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### Incidence and molecular mechanism of aberrant splicing to a G->C splice acceptor site mutation causing Smith-Lemli-Opitz syndrome

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## Letters to the Editor

*J Med Genet* 2000;37:376-377

### Phosphorylase kinase deficient liver glycogenosis: progression to cirrhosis in infancy associated with *PHKG2* mutations (H144Y and L225R)

EDITOR—Deficiency of phosphorylase kinase (Phk), a regulatory protein kinase in glycogen metabolism, is the most frequent cause of hepatic glycogen storage disease (GSD). Patients typically present as infants with hepatomegaly, growth retardation, and raised triglycerides, cholesterol, and transaminases. Compared to other types of liver GSD, the condition is usually mild and its course is benign such that patients may even become asymptomatic as they grow up. Hypoglycaemia and lactic acidosis, for example, are uncommon in Phk deficiency in contrast to glucose-6-phosphatase deficiency (GSD type I). Hepatic architecture typically remains normal, unlike GSD III (debranching enzyme deficiency) in which hepatic fibrosis is common, and unlike GSD IV (branching enzyme deficiency) which usually progresses to cirrhosis in infancy. Only two infants with Phk deficiency and cirrhosis have been reported. Development of fibrosis and even cirrhosis was found in five older Japanese patients, but it remains to be clarified whether this observation can be generalised and also applies to other ethnic groups.<sup>1-5</sup>

Phk is a complex enzyme consisting of four different subunits,  $(\alpha\beta\gamma\delta)_4$ , and isoforms or splice variants exist for each subunit. This gives rise to genetic and phenotypic heterogeneity of Phk deficiency.<sup>6</sup> A muscle specific form of Phk deficiency is caused by mutations in the gene for the muscle isoform of the  $\alpha$  subunit, *PHKA1*, which resides on the long arm of the X chromosome, whereas liver Phk deficiency can be caused by mutations in three genes: *PHKA2*, *PHKB*, and *PHKG2*. *PHKA2* encodes the liver isoform of the  $\alpha$  subunit. It is located on the short arm of the X chromosome and therefore involved in most (male) cases of liver Phk deficiency.<sup>7</sup> *PHKB*, located on human chromo-

some 16, encodes the  $\beta$  subunit that is expressed in all tissues. *PHKB* mutations therefore cause autosomal recessive Phk deficiency of both liver and muscle, but liver symptoms predominate and the biochemical muscle involvement is often not clinically apparent. Ten different mutations in the *PHKB* gene have been identified in seven patients.<sup>8-10</sup>

*PHKG2*, also autosomal, encodes the testis/liver isoform of the catalytic  $\gamma$  subunit. *PHKG2* mutations seem to be the rarest variant with only five patients described to date, all of them homozygous offspring of consanguineous parents.<sup>11 12</sup>

A genotype-phenotype correlation is emerging in liver Phk deficiency. The functional impact of *PHKB* mutations, both on residual enzyme activity and clinical condition, appears to be mildest. In contrast, *PHKG2* mutations seem to concentrate at the opposite end of the spectrum of severity with very low residual Phk activities, very high plasma lipids and transaminases, and other abnormalities that are uncommon in Phk deficiency, such as lactic acidosis, abnormal glucagon response, or hepatocellular adenoma. Most strikingly, the only two published cases of Phk deficiency who developed liver cirrhosis in infancy were both found to carry translation terminating mutations in the *PHKG2* gene.<sup>11 12</sup> In the present study, we have identified *PHKG2* mutations in another patient with liver Phk deficiency and early cirrhosis, further substantiating that *PHKG2* mutations are associated with a more severe phenotype and particularly with a high risk of cirrhosis.

A male infant, the first child of healthy, unrelated English parents, presented with abdominal distension from 5 months, and three early morning episodes of eye rolling, pallor, and clamminess at 7 months. Examination showed poor growth, muscle wasting, and hepatomegaly 11 cm below the costal margin. Abnormal findings included low prefeed plasma glucose (0.5 mmol/l), raised plasma alanine aminotransferase (587, normal <45 U/l), aspartate aminotransferase (>2000, normal <45 U/l), and  $\gamma$ -glutamyltranspeptidase (2150, normal <40 U/l), and raised plasma cholesterol (18.4, normal 2.5-4.4 mmol/l) and triglycerides (9.3, normal 1.5-1.8 mmol/l). Phk activity

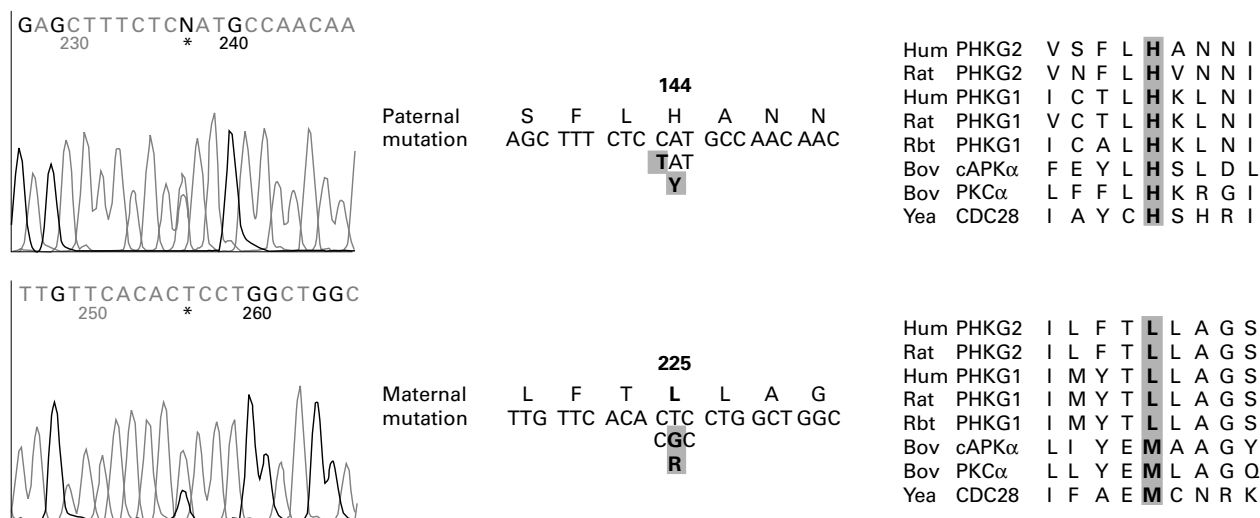


Figure 1 *PHKG2* missense mutations in a patient with Phk deficiency and liver cirrhosis. Sequencer tracings are shown on the left, with heterozygous sequence positions marked by asterisks. On the right, the high conservation of the mutated amino acids is illustrated by alignment of the corresponding sequence regions from the testis/liver (*PHKG2*) and muscle (*PHKG1*) isoforms of the Phk  $\gamma$  subunit from human, rat, and rabbit, and from bovine protein kinase A ( $\alpha$  isoform), bovine protein kinase C ( $\alpha$  isoform), and yeast CDC28 kinase as examples of other serine/threonine kinases. See refs 11 and 13 for sequence references and alignment of additional protein kinase sequences.

was 1.6 U/g Hb (normal, 6-30) in erythrocytes and <1 U/g wet weight (normal, >20) in liver. Hepatic branching and debranching enzyme activities were normal. Liver histology showed fibrous expansion of portal tracts with portal-portal bridging and gross parenchymal glycogen deposition with minimal fat. Overnight nasogastric feeds and two-hourly daytime feeds were necessary to prevent hypoglycaemia and lactic acidosis. Firm irregular hepatomegaly and raised plasma transaminases persisted. Repeat liver biopsy at 3 years showed that glycogen deposition had diminished but cirrhosis was present, with broad bands of interportal fibrosis encircling parenchymal nodules.

Genomic DNA was purified from a blood sample and the *PHKG2* gene amplified in four segments and analysed by direct sequencing in both directions, as described previously.<sup>12</sup> The patient was found to be heterozygous for two single nucleotide replacements: a C to T transition replacing histidine 144 with tyrosine, and a T to G transversion replacing leucine 225 with arginine (fig 1). H144 is not only conserved in both Phk  $\gamma$  subunit isoforms from several species, but also in the catalytic domains of nearly all serine/threonine protein kinases, where it resides in subdomain VI A.<sup>13</sup> Similarly, the counterparts of L225 in more than 100 protein kinase sequences (subdomain IX) are most frequently leucines, methionines, or other hydrophobic or uncharged amino acids, but never charged residues (fig 1).<sup>13</sup> Analysis of parental DNA showed that the H144Y allele had been inherited from the father and the L225R allele from the mother, together accounting for the patient's enzyme deficiency.

The patient described here is the first male case and the first to be found compound heterozygous for *PHKG2* mutations, whereas all previous *PHKG2* cases were female, homozygous offspring of consanguineous parents. All seven *PHKG2* mutations identified to date in different subjects are unique. Including the patient described here, three liver Phk deficiency cases with early cirrhosis have now been analysed for mutations, and *PHKG2* mutations were identified in all three. This suggests strongly that this rare complication of Phk deficient liver glycogenesis is generally caused by *PHKG2* mutations. Among the first group of three other patients not selected for clinical severity in which *PHKG2* mutations were identified, one had a mild course whereas two developed fibrosis but not cirrhosis at an early age together with other complications uncommon in Phk deficiency.<sup>11</sup> A feature shared by all previous cases and the present one is a very low residual Phk activity and pronounced secondary

biochemical abnormalities. Missense as well as truncating mutations are found among both the cirrhotic and the non-cirrhotic patients, so that no obvious correlation between mutation severity and cirrhosis is evident. These observations suggest that patients with *PHKG2* mutations have a much higher risk of severe liver disease than those with *PHKA2* or *PHKB* mutations. The malignant potential of cirrhosis in this small subgroup is unknown.

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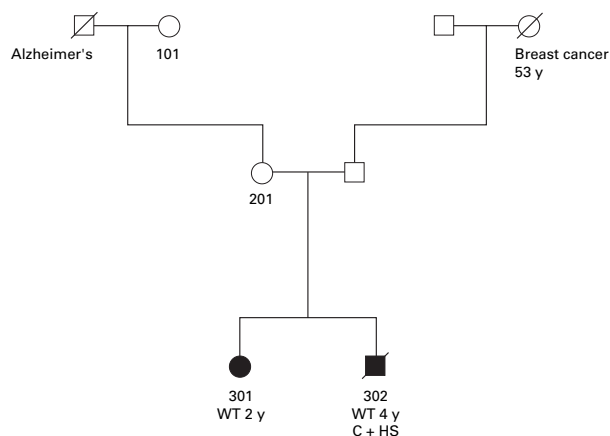
## Familial Wilms tumour resulting from *WT1* mutation: intronic polymorphism causing artefactual constitutional homozygosity

EDITOR—Familial Wilms tumour is rare, accounting for only 1-2% of cases, and is not usually associated with other tumour types or congenital malformation.<sup>1</sup> The pattern of inheritance has been interpreted as a dominant predisposition to Wilms tumour with incomplete penetrance of approximately 30%. Following the isolation of the *WT1* gene in 1990, it soon became clear that *WT1* mutation did not account for any of the large Wilms tumour pedigrees and was found in only the minority (~10%) of sporadic

Wilms tumours. Pedigree specific differences in age at onset, incidence of bilateral tumours, and presence of metastases provided further evidence for the existence of more than one gene for familial Wilms tumour.<sup>1</sup> Recently, two familial Wilms tumour gene loci, designated *FWT1* and *FWT2*, have been mapped by genetic linkage analysis to 17q12-21 and 19q respectively.<sup>2,3</sup>

While a series of Wilms tumour families were being analysed for linkage to the putative *FWT1* locus on 17q,<sup>2</sup> one proband was noted to carry a tumour specific 11p13 deletion (the *WT1* locus) and to suffer from genitourinary abnormalities (fig 1). Mutational analysis of *WT1* was therefore undertaken in this pedigree.

Family WILMS 5 was identified as a sib pair affected with unilateral Wilms tumour. The sister (No 301) presented at the age of 2 years with a tumour of fetal rhabdomyomatous type and remains well 12 years later. Her brother (No 302) presented at the age of 4 years with a



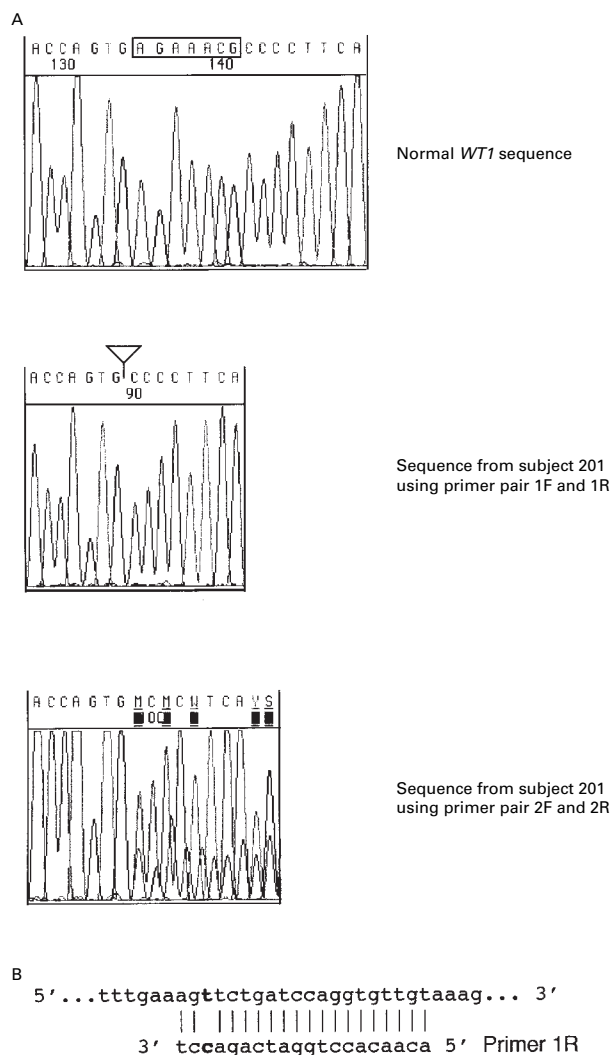
**Figure 1** Pedigree of WILMS 5. WT = Wilms tumour, C + HS = cryptorchidism and hypospadias. Subjects 201, 301, and 302 are carriers of an intragenic *WT1* mutation. 101 is not a carrier. It is unknown whether the mutation arose in 201 *de novo* or was inherited from her father. However, none of her three sibs nor any of their eight offspring were affected by Wilms tumour.

tumour of biphasic histology (stromal and blastemal). He relapsed shortly after finishing standard treatment and subsequently died from progressive tumour. He suffered from severe bilateral cryptorchidism and hypospadias, and surgery showed the presence of persistent Mullerian structures as well as testicular tissue. His constitutional karyotype was 46,XY whereas metaphases from the tumour displayed an interstitial 11p13 deletion and an isochromosome 1q. Both parents were phenotypically normal with no history of Wilms tumour. There was no family history of other cancers or congenital malformation.

