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## Letters to the Editor

### Sequence analysis of the homogentisate 1,2 dioxygenase gene in a family affected by alkaptonuria

EDITOR—Alkaptonuria (AKU) is a disorder of the catabolism of aromatic amino acids. A defect of homogentisate 1,2 dioxygenase (HGO) leads to an accumulation of homogentisic acid (HGA) and subsequently to deposition of polymerised HGA, a brown-black pigment, in connective tissue, primarily in cartilage.<sup>1,2</sup> This phenomenon is known as ochronosis. It results in debilitating arthropathy which typically becomes manifest in the fourth decade of life. Large amounts of HGA are excreted in the urine and cause its black discoloration upon oxidation. In 1891, homogentisic acid was first isolated by Wolkow and Baumann<sup>3</sup> from the urine of an AKU patient from a remote area of the Black Forest in south western Germany. In 1902, Garrod, aware of this biochemical finding, observed the autosomal recessive mode of inheritance of AKU and thereby showed for the first time that mendelian laws also apply to human genetics.<sup>4</sup> Garrod postulated that AKU results from an enzyme deficiency and introduced the concept of the “inborn error of metabolism”.<sup>5</sup>

Recently, the human gene encoding HGO was cloned by Fernández-Cañón *et al.*<sup>6</sup> Two different mutations of this gene were identified in two unrelated AKU affected families. These mutations cosegregated with manifest disease and could be shown to abrogate enzymatic activity of HGO protein.<sup>6</sup> Homozygosity for these mutations, therefore, was the cause of AKU in the two families. Two additional mutations in the HGO gene were found to cosegregate with AKU in two Slovakian pedigrees.<sup>7</sup> One of these mutations caused a frameshift in an upstream exon and was thus likely to result in a loss of HGO activity. For an additional mutation, complete cosegregation with AKU was reported in an extensively studied Canarian family.<sup>8</sup> Various different mutations of the HGO gene were found in 14 unrelated AKU patients.<sup>9</sup>

We performed sequence analysis of the HGO gene in an AKU affected family from the Black Forest. AKU with severe ochronosis including involvement of the sclerae was diagnosed at necropsy of a 71 year old farmer (fig 1, No 1). The diagnosis of AKU had not been established during the patient's lifetime. He died of recurrent myocardial infarction. Subsequently, the patient's family underwent physical examination. A sister (fig 1, No 2) and a first cousin (fig 1, No 3) were found to be affected by the disease. These patients have been suffering from arthritic symptoms of AKU since the fourth decade of life and show the typical discoloration of the urine and the ochronotic pigmentation of the sclerae.

However, the condition had until then been misdiagnosed as degenerative polyarthrititis. A brother (fig 1, No 4) of patient 1 was healthy as were the three children (fig 1, Nos 5, 6, and 7) of patient 2. Anamnestically, a brother (fig 1, No 8) and a first cousin (fig 1, No 9), who died in 1988 and 1995, respectively, were reported to have suffered from debilitating early onset polyarthropathy and the typical ochronotic involvement of the sclerae. They were very probably affected by AKU. No characteristic AKU symptoms were reported

for two brothers (fig 1, Nos 10 and 11) of patient 1, who died in 1940 and 1974, respectively.

The pedigree of the family was constructed using the parish registers of the region. Information was available back to the early 17th century. In the last three centuries the pedigree shows four consanguineous marriages of third and fourth cousins (fig 1, I), fifth and seventh cousins (fig 1, II), second and third cousins (fig 1, III), and third cousins (fig 1, IV).

DNA was extracted by standard procedures from liver tissue sections obtained during necropsy of patient 1 and from blood samples of two other affected subjects (Nos 2 and 3) and four healthy members of the family (Nos 4, 5, 6, and 7). The 14 exons of the HGO gene were amplified using primers hybridising in the flanking intronic sequences, as described by Fernández-Cañón *et al.*<sup>6</sup> and PCR products were directly sequenced from both sides.

Sequence analysis of the HGO gene showed that all three family members suffering from AKU were homozygous for a point mutation in exon 13, an adenine for guanine substitution at position c1269 (c1269A→G), which causes the replacement of methionine 368 by valine. The three healthy children of patient 2 and the healthy brother of patient 1 were heterozygous carriers of this mutation. Interestingly, the same mutation of the HGO gene was recently described in a French and another German AKU affected family.<sup>9</sup> Methionine 368 is conserved between *Aspergillus nidulans*, mouse, and man and may be essential for enzymatic activity of HGO protein.<sup>10,11</sup>

It is of anecdotal interest that our patients stem from the same remote, in former times genetically isolated, area of the Black Forest as the AKU patient from whose urine Wolkow and Baumann<sup>3</sup> first isolated homogentisic acid in

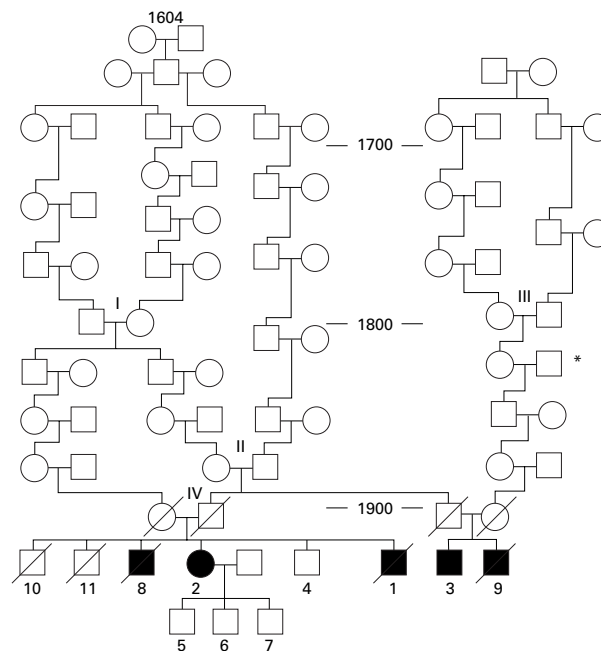


Figure 1 AKU affected family. Sequence analysis was performed for subjects 1-7. For patients 8 and 9, the family reported AKU related symptoms. They were, therefore, probably affected. Four consanguineous marriages in the last three centuries are indicated by Roman numerals. An asterisk marks the person who comes from the same kindred as the patient of Wolkow and Baumann.<sup>3</sup>

1891. Therefore, it is likely that their patient was homozygous for the defective *HGO* allele we found in our AKU family.

