Leigh syndrome transmitted by uniparental disomy of chromosome 9

EDITOR—Severe, isolated, and generalised deficiency of complex IV (cytochrome c oxidase, COX) can result in Leigh syndrome (LS) (MIM 256000), an early onset mitochondrial disorder characterised by rapidly progressive, symmetrical degeneration of the brain stem, diencephalon, and basal ganglia.1 2 SURF-1, a gene located on chromosome 9q34, has recently been identified as the gene responsible for numerous cases of LS.3 4

SURF-1 associated LS is usually inherited as an autosomal recessive trait. We report here a homozygous loss of function mutation of SURF-1 in two monozygotic LS female twins, owing to uniparental disomy of two almost identical maternal chromosomes 9.

The probands were born to non-consanguineous parents at 33 weeks of gestation by caesarean section. The mother was 46 years old. The pregnancy was uneventful until the 24th week, when persistent uterine contractions ensued. Two older sibs of the probands are alive and healthy. The father was 34 years old. He had a hypotonia, ophthalmoparesis, mild bilateral optic atrophy, and psychomotor regression, characterised by failure to thrive, psychomotor regression, and the Apgar score was 1-8 in both patients. Growth rate and psychomotor development were regular during the first 8 months of life. During the following months the patients developed a rapidly progressive clinical syndrome characterised by failure to thrive, psychomotor regression, hypotonia, ophthalmoparesis, mild bilateral optic atrophy, and ataxia. At 18 months both patients had mild lactic acidosis. MRI showed symmetrical paramedian lesions in the mesencephalon and brain stem, as typically found in LS. Both patients died of respiratory failure in the third year of life.

Needle muscle biopsies performed at 24 months of age showed a diffuse reduction of the histoenzymatic reaction to COX. Biochemically, COX activity in muscle homogenates was 12.1 nmol/min/mg in one patient and 3.6 nmol/min/mg in the second (normal values 68±20), while the activities of the other respiratory complexes were all normal. The COX defect was also detected in cultured fibroblasts of one patient (0.4 nmol/min/mg, normal value 25±11), but this assay was not performed in the second patient. Specific activities of the respiratory complexes in a muscle homogenate of the mother were all normal.

Automated sequence analysis of the nine exons of the SURF-1 gene in the probands showed the presence of a previously reported1 homozygous frameshift mutation (751C>T). This mutation destroys a BsiWI restriction site, which is present in the wild type gene. BsiWI RFLP analysis showed a heterozygous mutation in the mother, while no mutation was detected in the probands (fig 1). A de novo mutation in the paternal chromosome 9 identical to the mutation carried by the mother was considered unlikely. Non-paternity was excluded by linkage analysis with numerous microsatellite markers. To test the hypothesis of chromosome 9 specific paternal non-contribution, we then analysed three STSs (D9S1831, D9S1826, and D9S158), flanking the SURF-1 locus at 9q34. All three markers showed the presence of one maternal allele only, while the paternal allele was consistently absent. To verify whether the paternal non-contribution was the result of a microdeletion at 9q34, the cosmid P117B6, which contains the SURF-1 gene,5 was used as a probe in FISH experiments on metaphases from one proband. The probe detected two comparable signals on both chromosome 9 homologues (fig 2). These results excluded the presence of a deletion in a paternal chromosome, suggesting instead a mechanism of uniparental disomy (UPD) of two maternal chromosomes. To test this hypothesis, additional microsatellites distributed along the whole of chromosome 9 were analysed for a total of 22 markers (fig 1). With the exception of two small regions (D9S288-D9S286, and D9S167-D9S283-D9S287, see fig 1) the alleles were all homozygous; in 15 instances the obligate contribution of the paternal allele was unequivocally missing. In particular, homozygosity was detected for 10/10 markers encompassing the SURF-1 locus, in the interval defined by markers D9S1831-D9S158. We conclude that loss of the contribution of a second normal SURF-1 allele has led to the manifestation of LS in our patients.

UPD is defined as the exceptional inheritance of a pair of chromosomes from one parent only, as the result of gamete complementation, chromosome loss in trisomy, or duplication in monosomy. In isodisomy, the uniparental pair is a duplicate of the same chromosome DNA template, and causes an increased risk of a recessive disorder by reduction to homozygosity.1 In our patients the presence of two small heterozygous regions can be explained as the result of two crossing over events in otherwise identical maternal chromosomes 9. These data indicate that the double maternal contribution was the result of a non-disjunction
A case of Williams syndrome with a large, visible cytogenetic deletion

EDITOR—Williams syndrome (WS) is generally characterised by mental deficiency, gregarious personality, dysmorphic facies, supravalvular aortic stenosis (SVAS), and idio-pathic infantile hypercalcaemia. Patients with WS show allelic loss of STX1A, elastin (ELN), and LIMK1, with most exhibiting a submicroscopic deletion at 7q11.23, detectable by FISH. The common deletion size is about 1.5 Mb. Previous studies have shown that WS patients have consistent deletion sizes and share common proximal and distal breakpoints. Here we report a patient who has a large, atypical, visible chromosomal deletion of 7q11.23 and features consistent with, and in addition to, those typically seen in Williams syndrome.

The patient was originally referred to the genetics clinic at 5 months of age for evaluation of global developmental delay and dysmorphic features. She was delivered at 37 weeks' gestation by caesarean section weighing 2350 g (<5th centile). The initial course included a history of poor feeding in the newborn period. Clinical examination showed macrocephaly, cutaneous haemangioma, and craniofacial features consisting of a large anterior fontanelle, frontal bossing, depressed nasal bridge, cup shaped ears, hypertelorism, and prominent lips (fig IA). Neurological examination showed generalised hypotonia with heel cord and hamstring tightness. CT scan of the head and renal ultrasound were normal. Because of a grade III/VI systolic murmur, echocardiogram was performed, which showed a slightly thickened aortic valve. Cytogenetic analysis showed a 46,XX karyotype.

Re-evaluation at 4 years of age, showed short stature (90 cm, <3rd centile), continued significant development delay, and coarsened facial features with stellate irides. Repeat echocardiogram showed moderately severe supravalvular aortic stenosis. Cardiac catheterisation confirmed these findings with no evidence of leaflet leaflets or the rest of the ascending aorta, and no evidence of aortic insufficiency. Ophthalmological examination showed bilateral exotropia and hyperopia requiring corrective lenses.

UPD may also cause functional balance disruption of imprinted genes. The existence of imprinted genes on chromosome 9 is controversial, but it seems unlikely. Our patients did not show gross dysmorphic features or malformations apart from LS. With the limitations because of the brief survival and severe phenotype, this observation suggests that chromosome 9 does not contain maternally imprinted genes crucial for embryonic development.

We are indebted to Ms Barbara Gezian for revising the manuscript and to Mr Franco Carraza and Mr Matteo Granatiero for technical assistance. This work was supported by Fondazione Telethon-Italy (grant No 767 and grant No E672), Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Associazione Italiana Ricerca sul Cancro (AIRC), Ricerca Finalizzata della Sanità No ICS 120.2/RF96.348, and European Union Human Capital and Mobility network grant "Mitochondrial Biogenesis in Development and Disease".

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and very grey optic discs with surrounding peripapillary retinal pigment epithelial changes. Serum calcium levels were raised once (10.8 mg/dl, normal range 8.5-10.5 mg/dl), but have since been in the normal range. FISH analysis for the elastin locus on chromosome 7q11.23 showed a deletion consistent with Williams syndrome and repeat cytogenetic analysis showed a visible deletion in band 7q11.2 (46, XX, del(7)(q11.1q11.23)) (fig 2).

Follow up at 6½ years of age showed weight 19.8 kg (40th centile), height 107 cm (5th centile), and head circumference 52.5 cm (70th centile), with other notable findings that included prominent supraorbital ridging, periorbital fullness, stellate pattern to her irides, cup shaped ears in normal position, prominent, full lips, long philtrum, and a broad nose with anteverted nostrils (fig 1B). She had fifth finger clinodactyly and brachydactyly. Examination of her skin showed a haemangioma in the midline lumbosacral region, which had reportedly once extended from her occiput to her buttocks. She had a hoarse, raspy voice and frequent drooling. Developmentally, she functioned in the severe mental retardation range. She showed significant delays in communication; expressive language was severely delayed with rare speech (a few words) and limited sign language and receptive language at <1 year of age. She had atypical behaviour including diminished interest in social interaction with others, self-injurious behaviour, intermittent stereotypic behaviour, and sleep disturbance.

The patient had a sister (aged 8 years) and a brother (aged 10 years) who were healthy with normal development. There were no other family members with mental retardation, short stature, or birth defects. The parents were non-consanguineous. Blood samples were obtained from both parents and the proband for additional molecular studies.