Sequencing of exon 7 of *WT1*, which codes for the first zinc finger, showed a heterozygous 7 bp deletion in constitutional DNA from both affected sibs. This frameshift is predicted to lead to the introduction of 59 novel amino acids following Ser<sup>318</sup> with a termination codon before the zinc finger region, which is known to be critical for DNA binding by this putative transcription factor. Sequencing of the remaining exons showed no other mutations. Tumour DNA from 302 had only the mutant allele, as expected, since the tumour karyotype showed an interstitial deletion of 11p13. Tumour DNA from 301 showed an intragenic mutation of the second *WT1* allele, a 26 bp insertion also in exon 7, predicted to introduce a termination codon after two novel amino acids. Thus, in both sibs' tumours, *WT1* was behaving as a tumour suppressor gene, with loss of function mutations affecting both alleles.

Sequencing of lymphocyte DNA from the mother (No 201), who is phenotypically normal, showed homozygosity for the 7 bp deletion. This was an unexpected finding, since homozygous constitutional mutation of *WT1* in a murine knock out model causes absence of the kidneys and gonads and is incompatible with postnatal survival.<sup>4</sup> However, a single case of a Japanese girl with Denys-Drash syndrome (DDS), who is constitutionally homozygous for a missense mutation in zinc finger 2 has been described.<sup>5,6</sup> Her viability, in contrast to the prediction of the knock out model, may be because of the presence of a mutant form of *WT1*, still capable of protein-protein interactions. The truncated *WT1* protein produced in the WILMS 5 pedigree could also be capable of such activity through its N-terminal region. An alternative explanation is species specific differences in requirements for *WT1* function.

Further investigations were therefore carried out to resolve this discrepancy. DNA extracted from buccal cells and from skin fibroblasts exhibited the same homozygous genotype, ruling out the possibility of constitutional mosaicism. However, PCR analysis using a new primer set external to the original pair showed that subject 201 was in



**Figure 2** (A) Automated sequencing in the region of the deletion found in family WILMS 5. Top panel, normal sequence, showing the 7 bp that are deleted (boxed). Centre panel, sequence from subject 201 using primer pair 1, showing apparent homozygosity for the 7 bp deletion. Lower panel, sequence from subject 201 using primer pair 2, showing heterozygosity for the 7 bp deletion. (B) Sequence of the intron downstream of exon 7 from subject 201 around the annealing site for primer 1R. The mismatch at the 3' end of the primer is shown in bold. Mutational analysis for exons 1-10 of *WT1* was performed on DNA extracted from blood, buccal cells (201 only), and tumour material (301 and 302) as previously described.<sup>11</sup> Primer sequences for exon 7 were: pair 1 1F 5'-GACCTACGTGAATGATCACATG-3' and 1R 5'-ACAACACCTGGATCAAGACCT-3'; pair 2 (flanking primer pair 1) 2F 5'-CACCCCTCTTTGGAIATAC-3' and 2R 5'-CTGACCTCTGTAATAAAGGAT-3'. PCR conditions for primer pair 2 (449 bp product) were 1.5 mmol/l MgCl<sub>2</sub>, 55°C annealing. PCR products were sequenced using the ABI Prism Dye Terminator Cycle Sequencing kit with AmpliTaq FS using a modified method and capillary electrophoresed on a 310 Genetic Analyser (Perkin Elmer ABI).<sup>15</sup> The results were analysed using Factura software and by eye to identify heterozygotes. Samples containing mutations were resequenced using a second independent PCR product as a template. Subcloning and sequencing of PCR products was carried out as previously described, where necessary.<sup>11</sup>

fact constitutionally heterozygous for the deletion. The apparent homozygosity was the result of an intronic point mutation, close to the 3' end of the original reverse primer, preventing amplification of the normal allele (fig 2). This base change was also found in the maternal grandmother (subject 101). No material was available from the maternal grandfather so it was not possible to say whether the inherited *WT1* mutation had arisen *de novo* in subject 201 or was paternally inherited. Since primer pair 1 had been used in our previous screen for *WT1* mutations in leukaemia, a small study was undertaken to see if this intronic base

change was common. Constitutional DNA from 16 normal people and two with Wilms tumours was sequenced, but none was found to carry the G to T change, implying that this base change is either a rare polymorphism or is specific to family WILMS 5. This illustrates the importance of verifying homozygous constitutional mutations using a second pair of PCR primers or an independent method.

This is only the fourth Wilms tumour pedigree where an inherited *WT1* mutation has been identified. In the previously described pedigrees, one combined familial Wilms tumour with aniridia owing to transmission of a t(2;11)(q32;p13) with presumed complete deletion of the adjacent *WT1* and *PAX6* genes at 11p13.<sup>7</sup> A father and son both had Wilms tumour associated with a constitutional 1 bp deletion in exon 6 of *WT1*, causing truncation of the protein before the zinc finger region.<sup>8</sup> Only the son had urogenital abnormalities. In the third family, a nonsense mutation in zinc finger 2 was transmitted by an unaffected father to three daughters all affected by Wilms tumour.<sup>9</sup> The same mutation has also been reported in a person with Denys-Drash syndrome but there were no features of this syndrome in this pedigree.<sup>10</sup>

The mutation described in the pedigree in this report is novel, although mutations resulting in truncation of *WT1* in exons 6 or 7, just before the zinc finger region, are described in sporadic Wilms tumours and in acute myeloid leukaemia.<sup>11</sup> In this pedigree, germline *WT1* mutation was associated with severe genitourinary malformation in the male whereas the two females had normal development; the mother is clearly fertile and the daughter has gone through normal puberty. A similar sex difference in severity of genital malformation is seen in patients with Denys-Drash syndrome, now that the syndrome has been expanded to include genotypic females with the characteristic nephropathy and Wilms tumour. This pedigree also shows the variable nature of tumour behaviour resulting from a common initiating event.

It has been suggested that there is a genotype-phenotype correlation in the histological appearance of Wilms tumours with *WT1* mutations, with a predominance of differentiation into heterologous mesodermal derivatives, commonly muscle, cartilage, bone, or fat.<sup>12</sup> Subject 301's Wilms tumour did indeed show an extreme form of heterologous differentiation towards muscle, with the fetal rhabdomyomatous subtype. However, her brother's tumour had biphasic histology (stromal and blastemal) with no heterologous differentiation. This difference may be because of the nature or timing of the second *WT1* mutation during renal or tumour development. However, it is equally likely that different morphologies result from an identical initiating mutation owing to the inherent plasticity of renal development. This is clearly seen in Wilms tumours resulting from mutation in the *FWT1* gene, where histologies range from classical triphasic Wilms to the fetal rhabdomyomatous subtype (Rahman and Pritchard-Jones, unpublished observations).

Germline *WT1* mutations are also found in cases of Wilms tumour without a family history. The greatest number has been described in patients with DDS, but a few have been described in some patients with bilateral tumours or genitourinary abnormalities, particularly cryptorchidism, and in a small number of unilateral cases without congenital abnormalities.<sup>12-14</sup> In a few of these cases, the mutation is inherited from an unaffected parent. Germline mutations causing truncations in exon 7 of *WT1* have been described in four patients with sporadic Wilms tumour, all of whom had normal genitourinary development.<sup>12</sup> This is in contrast with the severe abnormalities found in subject 302 and further indicates the extreme variability in phenotypic expression of *WT1* mutations.

Given the excellent survival following treatment for Wilms tumour without the need for gonadotoxic chemotherapy, the rarity of familial *WT1* mutation could be because of pleiotropic effects of germline *WT1* on genitourinary development, causing reduced fertility in affected subjects. Constitutional *WT1* mutations are more commonly found in certain congenital malformation syndromes predisposing to childhood genitourinary cancers (WAGR, Denys-Drash syndrome, and Frasier syndrome). Nevertheless, sporadic Wilms tumours occurring in conjunction with genitourinary abnormalities or showing heterologous differentiation can be the result of germline *WT1* mutations. Screening of such patients and their relatives is advisable, particularly as unaffected subjects can carry mutations that might have severe consequences for their offspring. Ten of 21 patients with Wilms tumours showing heterologous differentiation had germline *WT1* mutations.<sup>12</sup> A recent report by the National Wilms Tumour Study Group found germline *WT1* mutations in 7/28 Wilms tumour patients with cryptorchidism and 4/12 with hypospadias, but only 1/110 patients without genitourinary malformation.<sup>13</sup> *WT1* mutational screening can therefore probably be safely reserved for cases selected on the basis of associated abnormalities or tumour histology.

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## Silver-Russell syndrome and ring chromosome 7

EDITOR—We read the recent paper by Miyoshi *et al*<sup>1</sup> with great interest. They report a child with Silver-Russell syndrome (SRS) and maternal uniparental disomy of chromosome 7 (mUPD7), with 30% mosaicism for an additional ring chromosome 7 of paternal origin. As the ring contained four CA repeat polymorphic markers which map to the region 7p13-q11 (D7S2552, D7S499, D7S494, and D7S2503), they concluded that imprinted genes within this region can be ruled out as candidates for SRS. While the data are interesting, we feel that they are inadequate to support this conclusion.

Firstly, the genetic distance spanned by the four markers used to define the extent of biparental inheritance is small (2.26 cM between 64.81 and 67.07 cM).<sup>2</sup> Furthermore, UPD was shown using marker D7S506 which is estimated to lie less than 0.1 cM distal to D7S2552. The trisomic region is therefore likely to be considerably smaller than 7p13-q11. There are now more accurate localisations for at least two of the biparentally inherited markers; D7S499 and D7S494 have been mapped to 7p11.2 and 7p11.2-q11.1, respectively.<sup>3</sup> The ring may therefore only include a small region close to the centromere, leaving 7p12-13 uninvolved. It is interesting to note that three genes (*IGFBP1*, *IGFBP3*, and *GRB10*), which have been proposed as candidates for SRS,<sup>4,5</sup> lie between two markers (D7S2497 and D7S506) which are outside the potentially excluded region.

There is also no evidence to show that genes on the ring chromosome are active. Indeed, follow up of patients with supernumerary marker chromosomes has found that they are frequently not associated with any phenotypic effect.<sup>6</sup> Finally, even if the ring chromosome is active, it can only be used to infer exclusion of a paternally expressed imprinted gene from the region. If the SRS gene is transcribed exclusively from the maternal allele, it will be overexpressed in this patient, whether or not the gene is located within the ring chromosome, since she has mUPD7.

In conclusion, caution should be used not to over-interpret the data reported by Miyoshi *et al*.<sup>1</sup> Nevertheless, the findings do support the continuing search for a candidate imprinted gene(s) for SRS on chromosome 7. They also emphasise the importance of searching for cytogenetic

abnormalities of chromosome 7 in SRS which will provide much needed clues to the region involved.

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This letter was shown to Dr Miyoshi *et al*, who reply as follows.

Dr Wakeling *et al* suggest that the ring chromosome 7 observed in our SRS patient must be smaller than we estimated. This is based on databases<sup>2,3</sup>; for example, the genetic distance between markers D7S506 and D7S2552 they cite is <0.1 cM. However, when using another database,<sup>7</sup> it is calculated to be 1.1 cM. Since this discrepancy is obviously derived from the different databases used, and since not many DNA markers have physically been assigned to the chromosomal region, we tried to perform FISH mapping of D7S506, which is the most centromeric marker among those showing UPD(7)mat in our previous study,<sup>1</sup> using a PAC clone (303F13) containing the marker as a probe. Consequently, the PAC was mapped to the 7p11.1-p11.2 region. This finding indicates that the four biparentally inherited markers (D7S2552, D7S499, D7S494, and D7S2503) we identified<sup>1</sup> are located centromerically to D7S506 and the short arm breakpoint of the ring chromosome must lie between them. In addition, FISH analysis using a probe for the elastin gene locus (VYSIS, UK) that was localised to 7q11.23 gave no fluorescence signals on the ring chromosome. Therefore, the extent of the ring is smaller than the 7p11.2-q11.23 segment.

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2 <http://cedar.genetics.soton.ac.uk/pub/chrom7/map.html>

3 Tsui LC, Donis-Keller H, Grzeschik KH. Report of the Second International Workshop on Human Chromosome 7 Mapping. 1994;[http://www.genlink.wustl.edu/workshops/chr77\\_report.html](http://www.genlink.wustl.edu/workshops/chr77_report.html)

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5 Miyoshi N, Kuroiwa Y, Kohda T, *et al*. Identification of the *Meg11/Grb10* imprinted gene on mouse proximal chromosome 11, a candidate for the Silver-Russell syndrome gene. *Proc Natl Acad Sci USA* 1998;**95**:1102-7.

6 Warburton D. De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis: clinical significance and distribution of breakpoints. *Am J Hum Genet* 1991;**49**:995-1013.