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## A new family linked to the *RP1* dominant retinitis pigmentosa locus on chromosome 8q

EDITOR—Retinitis pigmentosa (RP) is the term given to a group of inherited retinal degenerations affecting approximately 1 in every 4000 people.<sup>1</sup> Clinical presentation includes night blindness, a peripheral bone spicule appearance to the retina, constriction of retinal arterioles, and visual field loss. RP can be inherited in an autosomal dominant, autosomal recessive, or X linked fashion, with autosomal dominant RP (ADRP) accounting for around 20%.<sup>2</sup> Within the autosomal dominant category there is both clinical and genetic heterogeneity. Nine ADRP loci have been reported to date. Mutations in rhodopsin account for between 20 and 50% of ADRP<sup>3-6</sup> and those in peripherin/RDS for less than 5%,<sup>7,8</sup> while around 20% of large families are linked to a major locus on chromosome 19q.<sup>9</sup> The remaining six loci, for which genes have not yet been identified, are relatively rare and approximately 15% of families do not map to any known locus, indicating yet further genetic heterogeneity.<sup>6,10</sup>

The *RP1* locus on 8q was first identified by linkage analysis in a large pedigree known as UCLA-RP01, originating in the Appalachian mountains of Kentucky in eastern USA.<sup>11</sup> The phenotype in UCLA-RP01 is described as type 2 or R type ADRP, with regional and combined loss of both rod and cone photoreceptor sensitivities. A second unrelated family of Australian origin has also been linked to *RP1*.<sup>12</sup> Crossovers in these families place the locus in a 4 cM interval between markers D8S601 and D8S285. The phenotype in both families is reported as showing wide variation in severity and age of onset, and subjects in both were found to carry the gene yet manifest no symptoms. However non-penetrance is rarer at this locus (around 6% in the Kentucky pedigree) than at two other ADRP loci on 7p (*RP9*<sup>13</sup>) and 19q (*RP11*<sup>9</sup>) which have 10% and 35% non-penetrance, respectively.

We now report a new *RP1* linked family originating in south west England. The phenotype in this family is similar to that seen in the US and Australian families. Age of onset varies from 12 years to the sixth decade, with simultaneous loss of visual fields and night vision as the first symptoms. Fundus examination in affected subjects shows pale optic discs, peripheral pigment clumping, and attenu-

ated retinal blood vessels. The family includes four subjects under the age of 25 years who are asymptomatic, but who offered DNA samples for linkage analysis to help determine which RP locus was involved in the family. All four subsequently requested to know their results and our interpretation of these, particularly in order to help guide them in career choices. These results were given with supportive counselling, according to predictive test protocols, albeit emphasising the uncertainties of linkage analysis. One of these asymptomatic subjects was found to have inherited the high risk haplotype, but at the age of 16 years showed no detectable abnormality on fundus examination and electrodiagnostic testing (V.7 in fig 1). All members of the family have agreed to publication of the pedigree with linkage results attached.

Microsatellite markers from known ADRP loci were typed in genomic DNA samples from the family by PCR, with incorporation of <sup>32</sup>P labelled cytosine, followed by size fractionation on 6% polyacrylamide denaturing gels. For most markers a standard cycling profile of 30-35 cycles at 94°C, 55°C, and 72°C was used, with 20 seconds at each step. Allele frequencies were estimated from panels of between 18 and 34 chromosomes of normal partners of RP patients in this and other UK families. Lod scores were calculated from data files prepared on the LINKSYS (version 3.1) data management package then transferred to the LINKAGE (version 5.1) suite of programs. Linkage analysis was carried out both on a PC and on the Human Genome Mapping Project Resources Centre computing facility.

DNA from the family was tested for linkage to markers from each of the nine known ADRP loci, and crossovers were detected in affected subjects with all but those markers at the *RP1* locus at chromosome 8q11. Lod scores for markers from the other ADRP loci are given in table 1. Markers used to establish linkage to the *RP1* locus are as follows: 8pter/D8S87/6 cM (including 8cen)/D8S601/3 cM/D8S285/1 cM/D8S166/ 4 cM/D8S507/8qter. This order and genetic distances are approximately as given by Xu *et al*<sup>2</sup> and agree well with data from the Marshfield integrated human genetic map (<http://www.marshmed.org/genetics>).

When testing for linkage between the retinal disease in this family and *RP1* markers, lod scores were obtained by two different analyses. The first follows the method of Xu *et al*<sup>2</sup> and assumes full penetrance but excludes all subjects diagnosed unaffected who are under 25 years of age (marked with a question mark in fig 1). Analysis under this

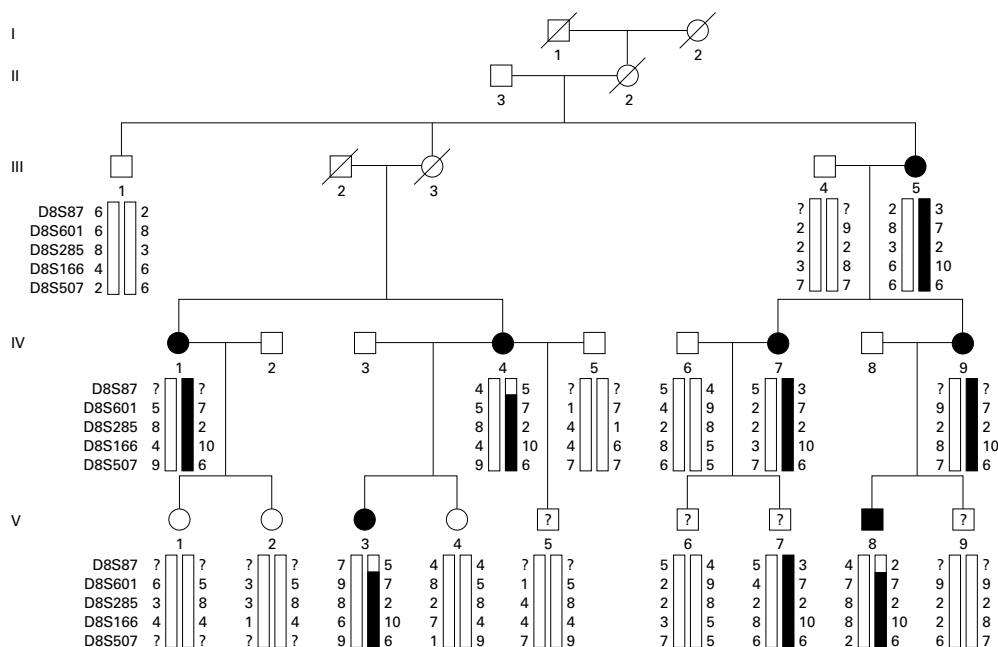


Figure 1 The UK *RP1* linked family. Filled symbols denote subjects confirmed as having RP. Haplotypes for chromosome 8q markers are shown below each symbol, with the affected haplotype denoted by a black bar. Symbols containing a question mark are apparently normal, but were below the age of 25 when last examined.

