Letters to the Editor

No evidence of germline PTEN mutations in familial prostate cancer

EDITOR—Prostate cancer is the second most common cause of male cancer mortality in the UK.1 Current indications are that like many common cancers, prostate cancer has an inherited component.2 Segregation analysis has led to the proposed model of at least one highly penetrant, dominant gene (with an estimated 88% penetrance for prostate cancer by the age of 85 in the highly susceptible population). Such a gene or genes would account for an estimated 43% of cases diagnosed at less than 55 years.2

One prostate cancer susceptibility locus (HPC1) has been reported on 1q24-253 and confirmed by Cooney et al4 and Gronberg et al.5 Latest estimates suggest that this locus would only account for 4% of families overall in the UK (upper 95% confidence interval (CI) limit of 31%).6 Another locus has been reported on 1q42.2-43 after a genome wide search of 47 French and German families.7 This locus is estimated to explain 50% of these families and appears to be distinct from the HPC1 locus as the two are estimated to be 60 cM apart. Confirmatory studies of this second locus have not yet been reported. A third locus has been reported. This locus, situated on the X chromosome, is estimated to explain approximately 16% of the families studied (including the families which were first typed to map the 1q24 locus).8 The heterogeneity lod score for linkage to this locus is 3.85 with the strongest evidence being a locus in proximity to the markers DXS297 and DXS12100.

While linkage studies have not identified chromosome 10 as the site of a predisposing gene, the long arm of chromosome 10 is the fourth commonest region showing loss of heterozygosity (LOH) in sporadic prostate cancers after 7q, 8p, and 16q.9 Deletion mapping studies have identified 10q23 to be the minimal region of loss.10-12 One candidate gene which maps adjacent to this region, MXI1, has been assessed for a role in familial prostate cancer susceptibility but no germline mutations were identified.13 PTEN/MMAC1 (Phosphatase and Tensin homologue deleted on chromosome Ten/Mutated in Multiple Advanced Cancers 1), a tumour suppressor gene, has recently been identified at 10q23 through mapping of homozygous deletions in tumour cell lines.14-15 Li et al16 and Steck et al17 found PTEN mutations in four out of four and one out of three prostate cancer cell lines respectively, suggesting a role in prostate carcinogenesis.11,12 Cairns et al18 found LOH at 10q in 23 of 80 prostate tumours. Sequencing identified a mutation in PTEN in 10 of these 23 tumours (43%).19 More recently, Wang et al19 found that 60 prostate adenocarcinomas, 10-15% of primary stage B prostate carcinomas had PTEN inactivation by homozygous deletion. A number of studies have examined the frequency of somatic mutations in this gene. For instance, somatic mutations have been found in glioblastomas, melanomas, and breast and prostate carcinomas.20-22 Germline mutations in PTEN have been shown to be the cause of Cowden disease,23-26 while PTEN deficient mice (PTEN +/-) show hyperplastic and dysplastic changes in the prostate and indeed develop prostate cancer.23-25

We hypothesised that germline PTEN mutations could be important in familial prostate cancer for the following three reasons: somatic mutations have been found in PTEN in prostate tumours; germline mutations in Cowden disease produce a phenotype (although with no evidence of an associated susceptibility to prostate cancer); and PTEN deficient mice exhibit prostate abnormalities. We have therefore screened the Cancer Research Campaign/British Prostate Group (CRC/BPG) UK Familial Prostate Cancer Study samples for evidence of PTEN mutations.

The CRC/BPG UK Familial Prostate Cancer Study has collected lymphocyte DNA from 188 subjects from 50 prostate cancer families. These families were chosen because each contained three or more cases of prostate cancer at any age or related sib pairs where at least one man was less than 67 (original criterion was 65) years old at diagnosis. In fact, the majority of the clusters consisted of affected sib pairs, with DNA often only available from cases. Twenty eight of the families had two affected males, 10 had three affected, nine had four affected, and three had five affected; the average age of onset was 66.9 years. Sample family pedigrees and DNA extraction protocols are described in Edwards et al.19 These families were previously analysed for linkage to HPC1 and showed no evidence for linkage.19 DNA extracted from a known Cowden disease patient was used as a positive control for mutational analysis. The study was approved by the Royal Marsden NHS Trust Local Research Ethics Committee.

Linkage analysis was performed after genotyping using three polymorphic DNA markers flanking PTEN (D10S541, D10S1765, and D10S2491). Lod scores for linkage to PTEN were calculated under the assumption that prostate cancer was caused by an autosomal dominant gene with Carter’s estimate of penetrance2 using the GENEHUNTER software23 and assuming the marker order of D10S1765/D10S2491 - 20.5 kb - PTEN - 0.2 cM - D10S541. Under this model of inheritance, there is an assumed lifetime penetrance (to 85 years of age) of 88% for prostate cancer in the highly susceptible population and with 0.6% of the general population carrying such a predisposition. The risk to age 85 in the non-susceptible male UK population was assumed to be 6.5%. Linkage analysis was performed under a model of homogeneity with PTEN being the only high penetrance predisposition gene for prostate cancer and also under a model of heterogeneity in which other high penetrance predispositions were assumed. For D10S1765 and D10S541, the allele frequencies were estimated from the family data, while for D10S2491 the alleles were assumed to be equally frequent as limited typing precluded estimation (at the time this was a new marker with no published allele frequencies). Non-parametric analysis of linkage was also performed using the NPL statistic of GENEHUNTER.23 Pairwise and multipoint linkage analysis showed no evidence of linkage to the PTEN region. Indeed, under homogeneity and tight linkage there was strong evidence against the hypothesis of a gene in the region of PTEN, which approached the conventional limit for exclusion mapping; the multipoint lod score was −1.96 close to the cut off of −2.0. Under heterogeneity, the overall heterogeneity lod score at the PTEN locus was 0.18 with an estimated 29% of the families being the result of PTEN with a 95% confidence interval of 0% to 79%. Non-parametric linkage analysis using the NPL statistic of GENEHUNTER was also performed to guard against a misspecified mode of inheritance; again, this showed some evidence for allele
sharing which did not reach formal significance in the region of PTEN (NPL=0.88, p=0.19). Further, there was no difference in age of onset in those families consistent with linkage versus those against linkage to PTEN and there was no trend in terms of the proportion of linked families by number of affected males (data not shown).

Linkage analysis of common cancers is complicated by the presence of heterogeneity. As the majority of our families consist only of sib pairs, the opportunity of confirming identity by descent among the affected brothers is extremely limited, thus compromising the ability of linkage analysis to extract convincing evidence of cosegregation. While there is no formal evidence for linkage, even under multipoint analysis for a familial prostate cancer gene in the PTEN region of 10q23, the point estimates for the proportion of families linked is 29% (95% CI (0.00-0.79)) showing lack of discrimination from the family material. With these data, and given the putative role of PTEN in prostate cancer carcinogenesis, we decided to pursue PTEN further by mutation testing families in which the brothers appeared to share at least one parental haplotype around PTEN.

All nine exons of the PTEN gene of the youngest diagnosed member from the remaining 37 families for whom DNA was available and a Cowden disease positive control were sequenced in both directions using dRhodamine labelled ddNTPs (Perkin Elmer). Primers were designed to amplify all coding sequence and at least 15 flanking base pairs.

The IVS8 +102T>G polymorphism in intron 8 reported by Wang et al was found in 27 (73%) of the 37 cases. Forty five percent of the alleles were the "wild type" G, while the remaining 55% of alleles were T. Fourteen (38%) of the cases were TT homozygotes, 13 (35%) were TG heterozygotes, and 10 (27%) were GG homozygotes; these figures are consistent with Hardy-Weinberg equilibrium ($\chi^2$ with 2 d.f.=3.1, p=0.21). No association was found between age of onset of prostate cancer and genotype (TT v TG v GG) when the cases were divided into two categories depending on age of onset being less than or equal to 60 years of age or >61 years of age or higher ($\chi^2$ with 2 d.f.=1.0, p=0.61).

A novel polymorphism (IVS4 −31insT) was found in intron 4 in 4 out of the 10 patients, but not in the patient’s brother who also had prostate cancer, suggesting that it is not important in prostate cancer susceptibility.

While a missense mutation, c.494G>A (p.165G>E), was found in exon 6 of the Cowden disease positive control, no coding mutations were found in the prostate cancer families. In some cases sequencing only extended 15 bp into non-coding sequence, so non-coding mutations which may possibly influence RNA splicing or stability may have gone undetected. The promoter and regulator regions of PTEN are also unknown at present, so any mutations in these regions were also not detected. The HPC1 locus in 1q24-25 has been shown to be preferentially involved in families with four or more affected cases and this might also be the case for PTEN.

Our linkage results suggest that it is unlikely that PTEN or any gene nearby in the 1q23.3 region plays any significant part as a high penetrance susceptibility gene for prostate cancer. This is consistent with the concept that somatic mutations in PTEN are a late event in carcinogenesis, as suggested by Feilotter et al, who found very few PTEN mutations in primary prostate cancer tumours, and the work of Cairns et al, who found a positive correlation between high prostate tumour grade and presence of PTEN mutation.
Mutation analysis of H19 and NAPIL4 (hNAP2) candidate genes and IGF2 DMR2 in Beckwith-Wiedemann syndrome

The H19 gene maps approximately 200 kb telomeric of IGF2 in humans; synteny is conserved in the mouse. Data from human tumours, BWS, and experimental manipulation of the mouse genome indicate that the regulation of H19 and IGF2 expression is closely and reciprocally linked. For some BWS patients with IGF2 LOI, biallelic IGF2 expression is associated with suppression of H19 expression and reversal of the normal (unmethylated) maternal allele methylation patterns, so that both parental IGF2 and H19 alleles display a paternal methylation pattern. However, in other cases with biallelic IGF2 expression, H19 and IGF2 allelic methylation is normal. A possible explanation for these observations is that the maternal H19 RNA is functionally inactivated, not affecting its own imprinting status, but leading to loss of repression of the maternal IGF2 allele. This hypothesis is consistent with data from the mouse.

The most centromeric imprinted gene in the 11p15.5 imprinted region is the candidate tumour suppressor gene TSSC3. The NAPIL4 gene lies 15 kb 5′ of TSSC3 and encodes a chaperone protein associated with chromatin assembly and has been shown to bind to core and linker histones facilitating transfer to the DNA template. Although NAPIL4 has not been shown to be imprinted to date, this has not been extensively investigated and tissue specific or developmentally regulated imprinting cannot be excluded. NAPIL4 lies within the interval associated with loss of heterozygosity in Wilms tumour (WT2) and centromeric to the BWSCR1 breakpoint cluster. It therefore fulfils one of the criteria for a BWS candidate gene. The possibility that chromatin structure affects the activity and imprinting status of genes is very strong and it is possible that mutations in NAPIL4 might appear to have an allele specific effect even if NAPIL4 is not itself imprinted. Recent studies on a BWS family with a maternally inherited inversion of 11p15.5 suggest that relaxation of IGF2 imprinting may result from an H19 independent pathway. In the family reported by Brown et al., a BWSCR1 breakpoint in the region of NAPIL4 was associated with IGF2 LOI and normal H19 expression. Importantly, NAPIL4 is expressed in normal kidney and some Wilms tumours (WT) lacking NAPIL4 expression show IGF2 LOI (Munroe et al., unpublished observations). While mutations in NAPIL4 have not been found in sporadic Wilms tumours, those associated with a genetic predisposition have not yet been examined, leaving open the possibility that NAPIL4 mutations in the germline or somatic mutations early in development may predispose to the changes seen in BWS and familial WT through an effect on the imprinting status of key genes such as IGF2.

Overexpression of IGF2 in mouse development mimics many features of BWS, further implicating IGF2 in BWS. The IGF2 gene has a conserved differentially methylated region (DMR) in exon 9. This region has been shown in mice to be consistently methylated on the expressed paternal allele. It has been postulated that the DMR is a methylation sensitive site for silencer binding. Hence, IGF2 LOI is not accounted for ∼40% of familial cases and 5% of sporadic cases. This suggests that further BWS genes remain to be identified. Most sporadic BWS patients show LOI of IGF2 and a candidate BWS gene might cause BWS by influencing IGF2 imprinting status.
could result from mutations that alter the sequence motifs for silencer binding on the maternal IGF2 allele.