In order to identify a FISH probe for the gene encoding the α/β subunits of the L type voltage dependent skeletal muscle calcium channel (CACNL2A), a PCR primer set (exnCa-A: 5’-CGGTGAGTGCTAAGACCTGAATG-3’, exnCa-B: 5’-CAGCCCTCATAGATGTCAGTAGG-3’) was designed from exon sequence obtained from the EMBL database under accession number Z28599. Primers were used at a final concentration of 1.5 µmol/l in a 20 µl reaction. The amplification was performed with an annealing temperature of 60°C for 30 cycles. Total human DNA (25 ng) was used as a template. The resulting 311 bp product was electrophoresed on a 1% low melting point agarose gel. The gel fragment was excised and diluted 1:3 with sterile water and was labelled with 32P-dCTP by standard methodology. The probe was hybridised to high density filters arrayed with clones from a human total genomic P1 library. Four positive coordinates were
submitted to the Baylor College of Medicine YAC core and
streaks, representing 12 P1 clones per coordinate, were
received. For each, single colonies were tested by PCR
using the exnCa primer pair. Clones from coordinate
101F1 were found to be positive for the primer sequence.
The positive clone was grown and DNA was prepared
using the Qiagen Plasmid Midi Kit following the amended
instructions for P1 clones distributed by Qiagen. A cosmid
size FISH probe was made by subcloning the P1 clone
using the SuperCos 1 Cosmid Vector Kit available from
Stratagene. A clone (CaE-1) specific to the exnCa primer
was isolated by testing subclones by PCR as described above.

A cosmid containing the D7S849 locus, known to be
linked to the CACNL2A locus, but not contained in the
previous P1 clone, was subcloned from a yeast artificial
chromosome (YAC) isolated from the CEPH Mark I YAC
library by the Baylor College of Medicine YAC core using
the D7S849 primer set. YAC DNA was prepared by a
standard caesium chloride protocol and subcloned using
the Qiagen Plasmid Midi Kit and was used (20
ng/µl) as a FISH probe against patient metaphase chromo-
somes.

Additional probes used in the FISH analyses included a
cosmid containing the 5' end of the elastin gene
(cELN272), a cosmid containing the full sequence of
LIMK1, and a bacterial artificial chromosome clone
(BAC) containing the STX1A gene (BAC 137N23;
Research Genetics, Huntsville, AL).

All FISH probes were labelled by nick translation with
digoxigenin-dUTP and detected with anti-digoxigenin
conjugated to rhodamine. Either a biotin labelled chromo-
some 7 alpha satellite centromere probe or a digoxigenin
labelled chromosome 7q telomere probe (Oncor, Inc,
Gaithersburg, MD) was used as a control to identify the
chromosomes 7. The centromere probe was detected using
avidin conjugated to fluorescein isothiocyanate (FITC).
Slides were counterstained with DAPI. FISH analyses were
performed as recently described.

This patient was deleted for all the FISH probes tested,
including the CACNL2A gene and the locus D7S849
(fig 3).

DNA was extracted from peripheral blood from the
patient and each parent using standard methodology. Poly-
morphic dinucleotide repeat markers were used to detect
deletions and determine the parental origin of the deletion
as previously described. A deletion was evident when the
proband failed to inherit an allele from one of the parents.
The following loci were examined (listed centromeric to
telomeric): D7S672, D7S1816, D7S489U, D7S2476,
ELN, LIMK1, D7S613, D7S2472, D7S1870, D7S489L,
D7S849, D7S675, D7S699, D7S440, and D7S634.

The patient was deleted for markers D7S489U (centro-
meric) to D7S440 (telomeric) and uninformative for
D7S634. The centromeric breakpoint was the same as seen
in classical WS patients. The patient's distal deletion
breakpoint was telomeric to the classical breakpoint
(D7S1870), beyond the marker D7S440, with the exact
distal breakpoint undetermined.

WS presents as a remarkable collection of features with
significant phenotypic variability among patients. Variabil-
ity in the phenotype could be the result of different sized
deletions around ELN or the variation in gene content or
gen activity of the hemizygous alleles on the non-deleted
chromosome. Our previous studies have shown the size of
the deletions in the majority of WS patients studied to be
consistent between the markers D7S489U and D7S1870. The
current patient represents a rare exception.

The gene encoding the α/δ subunits of the L type volta-
age dependent skeletal muscle calcium channel
(CACNL2A) was mapped to 7q21-q22. In addition, a
form of malignant hyperthermia susceptibility (MHS) has
been linked to CACNL2A by analysis of a (CA), repeat
polyorphism, D7S849, mapping to 7q11.23-q21.1. Given
the occurrence of hypercalcaemia and the reports of
masseter spasm and sudden death during surgical proce-
dures in WS patients and the mapping of CACNL2A
near the WS critical region on chromosome 7, Mammi et

Figure 2  GTG banded partial karyotype of the patient. The normal
chromosome 7 is shown on the left and the deleted chromosome 7 is shown
on the right (arrow).

Figure 3  Fluorescent in situ hybridisation using cosmid CaE-1 to the
CACNL2A locus. A 7q telomeric probe is used as a control. Two signals
are seen on the normal chromosome 7. Only the control signal is present on
the deleted chromosome 7 (arrow), indicating a deletion of the CACNL2A
locus.
The authors thank the family for their participation, Dr M Keaning (University of Utah) for the elastin containing cosmid, and Dr Tassabehji (St Mary’s Hospital, UK) for the LIMK1 containing cosmid. This research was supported in part by an American Heart Association grant in aid (LGS) and NIH grant RO3 HD 35112 (LGS).

aP2 investigated the inclusion of this gene in microdeletions in WS. Although CACNL2A2 was excluded from the common critical region in the WS patients studied,12 this locus was deleted in the current case of a visible deletion of 7q11.2. It is not known if this patient would be susceptible to malignant hyperthermia, but caution should be exercised if anaesthesia is ever necessary.

To date, this is the only case of a visible deletion of 7q11.2 in which the extent of the deletion has been delineated by molecular methods. The present deletion appears to extend distally beyond D7S849 and CACNL2A2. This patient has the characteristic craniofacial and cardiovascular abnormalities, including supravalvular aortic stenosis, described in patients with Williams syndrome. By the scoring system developed by Preus,12 this patient’s score was +9.75, well within the Williams syndrome range (+12.9 ± 4.18). She has typical complications reported in patients with Williams syndrome, such as esotropia, hyperopia, ename hypoplasia, microdontia, early hypercalcaemia, chronic otitis, short stature, and feeding problems. She has not developed scoliosis, kyphosis, or contractures. In addition, she has some atypical findings not seen in Williams syndrome including macrocephaly, retinal problems, severe mental retardation, and minimal lingual hypoplasia. However, the unique facial dysmorphism and clinodactyly. However, the unique

Facial dysmorphism and clinodactyly in one patient suggests the presence of a common critical region in the WS patients studied,12 this region. Thus, deletions in WS patients may be associated with proximal or distal deletions of 7q, deletions including our patient, perhaps because of more severe mental retardation. Additionally, seizures are deletions, including our patient, perhaps because of more severe mental retardation. Additionally, seizures are possible the result of hemizygosity for genes outside the classical WS deletion region.

In examining previously published reviews of cases of 7q deletion, there is considerable clinical variation.14–19 In an attempt to determine if specific phenotypic features are associated with proximal or distal deletions of 7q, deletions of 7q were grouped into terminal deletions1–12 and interstitial deletions.20–22 Interstitial deletions of 7q have been divided into three categories based on the region involved: (1) c1–q21-q22 (proximal), (2) q21–q31/32 (intermediate), and (3) q32–q34 (distal).23 Although correlations between phenotypes and deletions are difficult to establish owing to the variable breakpoints, there are some common but non-specific findings reported in patients with proximal deletions or rearrangements of 7q, including low birth weight, mental retardation, microcephaly, growth retardation, early feeding problems in infancy, abnormal EEG/seizures, hypotonia, and abnormal skull shape.24–25 Comparison of the phenotypes between WS and proximal deletion of 7q shows many common features, such as mental retardation, developmental delay, growth retardation, low birth weight, cardiovascular defects, eye abnormalities, facial dysmorphism and clinodactyly. However, the unique cognitive profile seen in WS patients, significant deficits in motor skills and impaired visuospatial recognition,26 loquaciousness, sociability, weak adaptive skills, dependency, hyperactivity, distractibility, inattention, and limited proveriance,27 is not seen in patients with large deletions, including our patient, perhaps because of more severe mental retardation. Additionally, seizures are common among visible deletion patients, but not generally seen as a feature in WS. This finding suggests that the genes responsible for seizures are outside the common WS deletion. The proximal breakpoint of this case is the same as the proximal breakpoint of the critical deletion region of Williams syndrome, indicating, perhaps, a common mechanism for the deletion in this case and the classical deletion.28

First molecular evidence for a de novo mutation in \textit{RS1 (XLRS1)} associated with X linked juvenile retinoschisis

**Editor—**Juvenile retinoschisis (RS, OMIM 312700) is an X linked recessive vitreoretinal disorder that variably affects visual acuity because of microcystic degeneration of the central retina.\(^1\) In approximately 50% of affected males, peripheral scissia may also occur. Major sight threatening complications include vitreal haemorrhages, retinal detachment, and neovascular glaucoma.\(^1\)

Recently, the gene underlying RS, designated \textit{RS1} (also called \textit{XLRS1}), was positionally cloned\(^2\) and more than 80 different mutations covering a wide mutational spectrum, including intragenic deletions, splice site, frame shift, nonsense, and missense mutations, were identified.\(^4,7\) Interestingly, missense mutations mainly cluster in exons 4 to 6 of the \textit{RS1} gene known to encode a highly conserved discoi-din domain thought to be involved in cell-cell interactions on membrane surfaces.\(^2\)

The high recurrence rate of some of the \textit{RS1} mutations (for example, Glu72Lys in more than 34 patients from different ethnic backgrounds) suggests a significant de novo mutation rate in RS.\(^1\) In this report, we provide the first molecular evidence of a de novo \textit{RS1} mutation (Glu72Lys) in a Greek family. The \textit{Pro203Leu} mutation is present in two brothers diagnosed with severe features of RS at the ages of 9 and 5 years, respectively. We show that the mother is a heterozygous carrier while neither of the maternal grandparents carry the \textit{Pro203Leu} mutation.