model gave maximum two point lod scores of 2.89, 1.09, and 2.66 with markers D8S601, D8S285, and D8S166 against RP respectively, each with no recombination. Multipoint analysis with this model using these three markers gave a maximum lod score of 3.01 at D8S601. The second analysis includes all of the subjects shown but allows for the possibility that apparently normal family members are non-expressing gene carriers. The original report on *RP1* linkage to chromosome 8<sup>11</sup> described a pedigree of over 80 affected subjects which included two dead obligate carriers reported to be asymptomatic and a further three living gene carriers with minimal symptoms in their fifties. The second documented *RP1* pedigree<sup>12</sup> had two asymptomatic gene carriers out of 11 affected subjects. We therefore assumed a penetrance of approximately 90%. In this way, maximum two point lod scores of 2.76, 1.32, and 2.53 were obtained with these three markers, while the peak multipoint lod score of 2.88 was again at marker D8S601. Haplotypes for the three markers used are shown in fig 1. Five other markers from the region were typed in the family but did not add significantly to the results presented.

The pedigree described here does not further refine the *RP1* locus, since flanking markers D8S601 and D8S285 detect no crossovers in the family. However, the assignment of a third ADRP family to this locus may imply that *RP1* is

among the more common dominant RP loci. Alternatively it is possible that either the Australian or US families are related to this one, given the extensive emigration from the UK (and particularly from the south west) to those countries over the last three centuries. The phenotype in the new family is similar to that described in both of the previously reported *RP1* families. *RP1* linked pedigrees consistently exhibit wide variation in severity and age of onset of symptoms and include asymptomatic gene carriers, but with a lower frequency of non-penetrance than at the *RP9* and *RP11* loci. The mechanism of partial penetrance at these loci has not yet been fully elucidated, but McGee *et al*<sup>14</sup> reported that penetrance of the mutated allele at the 19q *RP11* ADRP locus is moderated by the normal allele with which it is paired. The pedigree described here would be consistent with such a mode of inheritance. Sib pair IV.1 and IV.4, who appear to have inherited the same haplotype of markers for chromosome 8 from their normal father, both reported age of onset in their early thirties, while sib pair IV.7 and IV.9, who inherited different versions of chromosome 8 from their father, had onset of symptoms in their mid-thirties and at 12 years of age respectively. Analysis of the other *RP1* families may therefore further implicate allelic moderation as a phenomenon common to different forms of RP.

Table 1 Markers used to exclude the other known ADRP loci and lod scores obtained. All lod scores exclude a locus close to the marker tested in this family and each of the markers tested is within 3 cM of the candidate ADRP locus. Marker D7S514, which gives the weakest exclusion, lies within the 5 cM interval for the *RP10* locus

Locus	Marker	Lod scores						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
<i>RP18</i>	D1S498	∞	-4.81	-2.55	-1.56	-0.65	-0.24	-0.06
<i>RHO</i>	ACPP	∞	-4.99	-2.82	-1.88	-0.98	-0.51	-0.20
Peripherin/RDS	D6S271	∞	-4.41	-2.38	-1.56	-0.80	-0.41	-0.17
<i>RP9</i>	D7S460	∞	-6.32	-3.59	-2.46	-1.38	-0.76	-0.32
<i>RP10</i>	D7S514	∞	-1.12	-0.50	-0.28	-0.12	-0.06	-0.02
<i>RP13</i>	D17S831	∞	-4.72	-2.49	-1.53	-0.65	-0.24	-0.05
<i>RP17</i>	D17S807	∞	-3.26	-1.30	-0.57	-0.03	0.11	0.09
<i>RP11</i>	D19S572	∞	-4.04	-1.88	-0.98	-0.23	0.04	0.09

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## 21-hydroxylase deficiency in Italy: a distinct distribution pattern of *CYP21* mutations in a sample from southern Italy

EDITOR—Congenital adrenal hyperplasia (CAH) is an autosomal recessive genetic disease, the main cause of which is steroid 21-hydroxylase (21OH) deficiency.<sup>1</sup> The classical phenotype of the disease includes a severe salt wasting (SW) form in which both cortisol and aldosterone synthesis is impaired, and a mild form with normal aldosterone synthesis, commonly known as simple virilising (SV). In most white populations, an incidence of 1:5000 to 1:15 000 has been reported for the severe classical forms.<sup>2</sup> The late onset form (LO), also called non-classical (NC), is characterised by the appearance of symptoms of excessive androgen activity late in life with an incidence of 1:100 to 1:1000 persons.<sup>3</sup>

The steroid 21-hydroxylase locus has a complex structure with two genes, *CYP21P* and *CYP21*, located on the short arm of chromosome 6 within the HLA class III gene region downstream of each of the two genes encoding the fourth component of complement, *C4A* and *C4B*, respectively. Small exchanges of sequences, a process known as gene conversion, could create many of the disease causing mutated alleles by transferring some of the deleterious mutations from the pseudogene to the *CYP21* gene.<sup>4,5</sup> Owing to the complexity of the locus, complete characterisation of mutant *CYP21* alleles in patients with 21OH deficiency could be performed either by direct sequencing or by a mutation scanning method, such as non-radioactive DNA single strand conformation polymorphism analysis of all exons and exon/intron junctions.<sup>6</sup> To date, deletion and several sequence aberrations of the *CYP21* gene have been reported to cause steroid 21-hydroxylase deficiency in different populations including Italians, with 10 mutations being the most frequent.<sup>7-11</sup>

In cases of other well characterised recessive diseases, such as phenylketonuria and cystic fibrosis, clear heterogeneity of distribution of the relative frequencies of the disease mutation has been found in the Italian population.<sup>12,13</sup> With the aim of determining the *CYP21* mutation profile in a selected Italian sample, the screening of the 10 most frequent *CYP21* gene mutations was performed on a cohort of 21OH deficient patients, originating from southern Italy. The results of this study may also prove to be of practical use for the implementa-

tion of regional programmes of carrier screening or prenatal diagnosis using methods to screen simultaneously for the most frequent mutations.