To investigate the molecular mechanism of BWS, we have performed mutational analysis of the H19 and NAP1L4 genes and the DMR2 IGF2 region in BWS patients. Up to 21 subjects (11 male, 10 female) with BWS were investigated. BWS was diagnosed according to previously defined criteria: (1) three major features (anterior abdominal wall defects, macroglossia, and pre-/postnatal growth >90th centile), or (2) two major features plus three or more of: characteristic ear signs (ear lobe creases or posterior helical ear pits), facial naevus flammeus, hypoglycaemia, nephromegaly, and hemihypertrophy. Peripheral blood samples were obtained from all patients and high molecular weight genomic DNA was extracted as described previously.

The H19 genomic sequence was numbered as in Brannan et al. Sequencing was performed from nucleotides 650 to 3461. This included 170 bp of sequence 5’ to the transcription initiation site and all five exons and four introns. The H19 gene was sequenced using genomic DNA to derive overlapping template fragments approximately 500-700 bp long. Primer sequences used to cover the entire H19 genomic region in both forward and reverse directions (and PCR conditions) are available on request. The sequencing PCR reaction was run according to the ABI PRISM protocol and H19 sequence data were obtained using ABI software.

The H19 gene was sequenced in 15 BWS patients without uniparental disomy. These patients represented a variety of aetiologies of BWS; two patients were familial (without germline CDKN1C mutations) and 13 were sporadic. Of the latter, two had previously been identified as having a IGF2/H19 imprinting centre defect (ICD) with H19 promoter hypermethylation and silencing of H19 expression. Of the remaining 11 sporadic BWS patients with normal H19 methylation, seven were informative for allele specific IGF2 mRNA expression analysis; five had biallelic IGF2 expression with normal H19 expression and two had biallelic IGF2 expression and absent H19 expression (but this was not associated with H19 hypermethylation as in the putative ICD cases).

Table 1  Corrections of H19 genomic sequence identified in all patients (n=23) and controls (n=5)

<table>
<thead>
<tr>
<th>Site</th>
<th>Nucleotide position</th>
<th>Correct sequence</th>
<th>Polymorphism</th>
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<tr>
<td>Exon 1</td>
<td>1531</td>
<td>C→T</td>
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<tr>
<td>Exon 1</td>
<td>1569</td>
<td>T→A</td>
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<tr>
<td>Exon 1</td>
<td>1737</td>
<td>G→A</td>
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<tr>
<td>Intron 2</td>
<td>2441</td>
<td>+CGG</td>
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<td>Intron 2</td>
<td>2461</td>
<td>G→C</td>
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<tr>
<td>Intron 3</td>
<td>2599</td>
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<tr>
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Comparison of the H19 sequence in BWS cases and controls to the sequence published by Brannan et al. (Genbank Accession number M32053) showed sequence differences from the published sequence at five sites that were present in all patients and controls. These were considered to represent sequencing errors in the published sequence (table 1). In addition to the consistent sequence changes identified in all patients and controls, 10 polymorphic sequence variants were noted in BWS patients. Two of these nucleotide substitution sites resulting in a RFLP have been described previously (AluI site at nt 2883 and RsaI site at nt 3241), but eight novel sequence variants were identified (table 1): C→T at nt 1531, T→A at nt 1569, G→C at nt 2461, G→C at nt 2791, T→C at nt 2976, T→C at nt 2992, A→G at nt 3238, and T→C at nt 3281. In each instance, these changes were also identified in normal controls and were considered to represent simple polymorphisms.

The intron-exon structure of NAP1L4 has been reported previously and primers were designed to amplify each of the 14 exons (primer details and conditions are available on request). PCR products (5 µl) were denatured and electrophoresed through an 8% polyacrylamide gel containing 5% glycerol, using 0.5 × TBE as the running buffer. Electrophoresis was at 1 watt per gel for 12-18 hours depending on the size of PCR product. DNA bands were visualised by silver staining.

A total of 21 BWS patients were analysed for NAP1L4 gene mutations. These included the 15 patients analysed for H19 gene mutations and a further two familial and four sporadic cases with normal H19 methylation. Seven informative cases were known to have biallelic IGF2 expression analysis. The coding sequence of the NAP2 gene was amplified in 14 single exon fragments and analysed by SSCP and ABI sequencing. The only sequence variant identified was an A→C transversion at +7 of the 3’ exon 2 splice site. This sequence variant does not affect the splice site consensus sequence but causes gain/loss of an Rsai restriction enzyme cutting site (fig 1). This finding was used to screen for the splice site variant and this was identified in both BWS cases and controls. We concluded that this change represented a simple polymorphism.

Primers used to analyse the DMR were forward: 5’ TCT GCC COT GGA CAT TAG 3’ and reverse: 5’ GGC GTC TTG GGT GGT GAG TAG 3’. The PCR conditions were as for SSCP of NAP1L4 with an annealing

Figure 1  H19 gene A/G polymorphism at nt 3238.
temperature of 61°C. Half of the PCR product from each patient was cloned into InVitrogen TA cloning vectors according to the manufacturer’s instructions (InVitrogen, BV, The Netherlands). The rest of the PCR product from each patient was mixed with an equal volume of formamide loading dye, denatured at 95°C for five minutes, and electrophoresed on 8% PAGE. Sequencing of PCR clones obtained after the InVitrogen TA cloning was according to the ABI PRISM protocol described above. Thirty-eight patients with BWS (four with IGF2 LOI) were analysed by SSCP. The expected PCR product was 250 bp, and no differences in product were detected by SSCP between any of the patients. After cloning the PCR products, four clones per patient were sequenced. In total, 49 clones were successfully sequenced and all of these were normal.

It appears that mutations or epigenetic lesions in CDKN1C and IGF2 respectively are involved in the pathogenesis of BWS. In certain cases, for example, uniparental disomy, other genes may also be involved. While a significant proportion of familial cases (40%) are caused by germline CDKN1C mutations, the vast majority of sporadic cases show either uniparental disomy or loss of imprinting of IGF2.5 13 15 16 Manipulation of the mouse H19 gene shows that mutations in this locus may result in LOI of IGF2,22 suggesting that alterations in H19 expression could directly affect IGF2 imprinting in humans. BWS patients with LOI of IGF2 may have normal or absent H19 expression suggesting that a variety of mechanisms (including mutations) could inactivate H19 function and lead to IGF2 LOI.16 Despite frequent suggestions that H19 is a candidate BWS gene, H19 mutation analysis has not been reported previously. As germline H19 deletions have not been detected in BWS patients with H19/IGF2 imprinting centre defects (ICDs) (IGF2 LOI, H19 hypermethylation, and silencing) or in “H19 null” BWS patients (IGF2 LOI, absent H19 expression, but normal H19 methylation), the possibility of mutations within the H19 gene itself required investigation. In mice, deletion of the H19 transcription unit leads to loss of imprinting of the adjacent, normally silent, maternal allele of the IGF2 gene, whereas the imprinting status of the replacement transcription unit is retained.23 Similarly, deletion of sequences upstream and downstream of H19, including an endoderm specific enhancer, which affects its transcription, also affect the IGF2 epigenotype both in cis and trans, together with a switch in the characteristic pattern of IGF2 methylation.18 Taken together, these data suggest a role for H19 and its surrounding sequences in the regulation of IGF2 imprinting. We therefore analysed the H19 gene to determine if some BWS patients had H19 mutations causing a silencing of maternal H19 expression or an expressed but non-functional H19 RNA. Absent H19 expression might be associated with large deletions, promoter mutations, or intragenic mutations which decreased RNA stability. Our molecular analysis would be expected to detect most intragenic mutations and the report of a single nucleotide substitution in the Xist promoter associated with skewed X inactivation19 raised the possibility that H19 promoter mutations might account for the ICD or “H19 null” BWS patients. Most of the patients we studied had normal H19 promoter methylation analysis, excluding the possibility of a large promoter deletion in these cases. Sporadic BWS cases with normal H19 methylation and absent H19 expression could have H19 promoter mutations, but we did not detect any evidence of such changes in the 170 bp of sequence 5’ to the transcription initiation site that we sequenced. The finding that most patients were heterozygous for at least one H19 intragenic polymorphic sequence variant excludes the presence of a complete H19 gene deletion in most cases where expression from both alleles is suppressed. With the reservation that small, more distal 5’ flanking region mutations may have been missed in some patients, we conclude that H19 germline mutations cannot account for the loss of IGF2 imprinting observed in most BWS cases.16 The mode of action of H19 on IGF2 allele specific transcriptional control is unclear, as it does not give rise to a translation product. Although it has been postulated that H19 functions directly or indirectly as a modifier of chromatin structure in a way similar to that proposed for the action of XIST in X chromosome gene inactivation,22 deletion of the mouse H19 gene does not affect the imprinting status of the Mash2, Cdkn1c, or Kcnq1,24 suggesting that there are at least two imprinting control centres within this region.

In humans, the observation of biallelic IGF2 expression in a BWS family with a BSWSCR breakpoint27 is compatible with an H19 independent pathway of IGF2 imprinting control. BSWSCR1 rearrangements associated with LOI IGF2 may show loss of a parental allele specific methylation pattern at KVLQ1T1, but not more distally at IGF2/H19.18 This observation is consistent with two imprinting control centres in 11p15.5. Thus, a candidate BWS gene might function as a cis acting repressor of maternal IGF2 expression and map centromeric to BSWSCR1. NAP1L4 (hNAP2) lies at the centromeric boundary of the imprinted gene cluster on 11p15.5 17 and maps centromeric to the BSWSCR1 region in the candidate region for a cis acting regulator of IGF2 imprinting. To date, evidence of imprinting for NAP1L4, but its biological function as a histone chaperone protein provides a possible mechanism for altering the imprinted status of one or more genes through potential effects on chromatin silencing or activation. Under this model, only the maternal IGF2 allele would be responsive (because of specific methylation or chromatin structure imprints) to a cis acting repressor. Although NAP1L4 represented a strong BWS candidate gene, our failure to identify NAP1L4 mutations in a large cohort of BWS patients strongly suggests that NAP1L4 is not a major BWS gene.

Having excluded coding sequence mutations in NAP1L4 and major changes in both the coding sequence of H19 and its immediate promoter region as being frequent pathogenic lesions in Beckwith-Wiedemann syndrome, we then considered that mutations in the IGF2 DMR2 would represent another cause of IGF2 LOI. Consistent with its putative IGF2 silencer function, DMR2 is more consistently methylated on the paternal allele than on the maternal in tissues in which Igf2 is expressed, and the unmethylated (maternal) DMR2 sequence is bound by specific nuclear proteins (AM and WR, unpublished observations). However, although DMR2 mutations represented a logical explanation for the subset of BWS patients with IGF2 LOI and normal H19 imprinting, no mutations were identified. Thus, the frequent IGF2 LOI found in BWS must therefore originate in other lesions, either genetic or epigenetic. It still remains to examine the candidate of TSSC3, IMPT1, ASCL2, and other loci as they might emerge from this dense cluster of growth related genes. Such analysis will identify sequence mutations that lead to altered epigenetic modifications. However, epimutations (that is, changes in the epigenetic status without genetic modification) could be a mechanism for LOI which would be sporadic and potentially be reset in the germline. As mutations in further BWS candidate genes are excluded as a cause of LOI IGF2, the likelihood of epimutation being the major cause of BWS will increase.

We thank the many colleagues who referred patients. We are grateful to the Wellcome Trust (EM, PS), the BBSRC (PS, JAJ), Action Research (WR, WL, EM), Cancer Research Campaign (AM, WR), and East Anglian Regional Health Authority (EM) for financial support.
A novel mutation in the CFTR gene correlates with severe clinical phenotype in seven Hispanic patients

Editor—Cystic fibrosis (CF) is one of the most common autosomal recessive disorders, with an incidence of one in every 2000 to 3000 white people. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Over 800 CFTR mutations have been identified. Five mutations in Ashkenazi Jews, 1, 2, 24 in whites, and 15 in African-Americans account for 97%, 90%, and 75% of CF chromosomes in the respective populations. In contrast, 90% of CF Spanish alleles were contributed by as many as 75 mutations, indicating a heterogeneous CF genotype in Spain. The overall mutation spectrum and the frequency of common mutations in a particular population depend on the ethnic background.

