BRCA1* and *BRCA2* in women with breast cancer has enabled the frequency of mutations to be determined in women with different ages at diagnosis and extent of family history of breast cancer.[2 3] The CASH study into the attributable risk of breast and ovarian cancer estimated that 33% of all breast cancers diagnosed by the age of 29 years, and 22% diagnosed by the age of 30-39 years, are the result of an inherited mutation.[4] However, the proportion of breast cancer cases diagnosed by 40 years resulting from a *BRCA1* mutation was predicted to be 5.3%.[5] Previous population based studies of the prevalence of *BRCA1* mutations in early onset breast cancer have been in cases unselected for family history, and the majority of mutation carriers detected did have some degree of family history of either breast or ovarian cancer.[6–10] The aim of this study was to establish the prevalence of *BRCA1* mutations in a large series of British patients with a young age of onset and no known family history of the disease, since such patients are referred relatively frequently for genetic counselling. The presence of *BRCA1* mutations in a significant proportion of these patients would have important implications for the planning of a mutation screening strategy in diagnostic services.

Patients were ascertained from the Imperial Cancer Research Fund Clinical Breast Oncology Department at Guy's Hospital, and the family cancer clinics at the Genetics Departments of Guy's Hospital and the Royal Free Hospital, both in London, and from St Mary's Hospital, Manchester. The Manchester cases were initially ascertained as part of a population based series for other studies. Patients without a known family history of breast or ovarian cancer at referral were recruited into this study, and these patients were interviewed at home by a genetic nurse counsellor. A family history was taken by the clinical geneticist or genetic nurse counsellor, and the diagnosis confirmed in all probands with either the referring breast unit or the patient's GP or oncologist. Patients with a known family history of breast or ovarian cancer at referral were excluded from the study. The range of age of diagnosis was 22-35 years and the median age of diagnosis was 31 years. Blood samples were taken after the purpose of the study had been explained and informed consent obtained. Full genetic counselling was provided, following agreed protocols, and results were made available to those women wishing to be informed.

The 22 *BRCA1* coding exons were amplified using 24 pairs of primers (exon 11 was amplified in three overlapping fragments). The fluorescent chemical cleavage of mismatch (FCCM) protocol was adapted from the method of Rowley *et al.*[11] PCR products were labelled by incorporation of dUTP analogues which were labelled with either R110 (blue), R6G (green), or Tamra (yellow) fluorescent dyes (PE Applied Biosystems Inc), and heteroduplex molecules then subjected to hydroxylamine modification and piperidine cleavage. Using the three different dyes to label the 24 PCR products that cover the complete *BRCA1* coding sequence, three patients could be analysed for one fragment in one lane of the gel; thus three patients were completely screened in 24 lanes and six patients were screened on a 50 well gel. The FCCM technique has been reported to detect over 95% of mutations in a blind study of haemophilia A patients.[11] We evaluated the sensitivity of our *BRCA1* protocol by examination of the eight polymorphisms that have been reported in the *BRCA1* gene with a frequency of at least 5% (1186A/G, 2201C/T, 2430T/C, 2731C/T, 3232A/G, 3667A/G, 4427C/T, 4956A/G).[12] These polymorphisms were all detected reproducibly at the expected frequency in a panel of over 400 patients who were tested, as part of our continuing studies. In addition, FCCM detected the mutations in three known *BRCA1* positive samples (188del 11bp, 5242 C/A, and 5382insC) that were previously found in our laboratory by SSCP analysis.[13] The fluorescent chemical cleavage assay which we have developed therefore allows a rapid and sensitive mutation screen of *BRCA1*.

Four mutations that were likely to be pathogenic were detected in 110 patients (3.6%) and are listed in table 1. These included three sequence variants that would be predicted to result in premature termination of translation. The 185delAG frameshift mutation, which is prevalent in the Ashkenazi Jewish population, was identified in a British patient (91032) with Jewish ancestry. The 4693-4694delAA mutation was detected in a British patient (78750) who was diagnosed with breast cancer at the age of 26 years. The truncating mutation 3875-3878delGTCT was seen in a patient of Afro-Caribbean origin (94641), who was diagnosed with breast cancer aged 33 years and with a second primary cancer at 38 years. Testing for this mutation in the parents of the patient indicated that it was inherited from her father. A novel in frame deletion, 1965-1967delTCT, was detected in a patient of West African origin (103727) who was diagnosed with breast cancer at the age of 27 years. This mutation would be predicted to result in the deletion of a single amino acid, serine 616, but would not lead to premature termination of translation. This sequence change was absent in over 350 control chromosomes in our study and fulfils all the other criteria for pathogenic status.[13] The pedigrees of these four women are shown in fig 1. Although all four patients originally reported no family history of breast cancer, further investigation showed that the maternal great grandmother of patient 103727 was diagnosed with the disease at about 60 years of age. The lack of a history of breast or ovarian cancer in these families is likely to result from a combination of factors including paternal transmission of the mutation, chance, and reduced penetrance.

Eight other rare DNA sequence variants were identified (table 2). Three of these would not be predicted to alter the expression of *BRCA1* or the sequence of its encoded protein (Q1604Q, IVS22+8T/C, UGA+36T/C) and R1347G

Table 1 BRCA1 pathogenic mutations

Patient	*Exon*	*Nucleotide change*	*Amino acid change*
91032	2	185-186delAG	39 stop
103727	11	1965-1967delTCT	S616del
94641	11	3875-3878delGTCT	1264 stop
78750	15	4693-4694delAA	1529 stop

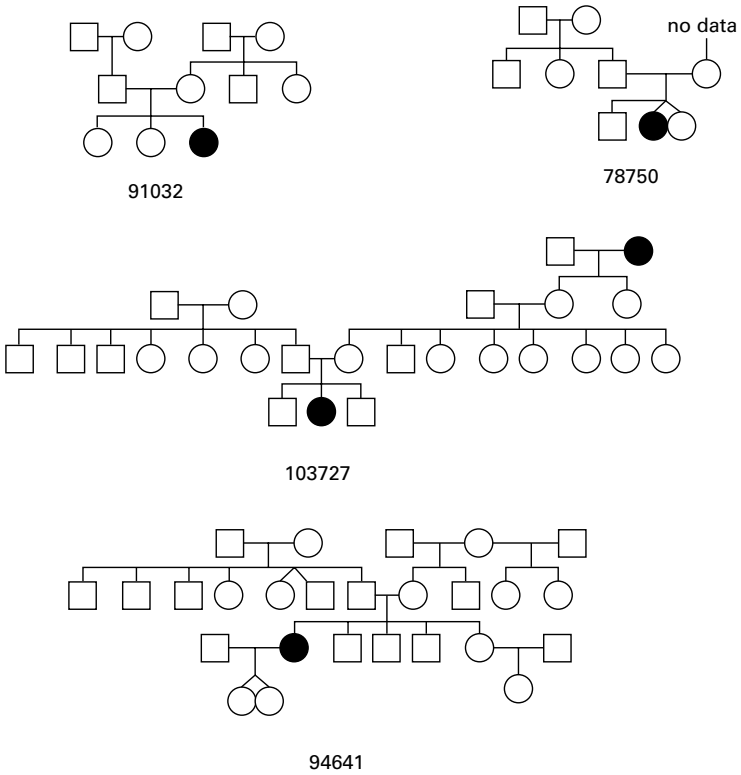

Figure 1 Pedigrees of cases in whom a pathogenic BRCA1 mutation was identified.

Table 2 BRCA1 unclassified variants and polymorphisms

Exon	*Nucleotide change*	*Amino acid change*	*Freq in controls*	*Conserved in mouse/dog*	*BIC entries*
11	2531 G/C	Q804H	0.0	+/−	0
11	3537 A/G	S1140G	0.0	−/+	15
11	4158 A/G	R1347G	0.01*	−/+	75
16	4931 A/G	Q1604Q	ND	+/+†	16
16	5029 C/T	P1637L	0.0	+/+	11
18	5075 G/A	M1652I	0.01	+/+	8
intron 22	IVS22+8 T/C	Unknown	ND	ND	2
3' untrans	UGA+36 C/G	Unknown	ND	ND	4

*As reported by Langston *et al*.[7]
†Nucleotide sequence is conserved in mouse and dog *BRCA1* genes.
ND = not determined.

was present in a patient with a frameshift mutation.[12] The pathogenic status of the other four (Q804H, S1140G, P1637L, and M1652I) remains inconclusive in the absence of a functional assay for the BRCA1 protein. At present, screening panels of ethnically matched controls is a useful means of excluding missense mutations as pathogenic mutations of high penetrance, and it would be helpful if this information was provided in the Breast Cancer database (www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/index.html).

Our detection of pathogenic *BRCA1* mutations in 3.6% of young breast cancer patients without a family history of breast or ovarian cancer is consistent with the estimate of Ford *et al*[5] that the proportion of breast cancer cases in the general population resulting from *BRCA1* is 5.3% below the age of 40 years. A recent population based study of young British breast cancer patients unselected for family history found *BRCA1* mutations in nine of 254 (3.5%) women diagnosed by the age of 36 years.[8] In North American studies of *BRCA1* mutations in women unselected for family history and diagnosed below the age of 35 years, Langston *et al*[7] detected mutations in 7.5%, Malone *et al*[9] in 6.2%, and Struewing *et al*[10] in 5.7%. Fitzgerald *et al*[6] detected *BRCA1* mutations in 13% of women diagnosed before the age of 30 years, but this included Ashkenazi Jewish patients who have founder mutations and some patients with a family history. Since we did not screen the promoter region of *BRCA1* or for deletions of entire exons,[14] we cannot exclude the possibility that some mutations were missed, and the pathogenic status of several sequence variants remains unresolved. However, the important practical implication of our study is that, given the time and expense involved, it would be reasonable to attach a low priority to *BRCA1* mutation screening of young isolated cases of breast cancer in the context of the provision of a publicly funded and cost effective diagnostic service. A screen of this cohort of patients for *BRCA2* mutations is in progress.

This study was supported by the NHS National Cancer Research and Development Programme (UK) (project No NCP/B11/34).

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J Med Genet 2000;**37**:794–796

Delineation of a new syndrome: clustering of pyloric stenosis, endometriosis, and breast cancer in two families

EDITOR—Familial tendencies have previously been observed for congenital pyloric stenosis, endometriosis, and breast cancer. These conditions have never been considered to have shared aetiological origins and consequently no previous attempts have been made to investigate an association. For example, when obtaining family history information for a child with pyloric stenosis, one would not routinely request a description of adult onset conditions such as endometriosis or breast cancer. Two families sharing an unusual clustering of these three conditions (pyloric stenosis, endometriosis, and breast cancer) were ascertained at the familial cancer clinics of the Women's College and Princess Margaret Hospitals in Toronto.

Family 1 (fig 1) contains four confirmed cases of breast cancer (below age 60), seven cases of endometriosis, five cases of congenital pyloric stenosis, nine cases of polycystic ovaries, and four cases of non-insulin dependent diabetes. In a second unrelated family, a woman previously diagnosed with premenopausal breast cancer, endometriosis, and pyloric stenosis reported one other case of congenital pyloric stenosis and four other cases of endometriosis in her family (fig 1). It is the similar and unusual presentation in these two families which suggests that the clustering of pyloric stenosis, endometriosis, and breast cancer may not be the result of chance.

A family history of breast cancer is known to be the most significant risk factor for developing the disease. Approximately 5-10% of all cases are hereditary and accounted for by mutations in cancer susceptibility genes *BRCA1* and *BRCA2*.[1 2] Family 1 met our criteria for *BRCA1* and *BRCA2* testing, with four known cases of breast cancer diagnosed below the age of 60. Mutation analysis by direct sequencing of the coding regions of *BRCA1* and *BRCA2* as well as 1700 adjacent non-coding intronic base pairs was performed by Myriad Genetic Laboratories (http://www.myriad.com). No *BRCA* mutation was identified for family 1. Family 2 was not tested for *BRCA1* or *BRCA2* mutations.

The aetiology of endometriosis remains uncertain, although familial trends have been described.[3] Four studies found that there is an increased risk among first degree relatives.[4–7] A small twin study found 6/8 monozygotic and 0/2 dizygotic twin pairs had endometriosis.[7] In family 2, five women in three generations had endometriosis and in family 1 seven women in three generations were given the diagnosis. The pattern of inheritance is consistent with sex limited, autosomal dominant inheritance.

A multifactorial genetic contribution for pyloric stenosis has been well established, although its pathological basis remains unknown. Twin studies have shown that there is a 25-40% concordance rate in monozygotic twins.[8 9] Based on pooled data from several family studies and assuming a population prevalence of 0.3%, a relative risk for first degree relatives compared to the general population was 18[10]; however, population based studies (unselected patients) have not been done. Pyloric stenosis has recently been linked to the locus of the neuronal nitric oxide synthase (*NOS1*) gene, based on 27 families.[11] The *NOS1* locus was also examined for other multifactorial conditions such as asthma (candidate gene)[13 14] and multiple sclerosis (no association).[15] Family 1 has five documented cases of surgically corrected pyloric stenosis in three males and two females. Family 2 has a parent and child with PS, both female.

In addition to endometriosis, family 1 contains nine women with polycystic ovary syndrome (PCOS), including one woman with non-insulin dependent diabetes mellitus (NIDDM). PCOS and NIDDM have been shown to have a shared aetiology.[12] Women with PCOS have a unique disorder of insulin action and are at increased risk of developing NIDDM, which occurs substantially younger (in the third to fourth decades) than it does in the general population.

