Can hair be used to screen for breast cancer?

EDITOR—The use of hair as a biopsy tissue has been considered for some time. For instance, in the case of breast cancer, raised zinc levels in head hair have been reported.\(^1\) Besides, x-ray diffraction patterns of hair are rich and have attracted much attention for 70 years.\(^2\) However, its potential use as a diagnostic indicator of disease was only suggested a short time ago.\(^3\) Most recently, James et al.\(^4\) reported that x-ray diffraction of hair taken from women diagnosed with breast cancer (and those at high risk by virtue of a proven \(BRCA1/BRCA2\) mutation) showed a diffuse ring. They claimed a 100% correlation with the disease, advocating the use of pubic hair as a simple non-invasive screening method for breast cancer. The use of pubic hair was suggested in view of possible damage to the head hair from cosmetic treatments. Despite this note of caution, the study of James et al.\(^4\) was based on 12 pubic hair samples with only eight from cancer affected subjects. Here, we report a detailed double blind study from 109 women belonging to five clinically distinct groups as well as a normal population group and show that there is no correlation between the diffuse ring and breast cancer or breast cancer predisposition.

The present work was initiated in November 1998 to provide an independent double blind study on clinically well controlled samples because of our concerns with the study of James et al.\(^4\) for which a major proportion of the samples was provided by two of us (AH and DGRE). Both diffraction and x-ray fluorescence data have been obtained. Diffraction data were collected on station 2.1 with the multiwire area detector and the fluorescence data on station 7.1 of the UK Synchrotron Radiation Source (SRS). The use of a multiwire detector allowed on line alignment of hair within seconds and a large enabling efficient diffraction data collection from a large number of samples. Six groups of subjects were selected including a normal population group. For each subject, head and pubic hair were collected and reference numbers assigned in a random manner. The identities of samples were kept blinded until all data were analysed. Each woman was asked to provide information on whether she had had any hair treatment (perm, dye, etc) or was on medication. No hair treatment was reported for pubic hair and thus here only results from pubic hair (108 samples) are discussed. Results of head hair are included in table 1 for completeness. The groups consisted of 27 unaffected controls aged 26–60 years, 21 isolated cases of breast cancer (<31 years) who had been screened negative for \(BRCA1/BRCA2\) mutations, and three cases aged over 60 years not so tested. The remaining groups came from a set of 43 families with proven \(BRCA1/BRCA2\) mutations: 25 affected mutation carriers, 10 unaffected mutation carriers, and 23 unaffected close female relatives who tested negative for the known mutations in the family.

Diffraction patterns could be grouped into two categories: one characterised by a diffuse ring at 4.78 ± 0.10 nm and one with no ring present (fig 1). The occurrence of this ring is well known from x-ray patterns of both keratins and muscle and is ascribed to lipid crystals resulting from degradation processes.\(^4\) Contrary to the observation of James et al.,\(^4\) the pubic hair of only 12 of 49 (25%) women with breast cancer showed the diffuse ring. This is only slightly larger than in the normal population group, where about 20% of the pubic hair samples showed the ring. In the case of the affected group who tested positive for a \(BRCA1\) or \(BRCA2\) mutation, 56% of the pubic hair samples showed no ring and only 24% of the pubic hair samples showed the ring. Table 1 provides a detailed summary of diffraction results for all six groups. A statistical analysis for pubic hair samples was performed to determine

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**Table 1** Proportion of subjects in each of six groups whose diffraction patterns show or do not show the diffuse ring

<table>
<thead>
<tr>
<th>Pubic hair</th>
<th>Head hair</th>
</tr>
</thead>
<tbody>
<tr>
<td>(</td>
<td>N = 217</td>
</tr>
<tr>
<td>Ring* (%)</td>
<td>( 5 (19)</td>
</tr>
<tr>
<td>No ring (%)</td>
<td>( 22 (81)</td>
</tr>
</tbody>
</table>

\( N = \) control from normal population. \( U = \) unaffected (known not to have breast cancer). \( A = \) affected (known to have breast cancer). \( +/- = \) tested positive/negative for mutations in \(BRCA1\) and \(BRCA2\) genes. \( \text{nh} = \) no family history.

*Includes complete and partial ring. James et al.\(^4\) suggest that pubic hair should be used for diagnostic purposes; 50% of all head hair samples show the ring in contrast to only 27% of pubic hair samples. This difference may arise from hair treatment among other factors.

\( \dagger \)One woman supplied only head hair.
whether there was an association between the presence of the diffuse ring and breast cancer. There were 49 samples from women with breast cancer and 59 from unaffected women. A χ² value of 0.86 was obtained. For 1% significance, a value of 6.63 and for 5% significance, a value of 3.84 is required. Thus, it can be concluded that there is no measurable association between the diffuse ring and breast cancer. The trace element (Zn, Cu, Fe, and S) analysis of intact hair showed no correlation with the ring structure in the diffraction pattern or with the subjects’ group. The women in the normal population group whose hair had shown the diffuse ring were examined and shown not to have breast cancer.

Our x-ray diffraction data do not support the recent claim that hair from breast cancer patients or those at high risk (BRCA1/BRCA2 mutation carriers) show a distinct diffuse ring. This conclusion for breast cancer diagnosis was also reached on a much smaller study of head hair only. In our study, diffraction patterns from 75% (37 of 49) of the breast cancer patients do not show this ring. Moreover, the χ² test shows no association between the diffuse ring and breast cancer, and, as such, the claim that x-ray diffraction of pubic hair can be used as a screening method for breast cancer or breast cancer predisposition is invalid.

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Mutation analysis of SMAD2, SMAD3, and SMAD4 genes in hereditary non-polyposis colorectal cancer

Editor—Transforming growth factor-β (TGF-β) family members are known to be involved in the regulation of cell proliferation, differentiation, and apoptosis.1 Members of the TGF-β family include TGF-βs, activins, and bone morphogenetic proteins (BMPs). Their signals are mediated to the cell nucleus by a network of transmembrane serine/threonine kinase receptors and their downstream effectors, the SMAD proteins.2 SMAD proteins play a key role in intracellular TGF-β signalling and inactivating mutations of SMADs, such as SMAD2, SMAD3, and SMAD4, provide resistance of cells to TGF-β induced growth inhibition.

To date, eight human SMADs have been identified. Two of them, SMAD2 and SMAD4, have been reported to be mutated in a subset of colorectal carcinomas.3–6 Germline mutations of SMAD4 have been found in patients with juvenile polyposis, a condition predisposing to colorectal cancer.7–10

SMAD3 mutations have not been reported in human cancers. In a recent study by Arai et al.,11 SMAD3 mutations were analysed in 35 sporadic colorectal and 15 HNPCC cancers and no mutations were found. Targeted disruption of the SMAD3 gene in mice has been reported to lead to development of colorectal cancer,12 though other studies have not detected a clear association.13–14 No genetic alterations in other SMADs have been reported in malignancy. Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited cancer susceptibility syndrome, associated with germline mutations in five DNA mismatch repair genes: MLH1, PMS1, PMS2, MSH2, and MSH6.15–19 Inactivation of both alleles of a mismatch repair gene results in microsatellite instability (MSI) that is a hallmark of HNPCC tumours.20–25 The genes responsible for microsatellite stable (MSS) HNPCC are still unknown.

Loss of growth inhibition by TGF-β is an important step in colon tumorigenesis and in HNPCC tumours with MSI. This is mainly the result of frameshift mutations within a microsatellite repeat within the TGF-β type II receptor (TGFβRII) gene.26 It has been proposed that mutations in TGFβRII could underlie the cancer predisposition in MSS HNPCC,27 and also that other genes involved in the TGF-β pathway are candidates for MSS HNPCC.28

Chromosomal deletions are common genetic alterations in cancer and they are targeted at tumour suppressor loci.29–32 Previous studies have shown that one copy of chromosome 18q is lost in over 70% of sporadic colorectal cancers.33–35 The DCC (deleted in colorectal cancer) gene has been suggested as a candidate target gene in this region and loss of expression of DCC has also been reported in colorectal cancers.36 However, mutations in the coding region of DCC seem to be rare37 and the position of DCC as a candidate tumour suppressor is not clear. Two other candidate genes, SMAD4 and SMAD2, have recently been identified at the same 18q region38 emphasising the possible role of the SMAD genes in colorectal tumorigenesis. The aim of the present study was to investigate whether germline mutations in SMAD2, SMAD3, and SMAD4 underlie microsatellite stable HNPCC.

Mutation screening was performed in 14 Finnish HNPCC kindreds from which lymphoblastoid cell lines were available. Based on genealogical evidence the families are unrelated, though the existence of early common ancestors cannot be excluded. One affected subject per family was included in the study. Of the kindreds, six fulfilled the Amsterdam criteria for HNPCC.39 Other patients represent familial HNPCC-like colorectal cancer (CRC); the number of patients with CRC or endometrial cancer ranged from two to six per family (average three, Table 1). All kindreds selected for this study have previously been shown to be MLH1 and MSH2 mutation negative.39 In three kindreds, DNA from tumour tissue had not been available. From 10 families one and in one family two colorectal cancer samples were available and no evidence of MSI had been detected (Table 1). The study

### Table 1

<table>
<thead>
<tr>
<th>Kindred</th>
<th>No. of Subjects</th>
<th>Sample</th>
<th>MSI Status</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD4</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>No</td>
<td>MSS</td>
<td>SMAD2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>No</td>
<td>MSS</td>
<td>SMAD3</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD2</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>No</td>
<td>MSS</td>
<td>SMAD3</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>No</td>
<td>MSS</td>
<td>SMAD4</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD2</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>No</td>
<td>MSS</td>
<td>SMAD3</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD4</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>No</td>
<td>MSS</td>
<td>SMAD2</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>No</td>
<td>MSS</td>
<td>SMAD3</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>Yes</td>
<td>MSS</td>
<td>SMAD4</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>No</td>
<td>MSS</td>
<td>SMAD2</td>
</tr>
</tbody>
</table>
Each gene was divided into five fragments, covering the whole coding region of the gene. The forward (F) and reverse (R) primers and size of each PCR product are listed in table 2.