For example, the frequency of the W1282X mutation is 1.2% in the white population, but it is as high as 60% in Ashkenazi Jews. That of AF508 is 70% in northern Europeans but it is less than 50% in Spanish and Hispanics. In order to provide accurate genetic counselling, it is necessary to determine the prevalent mutations in each ethnic group. A study of Hispanic CF patients from the southwestern United States showed that only 58% of Hispanic CF chromosomes have been studied, there is a shortage of genotype-phenotype correlation studies of rare mutations, particularly the ones that appear to be unique to Hispanic CF patients. This is partly because of the lack of an effective approach to identify the mutations accounting for the remaining Hispanic CF chromosomes. In addition on identification of mutations genotype-phenotype correlation studies can be facilitated.

The clinical diagnosis of CF has been recently reviewed. Although the structure and function of AF508 and W1282X mutant CFTR have been studied, there is a shortage of genotype-phenotype correlation studies of rare mutations, particularly the ones that appear to be unique to Hispanic CF patients. This is partly because of the lack of an effective approach to identify the mutations accounting for the remaining Hispanic CF chromosomes. In addition on identification of mutations genotype-phenotype correlation studies can be facilitated.

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For example, the frequency of the W1282X mutation is 1.2% in the white population, but it is as high as 60% in Ashkenazi Jews. That of AF508 is 70% in northern Europeans but it is less than 50% in Spanish and Hispanics. In order to provide accurate genetic counselling, it is necessary to determine the prevalent mutations in each ethnic group. A study of Hispanic CF patients from the southwestern United States showed that only 58% of Hispanic CF chromosomes have been studied, there is a shortage of genotype-phenotype correlation studies of rare mutations, particularly the ones that appear to be unique to Hispanic CF patients. This is partly because of the lack of an effective approach to identify the mutations accounting for the remaining Hispanic CF chromosomes. In addition on identification of mutations genotype-phenotype correlation studies can be facilitated.

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method for screening the mutations in the large CFTR gene and because a significant proportion (about 40%) of Hispanic CF mutations have not been identified. We recently described the clinical features of a patient homozygous for R1066C and a group of Hispanic patients with the 3849+10kbC>T mutation. In an effort to continue searching for unknown CF mutations in Hispanic patients, we have developed an effective method, temporal temperature gradient gel electrophoresis (TTGE), to screen DNA abnormalities in CF chromosomes and have identified several novel mutations. Here we report the discovery of 3876delA and its clinical presentation in seven Hispanic patients who were heterozygous for this novel mutation.

DNA samples of 44 CF patients, from 43 unrelated families, attending the Children’s Hospital at Los Angeles (CHLA) Cystic Fibrosis Clinic were selected for mutational analysis by TTGE, according to the CCI (Committee on Clinical Investigations) approved protocol No 90-117. Thirty of the 44 patients were Hispanics, including two sibs. In this study, “Hispanics” refers to people originally from Latin America and “Spanish” refers to people living in Spain. These patients have been genotyped by DNA diagnostic laboratories at the University of California San Francisco, Baylor College of Medicine, or Genzyme Corporation, and had either one or both CF alleles unidentified. Clinical information was obtained from CHLA CF Clinic records and by reviewing the patients’ medical charts and consulting clinicians familiar with the patients’ disease progression.

DNA was extracted from patients’ blood samples. All 27 exons were PCR amplified and analysed with TTGE. Exon 20, which contains the 3876delA mutation and its flanking intron regions were PCR amplified by using primers 5’GGTCAACCATTGAAATGGT3’ (forward) and 5’ATGAGAAACTGCACTGG3’ (reverse). The PCR reactions were performed under standard conditions as described previously. PCR products were denatured at 95°C for 30 seconds and slowly cooled down to 45°C for a period of 45 minutes at a ramp of 1.1°C/minute. The 450 bp PCR product containing the entire exon 20 was analysed by the TTGE method using the Bio-Rad DCode™ mutation detection system according to the published procedures. Electrophoresis was carried out at 130 V at a constant temperature increment of 1.3°C/hour. The temperature range (48°C to 56.5°C) for this exon 20 PCR fragment was determined empirically with the aid of computer simulation (MacMelt, Bio-Rad Laboratories).

The DNA samples that showed abnormal banding patterns by TTGE analysis were sequenced using the BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems) and analysed on ABI Prism 377 DNA Sequencer (Perkin-Elmer, Applied Biosystems) according to the manufacturer’s protocols. The sequencing data were analysed with ABI DNA sequencing analysis software (version 3.0).

TTGE analysis of exon 20 of the CFTR gene showed an abnormal banding pattern in seven, including two sibs, out of 29 unrelated Hispanic patients. Fig 1 (above) illustrates the results of TTGE analysis. Normal subjects showed a single band and patients showed three bands. Sequencing analysis showed a deletion of an A at nucleotide position 3876 of the CFTR gene (fig 1, below). All seven patients were heterozygous for 3876delA. Two of these patients were sibs (patients 3 and 4). The remaining 28 patients screened were from unrelated families. Thus, this novel mutant allele represents a frequency of 10.3% (6/(29 × 2))

![Figure 1 (Above) TTGE analysis of the DNA fragment containing CFTR exon 20. Lanes 1 and 4, fragments containing wild type exon 20. Lanes 3 and 4, exon 20 containing fragments amplified from patients 5 and 6. (Below) The upper panel shows the wild type DNA sequence surrounding the nucleotide position 3876 in the CFTR gene. The underlined “A” is deleted in heterozygous mutant DNA (lower panel).](http://jmg.bmj.com/10.1136/jmg.37.3.224)
in this Hispanic patient group. The 3876delA mutation was not detected in 14 unrelated non-Hispanic CF patients examined by TTGE.

Table 1 summarises the clinical findings of these patients. Five out of the seven patients with 3876delA mutation were females. Five of them had ΔF508 as the other mutant allele, one had 1949del84, and one had an unidentified mutant allele. The current age of the five surviving patients ranged from 4 to 26 years; two had died at the age of 19 and 20 years. The age of diagnosis ranged from 3 weeks to 7 months, except for patient 6 who was diagnosed at 16 months. In all cases, high levels of sweat chloride (84-142 mmol/l) were detected.

The pulmonary function was measured by percent predicted FEV1 and percent predicted FVC at diagnosis in pulmonary function. Patient 4 was too young to be tested for FEV1 and FVC. Pulmonary function of patients 2 and 3 had remained normal (>90% predicted values). All patients had infections of *Staphylococcus* and *Pseudomonas* (table 1). Five of the seven patients who carried this 3876delA mutation developed the syndrome of allergic bronchopulmonary aspergillosis (ABPA). ABPA is an immune mediated pulmonary disorder triggered by fungal colonisation, associated with signs of lung infiltrates, increased cough, respiratory distress, and wheezing. These five patients were from unrelated families. The two without ABPA were the sibs, who had similar clinical symptoms, including liver disease and gastro-oesophageal reflux. Overall, these patients showed very similar respiratory symptoms and common, yet somewhat variable, bronchopulmonary impairment.

All seven patients were pancreatic insufficient (PI) and required daily treatment of pancreatic enzyme supplement. Since the other alleles, ΔF508 and 1949del84, render a PI phenotype, these results suggest that 3876delA is also a deleterious mutation with a PI phenotype. Consistently, all patients had poor growth. Although patient 3 recovered from the earlier low growth centile at the age of 10, she suffered respiratory distress as a newborn, portal hypertension, and hyponatraemia, in addition to liver disease and gastro-oesophageal reflux. Patient 6 developed insulin dependent diabetes. Patient 5 had cor pulmonale, a late terminal event of CF. She had poor weight gain and died at the age of 20 of pulmonary failure as did patient 1. In summary, these observations are consistent with severe phenotypes, which correlate with the predicted disruption of NBD2 by 3876delA.

The deleterious frameshift mutation, 3876delA (L1258X), was detected in six out of 29 unrelated Spanish CF patients attending the CHLA CF clinic. This deletion accounts for about 10% of the total CF alleles in this group. The frequency of this mutation in the Hispanic CF patients attending CHLA is similar to that of G542X, only second to ΔF508. The severity of this group of CF patients is shown by the early age of diagnosis (average 6 months), the high sweat chloride (average 105 mmol/l), ABPA, PI, liver disease, cor pulmonale, and early death. Like those with ΔF508/AF508, ΔF508/W1282X, and W1282X/W1282X, patients with the 3876delA/ΔF508 mutation had variable pulmonary function. This indicates that other factors may be involved in this phenotype.

It will be important to follow up patient 4 on her later development of portal hypertension and more severe respiratory problems. Meconium ileus, distal intestinal obstruction syndrome (DIOS), and nasal polyps were not found in this group of patients. However, the observed poor growth and cholestasis suggest that 3876delA might be associated with a variable hepatointestinal status.

Patient 1 was a compound heterozygote for 3876delA and 1949del84. She had a high sweat chloride concentration of 142 mmol/l, early age of onset (2 months), and death at the age of 19. The 1949del84 mutation, which results in an in frame deletion of 26 amino acids located in the R domain, was originally discovered in a 6 month old Spanish patient who had ΔF508 on the other mutant chromosome. This patient had a severe clinical course as well: a sweat chloride concentration of 80 mmol/l, PI, and both respiratory and digestive problems.

It is intriguing that after extensive search for CF mutations in Spanish, European, Ashkenazi Jewish, and African-American populations, the 3876delA mutation was not discovered. Since most of our Hispanic CF patients are descendants of people from Mexico and South America, this could be a reflection of the unique genetic background of our Hispanic population.
America, the 3876delA mutation might be derived from native Mexicans. Further haplotype studies will be necessary to support this hypothesis. It is concluded that 3876delA is one of the common mutations in Hispanic CF patients and should be included in the routine mutational analysis of Hispanic CF patients. This mutation does not result in change of restriction site. The allele specific oligonucleotide (ASO) dot blot analysis would be a simple method for quick diagnosis of this mutation. The genotype-phenotype correlation will greatly assist genetic counselling regarding the prognosis of CF patients.

Like other ABC transporters, the CFTR chloride channel contains two nucleotide binding domains, NBD1 and NBD2, both of which have the consensus sequences for Walker A and Walker B motifs.18 Between the two NBDs is a highly positively charged regulatory (R) domain, whose phosphorylation by protein kinase A modulates the interaction between the NBDs.19 The 3876delA mutation resides in the very centre of the Walker A motif in the NBD2, resulting in the alteration of the highly conserved amino acid sequence in the second half of this motif, starting at amino acid S1248 (fig 2). It also causes a premature protein termination at L1258, 10 amino acids downstream of the mutation site, resulting in the loss of six conserved sequence blocks including the Walker B motif which hosts the γ phosphate binding pocket.1 According to the proposed secondary structure model for NBD2,20 the 3876delA mutation would abolish all the seven helices in NBD2. Since ATP hydrolysis at NBD2 terminates a burst of activities associated with opening the channel, loss of NBD2 would confer a loss of the gating control.

A recent study shows that the Walker A motif in NBD2 is more solvent accessible than that in NBD1, suggesting a difference in structure and function for the two NBDs.21 In addition to 3876delA, a few other mutations in NBD2, including G1244E, S1255P, S1255X, 3905insT, W1282X, N1303K, and G1349D, all result in a PI phenotype.22 It should be noted that S1255P, S1255X, 3905insT, and 3876delA are all clustered around the Walker A motif. However, whether the PI phenotype is caused by the loss of gating control in NBD2 is not clear. Evidence is emerging for the involvement of the C-terminus of CFTR in some other biological processes. It has been shown that there is a specific and tight binding between the CFTR C-terminus and the Na"⁻H"⁺ exchanger regulatory factor.23 The 3876delA mutation offers a good opportunity for understanding the molecular mechanisms of CF pathogenesis.