Haplotyping data from several polymorphic DNA loci flanking the \textit{RS1} gene confirm paternity and strongly suggest that the \textit{Pro203Leu} mutation originated on the X chromosome of the maternal grandfather.

Two brothers were referred to one of the authors (BL) presenting with unclassified vitreoretinal degeneration in both eyes. By history, retinal detachment had been diagnosed in the right eye in the older (III.1) at the age of 9 months. At the age of 9 years, best corrected visual acuity was 20/200 in the right eye (RE) and 20/40 in the left eye (LE). Fundoscopy showed a bullous peripheral scissia and a flat scissia at the entire posterior pole with inner leaf hole formation in the RE. In the LE, a macular scissia with marked vitreous veils could be seen. Electrotetroinography (ERG) recordings corresponding to the ISCEV Standard were consistent with the diagnosis of RS, that is, rod response was unrecordable in the RE and residual in the LE, there was a negative maximal response, and an unrecordable cone response in both eyes.

**Table 1 Polymorphic microsatellite markers used in the study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Locus</th>
<th>Primer sequence 1 (5’–3’)</th>
<th>Primer sequence 2 (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>207</td>
<td>DXS207</td>
<td>TCACTCCAGATCTGACATGC</td>
<td>AATTGACGACCTTGAAGGAG</td>
<td>12</td>
</tr>
<tr>
<td>389gt</td>
<td>RX324</td>
<td>TGGATGACGTTGACAGC</td>
<td>TATGGAAATGTTGCTCACAGC</td>
<td>13</td>
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<tr>
<td>418</td>
<td>DXS418</td>
<td>TGTGAGGGTTGTTGCTCTGC</td>
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<tr>
<td>RX324</td>
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<td>TTAACGAGGGATTGACACC</td>
<td>This study</td>
</tr>
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<td>ATMi4</td>
<td>ATM</td>
<td>TCTGACCTTTCAACAGAAGGCA</td>
<td>GCAAGATGTTGCTCTAGGCC</td>
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</tr>
<tr>
<td>AFM248YG9</td>
<td>D17S855/BRCAl</td>
<td>AAGAGGTAGGGTTGACCAGCA</td>
<td>AAGACGAGGGTTGCTCACAGC</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^*\text{(CA)}_{30}\) dinucleotide repeat derived from PAC clone dJ389A20 (http://www.sanger.ac.uk)  
\(\dagger\) GenBank Acc No U82828

In the younger brother (III.2), bullous cyst-like retinal changes in both eyes had been diagnosed at the age of 1 year. Four years later fundoscopy showed a bullous retinal detachment in the inferotemporal retina of the RE including the macula with some cystic changes in the area of the inferior temporal vascular arcade. In the LE, only pigmen-tary abnormalities and whitish subretinal deposits consistent with a collapsed scissia could be seen. Best corrected visual acuity was light perception RE and 2/100 LE. Because of severe nystagmus and reduced compliance, the ERG was not recorded.

Fundus examination and ERG were normal in the mother (II.1) and maternal grandfather (I.2).

Genomic DNA from the members of the Greek family was extracted using standard techniques. Haplotyping was done using microsatellite markers 207F/R (DXS207), 389gt, 418F/R (DXS418), and RX324 (DXS443) closely flanking the RS locus (table 1).\(^1,4,7\) Microsatellite marker 389gt was identified in PAC clone dJ389A20 as a (CA)\(_{30}\) dinucleotide repeat located 50 kb distal to DXS418 (genomic sequence available at http://www.sanger.ac.uk/). The repeat sequences were PCR amplified in the presence of \(^3\)P-dCTP (3000 Ci/mmol) using flanking oligonucleotide primers and conditions as given in the references (table 1). To confirm paternity, an additional two highly polymorphic microsatellite markers at the \textit{ATM} locus on 11q23 and the \textit{BRCA1} locus on 17q21 (D17S855) were used (table 1).

For mutational analysis, the six exons of the \textit{RS1} gene were PCR amplified from genomic DNA of patients III.1 and III.2 with intronic oligonucleotide primers flanking the respective coding exons and amplification conditions as described previously.\(^1\) Mutation detection was done by single stranded conformational analysis (SSCA). Amplification of the coding exons was carried out with \textit{Taq} polymerase (Gibco BRL) in a 25 µl volume in 1 × PCR buffer supplied by the manufacturer. PCR products were electrophoretically separated on a 6% non-denaturing polyacrylamide gel with or without 5% glycerol at 4°C. DNA fragments showing aberrant mobility shifts as well as the corresponding maternal and grandparental PCR products were directly sequenced using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham, Life Science).

Prescreening by SSCA of the six coding exons of the \textit{RS1} gene showed a similar aberrant band shift in exon 6 in the two brothers III.1 and III.2 (fig 1 and data not shown). Direct sequencing of PCR products identified a C to T transition at nucleotide position 608 of the cDNA. This is predicted to result in a proline to leucine substitution at codon 203 (fig 1). Subsequently, sequencing of \textit{RS1} exon 6 was performed in the mother, II.1, as well as in both materi-
Haplotype analysis has shown that the maternal grandfather of the two Greek RS patients carries the haplotype that becomes disease associated in his daughter and his two grandsons. This provides strong evidence that the Pro203Leu mutation is in fact a de novo event. It should be pointed out that the Pro203Leu mutation occurred at a CpG dinucleotide (codon 203: CCG to CTG) which, if methylated at the genomic level, is known to be frequently involved in C→T transitions.16 We cannot exclude that the unaffected grandfather is a mosaic for the Pro203Leu mutation with the mutant genotype being present in one or more tissues, excluding the ocular tissues but including a precursor of the germ cells. Assuming such a situation in the grandfather, the mutation could be transferred to his daughter and would then be perceived as a de novo germinal mutation.

Besides the Greek family, we were able to analyse the segregation of RS1 mutations in another four pedigrees where RS occurred in a single generation of large families. However, considering the small number of families tested, the present study supports an earlier notion that the new mutation rate in RS may be significant.17 Further segregation analyses in multigeneration families with “sporadic” or only a few cases of RS will be required to estimate more accurately the frequency of de novo mutations in X linked juvenile retinoschisis.

We thank the patients and their family for their kind cooperation. This work was supported by the Deutsche Forschungsgemeinschaft (We 1259/5-3 and Lo 457/3-1).

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**Figure 1** Analysis of a three generation Greek family with two cases of X linked juvenile retinoschisis (III.1 and III.2). Polymorphic markers at the ATM and the BRCA1 locus (D17S855) were used to confirm paternity. Haplotype analysis was performed using microsatellite markers DXS207, 389gt, DXS418, and DXS443 that closely flank the RS1 gene on Xp22.2. The order of markers is from telomere to centromere. Haplotypes associated with the Pro203Leu mutation in exon 6 of the RS1 gene are boxed. Note that the grandfather, I.2, shares the disease associated haplotype with his two grandsons III.1 and III.2 but does not carry the Pro203Leu mutation.

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The analysis of the two Greek brothers, I.1 and I.2, showed that the maternal grandfather, I.2, is responsible for the RS phenotype in the two Greek brothers. Although the mother is a heterozygous carrier neither of the maternal grandparents have the Pro203Leu mutation. Haplotype analysis with polymorphic markers closely flanking the RS1 locus provides strong evidence that the Pro203Leu mutation occurred at a CpG dinucleotide (codon 203: CCG to CTG) which, if methylated at the genomic level, is known to be frequently involved in C→T transitions.16 We cannot exclude that the unaffected grandfather is a mosaic for the Pro203Leu mutation with the mutant genotype being present in one or more tissues, excluding the ocular tissues but including a precursor of the germ cells. Assuming such a situation in the grandfather, the mutation could be transferred to his daughter and would then be perceived as a de novo germinal mutation.