Breast cancer, endometriosis, and pyloric stenosis in families 1 and 2 may be explained by separate genetic predispositions; however, the possibility that there is a common genetic basis exists. It is the complex interplay between environmental, hormonal, and genetic factors which poses a challenge to understanding the aetiology of

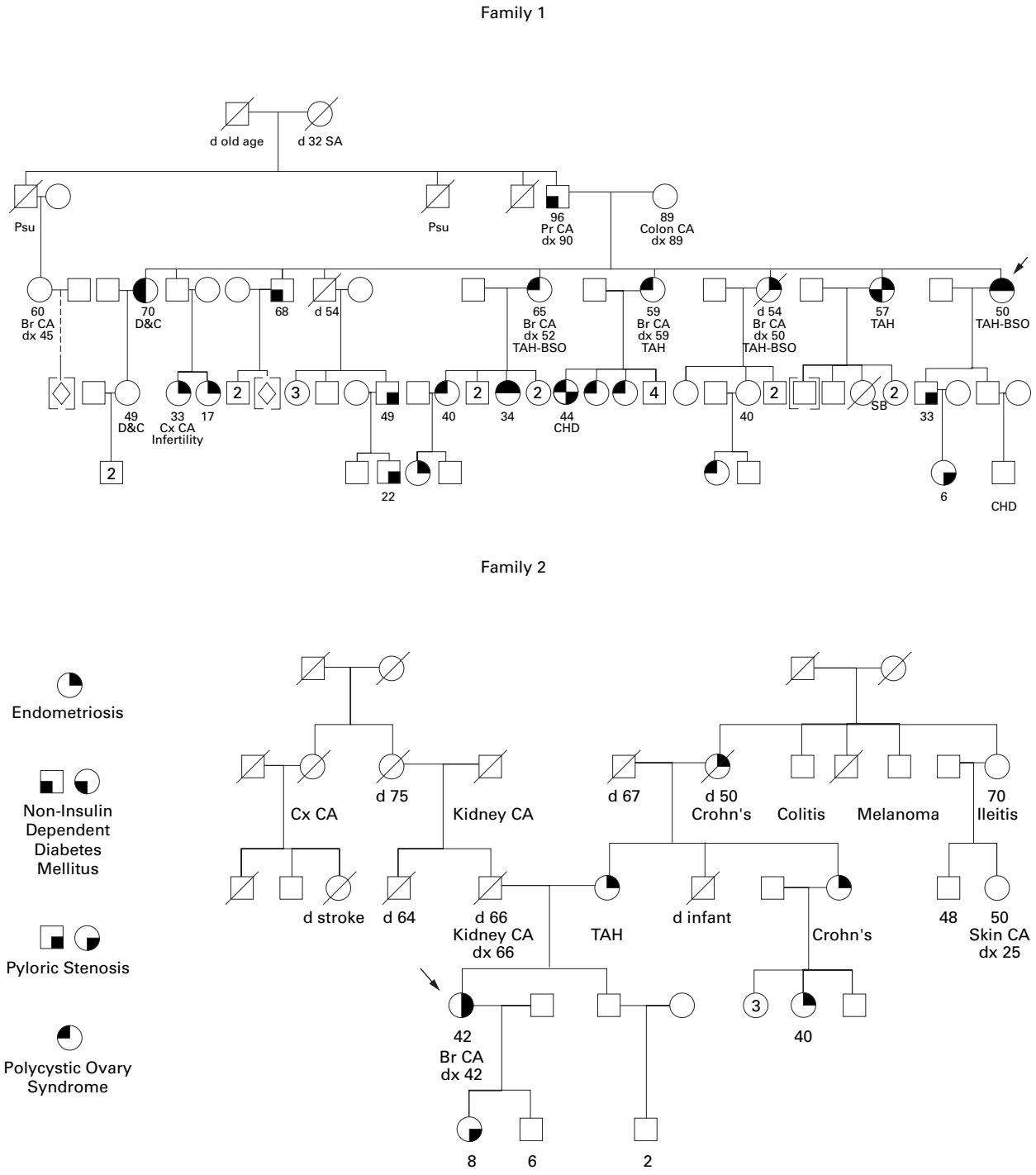

Figure 1 Pedigrees of families 1 and 2. Proband is indicated by an arrow. The age of the subjects appears directly below symbol. CA = breast cancer followed by age of diagnosis (dx); Psu = primary site of cancer was not known; Pr CA = prostate cancer; Cx CA = cervical cancer; TAH = total abdominal hysterectomy; BSO = bilateral salpingo-oophorectomy; NIDDM = non-insulin dependent diabetes mellitus; CHD = congenital heart disease; D&C = dilatation and curettage; SA = spontaneous abortion; SB = stillbirth.

each condition. A future study of pyloric stenosis in a case-control design may investigate any association with breast cancer or endometriosis.

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J Med Genet 2000;**37**:796–798

Punctate calcification of the epiphyses, visceral malformations, and craniofacial dysmorphism in a female baby

EDITOR—We report a fetus with striking craniofacial dysmorphism, brachydactyly, and cerebral and cardiac malformations in addition to punctate calcification of the epiphyses.

The mother was treated for tuberculosis seven years before the pregnancy but there were no known systemic illnesses or teratogenic influences during this pregnancy. The mother's first pregnancy resulted in a termination at 22 weeks of gestation for multiple congenital abnormalities, but further details are not known.

The baby was the second child born to a 21 year old mother. A termination was performed at 21 weeks of gestation because of multiple anomalies seen on antenatal scanning. Necropsy showed a female fetus (fig 1) with a weight of 1544 g, consistent with 17 weeks' gestation. The crown-heel length was 16.4 cm and right foot length was 18 mm. Facial examination showed an open right eye with exophthalmos, hypertelorism, a flat nasal bridge with hypoplasia of the alae nasi, flattening of the midface, a short philtrum with a well defined philtral groove, large lips, and a wide mouth with micrognathia. The right ear was simple and low set and the left ear was rudimentary

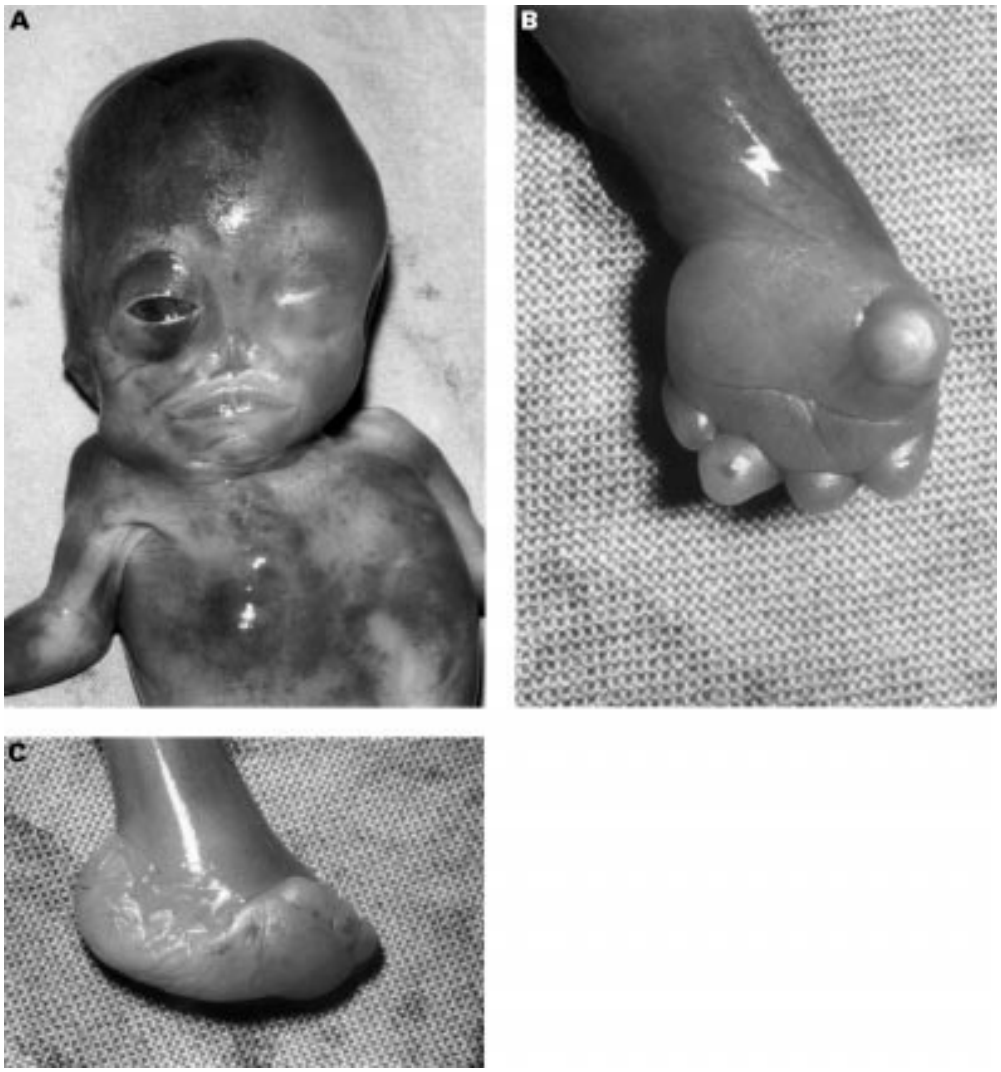

Figure 1 (A) Front view of fetus. (B) Hand of fetus. (C) Foot of fetus.

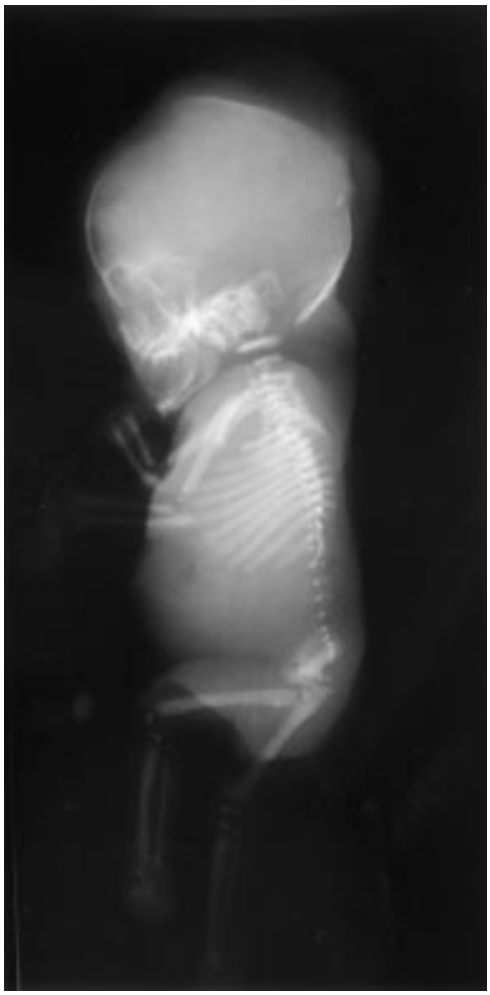

Figure 2 Radiograph of fetus showing punctate calcification of epiphyseal areas and stippling of carpal and tarsal bones.

and poorly formed. The palate was normal. Both hands had brachydactyly involving all digits with short thumbs. There were bilateral varus foot deformities and both feet were rockerbottom in shape. The remainder of the examination showed nuchal thickening, a short, broad chest, and a straight spine. The genitalia were female with a vaginal opening but there was clitoral hypertrophy and prominence of the labial folds.

Internal examination of the brain showed enlargement of both lateral cerebral ventricles, absent olfactory bulbs, and an absent pituitary gland. The heart had a common truncus arising from the right ventricle with a high ventricular septal defect. The right adrenal gland was absent. Histological sections showed that one umbilical artery was rudimentary without a lumen. Examination of the placenta showed no inflammatory changes. Unfortunately, no histological studies were performed on bone.

Chromosome analysis showed an apparently normal female karyotype but the quality of the preparation was sufficient only to exclude numerical and large structural abnormalities. Culture of the fetal cells failed and peroxisomal studies and a 7-dehydrocholesterol level were not performed.

Radiographs (fig 2) showed extensive calcification and stippling in all epiphyseal areas. There was undermodelling of the long bones with shortening of the humeri and relative widening of the diaphyses. There was poor calcification and sagittal clefting of the vertebral bodies. Radiographs of the hands and feet showed stippling in the carpal and tarsal bones and the fingers and toes were extremely short with a single phalanx in the second to fifth digits and almost absent ossification of the phalanges. The thorax was short and broad with 11 pairs of ribs and there was posterior dislocation of the hips. The couple chose not to attend genetic counselling clinic.

This baby had an unusual pattern of malformations including right exophthalmos, midface hypoplasia, rudimentary ears, cerebral ventriculomegaly, a common truncus arteriosus and ventricular septal defect, and extreme brachydactyly in addition to calcific stippling of the epiphyses. Epiphyseal stippling can be found in the primary chondrodysplasias,[1] but the visceral abnormalities in this baby are not typical of chondrodysplasia and the presentation is neither consistent with brachytelephalangic chondrodysplasia punctata (CP)[2 3] or humerometacarpal CP.[4 5] One plausible diagnosis is Pacman dysplasia, a rare condition in which stippling of the lower spine and epiphyses has been found with bowing of the femora, spinal clefting, and giant, multinucleated, osteoclast-like cells.[6 7] Although a patent ductus was described in one child, Pacman dysplasia has not been reported with severe visceral abnormalities or craniofacial dysmorphism as found in this baby.

Stippling of the epiphyses is well known to be causally heterogeneous,[1 8] but syndromes with punctate calcification and visceral malformations are rare. Two sibs were described with short stature, ocular colobomata, midface hypoplasia with a small nose, low set ears, and dysplastic distal phalanges in addition to stippled epiphyses.[9] A second report of a girl with similar facial dysmorphism, a right retinal coloboma and an incomplete atrioventricular canal, coarctation of the aorta, enlargement of the anterior horns of the lateral ventricles, and partial agenesis of the corpus callosum concluded that these anomalies comprised a separate syndrome.[10] Recently, a male baby with punctate epiphyses and coronal clefting of the spine was described with a Dandy-Walker malformation, hypogenesis of the corpus callosum and falx cerebri, a ventricular septal defect, and bilateral cleft lip and palate.[11] This child also had craniofacial dysmorphism with telecanthus, clouding of the corneas, a high nasal bridge and retromicrognathia, and brachydactyly.

These cases show a degree of similarity to this baby with calcific stippling in association with midface and nasal hypoplasia, dysplastic ears, brachydactyly, and cranial and cerebral malformations. However, the variable nature of the accompanying anomalies makes it difficult to be confident that these patients represent heterogeneity of the same condition. Despite the incomplete clinical information in our case, we believe that documentation of these abnormalities will prove useful in view of the rarity of the combination of features and the paucity of reports describing epiphyseal stippling and malformations.

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J Med Genet 2000;**37**:798–804

Unexpected high frequency of de novo unbalanced translocations in patients with Wolf-Hirschhorn syndrome (WHS)

EDITOR—Wolf-Hirschhorn syndrome (WHS), first described independently in 1965 by Wolf *et al*[1] and Hirschhorn *et al*,[2] is a well defined multiple congenital anomalies/mental retardation syndrome resulting from deletion involving chromosomal band 4p16.3 with a minimal critical region of 165 kb.[3]

Different mechanisms are responsible for WHS, including terminal 4p deletions, familial translocations, and de novo complex chromosomal rearrangements such as unbalanced translocations. The frequency of translocations in WHS as a result of parental chromosomal translocations is estimated to be 5 to 13%,[4 5] whereas the rate of sporadic translocations in WHS was suggested to be 1.6% (2/120).[5] A few isolated cases with de novo translocations[5–12] resulting in WHS have been described recently. The trisomic segment, however, often could not be defined owing to the lack of specific cytogenetic techniques.[5 6 8 13–15]

Here we report six patients with unbalanced translocations, t(4p;8p) and t(4p;7p), respectively, and discuss their phenotypic abnormalities.