This study is part of the project "Molecular and Clinical Studies of CF Patients in Southern California" supported by the Webber Berger Foundation.

2 Cystic Fibrosis Consortium. CFTR Mutation Table. 1999. Web site: http://www.gent.sickkids.on.ca/cfr-cg-bm/FullTable
The continuing failure to recognise Alström syndrome and further evidence of genetic homogeneity

EDITOR—A disorder may be prone to misdiagnosis or underdiagnosis when it is rare, has multiple presentations, a slowly evolving phenotype, or no pathognomonic test. Such would seem to be the case for Alström syndrome. We have recently diagnosed this disorder in seven members of six Pakistani families. In none of the affected subjects had the correct diagnosis previously been made. Instead the given diagnoses were Bardet-Biedl syndrome, Leber’s amaurosis, a type of retinitis pigmentosa, sporadic dilated cardiomyopathy, an unidentified mitochondrial disorder, and Usher syndrome. This experience is not unusual since in a recent British study of Alström syndrome patients, seven of 22 had initially been incorrectly diagnosed.

The clinical features of Alström syndrome are well illustrated in our families. Progressive visual impairment presented in the first 6 months of life as photophobia and nystagmus, advancing to a cone-rod dystrophy and registered blindness in the second decade. Truncal obesity and acanthosis nigricans were evident before 5 years, but became more obvious after puberty. Male external genitalia remained small (especially in males with obvious gynaecomastia). All patients were of short stature by the age of 8 but none manifested diabetes mellitus, although this has previously been reported as a common feature. Sensory deafness presented late in the first decade. Renal failure frequently develops during the third decade, this being the cause of death in three family members suspected to have had Alström syndrome. A dilated cardiomyopathy can also occur at any age and often spontaneously improves. It may present in the first year of life before other disease features and did so in two affected subjects in the families reported here.

After further pedigree determination, we found that four of the families were related (fig 1) and that the remaining two families were also part of a larger pedigree. Genotyping showed that polymorphic markers D2S292, D2S2113, D2S2110, and D2S2112 were homozygous in all affected subjects. Markers D2S136 and D2S286 delineate the critical region of 10 cM in our families. D2S136 lies 4 cM telomeric to the minimal critical region reported by Macari et al., while our centromeric boundary (D2S286) is coincident with that of Collin et al. Using MLINK, a maximum two point lod score of +4.62 was obtained for the marker D2S2113 at θ=0. Genetic linkage analysis thus confirmed our clinical diagnosis and the recent reports of an Alström syndrome gene location at chromosome 2p13.

Our experience suggests that Alström syndrome should be considered in all cases of early onset dilated cardiomyopathy and cone-rod dystrophy/atypical Leber’s amaurosis. Follow up should be undertaken to seek additional features of this autosomal recessive disorder with a 1 in 4 recurrence risk. To date, all published Alström syndrome families have shown linkage to chromosome 2p13, suggesting that the disorder is genetically homogeneous. Now that a locus has been identified, gene cloning and mutation detection can be anticipated which will allow diagnostic testing in any suspected case of Alström syndrome. Functional analysis of the cloned gene may also provide wider insights into the pathogenesis of dilated cardiomyopathy and maturity onset diabetes mellitus.

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1 http://nemec.hgmp. ac.uk/OMIM/203800

Figure 1 The simplest genealogical links between affected family members of family 1.
Clinical and radiographic features of a family with hypochondroplasia owing to a novel Asn540Ser mutation in the fibroblast growth factor receptor 3 gene

Editor—Hypochondroplasia is a mild, autosomal dominant skeletal dysplasia. The relative dearth of specific clinical manifestations and the absence of pathognomonic radiographic features often make the diagnosis of hypochondroplasia difficult.1–4 Short limbed dwarfism is rarely recognised before the age of 2 years and is usually mild with heights up to the low normal range. Muscular body build, macrocephaly with mild frontal bossing, and lumbar hyperlordosis are frequently reported. The radiographic features are variable and can be almost normal in mildly affected subjects.1 They most typically include no change or decrease in the interpedicular distance from the first to the fifth lumbar vertebral bodies, anteroposterior shortening of the lumbar pedicles, short iliac bones with flat acetabular roof, small sacrosciatic notches, short tubular bones, short and broad femoral necks, and relative elongation of the distal or proximal portion of the fibula.2 3 Proof that achondroplasia and hypochondroplasia are allelic disorders came with the discovery that both conditions map to the distal short arm of chromosome 4.5 6 Subsequently,
mutation analysis of the FGFR3 gene, located in the 4p16.3 region, showed a recurrent mutation (N540K) in several unrelated hypochondroplasia patients. Recently, two novel mutations in the same region of the FGFR3 gene causing hypochondroplasia have been identified: N540T in a Dutch family and I538V in a Swedish kindred. In some sporadic patients and families with clinical or radiographic features of hypochondroplasia, a causal involvement of the FGFR3 gene has been ruled out, suggesting locus heterogeneity.

We report here a novel N540S mutation in the FGFR3 gene and provide evidence that this mutation causes hypochondroplasia in a Belgian family. The proband is an 8 year old girl referred to the paediatric endocrinologist because of short stature. She was born after an uncomplicated pregnancy to young and non-consanguineous Belgian parents. Birth weight was 3350 g (50th centile) and length 49 cm (25th-50th centile). Psychomotor development was normal. She presented at the age of 8 years 9 months with mild disproportionate short stature. Anthropometric mea-

Figure 2  Radiographs of the spine and pelvis in the proband at the age of 8 years 9 months. (A) The pelvis is normal. (B) Anteroposterior view of the lumbar spine shows minimal increase in interpedicular distance from the first to fifth lumbar vertebra. (C) Lateral view of the lumbosacral spine illustrates mild anteroposterior shortening of the lumbar pedicles and accentuated lumbar lordosis with the sacrum tilted more horizontally.
measurements showed a height of 120.1 cm (3rd centile=123 cm), weight 23.8 kg (3rd centile), head circumference 52.5 cm (50th-75th centile), span 120 cm, lower segment 58 cm (upper to lower segment ratio 1.07), hand length 13.2 cm (3rd centile), and foot length 18 cm (3rd centile=18.7 cm). In addition, a prominent forehead, low nasal bridge, anteroposteriorly flattened thorax, and lumbar hyperlordosis were found on physical examination (fig 1). Radiographic study of the skeleton showed mild shortening of the tubular bones, minimal increase in lumbar interpedicular distance, anteroposterior shortening of the lumbar pedicles and vertebral bodies, and accentuated lumbar lordosis with horizontally tilted sacrum (fig 2). The 37 year old father is also short with a height of 167.9 cm (3rd-25th centile), head circumference 60.3 cm (98th centile=58 cm), span 175 cm, and lower segment 82 cm (upper to lower segment ratio 1.05). His clinical phenotype is characterised by macrocephaly with a prominent forehead, low nasal bridge, muscular build, and broad thorax. Radiographs of the skeleton showed mild shortening of the tubular bones, increase in lumbar interpedicular distance, anteroposterior shortening of the lumbar pedicles and vertebral bodies, long proximal portion of the fibula, and remarkably short femoral necks (fig 3).

Because the clinical and radiographic features in both father and daughter suggested the diagnosis of hypochondroplasia, sequence analysis of the tyrosine kinase I domain of the FGFR3 gene was performed. Genomic DNA was extracted from peripheral blood leucocytes by the Qiagen Blood miniprep kit (Qiagen Inc, Chatworth, CA). Oligonucleotide primers and PCR conditions for amplification of exon 11 and part of exon 12 were used as previously described.\(^8\) The amplified DNA fragments were cloned using the TA cloning kit (Invitrogen) and sequenced. This analysis showed heterozygosity for an A to G transition in both patients, resulting in substitution of serine for asparagine at position 540 (N540S) of the FGFR3 protein (fig 4). This nucleotide sequence change creates a cleavage site for the restriction endonuclease MwoI. Restriction analysis of amplified genomic DNA fragments confirmed that both patients were heterozygous for the mutation and that neither unaffected family members nor any of a panel of 100 unrelated, healthy controls carried the nucleotide change (data not shown).

Both subjects are mildly but variably affected. The radiographic changes in the daughter are subtle whereas the father, with a height in the low normal range, shows convincing radiological features of hypochondroplasia. It is highly likely that this mutation is responsible for hypochondroplasia based on the following strong arguments. First, the mutation is not present in the unaffected family members or in 100 unrelated, healthy controls. Second, the mutation resides in a highly conserved region when comparing all four human FGFRs.\(^13\) Third, the nucleotide change implies the replacement of the same amino acid as in the common N540K mutation, which has been clearly established to cause hypochondroplasia. Fourth, substitution of the same asparagin by threonine, a neutral and

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**Figure 3** Radiographs of the spine, pelvis, and knees in the father aged 37 years. (A) Short femoral necks on the frontal radiographs of the pelvis. (Hips rotated outwards are shown) (B) Normal increase of interpedicular distances in lumbar spine. Anteroposterior shortening of the lumbar pedicles and vertebral bodies. (C) Elongation of the proximal end of the left fibula (top shown by arrow).
polar amino acid similar to serine, has been reported in hypochondroplasia.

The identification of yet another novel mutation, resulting in the substitution of asparagine in position 540 of the FGFR3 protein and with hypochondroplasia as the phenotype, emphasises the important role of this specific site of the tyrosine kinase I domain in the pathogenesis of the disorder. Therefore, in patients with clinical/radiographic features of hypochondroplasia in whom restriction analysis or mutation detection methods do not show the presence of the common N540K mutation, sequence analysis of the tyrosine kinase I domain of the FGFR3 gene should be performed to exclude other nucleotide changes in that region.

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Screening British CADASIL families for mutations in the NOTCH3 gene

EDITOR—CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy) is a hereditary form of multi-infarct vascular dementia. Clinical symptoms often present in middle adult life (30-50 years of age) and include recurrent subcortical ischaemic strokes, migraine with or without aura, major psychiatric symptoms, and dementia. Magnetic resonance imaging (MRI) shows high intensity signal lesions, often confluent, and areas of cystic degeneration of the subcortical white matter and basal ganglia. Pathological examination shows multiple, small, deep cerebral infarcts, leucoencephalopathy, and a non-atherosclerotic, non-amyloid angiopathy involving mainly the small, deep, perforating cerebral arterioles. Severe alterations of vascular smooth muscle cells are evident on ultrastructural analysis.

The term CADASIL was adopted after linkage of French families with these symptoms to chromosome 19, but families with many of the features of CADASIL had been described by Worster-Drought et al. in the 1930s as familial presenile dementia with spastic paralysis, by Sourander and Walinder as hereditary multi-infarct dementia, and by Stevens et al. as chronic familial vascular encephalopathy. In 1996, the responsible gene was identified as NOTCH3, a member of the Notch family of signalling proteins originally identified in Drosophila.

Notch and Notch homologues control the ability of non-terminally differentiated cells to respond to differentiation/proliferation signals through local cell interactions. They are transmembrane proteins with distinct extracellular and intracellular domains. Notch is activated by binding of a ligand to the extracellular so-called epidermal growth factor (EGF) repeats. This is thought to release the intracellular domain which translocates to the nucleus to regulate the transcription of genes that ultimately determine cell fate.

To date, 26 separate mutations have been found in NOTCH3, as described by Joutel et al. in the French families and two additional mutations from American families, as reported by Meeks et al. Twelve of these mutations are clustered in exon 4. All of these mutations predict the introduction or replacement of cysteine residues in the extracellular EGF repeat domain. CADASIL has also been reported in Dutch, German, Swiss, Italian, American, and Japanese families.

We report here the results of linkage analysis and screening for mutations in British families with a diagnosis of CADASIL.