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Pathogenicity of homoplasmic mitochondrial DNA mutation and nuclear gene involvement

Editor—Seneca et al reported a homoplasmic deletion of a T nucleotide in a 5T stretch (15 940-15 944 base pairs (bp)) of mitochondrial DNA (mtDNA) in two families associated with clinical and pathological findings of mt cytopathy. Although this deletion was homoplasmic and did not fulfil the classical criteria of pathological mutation, Seneca et al suggested that it was pathological, as they could not identify any other heteroplasmatic mutations, deletions, or duplications in tRNA genes of mtDNA in these patients. However, this mutation was present not only in affected patients but also in asymptomatic relatives in both families. Therefore, this mutation does not cosegregate with the disease. It is difficult to confirm whether homoplasmatic mutations are pathological, as was recently indicated by Chinnery et al. There are currently no concrete criteria to determine what kind of homoplasmic mtDNA abnormalities are pathological. Maternal inheritance is an important characteristic to confirm their pathogenicity, which, however, was not significant in these two families. The mode of inheritance of this deletion is difficult to confirm, as it is currently unknown whether the single nucleotide deletion is inherited maternally like mtDNA point mutations. It is possible that it is inherited autosomal dominantly like mtDNA deletions. In such cases, cosegregation of the mutation in affected family members is important to determine its pathogenicity. A population based association study is another method for confirming significant role of homoplasmic or heteroplasmic mtDNA mutations. The association should also be confirmed by other studies on the same and different ethnic groups.

By directly sequencing a mutation hot spot of mtDNA (3130-3423 bp) from 30 patients with type 2 diabetes mellitus (DM), we identified a G3316A homoplasmic mutation. The prevalence of this mutation was significantly higher in patients with glucose intolerance than in those with normal glucose tolerance. This missense mutation in the ND-1 gene, which substitutes alanine for threonine, was present at an increased frequency in patients with type 2 DM compared with non-diabetic subjects in other studies in Japanese or European populations. The same mutation was also identified in a patient through screening patients with hypertrophic cardiomyopathy, suggesting a role of this homoplasmic mutation in the development of mt cytopathy (manuscript in preparation). Although homoplasmic mtDNA mutations do not fulfil the classical criteria for pathogenicity, another recent study indicated that homoplasmic mutations are significantly associated with type 2 DM (p=0.019). The findings suggest that the homoplasmic mutations are also of pathological importance in mt cytopathy. Investigations on Leber’s hereditary optic neuropathy (LHON) suggest a role of the nuclear gene in the pathogenesis of clinical symptoms of mt cytopathy. Previous investigations, however, failed to identify any nuclear gene abnormalities in patients with mt cytopathy. We consider that homoplasmic mutations are also important in the development of mt cytopathy, as nuclear DNA may be involved in its pathogenesis.

Concerning the A3243G mutation, we suspect that nuclear gene abnormalities may be responsible for the different clinical phenotypes of type 2 DM or MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) associated with the same A3243G mutation. Recent investigations indicate that nuclear encoded gene mutations are associated with Leigh syndrome. These observations highlight the importance of nuclear gene-mtDNA interaction in the pathogenesis of mt dysfunction. They also suggest that homoplasmic mtDNA mutations are important in developing mt cytopathy, such as mt myopathies, diabetes mellitus, or cardiomyopathies. Although difficulties exist in confirming a pathogenic role of homoplasmic mtDNA mutations, some homoplasmic mutations are probably associated with mt dysfunction causing mt cytopathy. We propose that investigations of mtDNA abnormalities in patients with mt dysfunction should include homoplasmic mutations which cosegregate with clinical or pathological manifestations of mt cytopathy or are present with an increased frequency in affected patients.
Identification and clinical presentation of β thalassaemia mutations in the eastern region of Saudi Arabia

EDITOR—The autosomal recessive disease β thalassaemia is a common single gene disorder that poses a serious health problem in many parts of the world. According to the Human Gene Mutation Database (http://www.uwcm.ac.uk/uwcm/mg/hgmd.html) and the β-Globin Gene Server (http://globin.cse.psu.edu) about 300 sequence variants in the β-globin gene have been identified up to the present. Mutations in the β-globin gene have been found at carrier frequency rates ranging from 1% in some areas of Saudi Arabia to 15% in others. Both β° and β' thalassaemia have been reported. Studies on the molecular pathogenesis of β thalassaemia have shown that the mutations encountered in Arab countries close to the Mediterranean basin are the same as those reported in other Mediterranean populations. In the Gulf region, in Saudi Arabia, UAE, and Iraq, the Asian pattern of mutations seems to be prevalent. The precise genetic changes prevalent in the different regions of the large country of Saudi Arabia and analysis of the genotype/phenotype relationship of the disease in Saudi patients still remain inadequately studied.

The present study aimed to investigate the mutational pattern of the β globin gene and to explore the relationship between these mutations and disease presentation in a group of patients with β thalassaemia major from the eastern region of Saudi Arabia. For this purpose, 31 children diagnosed with β thalassaemia major who over the past two years had regularly attended the paediatric clinics of Qatif Central Hospital or Dammam Maternity and Children Hospital were selected. Within this group of patients there were four pairs of sibs and one pair of first cousins. The whole β globin gene of all patients was amplified using standard PCR techniques and six specially designed different primers for amplification and sequencing. Nucleotide sequencing was performed by electronjection of the PCR products into an automatic capillary ABI Prism Genetic Analyzer type 310 (Perkin-Elmer, USA).

Results of the nucleotide sequencing have enabled accurate identification of disease causing mutations both in the homozygous and heterozygous states in each of the 31 patients diagnosed with β thalassaemia major. In total, eight disease causing mutations were detected: IVS-2-2CD8(-AA), 8 IVS-2+1(G→A),6 IVS-2+1(G→A), 6 −1CD44(−C), 11 +1CD8/9(+G), 11 IVS-1+5(G→C), 11 and IVS-1+5(G→T) 12 that comprised allele frequencies of 32.1%, 22.6%, 15.1%, 7.5%, 3.8%, 1.9%, and 1.9%, respectively. An overall β thalassaemia detection rate of 100% was achieved (tables 1 and 2), thus reflecting the efficiency of the technique. The accuracy of the genetic analysis has a special diagnostic importance in view of the fact that certain haemoglobinopathies, for example, sickle cell anaemia, spherocytosis, and autoimmune haemolytic disease, are known to produce symptoms that mimic those of β thalassaemia major. The silent polymorphic mutation CD2(CAC→GAT) was very frequent among this group of children as it was encountered in 16 out of 31 patients with β thalassaemia major. On the other hand, the polymorphisms IVS-2+26 (T→G) and IVS-2+74 (G→T) were less common and detected in only two patients each (Nos 29 and 37 and 17 and 45, respectively). A fourth polymorphism IVS-2+666 (T→C) was also detected in two patients (Nos 17 and 45). These sequence variants are not shown in table 2.

The spectrum of mutations identified here confirms the notion that, for historical reasons, there is an overlap between Mediterranean and Asian mutations in Saudi Arabia. Five of the eight mutations detected here have been reported to exist among patients from other parts of the country, albeit with different allele frequencies. We report here the novel identification in the Saudi population of three mutations: the Turkish frameshift mutation −2CD8(−AA), 4 the Kurdish frameshift mutation

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Type of mutation</th>
<th>Nucleotide change</th>
<th>Type of thalassaemia</th>
<th>Independent alleles</th>
<th>Origin of mutation</th>
<th>Original reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD39(C→T)</td>
<td>Nonsense</td>
<td>Substitution C→T</td>
<td>β° thalassaemia</td>
<td>17 32.1%</td>
<td>Mediterranean, European</td>
<td>6</td>
</tr>
<tr>
<td>IVS-1 3' end-25bp</td>
<td>Splice junction frameshift</td>
<td>Deletion of 25 bp</td>
<td>β° thalassaemia</td>
<td>12 22.6%</td>
<td>Asian, Indian</td>
<td>7</td>
</tr>
<tr>
<td>IVS-2+1(G→A)</td>
<td>Splice junction change</td>
<td>Deletion of 2A</td>
<td>β° thalassaemia</td>
<td>8 15.1%</td>
<td>Turkish</td>
<td>8</td>
</tr>
<tr>
<td>−1CD44(−C)</td>
<td>Frameshift</td>
<td>Deletion of C</td>
<td>β° thalassaemia</td>
<td>4 7.5%</td>
<td>Mediterranean, Tunisian, African-American,</td>
<td>9</td>
</tr>
<tr>
<td>+1CD8/9(+G)</td>
<td>Frameshift</td>
<td>Insertion of G</td>
<td>β° thalassaemia</td>
<td>2 3.8%</td>
<td>Asian, Indian</td>
<td>11</td>
</tr>
<tr>
<td>IVS-1+5(G→C)</td>
<td>Consensus change</td>
<td>Substitution G→C</td>
<td>β° thalassaemia</td>
<td>1 1.9%</td>
<td>Indian, Chinese, Melanesian</td>
<td>11</td>
</tr>
<tr>
<td>IVS-1+5(G→T)</td>
<td>Consensus change</td>
<td>Substitution G→T</td>
<td>β° thalassaemia</td>
<td>1 1.9%</td>
<td>Mediterranean, Blacks</td>
<td>12</td>
</tr>
</tbody>
</table>