Twenty eight patients from southern Italy with a total of 50 independent CAH chromosomes were enrolled in this study. Southern Italian origin was assessed on the basis of the birth place of the grandparents. Patients were diagnosed on the basis of increased plasma 17 $\alpha$ -hydroxyprogesterone (17OHP) and the ACTH provocation test. Each patient examined had high values of both baseline and ACTH stimulated 17OHP serum concentration, aggregating in the upper part of the nomogram reported in Wilson *et al.*,<sup>14</sup> and were classified as having one of the three clinical forms of CAH.<sup>3</sup> Seventeen patients suffered from the SW form, eight from the SV form, and three from the LO form. Where available, parents were also enrolled in the study.

Genomic DNA was prepared from peripheral blood leucocytes by standard procedures. Oligonucleotides were purchased from Pharmacia (Sweden) and their sequences and names have been reported elsewhere.<sup>6,15</sup> PCR amplifications were performed using approximately 100 ng of genomic DNA in the presence of 200  $\mu$ mol/l of each dNTP, 1 mmol/l MgCl<sub>2</sub>, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 50 pmol of each primer, and 2.0 U *Taq* polymerase (Promega) in a volume of 100  $\mu$ l. Selective amplification of the *CYP21* gene in two overlapping fragments was carried out as previously reported.<sup>6</sup> Genotyping was performed in a second, allele specific round of PCR essentially as described by Wedell and Luthman<sup>15</sup> using 1  $\mu$ l of the initial reaction. The following primers were used: for P31L: P92T, P92C, and P48; for I2 splice: P659G, P659A, P659C, and P48; for I173N: P1004A, P1004T, and Zfor; for cluster E6: P1388A, P1388T, and Zfor; for V281L: P1688T, P1688G, and Ofor; for 306insT: P1678T, P1678G, and Ofor; for Q318X: P1999T, P1999C, and Ofor; for R356W: P2113T, P2113C, and Ofor; for P453S: P2584T, P2584C, and Ufor. The I172N, V281L, and R356W mutations were analysed using an allele specific PCR protocol involving the use of a competitive allele non-specific primer placed outside the allele specific primer, that is, Lrev, Prev, and Vrev, respectively.<sup>6</sup> All PCR products, except those obtained from the screening of I2 splice, were analysed by agarose gel electrophoresis and ethidium bromide staining. In the case of I2 splice, PCR products were visualised after silver staining of 13% polyacrylamide minigels.

To determine the presence of the 8 bp deletion in exon 3 (del8bp), a fragment of 616 bp was amplified with the gene specific forward primer Efor and Hrev.<sup>6</sup> This fragment was

used as a template for a second PCR reaction using the primer pair del8for/del8rev<sup>16</sup> with amplification products analysed by electrophoresis on 12% polyacrylamide minigels followed by ethidium bromide staining. For those subjects carrying the del8bp mutation, specific amplification of the full length *CYP21* gene was directly performed from genomic DNA with Yfor/Zrev primers after removing the pseudogene by *TaqI* cleavage, as previously described.<sup>17</sup> In order to confirm homozygous point mutations, Southern blotting analysis was carried out whenever parents were not available; 10 µg of DNA was digested with the restriction endonuclease *TaqI*, and the products were analysed by electrophoresis on 0.8% agarose gels followed by Southern transfer to nylon membranes. The probe was a 2209 bp fragment of the *CYP21* gene, radiolabelled with [<sup>32</sup>P]dCTP by using the Megaprime Kit (Amersham). Hybridisation conditions were essentially those reported by Haglund-Stengler *et al.*<sup>18</sup> Autoradiography was carried out by exposure of filters at -80°C for 96 hours. The relative intensity of the 3.7 kb compared to the 3.2 kb fragment was determined using the GS-700 Imaging Densitometer implemented with the Molecular Analyst Software (Bio-Rad Laboratories).

Statistical tests for heterogeneity of distribution of *CYP21* mutations were performed using 2 × 2 contingency tables and Fisher's exact test.

A total of 98% of the chromosomes analysed were genotyped by the approach used in this study, showing the highest rate of mutation detection so far achieved in the characterisation of *CYP21* mutations in 21OH deficiency. The *CYP21* gene mutation profile is reported in table 1. I2 splice was present in more than half (56%) of the southern Italian CAH chromosomes analysed. This mutation occurred de novo on one chromosome analysed. Cluster E6 was identified in the Italian population for the first time, whereas P453S was not found in our patients. No subject analysed was found to be homozygous for a *CYP21* deletion since in every case a successful amplification of the *CYP21* gene was obtained. Three patients were hemizygous for gene deletion, accounting for 8% of the chromosomes genotyped. Mutated alleles carrying only one mutation represented 78% of CAH chromosomes analysed. Six different complex alleles, as deduced from the detection of more than one mutation on the same chromosome, were identified with a total relative frequency of 12%. For all patients except one, the two *CYP21* alleles were fully characterised: 18 (64.3%) are compound heterozygotes for different mutations and 10 (35.7%) are homozygotes.

Table 1 Relative frequencies of *CYP21* alleles in 50 southern Italian CAH chromosomes

Alleles	C forms		Total C and NC forms	
	No of alleles	Relative frequency(%)	No of alleles	Relative frequency(%)
I2 splice	27	61	28	56
del <i>CYP21</i>	4	9	4	8
I172N	3	7	3	6
V281L	0		3	6
Q318X	2	4.5	2	4
R356W	2	4.5	2	4
del8bp	0		1	2
I2 splice, I172N	1	2.3	1	2
I2 splice, cluster E6	1	2.3	1	2
I2 splice, V281L	0		1	2
P31L, I2 splice, del8bp	1	2.3	1	2
I2 splice, I172N, cluster E6, R356W	1	2.3	1	2
I172N, cluster E6, V281L, 306insT, Q318X, R356W	1	2.3	1	2
ND	1	2.3	1	2
Total	44		50	

C, classical; NC, non-classical.

