Letters to the Editor

SHOX point mutations in dyschondrosteosis

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Dyschondrosteosis (DCS) has been recently ascribed to mutations of the *SHOX* gene on the pseudoautosomal region of the X and Y chromosomes.^{1,2} Most cases are accounted for by large scale deletions³⁻⁷ and only two point mutations have been hitherto identified in exon 4 (R195 X and Y199X^{1,2}). Here, we show that point mutations in various regions of the *SHOX* gene also play an important role in the pathogenesis of the disease.

A total of 22 affected subjects belonging to eight families were included in the study. Inclusion criteria for affected status were short stature (2 SD below normal) with short forelimbs and distal radioulnar deformity on forearm x rays.

The 22 patients and their relatives were genotyped using microsatellite DNA markers of the pseudoautosomal region (CA-*SHOX*, DXYS233, DXYS234, DXYS228). Linkage studies supported the mapping of the disease gene to Xp22.3 in all families and hemizygosity at the CA-*SHOX* locus was observed in three families (families 1-3, data not shown).

In families 4-8, PCR amplification and sequence analysis of the five translated exons of the *SHOX* gene (table 1) led to the detection of five different mutations which consistently cosegregated with the disease (table 2). In family 4, a two base pair deletion in exon 2 led to a frameshift and a translation termination at codon 77. In families 5 and 6, base changes at nucleotides 334 and 445 respectively created a stop codon in exon 3 (Q112X and E149X). In family 7, a GT insertion at codon 161 led to a premature stop codon. All four mutations resulted in a premature translation termination. Finally, in family 8, a C→T transition in exon 4 changed an arginine into a cysteine in the protein (R173C). This mutation was found in the affected mother and her three affected children but neither in the healthy father nor in 90 control chromosomes.

We have reported on *SHOX* deletions and a nonsense mutation in DCS (seven deletions,

Table 2 *SHOX* point mutations identified in DCS patients

	Sequence change	Exon	Protein effect
Family 4	106-107 del CG	2	77X
Family 5	334 C→T	3	Q112X
Family 6	445 G→T	3	E149X
Family 7	481 ins GT	3	181X
Family 8	517 C→T	4	R173C

one nonsense mutation¹). Studying eight additional DCS families, we describe here three deletions and five point mutations of *SHOX*. Taken together, these data suggest that *SHOX* deletion is the most frequent common disease causing mechanism in DCS (10/16, 62.5%) and that point mutations also account for a significant fraction of our patients (6/16, 37.5%).

This study also supports the view that haploinsufficiency is the most frequent mechanism in DCS (10 deletions and five nonsense and frameshift mutations in 15/16 families). Among the five novel mutations reported here, only one was a missense mutation resulting in an amino acid change in the homeodomain of *SHOX*. Interestingly, this family was not clinically different from the others. Indeed, all patients had short stature and x ray deformity of the forearms although intrafamilial variability was consistently observed with males being usually less severely affected.

In conclusion, this study shows that point mutations of the *SHOX* gene account for a significant number of DCS patients and shows that haploinsufficiency is the most frequent disease causing mechanism in DCS.

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Table 1 Detailed PCR conditions for *SHOX* exon amplification. The exon numbers have been assigned according to Blaschke and Rappold⁸

	Primer sequence	Annealing temperature (°C)	Product size (bp)
Exon 2 5'	GAGACGCGCGCATCCACCA	64	
Exon 2 3'	GAGGCGCCGAACCCAGGAG	64	382
Exon 3 5'	AAAACCTCCCGGCCTCAG	64	
Exon 3 3'	GTGGCTGTACGCGTGCGCTGTG	64	332
Exons 4-5 5'	GGGAGGCTGGGCTGGGTTC	64	
Exons 4-5 3'	GGGGAAGGAGCAGCCAGGAA	64	378
Exon 6a 5'	TTACCATGTGTAGCCAGGAA	64	
Exon 6a 3'	ATCACCTGAGGTCAGGAGTT	64	376

Phenotypic heterogeneity of *CYP1B1*: mutations in a patient with Peters' anomaly

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Congenital glaucoma refers to a genetically heterogeneous group of distinctive clinical diseases characterised by increased intraocular pressure most often associated with increased corneal diameter, corneal oedema, and consequent visual impairment. Primary congenital glaucoma (PCG) is associated with a primary angle defect, whereas secondary congenital glaucoma is associated with a more generalised developmental anomaly of the anterior segment such as seen in Peters' anomaly. The inheritance of PCG is usually autosomal recessive.

Peters' anomaly consists of corneal opacity, defects in the posterior structures of the cornea, and iridocorneal and/or keratolenticular adhesions, and it most frequently occurs sporadically.¹ Over 50% of subjects develop glaucoma in childhood. Numerous aetiologies have been proposed including chromosomal abnormalities, teratogens,¹ and mutations in the eye developmental genes *PAX6*^{2–4} and *PITX2*.⁵ However, large subsets of Peters' anomaly cases are without molecular characterisation.

Primary congenital glaucoma has been linked to chromosomes 1p36 (*GLC3B*) and 2p21 (*GLC3A*), but only the *GLC3A* gene has been identified. This gene, *CYP1B1*, encodes a broadly expressed cytochrome P450 enzyme (*P4501B1*) whose natural substrate is unknown.^{6–7} It is proposed to be the major gene for disease causing mutations in primary congenital glaucoma.^{6–11} A variety of chain terminating and missense *CYP1B1* mutations have been described.^{6–9} We report two novel mutations in *CYP1B1* in a patient who had Peters' anomaly with secondary congenital glaucoma.

Case report

The subject, a male of Native Indian (Mohawk)/French Canadian background, presented with a history of bilateral cloudy corneas and tearing since birth. Examination at 3 weeks of age showed bilateral corneal oedema with central corneal opacities, superficial pannus (corneal vascularisation), and iridocorneal adhesions with a well formed anterior chamber (fig 1, above). Iris was present for 360° in both eyes. Corneal diameters were 10.75 mm in the right eye (OD) and 11.25 mm in the left eye (OS), which was slightly increased for age.¹² No view was available of the angle or optic nerve. Applanation tonometry under general anaesthesia documented intraocular pressures (IOPs) up to 49 mmHg in the right eye and 50 mmHg in the left eye, which were well beyond the normal range. The child was otherwise

entirely well with no other malformations and no significant family history.

A diagnosis of congenital glaucoma and Peters' anomaly with no significant family history was made. Bilateral trabeculotomies were performed to decrease the IOP followed by a left corneal transplant for visual rehabilitation. At surgery, the undersurface of the cornea showed a central, Y shaped, posteriorly projecting opacity that corresponded to a raised opacity on the anterior capsule of the lens. This overlapped the position of the fetal sutures and suggested the existence of previous keratolenticular adhesions during development. The lens also showed a pulverulent (powdery) cataract through which there was a good red reflex. Subsequently, the IOPs were difficult to control in both eyes, requiring frequent surgery and medication. At the most recent examination at 6 years of age, his right eye showed signs of advanced but controlled glaucoma with a cup:disc ratio of 0.8 to 0.9 OD. His left eye became phthisical (shrunken).

Microscopic examination of the left corneal button removed at transplantation showed that the corneal epithelium was of normal thickness, although there was some oedema of the basal cells (fig 1, below). Bowman's membrane was not recognisable and the anterior stroma was hypercellular with a disordered lamellar pattern. The posterior stroma was absent centrally, and there was a basophilic granular deposit containing a small amount of extracellular melanin. Descemet's membrane was not identified in this half of the button. However, transmission electron microscopy showed a recognisable Bowman's membrane, though abnormal and containing scattered keratocytes. Also a small segment of Descemet's was observed, although thin (approximately 1.5 µm) with a rather poorly defined banding pattern. No endothelial cells were seen.

DNA extraction followed standard protocols.¹³ Amplification of the coding sequence of *CYP1B1* used previously published primers.^{7–8} Additional primers for exon 3 were designed from the genomic DNA sequence (Acc No U56438). Forward primer: 5'-ctataaagcttctctgaagc-3', reverse primer: 5'-ttgtccaagaatcgagctgg-3'. Mutational analysis of the amplicons by single strand conformational polymorphism (SSCP) and direct cycle sequencing used protocols previously described.^{11–14} We identified two novel *CYP1B1* mutations in exon 2 making our subject a compound heterozygote for the missense mutation 3807T→C, which is predicted to result in the amino acid change Met1Thr (ATG→ACG), and the nonsense mutation 3976G→A, which

Figure 1 (Above) Photo of the right cornea in the first year of life with central corneal leucoma (opacity). (Below) Histopathology of the central cornea with scarring of anterior stroma, posterior stromal defect (D), and basophilic granular deposit. Where the full thickness of the stroma is seen (arrow), Descemet's membrane is not present. (Haematoxylin and eosin.)

Figure 2 Chromatograms of direct cycle sequencing of *CYP1B1*, exon 2 showing (left) substitution of 380T→C compared with normal, above and (right) 3976G→A resulting in a premature stop codon, also compared with the normal sequence shown above.

is predicted to truncate the P4501B1 polypeptide (Trp57Stop, TGG→TAG) and possibly cause nonsense mediated decay of the *CYP1B1* mRNA (fig 2). This latter mutation was seen in the mother's DNA who was clinically normal.

The father was not available for testing. These mutations were not seen in 100 normal controls of mixed ethnicity. Furthermore, mutational analysis of *PITX2* and *PAX6* using previously described protocols¹⁵⁻¹⁷ failed to document any pathogenic sequence change in this patient. Ten additional unrelated subjects with Peters' anomaly were screened for mutations in *CYP1B1*, *PITX2*, and *PAX6*. Only one patient showed a sequence change in *PAX6* and none in *CYP1B1*.

Discussion

Peters' anomaly is a congenital abnormality that shows a wide range of histopathological changes.¹⁸⁻²¹ It has been proposed that a normal Descemet's/endothelial unit is necessary for maintenance of the integrity of the corneal stroma.²² The posterior stromal abnormalities seen in our patient may be a consequence of the abnormal segment of Descemet's membrane. The nature of the basophilic deposit in the posterior stromal defect remains uncertain but may represent denatured lens protein associated with the presumed developmental keratolenticular adhesion noted at the time of the corneal transplant. The absence of Bowman's membrane on light microscopy, albeit not complete, is also a characteristic feature of Peters' anomaly.

It has been suggested that both Peters' anomaly and primary congenital glaucoma arise from defective neural crest cell migration in the 4th to 7th week of fetal development, during which period the anterior segment of the eye forms.²³ The relationship between these two diseases is otherwise not well understood. This is the first documentation of *CYP1B1* mutations causing a phenotype other than congenital glaucoma. We report two new *CYP1B1* mutations, the combination of which is associated with a different phenotype, Peters' anomaly. These findings strengthen the potential role of this gene in anterior segment eye development. One of the *CYP1B1* mutations identified (3976G→A, Met1Thr) would probably disrupt translation initiation, as the methionine codon surrounded by a Kozak sequence is crucial for ribosomal recognition.²⁴ The next conserved Kozak sequence where translation could be initiated occurs in frame, but would result in truncation of 131 amino acids from the amino terminus of the protein. Translation can be initiated at non-AUG codons, including an ACG, but this is extremely rare and requires favourable mRNA secondary structure.²⁵ The second mutation in this patient (3976G→A) results in a premature stop codon (Trp57Stop). Expression of this allele would therefore result in a protein truncated by 486 amino acids and is thus also likely to represent a functional null allele.