Calculation of allele frequency:

There are 31 patients with β thalassaemia major. No of alleles = 31 × 2 = 62 alleles.
There are 4 pairs of sibs; hence 62−8 = 54 alleles.
Subtract 1 allele for first cousin = 53 independent alleles.
Detection rate = 53/53×100 = 100%.
Patients 1+2, 3+4, 13+14, 21+22 are sibs. Patient 5 is a first degree cousin of patients 3 and 4. The mutation IVS-1+5(G→T) in Saudi patients from the neighbouring Sultanate of Oman.16 Interestingly, and by contrast, this mutation has recently been reported as the most common mutation in Saudi Arabia. In the clinical management of patients with β thalassaemia, the mutations apparently did not confer protection against the adverse phenotypic expression of the disease. A possible explanation is that the two mutations are encountered here in the compound heterozygous state with CD39(C→T), which is known to cause severe thalassaemia. However, we have detected the rare Asian Indian mutation +1CD8(-G) in the compound heterozygous state in two patients. It is likely that the Indian mutation may have been introduced to this region by gene flow from Turkey, Iran, and the Indian subcontinent. The introduction of the Mediterranean/Black mutation in Saudi Arabia can be explained by a similar historical reason, that is, gene flow facilitated by trade and expansion of Islam in the Mediterranean region and in Africa. Similarly, evidence of a common ethnic origin of the cystic fibrosis mutation 3120+1G→A in Arab, African, Greek, and African-American populations has recently been reported.15 However, we have detected the rare Asian Indian mutation +1CD8(-G) in the compound heterozygous state in two patients. It is likely that the Indian mutation may have been introduced to this region by gene flow since the mutation has recently been identified in patients from the geographically close island of Bahrain,14 which is a known historical centre for international trade between Arabia, India, and Asia. Similarly, we have found that the splice junction frameshift mutation IVS-1 3’end-25bp, first reported in Asia and India, was the second most prominent mutation with an allele frequency of 22.6% in this group of patients. This mutation has recently been reported as the most common mutation in Bahrain with the highest allele frequency of 40%.14 Therefore, all these findings provide genetic evidence that the eastern coast of Saudi Arabia, unlike the inner desert parts of the Arabian Peninsula, was particularly prone to gene flow from Turkey, Iran, and the Indian subcontinent. The most frequent mutation in the β globin gene detected in this study is the Mediterranean CD39(C→T) mutation with an allele frequency of 32.1%. This mutation has recently been reported to have an even higher allele frequency in Libya, where the mutation, together with IVS-1+6 (T→C) and IVS-1+110 (G→A), comprised an allele frequency of more than 90% in Libyan patients.15 The mutation IVS-1+5(G→C) is a known Asian mutation and has been detected in only one patient in this study. Interestingly, and by contrast, this mutation has recently been found to have the highest allele frequency of 61% in patients from the neighbouring Sultanate of Oman.16

The clinical presentation of β thalassaemia major in this group of children was mostly severe. Most patients suffered from failure to thrive leading to delayed puberty, increased plasma volume, pallor, lethargy, haemochromatosis, hepatosplenomegaly, and jaundice. Less common presentations of the disease noticed in this region of the country included cardiomegaly, repeated infections (such as pneumonia, peritonitis, and meningitis), deformity of the facial bones and teeth, osteoporosis, liver cirrhosis, ascites, and diabetes mellitus. In the clinical management of patients with β thalassaemia, blood transfusion comes second after bone marrow transplantation as the most delicate, laborious, and costly management of the disease. Therefore, for the sake of simplification, the presentation of the disease in this study is classified as severe, moderate, or mild if the number of annual blood transfusions needed is more than six, between six and three, or between two and none, respectively. Hence, 25 patients (81%) presented with a severe form, four patients (13%) with a moderate form, and only two patients (6%) presented with a mild form of the disease. The genotype/phenotype analysis indicated that the mutations CD39(C→T) and -1CD44(-C) in the homozygous state were consistently associated with severe presentation of β thalassaemia major. However, we observed variability of disease presentation from severe to moderate and mild caused by the mutations IVS-2+1(G→A), IVS-1 3’end-25bp and -2CD8(-AA) in the compound heterozygous state. It is possible that the mutation IVS-1+5(G→T) and -1CD44(-C) may alter the allele frequency in patients with the same genotype may well find its basis in the number of active α globin genes. Genetic analysis of the α globin gene has not been performed in this study. In two patients who are carriers of the mutations IVS-1+5(G→C) and IVS-1+5(G→T), known to cause β’ thalassaemia, the mutations apparently did not confer protection against the adverse phenotypic expression of the disease. A possible explanation is that the two mutations are encountered here in the compound heterozygous state with CD39(C→T), which is known to cause a severe form of the disease.

In summary, we report the first identification of three mutations, the Turkish frameshift mutation -2CD8(-AA), the Kurdish frameshift mutation -1CD44(-C), and the Mediterranean/Black mutation IVS-1+5(G→T) in patients with β thalassaemia major from the eastern region of Saudi Arabia. Documentation of the spectrum of β thalassaemia mutations could facilitate national screening and educational programmes which would be important with respect to the problem of the haemoglobinopathies in this region.

We are grateful to Dr Samia Flimban of the Maternity and Children Hospital in Dammam for supporting this study. Our thanks are extended to Miss Michaela Finsel for her assistance in the preparation of the manuscript. We gratefully acknowledge the financial sponsorship of this work by the Alexander von Humboldt-Stiftung, Bonn (Bad Godesberg), Germany. We are grateful to Dr Samia Flimban of the Maternity and Children Hospital in Dammam for supporting this study. Our thanks are extended to Miss Michaela Finsel for her assistance in the preparation of the manuscript. We gratefully acknowledge the financial sponsorship of this work by the Alexander von Humboldt-Stiftung, Bonn (Bad Godesberg), Germany.

### Table 2 The genotype, mean clinical indices, and disease presentation of Saudi children diagnosed with β thalassaemia major

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Genotype</th>
<th>Mean Hb A(1) (%)</th>
<th>Mean age at diagnosis (y)</th>
<th>Mean annual No of blood transfusions</th>
<th>Disease presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3, 4, 5, 6, 15 &amp; 30</td>
<td>CD39 (C→T) / CD39 (C→T)</td>
<td>3.3</td>
<td>0.8</td>
<td>12.8</td>
<td>All 8 severe</td>
</tr>
<tr>
<td>17</td>
<td>CD39 (C→T) / IVS-1 +5 (G→C)</td>
<td>2.5</td>
<td>0.3</td>
<td>12.0</td>
<td>Severe</td>
</tr>
<tr>
<td>26</td>
<td>CD39 (C→T) / IVS-1 +5 (G→T)</td>
<td>2.7</td>
<td>2.0</td>
<td>10.0</td>
<td>Severe</td>
</tr>
<tr>
<td>19 &amp; 45</td>
<td>CD39 (C→T) / IVS-2 +1 (G→A)</td>
<td>2.0</td>
<td>4.0</td>
<td>9.0</td>
<td>1 severe, 1 moderate</td>
</tr>
<tr>
<td>18</td>
<td>CD39 (C→T) / +1 CD8(-G) (+G)</td>
<td>4.0</td>
<td>5.0</td>
<td>12.0</td>
<td>Severe</td>
</tr>
<tr>
<td>32</td>
<td>CD39 (C→T) / IVS-1 3’end-25bp</td>
<td>3.8</td>
<td>1.3</td>
<td>12.0</td>
<td>Severe</td>
</tr>
<tr>
<td>8, 20, 21, 22, 23 &amp; 31</td>
<td>IVS-1 3’end-25bp / IVS-1 3’end-25bp</td>
<td>3.6</td>
<td>2.7</td>
<td>8.7</td>
<td>4 severe, 2 mild</td>
</tr>
<tr>
<td>44</td>
<td>IVS-1 3’end-25bp / +1 CD8(-G) (+G)</td>
<td>1.0</td>
<td>3.0</td>
<td>12.0</td>
<td>Severe</td>
</tr>
<tr>
<td>7, 10, 35 &amp; 36</td>
<td>-2CD8 (-AA) / -2CD8 (-AA)</td>
<td>2.3</td>
<td>3.3</td>
<td>10.0</td>
<td>3 severe, 1 moderate</td>
</tr>
<tr>
<td>29, 34 &amp; 37</td>
<td>IVS-2 +1 (G→A) / IVS-2 +1 (G→A)</td>
<td>2.0</td>
<td>4.3</td>
<td>7.3</td>
<td>2 moderate, 1 severe</td>
</tr>
<tr>
<td>13, 14 &amp; 33</td>
<td>-1CD44 (-C) / -1CD44 (-C)</td>
<td>4.5</td>
<td>0.4</td>
<td>12.0</td>
<td>All 3 severe</td>
</tr>
</tbody>
</table>

Patients 1+2, 3+4, 13+14, 21+22 are sibs. Patient 5 is a first degree cousin of patients 3 and 4.