DNA was available from four multiplex families from the central belt of Scotland, all of Scottish ancestry, a family from south west England of English ancestry, and one further isolated subject from Scotland with suspected CADASIL. We classified the diagnosis of CADASIL into definite, when there was neuropathological confirmation or evidence of genetic linkage to chromosome 19p12 or both, and probable, where there were clear clinical symptoms and MRI findings typical of CADASIL plus a positive family history. The clinical-demographic details and results described in the text below are summarised in Table 1. In spite of extensive genealogical investigations we were unable to find a common ancestor for any of the families described.

Families 1 and 2, both Scottish, had sufficient meioses available to perform analysis of genetic linkage to chromosome 19p12. We genotyped the families on a Perkin Elmer Applied Biosystems (PE ABI) 377 automated genotyper with 5’ FAM labelled primers for six polymorphic microsatellite markers, D19S226, D19S411, D19S885, D19S199, D19S923, and D19S841 flanking the NOTCH3 gene (GDB accession numbers 188569, 199752, 605844, 182271, 611676, and 593357, respectively), and analysed the results using Perkin Elmer Genescan software (version 2.1).

Clear genetic linkage to chromosome 19p12 was found in both families. Family 2 was originally reported not to be linked to chromosome 19. However, further characterisation of the phenotypes in these families by MRI scanning, which had not been performed at the time of the initial reporting, required us to reclassify several cases showing key recombinants. We also determined haplotypes at NOTCH3 in the other multiplex families and found the same patterns of microsatellite allele sizes for families 3 and 4.

We then sequenced exon 4 of the NOTCH3 gene (GDB accession number AF058883) in all our families, by automated sequencing of PCR products. After checking size and yield on 1% agarose gel electrophoresis, PCR products were purified using Centrikon™ columns. Purified PCR products were sequenced by automated cycle sequencing using PE ABI BigDye™ chemistry. The products were run on polyacrylamide gels on a PE ABI 377 automated sequencer and analysed using PE ABI Factura (2.0.1) and Sequence Navigator (1.1) software.

Table 1 Diagnostic details for families studied

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of cases</th>
<th>Neuropathology</th>
<th>Clinical symptoms</th>
<th>MRI</th>
<th>Family history</th>
<th>Linkage analysis</th>
<th>Mutations</th>
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<tbody>
<tr>
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<td>C499T</td>
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<tr>
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<td>1, 2</td>
<td>Yes</td>
<td>NA</td>
<td>C622T</td>
</tr>
</tbody>
</table>

NA: not available.

Clinical symptoms: (1) strokes, (2) psychiatric symptoms, (3) migraine with or without aura, (4) dementia.
The CADASIL disease mutation was found in exon 4 in all families in positions described earlier (Table 1). The sequencing results corresponded with the genetic linkage analysis for all affected and unaffected members of families 1 and 2. The results also agreed with the predicted affected status based on clinical and MRI data for members of families 3-6. The C499T mutation coding for the R143C amino acid substitution is shared between families 3 and 4. Since they share the same haplotype it is likely that the mutation has the same ancestral origin.

In addition to the mutations, the sequencing also detected polymorphisms. For example, the G684A polymorphism in exon 4 was found with a frequency that matches the 0.17 described by Joutel et al. In conclusion, our results confirm the broad geographical occurrence of CADASIL in Europe. It is conceivable that this rare Mendelian type stroke is still largely underdiagnosed and mutation analysis will lead to an increase in the number of diagnosed cases.

We thank Drs Durward and Bone, and Professor Behan (Southern General Hospital, Glasgow) and Dr J Gibson (Derriford Hospital, Plymouth) for access to DNA from families. This work was funded by the Stroke Association, Smith Charities, and Davidson Bequest. This work was funded by the Stroke Association, Smith Charities, and Davidson Bequest.

Correlation between mutations and age in cystic fibrosis in a French Canadian population

EDITOR—Cystic fibrosis (CF) is the most common, lethal, autosomal recessive childhood disorder in the white population, occurring in about 1 in 2500 live births. The incidence (1975-1995) is 1 in 936 live births, with a carrier rate of 1 in 15 in inhabitants of Saguenay-Lac-Saint-Jean (SLSJ), a geographically isolated region in north eastern Quebec.

One hundred and sixty three patients in 143 families are known at the “Clinique de la Fibrose Kystique” in Chicoutimi (Saguenay), which is the referral centre for the whole region (285 000 inhabitants) and has followed all the CF patients but one since 1973. Molecular characterisation has been performed on all identified living CF patients. Three mutations account for 94.4% of the CF chromosomes; these are the AF508 (64.1%), 621+1G→T (22.6%), and A455E (7.7%) mutations.

25 SLSJ offers a unique opportunity to investigate whether there is a relationship between CFTR genotype, mutation, and survival. Indeed, all the CF patients are followed by the same multidisciplinary team, one of the paediatricians having been affiliated with the clinic since its opening. Although therapy has improved over this period of time, there is continuity and homogeneity in the follow up and treatment. Furthermore, the presence of three mutations, two severe and one mild, at high frequencies allowed us to compare different classes of CFTR mutations.

Data collected on each patient were extracted from the files kept at the CF clinic in Chicoutimi where the patients come on a regular basis (approximately every two months) as outpatients for advice, follow up, and treatment. This medical visit consists of a physical examination, a growth and nutritional status evaluation, pulmonary function tests by spirometry, and a sputum culture. This phase of data gathering has been described in detail elsewhere.

We only considered the genotyped patients, alive or dead after 1973, diagnosed before they reached 5 years old, in order to have a more homogeneous group consisting of patients having sufficient clinical signs and symptoms for the diagnosis to be made early in life. The Kaplan-Meier
that of the patients carrying the 621+1G mutation between the three age groups, whereas there is a decline in survival analysis was used to test whether there were significant differences (p<0.05) in the cumulative survival of the CF patients carrying one of the three main mutations.

Table 1A shows the distribution of the 137 CF patients, including 15 dead (10.9%), by age groups and genotypes. The proportion of patients homozygous for F508 remains quite constant between the three age groups, whereas there is a decline in that of the patients carrying the 621+1G→T/F508 genotype and an increase of those having a F508/A455E or 621+1G→T/A455E genotype.

Because of the small numbers involved, the 15 genotypes were grouped according to the three main CFTR mutations (table 1B). The A455E mutation group contains all the patients with that mutation, independently of the second mutation composing their genotype. The CF patients carrying the 621+1G→T mutation, but not A455E, were included in a unique group. Patients homozygous or compound heterozygous for the F508 mutation, without the 621+1G→T or the A455E mutation, were grouped together. We observed an increase of A455E and a depletion of 621+1G→T at older age groups, whereas the frequency of the F508 mutation stayed constant (table 1B).

Fig 1 shows the cumulative survival by mutation. The Kaplan-Meier survival analysis could not be performed considering the three mutations because there were no deaths in the A455E group. No significant difference was found between the F508 and the 621+1G→T groups (p>0.05). Evaluating survival as a function of the CFTR genotype or mutation requires that patients have the same access to medical care and had been diagnosed very early (before 5 years old). Our approach is different from those who determined the genotypes of adult patients or patients diagnosed at a later age and found rare mutations. Indeed, those patients should have a less severe phenotype or disease evolution, which does, in part, correlate with milder mutations.

The F508 and 621+1G→T alleles are known to be severe mutations conferring pancreatic insufficiency when they are combined with another severe mutation. The A455E allele is a mild mutation associated with pancreatic sufficiency and exerts a dominant effect on the severe mutations. Compound heterozygotes for the A455E mutation have a milder pulmonary disease, no meconium ileus, and no late complications, such as diabetes and liver cirrhosis. Therefore, since pulmonary insufficiency is the major cause of mortality in cystic fibrosis, it is not unexpected that no CF patients carrying the A455E mutation have died unlike the 13% of those with two severe mutations.

Since our series is small, the results should be considered as preliminary. However, it appears that survival is not only related to good care and treatment, but is also, at least in part, genetically determined.)

The authors thank Mrs Simone Aubin, Claudette Larochelle, and Suzanne Migneault from the Clinique de Fibrose Kystique in Chicoutimi for their invaluable help. This study was supported in part by Organon Canada.

### Table 1 Distribution of cystic fibrosis patients diagnosed before the age of 5 by age groups in Saguenay-Lac-Saint-Jean, (A) by genotype, (B) by mutation

<table>
<thead>
<tr>
<th>0–10 years</th>
<th>10.1–20 years</th>
<th>Over 20 years</th>
<th>All ages</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>(A) Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F508/A508</td>
<td>15 (1)</td>
<td>40.5</td>
<td>21 (2)</td>
</tr>
<tr>
<td>F508/621+1G→T</td>
<td>12 (1)</td>
<td>32.4</td>
<td>16 (1)</td>
</tr>
<tr>
<td>F508/A455E</td>
<td>1</td>
<td>2.7</td>
<td>6</td>
</tr>
<tr>
<td>F508/I148T</td>
<td>1</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>F508/Y1092X</td>
<td>3 (1)</td>
<td>5.2</td>
<td>1</td>
</tr>
<tr>
<td>F508/Q890X</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>F508/R1158X</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>621+1G→T/621+1G→T</td>
<td>2 (1)</td>
<td>5.4</td>
<td>4</td>
</tr>
<tr>
<td>621+1G→T/A455E</td>
<td>1</td>
<td>2.7</td>
<td>4</td>
</tr>
<tr>
<td>621+1G→T/T1111G→T</td>
<td>2 (1)</td>
<td>5.4</td>
<td>2 (1)</td>
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<tr>
<td>621+1G→T/Y1092X</td>
<td>1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>621+1G→T/S489X</td>
<td>1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>621+1G→T/G85E</td>
<td></td>
<td>1 (1)</td>
<td>1.7</td>
</tr>
<tr>
<td>A555E/R117C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N1303K/I148T</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| *Including one accidental death.

The Kaplan-Meier survival analysis could not be performed as preliminary. However, it appears that survival is not only related to good care and treatment, but is also, at least in part, genetically determined.

The authors thank Mrs Simone Aubin, Claudette Larochelle, and Suzanne Migneault from the Clinique de Fibrose Kystique in Chicoutimi for their invaluable help. This study was supported in part by Organon Canada.

### Figure 1 Survival analysis (Kaplan-Meier cumulative) for three CFTR mutations in a French Canadian population.

Letters
The heritability of high myopia: a reanalysis of Goldschmidt’s data

EDITOR—The prevalence of myopia varies widely. In western Europe and the United States the prevalence of myopia is estimated as 10-25%, while in parts of Asia the prevalence is often much higher. High myopia (usually defined as a refractive error \( \geq -6.00 \) D) has a prevalence of 0.5-2.5% in western Europe and the USA and is second only to diabetes as the most common cause of blindness in the working age population. Here, to distinguish it from high myopia, myopia \(< -6.00 \) D will be referred to as “low myopia”.

There is compelling evidence that both environmental and genetic factors are involved in the aetiology of myopia. An influential series of population studies by Young et al. reported a dramatic increase in the prevalence of myopia in the generation of Alaskan Eskimos first exposed to compulsory education and a “westernised” environment during their childhood. Independent population studies targeting other isolated communities that had been exposed to similar changes in environment corroborated these findings. Parent-offspring heritability estimates in these studies were generally low (table 1), while sib-sib heritability was usually high, suggesting that environmental factors had dominated any influence of genetics in determining refractive error. Dominance of environmental factors over genetic factors is also evident in “form deprivation” myopia. This condition develops when visual clarity is severely compromised during a critical period of postnatal development.

The genetic contribution towards the development of myopia has been investigated in both population studies (table 1) and twin studies (table 2), and has been reviewed previously. While these investigations have suggested that myopia is genetically determined to some extent, there has been no consensus of opinion regarding whether or not myopia has different aetiological causes, nor on the mode or modes of inheritance of the disease. Nevertheless, the reviews of Bear and Goss et al. suggest that myopia is likely to be aetiologically heterogeneous and that, in the case of high myopia, both polygenic and various mono- genic forms of inheritance may be involved.