Our observations suggest that a normal *CYP1B1* product is required for differentiation of the anterior segment, and that some cases of Peters' anomaly and PCG may share a common aetiology. Homozygous *CYP1B1* null mutations have been described in cases of congenital glaucoma^{6-9 11}; however, insufficient clinical information is available to establish

phenotype-genotype correlations. We hypothesise that the severity of the Peters' phenotype described correlates with the extent of predicted protein truncation.

The spectrum of the phenotypes associated with *CYP1B1* mutations is broader than anticipated as is the genetic heterogeneity of Peters' anomaly. The findings reported here suggest that the role of *CYP1B1* is not solely confined to the pathogenesis of primary congenital glaucoma but may play a more general role in eye development. This work emphasises the genetic heterogeneity and the complexity behind anterior segment development disorders and early glaucoma.

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Role of *TP53* P72R polymorphism in human papillomavirus associated premalignant laryngeal neoplasm

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Table 1 *TP53* polymorphism in adult onset laryngeal papilloma patients and controls

Amino acid at position 72	Patients (n=42)	%	Controls (n=186)	%	p value
Arg	24	57	88	47	0.31
Pro/arg	18	43	75	40	0.58
Pro	0	0	23	12	0.01

No significant differences were detected between Arg and Pro/Arg amino acid sequences at position 72 in patients and controls. The Pro allele was more common in controls than in patients (Fisher's exact test, two tailed).

Laryngeal papillomatosis, a disease in which malignant transformation takes place in 3-7% of patients,^{1,2} is caused by low risk human papillomavirus (HPV) types 6 and 11. The mechanism by which HPV affects *TP53* involves the HPV E6 oncoprotein, which has been shown to bind *TP53* protein and cause its degradation through the ubiquitin pathway.³ It has been proposed that subjects with homozygous arginine (Arg) at position 72 in the *TP53* amino acid sequence are more susceptible to HPV associated uterine cervical and cutaneous tumorigenesis than are heterozygotes, and that the *TP53* Arg allele is susceptible to *TP53* E6 mediated degradation regardless of whether the infection is by a low risk or high risk HPV type.⁴ However, although these findings could not be confirmed in studies of large populations of women with premalignant and malignant cervical neoplasia,⁵ recently Zehbe *et al*⁶ reported an association between *TP53* codon 72 Arg homozygotes and development of cervical cancer.

Our study is based on Finnish adult onset (age at diagnosis ≥ 17 years) laryngeal papilloma patients treated at Helsinki University Central Hospital, Department of Otorhinolaryngology, during the years 1975-1994.⁷ Diagnosis of laryngeal papillomatosis was confirmed by detection of HPV DNA by polymerase chain reaction (PCR) from patients' laryngeal biopsies. Because only paraffin embedded biopsies (n=59) were available, and only 29 of them were HPV positive, results on fresh frozen HPV positive biopsies of an additional 13 randomly selected adult onset patients treated during the years 1997-1999 were included.

The *TP53* proline 72 arginine (P72R) polymorphism was analysed from lymphocyte DNA by sequencing the genomic PCR product

of exon 4 of these 42 adult onset patients. The results were confirmed by digestion of the PCR products. All data derived from the patient samples by sequencing and by digestion analysis were identical. Our reference population was 186 cancer free anonymous blood donors and their samples were analysed by digestion only. No differences existed in Arg and Pro/Arg allele frequencies between patients and controls (table 1).

Of the HPV 6 positive patients, 15/27 (56%) were Arg homozygotes, and of the HPV 11 positive, 7/10 (70%). In addition, three patients' biopsies were both HPV 6 and 11 positive, and two patients had some other HPV type in their laryngeal biopsy. Based on experimental studies with HPV 11 and analysis of skin cancers representing mixed HPV types, Storey *et al*⁴ proposed that in addition to high risk HPV types, Arg homozygosity predisposed to tumours promoted to low risk HPV types as well. This conclusion is not in accordance with our data. Although HPV 11 E6 may induce degradation of *TP53* Arg in vivo, this mechanism is not necessarily involved when laryngeal tumours are caused by HPV 6 or 11 virus types. These findings argue against a role for the P72R polymorphism in this HPV associated premalignant neoplasm and against the hypothesis that *TP53* Arg affects the susceptibility of *TP53* to E6 mediated degradation also in infections caused by low risk HPV types.

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Predisposing chromosome for spinocerebellar ataxia type 6 (SCA6) in Japanese

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The autosomal dominant cerebellar ataxias (ADCAs) are a group of neurodegenerative disorders which can be classified into three major categories on the basis of their clinical features and mode of inheritance.¹ ADCA type III is a pure cerebellar syndrome that is genetically heterogeneous and includes spinocerebellar ataxia type 5 (SCA5),² SCA6,³ SCA10,^{4,5} and SCA11.⁶ The gene responsible for SCA6 has been identified as coding for the α_{1A} subunit of the P/Q type voltage dependent calcium channel (*CACNA1A*). Moderate CAG expansion in the coding region causes the disorder, with the number of CAG repeats being originally reported as 21–27 in mutant alleles ($n=8$) and 4–16 in control alleles ($n=950$).³ Subsequent studies have indicated that the range of pathological expansion in SCA6 alleles varies from 20⁷ to 33.⁸ The *CACNA1A* gene was first identified during the search for specific mutations causing familial hemiplegic migraine (FHM) and episodic ataxia type 2 (EA2).⁹ The gene product has four transmembrane domains and glutamine repeats are located at the C-terminal side of the intracellular segment. Missense mutations of these transmembrane domains and deletions or splice mutations leading to a truncated protein are responsible for FHM and EA2, respectively. The *CACNA1A* gene is predominantly expressed in Purkinje cells and granule cells of the cerebellum and is essential for the survival and maintenance of normal function by these neurones.^{10,11} Biochemical mechanisms leading to the development of SCA6 are not fully understood. However, the fact that slowly progressive ataxia is often observed in EA2 indicates that a small glutamine expansion in the SCA6 gene also disturbs the function of P/Q-type calcium channels, leading to selective neuronal degeneration in the cerebellum.

The pathogenic expansion in SCA6 is relatively small compared with those in other SCAs caused by triplet repeat expansion, but there is still a significant inverse correlation between the age at onset and the number of repeats in SCA6.^{8,12–20} Some homozygotes for the SCA6 mutation show a more severe phenotype,^{13,15} but others do not.^{15,21} Unlike other SCAs with long CAG repeats, the expanded SCA6 allele is known to be relatively stable during meiosis and mitosis, with some exceptions.^{7,22} The cardinal feature of SCA6 is slowly progressive ataxia,³ but exceptions have been reported.^{14,19}

The frequency of SCA6 varies between white ethnic subgroups, with a range of 0% to 15.2%.^{16,17,19,23–25} In Japan, the frequency varies between regions, ranging from 5.9% to more than 30%.^{13,15,20,26,27} In Hokkaido, the north-

ernmost island of Japan, SCA6 accounts for 30% of 161 families with ADCA, the highest frequency reported to date.²⁸ These findings prompted us to search for a possible founder chromosome in Japanese SCA6, and to determine whether there are any alleles predisposing to the generation of SCA6 mutation.

Material and methods

Twenty one unrelated Japanese SCA6 families were investigated. Twelve non-consanguineous families⁸ and one consanguineous family²¹ have already been reported elsewhere, while eight families were newly added in this study. Thirteen of 21 families reside in Hokkaido, while the other eight families come from various other areas of Japan. The ancestors of the Hokkaido families moved to this island approximately a century ago from various, random other areas (data not shown). Altogether, 58 subjects were clinically affected, 35 were asymptomatic, and 10 had married into these families. In addition, 25 patients without family members available for testing were recruited from Hokkaido; a family history of ataxia was positive in 18 and negative in seven patients. Among the total of 83 patients, the mean age at onset was 49.6 (SD 11.6) years, ranging from 19 to 75 years.

After informed consent was obtained, high molecular weight DNA was extracted from peripheral white blood cells. According to the method of Zhuchenko *et al*,³ polymerase chain reaction (PCR) amplification of CAG containing segments in the *CACNA1A* gene was performed using primers S-5-F1 and S-5-R1. S-5-F1 was end labelled with 6-FAM (PE Biosystems). After PCR amplification of genomic DNA using a PE9600 thermal cycler (PE Biosystems), the CAG repeat polymorphism was analysed using an ABI PRISM 377 gene sequencer equipped with GeneScan[®] software version 2.0 (PE Biosystems). The number of CAG repeats was determined with reference to the product size of the sequenced alleles.

To construct haplotypes carrying the *CACNA1A* gene, D19S840, D19S1150, D19S226, and D19S885 were analysed. These four microsatellites cover a 4 cM interval containing the entire *CACNA1A* genome from the telomeric to the centromeric end.⁹ D19S1150 is located in intron 7 of the gene (fig 1). Polymorphism of these microsatellites was analysed using an automated gene sequencer²⁹ and the alleles of each microsatellite were numbered according to the product size. The CA repeat sequence of D19S1150 was determined using Genome Database information (accession No 1320259). After purification on a Microcon-100 spin column (Amicon), PCR

Figure 1 Polymorphic markers and locations examined in this study.

products of homozygotes for D19S1150 were directly sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Biosystems), with p858 FOR as the forward primer.

In addition to these microsatellites, we examined two single nucleotide polymorphisms (SNPs) in the coding region of the gene: one (A/B system) was a G to A substitution at position 2369 in exon 16, and the other (C/D system) was a G to A substitution at position 1457 in exon 8 (fig 1). A pair of primers, Yb-1 (5'-TCCACAGCTGCATCTCC AAG-3') and Yb-2 (5'-ACCCTCCCTTGAG CCCCT-3'), generated a 270 bp fragment covering the site of position 2369 in exon 16 (A/B system). This site was recognised by the *HgaI* restriction enzyme. The SNP at nt 1457 in exon 8 (C/D system) was detected by mismatch PCR. Another primer pair, Ym-1 (5'-ATACTCTGGCTTTTCTATGC-3') and Ym-2 (5'-TTTCATCCTCGGCGAGGATC A CCTCTTCTGCTTTTGAGATCGA-3'), generated a 170 bp fragment that included a *ClaI* restriction site. PCR was done for 30 cycles in a total volume of 20 μ l with 1.7 mol/l N,N,N-trimethyl glycine (Wako) under the same conditions as for amplification of microsatellites, except that denaturation, annealing, and extension were each done for 60 seconds. To facilitate introduction of a restriction site, *rTth* DNA polymerase[®] (PE Biosystems) was used with the Ym-1/-2 primer pair. The annealing temperature was set at 58°C for the Yb-1/-2 pair and 52°C for the Ym-1/-2 pair. After digesting the PCR products for one hour at 37°C with 1 U of either *HgaI* or *ClaI*, the alleles at each polymorphic site were determined by agarose gel electrophoresis.