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## Variants of *STAT6* (signal transducer and activator of transcription 6) in atopic asthma

EDITOR—Atopy is characterised by raised IgE levels and it underlies the clinical disorder bronchial asthma.<sup>1-3</sup> IL-4 and IL-13 play a key role in the development of asthma acting through the IL-4 receptor (IL-4R) and IL-13R, respectively.<sup>1-3</sup> Since these two receptors share the IL-4Rα

chain, this subunit is the crucial component required for IL-4 and IL-13 signalling.<sup>1-3</sup> Recently, three coding variants in the human *IL4R* gene have been identified and these showed genetic and functional association with atopic asthma in different ethnic groups. The Ile50 variant was associated with atopic asthma in a Japanese population<sup>4,5</sup> and Arg551 or Pro478 were associated with IgE levels in American<sup>6</sup> and German populations,<sup>7</sup> respectively, but perhaps through different mechanisms. Ile50 specifically and strongly upregulates cellular IgE synthesis through stat6 when tested by cDNA transfection into human and murine B cell lines.<sup>4,5</sup> Functional assay of

Table 1 Association between variant of *STAT6* gene and atopic asthma in the British population

Phenotype	No of cases	2964G/A genotype of <i>STAT6</i>		Odds (95% CI)	p value
		AA+ AG	GG		
Controls	150	62	88		
Atopic asthmatics	150	57	93	1.15 (0.72–1.82)	0.555
Non-asthmatics	119	51	68		
Asthmatics	181	70	111	1.19 (0.74–1.90)	0.470
Low IgE <120 IU/ml	167	63	104		
High IgE >120 IU/ml	133	58	75	0.78 (0.49–1.25)	0.302
ASE(-)	110	50	60		
ASE(+)	190	71	119	1.40 (0.87–2.25)	0.169
Non-atopics	99	43	56		
Atopics	201	78	123	1.21 (0.74–1.97)	0.442

Table 2 Association between variant of *STAT6* gene and atopic asthma in the Japanese population

Phenotype	No of cases	2964G/A genotype of <i>STAT6</i>		Odds (95% CI)	p value
		AA+ AG	GG		
Controls	00	91	9		
Asthma					
Adult atopic marked	100	84	16	1.93 (0.79–4.69)	0.134
Adult atopic mild	100	76	24	3.19 (1.40–7.08)	0.0043
Childhood atopic marked	100	90	10	1.12 (0.42–2.93)	0.809
Intrinsic adult	100	88	12	1.65 (0.68–4.00)	0.376
Low IgE	265	224	41		
High IgE	235	204	31	0.83 (0.50–1.37)	0.469
ASE(-)	183	159	24		
ASE(+)	317	270	47	0.87 (0.51–1.47)	0.597
Non-atopic	167	147	20		
Atopic	333	278	55	0.77 (0.44–1.31)	0.180

Arg551Gln IL-4R $\alpha$  showed impaired binding of the negative regulatory molecule, protein tyrosine phosphatase, SHP-1, and increased expression of CD23 was found in peripheral blood mononuclear cells after challenge with human IL-4.<sup>6</sup> However, this finding was not replicated by another group.<sup>8</sup> In contrast, Pro478 is in tight linkage disequilibrium with Arg551 in a German population and may change the structure of the receptor, leading to altered phosphorylation patterns of signal molecules and hence lower IgE levels.<sup>7</sup> These findings emphasise the genetic heterogeneity of atopy and question whether the molecular interaction between variants of IL-4R $\alpha$  and its associated signal transduction molecules, such as stat6, may be important.<sup>1–3</sup>

The Stats are a family of transcription factors evolutionarily conserved from *Drosophila* to humans.<sup>9</sup> To date seven different *STAT* genes have been identified in humans.<sup>9</sup> Stat6 activation correlates with functional responses induced by IL-4 and IL-13.<sup>1–3</sup> Mice deficient in Stat6 lack IgE production and Th2 inflammatory reactions.<sup>10–11</sup> Stat6 is therefore an essential molecule for IL-4 and IL-13 signal transduction. Moreover, it is assigned to chromosome 12q13–14, the site of genetic linkage to atopic asthma.

The aim of this study was to test whether variants of the *STAT6* gene relate to atopic asthma in both British (n=300) and Japanese (n=400) populations.

For the Japanese study, 100 adults from Osaka were randomly selected as controls from clients of a commercial medical examination company, with adjustment for the sex and age distribution of this area.<sup>12–13</sup> We recruited 400 patients, 100 in each group, with atopic asthma, intrinsic (non-atopic) asthma, atopic rhinitis, and atopic eczema from Kawai Clinic, Wakayama Red Cross Medical Centre, and Habikino Hospital, respectively. We selected patients with one principal atopic clinical disorder at the time of examination.

For the British study, 150 subjects were randomly recruited from an obstetric outpatient clinic in Oxfordshire. One hundred and fifty subjects with physician diag-

nosed atopic asthma were selected from the Oxford Chest Unit; both controls and patients were white.

All the asthmatic subjects had specialist physician diagnosed asthma with (1) recurrent breathlessness and chest tightness requiring continuing treatment, (2) physician documented wheeze, and (3) documented labile airflow obstruction with variability in serial peak expiratory flow rates >30%. Marked asthma was designated as chronic rather than episodic asthma, needing chronic therapy with steroids. The diagnosis of rhinitis was based on chronic nasal obstruction or congestion, rhinorrhoea, and sneezing, not caused by chronic infection. The diagnosis of atopic eczema was made on the basis of the morphological appearance of active skin disease, the distribution of skin lesions, the clinical course of the disorder, and a family history of atopy. All cases of eczema met the diagnostic criteria of Hanafin and Rajka.<sup>14</sup> There were no heavy smokers (>20 cigarettes per day) among these subjects.

Specific IgE against 15 airborne antigens was detected by solid phase immunoassay, MAST (Hitachi, Tokyo, Japan), and we used previously described criteria for a positive titre. A high concentration of total serum IgE (Pharmacia CAP system, Uppsala, Sweden) was taken to be greater than the mean +2 SD.<sup>13–14</sup> Atopy, defined as IgE responsiveness, was diagnosed as the presence of a high concentration of total serum IgE, a positive specific IgE titre (>0.35 kU/l) against one of 15 highly purified aero-allergens, or a combination of these two features.<sup>13–14</sup>

DNA samples were extracted using a commercial kit (Iso-Quick, Microprobe Corporation, Garden Grove, USA). cDNA was extracted using commercial kits (Pharmacia, Sweden). To detect variants, cDNA for the Stat6 coding region was amplified with primers 5'GAA GAC AGC AGA GGG GTT GC and 5'CTG TGG GGG TAGT AGA AGA G.<sup>15</sup> Sequencing was conducted with the big-dye system (ABI, UK) and images were visualised in the commercialised POP-6 gel using the automated sequencer (ABI Prism 310 Genetic Analyser). Primers were 5'TGT CTC CCC TGG TGG TC or 5'GGT CCC CAC CTC AGC CAT G.<sup>15</sup>

To conduct the genetic association study, PCR including 1.5 mmol/l of magnesium chloride and genomic DNA in a 30  $\mu$ l mixture was performed in a Perkin Elmer Cetus thermal cycler. Primers were 5'GGG AAG TTC AGG CTC TGA GAC CC and 5'AGC TCT GTA TGT GTG TGT G.<sup>15</sup> The underlined sequence was exchanged to incorporate the restriction site. The amplification products were digested with *Acl*I.

Contingency table analysis, odds ratios, 95% confidence intervals, and significance values were estimated by computerised exact methods (SPSS program version 8). Odds ratio (OR) was calculated between high and low risk genotypes and between high and low risk alleles. If the number in the column was less than 10, Fisher's exact test was used. Probability values from this test were corrected for multiple comparisons by multiplying the p value by the number of comparisons that were made (Bonferroni correction).

Mutation scanning enabled us to identify a G2964A variant in a 3' untranslated region of *STAT6*.<sup>5</sup> As shown in tables 1 and 2, the allele frequencies for *STAT6* in the two control populations were quite different, p(G)=0.76, p(A)=0.24 in the British population and p(G)=0.33 and p(A)=0.67 in the Japanese population ( $\chi^2=82.2$ , df=2, p<0.00001). There was no significant association between atopic asthma and this *STAT6* variant in the British population; no association was seen with any atopy or asthma phenotype. In the Japanese population, mild atopic asthma characterised by positive antigen specific IgE response (ASE) (<0.75 kU/l) or high total IgE (mean + between 1 SD and 2 SD) showed a strong association with this variant (OR=3.19 (95% CI: 1.40–7.08), p=0.0043, Pc=0.016).

Neither adult nor childhood asthma with a marked atopy phenotype (ASE > 3.5 kU/l and total IgE > mean + 2 SD) was associated with this variant of *STAT6*. Again, no atopy phenotypes were associated with this variant.

Recent genetic and functional studies highlight the importance of variants of *IL-4R $\alpha$*  in the development of atopy; Ile50 upregulates cellular IgE synthesis and is associated with atopic asthma in a Japanese population,<sup>4,5</sup> while Pro478 alters the binding affinity of IRS-1/2, and is associated with lower IgE levels.<sup>7</sup> No association was found between atopic asthma and these variants in a British population.<sup>5</sup> These findings emphasise the genetic heterogeneity of atopic disorders, even within one locus in different ethnic groups; they raise the question of interaction between variants of different components of the *IL-4/IL-13* signalling pathway. *BCL6* might repress *stat6* activated transcription<sup>16,17</sup> and a variant of *BCL6* has been associated with marked atopy.<sup>18</sup> Interactions among signal transduction molecules of *IL-4R $\alpha$*  might explain the complex relationships between genetic variants and phenotypes in atopy and asthma.<sup>1-3</sup>

The gene encoding human *STAT6*, spanning 19 kb on 12q13-14, consists of 23 exons.<sup>19</sup> Genome wide searches for atopic asthma showed a strong linkage to 12q14-24.<sup>20-22</sup> Our present data, showing association between variants of *STAT6* and asthma with mild atopy, support the candidacy of *STAT6* as an "asthma" locus on 12q in a Japanese population. Also, the marked difference in genotype frequencies between the British and Japanese populations may explain the "genetic heterogeneity" among and within ethnic groups.<sup>1-3</sup> Since G2964A variant is located in 3'UTR of the gene, the functional role of this variant remains unknown; it may be in linkage disequilibrium with so far unidentified but functional variants in the regulating or coding parts of *STAT6* or variants of the immediately adjacent genes. Further studies are needed to clarify the role of the *STAT6* variants in predisposing to asthma among different ethnic groups.

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*J Med Genet* 2000;37:382-384

## No linkage or association of the *IL-4R $\alpha$* gene Q576R mutation with atopic asthma in Italian families

EDITOR—Atopy, a familial clinical syndrome of asthma, rhinitis, and eczema, is characterised by IgE mediated allergy, which results from genetic and environmental events. The disease is immunologically defined by the presence of specific IgE antibodies to common allergens and raised total serum IgE concentrations. Also, because of its high prevalence, age dependent penetrance, and assumed heterogeneity, the mode of inheritance is unknown.<sup>1</sup> Several genes which affect IgE responsiveness have been reported.<sup>2</sup>

Among the genes that have been reported to affect the atopic phenotype, that for *IL4* regulates the production of IgE.<sup>3,4</sup> Through its receptor (*IL4-R*), *IL4* signals target cells and tissues to mount a response. The *IL4-R*  $\alpha$  chain binds *IL4* and mediates its effect through kinases attached to the intracellular domain.<sup>5</sup> The *IL4-R $\alpha$*  coding gene has been localised to the short arm of chromosome 16 (16p12.1).<sup>6</sup> Sharing of maternal markers flanking the *IL4-R $\alpha$*  gene was recently found in atopy.<sup>7</sup> A gain of function mutation in the *IL4-R $\alpha$*  gene was recently described, and it was reported to be associated with higher levels of expression of CD23 by *IL-4*, severe atopic dermatitis, raised serum IgE level, or a specific response to a common allergen in a case-control study with 20 affected and 30 unaffected adults in the North American population.<sup>8</sup> This association study needs confirmation in a different sample.



In the present study we have investigated the frequency of the *IL4-Ra* Q576R mutation in a large sample of Italian atopic asthmatic families to determine its involvement in genetic susceptibility to some atopic asthma related phenotypes.

A panel of 851 subjects was analysed, recruited from 192 families with one or more affected children, of whom 133 were attending the Allergy and Pulmonology Clinic of the Department of Paediatrics of the University of Verona, as described previously,<sup>9</sup> and 59 families attending the Institute of Paediatrics of the Hospital in Bolzano, both located in north east Italy. All the subjects were tested for clinical history, total serum IgE level, skin prick test (SPT), and bronchial hyper-responsiveness (BHR). Clinical asthma was defined according to the American Thoracic Society criteria,<sup>10</sup> including the response to a respiratory questionnaire. BHR to methacholine was defined as PC 20 < 25 mg/ml. Atopy was defined by the presence of one or both of the following criteria: (1) positive SPT to one or more common aeroallergens, such as house dust mites, cat, dog, *Alternaria*, grass pollen, *Parietaria*; (2) raised circulating total IgE (from 0 to 10 years of age: age adjusted standard curve, levels above the 90th centile; after 10 years of age: 200 kU/l). Among the families, 522 atopic subjects, 477 with a positive SPT, 288 with raised total serum IgE levels, 298 with clinical asthma, and 281 with BHR were analysed. The total is greater than the total number of people, as two or more phenotypes could occur together in the same person.

Genomic DNA was extracted from whole blood, according to standard protocols. To achieve an easy and rapid evaluation of the guanine for adenine substitution at nucleotide 1902 of the *IL4-Ra* gene, a new protocol based on RG-PCR and nuclease digestion<sup>11</sup> was developed. This method is faster than the single strand conformation polymorphism technique (SSCP) and DNA sequencing analysis previously described.<sup>8</sup> A mutant oligonucleotide which inserted in the product of DNA amplification a new restriction site for *DdeI* was synthesised. The primers used were a novel 21-mer forward oligonucleotide (5'-TCGGCCCCACCAGTGGCTCT-3') with an A1899C substitution, and the same reverse oligonucleotide previously described.<sup>8</sup> The PCR product is a 298 bp fragment. Restriction analysis with *DdeI* results in two fragments of 277 and 21 bp for the Q576 allele, and a single fragment of 298 bp for the 576R allele, as shown in fig 1. All the subjects were genotyped. The data were analysed using the affected sib pairs method implemented in SIBPAL (SAGE package) and the transmission disequilibrium test.<sup>14</sup> The frequency of the 576R mutation calculated in the family founders was 18% (PIC=0.25). In the normal controls, the allele R frequency was 17.5% (18/104 alleles). This frequency is similar to that reported for US controls (10%).<sup>8</sup>

Linkage analysis indicated no significant increase in allele sharing for atopy, SPT, IgE, BHR, or asthma, respectively. No allele transmission disequilibrium was observed. Association studies were performed with 52 adult unrelated controls in whom positivity for each of the phenotypes was excluded. Cases were collected from family founder members according to the phenotype (asthmatic: 45, BHR: 60, IgE: 71, SPT: 153, atopy: 175). The case-control analysis did not show a variation in the distribution of alleles between cases and controls in the phenotypes investigated. The statistical tests have sufficient power (80%) to detect an association with an odds ratio of 3 for IgE, SPT, atopy, and of 5 for asthma and BHR.