The PCR reactions were carried out in a 50 µl reaction volume including 2 µl of cDNA, 1 × PCR reaction buffer (Perkin Elmer Applied Biosystems Division), 200 µmol/l of each dNTP (Finnzymes), 0.8 µmol/l of each primer, and 2 units of AmpliTaq GOLD polymerase (PE/ABI). The MgCl₂ concentration was 1.5 mmol/l for SMAD2 fragments 1 and 2, SMAD3 fragment 3, and all SMAD4 fragments. For all other fragments the MgCl₂ concentration was 2.5 mmol/l. SMAD3 fragment 2 reaction also included 10% of DMSO. The PCR conditions are available upon request.

After PCR, 5 µl of the PCR product was run on a 3% agarose (NuSieve) gel to verify the specificity of the PCR reaction. The rest of the PCR product was purified using QIAquick PCR purification Kit (QIAGEN). Direct sequencing of the PCR products was performed using the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (PE/ABI). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FM Bioproducts) and analysed on an Applied Biosystems model 373A or 377 DNA sequencer (PE/ABI).

The screen for the presence of a base substitution in SMAD3 in controls, restriction enzyme digestion was performed. HpaI (New England BioLabs) digestion was used to detect A to G change in SMAD3 exon 3 at codon 170. New PCR primers for genomic exon 3 amplification were designed using the Primer3 server. The primers were: 5’-ATCGACACTGAGGCCACCTC (forward) and 5’-CCACCGTGCTCCTCCTC (reverse). The PCR reactions were carried out in a 50 µl reaction volume including 100 ng genomic DNA, 1 × PCR reaction buffer (PE/ABI), 200 µmol/l of each dNTP (Finnzymes), 0.8 µmol/l of each primer, and 2 units of AmpliTaq GOLD polymerase (PE/ABI), and 1.5 mmol/l of MgCl₂. The following PCR cycles were used for amplification: 10 minutes at 95°C, 40 cycles of 45 seconds at 95°C, 45 seconds at 56°C, one minute at 72°C, and final extension for 10 minutes at 72°C. HpaI cuts the PCR fragment (187 bp) that contains the substitution into two fragments (134 bp and 53 bp in size) whereas the wild type fragment lacks the restriction site and is not digested. The digestion was performed in 1 × NEBuffer (New England BioLabs) at 37°C overnight. After digestion, the PCR products were electrophoresed through a 3% agarose gel.

In this work we analysed SMAD2, SMAD3, and SMAD4 mutations in 14 familial colon cancer kindreds, 11 of these displaying at least one MSS tumour. The microsatellite analysis data derived typically from one single tumour per family suggest that these kindreds do not segregate DNA mismatch repair gene mutations, but does not exclude this possibility. Previous studies had evaluated MLH1 and
SMAD2, SMAD3, or SMAD4 mutations were not found in any of our patients using a cDNA based mutation analysis. It should be noted that like all other mutation detection methods, this method may miss a subset of mutations. Also, the potential existence of founder mutations in the Finnish population may have hampered our efforts to detect SMAD gene defects in HNPCC. However, it is likely that defects of SMAD2, SMAD3, or SMAD4, or other genes, may be too uncommon to unravel the molecular background of MSS HNPCC.

We thank Siv Lindroos, Liisa Sukki, and Paivi Laibo for technical assistance. This study was supported by grants from the Finnish Cancer Society, the Academy of Finland, Emil Aaltonen Foundation, Finnish Cultural Foundation, Sigrid Juselius Foundation, and Biocentre Helsinki.

References


Novel mutation in the MYOC gene in primary open angle glaucoma patients

Editor—Glaucoma is the world’s leading cause of irreversible blindness and is characterised by progressive optic disc cupping with corresponding visual field loss. Both intraocular pressure (IOP) and positive family history are risk factors for the development of the disease. Juvenile open angle glaucoma (JOAG) is a subtype of open angle glaucoma characterised by an early onset (10 to 35 years of age) and autosomal dominant inheritance with high penetrance, a characteristic which has led several authors to investigate affected families in an attempt to identify a gene or genes associated with this condition. With the use of genetic linkage analysis in families with JOAG, a genetic locus (GLC1A) was recognised on chromosome 1q21–q31. The gene associated with GLC1A has been identified and it codifies a 57 kDa protein named trabecular meshwork induced glucocorticoid response protein (TIGR), also known as myocilin (MYOC). The MYOC gene is composed of three exons of 604, 126, and 785 bp, respectively. During screening for mutations in the MYOC gene in 25 unrelated Brazilian patients with JOAG, an unreported mutation (Cys433Arg) was detected, present in seven of them.

Patients were followed at the Glaucoma Service of the State University of Campinas, Brazil. They underwent an ocular examination, including gonioscopy by Posner lens, application of 1% tropicamide, slit lamp biomicroscopy, optic nerve evaluation, and automated perimetry (Humphrey 630, program 30-2). JOAG was defined as the presence of characteristic bilateral optic nerve damage and visual field loss in the presence of an open angle in subjects younger than 36 years of age. Each patient included in this study came from different families according to interview data. The study was approved by the Ethics Committee of the State University of Campinas at the time of the ocular examination, the mean age of JOAG patients was 25.52 years (SD 6.99), ranging from 10 to 35 years, and the mean IOP was 29.96 mm Hg (SD 13.00). Thirteen patients (52%) were male and 12 (48%) were female; 13 (52%) were white, 11 (44%) were black, and one (4%) was Asian. Some of the patients (48%) were female; 13 (52%) were white, 11 (44%) were black, and one (4%) was Asian. Six patients (85%) of those with the mutation had a positive family history who had the mutation had the family investigated for its presence. Nine subjects were studied and four harboured the Cys433Arg mutation (fig 3). Three of them had glaucoma and one had ocular hypertension without optic nerve or visual field damage. The other members who did not have the disease were not affected with the Cys433Arg mutation.

One of the patients who showed the Cys433Arg mutation had the family investigated for its presence. Nine subjects were studied and four harboured the Cys433Arg mutation (fig 3). Three of them had glaucoma and one had ocular hypertension without optic nerve or visual field damage. The other members who did not have the disease were not affected with the Cys433Arg mutation. In order to discriminate between a founder effect and a de novo recurrence, haplotype analysis was performed in the six patients with a positive family history who had the new mutation and in one family using four microsatellite markers, mapped at band 1q21-25, closely linked to the GLC1A locus, D1S210, D1S2790, D1S1619, and NGA19. Polymerase chain reaction (PCR) was carried out following standard procedures and primer sequences were obtained from the Genome Data Bank. For allele scoring, PCR products were size fractionated on a 6% polyacrylamide-urea gel and autoradiographed. The analysis of four microsatellite markers showed that the Cys433Arg mutation is associated with a common haplotype, suggesting that these patients inherited their mutation from a common ancestor. A pedigree of the family analysed can be seen in fig 3, depicting a potential disease haplotype.
Figure 2. Direct sequencing of the PCR product showing a T-C substitution at codon 433 of the MYOC gene, which changes the amino acid Cys (TGT) to Arg (CGT). The arrow indicates the exact location of the mutation.

Figure 3. Pedigree of a JOAG patient’s family (the proband is indicated with an arrow). Solid symbols indicate affected subjects, all harbouring the Cys433Arg mutation. II.3, indicated with a dotted symbol, has not developed glaucoma, but has very high IOP levels and also shows the Cys433Arg mutation. Four microsatellite markers, located in the vicinity of the GLC1A locus, were used for haplotype analysis. The pedigree shows the genotypes at polymorphic microsatellite loci as well as a potential disease haplotype shown in the boxes.
Three novel SALL1 mutations extend the mutational spectrum in Townes-Brocks syndrome

EDITOR—Townes-Brocks syndrome (TBS, MIM 107480) was first described by Townes and Brocks in 1972 as an association of imperforate anus, supernumerary thumbs, malformed ears, preauricular tags, and sensorineural hearing loss. Several additional familial as well as isolated cases have been reported. TBS is caused by mutations of the putative zinc finger transcription factor gene SALL1. All SALL1 mutations identified to date in TBS patients are located 5' of the first double zinc finger encoding region.

Three of these are nonsense mutations at two different positions. The mutation 826C>T was found in three unrelated sporadic cases, and at position 1115 one patient carried an adenine (1115C>A) and another a guanine (1115C>G) instead of a cytosine. All seven other reported mutations are short frameshift deletions of 1, 2, 7, or 10 base pairs.
SALL1 encodes four double zinc finger domains which are characteristically distributed over the entire protein. All known mutations have been predicted, if the mutated transcripts are indeed translated, to result in prematurely truncated proteins lacking all double zinc finger domains presumed to be essential for SALL1 gene function. Since no mutations were found 3' of the most 5' located double zinc finger encoding region, it was assumed that only those mutations which remove all double zinc fingers could cause TBS, whereas mutations located further 3' in SALL1 could result in a different phenotype or no abnormal phenotype at all. Here we describe three novel mutations in three independent families illustrating that truncating mutations positioned further 3' of the previously described hotspot region in SALL1 also result in TBS.