Since 1996 we have performed clinical, molecular, cytogenetic, and molecular-cytogenetic investigations in a total of 22 patients with clinical signs of Wolf-Hirschhorn syndrome. These patients were all seen by one of the authors. In five of them (22%), the combination of cytogenetic and molecular investigations showed a de novo unbalanced translocation; in a sixth patient the de novo occurrence of the translocation could not be confirmed because no blood sample from the father was available.

The clinical findings of patients 1-5, who all presented with characteristic features of WHS, are summarised in table 1. Patient 6 (CL220585/87E1624) had some additional findings listed in table 1.

The combination of Hirschsprung disease and hydrocephalus was suggestive of CRASH syndrome in patient 6, but SSCP analysis of the *L1CAM* gene was normal.

Chromosome studies including GTG banding were performed on peripheral blood lymphocytes according to slightly modified standard techniques.[16]

DNA from cosmids pC847.351,[17] L21f12 (D4S180), and L228a7 (D4S81)[18] and inter-Alu PCR products[19] from YAC 877G6, 405D10, 794D12, 225D2, and 435A11 (CEPH) were labelled with digoxigenin using a BRL nick translation kit (Gibco, Life Technologies Inc, Gaithersburg, MD, USA). Labelled cosmid and YAC DNA was separated from unincorporated nucleotides by using 1800 ml Centricon 30 (Amicon Inc, Beverly, MA 01915, USA) filters. FISH was performed as described by Lichter *et al*.[20] Labelled cosmid DNA (50 ng) or YAC DNA (100 ng) was mixed with human Cot-1 fraction DNA to suppress repetitive sequences or block non-specific hybridisation. Hybridisation was detected using monoclonal anti-digoxin, anti-mouse IgG FITC conjugate and anti-rabbit IgG FITC conjugate (Sigma Immuno

Table 1 Clinical findings in patients 1-5 with t(4;8) and patient 6 with t(4;7)

	Patient 1 (CG/96E0600)	*Patient 2 (DT/92E1657)*	*Patient 3 (LF/96E0551)*	*Patient 4 (NR/97E877)*	*Patient 5 (AS/94E1416)*	*Patient 6 (CL/87E1624)*
Anthropometric data						
Birth (gestational weeks)	39	36	38	40	40	41
Weight (g)	2900 (−1.0 SD)	2120 (−1.2 SD)	1800 (−3.4 SD)	2240 (−3.6 SD)	2200 (−3.7 SD)	2950 (−1.6 SD)
Length (cm)	NR	46 (−0.4 SD)	42 (−2.9 SD)	47 (−3.2 SD)	45 (−4.3 SD)	48 (−2.5 SD)
OFC (cm)	31.5 (−2.4 SD)	30.5 (−1.9 SD)	NR	31.5 (−2.7 SD)	32 (−2.3 SD)	34 (−1.6 SD)
Age at examination (y)	1 1/12	3 5/12	3 6/12	2	5 10/12	12
Weight (g)	5240 (−3.8 SD)	9060 (−3.2 SD)	NR	NR	NR	29 000 (−2 SD)
Height (cm)	64.2 (−4.4 SD)	89 (−2.7 SD)	84.5 (−6.7 SD)	72 (−5 SD)	91 (−4.4 SD)	133 (−2.4 SD)
OFC (cm)	41.3 (−3.5 SD)	46.5 (−2.2 SD)	44 (−5.0 SD)	42 (−4.6 SD)	46 (−3.5 SD)	54 (mean)
Dysmorphic features						
Hypertelorism	+	+	+	+	+	+
Strabismus	+	−	+	+	+	+
Broad, large mouth	−	+	−	−	+	+
Downturned corners of mouth	+	+	+	+	+	+
Micrognathia		+	+	+	+	+
Cleft lip/palate	−	−	+	+	+	−
Dysplastic ears	+	+	+	+	+	+
Preauricular tag	−	−	+	−	−	−
Internal findings						
Heart defect	−	−	ASD	ASD	−	−
Genital anomalies	−	+	NR	−	−	+
Neurological development						
Sitting age (mth)	13	20	60	−	24	36
Walking age (y)	2	3 5/12	6 9/12	−	5	?
Simple words (y)	2 9/12	−	−	−	−	?
Understanding of speech	++	++	(+)	−	−	++
Seizures	−	+	+	+	+	+
Additional findings						Oligohydramnios Bilateral pneumothorax Flexion contractures of large joints Hypoplasia of corpus callosum Hirschsprung disease

Chemicals, St Louis, USA) and counterstained using propidium iodide or DAPI.

A cosmid derived from D4S96, the 8p telomere probe and 7p distal probe (Oncor, Gaithersburg, VA, USA) and the whole chromosome paints 4 and 8 (AGS, Heidelberg, Germany/Oncor, Gaithersburg, VA, USA) were hybridised according to the manufacturer's instructions. For the hybridisations, clone pGXba11/340[21] was used as centromeric control for human chromosome 4.

Genomic DNA was prepared from peripheral blood lymphocytes or EBV transformed lymphoblastoid cell lines from patients and their parents according to previously described techniques.[22 23]

Genotyping of chromosome 4p was performed using the following previously described microsatellite markers: D4S1182 (Acc ID GDB 197239[24]), D4S43 (Acc ID GDB 124360[24]), ADRA2C (Acc ID GDB 370834[25]), HOX7 (Acc ID GDB 156991[26 27]), D4S403 (Acc ID GDB 188106[28]), D4S2366 (Acc ID GDB 684453), D4S2639 (Acc ID GDB 685881), and D4S2397 (Acc ID GDB 683982). A new microsatellite, 75B9Rep, was generated by one of the authors based on sequence information of cosmid 75B9A (Acc No Z69651) which is derived from the distal end of the Huntington's disease cosmid contig.[29] Oligomers were designed from sequences flanking the compound dinucleotide repeat $(CT)_2G(CT)_{26}(CA)_{14}$. The following primer sequences were used for the amplification of the repeat, forward primer 5' CTGAACTTTATCCAATTAGTCTTG 3' and reverse primer 5' GAATCTTTCTGTCCCACGAT 3'. This primer set amplifies a 227 bp DNA fragment product. PCR reactions were performed in 10 µl volumes containing 100 ng DNA, 0.5 U Genecraft^R *Taq* polymerase, 1 × Genecraft^R PCR buffer, 2 µmol/l dNTP, 4 pmol/l of each primer, 1 µCi ^{32}P-dCTP (3000 Ci/mmol/lol, Amersham), and 2 mmol/l $MgCl_2$. Formamide (2%) was added to increase stringency of the reaction. After an initial denaturation of one minute, 30 cycles of one minute at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C were carried out in a Crocodile III^R (Appligene) thermocycler. The PCR products were resolved on 6% polyacrylamide gels flanked by a sequence ladder (M13, −40) as size marker. The Hu4/Hu5 (Acc ID GDB 249651) primer pair amplifies the CAG repeat of the huntingtin gene. Primer Hu4 has been published by Riess *et al*[25] (primer sequence: Hu4 5' ATGGCGACCCTGGAAAAGCTGATGAA 3'), and primer Hu5 has been published by Rubinsztein *et al*[30] (primer sequence: Hu5 5' GGCGGTGGCGGCTGTTGCTGCTGCTGC 3').

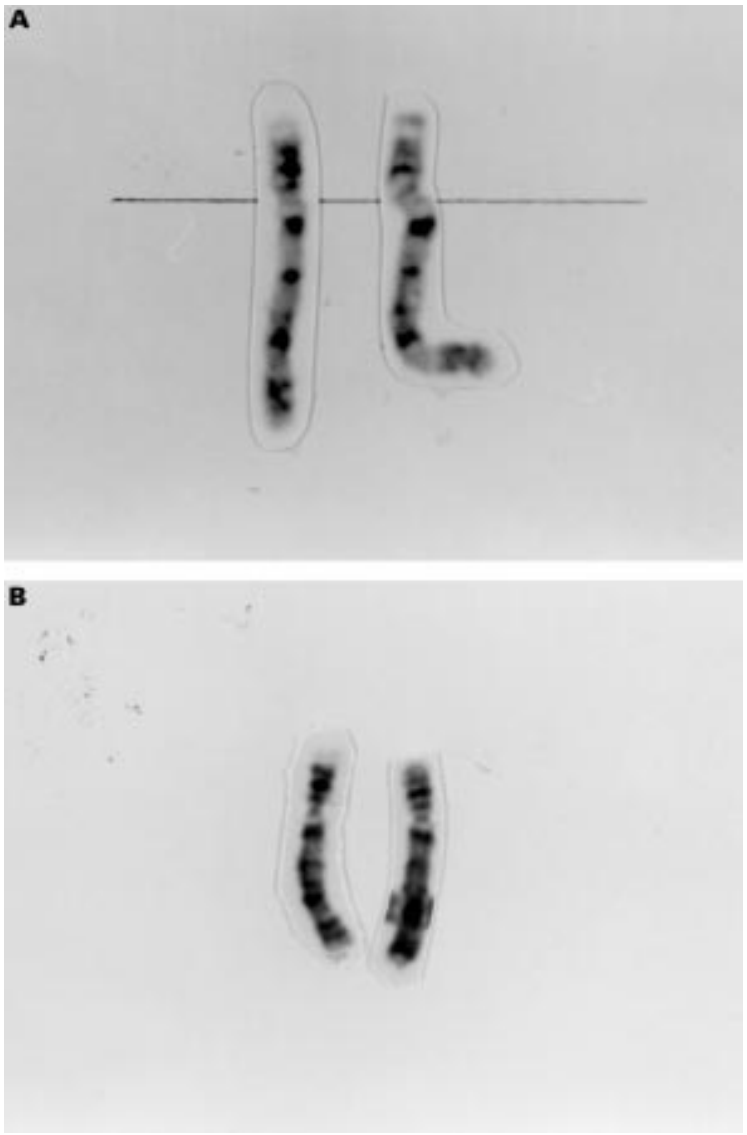

Figure 1 (A) Chromosomes 4 of patient 2 showing an additional G positive band in 4pter. (B) Chromosomes 4 of patient 4 showing same length of 4p but different G banded pattern.

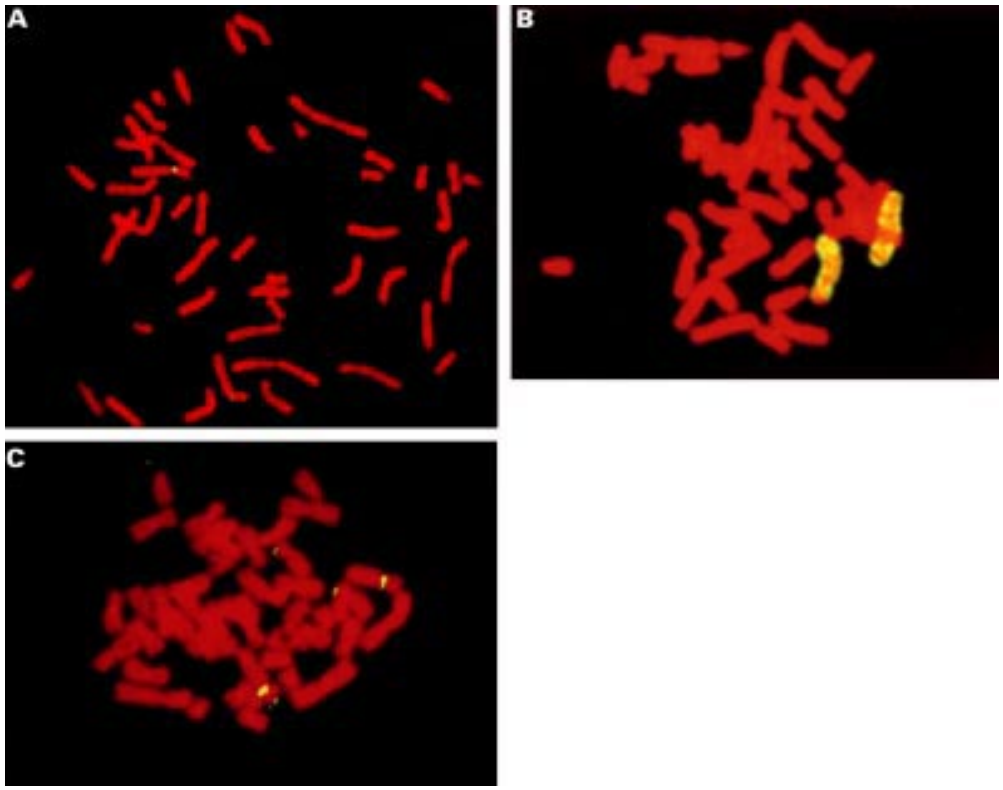

Figure 2 FISH results in patient 4. (A) FISH with cosmid pC847.351 showing lack of signal in one chromosome 4p. (B) FISH with wcp(4) showing fluorescent signals on the entire length of both chromosomes 4 except for the terminal region of one chromosome 4. (C) FISH with distal 8p probe showing two signals in the distal region of 8p and an additional signal in the distal short arm of one chromosome 4.

At the beginning of our study of 22 WHS patients, we reanalysed the chromosomes in lymphocytes by standard Giemsa banding techniques. At a banding resolution of at least 450 bands per haploid genome,[31] an additional small G positive band was observed on the distal short arm of one chromosome 4 in patients 1 and 2 (fig 1A) and an unbalanced translocation was suspected in patients 3, 4, and 5 (fig 1B). In patient 6, a deletion of 4p with breakpoints in 4p16.2 was suggested. Thus, we suspected an unbalanced translocation between the short arm of one of the chromosomes 4 and another unidentified chromosome in patients 1-5, while the cytogenetic aberration in patient 6 appeared to be a simple deletion.

Chromosomal analysis in all participating parents showed a normal karyotype. In patient 5 the father was not available. The remaining 16 patients of this study are described elsewhere (D Wieczorek, manuscript in preparation).

Different cosmids were used to confirm the clinical diagnosis of WHS in all patients. In patients 1, 2, and 5, a deletion of D4S96 (Oncor) and of cosmids pC847.351 and L228a7 on one homologue of chromosome 4 was noted (fig 2A). In patients 3 and 4, YAC 405D10 was also deleted on one homologue. In patient 5, the size of the deletion was determined by FISH only. Cosmids pC847.351, L21f12, and L228a7 and YACs 877G6 and 405D10 were deleted on one chromosome 4, whereas YACs 794D12, 225D2, and 435A11 were present on both chromosomes 4 (table 2).