As the informativity of Q576R was limited (PIC 0.25), further linkage analyses with additional *IL4-Ra* polymorphisms<sup>13</sup> may better define the putative involvement of the gene in hereditary susceptibility to atopic asthma. Wang *et al*<sup>14</sup> have recently noted that mutation

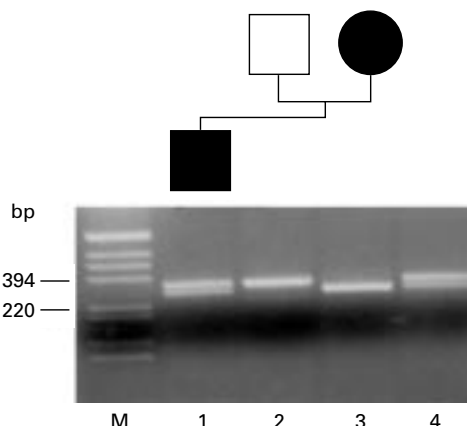


Figure 1 Detection of the *IL4-Ra* mutation with *DdeI* restriction analysis of PCR products after modification of the restriction site during PCR. (Top) Pedigree of family BZ78. Filled symbols denote affected family members. (Bottom) Electrophoretic analysis. Lanes 1-3: family members shown above, genotypes are QR, RR, QQ, respectively; lane 4: QR control. M: molecular weight marker (1 kb ladder, GIBCO-BRL).

Q576R does not have a direct effect on IL-4 signal transduction in murine cells and suggested that the hyper-sensitive induction of CD23 in cells derived from human allergy patients may be the result of different or additional alterations in the IL-4 signalling pathway. Another *IL4-Ra* gene mutation, Ile50Val, was reported to be associated with atopic asthma, increased total IgE, and increased mite specific IgE in a Japanese population, and to upregulate B lymphocyte growth and IgE production in response to IL4 challenge.<sup>15-16</sup> This mutation will now be investigated in the Italian group of patients.

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## Importance of the autosomal recessive retinitis pigmentosa locus on 1q31-q32.1 (RP12) and mutation analysis of the candidate gene *RGS16* (*RGS-r*)

EDITOR—Retinitis pigmentosa (RP, MIM 268000) is the term applied to a clinically and genetically heterogeneous group of retinal degenerations primarily affecting the rod photoreceptors. RP is characterised by progressive loss of vision, initially manifesting as night blindness and reduction in the peripheral visual field, and later involving loss of central vision.<sup>1</sup> Ophthalmoscopic examination typically shows pigmentary disturbances of the mid-peripheral retina. RP may be inherited as an autosomal recessive, autosomal dominant, digenic, or X linked trait. Autosomal recessive RP (ARRP) accounts for around 20% of all cases of RP, while sporadic RP, which is presumed to be recessive in most cases, accounts for a further 50%.<sup>2</sup>

Mutations causing autosomal recessive RP (ARRP) have been found in the genes encoding rhodopsin,<sup>3</sup> the  $\alpha$  and  $\beta$  subunits of rod phosphodiesterase,<sup>4,5</sup> the  $\alpha$  subunit of the cyclic GMP gated channel protein,<sup>6</sup> and the genes *RPE65*,<sup>7,8</sup> *RLBP1*,<sup>9</sup> *ABCR*,<sup>10</sup> and *TULP1*.<sup>11,12</sup> In addition, genetic linkage studies have identified ARRP loci at 1q31-q32.1,<sup>13,14</sup> 2q31-q33,<sup>15</sup> 6cen-q15,<sup>16</sup> and 16p12.1-p12.3.<sup>17</sup> (MIM numbers for the loci identified by these studies are 180380, 180071, 180072, 123825, 180069, 180090, 601708, 600132, 600105, and 602594 respectively. No MIM number has yet been assigned to the 2q31-q33 and 6cen-q15 loci.) Of these four loci, only linkage to the 1q31-q32.1 locus (RP12) and the 6cen-q15 locus has been reported in more than one pedigree.

The first report of linkage of ARRP to 1q31-q32.1 was in a large inbred Dutch family with ARRP in which most patients exhibited para-arteriolar preservation of the retinal pigment epithelium (PPRPE).<sup>13</sup> This was followed by linkage of a second, consanguineous, pedigree from Pakistan.<sup>14</sup> In both the Dutch and the Pakistani families there was evidence that only the branches of the family with PPRPE were linked to 1q31-q32.1, while other branches of each family had classical RP and were unlinked to 1q. In both cases the authors attributed this finding to non-allelic heterogeneity. The occurrence of two very similar eye conditions in one family would presumably be because of the high prevalence of recessive disorders in inbred populations. Recently the critical interval for the RP12 locus was reduced to a 3 cM region between the markers D1S412 and AFM207wb12.<sup>18</sup>

The human gene for a retinally expressed regulator of G protein signalling (*RGS16/RGS-r*) has been mapped to 1q25-1q31.<sup>19</sup> Since it has been shown that *RGS16/RGS-r* can interact with the  $\alpha$  subunit of transducin, a heterotrimeric G protein that is an integral part of the rod phototransduction cascade, the *RGS16/RGS-r* gene is an

excellent functional candidate for ARRP.<sup>20,21</sup> We sought to determine: (1) how important the 1q31-q32.1 locus is as a cause of ARRP by performing linkage analysis and homozygosity mapping in a panel of ARRP families from Europe and Asia, and (2) whether mutations in *RGS16/RGS-r* are responsible for ARRP at this locus (RP12). In order to perform linkage analysis and homozygosity mapping, 14 large/consanguineous families with ARRP from the UK (two families), Pakistan (nine families), and Spain (three families) were ascertained. In addition, PMK214, the family originally studied by Leutelt *et al.*,<sup>14</sup> was included for mutation screening of the candidate gene *RGS16/RGS-r*. Clinical examination of all affected subjects showed typical features of retinitis pigmentosa (pigmentary retinopathy, associated with symptoms of night blindness and loss of visual fields). Informed consent was obtained by local clinicians.

To identify the locus responsible for the disease in each family we performed a linkage or homozygosity analysis. The initial set of markers chosen to be analysed were those corresponding to the loci of genes responsible for ARRP, and those known to flank the ARRP loci for which no gene has as yet been identified (table 1). When evidence of linkage was obtained for the 1q31-q32.1 locus, further polymorphic markers from this region were analysed to determine whether recombinant subjects within the family would permit further refinement of the locus. Marker order was determined from the Génethon sex averaged genetic map.<sup>22</sup> Primers were obtained from the MapPairs set (Research Genetics, Huntsville, AL), or synthesised commercially according to data from GDB (Johns Hopkins University: <http://gdbwww.gdb.org/>).

Non-radioactive PCR was performed with 300 ng of genomic DNA, 10 pmol of each primer, 200  $\mu$ mol/l dNTPs, 1.5 mmol/l MgCl<sub>2</sub>, and 1 unit of *Taq* DNA polymerase. A three stage PCR consisting of 35 cycles of 94°C, 50-62°C, and 72°C, each for one minute, was used. The amplified products were then separated by electrophoresis on 6-8% non-denaturing polyacrylamide gels (Protogel, National Diagnostics) and stained with ethidium bromide. Two point lod scores were calculated using the LINKAGE package.<sup>23</sup>

Primers for heteroduplex analysis and direct sequencing of the five exons and the intron-exon boundaries of the *RGS16/RGS-r* gene were designed using the published

Table 1 Known loci for ARRP including some of the polymorphic markers used in this study

Locus	Gene	Reference	Markers used
1p31	<i>RPE65</i>	7	D1S1665, D1S1669
1p13-21	<i>ABCR</i>	10	D1S236, D1S2813
1q31-q32.1	<i>RP12</i>	13, 14	F13B, D1S2622
2q31-q33		15	D2S152, D2S157
3q21-q24	<i>Rhodopsin</i>	3	D3S1292, D3S1589
4p16.3	<i>PDEB</i>	6	<i>PDEB</i> intragenic, D4S412
4p14-q13	<i>CNGC</i>	5	D4S189, D4S405
5q31.2-q34	<i>PDEA</i>	4	D5S536, D5S412
6p21	<i>TULP1</i>	11, 12	D6S291, D6S276
15q26	<i>RLBP1</i>	9	D15S127, D15S124
16p12.1-p12.3		17	D16S287, D16S292

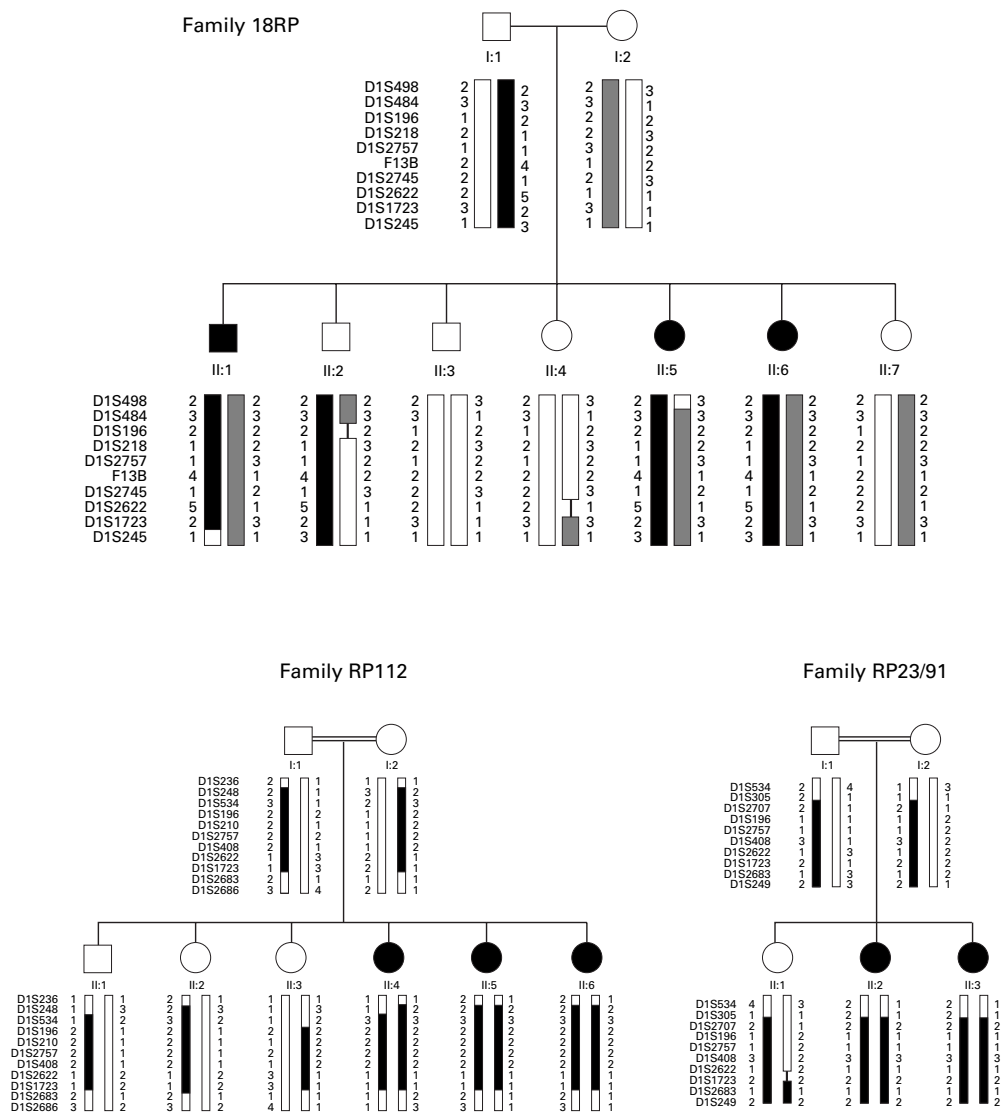


Figure 1 Pedigrees of families exhibiting evidence of linkage to 1q31-q32.1 showing haplotypes for polymorphic markers in this region. Maximum lod scores for each family were: 18RP  $Z_{max}=1.70$  (at  $\theta=0.00$ ) for the marker F13B, RP112  $Z_{max}=2.48$  at D1S2622, and RP23/91  $Z_{max}=1.63$  at D1S408. The RP12 locus lies between markers D1S2757 and D1S2622. Black or hatched bars situated between the marker alleles indicate the affected haplotype.

genomic sequence<sup>19</sup> (Genbank AF009356) as follows: exon 1F - GCCTGCCACCATCCTGCCTAC; 1R - CTCTCTCTCAAGCCTCTAGC (208 bp); exon 2F - CACCACCCAGAGCTCTTCTGG; 2R - GTTGCCAAGGTCCA CTTAGCG (312 bp); exon 3F - CAGCAATCACCAAGCAAGCC; 3R - CAGGCTCTCAGCAGTCTGG (281 bp); exon 4F - GATCCCCTAGTGTGCCAGCCTC; 4R - CTCTACACCTAGCCCTTCCTCC; (429 bp); exon 5F - CACTTCCTGATGCAGCATCCG; 5R - CTCTTCCC GCTGGCTTCC (372 bp). Polymerase chain reaction PCR was performed in a 50  $\mu$ l reaction with 1  $\mu$ g of genomic DNA, 20 pmol of each primer, 200  $\mu$ mol/l dNTPs, 0.75-1.5 mmol/l  $MgCl_2$ , and 1 unit of *Taq* DNA polymerase. A three stage PCR consisting of 40 cycles of 94°C, 55-60°C, and 72°C, each for one minute, was used. The resulting product was allowed to cool slowly to room temperature to maximise the formation of heteroduplexes.<sup>24</sup>

All five exons of *RGS16/RGS-r* were directly sequenced in two or more members of each potentially 1q linked family using an automated sequencer (ABI Biosystems model 373). PCR products were purified and then reamplified using the FS kit (Perkin Elmer). Variants were sequenced

in both the forward and reverse directions to confirm the location of sequence changes.

*AcuI* restriction digests were performed directly on 20  $\mu$ l of exon 4 PCR product by overnight incubation at 37°C. The resultant bands were photographed after electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Three of the 14 families studied showed evidence of linkage to the 1q31-q32.1 ARRP locus and were excluded by linkage analysis from all of the other ARRP loci (fig 1). Two of these pedigrees were of Pakistani origin (18RP and RP112) and one was Spanish (RP23/91). Analysis of additional polymorphic markers on 1q in these families did not permit further refinement of the RP12 locus.