All subjects available for investigations were examined for SALL1 mutations after giving informed consent, and all those available for investigations were clinically examined by a clinical geneticist. In all affected subjects chromosomal analysis before DNA studies had shown a normal karyotype.

In the first family (family 1), TBS occurred in a sporadic case. The male patient (patient 1 of this study), aged 44, came to the clinic because of sudden loss of visual acuity owing to optic neuropathy. Townes-Brocks syndrome was diagnosed because he showed bilateral dysplastic ears, bilateral triphalangeal thumbs, and congenital sensorineural deafness. As a child, he had undergone surgery for imperforate anus, and his feet were previously operated on because of toe malposition. X rays showed bilateral partial proximal synostosis of metatarsals IV-V as well as fusion of some tarsal bones (not shown). Unusual features of this patient include acute bilateral optic neuropathy as well as bilateral inguinal hernias and an umbilical hernia. The family history was negative for TBS. His parents were not available for investigation.

In the second family (family 2), TBS was diagnosed in the female index patient (patient 2 of this study) because of a bifid thumb on the left and preaxial polydactyly on the right side, bilateral, small, dysplastic ears, bilateral moderate to severe conductive hearing loss, bilateral renal hypoplasia, acquired hypothyroidism, anteriorly placed stenotic anus, and hypoplastic third toes with fifth toe clinodactyly. Recently, her mother gave birth to a boy who is also affected. He shows a bifid thumb on the right and complete preaxial polydactyly on the left side. He has bilateral cup shaped microtia with preauricular dimples and is reported not to respond to loud noises. The suspected hearing loss has not yet been fully evaluated. The baby boy has no anal malformation but a prominent midline perineal raphe. Congenital hypothyroidism was also diagnosed. The kidneys were normal on ultrasound. No other abnormalities are reported in these children. Both parents are clinically normal except for an anteriorly placed anus seen in the mother. The mother's family history is unremarkable. Her husband has a great aunt with unilateral preaxial polydactyly whose grandson also has preaxial polydactyly. The aunt's niece had a child with hemifacial microsomia. The family history was negative for TBS. His parents were not available for investigation.

In the third family, at least five persons in three generations are affected (fig 1A). The male index patient (III.2, patient 3) had imperforate anus with a prominent perineal raphe, bilateral cryptorchidism, prominent midline perineal raphe, and at least four independent clones were sequenced using vector specific primers. The kidneys were normal on ultrasound. No other abnormalities are reported in these children. Both parents are clinically normal except for an anteriorly placed anus seen in the mother. The mother's family history is unremarkable. Her husband has a great aunt with unilateral preaxial polydactyly whose grandson also has preaxial polydactyly. The aunt's niece had a child with hemifacial microsomia. The parents of the index patient are not consanguineous.

In the third family, at least five persons in three generations are affected (fig 1A). The male index patient (III.2, patient 3) had imperforate anus with a prominent perineal median raphe, bilateral cutaneous syndactyly of the third and fourth toes, bilateral hypoplastic second toes,lop ears, frontal bossing, retrognathia, glandular hypoplasia, and bilateral hypoplastic and dysplastic kidneys. Echocardiography showed a secundum type ASD. A mixed hearing loss was diagnosed at 1 year of age, assessed at the age of 2 as 50–60 dB (over the whole tone range). During childhood the boy showed considerable growth retardation (length and weight <3rd centile), probably secondary to chronic renal insufficiency. At the age of 6 haemodialysis was begun because of severe renal insufficiency. Intelligence is normal. His grandmother (I.1, patient 4) had late onset hearing loss first noticed at the age of 45. At 70 years of age, she had a hearing loss of 60–65 dB. Clinical examination showed lob ears with downfolded scapha helix and hypoplastic antihelix and insufficient rotation of both thumbs on opposition. Anamnestically, there were no anal abnormalities. She had never had any kidney complaints, but her kidneys were not specifically checked by ultrasound or biochemically. Her daughter (II.2, patient 5) showed sensorineural hearing loss (audiometry aged 37: mild perceptive hearing loss, 35 dB over the whole tone range). Her ears were operated on during adolescence ("bat ears"). Her anus was normal on physical examination, and her kidneys were normal on ultrasound. No further abnormalities were seen in I.1 or II.2. No clinical data are available on the brother of II.2 (II.3). He was reported by his spouse to have "strange ears" but he declined to be examined. However, he has two affected children. His son (III.4) had imperforate anus, "bat ears" with downfolded and hypoplastic margin of the scapha helix, perceptive hearing loss of 20 dB on audiometry at the age of 6, and showed insufficient rotation of the thumbs on opposition. His sister (III.3) had imperforate anus with a prominent perineal raphe,lop ears with downfolded upper margin of the scapha helix, and insufficient rotation of the thumbs on opposition.

Mutation analysis of SALL1 was performed by PCR amplification of all exons from genomic DNA (prepared from peripheral lymphocytes) followed by direct sequencing of PCR products as described elsewhere. Permanent lymphoblastoid cell lines were prepared as previously described. Cells were harvested by centrifugation and total RNA was isolated using Total RNA Reagent (Biomol) according to the manufacturer's instructions. Two µg of total RNA from each stage was reverse transcribed using Ready-To-Go"(W) You-Prime First Strand Beads (Amer sham Pharmacia) and 20 pmol of primer TR5.5 (5'GGCCACCATAGGTCGCATTC3'), and 1 µl of each first strand reaction was used as a template in a subsequent PCR reaction (conditions: 95°C for four minutes initial denaturation; 35 cycles of 94°C for one minute, 72°C for one minute, 72°C for two minutes (final extension) with primers TR4.2 (5'TGGATTTTGACATCTAGTGCACCGC3') and TR5.8 (5'TGAACAGGAAATGGAATGCTATGTC3'). A nested amplification was carried out using primers TF4.3 (5'GACACCCCCACACTCATGTCACCGC3') and TR5.5 (5'TGAACACGTGCTATGTCACCG3'). Conditions were: 95°C for four minutes initial denaturation; 35 cycles of 94°C for one minute, 64°C for one minute, 72°C for one minute; 72°C for two minutes (final extension) with primers TR4.2 (5'TGGATTTTGACATCTAGTGCACCGC3') and TR5.8 (5'TGAACAGGAAATGGAATGCTATGTC3'). A nested amplification was carried out using primers TF4.3 (5'GACACCCCCACACTCATGTCACCGC3') and TR5.5 (5'TGAACACGTGCTATGTCACCG3'). Conditions were: 95°C for four minutes initial denaturation; 35 cycles of 94°C for one minute, 64°C for one minute, 72°C for 45 seconds; 72°C for two minutes final extension. PCRs were performed on Primus 25 thermal cyclers (MWG). Amplification products were visualised on agarose gels, gel extracted, and DNA sequences of amplification products were verified by direct sequencing using primers TF4.3 and TR5.7. PCR products were also subcloned in pGEM-Teasy (Promega) and at least four independent clones were sequenced using vector specific primers.
II.1, II.2 (patient 5, fig 1A), III.1, and III.2 (patient 3, fig 1A). No mutation was found in the whole SALL1 coding region. However, within the intron 2 sequence, a heterozygous transition IVS2-19T>A (fig 1B) was found in III.2, and subsequently in I.1 and II.2, but not in the unaffected family members. This mutation was predicted to create an aberrant splice acceptor site. By comparing the surrounding sequences of the aberrant and the normal intron 2 splice acceptor site to consensus sequences, it was estimated that the aberrant splice site was as likely to be functional as the normal site. The mutation was excluded in 200 control alleles.

In order to test if the mutation indeed created a functional splice site, lymphoblastoid cell lines of patients II.2 and III.2 were examined by RT-PCR. In both patients, direct sequencing of RT-PCR products showed an identical pattern of two different overlapping sequences indicating that both the mutated and the normal transcript were present in similar amounts. Sequencing of the subcloned RT-PCR fragments showed the wild type allele (fig 1C) and
a mutated allele (fig 1D) carrying an insertion of 17 bp derived from intron 2 sequence between the aberrant and the normal splice acceptor sites. It is placed within the coding region for the most carboxy-terminal double zinc finger between exon 2 and exon 3 sequences (fig 1B-D fig 3). The frameshift resulting from the insertion is further predicted to cause premature termination of the SALL1 protein (1208 amino acids instead of 1324 in the wild type).

In addition to the mutations reported here, the following polymorphisms were detected in SALL1: IVS1+119G>A, IVS1+118C>G, IVS1+36delAC (all intron 1), 2574T>C, 3456C>T (both exon 2), IVS2-31delCT (intron 2), 3872A>G (N1291S), and 3915C>T (exon 3). The intronic polymorphisms occurred in more than 10% of all subjects (a deleted and una deleted) analysed for SALL1 mutations by our group. The exonic mutation N1291S is thought to be silent since it was found in two una deleted persons. The other exonic variations do not affect translation and are not segregating with the phenotype. Since the exonic sequence variations have only rarely been found, it is as yet unclear if they represent true polymorphisms or rare sequence variants.