Table 2 Genotype-phenotype correlation in 28 21-hydroxylase deficient patients

Genotype	No of patients	Phenotype
I2 splice/I2 splice	7	SW
I2 splice/Q318X	1	SW
I2 splice/R356W	3	SW
I2 splice/del <i>CYP21</i>	2	SW
I2 splice/I2 splice, cluster E6	1	SW
I2 splice/P31L, I2 splice, del8bp	1	SW
I2 splice, I172N, cluster E6, R356W/I172N, cluster E6, V281L, 306insT, Q318X, R356W	1	SW
I172N, cluster E6, V281L, 306insT, Q318X, R356W/del <i>CYP21</i>	1	SW
I2 splice/I2 splice	3	SV
I2 splice/I172N	1	SV
I172N/del <i>CYP21</i>	1	SV
I172N/Q318X	1	SV
I2 splice/I2 splice, I172N	1	SV
I172N/nd	1	SV
V281L/I2splice	1	NC
V281L/del8bp	1	NC
V281L/I2splice, V281L	1	NC

As for I2 splice frequency, this study provides the highest relative frequency found for this mutation both in Italian<sup>9</sup> and other European populations.<sup>8 10 11</sup> A statistically significant difference ( $p < 0.001$ ) was found between this relative frequency and that measured in the Italian population by Carrera *et al.*<sup>9</sup> (20%) and in the Spanish by Ezquieta *et al.*<sup>10</sup> (22%).

In comparison with previously characterised Italian patients,<sup>9</sup> Q318X was found with a lower relative frequency. On the other hand, R356W was found in our cohort as a single mutation allele with a relative frequency of 4%, whereas Carrera *et al.*<sup>9</sup> found it in only one complex allele together with five other mutations. The mutation cluster in exon 6 was found for the first time in the Italian population and was identified as part of a complex allele in three patients. However, these differences concern relatively rare mutations and are either not significant or only slightly so. Even if the size of our sample may not be considered large enough, it can be speculated that the statistically different frequency of I2 splice, as well as the other peculiar mutation frequencies found in our sample, could be secondary to ethnic differences. Recent evidence has been obtained concerning the genetic heterogeneity of the Italian population which is accounted for by prehistorical colonisation events.<sup>12 13</sup> Since no information is given about the origin of the Italian patients genotyped by Carrera *et al.*<sup>9</sup> the heterogeneity of distribution of relative frequencies of Italian CAH alleles will need further investigation.

Genotype-phenotype correlation was assessed for all subjects and the results are summarised in table 2. Only five mutations, I2 splice, *CYP21* deletion, I172N, Q318X, and R356W, represent 86% of CAH chromosomes associated with the classical forms of the disease. This finding could prompt the development of molecular diagnosis for phenotype prediction. Since the severity of the disease is thought to be determined by the activity of the less severely affected allele, both alleles of a 21OH deficient patient must be considered correctly to predict the phenotype, at least in the case of I172N and in other previously established severe mutations, such as Q318X and R356W.

However, the phenotype of patients does not always correlate with the genotype especially when the splicing mutation at nucleotide position 655 is observed. In vitro expression studies of the *CYP21* gene containing the intron 2 mutation led it to be considered as a severe mutation since aberrant mRNA splicing, associated with lack of enzymatic activity, was detected.<sup>4</sup> However, the in vitro studies might not reflect the in vivo situation in the adrenal cortex so,

although the majority of patients homozygous for this mutation show a strong association between I2 splice and the SW form, a SV phenotype was observed in three out of 10 patients according to previous reports.<sup>8 11 14</sup>

In view of these observations, the fact that I2 splicing mutation represents 61% of southern Italian CAH alleles associated with the classical forms of the disease (table 1) precludes the use of genotyping *CYP21* alleles for correct 21OH deficiency phenotype prediction aimed at the implementation of effective therapy. In any case, the possibility of performing prenatal diagnosis and carrier detection through the screening of the most frequent mutations still retains its validity.

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## Identification of a frameshift mutation in the gene *TWIST* in a family affected with Robinow-Sorauf syndrome

EDITOR—The original report by Robinow and Sorauf<sup>1</sup> described a large family with autosomal dominant craniosynostosis and hallucal duplication. The clinical features include craniosynostosis, plagiocephaly, flat face, hypertelorism, thin, long, and pointed nose, shallow orbits, strabismus, and broad great toes with a duplication of the distal phalanx. This autosomal dominant syndrome is listed as a separate entry in the McKusick catalogue<sup>2</sup> (MIM 180750), although it is clinically similar to Saethre-Chotzen syndrome (MIM 101400). The most characteristic additional feature in Robinow-Sorauf syndrome is a bifid or partially duplicated hallux. In the past, the relationship between these conditions has been controversial. Carter *et al*<sup>3</sup> emphasised the differences in the phenotype in two patients, and considered it as a separate entity in accordance with the report of Robinow and Sorauf.<sup>1</sup> In a further report, similar clinical findings were described as Saethre-Chotzen syndrome<sup>4</sup> or an unusual form of acrocephalosyndactyly.<sup>5</sup> Another phenotypically similar phenotype has been described as Pfeiffer syndrome.<sup>6</sup> Bifid distal hallucal phalanges have also been observed in aural-cephalosyndactyly syndrome, in which brachycephaly, facial asymmetry, delayed suture closure, and small pinnae were associated with cutaneous syndactyly 4/5 of the feet.<sup>7 8</sup> Based on the cytogenetic findings of Reardon<sup>9</sup> involving chromosome 7p21, there is now growing consensus that Robinow-Sorauf syndrome is a variant of Saethre-Chotzen syndrome involving the same gene.