Table 2 Extent and origin of the 4p deletion in patients 1–6

Distance from 4pter	*Microsatellite marker*	*Cosmid*	*YAC*	*Patient 1*	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*	*Patient 6*
~ 200 kb		pC847.351 (D4F26)		del	del	del	del	del	del
~ 1.2 Mb		pC678 (D4S96)		del	del	del	del	del	del
~ 1.9 Mb	L75B9 Rep			M	M	M			
~ 2.0 Mb	D4S1182			NI	M	NI			
~ 2.2 Mb	D4S43	33c6 (D4S43)		M	NI	NI			M
~ 3.0 Mb	Hu4/Hu5			M	M	NI			NI
~ 3.3 Mb		L21f12 (D4S180)					del	del	del
~ 3.8 Mb	ADRA2C			NI	M	M			
~ 3.8 Mb		L228a7 (D4S81)		del	del	del	del	del	del
~ 5.5 Mb	Hox7			ND	ND	NI			NI
~ 7.0 Mb			877G6					del	ND
~ 8.8 Mb	D4S2366			ND	ND	M			
~ 13.0 Mb			405D10			del	del	del	ND
~ 14.0 Mb			794D12			ND	ND	ND	ND
~ 16.5 Mb	D4S403			ND	ND	ND			
~ 19.8 Mb			225D2			ND	ND	ND	ND
~ 21.5 Mb			435A11					ND	ND
~ 23.0 Mb	D4S2639			ND	ND	ND			ND
~ 34.0 Mb	D4S2397			ND	ND	ND			ND

del: deleted; ND: not deleted; NI: not informative; M: maternally deleted.

FISH staining with a whole chromosome 4 paint (AGS/Oncor) in all six patients gave hybridisation signals along the entire length of both chromosomes 4 except for the distal part of the aberrant chromosome 4p (fig 2B). Both chromosomes 8 showed hybridisation signals along the entire length with whole chromosome paint 8 (AGS), but also on one chromosome 4 in patients 1-5. FISH with an 8p telomere probe (Oncor, Gaithersburg, VA, USA) showed signals on both chromosomes 8 and an additional signal on one chromosome 4 (fig 2C).

Based on these findings we determined the karyotype of patients 1 and 2 as 46,XX,der(4),t(4;8)(p16.3;p23.1).ish t(4;8)(wcp4−, wcp8+, 8ptel+, pC847.351−, D4S96−, 228a7−; wcp4−, wcp8+, 8ptel+) and in patients 3, 4, and 5 as 46,XX,der(4),t(4;8)(p16.2;p21).ish t(4;8)(wcp4−, wcp8+, 8ptel+, pC847.351−, D4S96−, 228a7−, 405D10−; wcp4−, wcp8+, 8ptel+).

FISH was also performed in the parents of patients 1-4 and the mother of patient 5. Normal results were detected with D4S96 (Oncor) and with the 8p telomere probe (Oncor). We conclude that the translocation t(4;8) occurred de novo in all patients. However, in patient 4 one could not exclude that the translocation may be inherited from the father.

To identify the chromosome involved in the translocation of patient 6, we used different paints and telomeric probes of G negative bands. Additional signals on chromosome 4 were noted with distal 7p probe (Oncor) (fig 3). We determined the karyotype in patient 6 as 46,XY,der(4),t(4;7)(p16;p22).ish t(4;8)(wcp4−, 7ptel+, pC847.351−, D4S96−, 228a7−; wcp4−, 7ptel+). Balanced translocations in his parents were excluded with FISH using D4S96 and 7ptel in combination with pGXba11/340.

The results of the analyses of polymorphic markers are listed in table 2. Blood samples from the father of patient 5 were not available. Therefore, the size of the deletion was determined by FISH only. DNA from patient 4 was not available to identify the origin of the deletion.

In patients 1, 2, 3, and 6, the maternal allele was absent and in patients 4 and 5 the origin of the deletion remains undetermined. In patients 1, 2, and 6, the deletion 4p was at least 3.8 Mb in size and in patients 3-5 the size of the deletion can be estimated to be 13 Mb.

In our study of 22 patients with Wolf-Hirschhorn syndrome, a de novo translocation was present in 5/22 (22.7%) and a familial translocation in 3/22 patients (13.6%) (manuscript in preparation). In one additional patient (patient 5) a de novo translocation was suspected, but could not be confirmed. To our knowledge, such a high rate of proven de novo translocations in WHS has not previously been reported. This could be explained by the refined molecular-cytogenetic techniques such as FISH analysis and microsatellite analysis used here. In particular, de novo unbalanced translocations between the short arms of chromosomes 4 and 8 seem to be not uncommon in WHS. Müller-Navia *et al*[10] described one fetus and Petit *et al*[11 12] another male patient with this chromosomal aberration.

Figure 3 FISH results in patient 6. FISH with distal 7p probe showing two signals in the distal region of 7p and an additional signal in the distal short arm of one chromosome 4.

The breakpoints of 4p and 8p differ in our patients, which makes sequence homologies between these chromosomal segments less likely. Thus, it is of great interest that Kogi *et al*[32] and Gondo *et al*[33] described a new class of tandemly repeated satellite DNA elements on chromosome 4p and 8p. They identified a tandem array of several RS447 sequence copies in 4p15 and distal 8p. This repeat was suggested to be responsible for formation of inversion duplication 8p[34] and might probably explain the higher frequency of translocations between 4p and 8p.

Moreover, other chromosomes also, for example chromosome 7 in patient 6, occur in de novo unbalanced translocations involving the short arm of chromosome 4. Our findings support the hypothesis of Reid *et al*[35] that cryptic complex chromosomal rearrangements are more common than usually disclosed by light microscopy of conventionally stained chromosomes. Selection bias was unlikely, as we investigated blood specimens of all children with known WHS and with a tentative diagnosis of WHS.

Determination of the origin of the deletion showed that in all four patients in whom this was possible the maternal allele was absent, which is in contrast to published data. To avoid bias owing to familial translocations, which are mostly inherited maternally in a 2:1 ratio,[36] the patients' parents were also investigated by conventional karyotyping and FISH analysis. We reviewed cases in which the origin of the 4p deletion was determined and found 27 paternally inherited and six maternally inherited deletions.[7 15 37–45] Thus, 82% of reported patients with WHS show a paternally derived deletion.

As to the published patients with maternally inherited deletions, the patient Val described by Anvret *et al*[39] had a more complex chromosomal rearrangement causing WHS. Further cytogenetic investigations were not possible. Patients 3 and 6 from the report of Dallapiccola *et al*[44] and case 2 of Thies *et al*[42] presented with a de novo, maternally inherited deletion with breakpoints in 4p16.1, 4p16.3, or 4p13, respectively. However, FISH analysis with whole chromosome 4 paint was not performed to exclude a cryptic translocation. In the patient described by Greenberg *et al*,[38] cryptic translocation in the mother was discussed, but not investigated. Patient 3 in the report of Kant *et al*[37] with maternal origin of the deletion had an unbalanced karyotype based on a cryptic translocation t(4;8) in the mother. Thies *et al*[42] described a third patient with de novo deletion and also a maternally inherited deletion. On the other hand, in two WHS patients with unidentified de novo translocations[40] the deletion was of paternal origin.

Different explanations for the preponderance of paternally inherited deletions are possible, for example, statistical deviation or real biological phenomena such as increased mutation rates in sperm or genomic imprinting.[41] An excess of rearrangements in male meiosis, related to differences between the mechanisms of sperm and egg production, have also been discussed.[44]

One aim of our study was to determine whether the WHS phenotype is influenced by the trisomic autosomal segment. The clinical findings in our patients 1-5, in previously published patients with familial translocations

Table 3 Clinical signs of patients with duplication 8p, with unbalanced translocation t(4p;8p), with monosomy 4p, with trisomy 7p, and reported patients

	*inv dup 8p**	*dir dup 8p†*	*t (4p;8p)‡*	*Our patients 1–5*	*WHS§*	*Our patient 6*	*Trisomy 7p¶*
Duplicated segment	At least 8p21.1-p21.3	8p22-p23.1 or 8p21.3-p22	8p21-pter – 8p23.1-pter	8p	7/24	7p	7p11.2-pter
Monosomic segment	8p23.1-pter in 6 cases	none	4p15.3-pter – 4p16.3-pter	4p	4p14-16	4p	7q36-qter
De novo	14/15	–	0/8	4/5	8/11	+	2/3
Sex	12 F; 8 M	3 F; 3 M	2 F; 6 M	5 F	14 F; 10 M	M	2 M; 1 F
Mental retardation	20/20	5/6	8/8	5/5	22/23	+	3/3
Muscular hypotonia	13/13	0/6	4/4	4/5	20/24	+	2/2
Seizures/EEG anomaly	1/2	0/6	5/8	4/5	18/24	+	NR
Normal birth weight	7/8	4/4	0/6	0/5	0/13	–	2/3
Low birth weight (<3rd centile)	1/17	0/4	6/6	5/5	13/13	+	1/3
Postnatal normal stature	3/6	3/5	0/6	0/5	4/23	–	0/2
Postnatal short stature	2/6	2/5	6/6	5/5	19/23	+	2/2
Absence of dysmorphic signs	10/10	2/3	0/8	0/5	0/23	–	0/3
Craniofacial anomalies							
Microcephaly	3/14	0/6	5/5	5/5	20/24	–	2/3
Macrocephaly	2/14	0/6	0/5	0/5	0/13	–	1/3
Wide open fontanelles	0/1	NR	NR	3/5	NR	+	3/3
Flat occiput	8/13	0/3	1/1	1/2	NR	–	NR
Prominent forehead	13/18	3/6	5/5	5/5	8/11	+	2/3
Prominent glabella	0/5	0/3	4/5	3/5	18/24	–	0/3
Hypertelorism	3/15	1/3	7/7	5/5	20/24	+	3/3
Hypotelorism	3/15	0/3	0/7	0/5	0/13	–	0/3
Strabismus	1/1	0/3	3/4	4/5	13/23	+	NR
Broad nasal bridge	11/17	3/6	6/6	5/5	20/24	+	1/3
Narrow nasal bridge	2/6	0/3	0/6	0/5	0/13	–	1/3
Anteverted nares	7/18	0/3	1/4	0/5	NR	–	2/3
Beaked nose	3/6	0/3	2/6	0/5	6/11	+	1/2
Short philtrum	1/6	1/6	7/7	3/5	13/13	+	1/2
Long philtrum	2/6	0/6	0/7	0/5	0/13	–	2/3
Large mouth	14/19	0/6	4/5	2/5	10/13	+	1/3
Downturned corners of mouth	1/5	0/3	3/4	5/5	13/13		1/3
Thin upper lip	9/17	2/6	2/5	4/5	6/13	+	3/3
High arched palate	10/13	0/3	1/3	0/2	2/11	+	NR
Cleft lip/palate	NR	0/3	4/5	3/5	8/24	–	NR
Abnormal dentition	12/13	1/5	3/3	1/1	1/11	–	1/1
Micrognathia	6/8	0/6	6/8	5/5	18/24	+	3/3
Large chin	2/8	1/6	1/5	0/5	0/13	–	1/3
Small ears	0/9	0/3	0/3	0/5	1/11	+	NR
Large ears	8/9	0/3	0/3	0/5	2/11	–	NR
Dysplastic ears	15/18	0/3	6/7	5/5	18/24	+	3/3
Brain anomalies							
Enlarged ventricles	2/6	0/3	1/2	0/2	NR	+	1/1
Agenesis of corpus callosum	4/6	0/3	0/2	0/2	NR	+	NR
Cortical atrophy	2/6	0/3	0/2	0/2	NR	+	NR
Congenital heart defect	5/12	0/6	3/5	2/5	6/24		2/3
Scoliosis	6/13	0/3	1/3	0/5	3/13	–	NR
Hypoplastic external genitalia	1/2	0/5	2/2	1/4	3/11	+	2/2
Hypospadias	NR	0/5	5/5	/	7/10	+	0/2
Renal anomalies	1/2	0/3	2/5	1/3	2/11	+	NR
Inguinal/umbilical hernia	3/13	0/3	2/2	0/3	NR	–	NR
Megacolon	NR	0/3	0/2	0/3	NR	+	1/1

*Refs 53–58, 61.
†Refs 60 and 61.
‡Refs 37, 46–51; 10 was not included because of lack of clinical description.
§Refs 6 and 62.
¶Refs 65–67.

t(4;8),[37 46–52] in patients with partial trisomy 8 owing to inversion duplication[53–59] or direct duplication,[60 61] respectively, and in patients with WHS[6 62] are compared in table 3. There are several clinical signs which are usually present in patients with partial monosomy 4p as well as in those with partial trisomy 8p, such as mental retardation, muscular hypotonia, prominent forehead, broad nasal bridge, large mouth, dysplastic ears, and congenital heart defects. Thus, these features are by no means specific enough to distinguish between the phenotypes.

The face in WHS is much more characteristic than in partial trisomy 8p and is not significantly influenced by the presence of this trisomic segment. All in all, it seems to us impossible to differentiate clinically between patients with WHS resulting from monosomy 4p and WHS resulting from unbalanced translocation 4p;8p. This agrees with the conclusions of other authors.[37 47–51]

As to patient 6, to our knowledge, no other patient with a de novo translocation t(4p;7p) has been described before.[63 64] However, many patients with trisomy 7p resulting from familial translocations have been reported. These patients are not listed in table 3 because a considerable influence on the phenotype by monosomic autosomal segments cannot be excluded. Only those patients with distal duplication 7p are included.[65–67] The characteristic, clinically recognisable phenotype in trisomy 7p encompasses delayed closure of fontanelles, sparse or even lack of eyebrows, a short nose with a low and broad nasal bridge, small upper and prominent, full lower lip, micrognathia, hypotonia, congenital heart defect, and delayed speech development.[65] Macrocephaly and enlarged cerebral ventricles were only described in one patient.[66] One might speculate that relative macrocephaly in patient 6, which is highly unusual in patients with WHS, may be the result of the duplicated segment in 7p. Delicado *et al*[66] noted intestinal malrotation with dilatation of the sigmoid and left colon in their patient, but excluded Hirschsprung's disease. Our patient presented with similar anomalies of the intestines. We assume that this clinical sign, which has not been described in WHS before, may be caused by the duplication 7p. In conclusion, the overall phenotype in

patient 6, in contrast to patients 1-5, appears to be considerably influenced by the trisomic region of 7p (table 3).

In summary, we show that de novo translocations causing WHS are more frequent than previously estimated. We recommend performing FISH analysis with wcp(4) in every WHS patient to exclude or confirm a de novo translocation as a common mechanism causing WHS. Also patients with cytogenetically visible deletions should be investigated by molecular-cytogenetic techniques to exclude cryptic rearrangements. Moreover, de novo translocations t(4p;8p) in WHS patients are more frequent than previously suspected.

Note added in proof

Since submission of the final version of this paper, 13 patients of this study have been published elsewhere.