All SALL1 mutations previously reported in TBS reside in exon 2, 5' of the coding region for the first double zinc finger domain (fig 3), and are predicted to result in SALL1 haploinsufficiency. Nonsense mutations seem to occur less frequently than small deletions, and both known nonsense mutations were found independently in two and three families indicating the existence of two hotspots at nucleotides 826 (mutated in three families) and 1115 (mutated in two families). In contrast, SALL1 small deletions as a group occur more often but they seem to represent private mutations only. 840delC is yet another short deletion located 5' of the coding region for the first double zinc finger domain (fig 3).

The phenotype of the patient carrying this mutation is typical for TBS, that is, he shows anal, ear, and thumb malformations. Interestingly, this man also shows acute optic neuropathy. While this has not been reported so far in TBS, this might represent another rare feature of the syndrome.

The two other mutations described in this report are the first SALL1 mutations located 3' of the region where all previously known mutations cluster. The 1509C>A mutation is neighbouring the 3' end of the double zinc finger 1 coding region (fig 3). While this mutation could result in a prematurely terminated SALL1 protein lacking double zinc finger domains 2-4, it is still unclear if the mutated transcript remains stable and the corresponding protein is indeed expressed. Therefore, this mutation could well result in haploinsufficiency if the mutated transcript is readily degraded. This mutation is the third nonsense mutation detected so far in SALL1, and it is the first one to be found in a family with dominant transmission since all other nonsense mutations known so far have been detected as de novo mutations in patients with severe features of TBS. Interestingly, one of the parents carries the mutation in the germline but not in lymphocytes. Yet the parental origin of the mutation needs to be determined in order to explain if the anteriorly placed anus seen in the mother could also reflect the presence of the mutation in other tissues. However, it is clear that the preaxial polydactyly reported in the father’s family cannot be related to the mutation which caused TBS in his children.

The most interesting mutation shown here is IVS2-19T>A (fig 1B-D). We were able to show that the predicted transcript resulting from aberrant splicing is indeed expressed. While quantitative PCR has not been performed, direct sequencing of the RT-PCR products indicates that the aberrant transcript is as abundant as the normal one. It seems therefore that splicing of the mutated
primary transcript occurs preferentially if not completely at the aberrant splice site. The predicted protein encoded by the mutated transcript contains intact coding sequences for all double zinc finger domains except for the double zinc finger 4 in which the carboxy-terminal finger motif is interrupted. How this mutation might lead to the phenotype remains to be elucidated. It has previously been shown in the Drosophila transcription factor Krüppel that a missense mutation replacing one of the conserved cysteine residues within the second of five tandemly arranged finger motifs results in a null allele. Therefore, the splice mutation reported here is likely to result in loss of biological function of the most carboxy-terminal double zinc finger domain. It remains unclear if this is sufficient to result in SALL1 haploinsufficiency causing TBS. The predicted mutant protein is 116 amino acids shorter than the wild type protein and contains different carboxy-terminal amino acids because of the frameshift. An alternative explanation for the effect of the mutation could therefore be that the changed three dimensional structure of the mutated protein results in a non-functional SALL1 protein which is unstable or not able to bind to its target sequences.

The phenotype of the severely affected family members reported here is not significantly different from other TBS cases in which classical truncating mutations were found. Therefore, we assume that all mutations shown in this report will lead to haploinsufficiency for SALL1, as suggested to be the common result of all mutations previously reported.1

Data access: GenBank: http://www.ncbi.nlm.nih.gov/. Accession numbers: Y18264 (SALL1 exon 1 and intron 1 genomic sequence (partial)), Y18265 (full SALL1 coding sequence), X98833 (SALL1 genomic sequence of intron 1 (partial), exons 2 and 3 and intron 2). Mutation accession numbers (Human Genomics Online Mutation Data Submission): H71415 (840delC), H71417 (IVS2- 19VTA), Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/OMIM (for Townes-Brocks syndrome, OMIM 107480). We thank all the patients and their families participating in this study for their cooperation and patience. We especially thank Gudrun Essers for EBV transformation, Marnike Hausmann for technical assistance, and Susanne Hirth, Sabine Ruth, and René Heise for DNA preparation and sequencing. This work was funded by the Wilhelm-Sander Stiftung (grant No 98.075.1 to JK). The first two authors contributed equally to this work.

Genotype-phenotype correlation in three homozygotes and nine compound heterozygotes for the cystic fibrosis mutation 2183AA→G shows a severe phenotype

EDITOR—Cystic fibrosis (CF) is the most common lethal childhood disorder in white populations and occurs at a frequency of about 1/2500 with regional variations. Over 1000 mutations in the CF transmembrane conductance regulator (CFTR) gene accounting for the disease have been identified so far and the most common gene mutation is ΔF508. The frameshift mutation 2183AA→G in exon 13 was first described in three Canadian CF patients1 and later was shown to have a significant frequency in patients from mid and southern Europe. The frequency among CF patients is 9.3% in north east Italy,2 2.4% in the Tyrol,3 1.2-1.5% in Belgium,4 1.8% in Greece,5 1% in Bavaria, Bulgaria, and France,6 and 0.4% in mid and northern Germany.7 We identified three homozygotes among 120 Turkish patients (2.5%), two born to first cousin parents, three compound heterozygotes among 185 Bulgarian patients (0.8%), and seven compound heterozygotes among 650 Spanish patients (0.5%). The mutation was detected by denaturing gradient gel electrophoresis or single strand conformational analysis followed by DNA sequence analysis.

We report here the genotype-phenotype correlation in 12 patients with CF with the mutation 2183AA→G (three homozygous and nine compound heterozygous for 2183AA→G and other mutations). The anamnestic, clinical, and laboratory data are summarised in table 1. Pancreatic insufficiency (PI) was assessed by the fat content of stools and requirement of pancreatic enzyme replacement therapy. Gastrointestinal symptoms (GI) are abdominal cramps and pain and frequent passage of foul and fatty faeces. The presence of pulmonary symptoms was defined as having at least one of the following clinical findings: increased rate of breathing, wheezing, dark coloured/profuse sputum, and recurrent attacks of coughing. Dehydration includes at least one of the following: decreased skin turgor and turgor, decreased output of urine, and sudden weight loss. The presence of bronchiectasis was evaluated by chest x rays and thin section computerised chest tomography.

Patient 1 was homozygous for 2183AA→G. She was admitted to hospital at 2 months of age and died within a week. Clinical findings were clearly of CF with pancreatic insufficiency. The second homozygous patient (patient 2) was examined for CF because all his four sibs had died of the disease before the age of 1 year. He had pulmonary insufficiency at 15 days. He had fatty and foul stools, bronchial hyperactivity, and early Pseudomonas colonisation. As a result of medical treatment, he no longer has steatorrhoea.
Table 1: Anamnestic, clinical, and laboratory data for CF patients carrying the mutation 2183AA-G

<table>
<thead>
<tr>
<th>Patient No</th>
<th>First CF Mutation</th>
<th>Second CF Mutation</th>
<th>Sex</th>
<th>Nationality</th>
<th>Onset of Symptoms</th>
<th>Meconium Ileus</th>
<th>Disease Progression</th>
<th>Data at Diagnosis</th>
<th>Data at Latest Visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>F</td>
<td>Turkish</td>
<td>25 d</td>
<td>No</td>
<td>Died</td>
<td>99</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Turkish</td>
<td>15 d</td>
<td>No</td>
<td>Died</td>
<td>80</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Turkish</td>
<td>2 mth</td>
<td>No</td>
<td>Severe</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>F</td>
<td>Bulgarian</td>
<td>At birth</td>
<td>Yes</td>
<td>Died</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Spanish</td>
<td>1 mth</td>
<td>Yes</td>
<td>Severe</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Spanish</td>
<td>At birth</td>
<td>Yes</td>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Spanish</td>
<td>3 mth</td>
<td>Yes</td>
<td>Severe</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Spanish</td>
<td>2 mth</td>
<td>Yes</td>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>F</td>
<td>Spanish</td>
<td>3 mth</td>
<td>Yes</td>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>F</td>
<td>Bulgarian</td>
<td>2 mth</td>
<td>No</td>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Bulgarian</td>
<td>At birth</td>
<td>Yes</td>
<td>Severe</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>2183AA-G</td>
<td>2183AA-G</td>
<td>M</td>
<td>Bulgarian</td>
<td>12 y</td>
<td>Yes</td>
<td>Died</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note: ND = not determined.
of these patients carry a third CF mutation. Spanish and Turkish patients were analysed for the IVS8-6(T) alleles and the mutation 2183AA→G was found to be associated with the allele 7T, except in patients 1 and 3, who were homozygous for the allele 9T. Spanish and Turkish patients were also studied for the microsatellite loci IVS8CA, IVS17bTA, and IVS17bCA. 12–14 While all six Spanish patients shared the same haplotype (16-31-13) for the mutation 2183AAA→G, Turkish patients were homozygous for two other haplotypes, 16-31-13 (patients 1 and 3) and 16-32-13 (patient 2). These three haplotypes are among the most common on normal chromosomes 15 and each can be derived from any of the other two. Alternatively, the mutation may have arisen independently in the two populations or even within the Turkish population. Despite the possible heterogeneous genetic background observed, in particular between the homozygous patients, the severity of the disease is similar.