We constructed haplotypes for the mutant SCA6 alleles (SCA6 chromosome) in 21 families based on their family structures and map order of 19p13 markers. Differences in allele frequency between the affected and control haplotypes were analysed using the χ^2 test and $p < 0.05$ was considered statistically significant. Unrelated normal Japanese subjects (mostly residents of Hokkaido) served as controls. In addition, three normal subjects who had married into the affected families from outside Hokkaido were included as controls. Both the SCA6 patients and controls were from the same ethnic background.

For phase unknown samples, such as the controls (unrelated normal subjects, $n=172$) and the SCA6 patients ($n=25$) for whom family samples were not available, estimation of haplotype frequency was performed by the maximum likelihood method using a simplified version of the GENEF computer program (J-M Lalouel, unpublished data). Procedures for generating the haplotype have been described in full by Jeunemaitre *et al.*³⁰ Briefly, two polymorphisms were chosen to generate the haplotype, followed by sequential inclusion of one polymorphism at a time. Haplotypes showing a frequency below $1/4N$ (where N is the sample size) were eliminated during the process, and then the haplotype frequency was re-examined. Simple χ^2 tests of homogeneity were applied for statistical comparison between cases and controls.

Results

Among the 21 families, 58 affected subjects and nine asymptomatic subjects of risk age carried the expanded CAG repeat. The geno-

Table 1 D19S840-D19S1150-C/D-A/B-(CAG)*n*-D19S226-D19S885 haplotypes of 21 unrelated SCA6 families

SCA6 haplotype							
Family Code No	Extragenic (tel)	Intragenic				Extragenic (cen)	
	D19S840	D19S1150	C/D	A/B	(CAG) <i>n</i>	D19S226	D19S885
P28	7	5	C	B	33	10	4
P76	7	5	C	B	25	10	2
P70	7	5	C	B	23	10	10
P83	7	5	C	B	23	5	2
P22	7	5	C	B	22	10	11
P27	7	5	C	B	23	10	2
P25	2	5	C	B	23	10	6
P57	2	5	C	B	25	10	2
P78	2	5	C	B	22	10	2
P79	2	5	C	B	24	10	2
P64	2	5	C	B	22	8	6
P60	2	5	C	B	22	11	2
P42	2	5	C	B	22	5	4
P20	2	5	C	B	23	7	2
P63	3	5	C	B	25	6	2
P80	7	1	C	B	22	10	2
P82	2	1	C	B	24	10	2
P81	2	1	C	B	21	5	2
P88	7	1	C	B	24	10	2
P89	ND	5	C	B	22	6	2
P90	2	5	C	B	22	8	ND

ND: phase not determined.

typing data for the 12 previously reported families⁸ are included in the present analysis. Among these 21 families, one asymptomatic subject of risk age was homozygous for 21 repeat alleles and the others were all heterozygotes with both expanded and normal alleles. No cases of the unstable transmission of expanded alleles were observed. The 25 other patients without family samples were all heterozygotes for SCA6 mutations. The mean CAG repeat size of the mutant alleles was 23.1 (SD 2.1) (*n*=93 SCA6 alleles), with the range being 21–33. There was a significant inverse correlation between age at onset and the number of CAG repeats (*n*=83 patients with known age at onset; $\gamma=-0.706$, $R^2=0.499$, $p<0.0001$, Pearson's product moment method). When polynomial analysis was used, a significant correlation was also obtained ($R^2=0.539$, $p<0.0001$). The number of CAG repeats in unrelated normal alleles ranged from 4–18 (*n*=388), with a peak of 13 (24.5% of the total); 64.2% of the control alleles had 11–13 repeats and 7.0% had 15 repeats or more.

After construction of the D19S840-D19S1150-C/D-A/B-D19S226-D19S885 haplotype in the affected families, we found that the same haplotype (major haplotype) cosegregated

with affected status in each family (table 1). In the D19S1150-C/D-A/B haplotype, either “5-C-B” (17 families, 81%) or “1-C-B” (four families, 19%) was selectively associated with SCA6 chromosomes. Sequencing showed that allele 1 of D19S1150 had (CA)₆AA(CA)₁₇₅ and allele 5 had (CA)₆AA(CA)₂₁.

We first compared the allele frequency of each polymorphism for unrelated control chromosomes with SCA6 chromosomes deduced from the affected families. Three intragenic markers, an intronic microsatellite (D19S1150) and two SNPs in exons 8 and 16, showed significant differences in allelic frequency between the affected chromosomes and controls ($p<0.0001$, table 2). Even two extragenic microsatellites, D19S226 and D19S885, showed a significant difference ($p<0.0001$ and $p<0.005$, respectively). These results indicate that there was significant linkage disequilibrium between SCA6 mutations and these markers.

In order to determine the profile of the *CACNA1A* gene haplotype, we then analysed the D19S1150-C/D-A/B polymorphism in controls (172 unrelated normal subjects) and 25 SCA6 patients for whom family data were unavailable. After genotyping, we performed

Table 2 Linkage disequilibrium between SCA6 mutation and 19p13 polymorphic markers from the SCA6 chromosomes of 21 unrelated families

Locus	Polymorphism No of alleles	SCA6 chromosome		Control chromosome	Difference	
		Associated allele	Frequency (No)	Frequency (No)	χ^2	<i>p</i> value
D19S840*	9	2(203 bp)	55% (11)	44.6% (318)	0.47	NS
		7(213 bp)	40% (8)	29.8% (318)	0.63	NS
D19S1150	9	5(158 bp)	81% (17)	16.8% (388)	51.23	$p<0.0001$
		1(150 bp)	19% (4)	1.8% (388)	22.63	$p<0.005$ †
nt1457 in exon 8‡	2	C	100% (21)	64.5% (344)	11.33	$P<0.001$
nt2369 in exon 16‡	2	B	100% (21)	71.8% (344)	8.18	$P<0.005$
D19S226	13	10(245 bp)	57% (12)	14.0% (318)	22.79	$p<0.0001$ †
D19S885*	7	2(175 bp)	70% (14)	32.3% (318)	10.47	$p<0.005$

*Data from SCA6 chromosomes from 20 unrelated families.

†Fisher's exact probability test.

‡Definitions of the biallelic system are shown in fig 1 [f1].

NS: not significant.

Table 3 Estimation of D19A1150- C/D-A/B haplotype frequency in SCA6 patients (n=25) and controls (n=172) using the GENE computer program

Haplotype	SCA6 patients	Control
D19A1150- C/D-A/B	(Allele = 50)	(Allele = 332)
1-C-B	0.0515	0
3-C-B	0.0225	0.0687
5-C-B	0.4858	0.1068*
6-C-B	0.0957	0.1282
9-C-B	0.0245	0.0968
10-C-B	0.0200	0.0435
3-D-B	0	0.0223
5-D-B	0	0.0205
6-D-B	0	0.0727
8-D-B	0	0.0281
9-D-B	0	0.1233
1-C-A	0.0285	0
5-C-A	0	0.0304
6-C-A	0.0715	0.0505
8-C-A	0	0.0198
9-C-A	0.020	0.0721
10-C-A	0	0.0248
3-D-A	0.0375	0.0144
5-D-A	0.0542	0.020
6-D-A	0.0328	0.0407
8-D-A	0	0.0123
9-D-A	0.0555	0
10-D-A	0	0.004
C/D-A/B	(Allele = 50)	(Allele = 344)
C-B	0.70	0.4470†
D-B	0	0.2710
C-A	0.12	0.1984
D-A	0.18	0.0836

* $\chi^2=46.69$ (df=1), $p<0.001$.

† $\chi^2=11.14$ (df=1), $p<0.001$.

haplotype estimation by the maximum likelihood method on samples for which the phase was not determined. Estimated haplotype frequencies were compared between SCA6 patients and controls (table 3). The results of this analysis were as follows: (1) the frequencies of 5-C-B and 1-C-B haplotypes were significantly higher in patients than in controls (49% *v* 11%, $\chi^2=46.69$, df=1, $p<0.001$ and 5% *v* 0%), indicating that the SCA6 mutant allele in these 25 patients was most likely to carry either haplotype 5-C-B or 1-C-B; (2) the frequency of the C-B haplotype was significantly higher in patients than in controls (70% *v* 45%, $\chi^2=11.14$, df=1, $p<0.001$); (3) C-B was the most frequent haplotype in controls (45% of 344 alleles); and (4) the D-B haplotype frequency was 27% in controls, but 0% in patients.

Discussion

The present study disclosed several findings about the genetic background of SCA6 in the Japanese. First, study of SCA6 families showed that only two haplotypes, "5-C-B" (81%) and "1-C-B" (19%), were significantly associated with the affected chromosomes (SCA6 chromosomes), and that the allele frequencies of each locus on these chromosomes was significantly different from those of controls. Second, 5-C-B was also the most frequent haplotype in probands (49%, n=50 chromosomes), indicating that one of the two haplotypes in each patient can be expected to be this common haplotype. Third, all of the affected haplotypes carried the C-B haplotype, which was the most frequent haplotype in control chromosomes (45%). The significantly high frequency of the 5-C-B haplotype among the probands implies that their SCA6 mutation also resides on this haplotype, as was found in the affected

families. However, since we could not determine directly which chromosomes (haplotypes) were the site of the SCA6 mutation, the possibility that haplotypes other than C-B carry the mutation cannot be completely excluded.

In Hokkaido, the majority of residents including the present subjects are descendants of immigrants from various areas of Japan and share a single ethnic background. Taking these historical data and the results of our genetic analyses into account, there is a possible founder effect in the subjects from Hokkaido and also in those from other areas of Japan. Judging from our data, these results favour the hypothesis that the expanded SCA6 alleles in the Japanese population originated from a chromosome with a C-B haplotype, which is the most frequent haplotype in controls (45% of alleles). The most plausible scenario is as follows. First, the SCA6 mutation occurred on a chromosome with the 5-C-B haplotype. At some point thereafter, the removal of four CA repeats occurred, an event which changed the haplotype from 5-C-B to 1-C-B. This is supported by the finding that the CAG repeat size of mutant SCA6 alleles is more variable on 5-C-B chromosomes than on 1-C-B chromosomes (22-33 *v* 21-24).

In the SCA6 allele, 7 and 11-13 CAG repeats are the predominant alleles in normal populations, regardless of ethnic background. Alleles with 15 CAG repeats or more are quite rare in European/American populations.^{3 17 18} However, alleles with 15-19 repeats are not infrequently observed in the Japanese population, having a range of 5.9%¹³ to 7.0% (present study, n=388). A recent study indicated that, in dominant SCAs caused by triplet repeat expansion including SCA6, the frequency of large alleles in a normal population is correlated with the relative prevalence in different ethnic groups.²⁷ These data suggest the possibility that such large alleles are a potential reservoir for full mutant alleles, which may explain the high prevalence of SCA6 in the Japanese. It would be worthwhile to determine whether such intermediate SCA6 alleles in the normal Japanese population have a C-B haplotype.