Clinical examination of the small Spanish RP23/91 family showed para-arteriolar preservation of the retinal pigment epithelium (PPRPE) as previously described in RP12 linked families.<sup>13,14</sup> A relative lack of pigmentation adjacent to retinal arterioles was also seen in the Pakistani RP112 family. This feature could not be conclusively shown in the 18RP pedigree, possibly because the three affected subjects are only 22, 14, and 13 years old respectively. The fundus photograph showing PPRPE in the

Table 2 Comparison of the disease haplotypes in the three families of Pakistani origin for polymorphic markers in the critical region on 1q31

Marker	RP12 Consanguineous	18RP		PMK214 Consanguineous
		Paternal	Maternal	
D1S2877	1	3	3	2
D1S2757	3	1	3	4
F13B	1	4	1	1
D1S2745	3	1	2	2
D1S1660	2	1	3	3
D1S2622	2	5	1	3
D1S1723	3	2	4	1

original paper of van Soest *et al*<sup>13</sup> shows heavy retinal pigmentation and was taken from a 41 year old patient.

In order to show that the three families of Pakistani origin were not distantly related and that RP12 is an important ARRП locus in that population, we compared the disease haplotypes for markers within and immediately surrounding the RP12 locus in each family (table 2). The maternal disease haplotype in family 18RP and the homozygous disease haplotype for PMK214 was identical for three adjacent markers (F13B, D1S2745, and D1S1660) within the RP12 locus. A founder effect could not be excluded in these two families. However, the paternal disease haplotype in 18RP and that seen in RP112 were entirely different, indicating that at least three different RP12 alleles may exist in these families.

These four families then underwent mutation screening of the five exons of the *RGS16/RGS-r* gene by heteroduplex analysis and direct sequencing. Heteroduplexes were observed with the exon 4 product in the parents from consanguineous families RP112, PMK214, and RP23/91, but not in their affected children. No heteroduplexes were observed in members of 18RP.

Direct sequencing of the exon 4 and exon 5 products showed polymorphisms in introns 3 (–10 a to g) and 4 (–35 c to t) respectively. The intron 3 a>g change, which creates a recognition site for the restriction enzyme *AclI*, was found to be present in the heterozygous state in the parents from families RP112, PMK214, and RP23/91 in whom a heteroduplex band had been observed, and in the homozygous state in their affected children. *AclI* restriction digest analysis was, therefore, used to show the segregation of this polymorphism in the large linked family PMK214. All seven affected members of this consanguineous pedigree were found to be homozygous for the intron 3 (–10 a to g) polymorphism. This indicates that *RGS16/RGS-r* cannot be excluded from the critical genetic interval for RP12 in this family. In addition, 10 Pakistani controls were subjected to *AclI* restriction digest analysis, two of whom were found to be heterozygous for the intron 3 polymorphism. No sequence variants within the coding regions of *RGS16/RGS-r* were observed in any subject.

While there are nine published loci for autosomal dominant RP (ADRP), *rhodopsin* mutations may account for up to 25% of cases.<sup>25</sup> In contrast, none of the 11 published genes/loci for ARRП has been estimated to account for more than 5% of recessive RP. In this study, two out of nine Pakistani and one out of five European families showed evidence of linkage to RP12 and exclusion from the other loci. While this result is based upon a small sample it suggests that mutations in the gene situated at this locus may be responsible for a significant proportion of ARRП, at least in the Pakistani population.

In rod photoreceptors, the GTP bound  $\alpha$  subunit of the heterotrimeric G protein transducin ( $T\alpha$ ) activates the effector enzyme, cGMP phosphodiesterase (PDE), by binding to its inhibitory gamma subunit (PDE $\gamma$ ). Hydrolysis of GTP by the intrinsic GTPase activity of  $T\alpha$  releases bound PDE $\gamma$  and results in inactivation of PDE $\alpha\beta$  during

the phase of recovery from photoactivation. In vitro experiments with purified  $T\alpha$  subunits have shown that the intrinsic GTPase activity of  $T\alpha$  is inadequate to account for the rapid hydrolysis of GTP that is observed in vivo.<sup>26</sup> It has been postulated, therefore, that a GTPase activating protein (GAP) that is capable of greatly accelerating this process must exist in rod outer segments.

RGS16/RGS-r, which is highly expressed in the retina, has been proposed to fill the role of the  $T\alpha$  GAP.<sup>19</sup> It has been shown in vitro that the rate of transducin GTPase activity in the presence of RGS16/RGS-r is sufficient to correlate with the in vivo recovery kinetics of the visual cascade, and that in addition RGS16/RGS-r facilitates signal termination by competing with and thereby displacing PDE $\gamma$  from  $T\alpha$ .<sup>20, 21</sup> This biochemical evidence suggests an important role for RGS16/RGS-r in retinal photoreceptors.

Since the human gene for *RGS16/RGS-r* has been mapped to 1q25-1q31,<sup>19</sup> and since in the present study it could not be excluded from the RP12 locus by analysis of the intron 3 polymorphism in the linked family PMK214, this gene must remain as a potential candidate for the ARRП linked to this locus. We failed to find any sequence changes in the coding region of the gene in our four families, though it is possible that disease causing mutations may lie in the promoter region of the gene, which was not screened.

This study must, however, reduce the prospect of *RGS16/RGS-r* mutations being found in other 1q linked families and lead to a renewed search for candidates within the RP12 critical interval.

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## Incidence and molecular mechanism of aberrant splicing owing to a G→C splice acceptor site mutation causing Smith-Lemli-Opitz syndrome

EDITOR—Smith-Lemli-Opitz (SLO) syndrome (MIM 270400) is an autosomal recessive disorder characterised by a wide spectrum of developmental abnormalities including craniofacial malformations, growth and mental retardation, and multiple congenital anomalies.<sup>1-3</sup> The disorder is caused by deficient activity of 7-dehydrocholesterol reductase (7-DHCR), the enzyme that catalyses the reduction of the C<sub>7</sub>-C<sub>8</sub> (Δ<sup>7</sup>) double bond of 7-dehydrocholesterol to produce cholesterol.<sup>4,5</sup> As a consequence, patients with SLO syndrome have low plasma cholesterol and raised plasma 7-dehydrocholesterol concentrations, thus constituting the biochemical hallmark used to confirm the clinical diagnosis of the syndrome.

Recently, we and others have identified the cDNA coding for human 7-DHCR and confirmed that SLO syndrome is caused by mutations in the corresponding 7-DHCR gene.<sup>6-8</sup> So far, 19 different disease causing mutations have been reported, which were identified after analysis of only 33 patient alleles. In our previous report, we described an aberrantly spliced mutant 7-DHCR cDNA with an insertion of 134 bp which we identified in two of three Dutch patients analysed with SLO syndrome, one of whom was a homozygote and the other a compound heterozygote.<sup>6</sup> Upon translation, this insertion not only introduces additional amino acids but also causes a frameshift, which leads to an inactive, truncated protein with a changed C-terminus.<sup>6</sup> Since the same insertion was also reported by two other groups,<sup>7,8</sup> and since the affected patients were not related to one another, this finding suggested that the insertion is the result of a frequently occurring mutation. To evaluate this, we analysed 17 additional patients with SLO syndrome for the occurrence of the 134 bp insertion using allele specific RT-PCR. Furthermore, we studied the underlying mechanism that gives rise to the aberrant splicing, which results in the 134 bp insertion at the cDNA level.

First strand cDNA was synthesised from total RNA isolated from leucocytes or primary skin fibroblasts<sup>9</sup> obtained from 17 patients who were diagnosed with SLO syndrome on the basis of clinical features and raised serum levels of

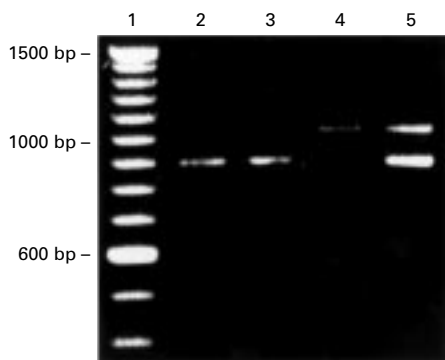
7-dehydrocholesterol. In the patients for whom fibroblasts were available, the biochemical defect was confirmed by the detection of reduced enzyme activity of 7-DHCR using a previously described ergosterol to brassicasterol conversion assay<sup>10</sup> followed by sterol analysis using gas chromatography-mass spectrometry.<sup>11</sup> To determine the presence of the 134 bp insertion, the first strand cDNA was used as template for the amplification by PCR of the second half of the coding region of 7-DHCR cDNA using primer set DHCR<sub>684-703</sub> and DHCR<sub>1563-1544</sub> (table 1). This primer set generates a single fragment of 915 bp in control subjects and patients with SLO syndrome, who do not harbour the 134 bp insertion (fig 1, lanes 2 and 3). In the previously described patient who was a homozygote for the insertion,<sup>6</sup> a single fragment of 1049 bp is amplified (fig 1, lane 4), while in a patient who is a compound heterozygote for the insertion, both fragments of 915 and 1049 bp are amplified. In compound heterozygotes, an additional intermediate sized fragment is frequently observed, which is a heteroduplex form composed of one strand of both fragments, as could be shown by mixing the 915 and 1049 bp fragments followed by one cycle of melting and annealing (not shown). Of the 20 patients thus analysed, including the three patients previously reported<sup>6</sup> and three pairs of sibs, one patient was a homozygote and 13 patients heterozygotes for the insertion. Of these 14 patients, eight patients (including the homozygote and two pairs of sibs) were of Dutch origin, four patients of German origin (including one pair of sibs), one patient of Belgian and one patient of Spanish origin.

In their recent paper, Fitzky *et al*<sup>7</sup> reported that two of their 13 patients with SLO syndrome were compound heterozygotes for the 134 bp insertion. They also indicated that the insertion was caused by a G→C transversion in the

Table 1 PCR primers used in this study

Primer*	Sequence (5'→3')
[-21M13]	TGTAAAACGACGGCCAGT
DHCR <sub>58-38</sub>	[-21M13]-GGTTCAAGAAGGAAAGTTCCC
DHCR <sub>684-703</sub>	[-21M13]-TCGGGAAGTGGTTTGACTTC
DHCR <sub>930-949</sub>	[-21M13]-CTGTGTCTGGCTGCCTTATC
DHCR <sub>449-967</sub>	[-21M13]-CTTACACGCTGCAGGGTC
DHCR-ins <sub>21-39</sub>	[-21M13]-CGTGTGTGACAGGCAGAGC
[M13rev]	CAGGAAACAGCTATGACC
DHCR <sub>979-960</sub>	[M13rev]-ACACCAAGTACAGACCCTGC
DHCR <sub>996-978</sub>	[M13rev]-CAGCTGCACGGGGTGTAC
DHCR <sub>1563-1544</sub>	[M13rev]-GGGCTCTCTCTCAGGTTACAG
DHCR-ins <sub>113-95</sub>	[M13rev]-GTCAAGCGGTGCTTTGCC

PCR conditions are available on request. \*Numbering of nucleotide residues according to previously published 7-DHCR cDNA sequence.<sup>6</sup>



**Figure 1** Identification of the 134 bp insertion by RT-PCR. The second half of 7-DHCR cDNA was amplified by RT-PCR from a control subject (lane 2) and three genotypically different patients with SLO syndrome using primer set DHCR<sub>684-703</sub> and DHCR<sub>1563-1544</sub> (see table 1). One patient contained two missense mutations (lane 3) while the other two patients were either homozygous (lane 4) or heterozygous for the 134 bp insertion (lane 5). Lane 1 contains a 100 bp DNA molecular weight marker.

splice acceptor site of intron 8 of the 7-DHCR gene, which is included in the 134 bp insertion of the corresponding cDNA, but they did not study the molecular background in further detail. Therefore, we amplified the corresponding DNA region by PCR from genomic DNA isolated from two control subjects and two patients with SLO syndrome to confirm that the insertion is caused by a mutation in a splice acceptor site and to determine the underlying mechanism that causes the specific splicing defect. To this end two separate primer sets were used. The first primer set consisted of a forward primer designed on the cDNA sequence 5' of the site of insertion (DHCR<sub>930-949</sub>) and a reverse primer designed on the 134 bp insertion sequence (DHCR-ins<sub>113-95</sub>), which amplified a genomic DNA fragment of approximately 2 kb. The second set consisted of a forward primer designed on the 134 bp insertion sequence (DHCR-ins<sub>21-39</sub>) and a reverse primer designed on the cDNA sequence 3' of the site of insertion (DHCR<sub>996-978</sub>), which amplified a genomic DNA fragment of approximately 120 bp. Taken together, these results indicated that the 134 bp insertion resulted from a partial retention of the 3' end of an intron with an approximate size of 2 kb.

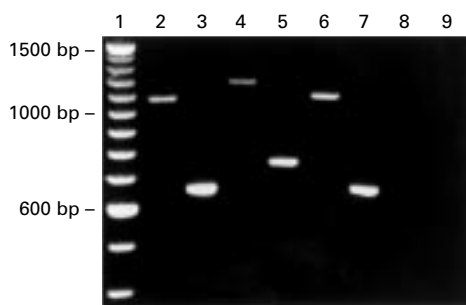
Indeed, sequence analysis of the two overlapping genomic DNA fragments which were amplified from the two control subjects identified consensus sequences for both a splice donor site and a splice acceptor site, which confirmed that the complete intron sequence, which comprises 1965 bp, was isolated (fig 2). Sequence analysis of the same overlapping genomic DNA fragments amplified from the two patients with SLO syndrome confirmed that the retention was caused by a G→C transversion at the -1 position of the splice acceptor site of the intron. This mutation disrupts the consensus sequence of the splice acceptor site and leads to alternative splicing of the transcript at a site located 134 bp 5' of the original splice site. The intron DNA sequence adjacent to this alternative site of splicing shows strong homology to the consensus sequence of a splice acceptor site and is preceded by a DNA sequence which has strong homology to the branch point consensus sequence, thus constituting quite a strong alternative splice acceptor site (fig 2).

The (compound) heterozygous presence of the 134 bp insertion enabled allele specific amplification by PCR of the two cDNAs, which could then be directly sequenced to identify the syndrome causing mutations on both alleles. To amplify the cDNA without insertion, primers DHCR<sub>979-960</sub> and DHCR<sub>949-9675</sub> which both span the site of insertion (after nucleotide 963), were used in combination with, respectively, a forward primer in the 5' (DHCR<sub>58-38</sub>) and a reverse primer in the 3' region (DHCR<sub>1563-1544</sub>). This amplification produced two overlapping fragments of 1073 and 650 bp, respectively (fig 3). The cDNA containing the 134 bp insertion was specifically amplified using the same 5' and 3' region primers in combination with the DHCR-ins<sub>113-95</sub> and DHCR-ins<sub>21-39</sub> primers designed on the insertion DNA sequence. This amplification produced two overlapping fragments of 1170 and 748 bp, respectively (fig 3). Using the latter two primer sets, the cDNAs containing the 134 bp insertion which were identified in the patients with SLO syndrome (see above) were amplified and analysed by sequencing. In all cases, the same G→C mutation at the -1 position of the splice acceptor site was identified.