The authors thank the European Community Concerted Action for the Coordination of Cystic Fibrosis Research and Therapy for providing the DGGE and sequencing oligonucleotide primers for the analysis of Spanish and Turkish patients. The work on Spanish patients was supported by FISS 96/2005 and Institucio Catalana de la Salut. The work on Turkish patients was supported by the Scientific and Technical Research Council of Turkey, The Academy of Sciences of Turkey, and Bogaçi University Research Fund. The work on Turkish patients was supported by the European Community Concerted Action for the Coordination of Cystic Fibrosis Research and Therapy. The authors thank the European Community Concerted Action for the Coordination of Cystic Fibrosis Research and Therapy for providing the DGGE and sequencing oligonucleotide primers for the analysis of Spanish and Turkish patients. The work on Spanish patients was supported by FISS 96/2005 and Institucio Catalana de la Salut. The work on Turkish patients was supported by the Scientific and Technical Research Council of Turkey, The Academy of Sciences of Turkey, and Bogaçi University Research Fund.

**Alkaptonuria in Italy: polymorphic haplotype background, mutational profile, and description of four novel mutations in the homogentisate 1,2-dioxygenase gene**

**Editor—Alkaptonuria (AKU, OMIM 203500) is a rare disorder caused by the deficiency of homogentisate 1,2 dioxygenase (HGO, EC 1.13.11.5).** HGO catalyses the conversion of homogentisate (HGA) to maleylacetoacetate in the phenylalanine/tyrosine catabolic pathway. As a consequence, affected subjects excrete HGA in their urine, which becomes dark upon exposure to air. The medical interest in this condition stems from its association with ochronosis, or the deposition of a brownish pigment in connective tissues including cartilage, where its accumulation can produce a debilitating degenerative joint disease. AKU occupies a unique place in the history of human genetics because it was the first disorder to be described as a Mendelian recessive trait. Recent advances in the understanding of the molecular basis of AKU have verified that loss of function mutations in the HGO gene are responsible for the disease. A few mutations have been repeatedly detected in patients from different European countries. Since these mutations segregated with specific haplotypes, they should be considered to be old mutations that have spread throughout western Europe with migration. However, allelic heterogeneity is the main feature emerging from the above and other studies. Most HGO mutations were found in just one family and did not involve CpG dinucleotides. Rather, a preferential occurrence in the CCC sequence motif and its inverted complement GG has recently been reported. Furthermore, some AKU chromosomes escaped mutation detection within the HGO coding region, suggesting the existence of HGO alleles whose defect might be related to gene expression. To determine the extent of allelic heterogeneity in Italian patients, we started a systematic search of AKU families through announcements at relevant National Congresses. We present here the results leading to the identification of four novel mutations. Our data should facilitate future mutation screening in Italian AKU patients and carrier identification by DNA typing. Ten affected subjects from five unrelated Italian AKU pedigrees were included in the study. In three families it turned out that the patients’ parents were consanguineous. Three patients were diagnosed at birth as having AKU through analysis of homogentisic aciduria. The remaining

1 Cystic Fibrosis Genetic Analysis Consortium. www.genet.sickkids.on.ca
5 Tzitos M, Kavanakis E, Antoniadi T, et al. Characterization of more than 85% of cystic fibrosis alleles in the Greek population, including five novel mutations. Hum Genet 1997;99:121-5.
6 Dörk T, Mekus F, Schmidt K, et al. Detection of more than 50 different CFTR mutations in a large group of German cystic fibrosis patients. Hum Genet 1994;94:533-42.

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Ten affected subjects from five unrelated Italian AKU pedigrees were included in the study. In three families it turned out that the patients’ parents were consanguineous. Three patients were diagnosed at birth as having AKU through analysis of homogentisic aciduria. The remaining
cases were diagnosed in adulthood on the basis of clinical and radiological examination. Seventeen normal relatives were also investigated. Both genomic DNA and HGO cDNA were obtained from peripheral blood by standard methods.

RNA was extracted from peripheral blood lymphocytes by the guanidinium thiocyanate-phenol-chloroform method.

cDNA synthesis was performed using both oligo dT and specific primer mapping in the 3'UTR of the HGO gene. From the complete sequence of a human HGO cDNA (AF045167), primers were designed to obtain overlapping amplicons spanning the entire coding sequence of cDNA. PCR products were heat denatured and subjected to electrophoresis on a non-denaturing 6% polyacrylamide gel and the alleles were non-isotopic detected. The PCR products were run on denaturing 6-8% polyacrylamide gels and the alleles were analysed by PCR with modifications to comply with the genomic level by SSCP screening of each exon amplified from genomic DNA. Five additional unrelated AKU families were analysed for mutations and polymorphisms in the HGO gene. The IVS9-56G→A and IVS9-17G→A HGO mutations in one Italian patient have already been described. Therefore, the mutations found in 12 AKU chromosomes of Italian ancestry are presented here (table 2). Since consanguineous marriage occurred in three families, only nine chromosomes may be considered to be independent in origin. In fact, patients from these families were homozygous for the AKU mutations G152fs (c621insG), G270R (c975G→A), and G360R (c1245G→C), respectively. The AKU patients in another family were compound heterozygotes for K248R (c909A→G) and IVS7+5G→A (c636+5G→A).

Finally, in family VRN, the AKU patient is most likely a compound heterozygote for G360R and an as yet unknown HGO mutation. Therefore, as many as eight different HGO mutations were found, four of which were novel. We also anticipate that the AKU mutation that remains to be identified will be novel (table 2) because we know that it is different from all previously characterised AKU mutations. We provisionally denoted this mutation as VRN. Three of the four novel mutations (K248R, G270R, and G360R) are missense mutations that affect HGO amino acid residues that are conserved in different species and are likely to be loss of function mutations. K248R is the consequence of an A to G transition at position c909 in exon 10, G270R results from a G to A transition at position c975 in exon 11, and G360R is caused by a G to C transversion at position c1245 in exon 13. This latter mutation was found twice in two unrelated patients. The other novel AKU mutation (IVS7+5G→A) is a G to A transition in the fifth nucleotide position of the donor (5') splice site sequence of intron 7, which most likely causes aberrant splicing of HGO. None of these four novel AKU mutations were observed, using SSCP screening, in a sample of 100 control chromosomes. Finally, the mutation G152fs originated from a one base insertion at position c621, which determined a frameshift eventually leading to premature arrest of the protein synthesis. This same mutation was described in two Slovak families. Although the loss of function nature of all these HGO mutations was not formally proven, SSCP and sequence analysis of the relevant DNA fragment in family members confirmed in all cases that the pattern of inheritance of AKU was compatible with the segregation of the HGO mutations.

### Table 2: HGO mutations and haplotypes identified in Italian AKU patients

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Type</th>
<th>Nucleotide change*</th>
<th>Associated polymorphism</th>
<th>No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G152fs</td>
<td>Frameshift</td>
<td>c621insG</td>
<td>T T 191 161 C T 187</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>IVS9-56G→A</td>
<td>Intron change</td>
<td>c836+5G→A</td>
<td>T A 199 161 C T 187</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>IVS9-17G→A</td>
<td>Intron change</td>
<td>c817-16G→A</td>
<td>T A 195 161 C T 181</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>K248R</td>
<td>Missense</td>
<td>c909A→G</td>
<td>A T 189 161 C T 191</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>G270R</td>
<td>Missense</td>
<td>c975G→A</td>
<td>A T 195 161 C T 187</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>G360R</td>
<td>Missense</td>
<td>c1245G→C</td>
<td>A A 193 161 C T 197</td>
<td>2</td>
<td>This study</td>
</tr>
<tr>
<td>VRN</td>
<td>Unknown</td>
<td>Unknown</td>
<td>A A 193 163 C A 189</td>
<td>1</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Positions of nucleotide changes are from the transcription start site (AF045167).
†The alphanumeric strings represent the haplotypes formed by the following HGO intragenic markers: IVS2+35T/A, c407T/A, HGO-3, HGO-1, IVS5+25T/C, IVS6+46C/A, HGO-2.
We managed to perform SSCP analysis of cDNA fragments from two families, one where the G360R mutation was segregating and the other where the patients were compound heterozygotes for the K248R and the IVS7+5G→A mutations. It is interesting to note that this latter supposedly splice site mutation did not affect the SSCP pattern of the cDNA amplicon defined by primer pair HS6/H53F spanning exons 7 to 9. Furthermore, IVS7+5G→A seemed to have no influence on HGO mRNA stability, as judged by the presence of both wild type and mutant bands in the cDNA amplicon defined by primer pair HS8/H52 containing the K248R site.

In the light of the recent report of a preferential occurrence of HGO mutations in the CCC/GGG sequence motif, we analysed the sequence context of the four novel mutations found in the present work. It could not be coincidental that G270R and G360R mutations take place in tri- and penta-G runs, respectively. Moreover, the G152fs mutation previously described in two Slovak families occurred in a tetra-G run. Whether the G152fs mutation we found in an Italian family has an independent origin remains to be determined. Haplotyping of the Slovak pedigrees as well as comparison with the Italian one could provide strong evidence that the CCC/GGG motif is a mutational hot spot in HGO.