Recurrent mutations of at risk chromosomes are considered to be potential founders in several CAG triplet disorders. In Huntington's disease (HD), haplotype studies on a cohort of families have shown that only 41% were derived from either one of two common ancestral haplotypes while the rest were from independent mutations.³⁰ De novo expansions from intermediate alleles have also been reported in HD.^{32 33} In Machado-Joseph disease (MJD/SCA3), haplotype analyses using intragenic SNPs have shown several ancestral mutations, and normal chromosomes with intermediate expansions in a prevalent population carry the same haplotype that is shared with the affected chromosomes in that population.³⁴ On the other hand, in DRPLA, a single predisposing haplotype was selectively associated with the affected chromosome and with normal chromosomes carrying a larger expansion.³⁵ The frequency of the allele with the pre-

disposing haplotype is considered to be correlated with the prevalence of DRPLA in different ethnic groups. Several different founder haplotypes for SCA6 have been identified in white populations.³⁶ In addition, de novo expansion from the intermediate alleles has been reported.⁷⁻²² Observation of these three triplet repeat diseases suggests that the number of founder haplotypes is associated with the degree of instability of the predisposing chromosomes, which leads to pathogenic repeat expansion.

Despite extensive ongoing investigation, the molecular mechanism responsible for the instability of expanded repeats remains unknown. Our study showed that the majority of Japanese SCA6 mutations are derived from a C-B haplotype pool. This implies the possibility that some *cis* acting factor plays a role in promoting instability of CAG repeats in the SCA6 gene. A similar mechanism has been postulated through the study of DRPLA.³⁵ Brock *et al*³⁷ reported that the expansibility of elongated CAG triplet repeats was strongly correlated with their location within CpG islands and with the GC content in the flanking sequence of CAG repeats. Their study provides insight into the molecular basis of *cis* acting factors, which modify the instability of expanded triplet repeats. However, in SCA6 as well as DRPLA, the molecular mechanism leading to full expansion from a particular predisposing chromosome is not fully understood. To understand the molecular mechanism of SCA6 mutation better, our conclusions need to be confirmed through the study of different ethnic groups.

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Prenatal testing for Huntington's disease: experience within the UK 1994-1998

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Table 1 Prenatal tests and their outcome (UK) 1994-1998

	1994	1995	1996	1997	1998	Total
Exclusion tests						
Outcome: low risk	13	4	3	8	6	34
Outcome: high risk	7	5	8	9	6	35
Outcome: uninformative	3	0	0	0	0	3
Terminations	7	5	8	8	6	34
Miscarriage	0	0	0	0	1	1
Total	23	9	11	17	12	72
Direct tests						
Outcome: low risk	8	4	12	10	12	46
Outcome: high risk	6	2	1	8	11	28
Terminations	6	2	1	5	11	25
Total	14	6	13	18	23	74
Total (all tests)	37	15	24	35	35	146

in a pregnancy. This gives an accurate result. If the status of the at risk parent has not been ascertained, then this may produce predictive information about that person.

In exclusion testing, the at risk grandparental chromosome 4 locus is excluded using linkage analysis. This test preserves the 50% risk of the parent, and allows a pregnancy at low risk to continue. In this situation, pregnancies that share the risk of the parent would be terminated. However, should the at risk parent not develop HD, a normal pregnancy would have been lost.

Given the technical feasibility of prenatal mutation testing and the severity of the disorder, it might be expected that prenatal diagnosis would be frequently requested.

Tyler *et al*¹ reviewed a group of referrals for exclusion testing in pregnancy, and surveyed a group of subjects at 50% risk of developing HD about their attitudes to prenatal testing. They concluded that the demand for such testing was likely to be small. We considered it important to assess this demand in relation to that for presymptomatic testing, and since the numbers recorded by individual centres were small, to collect the data on a UK basis.

In Britain, the UK Huntington's Disease Prediction Consortium was created to monitor the use of molecular testing in HD, to evaluate the developing service, and to ensure the highest standards were applied to the procedure.⁵ Several studies before the introduction of predictive testing reported the views of those at risk of HD. These showed that between 56% and 80% of at risk subjects would undergo predictive testing once it was available.⁶⁻⁸ Uptake of such testing has been considerably less than this, 9-15%¹ with some exceptions.⁹

The consortium has recorded all presymptomatic predictive tests since testing began in 1988, a total of 2937 up to the end of December 1997. Less than 50% of these have been unfavourable results. Data on all prenatal tests recorded in the period 1994 to 1998 form the basis for the study reported here. Anonymised data on each test performed in pregnancy were collected on an annual basis from each of the member centres of the United Kingdom Huntington's Disease Prediction Consortium. Information on referrals during pregnancy that did not lead to testing did not form part of this study.

One hundred and forty six prenatal tests were recorded in the years 1994 to 1998 (table 1). This includes one twin pregnancy.

Forty five percent of tests (65) occurred where the parent was aware that they had the mutation for HD. Nine of these parents were described as having clinical features of the disease at the time of the pregnancy.

Fifty four percent of tests (78) were carried out in pregnancies where the parent was at 50% risk. Eight of these were accomplished using mutation detection, that is, the at risk parent was prepared to find out that they too had the mutation as a result of investigation of the pregnancy. In three cases, presymptomatic predictive testing was performed during the pregnancy and before the pregnancy was investigated.

In two cases, the parent was at 25% risk, that is, the grandparent was asymptomatic but at 50% prior risk.

Sixty six (43%) unfavourable results have been produced, but only 61 terminations performed. In one of the four cases where the pregnancy continues, the pregnancy shares the parent's 50% risk as the result was produced using exclusion testing. In the remaining three cases, the mutation was detected in the fetus.

There was one report of miscarriage as a result of the prenatal investigation, in a twin pregnancy.

The decision to investigate a pregnancy for an adult onset disease (and possibly terminate that pregnancy) is never an easy one, and lately the families have expressed much hope that a cure will be found for the disease and therefore they would be able to avoid termination of a pregnancy. HD can be variable in severity, and undoubtedly experience of later onset disease, which may be less severe, can encourage couples not to investigate their pregnancies.

Many people who present for predictive testing already have at least one child.¹⁰ The trend is for these subjects not to test pregnancies that occur after they have received an unfavourable result, since they would then have children at 50% risk and children whose status was certain.

The trauma associated with termination of any pregnancy is considerable. Tolmie *et al*¹¹ described a group of UK families (an earlier group who are not included in this study) who had difficulty with their decision to terminate an at risk pregnancy following exclusion testing. Three of nine high risk pregnancies were continued. Clarke *et al*¹² showed the prob-

Figure 1 Direct testing and mutation testing 1994-1998.

lems of producing predictive information for an adult onset incurable disease. In cases where an unfavourable result is produced, but the pregnancy continues, these children will grow up with certainty of information about their status, having had no choice in the decision. In addition, their parents are aware of their status, and their prejudices and those of society will undoubtedly be disadvantageous for them. There are only four such cases in this series.

There are couples who have undergone tests on as many as five pregnancies in their efforts to ensure that no child of theirs would have to suffer the problems of being at risk as they had themselves (S Simpson, personal communication).

Many couples express their anxiety about any child of theirs growing up with an affected parent, even if it were known that the child would not be at risk because of prenatal testing. These people may have grown up in a family where alcoholism, suicide, and divorce are common.¹³ Those who have had an unfavourable result by predictive testing and then have chosen to have a pregnancy are in the minority in this group.

Other options are available to those who know they have the mutation and who wish to have children. In at least one case, after the termination of a high risk pregnancy, the couple chose artificial insemination by donor. However, couples who have attempted to adopt or foster have had considerable difficulties because of the at risk status of one of the partners.^{14 15}

It is now becoming possible to offer preimplantation diagnosis^{16 17} to couples who do not wish investigation of an established pregnancy. This technique remains at a research stage; only preliminary data are available for its use and the rate of successful implantation of the fetus remains low. Nevertheless some patients in the clinic setting have expressed interest, but accurate figures are not available.

For those at risk who decide not to undergo presymptomatic predictive testing, the opportunity to ensure that the gene is not transmitted to the next generation remains with the use of exclusion testing. For some subjects their commitment is absolute (see above) and they have repeated attempts to have a pregnancy with a

low risk. Forty nine percent of the total tests in this series were performed using exclusion testing, although there has been an increasing number of tests using direct mutation testing in the last three years (fig 1). These data provide evidence that many at risk subjects would prefer not to know whether or not they are going to develop HD, but such is their experience of the disease and their at risk status that they wish to prevent the birth of an at risk child.

Maat-Kievit *et al*¹⁸ described the experience in The Netherlands of 72 prenatal tests in Huntington's disease. As in our study, a trend towards an increasing number of direct tests has been observed, although exclusion testing is still seen as a useful tool for those who do not wish predictive testing.

In a survey of subjects from Germany¹⁹ who were at risk of HD, over 67% indicated that they would wish to undergo presymptomatic predictive testing themselves, but only 45% would wish to use prenatal diagnosis. Twenty seven percent of those questioned stated they could not use prenatal diagnosis because they felt they could not terminate a pregnancy.

In South Africa, 59 subjects who had undergone predictive, diagnostic, or prenatal testing for HD were reported.²⁰ The two affected pregnancies were aborted, from a group of 10 who had undergone prenatal tests.

In a review of international data, Evers-Kiebooms *et al*²¹ showed that more prenatal tests took place where the male was at risk. This may indicate that the female role of child care was recognised by the families, in that if the male were subsequently affected, child care would continue.

Only a minority of those at risk of Huntington's disease in the UK have chosen to prevent the transmission of the disease by the use of prenatal diagnosis. There may be lack of knowledge among the at risk population about the tests available²² and older family members and spouses occasionally deliberately withhold information about the presence of the disease within the families. This may also help to explain the low uptake of presymptomatic predictive testing in the UK, but it does not explain why the number of those requesting prenatal diagnosis is so small in comparison with the number of requests for predictive testing.

The families in general express great hope for the future²² for treatment or prevention of HD and the low uptake of prenatal testing may reflect this. The continued use of exclusion testing in pregnancies, despite the ready availability of accurate direct testing, also

shows the usefulness of exclusion testing for those who do not wish to obtain accurate information about their own status, but who are unwilling to risk transmission of the disease.

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Pregnancy outcome and long term prognosis in 868 children born after second trimester amniocentesis for maternal serum positive triple test screening and normal prenatal karyotype

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Measurement of maternal serum alpha-fetoprotein (ms AFP), human chorionic gonadotrophin (ms hCG), and unconjugated oestriol (uE₃) at the beginning of the second trimester of pregnancy is a well established screening test for Down syndrome (trisomy 21). Previous studies have described the association of abnormal levels of ms AFP and ms hCG with a variety of problems and complications of pregnancy, such as preterm delivery, fetal growth retardation, and fetal death,¹⁻⁵ and severe hypertensive disorders in pregnancy.⁶⁻¹⁰

Over the past years, we have noted in the genetic clinic that several children with syndromic and non-syndromic forms of MCA-MR were born after a pregnancy with a positive maternal serum triple screening test and a normal prenatal karyotype.

Therefore, we decided to perform the present study and collected data on the pregnancy outcome and the physical and psychomotor development of 868 children born after second trimester amniocentesis for positive maternal serum triple screening test with a normal prenatal karyotype. We found a significantly increased incidence of complex multiple congenital anomalies syndromes (17, 1.95%) in the children.

Material and methods

During the period from 1 January 1993 to 31 December 1995, 995 women had amniotic fluid analysed for aneuploidy based on a positive maternal serum triple test (trisomy 21 $\geq 1/250$). These samples were analysed at the Leuven Centre for Human Genetics and showed normal chromosome results and normal amniotic fluid AFP.