In the last decade, the molecular genetics of myopia has begun to be explored, and these investigations have increased our understanding of myopia considerably. Early studies in this area identified the myopias that occur as part of rare connective tissue diseases Marfan syndrome and Stickler syndrome types 1 and 2, tracing the conditions to defects in the fibrillin, \( \text{COL2A1} \), and \( \text{COL11A1} \) genes, respectively. In addition, the loci of the X linked Åland Island eye disease and the autosomal recessive Knobloch syndrome were mapped to Xp11 and 21q22.3, respectively. In the case of simple myopia (myopia that...
occurs in the absence of other abnormalities), Olmedo et al,30 and Pintado et al,30 were the first to provide evidence for genetic differences between high and low forms. More recently, genetic linkage studies have identified two discrete loci for dominantly inherited simple high myopia.29–31 The latter work has confirmed the aetiological heterogeneity of simple high myopia for the first time.

While there has been numerous attempts to estimate the heritability of myopia (tables 1 and 2), none of these studies has calculated a heritability estimate solely for high myopia. Given the likelihood of genetic differences between high and low myopia, we sought to calculate such a heritability estimate. In view of the probable polygenic nature of most high myopia,22 we also calculated a risk ratio for sibs (λs) for high myopia. In both cases, these calculations were based on a reanalysis of the data of Goldschmidt.3

**Methods.** The 1968 study by the Danish geneticist Ernst Goldschmidt,5 is particularly informative because the population groups investigated were large and chosen without bias. One of the two samples studied by Goldschmidt was the population of children born in Copenhagen in 1948. At the time of examination, these children were aged 13 to 14 years. The size of the population group was estimated to be 9700, and 9243 of these were surveyed (95%). The school medical records of the 9243 children were screened to identify those likely to be myopic (specifically those already wearing spectacles or with reduced visual acuity). Since all children in the population group were examined by a school medical officer on an annual basis, it seems unlikely that any cases of high myopia would have been missed. Subjective refraction was measured in sphere/cylinder form for 1039 of the 1111 children identified as potential myopes (for the 72 potential myopes who were not examined, Goldschmidt predicted that 62 were myopic based on their medical records). Cycloplegia was only used “where the indications were uncertain or there was an obvious discrepancy between the uncorrected and corrected vision”. Myopes with an ocular refraction in one eye of ≤−6.00 D were classified by Goldschmidt as high myopes, and the ocular refractions of their parents and sibs were also measured and reported.

Goldschmidt calculated the prevalence of myopia in his child population group to be 9.5%. However, for subsequent analyses regarding the different degrees of myopia, he confined his analysis to numbers of a representative cross-section of North American families. Unfortunately, however, the population group studied by Sperduto et al,13 was separated both in time (one and a half generations) and geography (Denmark versus the United States) from the population studied by Goldschmidt.3 The population prevalence of myopia in the sample of Sperduto et al,13 averaged 35%; however, it varied with both age and sex (range 18–68%). Furthermore, this population also contained

### Table 2 Heritability estimates for myopia from twin studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Heritability</th>
<th>No of twin pairs</th>
<th>Age</th>
<th>Calculation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorby et al,30</td>
<td>h² = 0.87</td>
<td>MZ = 78</td>
<td>4-14</td>
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<tr>
<td></td>
<td></td>
<td>DZ = 40 (a)</td>
<td></td>
<td>h² =</td>
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<tr>
<td>Nakajima et al,41</td>
<td>h² = 0.83</td>
<td>MZ = 39</td>
<td>12-17</td>
<td></td>
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<tr>
<td>Nakajima et al,41</td>
<td>h² = 0.73</td>
<td>DZ = 10 (a)</td>
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<tr>
<td>Kimmura et al,42</td>
<td>h² = 0.80</td>
<td>MZ = 33</td>
<td>15-20</td>
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<td>DZ = 16 (nls)</td>
<td></td>
<td>h² =</td>
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<tr>
<td>Hu et al</td>
<td>h² = 0.61</td>
<td>MZ = 49</td>
<td>7-19</td>
<td></td>
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<tr>
<td></td>
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<td>DZ = 37 (nls)</td>
<td></td>
<td>h² =</td>
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</tr>
<tr>
<td>Lin and Chen,43</td>
<td>h² = 0.25</td>
<td>MZ = 90</td>
<td>7-23</td>
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<td>h² =</td>
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<td>Teikari et al,44</td>
<td>h² = 0.58</td>
<td>MZ = 54</td>
<td>30-31</td>
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<td>DZ = 55 (a)</td>
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<td>h² =</td>
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<td>Angi et al,45</td>
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<td>MZ = 19</td>
<td>3-7</td>
<td>h² =</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>DZ = 20 (a)</td>
<td></td>
<td>h² =</td>
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</tbody>
</table>

h² = heritability, MZ = monozygotic, DZ = dizygotic, ls = like sexed, nls = non-like sexed, rMZ = correlation coefficient of MZ twins, rDZ = correlation coefficient of DZ twins, rMP = intrapair correlation coefficient in MZ twins (correlation in liability), rπ = intrapair correlation coefficient in DZ twins, ΛMZ and ΛDZ not defined by the authors.

The second population group studied by Goldschmidt consisted of Danish military conscripts. However, instead of quoting the prevalence of high myopia for this population, he reported the prevalence of myopia >−6.50 D. Thus, to enable comparison with this latter group, we have calculated the prevalence of myopia >−6.50 D for the boys in Goldschmidt’s population of 9243 children. Making the assumption that 50% of the children were male, the prevalence was (2 × 12/9243) × (877/815) = 0.28%. This assumption seems valid since Goldschmidt reported that exactly 50% of the 8981 children attending “normal” schools were male.

We calculated the heritability of high myopia from the family data for highly myopic children identified by Goldschmidt,3 using the method described by Falconer and Vogel and Motulsky,32 where heritability is defined as the ratio of additive genetic variance (VP) to phenotypic variance (VP). Briefly, midparent-offspring heritability was calculated as h = (n/0.5) where n was the correlation between midparent versus offspring ocular refractions. As suggested,11 offspring data were averaged for each family analysed. Sib-sib heritability was calculated as h² = 2r where r was the correlation between probands and the mean of the other sibs. Probands were included in the analysis since they were derived from a sample which was considered unselected. The 95% confidence intervals of the correlation were calculated and the null hypothesis of zero correlation was tested, as described by Altman.33 To avoid a source of potential bias, right and left eyes were analysed independently, since ocular refractions were highly correlated between the two eyes of subjects.

The risk ratio for sibs (λs) for high myopia was calculated for Goldschmidt’s child population according to the formula λs = risk to sibs of affected probands/population prevalence, as described.31 The population prevalence of high myopia for this group of children was taken as 0.45%.

It was not possible to calculate a risk ratio for sibs (λs) for low myopia from Goldschmidt’s study group because family data were only available for the highly myopic children. In fact the only previous study that reported sufficient data to enable such a calculation was that of Sperduto et al,13 This contained refractive data for a representative cross-section of North American families. Unfortunately, however, the population group studied by Sperduto et al,13 was separated both in time (one and a half generations) and geography (Denmark versus the United States) from the population studied by Goldschmidt.3 The population prevalence of myopia in the sample of Sperduto et al,13 averaged 35%; however, it varied with both age and sex (range 18–68%). Furthermore, this population also contained
high myopes and we were not able to exclude these from the analysis. Fortunately, the high myopes were vastly outnumbered by low myopes and thus were unlikely to affect the magnitude of the risk ratio for low myopia that was obtained. Because of the mode in which results were presented by Sperduto et al.,\textsuperscript{9} risk to sibs was inferred from families containing only two sibs, in which both, one, or neither of the sibs were affected by myopia. There were 255 such families (510 sibs) and the prevalence of myopia in this group was 39% (198 of the 510 sibs). With 57 families having two myopic sibs, the risk ratio for sibs ($\lambda_s$) for low myopia was therefore $=1.5$.

\textit{Results.} Goldschmidt\textsuperscript{5} screened the medical records of 95% of the children born in Copenhagen in the year 1948 and found that 9.5% of the children sampled were myopic. He then examined 93% of the myopic children in person to determine their precise ocular refraction (the children being aged 13–14 at the time of the survey). Thirty-nine children were classified by Goldschmidt as high myopes giving a prevalence of 0.45%. Family data were available for 36 of these 39 subjects. Of the three families for which data were not reported, one family did not want to cooperate with the investigation, while in the other two the probands lived in foster homes. Refractive data for one of the parents was not available for five of the 36 families, leaving 31 “complete” families available for reanalysis.

We calculated the midparent-offspring heritability estimates for ocular refraction in the 31 complete families to be 0.65 and 0.68 for right and left eyes, respectively (both significantly above zero at the $p<0.01$ level). In contrast, the sib-sib heritabilities for the 36 incomplete families described by Goldschmidt\textsuperscript{5} were not significantly different from zero ($h^2 = -0.09$ and $+0.12$ for right and left eyes, respectively). No improvement was gained by restricting the sib-sib analysis to just those sibs from the 31 complete families. The midparent-offspring heritability estimates calculated here for the families of Goldschmidt’s highly myopic probands are higher than those found previously (for unselected families, table 1), while the sib-sib heritability estimates for this sample are much lower than has been found previously (table 1).

The risk ratio for sibs ($\lambda_s$) for high myopia, calculated from Goldschmidt’s data, is 2.0. (Four sibs out of a total of 44 were highly myopic). Furthermore, if only sibs older than the probands are considered, the risk ratio is even higher ($\lambda_s=27.5$, since three out of 24 older sibs were affected), suggesting that the inclusion of sibs who have yet to develop high myopia leads to the risk ratio being underestimated.

The study by Sperduto et al.\textsuperscript{9} is the only one containing sufficient detail to enable a risk ratio for low myopia to be calculated. This study yields a $\lambda_s=1.5$ for low myopia. Importantly, because the population prevalence of myopia in the unselected sample studied by Sperduto et al.\textsuperscript{9} averaged 35%, the risk ratio could never have exceeded the theoretical maximum of 2.9 (numerator in $\lambda_s$ equation must be $\leq 1$, denominator $= 0.35$).

\textit{Discussion.} Several factors limit the reliability of the heritabilities and risk ratios reported here. Consequently, these results should be interpreted with caution and considered only as estimates. Some of the more important limiting factors are discussed below.

Results from an older Danish population group described in Goldschmidt’s 1968 paper suggest that many of the children he sampled had yet to develop myopia. In 18-20 year old Danish military conscripts, the prevalence of myopia was 14.5%, whereas it was only 8.5% in the 13-14 year old boys. Furthermore, while 0.6% of his conscript sample had myopia $> -6.50 \, \text{D}$, only 0.3% of the male children had this degree of myopia. Thus, it seems very likely that some children who would eventually become high myopes were not followed up in Goldschmidt’s analysis of parents and sibs. This effect of sampling a population group at an age before myopia has stabilised is likely to have led to the sib-sib heritability being underestimated, since heritability calculations use a correlation approach that relies on an analysis of quantitative data. Also, since ocular refraction continues to vary with age,\textsuperscript{6,7} parent-offspring heritability estimates might also be affected. Risk ratio estimates, in contrast, are based on an all or nothing approach, the presence or absence of a trait. Thus, risk ratios may be less susceptible to problems caused by age related variations in refraction than heritabilities.

Despite the large population targeted by Goldschmidt,\textsuperscript{5} only 36 highly myopic probands and their families were available for analysis. The use of such a small sample will inevitably have led to inaccurate in both heritability and risk ratio estimations.

It could be argued that the $\lambda_s$ values for high and low myopia reported here are not strictly comparable, since they relate to population groups that not only derive from different geographical areas, but that are one and a half generations apart. However, since the population prevalence of myopia in Goldschmidt’s population sample was at least 15%, the $\lambda_s$ for low myopia for this group could not have exceeded the theoretical maximum of 6.7 (1 divided by 0.15) and therefore would still be much less than that of high myopia.

The heritability estimates derived here from midparent-offspring and sib-sib analyses suggest that genetic factors might contribute to the determination of ocular refraction differently in highly myopic members of the population compared to the general population. However, it is unclear why the sib-sib heritability in the present sample should be so low, especially since sib-sib heritability has usually been thought to overestimate the role of additive genetic effects in determining ocular refraction.\textsuperscript{35} Falconer\textsuperscript{35} suggested that midparent-offspring heritability is likely to be the most reliable heritability indicator in human populations. According to this postulate, our results can be interpreted as suggesting that genetic factors exert a greater effect in determining high myopia than low myopia.