Table 2 also shows the haplotypes at the HGO intragenic markers IVS2+35T/A, c407T/A, HGO-3, HGO-1, IVS5+25T/C, IVS6+46C/A and HGO-2 which are associated with each AKU chromosome. Analysis of the HGO haplotypes harbouring the purported causative AKU mutations showed that the three intronic mutations were found in a common haplotypic background composed of the very same alleles at four SNPs as well as at HGO-1. Beltrán-Valeró de Bernabé et al referred to this as haplotype A, the most frequent in European populations. Two other mutations, G152fs and G270R, were detected in a gametic association, haplotype D, which is derived from haplotype A by variation at the SNP IVS2+35T/A. Closely related to haplotype A is also that derived from haplotype A by variation at the SNP c407T/A and IVS6+46C/A, respectively. On the other hand, the mutation VRN was probably within the so-called haplotype E. It has been postulated that haplotype E has a North African origin, an ethnic component that is known to have contributed to the modern Italian population. Whether the VRN mutation has originated in Italy or has been introduced into this country with the different migrations is at present unknown.

Only two of the AKU mutations found in Italians (G152fs and IVS9-56G→A) have been encountered in patients from other European countries. One of them, as indicated before, is the G152fs mutation that was identified in two Slovak pedigrees. It would be interesting to determine whether the G152fs mutation has an eastern European origin and appeared in Italy by migration. The IVS9-56G→A AKU mutation was also identified earlier in an AKU chromosome of a French patient. Interestingly the IVS9-56G→A mutation in the Italian and the French patients are associated with the same HGO haplotype. In this case, we postulate an Italian origin for this mutation since the French IVS9-56G→A carrier patient has Italian ancestors.

It is important to note that the mutations P230S, V300G, and M368V, which are relatively common mutations 7 12 15 19 were absent in our patients. P230S and V300G are typically associated with haplotype E and are thought to be North African in origin. The M368V mutation is widely distributed throughout Europe and is associated with haplotype A. It is also interesting that the mutation G360R was found twice within the same haplotype in two families from different ends of the Italian peninsula; patient VRN was from Calabria (southern Italy), whereas the affected sons of a consanguineous marriage were from South Tyrol, a German speaking region on the Austrian border. Additional HGO mutational and polymorphism analyses of AKU patients from many more different countries would be necessary to understand the population genetics of AKU and the migration of the different AKU alleles.

In conclusion, we report here an extensive description of the spectrum of AKU mutations in Italy, including the characterisation of their associated intragenic HGO polymorphisms. Four novel mutations were found, which include both missense mutations and subtle intronic changes. The Italian AKU sample confirms the high degree of allelic heterogeneity of the HGO gene and illustrates the complications of mutation screening in AKU patients. These data should facilitate the future identification of these AKU alleles in this and other populations.

Note added in proof

The mutation G270R has recently been described by Müller et al (Eur J Hum Genet 1999;7:645-51).

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Rough skin, brittle hair, and photosensitivity: a mild phenotypic variant of trichothiodystrophy

EDITOR—The trichothiodystrophies (TTD) are named primarily for the hair sulphur deficiency which is their most specific feature and which leads to brittleness of the hair. Other ectodermal tissues may be affected and typically the skin is ichthyotic and the nails dystrophic. Additionally, there may be a distinctive facies and physical and developmental retardation of varying degree of severity. Inheritance is autosomal recessive and at least three loci exist, of which two are known, the excision repair/transcription factor genes XPD/ERCC-2 and XPB/ERCC-3. We describe an 8 year old girl in whom the diagnosis of a mild and in some respects atypical form of TTD was made on the synthesis of clinical, pathological, and biochemical data. The genotypic basis of this clinical phenotype has yet to be established.

The patient was the second child of a dizygous twin pregnancy born to unrelated, healthy, white parents by emergency caesarean section at 32 weeks because of pre-eclampsia. The family history was unremarkable and her male co-twin was healthy. Birth weight was 2100 g (90th centile for this gestation). Birth length and head circumference were 51.5 cm and 32.5 cm respectively. The skin was dry and flaky from birth (but never “collodion”), and in using a towel her mother had to pat her dry, rather than to rub. Thickening of the palms and soles developed in the first year of life. The nails were brittle from birth. Hair growth has always been slow and she has never had a proper haircut, only trims. Desquamated cells from the external auditory canal failed to clear and she has required periodic syringing.

She was referred to our service at 5 years of age because of concerns related to persistent dermatitis, dermal photosensitivity suggested by easy burning in the sun, mild developmental delay, and distinctive facial appearance. We noted the following features: hair that was “wiry” in texture, fragile, and easy to extract; abnormal scalp hair distribution with temporal recession; prominent forehead with sparse eyebrows (fig 1); a generalised dryness of the skin with areas of keratoderma; and brittle, spoon shaped fingernails and toenails. The keratoderma was particularly marked on the soles and palms (fig 2) and at the popliteal and antecubital flexures. Apart from congenital absence of the second premolars, the teeth were normal. Her weight was 27 kg (90th centile), height 128 cm (97th centile), and head circumference 54 cm (98th centile). These measurements were consonant with the parental heights and weights, which were in the 90th-97th centile range. She was attending a normal school and was in the appropriate class for

Figure 1 Patient aged 8 years. The hair is short (has only been trimmed) and stands up irregularly. There is temporal recession.

Figure 2 Palmar keratoderma.
We used this occasion in one allele (c1381C>G, c2146del45) and a causative mutations identified in that case, these being a null mutation reported case of TTD (TTD183ME), who was a compound with reference to previously published values (table 1).

Table 1 Hair cysteine analysis results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean cysteine content (µmol/g hair dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously published controls¹</td>
<td>1407–1512</td>
</tr>
<tr>
<td>Control hair (age/sex matched)</td>
<td>1442.8</td>
</tr>
<tr>
<td>Patient’s hair</td>
<td>1172.8</td>
</tr>
</tbody>
</table>

unpublished data), but we expected that the XPD gene, being larger than XPA and with more introns, might prove more difficult.¹ Buccal smears were taken from the patient and her co-twin and sent by express mail from Melbourne to San Francisco. Samples were then solubilised in alkali extraction buffer and subjected to genomic amplification by PCR, using primers that flank those genomic regions in which mutations noted above had previously been identified. The PCR products were subcloned into a cloning vector and individual inserts sequenced. The unaffected co-twin had no causative mutations in the regions investigated, although there were two common neutral polymorphisms (c687G>A, c711C>T). The index case had neither of these polymorphisms and neither was the c1381C>G, c2146del45 null allele identified. We sequenced about 300 nucleotides around the region of the causative mutation (c2173G>C) and found only wild type sequences.

This child presents a mild phenotypic form of trichothiodystrophy (TTD). The scalp hair was abnormal in its texture, strength, and distribution, and the electron microscopic (but not light microscopic) picture was similar to that of the classical form of TTD. Hair cysteine content was reduced and the A layer of the cuticle cells defective, substantiating the diagnosis. Other clinical observations included keratoderma, nail dystrophy, and mild developmental delay. Linear growth was unaffected.

TTD can present with a notably wide clinical range.⁵ In its mildest form, the disorder may be confined clinically to an abnormality of the hair.⁶ The “tiger tail” hair sign is typical but not universal,⁷ as indeed our case exemplifies. A further grade of affection is illustrated in our patient, with mild to moderate involvement of the ectoderm and certain of its appendages, as well as (if indeed this is causally related) a minor cognitive component. At the other end of the spectrum, TTD is a seriously disabling disease, with a severe skin affliction and mental defect and growth retardation.

The TTDs comprise one of a group of related disorders resulting from abnormalities in components of the nucleotide excision repair (NER) system, these factors having a second role as subunits of the basal transcription factor TFIIB.⁸ Nine TTD complementation groups exist, corresponding to the genes XPD/ERCC-2, and an uncloned gene TTD. Xeroderma pigmentosum (XP) and a form of Cockayne syndrome (CS) with XP-like symptoms are also the result of defects

age, but was receiving remedial help with reading and perceptual motor skills. Her co-twin did not display any of the above features on examination and was reported to be performing satisfactorily at school. On review at the age of 8½ years, her hair was becoming thicker but the growth rate remained very slow. The keratoderma continued to be a problem and was managed by a combination of skin creams. The skin photosensitivity was being dealt with by ultraviolet barrier cream and sun protection. Formal testing with monochromatic light across the ultraviolet A and B spectra did not produce unusual erythema, indicating that the photosensitivity was of mild degree. Eye examination was difficult because of photophobia, but no abnormal retinal pigmentation was observed. She was having private tuition for reading and arithmetic.

Hair examination on polarised light microscopy showed no alternating dark and light band “tiger tail” pattern. On scanning electron microscopy, marked irregularity and flattening of hair shafts was observed. Some areas showed twisting and variation in shaft diameter. Transmission electron microscopy showed a defective A layer in the outermost cuticle cells (fig 3), a characteristic of TTD.¹ The hair cysteine content of the patient and of an age and sex matched control was assessed on hair cuttings scoured as in Church et al and measured according to a modification of the method in Strydom and Cohen.³ All hair shafts showed decreased cysteine content compared to the control specimens and with reference to previously published values (table 1).