Maternal serum triple tests and amniocenteses were performed in different centres. In October 1998, a questionnaire (available on request) was mailed to the 995 women with a list of questions about the outcome of pregnancy, the perinatal history, and the physical and psychomotor development of their children.

A total of 870 patients (87.5%) answered the questionnaire. They gave birth to 868 children (864 singletons, six twin pregnancies, four spontaneous abortions, and four intrauterine deaths). Further medical information was also obtained from the relevant obstetricians and paediatricians. All children with a major congenital malformation, as an isolated finding or as part of a multiple congenital malformation (MCA) syndrome or sequence, were

examined by the same clinical geneticist (JPF) and/or the same fetal pathologist (PM).

Results

Isolated (minor and major) congenital anomalies were present in 28 children (3.23%) (table 1). Twelve children (1.38%) had an isolated major congenital malformation and half of these (0.69%) were isolated congenital cardiac defects. The most interesting result of the study is the high incidence of so called multiple congenital anomalies (MCA) syndromes. In 17 children (1.95%), a complex MCA syndrome was diagnosed in the neonatal period: six MCA syndromes with monogenic inheritance, two chromosomal syndromes not diagnosed in the prenatal period (one 22q11 deletion, one 16q deletion mosaic), and nine children with MCA-(MR) syndromes/sequences of hitherto unknown aetiology (table 2). Two of these 17 children died in the perinatal period (a patient with Coffin-Siris syndrome and one with Fryns syndrome); two others (a child with 16q deletion mosaicism and one with MCA/MR syndrome and complex cardiopathy) died at the age of 18 months and 1 year, respectively. Of the 13 surviving MCA children, seven are moderately to severely mentally retarded (table 2, children 1 (3), 3 (4), 3 (6), 3 (7), and 3 (8)) and two mildly to moderately mentally retarded (table 2, children 2 (2) and 3 (2)). Only the two children with Pierre-Robin sequence, one child with Bartter syndrome, and one child with Wiedemann-Beckwith syndrome are mentally normal.

Table 1 Types of isolated minor and major malformations

<i>Minor malformations</i>	
Unilateral club foot	3
Unilateral hip dysplasia	2
Multiple cutaneous haemangiomas	2
Preauricular appendix	1
Unilateral ptosis	1
Unilateral cataract	2
Skull asymmetry	1
Thyroglossal cyst	1
Hypospadias grade II	3
Total	16 (1.84%)
<i>Major malformations</i>	
<i>Cardiopathies</i>	
Tetralogy of Fallot	1
ASD	1
VSD	4
Urological malformation with ureter duplex	2
Communicating hydrocephalus	1
Liver haemangioendothelioma	1
Spondylocostal dysostosis	1
Unilateral hand malformation with complete syndactyly of two fingers (superdigit)	1
Total	12 (1.38%)

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Table 2 Diagnosis in the 17 children with complex malformations

	No
1 Monogenic syndromes	
(1) Coffin-Siris syndrome (perinatal death) (MIM 135900)	1
(2) Fryns syndrome (perinatal death) (MIM 229850)	1
(3) Microcephalia vera (MIM 251200)	2
(4) Bartter syndrome (MIM 600359)	1
(5) Wiedemann-Beckwith syndrome (MIM 130650)	1
Total	6
2 Chromosomal syndromes	
(1) 46,XY/46,XY,del(16)(q11→qter)	1
(2) 22q11 deletion (velocardiofacial syndrome) with tetralogy of Fallot	1
Total	2
3 MCA/MR syndromes/sequences	
(1) Pierre-Robin sequence	2
(2) Spastic diplegia-adducted thumbs	1
(3) MCA syndrome with pre- and postnatal growth retardation, body asymmetry, renal malformation, conductive deafness	1
(4) MCA/MR syndrome with macrocephaly, ventriculomegaly, VSD	1*
(5) MCA/MR syndrome with microbrachycephaly, facial dysmorphism, ASD, VSD	1
(6) MCA/MR syndrome with complex cardiopathy, facial dysmorphism, pre- and postnatal growth retardation (died at 1 year)	1
(7) MCA/MR syndrome with facial dysmorphism/AVSD	1
(8) MCA/MR syndrome with pre- and postnatal growth retardation, VSD, hypospadias grade 3	1
Total	9
Total	17 (1.95%)

*Same malformation complex in deceased sister.

Table 3 Results of the study group compared with chromosomal anomalies in women with positive triple test (unpublished data)

	Normal karyotype* (n=868)			Abnormal karyotype† (n=1316)		
	No	%	95% CI	No	%	95% CI
MCA/MR syndromes‡	17	1.95	1.03–2.88			
Chromosomal anomalies§				29	2.2	1.41–3.00

*Normal karyotype: 868 liveborn children after amniocentesis with normal karyotype and normal amniotic fluid AFP for maternal serum positive triple test.

†Abnormal karyotype: 1316 women in a two year period with amniotic fluid sampling for maternal serum positive triple test.

‡MCA/MR syndromes: multiple congenital anomaly syndromes (table 2[†2]) or complex malformations.

§Chromosomal anomalies: all unbalanced structural or numerical autosomal anomalies (exclusion of sex chromosomal anomalies and balanced translocations).

Discussion

The general incidence in liveborns of most of the complex malformation syndromes diagnosed in this study, for example, Fryns syndrome, Coffin-Siris syndrome, and Wiedemann-Beckwith syndrome, is very low (less than 1 in 10 000 to 1 in 20 000 live births). So far, only a few case studies have been reported on positive maternal serum triple test screening and the occurrence of a MCA/MR syndrome, for example, Rubinstein-Taybi syndrome, in the liveborn child.¹¹

The findings in the present study show that the incidence of rare so called MCA/MR syndromes, diagnosed at birth, is significantly increased in children born after maternal serum positive triple test screening (with a normal karyotype after amniocentesis).

The number of MCA/MR syndromes (17 out of 868 children = 1.95%) found in our study group compares well with the number of autosomal unbalanced structural or numerical anomalies found in a control group of 1316 women referred to our centre for maternal serum positive triple test (29 of 1316 = 2.20%) (unpublished data) (table 3). This indicates that triple test screening for Down syndrome with the evaluation of ms hCG, μE_3 , and AFP in the second trimester of pregnancy selects a broader group of pregnancies at risk for serious MCA (MR) syndromes.

We conclude that a positive triple screening test result selects a group of pregnancies at risk for serious multiple congenital anomaly syndromes with the same efficiency as for numerical chromosomal abnormalities. These data need confirmation by further studies.

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A syndrome of overgrowth and acromegaloidism with normal growth hormone secretion is associated with chromosome 11 pericentric inversion

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An acromegalic phenotype in late childhood or early adulthood is shared by a variety of clinical conditions, including growth hormone (GH) excess.¹ Exclusion of an abnormality of the somatotrophic axis in a young patient with acromegaloid features should lead the differential diagnosis towards diagnoses such as pachydermoperiostosis (MIM 167100)²⁻⁵ or insulin mediated pseudoacromegaly, a disorder associated with severe insulin resistance.⁶ In the absence of insulin resistance and findings characteristic of pachydermoperiostosis, such as thickening of the periosteum (visible mostly in skull x rays) or the skin, acrolysis, or alopecia,⁴⁻⁷ another genetic syndrome associated with acromegaloid features may be considered.⁸⁻¹³ These are rare conditions, having each been described in individual kindreds, and their causes remain unknown. Inheritance, when present, appears to be as an autosomal dominant trait. They are almost always associated with abnormalities of the skin, the mucosa, and its appendages, such as keratitis,⁹ thickened mucosa,¹⁰ hypertrichosis,¹² and cutis verticis gyrata.⁸⁻¹³

In this report, we identify a chromosomal anomaly that was confirmed by fluorescence in situ hybridisation (FISH) in a patient with acromegaloid features and his family. The patient, his mother, and sibs participated in protocol 97-CH-0076 (National Institute of Child

Health and Human Development, National Institutes of Health (NIH)) and consented to cytogenetic and DNA studies, and the use of the proband's photographs for the purposes of medical education and publication.

Case report

The proband was a 14 year 3 month old male (fig 1) who was referred to our clinic with the diagnosis of possible acromegaly. He was born at term after an uncomplicated pregnancy. His birth weight was 5018 g (over the 95th centile for a newborn and on the 50th centile for a 2½ month old boy), and his length was 60 cm (over the 95th centile for a newborn and on the 50th centile for a 3 month old boy). At birth, he had a submucosal cleft palate and a diaphragmatic hernia for which he underwent surgical repair. Later, as a child, he developed sleep apnoea, but the rest of his health and development were normal. He entered and completed puberty normally and continued to grow in parallel to but above the 95th centile. The patient's mother, one sib, and an uncle (who had died of complications of sleep apnoea) had palatal clefts, overgrowth, and acromegaloid features of variable severity (fig 2).

The patient's physical examination showed symmetrical overgrowth (both height and weight over the 95th centile); his facies and body habitus were acromegaloid but the

patient had normal tooth spacing and an absence of acral enlargement (fig 1). The function and size of the lacrimal glands were normal. Oral examination showed a normally sized tongue. The size of the submandibular salivary glands was normal on examination of the neck. Thyroid, heart, and abdominal examinations were normal with no evidence of nodules, cardiac dysfunction, or organomegaly, respectively. The skin texture was normal, albeit somewhat oily; the patient had acne, as shown in fig 1B. Genital examination and pubic hair were normal (Tanner V); testicular volume was 25-30 ml, bilaterally.

The evaluation of the patient's somatotrophic axis is presented in table 1. All assays were performed at Endocrine Sciences (Calabasas Hills, CA) or Covance Laboratories (Vienna, VA). It included responses to both stimulation (arginine, clonidine, and L-Dopa) and suppression (oral glucose tolerance test (oGTT)) tests of GH, thyrotrophin releasing hormone (TRH) stimulation testing, and insulin-like growth factor type 1 (IGF-1) levels. The patient's 24 hour, every 20 minute GH secretion pattern was also obtained (data not shown). Radiological and skin biopsy examinations were also performed to exclude pachyder-

moperiostosis. Pituitary imaging and sonograms of the abdomen, liver, kidneys, and testes (data not shown) were also obtained. During the patient's evaluation at the NIH, his bone age was estimated; it was then compared with previous recordings of the patient's bone age at different chronological ages, obtained elsewhere (table 2).

The patient lacked the characteristic for acromegaly mandibular and acral enlargements (fig 1), but he had prominent supraorbital ridges and generally coarse facies. His laboratory evaluations, including oGTT, TRH stimulation, 24 hour GH secretion, and IGF-1 levels, were normal (table 1). Pituitary magnetic resonance and other imaging studies, which included an echocardiogram and renal ultrasonography, were also normal (data not shown). The patient did, however, have an advanced bone age (table 2). The clinical information that was available for the other members of the family is summarised in fig 2.

To search for features of pachydermoperiostosis, radiological imaging of the skull and the extremities and a skin biopsy were obtained. The former showed normal bones, without any signs of periosteal proliferation or new bone growth; the fingers were normal, without any

Figure 1 Clinical findings in the patient: (A) Symmetrical overgrowth. (B) Acromegaloid facies. (C) Lack of prognathism despite coarse facies and prominent supraorbital ridges. (D) Normally spaced teeth. (E, F) Absence of acral enlargement.