We thank Professor Passarge for continous support and for reading the manuscript, and Professor Horsthemke for helpful discussion. In addition, we thank Barbara Henke, Barbara Ulrich, Elke Jürgens, and Gudrun Rodepeter for excellent technical assistance and the parents for taking part in this study. We thank Dr Herdit Schüler for the results of previous chromosomal analysis in patient 1, Dr A J H Hamers for performing FISH with D4S10 in patient 2, Dr J J M Engelen for FISH in patient 4, and Dr Schröder and Professor A Gal for SSCP analysis of the *L1CAM* gene in patient 6. We also thank Tracy Wright for the cosmids pC847.351, 33c6, 21f12, and 228a7. In addition, we thank CEPH for providing the YACs. DW and GG-K were supported in part by a young investigators grant from the Medical Faculty University of Essen (IFORES 107 402.0) and by the Deutsche Forschungsgemeinschaft (Wi1440/4-1).

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Mosaicism for a dup(12)(q22q13) in a patient with hypomelanosis of Ito and asymmetry

EDITOR—Hypomelanosis of Ito (HI) (MIM 146150) is an aetiologically heterogeneous physical finding characterised by a swirling pattern of hypopigmentation of the skin, typically distributed along the lines of Blaschko, reflecting pigmentary mosaicism. Chromosomal abnormalities are common and very heterogeneous. They include X;autosomal translocations and mosaicism of aneuploidy, segmental trisomies, or monosomies.[1–5] As HI is not a specific disorder, it has also been referred to as pigmentary mosaicism.[6] This report adds a further chromosomal anomaly which has not been described in pigmentary mosaicism previously.

The proband was the first son born to a 29 year old mother and a 30 year old father. There was no family history of recurrent abortions, consanguinity, or mental retardation. He was born by caesarian section at term after an uncomplicated pregnancy with a weight of 3560 g (50th centile), a length of 50 cm (50th centile), and an occipitofrontal circumference (OFC) of 32 cm (<3rd centile). Apgar scores were 8, 9, and 10. No complications were reported in the perinatal period. Despite delayed psychomotor milestones (sitting at 9 months, walking at 2 years, first words at 3 years), the first evaluation was only done at the age of 5 years. At the age of 10 years he was referred to our endocrinological outpatient clinic because of cryptorchidism and glandular hypospadias. At this time he was found to be a shy and uncommunicative boy. He showed moderate mental retardation and attended a school for handicapped children. He had a height of 1.3 m (3rd centile), a weight of 26 kg (3rd centile), and an OFC of 50 cm (<3rd centile). On physical examination, mild facial asym-

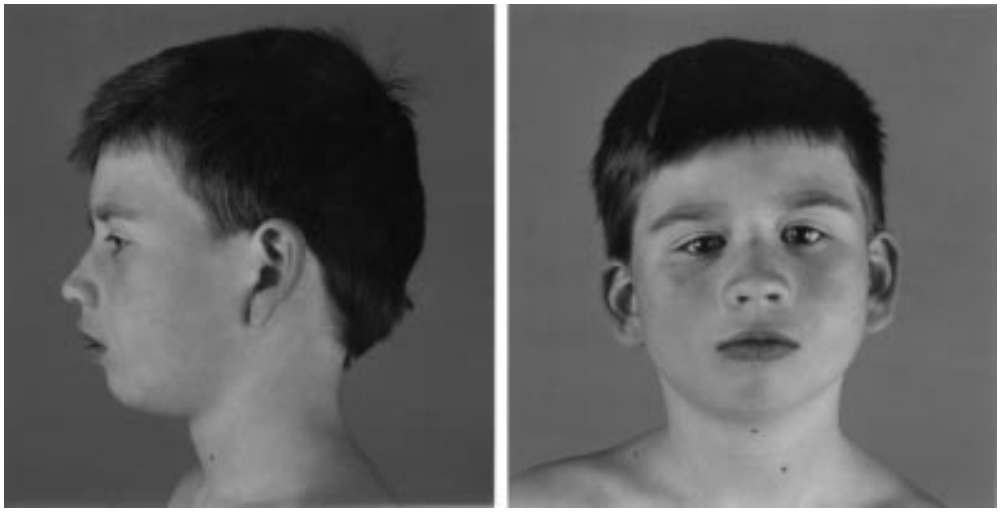

Figure 1 Patient at the age of 10 years.

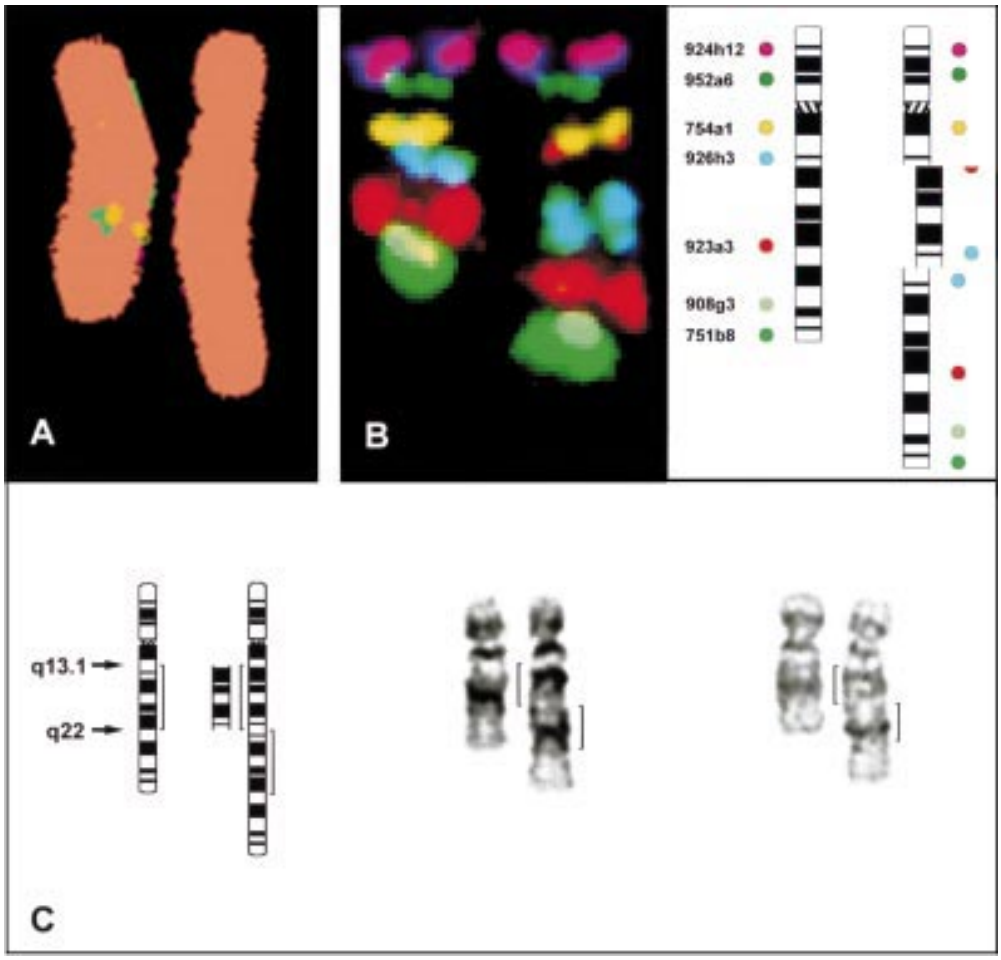

Figure 2 FISH and GTG banding. (A) M-FISH painted the elongated long arm of chromosome 12 homogeneously, indicating a duplication of chromosome 12 material. (B) Application of a multicolour chromosome 12 specific bar code allowed the identification and mapping of the duplicated segment. On the derivative chromosome 12, two signals corresponding to YAC 926h3 (blue) are located close to each other. The 12q21-22 specific YAC 923a3 displayed a weak red signal proximal to the 926h3 double spot in addition to a normal red signal distal to the blue double spot. Thus, the rearrangement represents an inverted duplication of the segment 12q13-q21/22 with upward direction, dup(12)(pter-q13::q22-q13::q22-qter). (C) Breakpoint analysis using GTG banding of chromosome 12, which is in concordance with the results of FISH analysis.

metry, epicanthic folds, a broad nose with a broad nasal tip, retrognathia, dysplastic ears, and a low set posterior hair line were noted (fig 1). Areas of linear hypopigmentation were distributed along the lines of Blaschko and were more evident on the legs. He also showed hemihypertrophy of the right leg, bilateral pes equinovarus, and pectus excavatum. There were no organic defects.

Chromosome analysis of peripheral lymphocytes showed the karyotype 46,XY[91]/46,XY,add(12q)[9]. The GTG banding pattern was suggestive of an interstitial duplication involving chromosomal segment q13-q21 or q13-q22 (fig 2). Parental karyotypes were normal. Fibroblast cultures were obtained from a normal and a hypopigmented skin area on the left arm. Fifty cells from each first subculture were analysed and identified 70% abnormal cells in the normal skin and 15% abnormal cells in the hypopigmented skin fibroblast culture. In addition, 2/50 cells from the normally pigmented skin showed the karyotype 46,XY,del(7)(q31).

Multiplex-FISH (M-FISH) allows the identification of chromosomes with distinct colours and was performed as described elsewhere.[7] The M-FISH analysis showed that the der(12) consisted of chromosome 12 material only, excluding an interchromosomal rearrangement. No other abnormalities were noted. In order to characterise the der(12) in more detail a chromosome 12 specific multicolour bar code was constructed with YAC clones from the CEPH library, as previously described[8] (table 1). On the der(12), two signals (blue) were observed for the 12q13 specific YAC 926h3 (fig 2). These signals were located close to each other indicating a duplication of the respective region. The 12q21-22 specific YAC 923a3 displayed a normal signal (red) distal and a weak signal proximal to the 926h3 double spot, indicating that this particular YAC is partially duplicated and spans a breakpoint of the duplication. The multicolour banding pattern clearly showed an inverted duplication involving the segment 12q13-q21/22 and allowed the differentiation between the two possibilities of inverted duplications (pter-q13::q22-q13::q22-qter versus pter-q21/22::q22-q13::q22-qter). Using the GTG banding pattern in addition to the FISH results, the structure of the der(12) could be designated as dup(12)(pter-q13::q22-q13::q22-qter) (fig 2). The patient was thus mosaic for a pure trisomy of the segment 12q13-q21.

Table 1 Barcode set 12-2

YAC	*Band*	*Fluor*	*Pseudocolour*
924h12	p12	Cy3.5, FITC	Lilac
952a6	p11.2-p12	Cy3	Green (dark)
754a1	12q12	Cy5.5	Yellow
926h3	12q13	Cy3.5, Cy3	Blue
923a3	12q21/q22	FITC	Red
908g3	12q24.1-q24.2	Cy5	Green (light)
751b8	12q24.3	Cy3, FITC	Green

In many kinds of de novo chromosomal aberrations, it is impossible to determine the chromosomal origin of the extra material by banding patterns alone and characterisation of the rearrangement often needs FISH analysis with single copy probes. In the present case, a partial trisomy 12q was suspected from the G banding pattern and was confirmed by M-FISH within a single hybridisation experiment. Chromosome specific bar codes obtained from well characterised YAC clones have been shown to be useful for characterising chromosomal rearrangements.[8] By performing multicolour FISH using a set of chromosome 12 specific YAC clones, the type of duplication in the proband was identified as inverted. Moreover, we could also differentiate between the two possibilities of inverted duplication occurring upwards or downwards on the chromosome arm. Using YAC mapping data and GTG banding pattern analysis, the rearrangement in the proband was defined as an inverted duplication in an upward direction involving the segment 12q13-q22. This case illustrates the potential of multicolour chromosome specific bar codes for the characterisation of intrachromosomal rearrangements by a single hybridisation experiment. Other methods, such as CGH and reverse painting, in general allow the accurate mapping of over-represented regions. However, CGH analysis would have failed in this case because of the mosaicism with a high percentage of normal cells. At best, reverse FISH could only allow the differentiation between direct and the two types of inverted duplication. In contrast, the fortuitous determination of a breakpoint YAC from the bar code enables mapping of the duplication at a molecular level, which would be impossible by CGH or reverse painting.

The trisomy 12q13-q22 was detected in 10% of peripheral lymphocytes, in 70% of fibroblasts obtained from the normally pigmented skin, and in 15% of fibroblasts from the hypopigmented skin. Thus, the proportion of abnormal cells in the two different skin areas did not correlate with the skin pigmentation in the patient. This is not surprising as fibroblasts were investigated and it is expected that melanocytes, as the carriers of the biochemical defect, have a different distribution, because the two cell types have different embryological derivations. As a consequence, the selection of the skin biopsy area should not be guided by the localisation of skin pigmentation abnormality, and biopsy of more than one site may be required to identify the chromosomal anomaly in fibroblasts. A deletion 7q was found in two fibroblast cells of the normally pigmented skin, but not in other cells. We do not know if deletion 7q is the result of a cultural artefact or a third cell line in skin fibroblasts. The low proportion of abnormal lymphocytes shows the importance of analysis of a large number of cells. Moreover, a normal karyotype in lymphocytes of HI patients does not rule out a chromosomal defect, as has been shown for many HI associated chromosomal anomalies, which were detected only in fibroblasts but not in blood cultures.[5] Like the proband described here, more than 70% of HI patients have one or more abnormalities of the central nervous system, the musculoskeletal system, facies, epidermal structures, and inner organs. The variability of the features associated with HI can be interpreted as a consequence of different chromosomal imbalances. Our proband also showed asymmetrical clinical features, which can also be considered as a non-specific sign of chromosomal mosaicism and as an indication for repeated chromosomal analysis of different tissues, especially if it is associated with mental retardation or dysmorphic features or both.[9]

The proband's phenotype is different from other patients with trisomy of a more distal part of 12q. Trisomy for the segment 12q13-q22 has not been described previously and a characteristic phenotypic pattern cannot be determined. To our knowledge, only three other patients have been reported with an interstitial duplication 12q.[10 11] All of them showed mosaicism suggesting that non-mosaic interstitial trisomy 12q may be lethal.

Cytogenetic diagnosis of structural mosaicism enables accurate genetic counselling of families. Given that the parents have normal karyotypes, as in the present family, the recurrence risk is very small. Mosaicism of structural abnormalities with a 46 chromosome complement is rare, and a significant proportion of such cases are direct or inverted duplications.[12] If chimerism were excluded, a postzygotic origin of the rearrangement in a chromosomally normal conceptus can be postulated for intrachromosomal duplications, which would rule out the small risk of parental gonadal mosaicism.

In summary, the present case stresses the importance of careful chromosomal analysis of different tissues in patients with pigmentary anomalies or asymmetrical clinical findings or both, and has shown the usefulness of multicolour FISH with single copy probes resulting in chromosome specific bar codes to characterise intrachromosomal rearrangements.

This study was supported by the Deutsche Forschungsgemeinschaft (Sp 460/3-1) and the Friedrich-Baur-Stiftung.

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An analphoid supernumerary marker chromosome derived from chromosome 3 ascertained in a fetus with multiple malformations

EDITOR—We report a case in which a termination of pregnancy for fetal abnormality at 18 weeks' gestation showed a supernumerary marker chromosome. This extra chromosome did not hybridise to any alphoid probes and was found to have a chromosome 3 origin when investigated by M-FISH.