Recently, mutations in the gene *TWIST* have been identified in patients with Saethre-Chotzen syndrome.<sup>10-12</sup> The gene is localised on chromosome 7p21 and encodes a transcription factor containing a basic helix-loop-helix (bHLH) motif.<sup>13</sup> Here we report the identification of a frameshift mutation in *TWIST* in a family with clinical features of Robinow-Sorauf syndrome. This supports the assumption that Robinow-Sorauf syndrome is an allelic variant of the Saethre-Chotzen syndrome. Other *TWIST* mutations have been identified in the family originally described by Young and Harper<sup>5 12</sup> and in another case described by El Ghouzzi *et al*.<sup>10</sup>

The pedigree of the three generation family is shown in fig 1A. The proband (III.1) was referred for diagnostic evaluation and genetic counselling at the age of 15 months because of craniosynostosis and broad thumbs and halluces. He was born at term after an uneventful pregnancy (weight 3450 g, length 55 cm, head circumference 36 cm). A younger brother is healthy. At the age of 2 days, seizures occurred which were treated with phenobarbital. At the age of 4 months the paediatrician noticed a protruding fontanelle without signs of brain pressure. Our first clinical examination at the age of 15 months showed the following abnormalities: plagiocephaly, downward slanting palpebral fissures and marked bilateral ptosis, a protruding anterior fontanelle, and broad thumbs and halluces with valgus deformity (fig 1B). Head circumference was 47 cm (50th centile). Developmental milestones were normal. Radiological examination and CT scan of the skull showed pansynostosis. X ray of the feet showed a partial duplication of the distal phalanx on both sides (fig 1C). The boy underwent neurosurgical treatment at the age of 40 months (biorbital advancement and reconstruction of the forehead and orbits). At our last clinical examination at the age of 6 years, he was in a good condition and head circumference was 52 cm (50th centile). Intellectual development was normal.

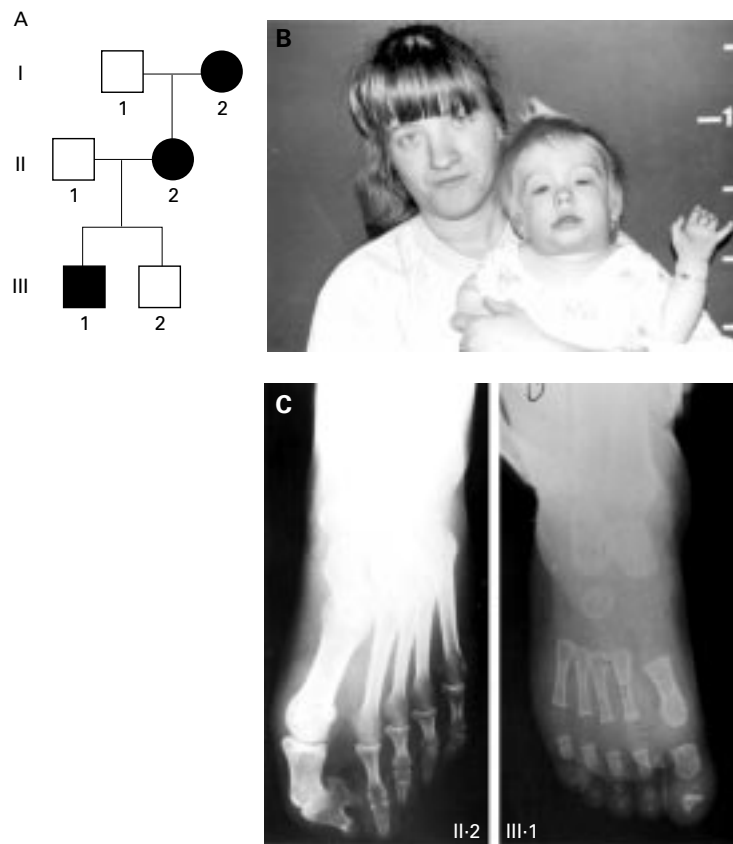


Figure 1 (A) Abbreviated pedigree of the family studied. (B) Front view of the face of the proband (III.1) and his mother (II.2). Note bilateral ptosis (II.2 and III.1) and downward slanting palpebral fissures (III.1). (C) X ray of the feet; left mother (II.2), right proband (III.1). Note duplication of the distal phalanx of the halluces.

The family history showed that the proband's mother (II.2, fig 1B) had been surgically treated for premature closure of the coronal and sagittal sutures at the age of 3

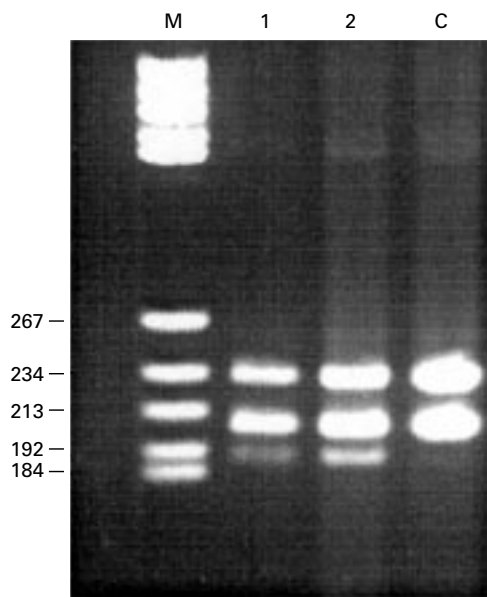


Figure 2 Confirmation of the frameshift mutation (460-461insA) by gel electrophoresis. DNA fragments generated by *Bsa*JI digestion of the PCR product representing the 3' portion of the *TWIST* gene (product size 461 bp<sup>10</sup>) (lanes 1, 2, C). M: size marker. Mother (II.2, lane 1) and son (III.1, lane 2) showing the mutant DNA fragment of 190 bp in addition to the wild type fragments (231 and 201 bp). C: unaffected subject showing the absence of mutation. DNA fragments 42 and 30 bp were not resolved by electrophoresis.

years. She had similar facial features to her son (downward slanting palpebral fissures, ptosis) and broad big toes in valgus position. The combination of craniosynostosis, facial features, and duplication of the big toes was suggestive of Robinow-Sorauf syndrome. The maternal grandmother was reported to have bilateral ptosis without other signs of the disorder. She refused to participate in molecular studies.