	Proband (III.2)	Mother (I.1)	Sister (III.1)	Half brother (III.3)	Maternal uncle* (II.2)
Height (centile)	> 95	> 95	> 95	> 95	> 95
Coarse facies	+++	++	+	++	+++
Tongue enlargement	–	–	–	–	+
Prognathism	–	+	–	–	+
Cleft palate	Submucosal	Complete	Submucosal	–	Complete
Sleep apnoea	++	+	–	?	++
Hernia	Diaphragmatic	–	Umbilical	–	–

* Information on II.2 was derived from his sister and from his necropsy report.

Figure 2 Pedigree and clinical information on members of the family with acromegaloidism and *inv(11)(p15.3;q23.3)*.

Table 1 Clinical profile of the proband

Time (minutes)	oGTT			TRH test		
	Glucose (mg/dl)	Insulin (μ IU/ml)	GH (ng/ml)	TSH (μ IU/ml)	PRL (ng/ml)	GH (ng/ml)
0	87	16.1	2.8	2.3	9.4	0.2
15				12.8	62.3	0.6
30	97	26.6	0.8	18.4	56.1	0.3
45				15.6	46.8	0.2
60	116	34.6	0.3	16	32.2	0.1
90	109	34.9	0.3			
120	101	21.1	0.9			
180	94	21.9	0.1			
Other tests						
Peak GH to clonidine		10.1 ng/ml				
Peak GH to arginine		15.6 ng/ml				
IGF-1		429 ng/ml (age related normal range 286–660, mean 403)				
IGFBP-3		3 mg/l (age related normal range 2.2–5.9, mean 4.2)				
IGF-2		348 ng/ml (age related normal range 245–737, mean 491)				
GHRH		19 pg/ml (normal values are less than 50 pg/ml)				
GHBP		139 pmol/l (age related normal range 52–783 pmol/l)				
C peptide		1.2 ng/ml (normal fasting values 0.4–2.1 ng/ml)				

widening indicative of clubbing (data not shown). A biopsy of skin obtained from the left forearm showed normal histology (data not shown). Adnexal structures were of the expected number and size (hair follicles and eccrine glands). Furthermore, the mucin content in this biopsy did not differ from normal controls.⁴

Table 2 Bone versus chronological age in the proband

Chronological age	Bone age
11 6/12	14
13 4/12	16
14 9/12	19

The patient's mother and sisters were also briefly examined, although they declined further investigation. The mother and one of the

Figure 3 G banding high resolution karyotype of the proband; the arrows point to two paradigms of the *inv(11)(p15.3;q23.3)* abnormality.

Figure 4 FISH on cultured lymphocytes from the proband with two probes that hybridise to the 11p telomere (11pter) and to the *MLL* gene on 11q23, respectively, showed that, on one chromosome 11, the two probes were positioned proximal to each other (indicated by the arrow), whereas they hybridised to their expected positions on the other chromosome 11 (upper right corner of the picture).

sisters had a height in excess of the 95% centile. Both shared cleft palate defects with the proband but had less obvious acromegaloid features. The mother's GH, GHRH, and IGF-I levels and oGTT responses were also normal (data not shown).

High resolution karyotype analysis was obtained by standard methods from the patient, his mother, father, and three sisters. Probes for fluorescence in situ hybridisation (FISH) were nick translated and hybridised on metaphase chromosomes that were prepared from peripheral lymphocytes, as previously described.¹⁴⁻¹⁶ Ten metaphases were scored for every probe examined. The commercially available probe for the *MLL* gene at 11q23¹⁷ was labelled with digoxigenin (Oncor Inc, Gaithersburg, MD) and detected with fluorescein (Oncor, Gaithersburg, MD). Chromosome identification was accomplished through cohybridisation with a chromosome 11 telomere specific probe (11pter) that was labelled with Spectrum Green (Vysis, Naperville, IL). Images were obtained with a Zeiss Axiophot microscope, equipped with a "Cytovision" imaging system (Applied Imaging, Pittsburgh, PA), as previously described.¹⁶

The peripheral blood karyotype of the proband showed that, on one of the chromosome 11 pair, the chromatin near the tip of the p arm had a pattern similar to that of the distal 11q region (fig 2), suggesting a pericentric inversion of chromosome 11 or a 46,XY,inv(11)(p15.3;q23.3) karyotype. FISH with two probes that hybridise to the 11p telomere (11pter) and to the *MLL* gene on 11q23,¹⁷ respectively, showed that, on one chromosome 11, the two probes were positioned on the p arm, with the *MLL* probe centromeric to the 11pter probe; the two probes hybridised to their expected positions (11pter on the distal p arm and *MLL* on the distal q arm) on the other chromosome 11 (fig 4). Classical and molecular cytogenetic analysis showed the same abnormality in the patient's mother and one of his sisters (data not shown); these studies were normal in the patient's father and his other two sisters, none of whom had this phenotype.

Discussion

No chromosomal abnormality or genetic locus has previously been described in the various syndromes associated with acromegaloidism. The present report identified a pericentric inversion of chromosome 11 that segregated with acromegaloid features and other abnormalities in one family. This finding may assist in the identification of gene(s) responsible for these conditions, only after proper clinical classification of acromegaloidism.

Acromegaloidism describes a highly heterogeneous group of disorders. In general, acromegaly and gigantism, the two syndromes of GH excess, share several features with pseudoacromegaly caused by severe insulin resistance.⁶ The distinction between these two groups of disorders is made by the laboratory finding of insulin resistance in pseudoacromegaly.⁶ Pachydermoperiostosis and the other

syndromes associated with acromegaloidism have neither insulin resistance nor GH excess, the exception being an apparently coincidental GH producing pituitary adenoma found in a patient with pachydermoperiostosis.¹⁸

The differentiation of pachydermoperiostosis from the other conditions is made radiologically or histologically or both. Patients with this condition have evidence of periosteal thickening of the skull or the long bones, digital clubbing, and/or acrolysis, similar to that seen in hypertrophic pulmonary osteoarthropathy or inherited acropathy.^{3 4 7 19} In addition, several cutaneous findings, including papular mucinosis, hyperkeratosis, and generalised hypertrophy of epidermal appendages,^{3 5 7 8} are distinctive features suggestive of pachydermoperiostosis. Cutis verticis gyrata (multiple furrows on the scalp and/or forehead) is also often seen in association with pachydermoperiostosis.^{4 19} Periostosis without acromegaloid features or any skin involvement has been reported and is referred to as Currarino disease²⁰; however, this condition should not be considered in the differential diagnosis of acromegaloid syndromes.

Other reports of families with an acromegaloid phenotype include a Kirghizian family with arthritis, osteolysis, keratitis, and other ectodermal findings with autosomal recessive inheritance (MIM 221810),⁹ a Canadian family with thickened oral mucosa and acral hyperextensibility (MIM 102150),¹⁰ and an Irish kindred with coarse facies and generalised hypertrichosis since childhood.¹² The family reported by Dallapicola *et al.*¹¹ was similar to the one reported by Hughes *et al.*¹⁰ As in most of the other patients with non-pachydermoperiostosis acromegaloid syndromes,⁹⁻¹² the features of acromegaloidism in our family were confined to the face. On the other hand, the proband had a history of a cleft palate and a diaphragmatic hernia. Neither of these congenital anomalies has been reported in other patients with acromegaloid syndromes. Although the diaphragmatic defect was not present in any of the patient's affected relatives, variable cleft palate was present in all of them.

The aetiology of acromegaloidism in our family remains unclear. The overgrowth and advanced bone age of the proband support the notion of aberrant expression of an unknown growth factor. The presence of an unidentified growth factor in the serum of patients with acromegaloidism has been suggested before.²¹ A substance of approximate molecular weight of 70 000 daltons was found in the sera of five subjects with acromegaloid features. Its growth promoting activity was shown by determining its effect on human erythroid cell progenitors *in vitro*. It was shown to be independent of epidermal, nerve, or fibroblast growth factors and growth hormone.²¹ Although the present study could be used to suggest that perhaps the gene coding for this factor is on chromosome 11, the patients studied by Ashcraft *et al.*²¹ had phenotypes different from those of our patients; at least one of them had acral thickening similar to that seen in acromegaly or pseudoacromegaly and another had, perhaps, true acromegaly.²¹

It is also possible that the genetic defect in our kindred is not a growth factor per se, but rather a signalling molecule that participates in a related pathway. It was recently found, for example, that patients with insulin resistance and pseudoacromegaly⁶ have a defect in phosphoinositide 3-kinase signalling,^{22 23} leading to insulin resistance and activation of mitogenic pathways stimulated by the insulin receptor.²²

The genetic defect responsible for the phenotype in our family could also involve inappropriate repression of a gene because of fusion of genetic material from another sequence on chromosome 11, the interruption of an inhibitory state (imprinting or a related state), or the deletion of suppressive elements. Similar abnormalities have been described in patients with chromosome 11 cytogenetic defects that involved growth factors and other genes mapped to this chromosome²⁴ and would also explain the variability of the phenotype seen in our family. Examples include Beckwith-Wiedemann syndrome that maps to 11p15.5 and is believed to result from relaxation of imprinting of this area leading to IGF type 2 (IGF-2) overexpression.²⁴ The 11p15.3 breakpoint of the pericentric chromosomal inversion in our kindred, however, is quite distal to the IGF-II and related loci.^{25 26} A search of the available databases²⁷ failed to find any other imprinted sequences or known growth factor genes that map to the breakpoints of the inversion.

In conclusion, we present a family with variable clinical expression of acromegaloidism associated with other developmental defects that segregate with a pericentric inversion of chromosome 11. This report may lead to the identification of new gene(s) on chromosome 11 that are important for midline fusion and growth of facial structures.

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Maternally inherited duplication of the possible imprinted 14q31 region

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The existence of parent of origin differences in the expression of some genes, a process known as genomic imprinting, has been recognised and documented over the past several years. This epigenetic marking process results in the differential expression of normal genes, depending on whether they are of maternal or paternal origin. A number of human disorders have been identified as resulting from alterations in genomic imprinting.¹ One type of genetic abnormality which can unmask genomic imprinting is uniparental disomy (UPD), in which both chromosomes of one pair are inherited from one parent, with no contribution from the other.² Distinct phenotypes exhibited by patients with maternal and paternal UPD(14) strongly suggest that at least some genes on human chromosome 14 are subject to imprinting effects.³⁻⁶ This finding is supported by studies in the mouse indicating that the distal portion of chromosome 12, recognised as a candidate imprinted region, is syntenic with human chromosome 14q.⁷

Nevertheless, no imprinted genes have yet been identified on chromosome 14, and the location of the imprinted region on human chromosome 14 remains unclear.

Analysis of parental origin effects in human trisomy for chromosome 14q⁸ or monosomy for the same chromosome⁹ makes the region 14q23-q32 a candidate region for containing imprinted genes.¹⁰ Moreover, the observation of a partial duplication of 14q in a developmentally delayed girl with minor abnormalities and her phenotypically normal father led Robin *et al*¹¹ to propose that the 14q24.3-q31 region may be imprinted. As genotype-phenotype correlations in patients with aneuploidy for this region may help the identification of imprinted genes on human chromosome 14, we report here a direct 14q31 duplication observed in a child with mild developmental delay and his phenotypically normal mother. This duplication was also detected in five relatives with a normal phenotype and maternal inheritance through three generations.