Significantly, the high risk ratio for sibs ($\lambda_s$) for high myopia found here also suggests an important role for heredity in determining extremes in ocular refraction. Furthermore, our data are consistent with the idea that high myopia is more likely to be determined by familial factors than is low myopia.

Very recently, Young et al.\textsuperscript{36} described two discrete loci for simple high myopia in pedigrees showing an apparently autosomal dominant pattern of inheritance. Interestingly, in three of the four families in which Goldschmidt’s highly myopic probands had an affected sib, one or more of the parents was also affected. Moreover, in the nine families that had at least one affected parent, 30% of sibs were affected. Both of these results are suggestive of a dominant pattern of inheritance, and so dominant genes may be responsible in large part for the high $\lambda_s$ for high myopia. However, since this dominant transmission pattern was only evident in nine of the 36 families in Goldschmidt’s cohort, it seems likely that other modes of inheritance of high myopia are more common than dominant inheritance. The failure to identify a major dominant myopia gene by segregation analysis\textsuperscript{32} is consistent with this view, although a recent study of the inheritance of corneal astigmatism\textsuperscript{37} suggests that this type of segregation analysis is not always capable of disclosing dominant gene effects.

The frequency distribution of ocular refraction is leptokurtotic rather than normal, and skewed towards myopia.\textsuperscript{1,38–40} However, a reanalysis of Goldschmidt’s sam-
ple of 815 children shows that for this population, the frequency distribution of the number of myopic eyes can be described as a single inverse exponential function, except for myopia >−9.00 D (fig 1). It is tempting to speculate that the frequency distribution of the number of myopic eyes can be approximated from −11.00 D to −30.00 D. For myopia of −9.00 D and above, the inverse exponential function predicts fewer cases than were actually observed, suggesting a possibly bimodal distribution.

The risk ratio for sibs for high myopia was approximately 20, compared to 1.5 for low myopia. In addition, the midparent-offspring and sib-sib heritabilities for the families of Goldschmidt’s high myope population differed from those reported for unselected families. Finally, it was noted that the frequency distribution of myopia in Goldschmidt’s unselected child population group conformed well to a biphasic distribution, consistent with the occurrence of two aetiologically distinct, major forms of myopia. Taken together, these results suggest that genetic factors play a significant role in the development of high myopia.
Progressive neurological deterioration in a child with distal arthrogryposis and whistling face

EDITOR—Freeman-Sheldon syndrome (FSS) (McKusick 193700), described in 1938, is characterised by a whistling face with a long philtrum, a puckered mouth, microstomia, H shaped cutaneous dimpling on the chin, multiple joint contractures with camptodactyly, ulnar deviation of the fingers, bilateral talipes equinovarus, and kyphoscoliosis. Burian rediscovered the entity and called it the “whistling face syndrome”. There is genetic heterogeneity. Both autosomal dominant and recessive inheritance have been described.

The psychomotor development of affected children is usually normal in the autosomal dominant forms, although mild motor delay attributable to joint anomalies has been reported occasionally. A few patients with severe developmental retardation have been described, all with autosomal recessive inheritance.

We describe a child who presented from birth with distal arthrogryposis, profound mental retardation, severe hypotonia, and whistling face. The severe neurological involvement precludes him from having FSS according to the classification of Bamshad et al. We suggest that patients with a whistling face, distal contractures, and severe neurological involvement should be diagnosed as having a separate autosomal recessive syndrome.

The patient was the first child of non-consanguineous, healthy, Moroccan Jews. The pregnancy was attained by in vitro fertilisation and was unremarkable. He was born at 42 weeks’ gestation. Apgar scores were 4 at one minute and 6 at five minutes; pH was 7.11 and improved with bicarbonate infusion. A single seizure on the second day did not recur following phenobarbital therapy. Hypotonia with scarce spontaneous movements but increased reflexes was first noticed then. Length, weight, and head circumferences were on the 50th centile.

He was first evaluated at the age of 6 months when his weight had dropped below the 5th centile. Physical examination showed a puckered mouth, mild retrognathia, camptodactyly of fingers 2 and 3, adducted thumbs, and rocker bottom feet (fig 1). Brain CT and MRI scans, EEG, echocardiogram, karyotype, and muscle biopsy including electron microscopy were normal. A diagnosis of the fetal hypokinesia sequence was considered at that time. A metabolic evaluation, including blood lactic, pyruvic, amino, pyhtanic, and very long chain fatty acids and urinary organic and bile acids, was normal.

At the age of 36 months clinical examination showed whistling face, micrognathia, a small mouth with a long philtrum and downturned upper lip, blepharophimosis, prominent and narrow forehead, and bitemporal balding. Height was on the 20th centile, head circumference was on the 50th centile, and weight was on the 2nd centile. Limb movements were scarce and there was no head control. He was able to follow with his eyes but could not smile. Eye examination was normal. Bronchoscopy showed severe laryngomalacia. There were frequent desaturations with CO₂...
retention. The muscles were atrophic but reflexes were increased. There has been no neurological development since birth.

A repeat brain MRI showed generalised cerebral, cerebellar, and brain stem atrophy (fig 3). Further evaluation including isoelectric focusing of transferrins and spectral karyotyping of his chromosomes was normal.

Freeman-Sheldon syndrome (FSS) was classified among the congenital arthrogryposis syndromes assuming a possible myopathic origin of both facial anomalies and joint contractures. However, EMG and structural abnormalities have not always been detected in the muscles involved. In addition, the most frequently described muscle abnormality, substitution of muscle tissue with connective tissue, may be a consequence rather than a cause of joint immobility.

Congenital arthrogryposis is part of the fetal akinesia sequence. There are multiple pathogenic factors involved in fetal hypokinesia, such as central nervous system malformations, spinal cord disease, neuromuscular disorders, and a restricted fetal environment. There are also numerous syndromes that present with this sequence. In the Marden-Walker and Pena-Shokeir syndromes the cause of the akinesia is unclear. Bamshad et al recently included FSS as one of the distal arthrogryposes and designated it DA2. Their definition of a distal arthrogryposis is “an inherited primary limb malformation disorder characterised by congenital contractures of two or more different body areas and without primary neurologic and/or muscle disease that affects limb function”. Indeed in most of the reported cases intelligence was described as normal; this includes all cases with autosomal dominant inheritance and some of the cases with autosomal recessive inheritance.

Recently, a variant of FSS with dominant inheritance has been mapped to chromosome 11p15.5-pter. A small group of patients with severe central nervous system involvement and presumed autosomal recessive inheritance has been described (table 1).

Illum et al reported two sisters and a brother from a sibship of four who were born with multiple joint contractures, camptodactyly, an expressionless face with a puckered mouth, and restricted mouth opening. There were widespread calcium deposits in the leptomeninges, on the surface of the cerebral convolutions, and throughout the brain. Calcification was also found in skeletal muscles, Schrander-Stumpel et al, Di Rocco et al, Zampino et al, and Hageman et al reported other similar cases. Schrander-Stumpel et al reported three unrelated patients who had distal arthrogryposis, severe developmental retardation, and a “whistling face” associated with the Pierre-Robin sequence. Zampino et al described a sporadic case of the whistling face syndrome in a boy who also had severe hypertonia, swallowing problems, poor weight gain, and cerebellar and brain stem atrophy. They suggested that primary brain anomalies may explain many of the syndrome’s manifestations. They suggested it might be more appropriate to speak of the Freeman-Sheldon...

Figure 2 The patient at the age of 3 years. Note the whistling face, long philtrum, blepharophimosis, narrow forehead, extreme failure to thrive, flat chest, camptodactyly, and adducted thumbs.

Figure 3 MRI at the age of 3 years. T1 weighted coronal image. Prominent cerebral and cerebellar atrophy.

Table 1 Comparison of the present patient to those previously reported

<table>
<thead>
<tr>
<th>Clinical symptoms</th>
<th>Illum et al</th>
<th>Schrander-Stumpel et al</th>
<th>Di Rocco et al</th>
<th>Zampino et al</th>
<th>Present patient</th>
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<td>Polyhydramnios</td>
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<td>Died 6 mth</td>
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<td>+</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>+</td>
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<td>+</td>
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Chromosomal duplication of band 10p14 segregating through four generations

EDITOR—In recent years the increased resolution that can be obtained with GTL banded prometaphase chromosomes has led to the recognition of abnormalities involving small regions of the karyotype. Some of these abnormalities involve deletion or duplication of only one or two chromosomal bands and are associated with a sufficiently mild phenotype as to be directly inherited. Nevertheless, directly inherited duplication of visible chromosomal material is an uncommon category of chromosomal abnormality that has been reported for a small number of specific regions of the karyotype, including 2q11.2-q21.1, 6p23-pter, 6q22-q24, 7p12-p13, 8p23.1, 9p22-p24, 14q31-q32, 15q12, and 18p. Some of these duplications are without apparent phenotypic effect, while in other cases there are mild phenotypic abnormalities. Genetic imprinting has been shown to have an effect on the phenotypic expression of dup(15)(q12) and also dup(6)(q24), and is a point for consideration in other small duplications.

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Tandem duplications occur when a second copy of a chromosomal region is inserted adjacent to the original region. They have been reported for a number of chromosomal segments, although not previously for band 10p14. Microduplication and microdeletion of chromosomal material is presumed to occur as a meiotic event following uneven crossing over between short sequences of highly similar DNA inserted at two close, but not contiguous, sites along the chromosome. Tandem duplication is generally de novo with rare cases reported of direct inheritance. To be balanced, an intrachromosomal duplication would have to be associated with chromosomal deletion of the specific region in the other homologue and to have occurred postzygotically. To our knowledge, this type of balanced chromosomal rearrangement has not been reported as a constitutional abnormality. We describe here a previously unreported small chromosomal duplication of band 10p14 segregating in a large pedigree with apparently direct inheritance in at least eight subjects, three of whom presented independently as index cases.

The pedigree of the family is shown in fig 1, with the three probands indicated. Proband 1 (IV.9 in fig 1, fig 2) was referred for chromosome studies at the age of 1 year 9 months with the clinical indications of intrauterine growth retardation, mild developmental delay, and small testes. A cleft palate had since been repaired. The child was in foster care and the foster parents reported the biological parents to be intellectually handicapped and the mother to have a very aggressive personality. There are two female sibs, both of whom are regarded as being intellectually normal, although neither has been seen by us. On review at 2 years 8 months, the child was small with height 88 cm (approximately 5th centile), weight 11 kg (3rd centile), and head circumference 47.7 cm (2 cm >3rd centile). He was being treated for seizures. He had no speech and no real vocalisation. Facial dysmorphism was definite but not marked, with the particular observations of a periorbital fullness, a flat nasal bridge with inner canthal folds, and a flattish midface (fig 2). The testes were abnormally small. There were bridged palmar creases on both hands and a degree of proximal implantation of the fourth toe on the right. Joint hypermobility was noted at the ankles.

Proband 2 (IV.5, fig 1) was referred with developmental delay. On review at the age of 2 years 2 months, he had no speech and displayed hyperactive aggressive behaviour. There was no definite facial dysmorphism. His father (III.6) had an essentially normal facies and habitus, other than an upper spinal kyphosis. He could converse sensibly, intelligibly, and appropriately. He had had special schooling and had previously worked in a sheltered workshop, although at the time of interview he was in normal employment as a forklift driver. He was prone to anger, which he himself recognised as a problem. He expressed affection and concern for his son, having an insight into the difficulties that the chromosome abnormality had caused. However, he has since left the family, and the three children (IV.5-7) are now being brought up by their retarded mother and normal maternal grandparents.