EDTA blood was obtained from the index patient (III.1) and his mother (II.2) and DNA extracted according to standard procedures. Primers used for the PCR amplification of the coding region of the *TWIST* gene were those described by Howard *et al*<sup>11</sup> (TW1f: 5'-GAG GCG CCC CGC TCT TCT CC-3' and TW1r: 5'-AGC TCC TCG TAA GAC TGC GGA C-3'; amplicon 378 bp) and El Ghouzzi *et al*<sup>11</sup> (TW2f: 5'-GCA AGC GCG GCA AGA AGT CT-3' and TW2r: 5'-GGG GTG CAG CGG CGC GGT C-3'; amplicon 461 bp) following their protocols. The resulting PCR products were run on a 1.2% agarose gel, excised from the gel, and the DNA was isolated with a Gel Extraction Kit (Qiagen). Isolated PCR products were sequenced in both directions using an ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin Elmer) on a model 373 automated DNA sequencer (Applied Biosystems).<sup>13</sup>

Sequence analysis of the patient's DNA showed a single base insertion (460-461 insA) localised in the second triplet of the helix II domain of the transcription factor gene *TWIST*. This frameshift mutation leads to a stop at position 864 and elongates the putative protein product by 88 amino acids. This mutation was confirmed by a restriction enzyme digestion of the 461 bp PCR product (TW2f/r), because the insertion generates a new *Bsa*JI restriction site. The normal sized fragments resulting from digestion

are 231, 201, and 30 bp (fig 2, lane C). The 231 bp fragment is split into a 190 bp and 41 bp fragment by the mutation. Both mother and son showed the wild type fragments in addition to the aberrant 190 bp DNA fragment (fig 2, lanes 1 and 2). DNA fragments 41 and 30 bp were not resolved by the electrophoretic conditions applied.

In Saethre-Chotzen syndrome, nonsense, missense, duplication, and deletion mutations have been identified in the coding region of *TWIST* in familial and isolated cases.<sup>15-17</sup> These aberrations occurred in different functional domains of the gene. Most of the known mutations are detected within the DNA binding helix I or loop domain.<sup>15-16</sup>

The mutation identified in the family reported here is caused by an insertion of a single adenosine at position 460-461 (460-461insA) which leads to a frameshift. Recently, three additional missense mutations affecting the helix II domain (460A→G, 475C→T, 481C→T) have been described.<sup>10-12-15</sup> None of these patients showed hallucal reduplication of the distal phalanx. Furthermore, in the family with Robinow-Sorauf syndrome originally described by Young and Harper,<sup>5</sup> a mutation in the coding region of *TWIST* has been described.<sup>12</sup> In another patient with a mutation in *TWIST*, a hallucal reduplication was also observed.<sup>10</sup>

In conclusion, the mutational spectrum in Saethre-Chotzen/Robinow-Sorauf syndrome does not allow phenotype-genotype correlation. It is not clear whether phenotypic expression is influenced by pleiotropy as suggested by mutant *M-twist* heterozygous mice.<sup>18</sup>

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## Unexpected Angelman syndrome molecular defect in a girl displaying clinical features of Prader-Willi syndrome

EDITOR— Prader-Willi syndrome (PWS)<sup>1</sup> and Angelman syndrome (AS)<sup>2</sup> are clinically distinct neurobehavioural disorders both resulting from altered expression of specific imprinted genes located in the 15q11q13 chromosomal region. PWS is usually caused by a deletion in the paternally inherited chromosome 15 or by maternal uniparental disomy (UPD) of chromosome 15, whereas maternal deletion or paternal UPD is responsible for AS.<sup>3-4</sup> AS patients exhibit severe mental retardation with absence of speech, frequent and inappropriate laughter, ataxic gait with raised arms, and a frequent history of seizures. Most patients have a typical face with a wide open mouth, protruding tongue, and prominent chin.<sup>5</sup> Clinical history and physical examination are different in patients with PWS, who have neonatal hypotonia almost invariably associated with poor sucking requiring nasogastric feeding, hypogonadism, short stature, mild to moderate mental retardation, and childhood onset obesity owing to hyperphagia beginning between 1 and 6 years.<sup>6</sup> However, although all these clinical characteristics are well defined,

molecular confirmation is recommended considering that other diseases share identical clinical features, for example fragile X syndrome and PWS,<sup>7-8</sup> or Rett syndrome and ATR-X syndrome and AS.<sup>9</sup> The molecular diagnosis of PWS and AS is based on the analysis of the differential parental specific DNA methylation imprint within the 15q11-q13 chromosomal region. This investigation is currently performed by Southern blotting using methyl sensitive restriction enzymes and either probe SNRPN or PW71.<sup>10-11</sup> Because most of the PWS and AS patients have a molecular defect of the same chromosomal region, a single molecular test is used for these two different diseases.

We report on a 5 year old girl born to non-consanguineous, healthy parents, whose clinical history was suggestive of PWS. After an uneventful pregnancy she was delivered by caesarean section because of fetal distress. Apgar scores were 3 at one minute and 6 at five minutes, birth weight was 3040 g, length 50 cm, and head circumference 34 cm. The neonatal period was characterised by hypotonia with feeding difficulties associated with severe gastro-oesophageal reflux. From the age of 2, she became progressively obese as a consequence of hyperphagia. CT scan was normal at 20 months. At 5 years old, physical examination was normal except for the obesity (fig 1). Her weight was 32 kg (>97th centile), length 113 cm (90th centile), head circumference 51 cm (50th centile), and formal developmental assessment showed a developmental quotient of 30 (gait DQ=36, visuomotor coordination DQ=30, socialisation DQ=25) associated with very poor

language skills. No dysmorphic features were observed and she was hyperactive. She has never had any seizures and her gait is not ataxic.

DNA methylation testing was carried out to confirm PWS. Genomic DNA was isolated from peripheral blood lymphocytes by conventional methods. Hybridisation of genomic DNA from the proband was performed using probes SNRPN and PW71B, as described previously.<sup>10 11</sup> Unexpectedly, both probes showed a single band of paternal origin and no band of maternal origin (data not shown). This pattern showed a complete lack of maternal imprinting as classically observed in AS patients. Consequently, although this patient displays clinical findings of PWS, she has the typical molecular abnormalities of an AS patient.