Figure 1 Partial R and G banded karyotype from the proband showing the normal (left) and the duplicated (right) chromosomes 14.

Case report

Our patient, a 2 month old boy, was referred following a cyanotic episode. He was the first child of healthy, unrelated parents. The family history was uneventful and he was born by caesarean section. Birth weight, length, and head circumference were 3515 g, 54 cm, and 33 cm, respectively. At the age of 2 months, clinical examination showed dysmorphic features (macrosomia, metopic suture, neck hyperextension without opisthotonos, large nose, prominent philtrum, gingival hyperplasia, and bilateral palmar simian crease) and axial hypotonia with moderate distal hypertonia. EEG and MRI scan of the brain, medullary ultrasound, abdominal ultrasound, pulmonary and skeletal x ray, ECG and retinal examination were all normal. The patient walked at 20 months. By the age of 27 months, clinical evaluation of the patient showed very slight dysmorphism, slight overgrowth (+2 SD),

moderate psychomotor delay with specific speech delay, hypotonia, and behavioural and sleep disturbance.

Routine R and G banding techniques, performed on metaphases from PHA stimulated lymphocytes of the proband, showed an elongated chromosome 14 with an extra G positive band on the long arm (fig 1). High resolution banding suggested that the patient had a duplication of band 14q31. The same 14q duplication was found in the proband's mother and four other members of his family, all of whom had a normal phenotype (fig 2A).

Fluorescence in situ hybridisation (FISH) was performed with various probes, in order to characterise the rearrangement. A chromosome 14 specific paint, used as recommended by the manufacturer (ONCOR), confirmed that the extra material originated from chromosome 14 (data not shown). FISH experiments using YAC probes estimated to cover the 14q31 band were performed, in order precisely to define the duplicated chromosomal region in the proband. The YAC clones 813e6, 930c12, 749g4, 815d5, 872e3, 756a4, 945d2, 856g8, and 965b9, obtained from the CEPH (Centre d'Etude du Polymorphisme Humain), were labelled with biotin-14dCTP or digoxigenin-11dUTP by random priming (Bioprime DNA labelling system, Life Technologies and Dig high prime, Roche Diagnostics), then hybridised on metaphase chromosomes or interphase nuclei. The hybridised biotin signals were made visible with fluorescein labelled avidin, and the digoxigenin signals were visualised with rhodamine labelled anti-digoxigenin antibody following standard protocols.¹² The results are summarised in fig 2B. The more proximal YACs (813e6 and 930c12), as well as the more distal ones (945d2, 856g8, and 965b9), gave signals of equal intensity on the normal and the duplicated chromosomes 14, indicating the presence of only two copies in the patient's genome. In contrast, YACs 749g4, 815d5, 872e3, and 756a4 showed two signals

Figure 2 (A) Pedigree of the proband (indicated by an arrow). (B) The different YACs used for delineating the extent of the duplication are listed on the right of the chromosome 14 diagram, with their normal (-) or duplicated (+) status.

Figure 3 Double FISH experiment both with a YAC included in the duplication (YAC 815d5, green signal) and a YAC located outside the duplication (YAC 965b9, red signal). Chromosomes and nuclei were counterstained in blue with DAPI. (A) Metaphase of the proband showing the superimposition of the green and red signals, resulting from the condensation of the metaphase chromosomes. (B) Partial karyotype from the same metaphase, showing the normal (left) and the duplicated (right) chromosomes 14. Both green and red signals have been dissociated in order to evaluate their size better. The green signal (YAC 815d5) located on the duplicated chromosome 14 is larger than the one located on the normal chromosome 14. It is also larger than each red signal (YAC 965b9) on both chromosomes. (C) In interphase nuclei, two red (YAC 965b9) and three green (YAC 815d5) signals are visible. The three green signals correspond to one copy on the normal and two copies on the duplicated chromosome 14.

of different sizes, the larger one being located on the duplicated chromosome 14. In order to confirm these results, two YACs with different FISH patterns were cohybridised on the same

Figure 4 The orientation of the duplication was studied by dual colour FISH on interphase nuclei, using YACs located at the borders of the duplicated segment: YAC 749g4 (green signal) at the proximal edge and YAC 756a4 (red signal) at the distal edge. Alternating of red and green signals shows the direct orientation of the duplication on the rearranged chromosome.

metaphase preparations: YAC 815d5 (green signal) presumed to be included in the duplication and YAC 965b9 (red signal) presumed to map outside the duplication. As expected, the green signal (815d5) on the duplicated chromosome 14 was larger than the red signal (965b9) on the same chromosome (fig 3A, B). The same experiment was performed on interphase nuclei, where the undercondensation of the chromatin allows increased resolution. This allowed the detection of two red signals (YAC 965b9) and three green signals (YAC 815d5), clearly showing a duplication of the region covered by YAC 815d5 (fig 3C). Different YAC probes were hybridised with metaphase spreads from subjects III.2 and III.3, confirming the presence of the same 14q duplication in the proband and his relatives.

In order to determine whether the duplication is direct or inverted, the proximal and distal duplicated YACs (749g4 and 756a4 respectively) were labelled differently, hybridised to interphase nuclei, and visualised with specific fluorochromes. Alternate green and red signals observed in nuclei show that the two copies of the duplication are organised in direct orientation on chromosome 14 (fig 4).

In order to gain insight into the mechanism that gave rise to this duplication, we used mic-

Figure 5 Polymorphism analysis with two informative markers included in the 14q31 duplication, D14S1052 and D14S67. For the proband (IV.1), his mother (III.2), and his uncle (III.3), all carrying the duplication, only two distinct alleles are identifiable, suggesting the monochromosomal origin of the rearrangement. The arrows indicate the allele shared by the three subjects, corresponding to the abnormal chromosome.

rosatellite analysis to determine whether the duplication originated from one or two homologous chromosomes 14. Genomic DNA from the proband and other family members was extracted from whole blood samples using standard methods. DNA polymorphisms were analysed, as previously described,¹³ using polymerase chain reaction (PCR) amplification of short sequence repeats from the following markers: D14S68, D14S67, D14S1052, and D14S1033 (Genethon). For three informative markers located in the duplicated region (D14S68, D14S67, and D14S1052), only two different alleles were observed in the patient (IV.1), his mother (III.2), and his uncle (III.3) (fig 5). One of these alleles is shared by these three members of the family and probably represents the abnormal chromosome 14. This result suggests a monochromosomal origin of the duplicated segment, probably resulting from unequal sister chromatid exchange.

Finally, on the basis of the genetic map of chromosome 14,¹⁴ we were able to estimate the approximate length of the duplicated segment to be between 3 and 9 cM. These data are in agreement with our cytogenetic observations, as the duplication corresponds approximately to cytogenetic band 14q31, whose physical size is estimated to be 8.8 megabases.^{15 16}

Discussion

In this study, we describe a phenotypically abnormal patient carrying a duplication of the 14q31 band, which occurs in the potentially imprinted region on this chromosome.^{8 9 10 11} The duplication, which was also found in five other family members, was maternally inherited through three generations. Apart from the proband (IV.1), who has minor dysmorphic features and psychomotor delay associated with behavioural and sleep disturbance, all family members carrying the abnormal chromosome are phenotypically normal. This suggests that the abnormal phenotype of the proband is probably unrelated to the dupli-

cated segment. Moreover, duplication of the 14q31 band, when maternally inherited, does not seem to be associated with an abnormal phenotype.

It has been suggested by Robin *et al*¹¹ that a similarly duplicated chromosome 14 (duplication 14q24.3–q31), if paternally transmitted, does induce an abnormal phenotype. This parent of origin dependent phenotypic disparity associated with similar duplications suggests a possible imprinting effect. The fact, however, that the duplications are not identical allows three different hypotheses to be formulated with respect to imprinting effects.

Firstly, the imprinted genes could be located outside the 14q24.3–q31 interval. This region may contain genes that do not have a deleterious effect if overexpressed, and duplication will therefore produce no phenotypic effect. In that case, the association of the 14q duplication with an abnormal phenotype, both in the proband described here and in that described by Robin *et al*,¹¹ would be merely coincidental.

Secondly, the imprinted genes could be located in band 14q24.3 and not in band 14q31. In that case they would be duplicated in the patient described by Robin *et al*¹¹ but not in the family described here. In such a situation, no parent of origin phenotypic effect would be associated with a 14q31 duplication. Then, a 14q31 duplication of paternal origin would be associated with a normal phenotype, as we show to be the case here for a 14q31 duplication of maternal origin. This hypothesis is supported by the fact that G positive bands, such as the 14q31 band, contain very few genes.¹⁷

Thirdly, imprinted genes could be located in band 14q31 and duplicated both in the family reported here and in that of Robin *et al*.¹¹ If this were the case, both observations are in agreement and suggest that imprinted genes from this region are paternally expressed. However, the presence of paternally expressed genes only is not consistent with the observation that the maternal UPD(14) phenotype is moderate compared to the paternal UPD(14) phenotype. This would suggest that maternally expressed genes are located elsewhere in the 14q23–q32 region. Unfortunately, it is not possible to choose between these three hypotheses because no case of paternally inherited 14q31 duplication has yet been described.

So far, only a few genes have been mapped to the human 14q31 band (NCBI <http://www.ncbi.nlm.nih.gov>), and none of them has been shown to be imprinted. Those that have been mapped include the genes encoding galactosylceramidase (*GALC*), the thyroid stimulating hormone receptor (*TSHR*), the suppressor of lin-12 (*C elegans*)-like (*SEL1L*), neurexin III (*NRXN3*), and a member of the G protein coupled receptor superfamily (*GPR68/OGRI*).

Further studies of chromosome 14 aneuploidy are needed in order to define the imprinted region better and identify the corresponding genes.

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A novel missense mutation in the GTPase activating protein homology region of *TSC2* in two large families with tuberous sclerosis complex

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder (OMIM 191092) characterised by autism, seizures, mental retardation, benign tumours of the brain, heart, kidney, lung, and skin, and malignant tumours of the kidney.¹ TSC has a wide range of phenotypic variability, with some subjects severely affected and others only mildly affected. There are two TSC genes, *TSC1* on chromosome 9q34² and *TSC2* on chromosome 16p13.³ Approximately two thirds of cases of TSC appear to result from de novo germline mutations.

In 1994, an extended four generation family with 19 affected members was reported in which 34 members (17 affected with TSC and 17 unaffected) underwent both physical and psychiatric assessments.⁴ The majority of the affected subjects had mild physical expression of TSC, but there was significant clustering of neuropsychiatric disorders among affected subjects compared with their unaffected relatives. The disorders that were over-represented included mood disorder, anxiety disorder, and autism. The largest difference was observed in anxiety disorder, which was seen in 10 of the affected subjects and in two of the unaffected subjects ($p=0.016$). One affected child had

pervasive developmental disorder and one had autism. Analysis of this family suggested that TSC could present phenotypically with mild physical signs and symptoms, but with significant neuropsychiatric disease. Linkage to the *TSC2* gene locus on chromosome 16p13.3 was shown with a lod score of over 3.⁴

We report here the identification of a missense mutation in exon 34 of the *TSC2* gene in affected members of this family. We also examined a second four generation family⁵ from the same geographical area as the first family but not known to be related to them. The same exon 34 mutation was found in affected members of the second family.