Proband 3 (III.4, fig 3) was referred for chromosome analysis with the indications of intellectual impairment and short stature at the age of 14 years. On clinical examination at the age of 16, he was borderline microcephalic (head circumference 52.7 cm, 2nd centile). He had deep set eyes, a high nasal bridge with a broad nasal tip, large mouth, and low set ears (fig 3). He had hypermobile joints. He would not allow examination of his genitalia. He was in special schooling and exhibited “boisterous” behaviour. His mother (II.2) had borderline mental abilities and had facial features similar to her son’s (fig 3B). She had a nerve deafness. She reported her daughter III.1 to be mentally retarded. One grandchild (IV.4) is said by II.2 to be developmentally delayed, but neither he nor his mother (III.2) was available for study.

Metaphase cells were prepared for chromosome analysis from peripheral blood samples using standard techniques. Cells were synchronised using either excess thymidine or FudR with BrdU release. Chromosome analysis of the three probands showed in each an apparent duplication of band...
Chromosome studies on the parents of proband 2 had initially been interpreted to show mosaicism for the dup(10)(p14) in the father (III.6). Given the structure of the pedigree, as it subsequently came to light, and upon review of the cytogenetic material, his karyotype was reinterpreted as non-mosaic 46,XY,dup(10)(p13p15) (fig 4C). The duplication of band 10p14 could be observed in chromosomes around the 850 band level. In shorter chromosomes at 400-500 band levels, the duplication is observed as a more intense band than the normal 10p14 band present in the homologue, making it difficult to distinguish the duplication from banding variation. Chromosome studies on the mother of proband 3 also showed dup(10)(p13p15) (fig 4E). Her daughter had been referred for cytogenetic study in 1987 and a normal karyotype was reported at that time, but upon review of the archived slides her karyotype was reinterpreted as 46,XX,dup(10)(p13p15)mat. Chromosome painting was carried out using a chromosome 10 specific paint (Cambio, Cambridge, UK). Chromosome painting (fig 5) of metaphase cells from proband 1 (IV.9) using a chromosome 10 specific paint showed uniform hybridisation over both chromosomes 10 (with the exception of the heterochromatic pericentromeric region). This indicated that the rearrangement in the distal region of 10p was not the result of either interchromosomal insertion or translocation.

Probes previously mapped to 10p14 (JC2080) and 10p15 (JC2216) (obtained from Dr Jen-I Mao)\(^1\) were labelled with either biotin or digoxigenin by nick translation following the manufacturer’s recommended method. Fluorescence in situ hybridisation (fig 6) on metaphase chromosomes from proband 3 (III.4) using probe JC2216 (10p15) showed hybridisation of comparable intensity to the terminal region of both the normal and duplicated chromosomes 10. In comparison, hybridisation with the probe JC2080 showed hybridisation to band 10p14 with increased intensity of signal present on the duplicated chromosome compared with the normal chromosome.

Comparative genomic hybridisation was performed using a modified protocol\(^2\) of the procedure described by Kallioniemi et al.\(^3\) Genomic DNA was extracted using the nucleon kit (Amersham) according to the manufacturer’s instructions and directly labelled by nick translation using a Vysis kit according to the manufacturer’s instructions. Test DNA was labelled with green fluorochrome and normal reference DNA was labelled with red fluorochrome. DNA samples obtained from proband 1 (IV.9) and from proband 2 (IV.5) were used in CGH experiments. For both samples, deviation of the profile towards the right occurred in the region of 10p14, indicating extra copies of DNA sequence in the test samples for this region (fig 7A).

Thus, we have described a chromosomal duplication of the band 10p14 that was ascertained independently during routine clinical cytogenetic analysis of GTL banded prometaphase chromosomes in three subjects, referred

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**Figure 2** IV.9 aged 2 years 8 months showing minor dysmorphism. Note the periorbital fullness, antverted nares, and midfacial flatness with relative prognathism.

**Figure 3** II.2 and III.4 at ages 16 and 47, respectively, showing only subtle dysmorphism. The clearest observation is of maxillary flatness.
because of mental retardation. In two cases the abnormality was shown by cytogenetic studies to be directly inherited from a parent who also showed similar clinical features to those seen in the child. Review of the genetic files of the three probands showed that they all belonged to a single kindred (fig 1). Although some family members interviewed were aware of mental retardation and behavioural problems elsewhere in the family, they did not know of the chromosome abnormality. There had been considerable family dysfunction, and some of the presumed unaffected family members have been at pains to avoid contact with their relatives.

Chromosome painting indicated that the abnormality in the distal region of 10p involved only chromosome 10 material, with neither insertion nor translocation of chromosomal material from another chromosome. CGH studies on DNA from two of the probands showed that there was increased copy number for the DNA in the distal region of 10p. This confirmed the interpretation made on the basis of the GTL banding of prometaphase chromosomes, namely, that the chromosome rearrangement is a tandem duplication of band 10p14 with breakpoints in 10p13 and 10p15. FISH studies using probes mapped to bands 10p15 and 10p14 were confirmatory.

In terms of dysmorphology, the phenotype associated with this karyotype is mild, shading into apparent normality in case III.6. Hyperactive, impulsive, and intemperate behaviour is common and cognitive impairment is universal. A number of examples of 10p trisomy are on record, but to our knowledge only two show duplication for a very similar region. Stone et al described dup(10p14→10pter) in a father and two daughters, and Benzacken et al reported dup(10) (p14→pter) resulting from a de novo unbalanced translocation. This is a larger region than the duplicated segment in the present kindred, but there is some phenotypic overlap. All have had mental defect. In respect of physical attributes, midfacial hypoplasia, epicanthic folds, and anteverted nares are in common in our cases and in those of Stone et al. Optic nerve defects, subtle or major, were described in the three affected subjects in the family of Stone et al and agenesis of the corpus callosum was shown in the patient of Benzacken et al. These defects have not been specifically sought in our cases.

The presumed parent in generation I who has apparently transmitted the duplication to at least two offspring (II.2 and, inferentially, II.5) may have been a non-mosaic carrier of the duplication, although given family reports that I.1 and I.2 were both of normal intelligence, the possibility of mosaicism remains an open question. All other obligate carriers in this kindred must have duplication of 10p14 in non-mosaic state, inherited by direct transmission. Segregation of the duplication from a parent to an affected offspring is proven in 8/21 meiotic segregations, with at least two segregations uncertain. There is no obvious selection against the duplication, and the segregation pattern is comparable to that of an autosomal dominant condition. This suggests that the duplication does not interfere with meiotic pairing and segregation, and that in utero viability is little, if at all, compromised. Fertility is apparently not affected, albeit there is the observation of small testes in one child at the age of 2 years 8 months. The duplication has been transmitted both maternally and paternally, with no clear distinction of phenotypic patterns in the two classes of affected offspring, arguing against an imprinting effect. While direct transmission of chromosomal duplications (and deletions) is well described, this is the first report of a cytogenetically detectable chromosome duplication associated with phenotypic abnormality segregating.
through as many as four generations of one family. This genetic abnormality has been the cause of an extraordinary amount of inherited mental deficiency and psychopathology in the family.

Patients III.6 and IV.5 were referred by Dr. Kelton, patient III.4 by Dr. Anderson, and patient IV.9 by Dr. Zehnwirth.

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**R J McKinlay Gardner**  
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**References**


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**Figure 6** FISH studies on metaphase cells with duplication 10p14 using (A) probe JC2216 which maps to 10p15, showing hybridisation of equal intensity to both the normal and the duplicated chromosomes 10, and (B) probe JC2080 which maps to 10p14, showing increased hybridisation on the duplicated chromosome compared with the normal chromosome 10.

**Figure 7** CGH profile for chromosome 10 obtained from hybridisation of labelled genomic test DNA hybridised together with normal female genomic DNA. (A) Test DNA obtained from IV.9. (B) Test DNA obtained from IV.5 (n=number of chromosomes included in the profile). Deviation of the profile towards the right is consistent with extra DNA sequence copy number present in the test DNA compared with the reference DNA in the distal short arm region of chromosome 10.
Somatic mosaicism associated with a mild Alport syndrome phenotype

EDITOR—Alport syndrome (AS) is a hereditary nephritis characterised by haematuria, proteinuria, and chronic renal failure associated with progressive high tone sensorineural deafness and characteristic eye lesions (macular flecks and anterior lenticonus). At the molecular level, X linked AS, which is the most common form, is caused by mutations in COL4A5, a type IV collagen gene expressed in the glomerular basement membrane of the kidney. Mutations in COL4A5 cause progressive kidney damage usually leading to renal failure in affected males in early adulthood (20-25 years, juvenile form). A small proportion of COL4A5 mutations cause a later onset form of AS with ESRF in males at >31 years, although nephritis is apparent much earlier than this. Heterozygous females are generally mildly affected and often do not develop renal failure. We report here the identification of three apparently mosaic parents (two mothers and one father) of affected subjects. In the case of the mosaic father, an unusually mild AS phenotype was observed which may be a consequence of his mosaicism.

The COL4A5 gene is composed of 51 exons spread over 250 kb of genomic DNA, which generate a 6.5 kb transcript encoding a 1685 amino acid protein and has been the subject of several large mutation studies. We have recently completed a screen of all 51 exons of COL4A5 in 153 patients with suspected X linked AS using single strand conformation polymorphism (SSCP) analysis followed by direct sequencing of fragments showing mobility shifts, and have identified mutations in 77 of these families. Where samples were available, other family members were analysed by SSCP both to provide accurate carrier diagnosis and to estimate the proportion of de novo mutations. In total, the mothers of 25 affected males and both parents of three affected females were screened, showing five de novo mutations and three instances of mosaicism.

In patient 15, AS is caused by a mutation in exon 26, 2208G→C, which changes glycine 669 to alanine and interrupts the Gly-X-Y repeat structure of the collagen triple helix. The proband is an 11 year old male who presented with haematuria at 9 years, but has no reported deafness or eye lesions and has not yet developed renal failure. His mother, who also has haematuria but no other signs, was found by SSCP analysis to possess a reduced amount of the mutant allele and this was confirmed using DNA extracted from a second blood sample (fig 1A). As an additional control, an equimolar mixture of DNA from her son and an unrelated, unaffected male was analysed to show that the two alleles amplified equally (data not shown). DNA from both maternal grandparents has also been analysed and neither possesses the mutation (fig 1A). On the basis of these results we can be unequivocal that the mutation causing AS in this family arose somatically in the mother.

A splice site mutation (849-3c→a), which results in the in frame skipping of exon 12, causes AS in patient 47. This man was diagnosed with haematuria and a renal biopsy showed irregular thickening and splitting of the glomerular basement membrane typical of AS before he developed end stage renal failure at the age of 17 years. He shows no loss of hearing or characteristic eye signs. His mother (who...
does not have haematuria) possesses a very small amount of the mutated allele (fig 1B), while his maternal grandmother does not carry the mutation at all. It seems likely, therefore, that the mutation causing AS in patient 47 arose somatically in his mother, although DNA from the maternal grandfather (unavailable) would need to be examined to confirm this.

In patient 11, AS results from the mutation 2114G→A (G638S) in exon 25 of COL4A5. In this case, the proband was a young female heterozygous for the mutation, who presented with haematuria at 4 years and in whom a diagnosis of AS was supported by a typical renal biopsy, although no other phenotypic features were observed. SSCP analysis of DNA taken from her father showed him to possess roughly equal amounts of the normal and mutant alleles (data not shown). This result has been confirmed with DNA extracted from a second blood sample by sequencing (fig 1C). It is unlikely that a Klinefelter karyotype (XXY) could explain these results, as such people are sterile; however, somatic mosaicism of XY/XXY cannot be ruled out. This result is interesting because while patient 11 was diagnosed at an early age, her father surprisingly showed no signs of AS until he went into renal failure at the relatively late age of 43. It may be that mosaicism in this father causes a mild form of AS, since some of the cells in his kidney may be expressing the normal α5 protein, resulting in a phenotype more akin to that of a heterozygous female than that of a hemizygous affected male. This is the first report of mosaicism apparently affecting the severity of AS.

Overall, this study has uncovered three probable somatic mosaics out of a total of 77 mutations found. This number is actually reduced to 3/28 when one includes only those cases where the parents were examined, giving a figure of 10.7% mosaic. This number falls into the range reported for a variety of diseases (0-30%) reviewed by Zlotogora.

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