To conclude, using allele specific 7-DHCR cDNA amplification, we analysed 20 patients with SLO syndrome and identified one patient as a homozygote and 13 patients, including three pairs of sibs, as heterozygotes for the G→C



**Figure 2** Molecular basis of the 134 bp insertion. (A) Sequence analysis of the genomic DNA region from which the 134 bp insertion originates and amplified by PCR from control subjects identified an intron of 1965 bp (small lettering) with consensus splice donor, branch point, and splice acceptor site sequences (italics and underlined). The complete nucleotide sequence of the intron can be obtained from GenBank under accession number AF132981. (B) In the patients, a G→C mutation at the -1 position of the authentic splice acceptor site (double underline) results in alternative splicing at a cryptic splice acceptor site located 134 bp 5' of the authentic splice acceptor site and preceded by a consensus branch point sequence (italics and underlined). The 134 bp intron sequence, which is retained as a result of the alternative splicing, is indicated in bold. Original exon sequences are indicated in capitals. Consensus sequences are defined as follows: splice donor site, [exon]..(C/A)AG ↓ gt(a/g)agt..[intron]; branch point, (t/c)n(t/c)t(g/a)ac (18-40 bp 5' of splice acceptor site); splice acceptor site, [intron]..(t/c)<sub>n</sub>(t/c)ag ↓ G/A..[exon].



**Figure 3** Allele specific amplification of cDNAs with and without the 134 bp insertion. The 7-DHCR cDNA without the 134 bp insertion was specifically amplified in two overlapping fragments from a patient heterozygous for the insertion (lanes 2 and 3) and a patient with two missense mutations (lanes 6 and 7) using primer sets DHCR<sub>-58-38</sub> and DHCR<sub>-979-967</sub> (lanes 2 and 6), and DHCR<sub>-949-967</sub> and DHCR<sub>-1563-1544</sub> (lanes 3 and 7), respectively. The cDNA containing the 134 bp insertion was specifically amplified in two overlapping fragments from the heterozygous patient using primer sets DHCR<sub>-58-38</sub> and DHCR-ins<sub>113-95</sub> (lane 4), and DHCR-ins<sub>21-39</sub> and DHCR<sub>-1563-1544</sub> (lane 5). Using the latter two primer sets, no fragments were amplified from the patient who had two missense mutations and lacked the splice site mutation causing the insertion (lanes 8 and 9).

transversion which results in the partial retention of intron 8 owing to alternative splicing.<sup>7</sup> After excluding the three sibs, this amounts to an incidence of ~35% (12/34 alleles) for the mutant allele in our group of patients. When patients previously reported by others are also included,<sup>7,8</sup> however, the incidence of this allele approximates ~25% (15/59 alleles). This makes this allele the most frequently occurring among SLO patients, since all other causative mutations identified to date have been recurrent among a limited number of patients<sup>6-8</sup> (unpublished results). The RT-PCR based methods presented in this paper provide an easy and rapid screening at the cDNA level for this frequently occurring mutant allele. The allele specific amplification of cDNAs in case of heterozygosity for the 134 bp insertion allows the establishment of compound heterozygosity without subcloning of the cDNAs, which is valuable in cases where no parental material is available.

The relatively high incidence of the splice acceptor site mutation among patients with SLO syndrome remains

unexplained at present, but could be the result of a founder effect. This is supported by the complete absence of any of the quite common polymorphisms,<sup>7</sup> 189A>G, 207C>T, 231C>T, 438C>T, 1158C>T, and 1272T>C, in all 15 mutant alleles identified in our patients. On the other hand, this seems less likely as the allele was identified in unrelated patients of Dutch, German, Belgian, Spanish, and North American origin<sup>6-8</sup> (this paper).

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## Clinical and molecular cytogenetic studies of a large de novo interstitial deletion 16q11.2-16q21 including the putative transcription factor gene *SALL1*

EDITOR—Interstitial deletions of the long arm of chromosome 16 share common clinical features including growth retardation, failure to thrive, microcephaly, high and prominent forehead, prominent metopic suture, large anterior fontanelle, hypertelorism, broad nasal bridge, low set and dysplastic ears, cleft palate, micrognathia, short neck, narrow thorax, broad first toes, mental retardation, muscular hypotonia, congenital heart defects, and gastrointestinal as well as renal anomalies.<sup>1</sup> More than 26 patients with different interstitial long arm deletions of chromosome 16 have been reported.<sup>2-29</sup> Recently, mutations in the transcription factor gene *SALL1* on chromosome

16q12.1<sup>30</sup> were shown to result in Townes-Brocks syndrome, an autosomal dominantly inherited malformation syndrome characterised by malformations of the anus, hands, and ears as well as deafness.<sup>31</sup> We describe a fetus with the 16q deletion syndrome and additional features, including unilateral radial aplasia, ulnar hypoplasia, preaxial hexadactyly, and segmentation defects of the vertebral column. Some of these features overlap with the malformations seen in Townes-Brocks syndrome. We therefore investigated the hypothesis that the *SALL1* gene was included within the deletion.

The 31 year old, gravida 2, para 1 (her first child is a healthy boy) was referred at 24 weeks gestation because her fetus had cleft lip and palate detected by ultrasound screening. Biometry showed asymmetrical growth retardation with a thoraco-abdominal diameter of 43 mm (<5th centile) corresponding to 19 weeks' gestation, while the BPD (60 mm) and femur length (41 mm) were within normal limits. Careful examination by ultrasound showed multiple fetal abnormalities. Dilatation of the lateral ventricles, a dilated third ventricle, and a cavum septum pellucidum were noted. Aplasia of the right radius was



Figure 1 Postmortem picture of the fetus presented. Note the left sided cleft lip/cleft palate, right sided radial aplasia, and a narrow chest.

suspected. The left kidney was absent. The right kidney was supplied by two arteries. The heart showed tetralogy of Fallot with absent pulmonary valve, agenesis of the ductus arteriosus, and a right sided aortic arch. The lungs were hypoplastic. Amniotic fluid, placenta, and umbilical vessels were normal. A fetal blood sample was taken for chromosome analysis. Necropsy examination after the parents opted for termination of the pregnancy in the 25th week of gestation showed a male fetus 31 cm in length and 690 g in weight (corresponding to the 24th/25th week of gestation). The fetus had a large neurocranium. Facial features included a prominent nasal bridge, a broad nose, hypertelorism, and upward slanting palpebral fissures. The ears were dysplastic, small, and deeply set with a single, left sided preauricular tag. The fetus had left sided cleft lip and palate. The chest was narrow. There was anal stenosis. In the upper extremities, the right radius was absent, while the ulna and thumb were hypoplastic. The left hand displayed preaxial hexadactyly with a finger-like thumb. Crowding of the toes was seen on both feet (figs 1 and 2). X rays confirmed the right radial aplasia and a shortened and thickened ulna. There was a single ossification centre (1 mm in diameter) within the hypoplastic right thumb, and a single ossification centre (1 mm in diameter) within the additional left thumb. The distal ossification centre of the additional left thumb was missing. The spine showed multiple segmentation defects (fusion of the third and fourth thoracic vertebral bodies, malformation of the eighth thoracic vertebral body, and aplasia of the third to fifth sacral vertebral bodies). Examination of the heart disclosed a perimembranous ventricular septal defect (4 mm in diameter), aplasia of the semilunar pulmonary valve, agenesis of the ductus arteriosus, and overriding of the right descending aorta. The left kidney was absent.

Chromosome analysis of fetal lymphocytes showed a large interstitial deletion of the long arm of chromosome 16 in all metaphases analysed. The result was confirmed by chromosome analysis of fetal fibroblasts. The karyotypes of the parents were normal. To define the exact site of the deletion, comparative genomic hybridisation (CGH) and fluores-



Figure 2 Lateral view of the fetal head. Note the dysplastic, small, and deeply set ears with a single left sided preauricular appendage, and retrognathia.

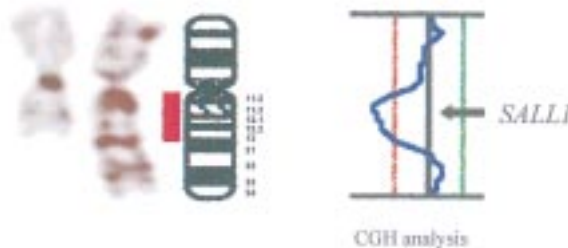


Figure 3 G banded chromosomes of fetal fibroblasts and CGH (comparative genomic hybridisation) analysis using fetal DNA showing an interstitial deletion 16q11.2-q21 according to ICGSN<sup>34</sup> as illustrated by the ideogram. Fifteen metaphases were used for CGH analysis. The bar located on the left of the ideogram indicates the deleted region.

cence in situ hybridisation (FISH) analysis were performed. CGH was performed with fetal DNA as described elsewhere.<sup>32</sup> CGH analysis defined the deletion to encompass bands 16q11.2 to 16q21 (fig 3). FISH analysis was performed using YACs from within and outside the deletion. YACs 922f01 (D16S415), 965g04 (D16S514/ D16S739), and 957h03 (D16S400/D16S3129) residing in 16q12-16q21 were shown to give only one signal on the normal chromosome 16, but none from the deleted chromosome 16. The STS numbers mapped to the corresponding YAC clones are given in parentheses. YAC 821g09 (D13S3021) located in 16q22 was not deleted. In addition, FISH using a PAC clone (reference: LLNLP704M031126Q4) including the *SALL1* gene<sup>33</sup> showed heterozygous deletion of *SALL1* (fig 4). DAPI banding confirmed the loss of the whole heterochromatin of chromosome 16 harbouring the deletion. The resulting fetal karyotype was defined as 46,XY,del(16)(q11.2;q21) according to ISCN.<sup>34</sup>

The phenotype of the 16q deletion syndrome was attributed to the deletion of critical bands at 16q11.2-q13,<sup>9 12 25</sup> 16q21,<sup>11 16 19 21 24</sup> and 16q22.1.<sup>14</sup> Specific features, such as congenital cataract<sup>20</sup> and iris coloboma,<sup>28</sup> result from more distal deletions of 16q22.3 and 16q23.1-24.2. Some of the features documented in patients with proximal deletions of chromosome 16q are also seen in Townes-Brocks syndrome.<sup>31 35 36</sup> The clinical presentation of TBS is highly

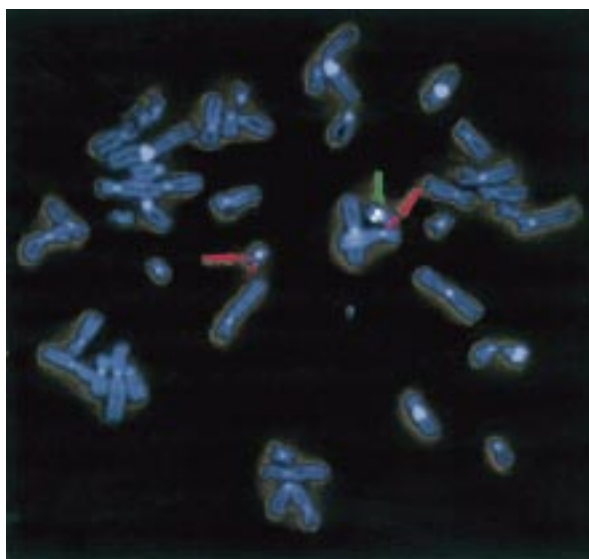


Figure 4 Two colour FISH analysis of fetal metaphase spreads (fibroblasts) using a PAC clone including *SALL1*.<sup>33</sup> The signal from the PAC clone, mapping to 16q12.1, gives only one signal on one chromosome 16, while the YAC clone (CEPH 765b06) located on the short arm of chromosome 16 (16p13) shows signals on both chromosomes.



Table 1 Clinical characteristics of patients with a deletion of the long arm of chromosome 16 including 16q12.1 or 16q13

	Present case q11.2q21	Reference						
		12	17	18	25	11	16	19
		q12.1q13 (twins)	q12.1 (sibs)	q11.1q13	q12.1q13	q13	q13	q13q21
Auricular malformation	+	++	++	+	+	+	+	+
Deafness	?	+ -	- -	?	SN	-	SN	-
Hands	Polydact	Small	T T	Small	SC	SC	Small	Polydact
Heart defects	+	- +	- -	+	-	+	-	+
Anal malformations	+	+ -	- -	-	-	-	Ectopic	+
Kidney hypo/agenesis	+	- -	++	?	-	+	+	-
Malposition of toes	+	++	++	+	+	+	-	-

SN = sensorineural deafness; polydact = preaxial polydactyly; T = proximally placed thumbs; SC = simian creases.

variable<sup>37</sup> within and between affected families. Characteristic features of TBS are anorectal abnormalities (imperforate anus, ectopic anus), abnormalities of the hands (preaxial polydactyly, triphalangeal thumbs, hypoplastic thumbs), abnormalities of the feet (syndactyly, club foot), deformities of the outer ear ("lop ears") and preauricular tags, and deafness.<sup>38</sup> Renal malformations include agenesis as well as hypoplastic or polycystic kidneys and may lead to renal failure.<sup>39</sup> The frequency of cardiac defects in TBS is high in those patients evaluated for cardiac defects by echocardiography.<sup>40</sup> Mental retardation has rarely been reported.<sup>41-42</sup>

TBS is caused by mutations of the *SALL1* putative transcription factor gene located on chromosome 16q12.1.<sup>30</sup> All mutations so far identified in TBS patients<sup>33</sup> are predicted to result in a prematurely terminated *SALL1* protein lacking all double zinc finger domains thought to be essential for *SALL1* gene function. Therefore, TBS is strongly suspected to result from haploinsufficiency for *SALL1*. This hypothesis is strengthened by the observation of a chromosomal translocation and a pericentric inversion in TBS patients, both involving a common breakpoint at 16q12.1.<sup>43-44</sup> The features of the fetus reported here show overlap with TBS. Our FISH studies prove that one allele of *SALL1* is indeed deleted. Therefore, we suggest that the ear anomalies, hand anomalies (preaxial polydactyly, hypoplastic thumb), anal stenosis, and the renal agenesis observed in the fetus result from heterozygous deletion of *SALL1*. The cardiac defects might also result from the *SALL1* deletion, since two TBS patients with tetralogy of Fallot have been described previously.<sup>45-46</sup> The anomalies of the radius and ulna seen in the fetus most likely result from the deletion of a contiguous gene in the deleted region. Vertebral segmentation defects have only recently been reported in a TBS patient.<sup>47</sup> However, a *SALL1* mutation has not been found in these patients.