Analysis of 20 R banded metaphases from the proband showed a mosaic 47,XX,+mar/48,XX,+2mar karyotype, whereas the karyotypes of both parents were normal. Dual colour hybridisation with probes D15Z1 (PCR generated probe)/SNRPN (Oncor<sup>®</sup>) and D15Z1/D15S10 (Oncor<sup>®</sup>) showed no 15q11q13 microdeletion. Hybridisation of D15Z1 with the small marker chromosome (SMC) indicated that it originated from chromosome 15. Further characterisation of this SMC showed that it was a small inv dup(15) without the PWS/AS locus. In the absence of deletion and because these markers usually do not have any phenotypic consequences and may not be directly responsible for the clinical manifestations in the proband,<sup>12 13</sup> a test for uniparental disomy was performed. Using PCR based analysis, 10 microsatellites markers at the D15S542, D15S543, D15S11, D15S10, GABRB3, GABRA5, ACTC, CYP, FES, and D15S87 loci were analysed by standard semiautomatic methods on an automated ABI PRISM 377 (Perkin Elmer) using fluorescent primers. Of the 10 microsatellites tested, five were fully informative in the family. No maternal allele was observed at D15S542, GABRB3, ACTC, CYP, or D15S87 loci which appeared homozygous for all dinucleotide repeat polymorphisms (table 1). Hence, our proband has inherited two copies of one paternal chromosome 15 (isodisomy) and has a pat UPD(15), as seen in 3-4% of AS cases.<sup>14 15</sup>



Figure 1 The proband aged 5 years. (Photograph reproduced with permission.)

Table 1 Results of microsatellite analysis. Allele designations are arbitrary

CA	Proband	Father	Mother
D15S542	a/a	a/a	b/c
D15S543	b/b	b/b	a/b
D15S11	a/a	a/a	a/a
D15S10	b/b	a/b	b/b
GABRB3	b/b	b/c	a/d
GABRA5	b/b	a/b	a/b
ACTC	b/b	a/b	c/d
CYP	a/a	a/c	b/c
FES	c/c	a/c	b/c
D15S87	c/c	b/c	a/b

To our knowledge, only two phenotypically atypical AS adults and one child have been reported.<sup>16-18</sup> By contrast to our patient, they showed the expected clinical findings of AS with additional PWS features including moderate obesity. Both adults had a cytogenetic deletion of 15q11q13 and the child had a paternal UPD(15). However, our proband lacked the major signs of AS including movement or balance disorder, frequent laughter, inappropriate happiness, and seizures, and only had speech impairment and hyperactivity. The main features typical of PWS were the history of neonatal hypotonia with feeding difficulties and the occurrence of hyperphagia with resulting obesity from the age of 2 years. However, after the molecular results, an EEG was performed that showed the typical slow wave bursts, providing further evidence for a diagnosis of AS. At first sight, the presence of a UPD(15) instead of a deletion does not explain this particular clinical overlap. No striking differences have been reported in AS patients so far<sup>19</sup> (although UPD patients may have a slight increase in weight when compared to deletion patients<sup>20</sup>), and the minor phenotypic differences between UPD and deletion either in PWS or AS patients has never led to a misdiagnosis.<sup>21</sup> In a similar way, the finding of the inv dup(15) could not account for this phenomenon as such SMCs do not usually give rise to severe clinical manifestations when they do not include the PWS/AS region.<sup>22</sup> However, they are known to be frequently associated with maternal or paternal UPD through different mechanisms.<sup>23</sup> In this case the presence of isodisomy rather than heterodisomy is in favour of a postzygotic duplication of the single chromosome 15 in a 46,XX,-15,+inv dup(15) zygote.

The biological basis for this case remains to be resolved, but a simple explanation could be a coincidental association of an incomplete AS phenotype with obesity and neonatal hypotonia. Although clinical PWS and AS overlaps are infrequent events, they nevertheless underline the importance of molecular testing in the diagnosis of these syndromes.<sup>3</sup> The study of such atypical cases will probably contribute to a better understand of their physiopathology.

Drs Dupont and Cuisset contributed equally to this work.

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### Correction

In the February 1999 issue of the Journal, we regret that the paper by Hamel *et al* (pp 140-143) contained errors in two of the figures. In fig 1, the markers used in the construction of the haplotypes were omitted, and part B of fig 3 was omitted. The corrected figures are reproduced below.

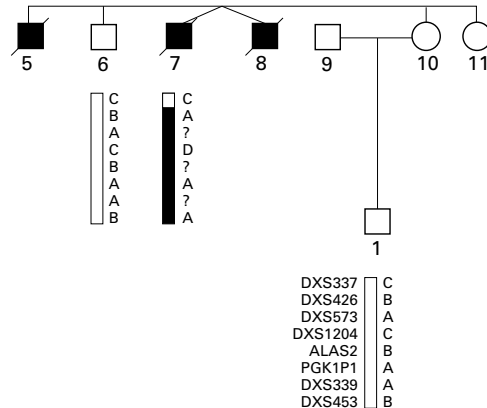


Figure 1 Part of the family pedigree showing informative markers from which the haplotypes were constructed.

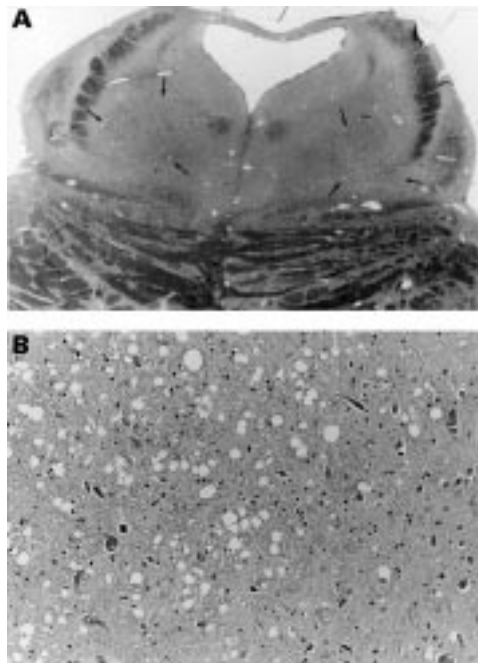


Figure 3 (A) Histological section of the dorsal part of the pons in patient IV7; note the symmetrical, spongy change in the area of the central segmental tract (see arrows). (B) Higher magnification of this tract shows pronounced microvacuolar change without gliosis or inflammatory infiltrate (combined LFB-HE staining).