-1CD44(-C), and the Mediterranean/Black consensus mutation IVS-1+5(G→T). It is highly unlikely that these mutations arose independently in the Saudi population. On the contrary, there is historical evidence that the Arabian, Turkish, and Kurdish populations have interacted in the past through trade and the introduction of Islam in Turkey. The introduction of the Mediterranean/Black mutation in Saudi Arabia can be explained by a similar historical reason, that is, gene flow facilitated by trade and expansion of Islam in the Mediterranean region and in Africa. Similarly, evidence of a common ethnic origin of the cystic fibrosis mutation 3120+1G→A in Arab, African, Greek, and African-American populations has recently been reported. However, we have detected the rare Asian Indian mutation +1CD8(-G) in the compound heterozygous state in two patients. It is likely that the Indian mutation may have been introduced to this region by gene flow since the mutation has recently been identified in patients from the geographically close island of Bahrain, which is a known historical centre for international trade between Arabia, India, and Asia. Similarly, we have found that the splice junction frameshift mutation IVS-1 3’end-25bp, first reported in Asia and India, was the second most prominent mutation with an allele frequency of 22.6% in this group of patients. This mutation has recently been reported as the most common mutation in Bahrain with the highest allele frequency of 40%. Therefore, all these findings provide genetic evidence that the eastern coast of Saudi Arabia, unlike the inner desert parts of the Arabian Peninsula, was particularly prone to gene flow from Turkey, Iran, and the Indian subcontinent. The most frequent mutation in the β globin gene detected in this study is the Mediterranean CD39(C→T) mutation with an allele frequency of 32.1%. This mutation has recently been reported to have an even higher allele frequency in Libya, where the mutation, together with IVS-1+6 (T→C) and IVS-1+110 (G→A), comprised an allele frequency of more than 90% in Libyan patients. The mutation IVS-1+5(G→C) is a known Asian mutation and has been detected in only one patient in this study. Interestingly, and by contrast, this mutation has recently been found to have the highest allele frequency of 61% in patients from the neighbouring Sultanate of Oman.

The clinical presentation of β thalassaemia major in this group of children was mostly severe. Most patients suffered from failure to thrive leading to delayed puberty, increased plasma volume, pallor, lethargy, haemochromatosis, hepatosplenomegaly, and jaundice. Less common presentations of the disease noticed in this region of the country included cardiomegaly, repeated infections (such as pneumonia, peritonitis, and meningitis), deformity of the facial bones and teeth, osteoporosis, liver cirrhosis, ascites, and diabetes mellitus. In the clinical management of patients with β thalassaemia, blood transfusion comes second after bone marrow transplantation as the most delicate, laborious, and costly management of the disease. There-
Rapid screening for the most common \(\beta\) thalassaemia mutations in south east Asia by PCR based restriction fragment length polymorphism analysis (PCR-RFLP)

EDITOR—The heterogeneity of the molecular lesions which underlie the failure of erythropoietic cells to synthesise normal haemoglobin in \(\beta\) thalassaemia is a complicating factor in its molecular diagnosis. However, although more than 100 different mutations have been identified, mostly single base substitutions or small deletions and insertions in the \(\beta\) globin gene, in many populations the bulk of \(\beta\) thalassaemia is caused by a population specific spectrum of only a small number of mutations. The strategy for the detection of mutations in patients, therefore, normally involves screening in the first instance for a small number of mutations that are the most common for the population concerned.

Two of the most commonly used screening procedures are dot blot or reverse dot blot hybridisation and the amplification refractory mutation system (ARMS). While these procedures are satisfactory in the hands of the more experienced specialised laboratories, the exacting conditions required for the performance of allele specific hybridisation or PCR amplification steps have made them less reproducible in less advanced laboratories. In south east Asia, where in some regions the frequency of \(\beta\) thalassaemia can be as high as 10%, many laboratories in medium sized provincial hospitals and universities are suitably equipped for routine molecular biology laboratory testing, but do not have the skill to comply with the strict conditions demanded by the above procedures, a situation which is perhaps shared by many other countries in which \(\beta\) thalassaemia is common. We have endeavoured, therefore, to develop a more robust and accurate alternative method for the detection of \(\beta\) thalassaemia mutations in south east Asia, based on restriction endonucleases that recognise naturally occurring or PCR-generated restriction sites associated with the \(\beta\) thalassaemia mutations. The approach is widely used in the detection of pathological mutations in mitochondrial DNA and has also been applied in the molecular diagnosis of \(\beta\) thalassaemia.

A strategy has been devised to allow rapid detection of nine of the most common mutations in south east Asia which requires the amplification of two segments only of the \(\beta\) globin gene. The mutations are at positions IVS-1 nt5, IVS-1 nt1, codon 26, codon 15, codon 17, codon 19, codon 30, IVS-1 nt2, and codon 41-42 of the \(\beta\) globin gene (fig 1 (top), table 1), which together account for around 70-90% of \(\beta\) thalassaemia mutations in most populations of south east Asia.

The PCR amplifications use primer sets TLF62028-TLR62320 and TLF62392-TLR62703. Primer TLF62320 includes a G at the position equivalent to nt8 of intron 1 instead of the normal A to create a GCTAGC site for Cac8I in the presence of the IVS-1 nt5 G>C mutation. A Cac8I site is also created in the presence of the IVS-1 nt2 T>C mutation which represents less than 1% of the \(\beta\) thalassaemia alleles in Indonesia. The mutations G to T at IVS-1 nt1, G to A at codon 26, and A to G at codon 19 abolish the natural occurring sites for BsaI, MnlI, and MaeIII, respectively. The mutations G to A at codon 15, A to T at codon 17, and G to C at codon 30 create sites for SfiI, BglI, and Bsp126I, respectively. The detection of the 4 bp deletion of codon 41-42 is essentially

Table 1  PCR primers and restriction endonucleases used in the detection procedure

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer sets*</th>
<th>Mutation</th>
<th>Restriction endonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal Mutant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>TLF62028-TLR62320</td>
<td>IVS-1 nt 5</td>
<td>Cac8I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IVS-1 nt1</td>
<td>BstI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 26</td>
<td>MnlI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 15</td>
<td>SfiI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 17</td>
<td>BglI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 19</td>
<td>MaeIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 30</td>
<td>Bsp126I</td>
</tr>
<tr>
<td></td>
<td>TLR62392-TLR62703</td>
<td>IVS-1 nt 2</td>
<td>Cac8I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Codon 41</td>
<td>TaqI</td>
</tr>
</tbody>
</table>

*The primers used were: TLF62028-5'ACCCTCACCCCTGGAGCCAC3' (common C in Old et al); TLR62320-5'CTATTGGTCCTCTAAACCTGCTT GTAACCTTGTA3'; TLF62392-5'TATTTTCCACCCCTAGGGTCTGGTGCTAACCCTT GGACCAGAGCTC3'; TLR62703-5'CCTCTCCTAT GACATGAACCTTA3'; modifications in nucleotide sequence are indicated by bold letters.

†Bold numbers indicate fragments of distinguishable sites.
as described by Chang et al; a C has been introduced at the second position of codon 41 in the sequence of primer TLF62392, which together with the 4 base deletion creates a TCGA site for TaqI.

As shown in fig 1 (A-D) the method produces unambiguous results. Thus, for the detection of the IVS-1 nt5 mutation, the normal allele (indicated by a 293 bp undigested product) can be distinguished readily from the mutant allele (a 257 bp digested fragment). Heterozygosity could be easily detected (fig 1A). Similarly, the 122 bp fragment of the mutant allele could be distinguished from the 60 and 62 bp fragments of the normal allele among the digestion fragments of MnlI in the detection of the HbE codon 26 mutation (fig 1B). Definitive electrophoretic patterns were also obtained in the detection of IVS-1 nt1 (fig 1C), codon 41-42 (fig 1D), codon 15 (fig 1E), codon 19 (fig 1F), and IVS-1 nt2 (fig 1G) mutations. Similar results were obtained in the detection of codon 17 and codon 30 mutations (data not shown). In all the above cases, the PCR-RFLP results agreed with those of ARMS and were confirmed by DNA sequencing.

The detection procedure described here does not involve an allele specific hybridisation or an allele specific PCR amplification step and is thus less prone to non-specific reactions which could lead to false positive/negative results. We have applied our new procedure to the detection of the underlying mutations in a number of difficult samples in which ARMS gave ambiguous results. In all cases the PCR-RFLP method proved to be more reliable and gave definitive identification of the underlying mutation, as confirmed by DNA sequencing data (data not shown). The nine mutations included here account for 70% of β thalassaemia alleles in Thailand, 90% in Malaysia, 53% in India, and 68-90% in Indonesia depending on the ethnic population. The procedure, therefore, is suitable as the front line screening for the molecular diagnosis of β thalassaemia in south-east and perhaps also in eastern and southern Asia.
Clinical and molecular findings in a patient with a deletion on the long arm of chromosome 12

Editor—Reports of congenital abnormalities resulting from deletions on the long arm of chromosome 12 are rare.1,4 A number of genes have been mapped to 12q, which include a gene for Noonan syndrome (NS) in some families1 and the gene for insulin-like growth factor I (IGF-I).4 The patient presented here was referred for assessment regarding the diagnosis of NS in view of her short stature, dysmorphic facies, and developmental delay and was found to have a de novo interstitial deletion on chromosome 12q. Despite some of the similar clinical findings to NS in this patient, molecular analysis showed that the deletion mapped approximately 17 cM centromeric to the critical region for NS. To our knowledge, there have been no similar cases of interstitial deletion reported on chromosome 12q.