Hoo *et al*<sup>17</sup> presented two mentally retarded sisters with a deletion of 16q12.1. Features described in these sisters included dysplastic ears with abnormal folding of the helices, proximally placed thumbs, and bilateral, symmetrically small kidneys. Twins with a deletion of 16q12.2-13 showed ectopic anus, congenital heart defects, and dysplastic ears.<sup>12</sup> A 10 year old boy with a de novo interstitial deletion 16q12.1q13 presented with micrognathia, a median cleft, low set dysplastic ears, a preauricular appendage, fusion of the third and fourth and the fifth and sixth vertebrae of the neck, hypospadias, hydrocephalus, sensorineural hearing deficits, and additional dysmorphic features.<sup>25</sup> Further clinical features described in patients with deletions of the proximal long arm of chromosome 16 are summarised in table 1. Whether or not *SALL1* is deleted in those patients reported with features similar to TBS and with deletions including 16q13 but not 16q12.1 remains to be elucidated.<sup>11-16-19</sup> Some families have been reported in which affected subjects show features typical of both TBS and Goldenhar syndrome/oculoauriculovertbral spectrum.<sup>48-49</sup> One family was de-

scribed with hemifacial microsomia and radial ray defects.<sup>50</sup> The affected members of this family displayed abnormal ears, multiple preauricular tags and pits, and unilateral micrognathia. In addition, triphalangeal thumbs, unilateral preaxial polydactyly, and a slightly anteriorly placed anus were present. Autosomal dominant inheritance was suggested in this family. Unilateral microphthalmia and facial asymmetry were observed in a family with anteriorly placed anus, irregular toes, and a digitalised thumb.<sup>51</sup> Gabrielli *et al*<sup>48</sup> described an infant with facial asymmetry, abnormal ears, preauricular tags, and anteriorly placed anus without triphalangeal thumbs or vertebral anomalies. Recently, a *SALL1* mutation was detected in the patient described by Gabrielli *et al*,<sup>48</sup> showing that *SALL1* mutations can also result in a Goldenhar-like phenotype.<sup>33</sup> It remains to be seen if the affected subjects in the families reported by Johnson *et al*<sup>49</sup> and Moeschler *et al*<sup>50</sup> also carry mutations in *SALL1*.

In summary, our results suggest that haploinsufficiency for *SALL1* might contribute to TBS-like features seen in the fetus presented here. In addition, certain clinical features in patients with proximal deletions of 16q including q12.1 are likely to result from haploid *SALL1* deletions. Nevertheless, the interstitial deletion in this fetus probably includes several hundred genes, arguing against a simplistic additive genetic model to explain the observed phenotype. Moreover, additional features of the 16q deletion syndrome, which are not part of the phenotypic spectrum of TBS, are likely to be caused by the deletion of additional genes located within the deletion. Some clinical features might be non-specific and cannot be attributed to the deletion of a single gene. FISH studies should indicate whether *SALL1* is deleted in those patients reported with features similar to TBS but with deletions including 16q13 but not 16q12.1. If a *SALL1* deletion cannot be confirmed in these patients, a TBS-like phenotype in these patients might still result from a position effect influencing *SALL1* gene expression.

Reference address for YACs: <http://www.mpimg-berlin-dahlem.mpg.de>; <http://www.mpimg-berlin-dahlem.mpg.de/~ctyogen/CHRM16.HTM>. We would like to thank Karin Lehmann and Gundula Leschick for expert technical assistance. Dr Friedrich C Luft helped us with the text. This work was funded in part by a grant from the Wilhelm-Sander-Stiftung to JK.

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## Oculocutaneous albinism type 2 with a *P* gene missense mutation in a patient with Angelman syndrome

EDITOR—Oculocutaneous albinism type 2 (OCA2) is an autosomal recessive disorder characterised by defective melanin production of the skin, hair, and eyes,<sup>1</sup> which is caused by mutations of the *P* gene.<sup>2,3</sup> The specific function of *P* has not been clarified, although it is likely to act as a transporter in the melanosomal membrane.<sup>2,4</sup>

The *P* gene is located in 15q11-q13, which is deleted in the majority of patients with Angelman syndrome (AS) and Prader-Willi syndrome (PWS).<sup>2,3</sup> The *P* gene is not imprinted and both alleles are expressed. PWS and AS patients with typical deletions are thus hemizygous for *P*. It is also well established that AS and PWS deletion patients usu-

ally show hypopigmentation of the skin and hair, and *P* is suggested to be responsible for this hypopigmentation as well,<sup>6,7</sup> although the mechanism has not yet been established.

A small intragenic deletion and a V443I missense mutation of the *P* gene were identified in the maternally inherited alleles of two PWS plus OCA2 patients who had a paternally inherited deletion of 15q11-q13.<sup>2,3</sup> Here we describe the first evidence that a *P* gene mutation is responsible for OCA2 associated with AS.

The male patient was born at 38 weeks' gestation to unrelated Japanese parents. There were no complications of pregnancy or delivery, but the birth weight, 1850 g, was small for gestational dates. Generalised albinism was noted at birth. Motor development was delayed, and he had a generalised tonic-clonic convulsion at the age of 18 months. He was admitted to hospital at the age of 19 months because of non-convulsive epileptic status, as reported previously.<sup>8</sup> Physical examination showed the



Figure 1 Phenotypic appearance of the patient at (A) 2 years of age and (B) 12 years of age.

typical facies of AS, including prominent mandible, protruding tongue, and relatively small head circumference ( $-1.72$  SD). His skin and hair pigmentation was markedly reduced consistent with albinism (fig 1A). His hair colour was fair-blond. Ophthalmological examination showed reduced pigmentation of the iris and a tigroid appearance of the retina with mildly reduced pigmentation. There was no nystagmus or other visual problems. He began walking unassisted at the age of 9 years with an ataxic gait typical of AS with the "hands up" position. He had severe mental retardation and could not speak. His skin, hair, and eye pigmentation gradually increased and he had dark hair by 12 years of age (fig 1B). His father and brother showed mild hypopigmentation of the skin and hair compared with other family members.

High resolution chromosome analysis and fluorescence in situ hybridisation (FISH) using D15S10 (Vysis, Downers Grove, Illinois) as probe showed a karyotype 46,XY,del(15)(q11q13) in the patient. Parental origin of the deletion was studied by the DNA methylation test using *SNRPN* exon 1 as probe.<sup>9</sup> Genomic DNA was extracted from the patient and parents by a standard procedure.<sup>9</sup> The *SNRPN* DNA methylation test indicated that the patient had only a paternal methylation pattern, indicating a maternal origin of the deletion (data not shown). These molecular cytogenetic results confirmed the clinical diagnosis of AS.

To screen for a *P* gene mutation, all 25 exons and flanking introns of the *P* gene were amplified by polymerase chain reaction (PCR) using the primers described by Lee *et al*,<sup>4</sup> agarose gel purified with GFX PCR and Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala,

Sweden), and direct sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequence reactions were run on a Perkin-Elmer 310 Automatic Sequencer and analysed by use of LASERGENE software (DNASTAR, Madison, WI). A missense mutation A481T (1441G>A) as well as non-pathological polymorphisms (IVS11-4A>G, G780G (2340C>T), S788S (2364G>A)) of the *P* gene were identified in the patient (fig 2). The 1441G>A mutation is located in exon 14 of *P* and causes amino acid substitution of alanine for threonine at codon 481. The non-pathological polymorphisms have been previously described in other people.<sup>4</sup> No wild type sequence was identified at these positions in the patient, as expected with hemizygosity at this locus. The father was shown to be heterozygous for the A481T mutation, whereas the mother was homozygous for the wild type allele (fig 2).

In this study the AS patient with OCA2 was shown to have a typical large deletion on the maternally inherited chromosome 15q, and a missense *P* mutation (A481T) on the paternally inherited chromosome. Most AS and PWS deletions are of similar size and *P* is always deleted in these patients.<sup>5</sup> Since the patient has only the A481T mutation allele, the mother is homozygous for the normal allele, and the father is heterozygous for the A481T mutation allele and the normal allele, the maternally inherited *P* allele must be deleted in the patient. This was confirmed for the deletion origin by methylation analysis with the adjacent imprinted region of 15q11-q13.

AS patients with the common 15q deletion are frequently associated with hypopigmentation, whereas other classes of AS patients are less often hypopigmented.<sup>10-11</sup> Similarly,

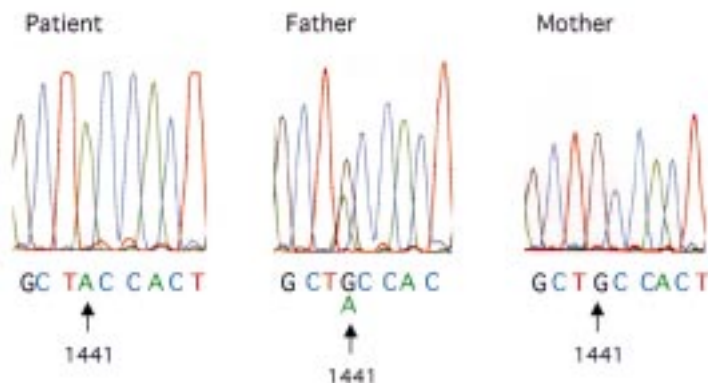


Figure 2 Segregation of the missense mutation in the patient and parents. The arrow indicates the position of the base substitution. The number below the arrow (1441) indicates the corresponding cDNA nucleotide position adapted from Lee *et al*.<sup>4</sup>

hypopigmentation in PWS also correlates with *P* deletion.<sup>7</sup> A hemizygous deletion of *P* cannot completely explain the mild degree of hypopigmentation more commonly seen in AS or PWS because heterozygous carriers of severe autosomal recessive OCA2 patients do not usually have hypopigmentation. Therefore, one can speculate that a pigmentation modifier gene(s) may be present in the deleted region of 15q11-q13.

The A481T mutation has previously been found in an African-American patient with *P* related autosomal recessive ocular albinism,<sup>3</sup> as well as in one of 50 unrelated controls.<sup>4</sup> The frequency of OCA2 is approximately 1/40 000 and 1/10 000 in whites and in African-Americans, respectively.<sup>1,12</sup> Carrier frequency is thus estimated to be 1/50-1/100. The carrier frequency of A481T reported by Lee *et al*<sup>1</sup> may have been overestimated, since previous studies have not shown that A481T is a predominant mutation in OCA2.<sup>3,12,13</sup>

The function of the A481T allele was shown by transfecting A481T mutant *P* cDNA into *p* null mouse melanocytes,<sup>14</sup> which showed that the A481T allele had approximately 70% function of the wild type allele. Therefore, since our patient is hemizygous, he may only have 35% of *P* gene function in melanin production compared with the wild type.

Our patient showed a severe deficiency of pigmentation during infancy to early childhood, but melanin production gradually increased and he had dark hair by 12 years of age. This is in keeping with the natural history of OCA2, as gradual increase in pigmentation during childhood is commonly seen in OCA2 patients. These findings are also consistent with the finding that the A481T allele has the potential for producing significant amounts of melanin as shown in the tissue culture experiments. Many mammalian genes are known to have an effect on pigmentation<sup>15</sup> and are developmentally controlled, although the precise mechanisms have not been established. Possible deletion of other pigmentation modifier gene(s) located in 15q may also explain the profound hypopigmentation in our patient during early childhood.

In conclusion, we describe the first genetic evidence that the *P* gene mutation is responsible for OCA2 associated with AS. These findings increase the spectrum of clinical conditions (AS or PWS plus OCA2, OCA2, ocular albinism) associated with *P* gene mutations.

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## Anticipation in progressive diaphyseal dysplasia

EDITOR—Progressive diaphyseal dysplasia (MIM 131300) is an autosomal dominant osteochondrodysplasia with late onset and marked variability in expression of clinical and radiological features.<sup>1</sup> We have recently described a patient with this diagnosis and suggested the presence of anticipation,<sup>2</sup> the tendency of a familial disorder to occur earlier in the younger than in the older generations of a family.<sup>3</sup>

In order to assess whether this hypothesis were true, we examined 24 other people from the same family (fig 1) who were invited and agreed to participate. We excluded the proband and tried to avoid ascertainment bias by including in the study all family members who were alive in four generations. Although the diagnosis of progressive diaphyseal

dysplasia had not been previously recorded in any of them, some family members had assigned to themselves the status of affected or unaffected. This was often discordant with the clinical and radiological data. It is also likely that people with bone complaints were more eager to consent to medical observation.

All family members were personally examined. The presence or absence and the age of onset of pain in the limbs, easy fatiguability, headache, poor appetite, and difficulty in running were recorded. Height, weight, and OFC were determined. Skull, spine, and limb morphology was examined and exophthalmos, muscle mass and strength, joint movement, tendon reflexes, and walking were assessed. Bone radiographic surveys, haemoglobin, white blood cell count, erythrocyte sedimentation rate, and serum alkaline phosphatase were performed.

The diagnosis of progressive diaphyseal dysplasia was clinically and radiologically established in 15 family members of the proband (table 1). Five of these have been previously described.<sup>2</sup>

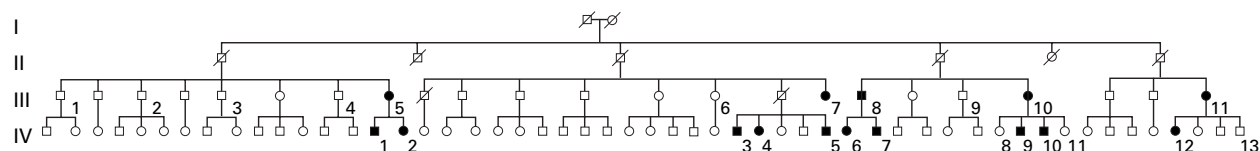


Figure 1 Pedigree showing 15 cases of progressive diaphyseal dysplasia in generations III and IV of one family. Only those personally examined are numbered and included in the statistical analysis.