Since the patient came from the same city as a previously reported case of TTD (TTD183ME), who was a compound heterozygote at the XPD/ERCC2 locus, we first tested the mutations identified in that case, these being a null mutation in one allele (c1381C>G, c2146del45) and a causative mutation in the other (c2173G>C).¹¹ We used this occasion to test the robustness of buccal smears for diagnostic purposes. We had previously used buccal smears successfully for the diagnosis of xeroderma pigmentosum complementation group A (XPA) patients (J E Cleaver, M McDowell, unpublished data), but we expected that the XPD gene, being larger than XPA and with more introns, might prove more difficult.¹ Buccal smears were taken from the patient and her co-twin and sent by express mail from Melbourne to San Francisco. Samples were then solubilised in alkali extraction buffer and subjected to genomic amplification by PCR, using primers that flank those genomic regions in which mutations noted above had previously been identified. The PCR products were subcloned into a cloning vector and individual inserts sequenced. The unaffected co-twin had no causative mutations in the regions investigated, although there were two common neutral polymorphisms (c687G>A, c711C>T). The index case had neither of these polymorphisms and neither was the c1381C>G, c2146del45 null allele identified. We sequenced about 300 nucleotides around the region of the causative mutation (c2173G>C) and found only wild type sequences.

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Figure 3 Cross section of hair shaft on transmission electron microscopy. (Left) Normal, showing central cortex and cuticle cell layers peripherally. The cuticle cells are elongated and show a dark continuous band (A layer) on their internal border (arrow). (Right) TTD, present case. The outermost cuticular layers are disrupted and the normal architecture is lost. The A layer (arrowhead) is only intermittently discernible.
The first description of lethal pterygium syndrome with facial clefting (Bartsocas-Papas syndrome) in 1600

EDITOR—A recently reviewed 12 page pamphlet dated 1600, housed at the Bodleian Library, Oxford, contains a detailed account of a severely malformed infant born in Herefordshire in January 1600.1 The child, whose gender was uncertain to the observers, was born to first cousins. “A most straunge, and true discoursse, of the wonderfull judgement of God. Of a monstrous deformed infant, begotten by incestuous copulation, betwenee the brother’s sonne and the sister’s daughter, being both vnmarried persons”.

Adhering closely to the language of the day the infant is described thus.

Head longer than ordinary children with no hair on the head or eyebrows.

Both eyes standing far out of the head, unequal to each other - right eye very small, like a black sloe stuck half out in the flat face - no eyelid or eyelid - “as it were a bullet sticking in a plain wall”; the left eye was very big and eminient, sticking out like the other but with eyelids which were show “tiger tail” pattern. Which, if either, locus underlies her case remains open to question.

We thank Dr Richard McNeill who referred the patient, Dr C W Chow and Professor J P Dowling who performed hair electron microscopy, Dr C Baker who did the skin light testing, and Mr Gary Corino of the Commonwealth


Letters

Sex reversal and diaphragmatic hernia in phenotypically female sibs with normal XY chromosomes

EDITOR—True agonadism, characterised by the absence of gonads in both XY and XX patients, is a rare, mostly sporadic, and isolated condition. Its association with diaphragmatic hernia seems to be extremely rare and has always been described associated with multiple congenital malformation (MCA) syndromes of unknown origin. Sex reversal and diaphragmatic hernia have been described once in normal, healthy, male children (II.2, II.3). At 34 weeks’ gestation of the fourth pregnancy, ultrasonography detected dextrocardia owing to a left diaphragmatic hernia involving the stomach, left lobe of the liver, and numerous intestinal loops. Chromosomal analysis was performed on both lymphocytes from fetal blood and fibroblasts from amniotic fluid. They were normal male: 46,XY.

Letter

Not clear if the child was male or female - had perfect members of neither - urine issued out of a small hole in the proper place - sometimes seemed more male, sometimes more female.

Knees up to the belly, legs fixed to buttocks; the calf of the leg growing to the buttock, and thigh unto the small of the leg; but the right leg quite down to the heele, within two fingers breadth.

Right foot bowed towards the left and joined to it by a string of flesh at the ends of the big toes - the rest of the toes covered with one skin as were the hands.

The body bigger and longer than other children.

The child died on the third day and was said not to sleep because “it had no eyelids”. In summary, the child appears covered with one skin as were the hands.

The concept and law relating to incest was very different in 17th century England compared with today. Incest was not a criminal offence but was prohibited by Canon Law and so heard in the Church Courts. Its scope was wide, embracing not only cases of consanguinity (including first cousin unions) but also cases of “affinity”, for example, a sexual relationship between a woman and her brother in law (although this could be dispensed with in specific cases if proper approaches were made before marriage). It was not until the late 19th century that marriage with a “deceased wife’s sister” was allowed as a matter of course.

Seventeenth century Scottish law was different and incest was a criminal offence, leading to a number of executions for sexual relations even in the category of “affinity”. Changes and rationalisation in the Law of Incest came about precisely at the time when the science of genetics was born, the publication of Francis Galton’s Natural Inheritance in 1889 being particularly significant.
Intubation was performed immediately after birth and the infant was transferred to the intensive care unit. HFO and NO treatments were given. Apart from the respiratory distress syndrome, the clinically female infant was normal except for bilateral membranous syndactyly of the second and third toes. This was absent in all three sibs but present to a lesser degree in her father. Cardiac and abdominal ultrasonography were normal. The diaphragmatic hernia was surgically repaired at 2 weeks of age, when an almost total absence of the left diaphragm was noted. This required the fitting of a Goretex® plate. The subsequent course was favourable and ventilatory support was discontinued at 1 month of age. At 9 months the patient was in good health; weight was 6 kg (−2 SD), height 66 cm (−1 SD), OFC 43 cm (−1 SD), and neuromotor development was normal.

Further investigations were performed in the proband who had completely feminised external genitalia, a normal male karyotype, severe diaphragmatic hernia, and whose sister had died of diaphragmatic hernia. Cholesterol and 7OH cholesterol were normal so that Smith-Lemli-Opitz syndrome (MIM 270400) could be excluded. Skeletal x rays showed somewhat curved clavicles but no anomaly that could indicate campomelic dysplasia (MIM 114290). Hormonal profile in the neonatal period showed very low testosterone (0.21 ng/ml, normal 2.4-4 ng/ml) and dehydrotestosterone (0.04 ng/ml), with normal FSH (1.8 IU/l) and LH (1.1 IU/l) levels, as seen in both complete XY gonadal dysgenesis and agonadism. Ultrasonography of the visceral organs showed the presence of an apparently normal uterus. Pelvic endoscopy was performed at 6 months of age and confirmed the presence of a normal uterus and fallopian tubes; however, no gonads were found, not even in an ectopic position. High resolution G banding chromosome analysis confirmed the normal male karyotype; in particular no anomalies were observed in the regions of localised or putative sex determining genes, such as Yp11.3 (SRY), Xp21.3 (DSS/DAX-1), 11p13 (WT1), 17q24 (SOX9), 9p21.3 or 10q.1 SRY was present and PCR analyses of both the promoter and the unique exon showed that they were present and of normal size. Furthermore, fluorescent in situ hybridisation (FISH) of the Y chromosome performed on paraffin preserved tissue from the dead sib detected retrospectively the presence of a Y chromosome. SRY analyses could not be performed because of the small amount of tissue preserved, and it was not possible to study retrospectively the internal genitalia of this normally feminised infant.

As diaphragmatic hernia has been described in one case of Denys-Drash syndrome (DDS, MIM 194080), renal function was carefully investigated in the proband (whose renal ultrasonography was normal) and was found to be normal. Moreover, renal tissue of the dead sister was re-examined and found to be normal. Finally, in the proband no anomaly was found in exons 7, 8, or 9 of the WT1 gene, which is responsible for DDS and Frasier syndrome (FS, MIM 136680).

Here we report two sibs with a normal male karyotype and diaphragmatic hernia. Clinical and laboratory investigations of sex reversal established the diagnosis of true agonadism, which is a rare, mostly sporadic condition characterised by the absence of gonadal structure. Agonadism has almost always been diagnosed in cases with a normal male (XY) karyotype, female external genitalia, and absence of extragenital anomalies. However, the condition could have been overlooked in karyotypically female patients because of the absence of sex reversal. In XY patients, agonadism has to be differentiated from XY pure gonadal dysgenesis (Swyer syndrome resulting from SRY mutations (MIM 306100)), in which the gonads are
reduced to streaks but are present, and both external and internal genitalia are normal female. In XY patients, agonism was termed embryonic testicular regression syndrome because, from an embryological point of view, it may be the result of secondary precocious regression rather than true agenesis of the primary undifferentiated gonads. Thus, early testicular tissue action (anti-Müllerian hormone (AMH) and testosterone) could explain the range of virilisation in some agonal XY patients. Because of the few familial cases of XY embryonic testicular regression syndrome, genetic determinism has been suggested.

Agnondism has seldom been observed with an XX chromosomal constitution in either sporadic (n=1) or familial cases (both XY and XX affected sibs of consanguinous parents).\textsuperscript{10} External genitalia were normal female in all five patients, but Müllerian ducts were either absent,\textsuperscript{11} hypoplastic,\textsuperscript{12} or normal,\textsuperscript{13} whereas no transient testicular anti-Müllerian effect can have acted in these XX patients. Thus, the question is raised of the involvement of autosomal genetic factors maintaining both gonads and Müllerian ducts (the latter independently of AMH action).