The patient, a 15 year old girl was the first born child to a 23 year old mother and an unrelated 21 year old father. Her younger brother, aged 11 years, was fit and well. The family history was unremarkable. The pregnancy was normal. She was born at term as an emergency caesarean section for fetal distress. Her birth weight was 2860 g. She was admitted to the special care baby unit for 24 hours for observation. The neonatal period was complicated by pyloric stenosis for which surgery was carried out on day 16. During the postoperative period, she developed severe candidiasis and lactose intolerance. At the age of 1 year she was challenged with milk and seemed to have recovered from the intolerance. Developmentally she was able to sit unsupported at the age of 8 months and walked independently at the age of 2 years. Her speech was delayed and she was unable to say two words together until the age of 3 years. She has had no visual or hearing problems. Her height has always been below the 3rd centile. At the age of 10 years, just as she was entering puberty, she was diagnosed as having growth hormone deficiency. Her peak growth hormone level on glucagon stimulation test was 7 mU/l. She was started on treatment with growth hormone and she showed a good response with sustained growth over a three year period. She had no other history of bleeding problems. Physical examination at the age of 15 years indicated that her height and weight were just below the 3rd centile and her head circumference was on the 3rd centile. She had ptosis, hypertelorism, and low set and posteriorly rotated ears (fig 1). Her hair was thin, fine, and sparse. Her posterior hairline was trident and low. Her neck was short with mild webbing. Cardiac examination showed a grade II ejection systolic murmur at the second left intercostal space. Echocardiogram showed normal dimensions and arrangements of the cardiac chambers and heart valves. The velocity of blood flow in the proximal pulmonary artery was slightly increased and this was thought to be the origin of the systolic murmur. Neurological examination was normal, but she had relatively poor coordination. Coagulation studies, including intrinsic clotting factor assays, were normal. Growth hormone secretion was normal with a peak response of 40.7 mU/l during the insulin tolerance test. Basal levels of IGF-I were normal (IGF-I 371 ng/ml, normal range 70–420 ng/ml).

Chromosome analysis by GTG banding was carried out on peripheral lymphocyte cultures. This showed an interstitial deletion of chromosome 12 (fig 2), karyotype 46,XX,del(12)(q21.2q23.2). Parental chromosomes were normal. FISH studies were not performed.

Venous blood was sampled from the patient, her normal brother, and her unaffected parents and genomic DNA was isolated. Seventeen microsatellite markers (D12S291, D12S96, D12S90, D12S335, D12S313, D12S80, D12S92, D12S337, D12S88, D12S81, D12S82, D12S101, D12S218, D12S346, IGF-I, D12S78, D12S84) of chromosome 12q were analysed. PCR amplification of 50 ng genomic DNA was performed in a 15 µl reaction mixture containing 30 ng of each primer, 200 µmol/l dNTPs, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 10 mmol/l Tris-HCl, 0.01% gelatin, 0.1% Triton X-100, and 0.1 U

Figure 1 Photographs of the patient at the age of 15 years showing (A) hypertelorism and ptosis and (B) low set, posteriorly rotated ears. (Photographs reproduced with permission.)

Figure 2 Chromosomes 12 of the patient showing her normal (left) and deleted (right) chromosomes 12 and an ideogram of chromosome 12 (centre). The breakpoints are indicated by arrows on the ideogram.
SuperTaq DNA polymerase (HT Biotechnology Ltd). In all reactions, 30 cycles (one minute at 94°C, two minutes at 55°C, and two minutes at 72°C with a final seven minute elongation step) were carried out in an automated thermal cycler (Hybaid). The amplified products were separated by electrophoresis on 10% polyacrylamide gels. The gels were silver stained and dried. Haploinsufficiency of markers D12S337, D12S88, D12S82, and D12S101 was found. Haplotype analysis indicated that the deletion was paternal in origin and mapped between markers D12S313 and D12S218. The deletion represented a minimum region of about 18 cM positioned approximately 17 cM proximal to D12S218. The deletion showed that the deletion was proximal to D12S218 and about 18 cM positioned approximately 17 cM proximal to the critical region for NS (fig 3).

We have reported a girl with short stature, dysmorphic facies, developmental delay, and a de novo interstitial deletion on chromosome 12q. Interstitial deletions of chromosomes 12q are rare. There have only been three previous cases reported to our knowledge with deletions on chromosome 12q, a male with a deletion (12)(q13.3q21.1),1 a female with a deletion (12)(q15q21.2),2 and a male with a deletion (12)(q12q13.12).3 A female with a derivative chromosome 9 and a recombinant chromosome 12 resulting from a balanced complex rearrangement involving chromosomes 8, 9, and 12 was suspected of having a submicroscopic deletion of chromosome 12q.

Common characteristics between our case and the reported cases were hypertelorism,1–4 posteriorly rotated ears,1–3 sparse, fine hair,2–4 developmental delay,1–4 growth retardation, and pectus deformity (table 1).1–4 From the UK cytogenetic databases, a male patient with a deletion on chromosome 12, del(12)(q21.33q24.1), had been seen by the clinical geneticists in Edinburgh. He was found to have marked developmental delay, short stature, hydrocephalus, prominent eyes, and long, thin fingers. He did not have any facial features of NS and no congenital heart defect (Dr David Fitzpatrick, Department of Clinical Genetics, Western General Hospital, Edinburgh, personal communication). Isolated deficiency of IGF-I, the gene for which has been mapped to chromosome 12q22-24.1,5 has been found in pygmies of the Central African Republic.6 In view of our patient’s short stature, dysmorphic facies, and increased pulmonary valve blood flow, it was suspected that the deletion might involve either the IGF-I gene or the critical region for NS on chromosome 12q. Analysis using microsatellite markers showed that the deletion was proximal to the critical region for NS and also proximal to the IGF-I gene (fig 3), making either scenario unlikely. Some of the genes which lie in the area deleted are shown in fig 3. The patient reported by Tonoki et al7 had features similar to those found in NS and the authors suggested that the deleted region in their patient may contain a gene for NS. Similarly, the deleted segment in our patient may also contain a gene for NS. Since the deletions in these patients do not overlap, it would mean that three genes responsible for NS would have to be situated on chromosome 12q, a considerable distance apart. This seems an unlikely event. Although the deletion in our patient lies outside the critical region, it does overlap with that identified by Krauter et al.8

Figure 3 Results of analysis of microsatellite markers in the patient showing (A) genetic map of chromosome 12q with results of analysis of microsatellite markers (right) and their distance apart (left), and (B) haplotype in the patient and her family; the thick black bar represents area deleted. The names of the genes in the area deleted are given in Krauter et al. DEL=deleted, U=uninformative, HET=heterozygous. IFNg=interferon gamma, RAP1B=ras related protein, ATP2B1=human PMCA1, DCN=decorin, BTG1=B cell translocation gene 1, HAL=histidine ammonia lyase, IGF-I=insulin-like growth factor I.
Table 1 Clinical features reported in patients with interstitial deletions of chromosome 12q

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Meinecke and Meinecke¹</th>
<th>Watson et al²</th>
<th>Masuno et al³</th>
<th>Tonoki et al⁴</th>
<th>Present patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>46,XY,del(12)(q13.3q21.1)</td>
<td>46,XX,del(12)(q15q21.2)</td>
<td>46,XX,+r(12),+del(9)(9pter-9q34:12q15-12qter), +rec(12)(12pter-12q15::9q32-9qter)mat</td>
<td>46,XY,del(12)(q12q13.12)</td>
<td>46,XX,del(12)(q21.2q23.2)</td>
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<tr>
<td>Hypertelorism</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ptosis</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Posteriorly rotated ears</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short neck/webbed neck</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Low posterior hairline</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sparse, fine hair</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth retardation</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pectus deformity</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other features</td>
<td>Bilateral cleft lip and palate, upward slanting palpebral fissures, macrostomia, micrognathia, overlapping toes and rocker bottom feet, atrial and ventricular septal defects</td>
<td>Broad forehead, frontal bossing, flattened occiput, sunken eyes, beaked nose, thin upper lip, high arched palate, some syndactyly of toes, cutis marmorata</td>
<td>Patent foramen ovale, trigonocephaly, broad nasal root, triangular face, fifth finger clinodactyly, brain CT scan abnormal</td>
<td>Cleft palate, inguinal hernia, undescended testes, hypocalcaemia, iron deficiency anaemia, strabismus, downward slanting palpebral fissures, epicantus, short nose, anteverted nostrils, long philtrum, micrognathia, shield shaped chest, small hands and feet, scoliosis, clinodactyly, little fingers</td>
<td>Pyloric stenosis, slightly increased pulmonary artery blood flow</td>
</tr>
</tbody>
</table>

+ present, − absent.

region for NS, it is possible that genes in this region may regulate genes in the NS critical region. Interestingly, our patient has a relatively mild clinical phenotype despite the size of the deletion. This suggests that the region deleted in this patient may only contain a small number of critical developmental genes. Alternatively, the effects of the genes deleted in this patient may be compensated for by the corresponding genes present on the non-deleted chromosome 12.