An anomaly ultrasound scan was performed because of raised alphafetoprotein and beta HCG levels at 17 weeks' gestation in a 32 year old, primigravida mother. The scan showed a large and cystic left kidney, banana sign, and absent cisterna magna, and signs of an open sacral spina bifida. The pregnancy was terminated and necropsy showed a male fetus consistent with 18 weeks' gestation with no dysmorphic facial features. A high arched palate with a small amount of postnuchal oedema was noted as well as a single transverse palmar crease on the right hand. Inspection of the back showed a 1.3 cm long lumbosacral myelomeningocele with protruding lower lumbar spinal cord. On internal examination the cerebral hemispheres were fully cleaved and appeared fluctuant suggesting the possibility of internal hydrocephalus. The posterior fossa of the brain was reduced in anteroposterior diameter as well as appearing deep and funnel shaped, and the extension of the cerebellar tonsils was below the level of the foramen magnum. These findings are consistent with Arnold-Chiari malformation. There was marked asymmetry of the kidneys; the right kidney showed normal fetal lobation and shape but the left kidney was very large and had thin, translucent, subcapsular cysts, especially at the lower pole. The cut surface showed a poor demarcation between the cortex and medulla and the presence of cysts in most of the renal parenchyma. These findings are consistent with cystic renal dysplasia. The placenta was unremarkable and the cord had three normal blood vessels.

The chromosomes of the abortus were examined from fetal skin fibroblasts derived using the method of Fisher *et al*.[1] The metaphases from the fetal fibroblasts and parental blood were GTL banded using a modification of the method of Seabright.[2] The abortus showed a male karyotype with a metacentric supernumerary marker chromosome approximately the size of a G group chromosome in 17 out of 30 (57%) metaphases examined in primary cultures. In subsequent passaging of the cultures, the proportion of the cells with the marker rapidly diminished. Both parents had apparently normal karyotypes. A fibroblast cell line (DD3329) and lymphoblastoid cell lines (DD3389 father, DD3390 mother) from both parents are available from ECACC, Porton Down, Salisbury, Wilts, SP4 0JG, UK.

Fluorescence in situ hybridisation (FISH) with nick translated biotin or digoxigenin (Boehringer-Mannheim UK) labelled centromere specific alpha satellite probes were used based on a technique by Pinkel *et al*.[3] The in situ hybridisation was detected using one layer of FITC conjugated anti-avidin for biotin labelled probes or TRITC conjugated anti-digoxigenin for digoxigenin labelled probes. Diamino-2-phenylindole (DAPI) at the rate of 0.05 mg/ml suspended in an antifade solution (Vectashield, Vector Labs, UK) was used to counterstain the chromosomes. A Carl Zeiss Axioskop epifluorescent microscope fitted with a Pinkel Fluorescent No 83 filter series (Chroma Technology) was used to examine the hybridisation, while a cooled charged couple device camera captured the images. Smartcapture software (Digital Scientific, Cambridge, UK) was used to analyse and visualise the digitised data. The normal homologues acted as internal controls for the FISH. The marker was screened with a library of alphoid centromere specific probes at 1 × SSC in 50% formamide stringency, but failed to hybridise to any of the probes, suggesting that what appeared to be the marker's primary constriction did not contain alphoid repeats. This was confirmed when an all centromere alphoid mixture used at low stringency (2 × SCC at room temperature) showed strong signal at all centromeres except for the marker (fig 1a).

Multiplex fluorescence in situ hybridisation (M-FISH)[4] was performed on the marker using the Spectra Vision Assay™ (Vysis). The protocol and probe set was as specified in the SpectraVision™ Assay protocol. The images were captured on a Provis microscope (Olympus) equipped with a motorised eight position turret with an epifluorescence filter set designed for the fluors used. Analysis was performed using M-FISH software supplied by Perceptive Scientific International Ltd (PSI). Using M-FISH, the marker was identified as being from chromosome 3.

FISH with the 3p and 3q subtelomere probes showed hybridisation to the 3q subtelomere probe on the ends of both arms (fig 1b), and wcp3 hybridised to the whole of the marker (fig 1c). Subsequently CGH[5 6] was applied using DNA extracted from fetal skin and testis. The CGH profiles were analysed using Vysis Quips CGH software following hybridisations to 10 metaphases from each tissue. The CGH profiles showed a significant gain of material in distal 3q26 in fetal skin and in DNA extracted from testis, a tissue not cultured in vitro. These profiles suggested that the tetrasomy may not include the most distal 3q bands (q28 and q29); however, CGH profiles at the extreme ends of chromosomes are known to be problematical because of variable repeat sequences. Conventional FISH with YAC 919f12 (3q29) confirmed that the marker contained two copies of this sequence (fig 1d). Owing to the instability of the marker in culture, we were unable to perform any investigations with constitutive centromere binding proteins. The conventional cytogenetics was re-evaluated and suggested that the marker was an inverted duplication from chromosome region 3q26.2→qter.

Molecular analysis was undertaken to check for the biparental inheritance of the two normal chromosome 3 homologues and to find the parental origin of the marker chromosome. DNA was extracted from fetal tissue and peripheral blood from the parents. Primer sets were used to detect polymorphic microsatellite repeat sequences along the length of chromosome 3 and it was found that the marker was maternal in origin and that the fetus had inherited one normal chromosome 3 from each of his parents and so excluded uniparental disomy 3.

As far as we are aware, the marker described here is the first instance of an inverted duplication causing tetrasomy for chromosome region 3q26.2→qter. Our patient had a prenatally detected lumbosacral myelomeningocele, Arnold-Chiari malformation with possible hydrocephalus, and cystic renal dysplasia, and as a result was terminated at 18 weeks' gestation. Arnold-Chiari malformation is seen in approximately 1 in 1000 livebirths[7] and is often associated

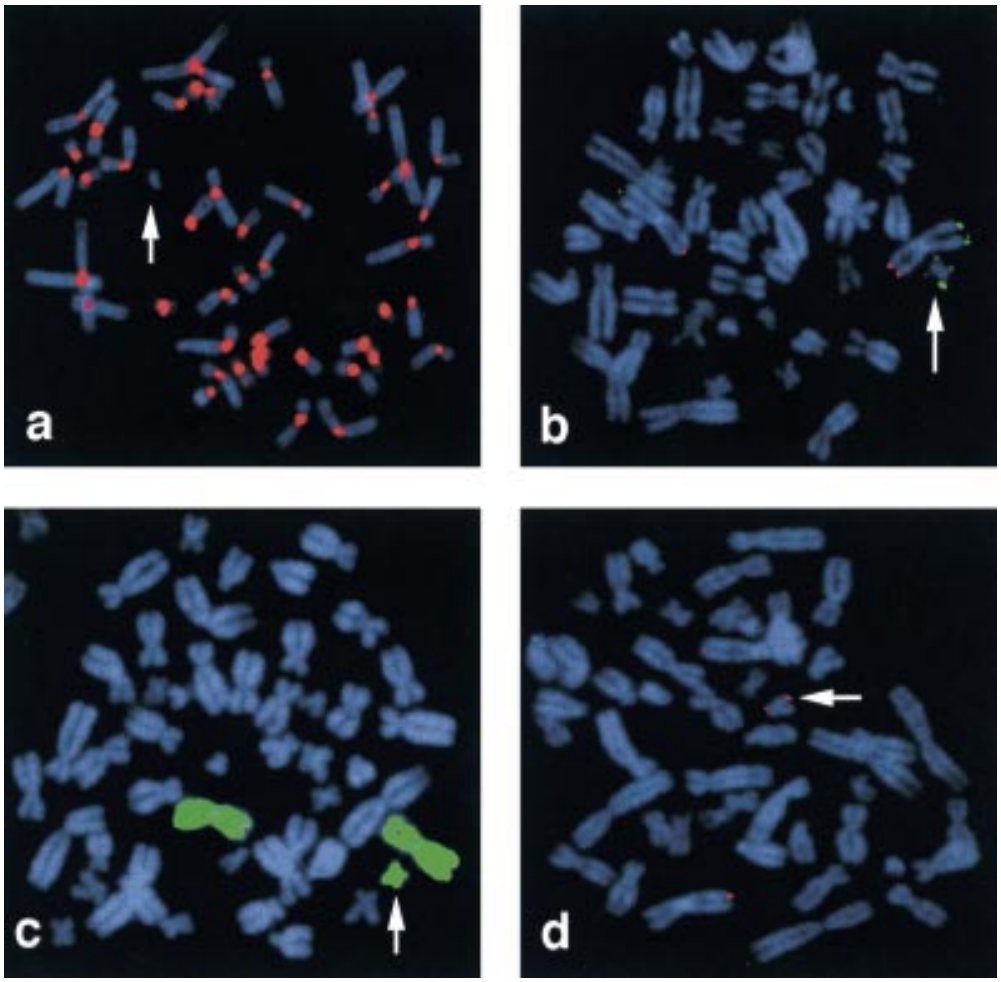

Figure 1 Molecular cytogenetic characterisation of the marker chromosome. Arrow indicates marker chromosome. (a) All centromere alphoid mix. (b) 3q subtelomere probe (196f4) green, 3p subtelomere probe (dJ11286B18) red. (c) Whole chromosome paint 3. (d) 3q29 probe (YAC 919f12).

with spina bifida with myelomeningocele and hydrocephalus. Schinzel[8] observed lumbosacral myelomeningocele and Arnold-Chiari malformation in single incidences of dup 3q23→25→q27 to qter. The marker breakpoint is thought to be at 3q26.2, so the marker contains two copies of 3q26.3, which Ireland *et al*[9] considered to be the location of the Cornelia de Lange syndrome gene and the duplication 3q syndrome critical region.[10] The only features seen which may be associated with de Lange or duplication 3q syndromes, and may also be coincidental, were high arched palate, a transverse crease on the right hand, and left cystic renal aplasia.[11] However, our case does prove the need to do a detailed karyotype where upper and mid neural tube defects are associated with other abnormalities.

Portnoï *et al*[12] reported a similar supernumerary marker chromosome in a healthy 22 year old male of normal intelligence. He was not dysmorphic, but was referred because of skin pigmentary anomalies showing hyperpigmented brown macular streaks following the lines of Blaschko, the onset of which occurred aged 10 to 12 years. The normal skin fibroblasts showed no evidence of the marker, but blood and hyperpigmented fibroblasts showed 30% and 6% cells respectively with the marker. Their marker was analphoid, acrocentric with a breakpoint 3q27.1. The lower level of mosaicism in vivo, proven tissue specificity, and the smaller size of the marker may account for the ameliorated phenotype in this patient compared with our fetus. Another neocentromere located at 3q26 was reported by Wandell *et al*[13] and was observed in a father and daughter, ascertained because of developmental delay in the child along with hypertelorism, epicanthus, and a large head. The father had borderline mental retardation. In this case the normal centromeric region was deleted from the chromosome 3 and had formed a small linear marker chromosome. The two distal portions of the deleted 3 had rejoined and a neocentromere was present at 3q26. Interestingly, the neocentromere formed microtubule associated kinetochores of the same size as other large chromosome kinetochores, but was found to be weakly positive with anticentromere (CREST) antibodies, whereas the normal centromere on the small marker chromosome showed a reduced kinetochore size but a strong CREST antibody signal.

Our marker increases the haploid autosomal length of the cell by about 1.5%, but is mosaic (57%) in primary cultures. Other analphoid markers which give rise to tetrasomies of the duplicated regions are also found to be unstable in long term or fibroblasts cultures and are often lost altogether.[14] It seems remarkable that these markers seem to be stable for many cell generations in vivo only to be lost so rapidly in culture. This instability in vitro makes it difficult to judge how much effect our marker had on the phenotype, although we know that it was present in tissues from two different embryonic lineages (fetal skin and testis).

The centromere is an essential structure of the chromosome and chromosomes lacking an active centromere will eventually be lost during subsequent cell divisions. The

centromeric DNA is composed of highly repetitive A+T rich sequences. The most investigated is alpha satellite DNA which in humans is a 171 bp sequence tandemly repeated many times such that between 2 and 4 Mb may be present in a typical centromere.[15] There seems to be no similarity in the primary DNA sequence between species and a lack of centromeric DNA conservation throughout evolution makes it difficult to equate its sequence to function.[16] Nonetheless, the repetitive nature of the DNA and its A+T content appears to be a consistent feature of many organisms and suggests that it is significant in centromere function.[17]

Supernumerary marker chromosomes (SMCs) have a prevalence of less than 1 in 1000 in the general population[18] and in recent years in situ hybridisation using alpha satellite probes allows the origin of most of the SMCs to be identified. However, a minority do not hybridise to any of the alphoid probes,[19] but nevertheless these analphoid markers are more or less stable in vivo and in vitro,[14 20] suggesting the presence of some centromeric properties, unlike a true acentric chromosome. Two main explanations have been suggested. Firstly, a complex rearrangement has deleted the normal centromere to such an extent that, although it can still function, the highly repetitive alpha satellite probes cannot hybridise to it. Secondly, when the normal centromere was lost, a latent centromere (or neocentromere) was activated in a region not normally associated with centromeric function.[16 21 22] This latter explanation is currently more favoured. Unfortunately, as marker chromosomes tend to be found by chance, only the endpoint is seen, never the intermediate steps nor the mechanism in action by which the neocentromere may be formed.[23]

Recent sequencing of the centromeric region of a chromosome 10 derived analphoid marker has shown that compared with the sequence of a normal centromere the marker centromere is lacking in repetitive sequences. The evidence from this neocentromere, and that from the deactivation of centromeres in dicentric chromosomes, is more proof that repetitive sequences per se do not dictate centromere function. In sequencing the chromosome 10 neocentromere, it was found that although the A-T content was no different from that of the rest of the genome, there was evidence of A-T rich islands but the significance of this remains unknown. Nor did the neocentromere sequence differ significantly from the homologous region in the normal chromosome 10 and there was no major feature present similar to known centromeric DNA. The complex rearrangement hypothesis seems unlikely because so much of the material that makes up a normal centromere is missing. So it does seem that a neocentromere forms from a latent centromere activating in a region not known to be centromeric.

Fewer than 35 neocentromeric markers have been reported to date, but they are probably more frequent than this figure suggests because of past difficulties in identification, and it is likely more will be recognised and characterised in the future.[14] Although at least 11 different chromosomes have been implicated in the formation of neocentromeres, it is interesting that our case is the third to be reported in which a neocentromere has been activated in the region near 3q26. Alphoid SMCs have duplicated material from around the centromere, whereas neocentromeric markers will allow us to investigate the effects of duplicated genetic material from other chromosomal regions.