On the other hand, agonism has also been described associated with variable extrauterine anomalies in both sporadic\textsuperscript{12-19} and familial cases (table 1), of which one sporadic\textsuperscript{12} and one familial\textsuperscript{13} cases were also associated with diaphragmatic hernia. However, the remaining features in these patients are sufficiently different from those we describe to exclude comparison. In the patient described by Oyer et al.,\textsuperscript{15} Müllerian derivative defects and bicuspid aortic valve were observed. In the sibs born to unrelated parents described by Sorgo et al.\textsuperscript{20} and Kennerknecht et al.,\textsuperscript{21} Müllerian ducts were present although rudimentary, and diaphragmatic hernia was associated with a heart defect in a patient with an XY chromosomal constitution, whose XX sib presented with omphalocele, heart defect, and cleft palate.

Diaphragmatic hernia and sex reversal has been described associated with multiple malformations in three unrelated phenotypic girls with a normal XY karyotype.\textsuperscript{22,23} However, the multiple associated malformations, cyanotic complex heart defect (n=2),\textsuperscript{23} hypoplastic left ventricle, ectopic spleen, and horseshoe kidney (n=1),\textsuperscript{24} and above all the present of testis tissue are sufficiently different from our observations to distinguish these conditions.

We also looked for WT1 mutations (which were first described in patients with Wilms tumour,\textsuperscript{25,26} then in DDS syndrome,\textsuperscript{27} and more recently in Frasier syndrome\textsuperscript{28} ), because of the description of a WT1 point mutation (arg 366 his) in a phenotypic girl with a normal male karyotype, a large left posterosilateral diaphragmatic hernia, and features of DDS (dysgenetic ovaries, double uterus, double vagina, and severe glomerulopathy), who died at 5 hours of age from respiratory distress.\textsuperscript{29} Furthermore, transgenic mice with a homozygous WT1 deletion consistently have diaphragmatic hernia and heart defects besides their urogenital malformations,\textsuperscript{20} although no WT1 mutation has been found in patients with isolated diaphragmatic hernia.\textsuperscript{30} WT1 is expressed in abdominal and lung mesothelium and encodes four isoforms of zinc finger transcriptional factors that could regulate several genes involved in the development of the diaphragm as well as those implicated in sex determination and differentiation, such as SRY, AMH, and the androgen receptor genes.\textsuperscript{31} Our patient did not fit all the diagnostic criteria for either DDS or FS; she had gonadism rather than gonadal dysgenesis, her Müllerian structures were normally developed while they are often hypoplastic or absent in DDS or FS, she has not presented any renal anomalies up to the age of 1 year, and no mutation has been found in WT1 exons 7, 8, or 9. However, the absence of identified gonads could be an extreme form of gonadal dysgenesis,\textsuperscript{20} testicular regression could have been precocious enough to prevent AMH secretion, and renal dysfunction owing to WT1 mutations could appear later (as seen in FS). Since, to our knowledge, no other familial cases of true gonadism associated with diaphragmatic hernia have been published, and since we did not find WT1 mutations known to be implicated in DDS and FS syndromes in our patient, we suggest that such cases could represent a new sex reversal syndrome that could be either autosomal recessive or X linked, and result from either unreported mutations of WT1 or anomalies of other developmental genes.

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Clinical variability of Stickler syndrome with a COL2A1 haploinsufficiency mutation: implications for genetic counselling

Editor—Sned and Yates have recently reviewed clinical and molecular findings in Stickler syndrome, the autosomal dominant connective tissue disorder characterised by ocular manifestations, facial abnormalities, cleft palate, sensorineural hearing loss, and degeneration of epiphyseal and articular cartilage (hereditary progressive arthro-ophthalmopathy). Mutations in the structural genes for collagen II (COL2A1) and collagen XI (COL11A1, COL11A2) have been identified in patients with a Stickler syndrome phenotype. Based on locus heterogeneity, a subclassification of COL2A1 associated Stickler syndrome type I, COL11A1 associated Stickler syndrome type III, and COL11A2 associated Stickler syndrome type II was established (OMIM 108300, 120280, and 184840). A clinical subclassification based on the presence or absence of an ocular phenotype, and on the

Figure 1 The proband in the newborn period (note midface hypoplasia, micrognathia, apparent exophthalmos, and deep set ears) and at 5 years of age. The face is slightly flat with maxillary hypoplasia and retrognathia. Her height is on the 90th centile; note also mild neck webbing and flat feet. (All photographs reproduced with permission.)
features of the ocular phenotype, correlates reasonably well with the genotype.19

Clinical variability in Stickler syndrome is well known,6 14 15 but correlations with specific mutations are scarce. We report a novel COL2A1 gene mutation found in a patient with flat face, cleft palate, myopia, and hearing loss (Stickler syndrome) and unexpectedly also in her father and her paternal grandmother who were considered healthy. The patient is the first child of healthy, non-consanguineous Swiss parents. The pregnancy was uneventful and she was delivered at term by caesarean section because of breech position. Birth weight was 3890 g (90th centile), birth length 50 cm (50th centile), and head circumference 37 cm (>97th centile). Macrocephaly and facial dysmorphism were noted in the neonatal period, including a flat midface, deep set ears, exophthalmos, palpebral oedema with telangiectasia, micrognathia, and median clefting of the soft and part of the hard palate (fig 1). No other abnormalities were recognised at that time. At the age of 5 years, midface hypoplasia and micrognathia were still evident. In addition, she presented with mild bilateral hypoaacusis, bilateral myopia (4 dioptres), slight webbing of the neck, minimal pectus carinatum, and flat feet (fig 1). Her growth was on the 90th centile. The clinical signs and symptoms as well as radiological findings of mild spondyloepiphyseal dysplasia suggested the diagnosis of Stickler syndrome.

All 54 exons of the COL2A1 gene, including the flanking splice sites, were amplified and screened by SSCP analysis. Exon 12 gave an abnormal pattern and was subcloned and sequenced, showing a deletion of 2 bp (nt 697-698), for which the patient was heterozygous. The mutation predicts a downstream premature TGA-stop codon in exon 13 (fig 2). The father’s DNA gave a similar heteroduplex pattern, and direct sequencing confirmed his heterozygous mutation carrier status. He had been regarded as unaffected, but re-evaluation showed some features consistent with Stickler syndrome including mild bilateral myopia (2 dioptres), a high arched palate, and a partially split uvula. His body habitus showed no distinctive features except for his height of 190 cm (>97th centile) (fig 3); hearing was normal and there was no history of arthropathy. Childhood photographs were reviewed and found to be unremarkable. We subsequently investigated the paternal grandparents and found that the grandfather carried the same COL2A1 gene mutation in the heterozygous state in her blood leucocytes. She considered herself healthy and declined further investigations. Her physician observed no features of Stickler syndrome. To rule out the presence of a second COL2A1 mutation in the proband leading to a more severe clinical phenotype, all 54 exons and the flanking intron splice site sequences were analysed by direct sequencing in the proband as well as in her father and grandmother. Except for a few already known common polymorphisms, no additional sequence abnormality was observed.

The 2 bp deletion in exon 12 of the COL2A1 gene identified in this family leads to a frameshift with a premature stop codon in exon 13. This causes the synthesis of truncated procollagen α1(II) chains, which are unable to participate in collagen II triple helix formation, without exerting other negative effects, a mechanism referred to as functional haploinsufficiency.16 17 To date, at least 10 COL2A1 gene mutations have been identified in patients with Stickler syndrome type I.15 18–24 Except for a 28 bp deletion of exon 12,14 all mutations generate unique premature stop codons leading to functional haploinsufficiency. Findings in our proband provide further evidence that COL2A1 haploinsufficiency is a common molecular mechanism in patients with Stickler syndrome. However, findings in this family cast some new light on the role of such mutations, as the mutation was detected in two family members who were considered healthy. Apparently, COL2A1 haploinsufficiency mutations do not necessarily result in the full Stickler phenotype but may cause a mild phenotype or remain below clinical expression.

As briefly mentioned by Snead and Yates,1 variable clinical expression of Stickler syndrome complicates the genetic counselling scenario. In a child with apparently sporadic Stickler syndrome, caution must be used before assuming a de novo mutation; accurate radiographic and clinical examination of the parents, including formal testing of hearing and vision,1 is indicated, and a mutation search should be undertaken whenever possible. On the positive
side, counselling must take into account the possibility that inheritance of such a mutation may have only minimal clinical consequences or remain silent, but it can be difficult for prospective parents to make use of information with this degree of uncertainty.

Some indications for future research may be derived. First, the question might be asked of whether variability is more frequent in COL2A1 haploinsufficiency mutations than in the other COL11A1 and COL11A2 mutations associated with Stickler syndrome. Second, a systematic ascertainment of families segregating such COL2A1 haploinsufficiency mutations might allow the penetrance of single clinical traits, such as cleft palate or severe myopia, to be determined. It cannot be excluded at present that among carriers of such mutations, subjects with the full blown Stickler phenotype are the exception rather than the rule; this must be investigated. Such insight would be helpful for counselling and parental decision making.

We are grateful to our young patient and her parents for cooperation and for permission to publish their photographs; to her grandparents and Dr Z Schnei-der for clinical information; and to Pia Hermanns, Jutta Busch, and Sabine Fleig for expert technical assistance. Our investigations were supported by a grant from the Swiss National Foundation (32-45401.95 and 32-57272.99) to Andreas Winterpacht and Bernhard Zabel.

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Figure 3 The patient’s father at 41 years of age. He is 190 cm tall and thus on the 97th centile for the Swiss population. He has mild myopia (2 dioptres), a compatible but non-specific finding. Little in his physical appearance suggests a collagen disorder; the only telltale finding was a partially bifid uvula.