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Amelogenesis imperfecta, sensorineural hearing loss, and Beau’s lines: a second case report of Heimler’s syndrome

EDITOR—Hearing loss owing to genetic causes has a reported prevalence of 1 in 1000 births and among these 15–30% are associated with other abnormalities, although only a small number are associated with oral and dental disorders.¹ Heimler et al² reported two sibs with a combination of sensorineural hearing loss, amelogenesis imperfecta, and nail abnormalities (McKusick No 234580). We describe a further case here and extend the phenotypic spectrum of this syndrome.

A 12 year old girl presented with a combination of unilateral sensorineural deafness and amelogenesis imperfecta. She was born at term following a normal pregnancy and had a birth weight of 3200 g (50th centile). There was no consanguinity within the family; her mother was aged 25 and her father was aged 29 at the time of conception. She had no major illnesses in the first years of life and all her developmental milestones were achieved within normal limits. At the age of 7, it was discovered that she had unilateral hearing loss and subsequent investigation showed that she had profound sensorineural deafness on the left, hearing on the right being normal. At the time her mother felt that she may have had reduced hearing in the left ear for about two years before the diagnosis was made. At the age of 8 years, there was evidence of extensive enamel discoloration in her permanent dentition and amelogenesis imperfecta was diagnosed. Her primary dentition was reported as having erupted on time and the remaining primary teeth had a normal appearance. There was no history of intellectual impairment and she was doing well in main-
stream education. Her family history was unremarkable; her parents were unrelated and there was no history of sensorineural deafness or enamel defects on either side of the family. She has one younger brother who is unaffected.

On examination she was on the 10th centile for height and the 25th centile for weight. She had a fair complexion (fig 1) and examination of her nails showed multiple transverse grooves (Beau’s lines) on several fingers and toes (fig 2A, B). There was no evidence of leuconychia. Examination of her teeth showed dental overcrowding and extensive enamel discoloration (fig 3). Ground sections of permanent premolars, extracted for orthodontic purposes, showed dentine and enamel of normal thickness. It seems likely therefore that the amelogenesis imperfecta is a hypomineralised rather than a hypoplastic variant.

The combination of sensorineural hearing loss, amelogenesis imperfecta, and nail abnormalities was first reported by Heimler et al. They suggested that the syndrome could be the result of a single gene affecting derivatives of the ectodermal tissue because the abnormalities described have a common embryological origin in the ectoderm. This theory is made more probable by the reporting of a second case here.

Heimler et al. described two sibs who both had profound bilateral sensorineural hearing loss, amelogenesis imperfecta of the permanent dentition, and Beau’s lines. Inheritance of this syndrome has been postulated to be autosomal recessive and our case does not allow us to draw any further conclusions on this. To date, no other cases of Heimler’s syndrome have been identified (A Heimler, personal communication). The case described here had unilateral sensorineural hearing loss, a hypomineralised form of amelogenesis imperfecta, and Beau’s lines and it is likely that this is a similar association to the originally described syndrome, which extends the phenotypic spectrum (table 1).

It is interesting that the hearing loss was diagnosed at a relatively late age and the mother’s observations suggest that it had previously been normal. In the original report, deafness was diagnosed in the second sib at the age of 2 1/2 years, hearing having been normal for the first two years. It thus seems that the hearing loss in this condition is not congenital.

Amelogenesis imperfecta is classified according to the predominant clinical and radiographic appearance of the enamel defect and on the mode of inheritance of the trait. The amelogenesis imperfecta described here is unusual in that only the permanent teeth are affected, which makes non-genetic causes less likely. The diagnosis of hypoplastic amelogenesis imperfecta in the original case report was

<table>
<thead>
<tr>
<th>Table 1: A comparison of phenotypes between the originally described cases and a new case of Heimler’s syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Sensorineural hearing loss</td>
</tr>
<tr>
<td>Primary dentition</td>
</tr>
<tr>
<td>Permanent dentition</td>
</tr>
<tr>
<td>Nail abnormalities</td>
</tr>
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</table>
made on radiographic evidence and a histopathological examination was not performed. The hypomimeralised amelogenesis imperfecta in our case illustrates the variation in phenotype that can occur in this condition.

Beau’s lines are transverse lines across the nails that can arise from severe illnesses, such as sepsis, AIDS, or bullous pemphigoid, and can also be physiological in menstruating women.4 They can be quite subtle as in this case where they only became apparent once nail varnish had been removed. Our patient had no evidence of punctate leuconychia which was described in the original family, which could suggest that this feature may have been an incidental finding in the original report, as it is a common normal variant. Our case also emphasises the importance of careful examination of the nails in patients with a combination of impaired hearing, which may only be unilateral, and dental pathology.

PTEN and LKB1 genes in laryngeal tumours

EDITOR—PTEN, a tumour suppressor gene located in chromosome 10q23, is homologous to tyrosine and dual specificity phosphatases with a high degree of substrate specificity. This enzymatic activity is necessary for PTEN/MMAC1 tumour suppressor function.1–4 Mutations of this specificity. This enzymatic activity is necessary for chromosome 10q23, is homologous to tyrosine and dual on colorectal cancer,13 have reported a low frequency of LKB1 are often targets of somatic mutations. In the case of the inactivating nature, often causing truncation of the protein

Our patient had no evidence of punctate leuconychia which was described in the original family, which could suggest that this feature may have been an incidental finding in the original report, as it is a common normal variant. Our case also emphasises the importance of careful examination of the nails in patients with a combination of impaired hearing, which may only be unilateral, and dental pathology.

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Table 1 Clinical data of the patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking</th>
<th>Diagnosis*</th>
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<td>Laryngeal carcinoma</td>
<td>T3N1M0</td>
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<tr>
<td>4</td>
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<td>16</td>
<td>4</td>
<td>F</td>
<td>No</td>
<td>Papilloma</td>
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*All laryngeal carcinomas were histologically squamous cell carcinomas.
†Neck metastasis.
‡With malignant transformation.
Direct sequencing of PCR products was performed with the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (Perkin-Elmer Applied Biosystems Division, Forster City, CA). Cycle sequencing products were analysed on an Applied Biosystems model 377 DNA sequencer (PE/ABI) or 310 Genetic Analyzer (PE/ABI).

Mutation analysis of the LKB1 gene was performed by single strand conformation polymorphism (SSCP) analysis. Primer sequences and PCR conditions used in this study have been described previously.19 After PCR, 5 µl of each reaction product was mixed with 5 µl of denaturing loading buffer (98% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured for five minutes at 94°C, and subjected to electrophoresis on 0.4 mm x 30 cm x 45 cm gels containing 6 × mutation detection enhancement solution (AT Biochem, Malvern, PA) and 0.6 × TBE buffer. Electrophoresis was conducted at 4 W overnight. PCR fragments were visualised in gels by silver staining. In order to validate the SSCP results, amplicon of exon 1 in the LKB1 gene from all samples were directly sequenced (as described above).

To determine whether the PTEN and LKB1 genes are mutated in laryngeal tumours, we screened 16 laryngeal tumour samples by genomic sequencing and SSCP assay, respectively. We performed direct sequencing of amplicon for PTEN mutation analysis; no mutations in the coding sequences and exon/intron boundaries were observed. To screen for LKB1 mutations, SSCP assay was applied in conjunction with PCR. Difference in mobility pattern on the SSCP assay usually suggests a variant, the nature of which should be examined by sequencing. In our hands SSCP analysis of LKB1 has always shown known mutations and it is reasonable to assume a 70% to 80% sensitivity, which is commonly reported for the method.19 To validate the SSCP results, exon 1 was directly sequenced from each sample. No mutations in exon 1 and the respective exon/intron boundaries were observed. The mutation detection methods used in this study would not have detected some mutation types, such as large genomic deletions or alterations in the promoter region. Yet the complete absence of changes indicates that somatic mutations of PTEN and LKB1 are not frequent in laryngeal tumours.

The molecular events which induce laryngeal tumorigenesis, especially laryngeal carcinogenesis, are not well characterised. Proto-oncogenes seem to be the target of the risk factors (cigarette smoking, alcohol abuse, ionising radiation, and human papillomavirus infection) that are commonly considered to be associated with laryngeal squamous cell carcinoma. Several tumour suppressor genes have been shown to play important roles in human tumours, including head and neck cancers. Mutations of the p53 gene are frequent events in primary squamous cell carcinomas of the head and neck as well as in SCCHN cell lines. While many cancer genes are associated with laryngeal tumorigenesis,20 our study suggests that PTEN and LKB1 are not among them.

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