We are grateful to Miss Sarah Beal for excellent technical assistance and we would like to acknowledge Andrew Sharp for the parental origin studies. We wish to thank Dr L Kearney and the National Institutes of Health and Molecular Medicine Collaboration for the subtelomeric probes and also wish to thank the Wellcome Trust for their financial assistance in the purchase of the image enhancement equipment. The CGH profiles were obtained during the "Advanced Molecular Cytogenetics" course held at Cold Spring Harbor Laboratory, March 1999. JAC would like to thank the course tutors, Dr Thomas Ried and Dr Evelin Schröck, for their expert help during the course, and the Wellcome Trust for financial assistance to attend Cold Spring Harbor.

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J Med Genet 2000;**37**:810–815

Why patients do not attend for their appointments at a genetics clinic

EDITOR—When a patient does not attend a scheduled appointment, or cancels so late that a replacement cannot be found, there is a cost to the health care system in terms of personnel time, extended waiting lists, and the loss of potentially beneficial services to patients who miss their visit. These costs are particularly important for genetics clinics because a great deal of preparation is often required before a clinic visit. Preparation may include sending out a family history questionnaire from which a pedigree diagram is constructed, and a review of the medical reports and charts of the patient and other family members. In the case of rare genetic conditions, a preliminary review of publications/computer database search may be conducted and research laboratories may be sought which would be willing to receive patient samples. Furthermore, genetics departments typically set aside at least an hour for each new patient visit.

Failed appointment rates at community and university medical clinics have been reported to range between 10 and 30%.[1 2] Studies involving hospital clinics set in low socioeconomic status populations have shown no show rates in the upper end of this range, whereas family practice clinics have reported fail rates as low as 5%.[3]

There is some evidence to suggest that missed appointments may be more likely among certain demographic groups, such as young adults and adults with young children,[3] patients with lower socioeconomic and educational status, and those with larger families.[1] Moreover, geographical distance from the clinic or the inability to obtain transport or both have been found to impede appointment keeping.[1] Sex and race have not been associated with compliance.[1]

Problems with communicating to patients about the timing or nature of an appointment and in providing them with information about their diagnosis may lead to missed appointments,[1 4] and a strong recommendation by the referring physician has been shown to have a major benefit on compliance.[5–7]

There may be a relationship between clinic attendance rates and certain attitudinal factors. There is evidence that patients are more likely to miss their appointments if they perceive the appointment as less urgent[1 3] or less helpful.[4] Other potential psychological determinants of health care use are variables from the Health Belief Model (HBM),[8] including people's perceived risk of developing a particular health condition, perceived severity of the health condition, and the perceived benefits, weighed against the costs, of an associated health behaviour. The HBM has been applied to a variety of health behaviours, such as breast cancer screening practices.[5 6 9] Patients' beliefs about the personal costs of medical clinic visits have also been shown to affect appointment keeping rates.[10]

The Children's Hospital of Eastern Ontario (CHEO) genetics clinic provides diagnostic and counselling services to patients of all ages, including routine advanced maternal age (AMA) counselling, personal or family history of known genetic disease, and the assessment of subjects whose condition is of unknown cause. At a time when clinical demands on our programme are increasing, we became concerned about the negative impact of no shows on our ability to deliver efficient and timely services. On that basis we undertook a study in order to determine the approximate rates of appointment cancellations and no shows at different Canadian genetics clinics, and to identify factors that may be associated with missing clinic appointments. It was hoped that some associated variables might be amenable to modification and lead to improved attendance rates.

Twenty genetics clinics across Canada responded to a survey regarding the frequency of broken appointments (no shows and cancellations). The centres provide genetic services free of charge as part of their respective provincial health services. The non-attendance rate at the CHEO genetics clinic was also determined. The clinics were separated into three groups according to number of patients seen per year; eight clinics had fewer than 500 patient visits per year (small), eight saw between 500 and 2000 patients per year (medium), and five clinics saw more than 2000 patients per year (large). Representatives of each genetics clinic, usually a medical geneticist or clinic administrator, completed a single page postal questionnaire designed to assess their estimated rates of missed appointments, the extent to which they considered these rates to be a problem, and the strategies they used to reduce non-attendance.

The CHEO genetics clinic operates according to the following pre-appointment procedure. Patients are referred to the clinic by their physician. The clinic receptionist schedules the appointment and, for non-AMA cases, sends the patient a family history questionnaire and consent form for release of medical information. Before the clinic appointment, the patient's case is reviewed with relevant documents and, for non-AMA patients, a family pedigree is drawn. Non-AMA patients are contacted by telephone 24-48 hours before the scheduled visit in order to confirm their attendance (AMA patients do not receive a reminder telephone call). At all stages, patients are asked to cancel if they do not plan to attend their clinic visit.

Data were collected by telephone from two groups of patients originally scheduled for clinic between 1 February 1998 and 30 April 1999: 75 who attended their appointments at the CHEO genetics clinic and 62 who either did not show up for their appointments or who cancelled with less than 12 hours notice. It should be noted that late cancellations (less than 12 hours notice) were counted as no shows because the ensuing consequences were considered to be equivalent. The other surveyed genetics clinics provided separate rates for cancellations in general and for "pure no shows".

A parent was interviewed if the index patient was under 18 years of age. All participants (total n=137) were English or French speaking and lived in the Ottawa-Carleton regional catchment area of approximately 1 million.

Two slightly different versions of the survey instrument were used, one for each group of participants. The instrument was developed by the authors to assess information in four main content areas: (1) demographics (age, marital status, children, education, family income, language spoken at home); (2) referral and genetic service information (reason for referral, the degree to which patients understood these reasons, the quality of explanations provided by referring physicians regarding these reasons, whether or not patients were referred at their own request, and the degree to which referring physicians recommended the genetics appointment); (3) environmental factors (transport, distance from home to the clinic, and arrangements for child care and taking time off work); and

Table 1 Rates of no shows and cancellations at Canadian genetics clinics

	Size of centre*			
	Small (n=8)	Medium (n=8)	Large (n=5)	Total (n=21)
Mean no show rate (%)	9.3	4.7	5.7	6.6
Mean cancellation rate (%)	7.3	5.9	7.0	6.7
Mean combined no show and cancellation rates (%)	15.1	10.0	10.8	12.0
% of centres that indicated non-attendance is a problem	50	75	80	67

*Small = <500 visits/year; medium = 500–2000 visits/year; large = >2000 visits/year.

(4) psychosocial factors (including perceived importance of the clinic visit as well as Health Belief Model variables). A variety of response formats were used, including yes/no, Likert scales, and open ended questions which were later categorised for analysis. The bilingual survey took approximately 15 minutes to complete and was administered by a trained research assistant.

Descriptive statistics were performed to assess the nature of the scheduled appointment at the clinic, as well as the reasons provided by non-attendees for missing their scheduled visit. Attendees and non-attendees were compared on the basis of variables in the four main content areas described above, using independent sample *t* tests and chi-square analyses as appropriate. Two by two factorial analyses of variance were also conducted in order to assess potential interaction effects between group membership and other relevant variables.

Representatives of 27 Canadian genetics clinics were sent copies of the no show survey and 20 (74%) completed and returned the survey. A summary of no show and cancellation rates for the three sizes of clinics is provided in table 1; data from the CHEO genetics clinic are included. Approximately half the data were estimated while the remainder were based on actual records of missed visits. The rate of combined no shows and cancellations at individual centres ranged between 2% and 25%, with an overall mean of 12%. Non-attendance rates were perceived as a problem by most genetics clinics across Canada. However, large and medium centres perceived a greater problem than did smaller centres, despite having lower non-attendance rates (mean rates were 10.8%, 10.0%, and 15.1% for large, medium, and small centres, respectively). If cancellations are eliminated, the mean overall rate of no shows is 6.6%.

For comparison purposes, failed appointment rates were obtained for a variety of other medical clinics at CHEO, including audiology, child development service, neuromuscular medicine, occupational therapy, physiotherapy, rehabilitation medicine, speech/language, and spina bifida clinics. The no show rates for 1998 ranged between 6% and 15%, yielding an overall mean of 11%.

The no show prevention strategies cited by the genetics clinics across Canada were compared. The centres were subdivided by their total non-attendance rate into three groups: low rate (5-11%), medium rate (12-18), and high rate (19-25%). The impact of the strategy most commonly cited (reminder phone call) could not be assessed because it was used virtually universally. However, the strategy of sending reminder letters to the patient was used most often by centres which reported low non-attendance rates.

A total of 258 households (120 potential attendees and 138 potential non-attendees) were telephoned regarding the study, of which 137 (53%) participated in the survey (75 attendees and 62 non-attendees). Among the subjects/families who did not participate, 24 (20%) could not be reached because of repeated busy signals or answering machines (no messages were left). Another 24 (20%) asked the interviewer to phone back at another time, and gave a similar response upon subsequent phone calls, even when the call was made at a prearranged time. Nineteen (17%) of the telephone numbers were either not in service or were wrong numbers, and two of the households (2%) reported that the patient (or parent of the patient) lived elsewhere and did not have a telephone. An additional 12 (10%) subjects had a language barrier. Finally, 30 (25%) of the non-participants stated that they were not interested in doing the survey. There were more no shows (76; 63%) than clinic attendees (45; 37%) among the households that did not participate in the survey.

Table 2 Demographic characteristics of the sample

	Attendees (n=75)		Non-attendees (n=62)	
Variable	No	% of group	No	% of group
Age of respondent				
20–29 years	6	8	9	15
30–34 years	12	16	13	22
35–40 years	43	58	34	56
>40 years	13	18	4	7
Marital status				
Married/common law	65	87	50	81
Single	5	7	7	11
Divorced, separated, or widowed	5	7	5	8
No of children				
0	21	28	10	16
1	24	32	18	29
2–3	27	36	29	47
>3	3	4	5	8
Planning more children*				
Yes	42	56	28	45
No	23	31	31	50
Language spoken at home				
English	48	64	42	68
French	19	25	8	13
Both English and French	3	4	8	13
Other	5	7	4	6
Education†				
Less than high school	4	5	4	7
High school	15	20	20	32
Some college or university	12	16	15	24
College or undergraduate degree	30	40	17	27
Graduate degree	14	19	6	10
Family income‡				
<$20 000	6	9	4	8
$20 000–$40 000	16	25	18	36
$40 000–$75 000	19	30	20	40
>$75 000	23	36	8	16

*Attenders had a higher overall education level than non-attenders, t (135) = −2.29, p<0.05.
†Attenders were more likely to be planning more children than non-attenders, $\chi^2(2) = 6.58$, p<0.05.
‡Numbers are smaller for this variable owing to missing data.

Table 2 provides a description of participants on the basis of key demographic variables. The average age of respondents was 36 years; where the respondent was a parent of the patient, the average age of patients was 5 years. The large majority of respondents were married/common law (84%) and had children (77%).

Attendees and non-attendees did not differ significantly on the basis of age (p=0.21), marital status (p=0.59), presence of children (yes or no; p=0.21), number of children (p=0.09), language spoken most often at home (p=0.11), or family income (p=0.11). Two demographic variables did yield significant results. Non-attendees had a lower mean level of education than attendees (p<0.05), and patients who were planning to have children (or more children) were more likely to have attended their clinic visits than those who were not planning on starting or expanding their families (p<0.05) (table 3).

Table 3 outlines the main reasons given by non-attendees for missing appointments. The reasons differed significantly between those who were referred for prenatal diagnosis (PND) services and those referred for other reasons (p<0.05). The main reasons given by all

Table 3 Reasons given by non-attendees for missing genetics clinic appointments

	Reason for referral to genetics			
	PND* (n=31)		Non-PND* (n=31)	
Reason given for missing appointment	No	%	No	%
Too busy/no time off work	8	26	14	45
Forgot/scheduling conflict	4	13	7	23
Believed appointment was non-mandatory or unimportant	4	13	1	3
Wanted to wait for lab results	3	10	1	3
Other	1	3	6	19
Unsure/afraid re risks of amniocentesis	4	13	1	3
Recent miscarriage	4	13	0	0
Morning sickness on day of appointment	3	10	1	3

PND = prenatal diagnosis.
*Reasons differed between PND and non-PND patients: $\chi^2(7) = 15.64$, $p<0.05$.

non-attendees for missing appointments were being "too busy" or unable to get time off work, forgetting the appointment or having a scheduling conflict, and believing the appointment to be non-mandatory or unimportant. Reasons associated with the PND group included being unsure or afraid of the risks associated with amniocentesis, having recently suffered a miscarriage, experiencing morning sickness on the day of the appointment, and wanting to wait for laboratory test results (such as maternal serum screening) before deciding whether to be seen at the genetics clinic.

Slightly less than half (46%) of all participants were scheduled to meet a medical geneticist at the clinic, and the remainder were scheduled to meet a genetic counsellor. There was no significant difference between attendees and non-attendees with respect to the type of health professional they were scheduled to see at the clinic ($p=0.86$). Approximately half (51%) of all participants were scheduled for appointments associated with advanced maternal age prenatal screening. Another 42 patients (31%) were being seen in order to seek a specific diagnosis; 23 patients (17%) were scheduled to discuss genetic risk information/carrier screening, and only two respondents (1.5%) indicated that they did not know the reason for their referral to the genetics clinic. Attendees and non-attendees did not differ on the basis of their reasons for referral to the clinic ($p=0.46$). Moreover, attendees were no more or less likely than non-attendees to have been referred to the clinic at their own request ($p= 0.92$), to understand well the reasons for their referral ($p=0.17$), or to indicate that their physicians had highly recommended the appointment ($p=0.15$) or had explained the reason for referral well ($p=0.13$).

It was hypothesised that some of the referral related variables (table 4) may have been confounded with patients' education level, and so additional analyses were performed in order to separate these effects. A dichotomous version of the education variable (originally in a Likert scale format) was created by cutting scores at the median, yielding the two categories of "high" (at least a college diploma or university undergraduate degree) and "low" (high school education or less). Two by two analyses of variance showed a significant group by education level interaction with regard to patients' reported degree of understanding of the reasons for their referral ($p<0.05$) and the reported quality of the explanation of these reasons by their referring physicians ($p<0.05$). An examination of cell means indicated that among patients with a higher education level, attendees understood their reasons for referral better and claimed their physicians had explained these reasons better than did non-attendees. No such group difference existed among patients with a lower education level.

Table 4 Comparison of attendees and non-attendees on referral/genetic service variables

	Attendees (n=75)		Non-attendees (n=62)	
Variable	No	% of group	No	% of group
Reason for referral to genetics				
Prenatal diagnosis	39	52	31	50
Carrier testing/risk information	12	16	11	18
Diagnostic evaluation	24	32	18	29
Other	0	0	2	3
How well was reason explained?*				
Very poorly	0	0	1	2
Poorly	2	3	3	6
Adequately	8	14	10	20
Well	14	25	12	25
Very well	33	58	23	47
How well did you understand the reason?*				
Not at all	0	0	2	3
Poorly	2	3	2	3
Adequately	3	5	4	7
Well	9	15	12	22
Very well	48	77	35	65
Referred at own request				
Yes	26	35	22	36
No	49	65	40	64
How strongly referring physician recommended the genetics appointment*				
Not strongly	5	8	7	13
Somewhat strongly	20	30	22	40
Quite strongly	15	23	9	16
Very strongly	25	38	16	29
Recommended against	1	1	1	2

*Numbers are smaller for these variables owing to missing data.