Table 1 Clinical, laboratory, and radiographic data from 15 patients with progressive diaphyseal dysplasia

	Patient														
	III.5	III.7	III.8	III.10	III.11	IV.1	IV.2	IV.3	IV.4	IV.5	IV.6	IV.7	IV.9	IV.10	IV.12
Age at onset of symptoms (y)	30	20	20	15	21	15	14	3	10	10	8	5	18	13	2
Age at examination (y)	54	64	51	42	47	25	16	36	34	29	23	8	20	16	26
Pain in limbs	PMo	PMi	PMi	PMi	PMi	PMi	PMi	PMo	PMo	PMo	PMo	PMi	A	A	PS
Easy fatiguability	PMo	PMi	A	PMi	A	PMi	A	PMo	PMi	PMo	PMo	A	A	PMi	PMo
Headache	A	A	A	PMi	A	A	PMi	A	PMi	A	A	A	PMi	A	A
Poor appetite	A	A	A	PMi	A	PMi	A	A	A	A	A	A	A	A	PMi
Poor muscle mass	A	A	A	A	A	A	A	PMi	A	PMo	A	PMi	A	A	PMo
Waddling gait	PMi	PMi	A	A	A	PMi	A	PMi	PMi	PMo	PMo	A	A	A	PS
Radiographic bone changes	PMo	PS	PMo	PMo	PMi	PMo	PMi	PS	PS	PS	PS	PMi	PMi	PMi	PS
Haemoglobin (g/dl)	13.6	13.2	14.9	12.9	12.9	15.3	13.3	13.7	14.5	14.6	13.3	12.8	13.9	14.7	13.7
Erythrocyte sedimentation rate mm/h	14	28	1	3	11	9	11	13	16	15	6	11	10	4	16
Alkaline phosphatase (U/l)	27	42	81	24	33	28	20	35	52	57	203	105	46	71	97

P, present; A, absent; S, severe; Mo, moderate; Mi, mild.

We were able to determine the age of onset of the disease in these 15 patients (table 1), five from generation III and 10 from generation IV. Age of onset of the disease was defined as that of the initial symptom. The earlier age of onset in the younger generation was statistically significant (table 2). A similar analysis applying the same criteria to the data previously described in another extended family with progressive diaphyseal dysplasia<sup>4</sup> gave identical results (table 2). Other published pedigrees do not describe large families and often do not mention the age of onset of the disease.<sup>5-10</sup> However, the available data, excluding once again the probands, is in accordance with the previous results (table 2).

A previous suggestion that anticipation was enhanced in father to son transmission<sup>2</sup> is not supported by these new data; the patient with the earliest age of onset and one of the most severely affected is a female (IV.12, fig 1, table 1), who inherited the disease from her mother.

As has been previously pointed out, statistical anticipation without biological anticipation would occur if the disorder reduces fertility.<sup>3</sup> However, the mean number of children in the family members of generation III is 2 for those that are healthy and 2.8 for those that are affected. The small number of people of generation IV who have already had children does not merit consideration.

Another type of bias could be that some of the younger persons might be classed as unaffected but might develop the disease later. This would inevitably increase the mean age of onset as assessed in the younger generation. However, the reported ages of onset of progressive diaphyseal dysplasia are between 4 and 32 years and in 25 patients all but four had ages of onset younger than 20, the exceptions being the ages of 21, 25, 30, and 32 years old.<sup>2,4</sup> In order to avoid this bias,

Table 2 Mean age of onset of progressive diaphyseal dysplasia in the older and younger generations in the family described here (family 1), the family reported by Naveh et al<sup>4</sup> (family 2), and other pedigrees<sup>5-10</sup>

		Number	Mean	SD	p
Family 1	Older generation	5	21.2	5.4	0.001903
	Younger generation	10	9.8	5.3	
Family 2	Older generation	5	18.0	10.2	0.020079
	Younger generation	5	4.2	3.1	
Families 1 and 2	Older generation	10	19.6	7.9	0.000195
	Younger generation	15	7.9	5.3	
Other pedigrees	Older generation	7	21.4	10.5	0.003419
	Younger generation	5	3.0	1.9	

we did not include the data on three persons, one of whom is affected, from generation V. The ages of the unaffected persons from generation IV are 16, 23, and 24 years.

Although anticipation was only accepted as a true biological concept in the last decade, the list of conditions exhibiting it is growing rapidly.<sup>3,11</sup> Most of them are neuropsychiatric diseases and trinucleotide repeat expansions are usually suggested to be the cause.

This report suggests that anticipation occurs in progressive diaphyseal dysplasia and widens the disease spectrum of this concept to bone dysplasias. A dynamic mutation with trinucleotide repeat expansion may or may not be the cause of this osteochondrodysplasia of dense bone with unknown pathogenesis. The search for the molecular explanation of this and other rare genetic disorders that cause dense bones could disclose approaches to more common health problems such as osteoporosis.<sup>12</sup>

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## Hereditary neuropathy with liability to pressure palsies: two cases with a reciprocal translocation t(16;17)(q12;p11.2) interrupting the *PMP22* gene

EDITOR—Hereditary neuropathy with liability to pressure palsies (HNPP) or tomaculous neuropathy is an autosomal dominant disease.<sup>1</sup> HNPP patients present with acute or recurrent transient muscle palsies and paraesthesias, usually after minor trauma. HNPP is characterised from the pathological point of view by the presence of sausage shaped swelling of the myelin sheath or tomacula<sup>2</sup> in sensory and motor nerves.<sup>3</sup>

A deletion on the proximal short arm of chromosome 17 was detected in affected members of HNPP families.<sup>4,5</sup> The deleted region is the same as that duplicated in Charcot-Marie-Tooth disease type 1 (CMT1A). While the CMT1A phenotype is mostly the result of extra dosage of the genes contained in this duplicated region, the HNPP phenotype results from monosomy of the same region in over 85% of cases.<sup>6</sup> This duplication/deletion event is thought to occur because of the unequal crossing over between two large repeats bordering the CMT1A region,<sup>7</sup> which encompasses 1.5 Mb of 17p11.2.<sup>8</sup>

The association between CMT1A and the peripheral myelin protein 22 (*PMP22*) gene, located within the CMT1A duplicated region, was established by the finding of point mutations in this gene<sup>9,10</sup> in CMT1A patients. The *PMP22* gene spans approximately 40 kb and contains four coding and two 5' untranslated (1A and 1B) exons.<sup>11</sup> In 35 unrelated patients with inherited peripheral neuropathies, 27 mutations in the *PMP22* gene have been described, affecting the different domains of the protein,<sup>12</sup> some of them leading to a truncated protein. The clinical phenotypes of the patients carrying mutations in the *PMP22* gene are variable, giving some clues about correlations between phenotype and genotype. Five mutations in the *PMP22* gene have been described that result in a HNPP phenotype: two frameshift mutations,<sup>13,14</sup> two non-sense mutations,<sup>15,16</sup> and a splice site mutation.<sup>17</sup>

We report here a pedigree with two affected members (mother and son) with HNPP, both of whom carry a reciprocal translocation t(16;17)(q12;p11.2), which we have

studied by FISH. The breakpoint on chromosome 17 in both patients lies within exons 1a to 3 of the *PMP22* gene.<sup>11</sup>

The proband is a 24 year old male referred to our clinic for molecular analysis to confirm the clinical diagnosis of HNPP. The mother confirmed delayed milestones during infancy, followed by clumsiness and difficulties with running and schooling during childhood and adolescence. At the age of 21, he had two episodes of numbness and muscular weakness of the left arm following compression while sleeping and weight lifting, without full recovery. Clinical examination showed distal muscle atrophy and weakness of the left arm involving the long distal extensors of the upper limb, the abductor of the thumb, and the supinator of the arm. He was able to walk on his toes and heels and had normal reflexes. No other signs of muscle weakness were noted. Nerve conduction studies showed delayed and reduced sensory action potentials with prolonged motor latencies and slow motor conduction velocities (table 1). Clinical and electroneurographic studies were also carried out in the parents and his twin sister. The mother was clinically asymptomatic, although she reported episodes of paraesthesias with complete recovery involving the upper limbs. Electroneurographic findings were consistent with peripheral neuropathy (table 1) and the diagnosis of HNPP was established in the family. No other members were affected.

Chromosome spreads were obtained from peripheral blood of each patient and a G banding karyotype was performed. Conventional cytogenetics showed the presence of a reciprocal translocation between chromosomes 16 and 17 (t(16;17)(q12;p11.2)). FISH analysis was performed as described elsewhere<sup>18</sup> using YAC 181g9 (CEPH) and cosmids c49-E4 (GDB 437232) and c103-B11 (GDB 437233) (both cosmids kindly provided by Dr P I Patel) as probes. FISH analysis showed that YAC 181g9, which encompasses marker D17S122 to marker D17S879 and contains *PMP22*, crossed the translocation breakpoint. FISH with cosmids c49-E4 and c103-B11 showed that the breakpoint was within cosmid c49-E4, which contains the genomic region encoding the first three exons of the *PMP22* gene (fig 1). Therefore, we concluded that the *PMP22* gene<sup>11</sup> is interrupted in these two patients with the HNPP phenotype.

This is the first case of HNPP being caused by a reciprocal translocation that interrupts the *PMP22* gene. Cytogenetic studies and FISH confirmed the diagnosis in the patient. The two cases presented here not only confirm that the HNPP phenotype was the result of the interruption of the *PMP22* gene, but also show the variable penetrance of the phenotype in two related patients carrying the same mutation. It is therefore unlikely that other genes within the commonly deleted region have contributed to the phenotype. This suggests that modifiers in other genomic regions are involved in the clinical variability of peripheral neuropathies.

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Table 1 Electroneurographic findings in two patients with hereditary neuropathy with liability to pressure palsies (HNPP)

	Motor and sensory nerve conduction					
	Ulnar nerve		Median nerve		Peroneal nerve	
	P	M	P	M	P	M
MCV (m/sec)						
R	40	40	53	60	—	27
L	39	48	52	44	32	31
N	(>52)		(>52)		(>48)	
DL (m/sec)						
R	3.5	2.8	4.5	4	—	—
L	3.2	3	3.7	3.8	—	—
SCV (m/sec)						
R	35	43	27	33	—	—
L	38	37	37	34	—	—
SAP amplitude (µV)						
R	2	4	2	2	—	—
L	3	2	3	3	—	—

P, proband; M, mother; R, right; L, left; N, normal value; MCV, motor conduction velocity; DL, distal latency; SCV, sensory conduction velocity; SAP, sensory action potential; —, not done.

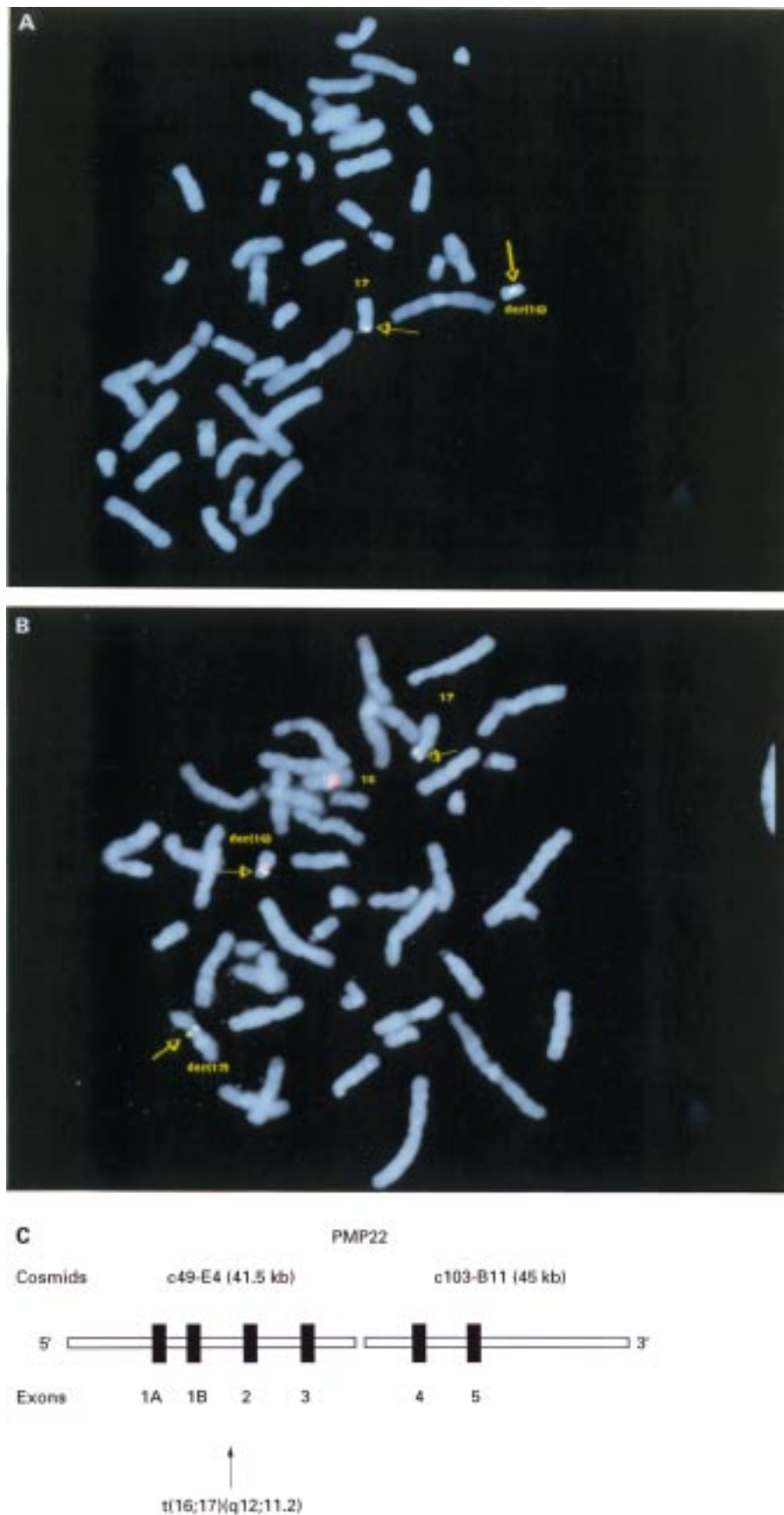


Figure 1 Hereditary neuropathy with liability to pressure palsies (HNPP) resulting from a  $t(16;17)(q12;p11.2)$  reciprocal translocation. (A) FISH with cosmid c103-B11 shows signals on chromosomes 17p11.2 and der(16) and does not cross the breakpoint. (B) FISH with cosmid c49-E4 (green) shows signals on chromosomes 17p11.2, der(16), and der(17), indicating that this cosmid crosses the breakpoint and that the PMP22 gene is interrupted by the reciprocal translocation. Probe D16Z1 (red) hybridises to the centromeric region of both chromosome 16 and der(16) (C) Schematic representation of the genomic region encompassing the PMP22 gene, indicating the  $t(16;17)(q12;p11.2)$  breakpoint (arrow).

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