Methods

To search for mutations in the coding regions of the *TSC2* gene we used single strand conformation analysis (SSCP). The primers amplifying each of the 41 exons of *TSC2* and the PCR conditions have been described previously.⁶ The PCR products were run on MDE gels (AT Biochem). To maximise the detection of variant bands, each PCR product was run on two gels, one without glycerol and one with 5% glycerol. Samples in which variant bands were detected were reamplified and sequenced. This

Figure 1 (A) Detection of variant bands in exon 34 using single strand conformation polymorphism analysis. The first two lanes (indicated below the figure with "1") contain DNA from patient 355. The other lanes (labelled 2, 3, and 4) contain DNA from three controls unrelated to this family. The arrows on the left indicate the variant bands and the lines on the right indicate the position of the wild type bands. (B) Sequencing of exon 34 from patient 355. The arrow indicates the double peak of A and C in germline DNA. (C) Sequencing of other affected and unaffected members of the families. Subjects 202, 210, 304, 305, 307, 325, and 328 are affected members of the first family. Subjects 715 and 717 are affected members of the second family. Subjects 201, 212, and 302 are unaffected members of the first family.

study was approved by the Institutional Review Boards of Fox Chase Cancer Center, the University of California at Los Angeles, and the University of California, Irvine.

Results

We found variant bands in exon 34 in DNA from the index patient (patient 355) from the first family (fig 1A). Variant bands were not found in any other exon of *TSC2*. DNA sequencing of exon 34 showed an A to C change at position 4508 (fig 1B). We then analysed 12 affected family members and three unaffected family members from the first family. All of the affected subjects, and none of the

unaffected family members, had the exon 34 change (fig 1C).

We next tested affected and unaffected members of a second, four generation, TSC family, TS-15. This family, from the same geographical area as the first family, includes 24 affected subjects.⁵ The same exon 34 change found in the first family was found in both of the affected members that we tested and none of six unaffected members. We also analysed *TSC2* exon 34 from 57 unrelated subjects without a personal or family history of TSC. None of these controls had the A4508C change, indicating that this is not a common genetic polymorphism.

Discussion

The A4508C mutation is predicted to change amino acid 1503 from glutamine to proline. This amino acid is identical among the human, mouse, rat, fugu, and *Drosophila* homologues of tuberin, the product of the *TSC2* gene (fig 2). Exons 34 to 38 of *TSC2* encode a region of tuberin with homology to rap1 GTPase activating protein (GAP).³ Tuberin has been shown to have GAP activity for both rap1⁷ and rab5.⁸ The A4508C is the first missense mutation identified in exon 34 of *TSC2*. Ten *TSC2* missense mutations in the GAP domain have been previously reported (one in exon 35, one in exon 36, five in exon 37, and three in exon 38). These are described in detail in the online TSC Variation Database (<http://expmed.bwh.harvard.edu/ts/>). Only one of the previously reported GAP domain missense mutations (in exon 38) was found in a familial, rather than a sporadic, case of TSC.

In the first family with the exon 34 mutation, mild physical signs of TSC were associated with significant neuropsychiatric symptoms including pervasive developmental disorder and autism.⁴ The second family has not yet had formal neuropsychiatric evaluations. However, like the first family, many affected subjects appear to have a mild form of TSC. For example, hypomelanotic skin macules (white spots) or white skin freckles were the only known manifestation of TSC in 10 of the 19 affected subjects in the first family (52%) and 12 of the 23 affected subjects in the second family (52%).

Several of the mildly affected subjects in these families might not have been recognised as having TSC had they not been related to the index patients. This raises the possibility that people in the general population with neuropsychiatric disease who lack the classical signs of TSC could also have germline missense changes within the GAP domain of *TSC2*. TSC is associated with autism, hyperactive behaviour, sleep disorders, and aggressive behaviour.⁹ Both mutations and polymorphisms in the TSC genes could therefore be considered candidate susceptibility or genetic modifier alleles for any of these disorders. *TSC2* may be of particular interest as a possible susceptibility locus for autism because autism appears to have a strong genetic component.¹⁰ Multiple genome wide screens have been performed,¹¹ two of which have identified

Figure 2 Evolutionary conservation of *TSC2* exon 34. Residues in red are identical to the human sequence, and those in green are similar. The arrow indicates the glutamine residue affected by the mutation.

potential autism susceptibility loci on chromosome 16p,^{12,13} the location of *TSC2*.

In summary, we have identified a novel missense change at a highly conserved residue within the region of GTPase activating domain homology of the *TSC2* gene in two four generation TSC pedigrees with a total of more than 40 affected members. This is, to our knowledge, by far the largest known group of TSC patients carrying the same mutation. Therefore, we anticipate that these families will be important in the future identification of modifier gene effects in TSC. In one family, an association of TSC with significant neuropsychiatric disease has already been documented. Further studies will be required to understand biochemically the functional consequences of this exon 34 missense mutation and to characterise more completely the clinical and neuropsychiatric manifestations of TSC in these families. Understanding the relationship between naturally occurring germline *TSC2* mutations and neuropsychiatric disease could elucidate the underlying biology of TSC and potentially facilitate studies aimed at prevention and/or early diagnosis.

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Interstitial deletion of 3p22.2-p24.2: the first reported case

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Autosomal deletions or chromosomal haploinsufficiency syndromes are observed in 1 in 7000 live born infants¹ and may cause multiple malformations, growth failure, and mental retardation. Deletions on the short arm of chromosome 3 have been reported in 35 cases and have been divided into two groups: deletion 3p syndrome² with breakpoints between 3p24 and 3p25 and proximal deletion 3p syndrome³ with different breakpoints between 3p11 and 3p21.2. The first reported case of an interstitial deletion of chromosome 3p22.2-p24.2 in a 6 year old male with developmental delay is presented here.

Case report

The proband was the fourth child born, in England, to healthy, unrelated, white parents. There was no family history of note. He was born vaginally following spontaneous onset of

labour at 41 weeks of gestation after an uneventful pregnancy and weighed 3140 g (10th centile). A murmur was noted shortly after delivery and echocardiography confirmed the presence of a small, perimembranous ventricular septal defect. His early milestones were reported as normal, but he was referred for assessment of developmental delay when aged 16 months. He made good progress following input from a child development unit. He walked at 23 months and had speech delay. He was reassessed three months after arrival in New Zealand at the age of 3.5 years. He had global developmental delay and it was felt he had some hearing impairment. His language skills were poor, only speaking occasional two to three word sentences by the age of 4 years, although his comprehension was felt to be good. He was a sociable child with no behavioural difficulties. He needed nappies at

Figure 1 Facial appearance of the patient.

Figure 2 Profile showing midface hypoplasia and posterior angulation of the ear.

Figure 3 Appearance of the proband's feet with incurving of the fourth toe and wide gap between the first and second toes.

Figure 4 A partial karyotype showing the $\text{del}(3)(\text{p}22.2\text{p}24.2)$ chromosome and ideogram.

night and help with toileting in the daytime. He had no other health problems.

On examination at the age of 5.5 years, his height was 100.5 cm (4 cm <3rd centile), weight 16.2 kg (3rd-10th centile), and head circumference 52.7 cm (75th centile). He had mild bilateral fifth finger clinodactyly. He had midface hypoplasia and a prominent forehead, shown in figs 1 and 2. He had posterior angulation of his ears and a thin upper lip. His nipples were widely spaced. His big toes were short and relatively broad and his fourth toe curled under the third toe (fig 3). The fifth toenail was hypoplastic and there was a wide gap and deep groove between the first and second toes. There were no other dysmorphic features and the remainder of the examination was unremarkable.

Audiological assessment showed a right unilateral sensorineural hearing loss (40-45 dB loss). X ray of his foot was carried out because of the unusual appearance of the toes. No significant bony abnormality was seen.

CYTOGENETIC AND FISH STUDIES

Cytogenetic G banded studies on the peripheral blood lymphocytes showed a de novo interstitial deletion of bands p24.2 to p22.2 in the proximal short arm of chromosome 3 (banding level of 550). The resulting karyotype was a de novo 46,XY,del(3)(p22.2p24.2) (fig 4). This finding was confirmed on examination of 60 cells.

Fluorescence in situ hybridisation (FISH) studies were used to confirm the cytogenetic deletion. A whole chromosome 3 painting probe (Oncor, Inc) showed continuous hybridisation along both the short and long arms of both homologues of chromosome 3. Locus specific YAC clones (966g05 locus D3S3714/D3S3680, 938g11 locus D3S1266, 937h04 locus D3S3564, 792d07 locus D3S3678, 802g01 locus D3S658, Max Planck Institute for Molecular Genetics) overlapping the cytogenetic breakpoints showed loss of signal for the locus D3S1266 and D3S3564. The final karyotype has been interpreted as: de novo 46,XY,del(3)(p2.2p24)[60]. ish(wcp3, D3S3714/D3S3680x2, D3S1266-, D3S3564-, D3S3678x2, D3S658x2).

Discussion

Terminal 3p deletions have been described in 24 cases and interstitial proximal 3p deletions^{3 5-14} in 11 cases and are felt to show distinct phenotypes. In the terminal 3p deletion syndrome, the patients have pre- and postnatal growth retardation, mental retardation, and developmental delay. A number of craniofacial anomalies are also described, including flat occiput, triangular face, hypertelorism, epicanthic folds, synophrys, ptosis, broad and flat nose, and downturned corners of the mouth. The patients also have small hands and supernumerary digits. In the proximal 3p deletion syndrome, the patients have a characteristic facial appearance with narrow forehead, epicanthic folds, short palpebral fissures, broad nasal bridge, and low set, poorly formed ears. Developmental delay is also described. Joint abnormalities may be present

including decreased joint mobility, ulnar deviation of the hands, camptodactyly, and calcaneovalgus deformity of the feet.

There have been no deletions published spanning the region 3p22.2-p24.2. In the case reported here, the proband had a number of mild dysmorphic features, global developmental delay, and short stature. These features are frequently described in patients with chromosome deletions and they are likely to be related to the monosomy of the region p22.2-p24.2. This is apparently the first case report with this deletion, which is surprising as, although the features are relatively mild, the combination of growth failure and developmental delay usually leads to a paediatrician checking the karyotype, as these are features associated with chromosomal abnormalities. In this case, it may be that the early response of the patient to effective intervention by the child development team delayed any further testing. The deletion may usually be associated with a milder phenotype and so chromosome analysis is not undertaken. There are other possible explanations for the mild phenotype. A cryptic rearrangement and low level mosaicism were excluded using locus specific probes and an extended examination of 60 metaphases. However, it is not possible to rule out epigenetic modification of the phenotype because of duplication elsewhere in the genome or that the phenotype is mild because the deletion is in a "protected" genomic region.

A meaningful correlation between this deletion and the clinical phenotype is not possible until further cases are described. It may then be possible to contribute further to the knowledge of the morbid anatomy of the human genome.

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