We hypothesised that patients with a higher education level would more likely have been referred to the genetics clinic at their own request, because of their presumed greater knowledge about genetic health services. A chi-square analysis showed this not to be the case ($0.55<p<0.60$).

The relationship between non-attendance and environmental variables is shown in table 5. Patients who reported having to arrange for child care in order to attend a clinic visit were more likely to have missed their scheduled appointments than those who indicated no such requirement ($p<0.05$). Although having to take time off work to attend a clinic visit was not associated with a greater likelihood of non-attendance ($0.70<p<0.75$), those respondents who were not paid for such time off were more likely to have missed their appointments than those who were able to take paid leave from work to attend the clinic ($p<0.05$). No significant differences between attendees and non-attendees were found in methods of transport to the clinic ($0.20<p<0.25$) or travel time to the clinic ($0.10<p<0.15$). Failure to make contact by telephone to remind the patient of the clinic visit occurred in 10 of the no shows and none of the attendees.

The relationship between non-attendance and psychosocial variables is shown in table 6. Respondents who attended their appointments perceived the clinic visit to be more important than respondents who did not attend ($p<0.01$). With regard to the Health Belief Model variables, no significant group differences were found in terms of perceived severity of the health condition (or potential health conditions) which were to be discussed during the appointment ($0.65<p<0.70$). Moreover, the two groups did not differ significantly with respect to their perceived risk of having (or eventually developing) these health conditions ($0.60<p<0.65$).

Non-attendance was significantly related to perceived benefits and disadvantages of the genetics appointment. Specifically, patients who missed their appointments perceived the potential benefits of the clinic visit to be less

Table 5 Comparison of attendees and non-attendees on environmental variables

Variable	Attendees (n=75)		Non-attendees (n=62)	
	No	*% of group*	*No*	*% of group*
Have to arrange for child care to attend the clinic?*				
Yes	12	16	19	31
No	63	84	43	69
Have to take time off work to attend the clinic?				
Yes	39	52	30	49
No	36	48	31	51
If yes, paid for this time off?*				
Yes	23	60	10	33
No	15	40	20	67
Method of transport to clinic				
Own vehicle	65	87	48	77
Get a ride	7	9	6	10
Bus	3	4	6	10
Walk	0	0	2	3
Travel time to clinic				
<15 minutes	4	5	4	7
15–30 minutes	35	47	24	39
30–45 minutes	18	24	10	16
45–60 minutes	7	9	7	11
>60 minutes	11	15	17	27

*p<0.05.

Table 6 Comparison of attendees and non-attendees on psychosocial variables

Variable	Attendees (n=75)		Non-attendees (n=62)	
	No	*% of group*	*No*	*% of group*
Perceived importance of clinic visit*				
Not at all important	1	1	6	10
Of little importance	4	5	5	8
Neutral	8	11	10	16
Somewhat important	16	21	2	3
Very important	46	62	27	43
Perceived severity of genetic condition				
Not at all serious	8	11	11	18
Of little severity	14	19	13	21
Neutral	18	24	5	8
Somewhat serious	12	16	14	23
Very serious	22	30	18	30
Perceived risk of genetic condition				
Very low	16	21	19	32
Somewhat low	13	17	11	18
Medium	26	35	12	20
Somewhat high	11	15	7	12
Very high	9	12	11	18
Perceived benefits of clinic visit*				
No benefits	1	1	7	12
Few benefits	4	5	4	7
Neutral	10	13	10	17
Some benefits	14	19	13	22
Many benefits	46	62	25	42
Perceived importance of disadvantages of clinic visit*				
Not at all important	56	74	30	52
Of little importance	6	8	5	8
Neutral	8	11	8	14
Somewhat important	2	3	3	5
Very important	3	4	12	21

*p<0.01.

important (p<0.01) and the potential disadvantages to be more important (p<0.01) than patients who kept their appointments.

The results of this study help to quantify the problem of missed appointments at genetics clinics across Canada. Over a one year period, approximately 11% of patients scheduled for visits at genetics clinics either cancelled or did not show up for their appointments. When cancellations are eliminated, this number decreases to 6%, representing the rate of "pure no shows". In comparison, 11% of patients failed to show up for their appointments at other non-genetics outpatient clinics at the Children's Hospital of Eastern Ontario (CHEO). These values are relatively low compared with the 10-30% range frequently reported.[1 2] Family practice centres have fewer broken appointments than adult medical centres.[3] Perhaps the family orientation of paediatric and genetics clinics is more akin to family practice and reduces the likelihood that appointments will not be kept.

It should be noted that in the survey of the CHEO genetics clinic, the non-attendees group included cancellations with less than 12 hours notice which, owing to their negative consequences, were considered equivalent to no shows. This distinction between early and late cancellations was not requested from the other genetics clinics surveyed in this study (they were simply asked to report two separate numbers for "pure" no shows and cancellations, respectively). If late cancellations had been included in the no show rates provided by the other clinics, the resulting no show rates would probably have been somewhat higher than reported.

Although an 11% rate of failed appointments at Canadian genetics clinics is comparatively low, it is still a sizeable barrier to the optimal provision of genetic health services. Unlike many clinics, where multiple bookings are common and visits are usually short, genetics clinics expend extensive resources for pre-appointment planning and generally allocate an hour for a clinic visit.

This study suggests a number of factors which may contribute to the problem of missed appointments. The most commonly stated reasons for missed appointments at the CHEO genetics clinic were scheduling conflicts and inability to get time off work. As these are predictable factors, it is surprising that more than half of respondents cited them, despite being called 24-48 hours before the appointment. The question arises as to whether the answers provided by patients over the telephone concealed underlying reasons such as anxiety, confusion, or personal belief systems, which lead to last minute decisions not to attend, or were too personal to admit to the secretary or the research assistant.

Some psychosocial variables appeared to contribute to the non-attendance rate. Patients who attended their scheduled appointments thought their visit to the genetics clinic to be more important than those who did not attend. This is similar to previous research which has found perceived urgency of medical clinic visits to be significantly related to compliance,[1 3] though urgency and importance may represent relatively distinct constructs.

Patients who attended the clinic also perceived more benefits and fewer barriers associated with their appointments than did non-attendees. This supports the Health Belief Model[8] and previous research, which has indicated such health beliefs to be important predictors of health behaviours.[6 9 10] Apparently, for non-attendees, the potential costs of the clinic visit (such as the anxiety or guilt that may be experienced after learning of one's carrier status) outweighed the possible benefits (such as relief from uncertainty or clarification of one's options). In contrast, the perception of the benefits of attending the genetics clinic appeared to outweigh the perceived disadvantages for those who kept their appointments.

We found that certain psychosocial factors may play a role in non-attendance, but not all of the anticipated effects were found. For example, perceived severity of the health condition (or potential health condition) for which patients were referred to the clinic, along with the perceived risk of having or eventually facing that health condition, were unrelated to clinic attendance. The former result is not surprising, given that the "perceived severity" component of the Health Belief Model has been shown to have limited value in predicting a variety of health behaviours.[11] However, a high perceived risk has been found to predict a number of health behaviours, including mammography,[5 12] genetic testing for susceptibility to breast cancer,[13 14] and

cystic fibrosis carrier screening.[15] One possible explanation for the absence of such an effect in this study lies in the nature of the "health condition" in question. Previous research has examined patients' health beliefs with respect to a single, specific illness or procedure. In the present study, patients were referred to the genetics clinic for a variety of reasons; consequently, a more general question regarding perceived risk was included in the survey which permitted patients to respond according to their own situation. This lack of specificity may have led to a varied interpretation of the question, thus reducing the strength of the "perceived risk" and "perceived severity" variables.

If the above health beliefs are the true reasons that underlie the stated explanations (for example, could not get time off work) given by some patients for missing their appointments, this holds important implications for the provision of adequate public education regarding genetics related health issues. It is widely accepted that a thorough explanation of the risks and benefits associated with a given medical intervention is required in order for patients to provide informed consent. It is not clear how such standards extend to different contexts, such as a genetics clinic appointment. Greater public awareness of the services provided by genetics professionals will provide patients with a balanced and accurate understanding of the importance, risks, and benefits of a scheduled appointment. The fact that patients with a higher education level (at least an undergraduate university degree) were more likely to attend their appointments than those with less education reinforces this need for greater public education. Studies have found that people of lower educational status are more likely to miss their medical appointments.[1] People with a university education probably have greater access to (and experience with) health related information found in scientific journals, books, and news magazines. Delivery of health information to those who have less formal education may facilitate the use of health services by more subjects and families. An alternative interpretation of the education effect is equally possible: those working in higher level positions may simply have greater job flexibility and may thus have had less difficulty attending their clinic appointments.

Other demographic variables, including age, marital status, number of children, language spoken most often at home, and family income, did not differentiate between attendees and non-attendees. This is encouraging, in that most of these variables are not easily targeted by clinic based interventions to improve attendance rates. One exception may be language, which has been subject to limited previous research and is an important variable to assess in a country with two official languages; in our genetics clinic services are regularly provided in French and English. Our finding that language does not appear to be a barrier to attendance is encouraging, but the question may need to be addressed for patients who do not speak either official language. People not fluent in English or French were excluded from the sample. There were 12 people who did not participate in the survey because of a language barrier. Nine of these were in the group who missed their appointments, so this is an issue that may merit further investigation, as a language barrier would represent a potential target for intervention.

It should be noted that the lack of significant results for many demographic variables may be in part because of the narrow response ranges associated with the corresponding survey items. For example, the mean age of survey respondents was quite young (36 years) and people over the age of 45 were virtually absent from the sample. Studies of adult non-genetics clinics would encompass a wider range of patient ages, leading to a greater likelihood of significant age effects. Moreover, the majority of participants were married (84%) and had children (77%). Only 10 participants (7%) reported a family income of less than $20 000, indicating a clear under-representation of low socioeconomic status families. The lack of any association with family income may also reflect universal access under the Canadian health care system. Previous studies showing significant effects of socioeconomic status have generally been conducted in the United States, a country which lacks universal health care.

The quality of information given to patients by their referring physicians about the referral to genetics was not associated with the overall rate of missed appointments. However, it did play a role in the specific subgroup of patients with a higher education level. Among those participants, the attendees reported that they understood the reasons for their referral better, and that their physicians had explained these reasons better, than those who did not attend. It appears that while patients with a higher education level are more likely to attend their appointments, they are also more likely to require thorough explanations of the rationale for the appointment before deciding whether or not to attend. Thus there may be some argument for improving the quality of pre-appointment information provided to patients by health care professionals.

These results lend some support to previous research indicating that patients who understand the reasons for their referral relatively well are more likely to attend their clinic appointments.[4] Contrary to previous studies,[5 7] however, the degree to which the referring physician recommended the genetics clinic appointment did not influence the likelihood of attendance.

Finally, although difficulties with scheduling conflicts, transport, and inability to take time off work may often at least in part conceal more important underlying reasons for non-attendance, they may sometimes compromise appointment keeping. Patients who had to arrange for child care in order to attend the clinic were more likely to miss their appointments, and this confirms previous results indicating that broken medical appointments are more likely among larger families and those with younger children.[1 3] The need for child care is an important barrier to the provision of health services at a genetics clinic because families comprise most of the patient population. Provision of day care facilities within medical institutions might prove cost effective, given that all clinics experience a similar, if not higher, no show rate.

The need to take time off work for a clinic appointment does not appear to present a significant barrier to appointment keeping, except for those who are required to take time off without pay. This problem could be solved if clinics extended their hours of operation, so as to accommodate work schedules.

Contrary to previous research,[1] other practical matters such as distance from the clinic and available method of transport were unrelated to rates of failed appointments at the CHEO genetics clinic. Such effects were lacking, despite the fact that patients reported a wide range of travel times, including 28 patients (20%) who indicated that it took them more than an hour to get to the hospital. It may be that once the patient has made an assessment that the appointment is important, travel time may be irrelevant. We cannot draw any conclusions regarding any effects of transport method, because the vast majority (83%) of respondents drove their own vehicles to the clinic. This probably reflects the high mean socioeconomic status of the current sample; studies involving less privileged people

might show that lack of a convenient mode of transport is a significant obstacle in appointment keeping.

Our patient sample does not reflect the general population and is a limitation of the present study. Participants were highly educated and reported relatively high family incomes, which may account for the slightly lower rate of non-attendance at this clinic, as compared with the rates seen in previous studies which have focused mainly on low socioeconomic status populations. It is possible that factors other than those we have evaluated, or that we have found negative in our sample, may contribute to non-attendance rates in patients with a lower educational and socioeconomic status. Nevertheless, our results support some findings of previous studies which included low socioeconomic status populations.[3 4 10] Moreover, the fact that our non-attendance rate was consistent with those reported by genetics clinics across Canada suggests that these rates reflect an accurate portrayal of the problem in the context of genetics health care in Canada.

Other limitations of this study include the limited generalisability of results to the United States health care system, as well as the telephone interview methodology. For non-attendees in particular, the latter approach may have restricted the degree of candour in participants' survey responses; future studies may benefit from the use of a more anonymous data collection format. Finally, approximately half of the surveyed genetics clinics provided estimated as opposed to actual rates of non-attendance. Estimation may not accurately reflect no show rates; however, a comparison of means indicated that the actual and estimated numbers were similar.

Results of this study suggest a number of potential targets for improving attendance rates at genetics and other outpatient medical clinics. Better education of patients about their medical condition, the nature and purpose of specific options available to them, and the costs and benefits associated with such options, is clearly indicated. To be maximally effective, such education should come from a variety of sources, including the mass media, pamphlets distributed to pharmacies and medical clinics of all types, and, most importantly, open and detailed communication between patients and physicians. Some clinics could also attempt to extend or modify their hours of operation and on site child care initiatives could be explored. These larger scale approaches, in combination with more traditional methods such as telephone and mailed appointment reminders, may help to replace lengthy waiting lists and wasted physician time with more efficient and far reaching health care services.

Support for this project was provided by the Children's Hospital of Eastern Ontario (CHEO) Research Institute. We thank the Canadian genetics centres for their contribution to data collection in the first phase of this study. We also thank Heather Tindell and Laura Van Houten for their role in collecting data at CHEO, as well as the participants for their time and cooperation.

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CORRECTION

In the April 2000 issue of the journal, in the paper by Mortier *et al* on "Report of five novel and one recurrent *COL2A1* mutations with analysis of genotype-phenotype correlation in patients with a lethal type II collagen disorder", the mutation T1191N should have been T1190N throughout.