

Letters to the Editor

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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 al*⁴ 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 DXS1200.

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.^{14–16} Li *et al*^{14,15} and Steck *et al*¹⁶ found *PTEN* mutations in four out of four and one out of three prostate cancer cell lines respectively, suggesting a role in prostate carcinogenesis.^{15,16} Cairns *et al*¹⁷ found LOH at 10q in 23 of 80 prostate tumours. Sequencing identified a mutation in *PTEN* in 10 of these 23 tumours (43%).¹⁷ More recently, Wang *et al*¹⁸ found that of 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.^{15,16} Germline mutations in *PTEN* have been shown to be the cause of Cowden disease,^{19,20} while *PTEN* deficient mice (*PTEN* +/-) show hyperplastic and dysplastic changes in the prostate and indeed develop prostate cancer.^{21,22}

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 consist 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*.¹³ These families were previously analysed for linkage to *HPC1* and showed no evidence for linkage.⁶ 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 in 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 +32T>G polymorphism in intron 8 reported by Wang *et al.*²⁴ 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 (χ^2 with 2 df=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 *v* 61 years of age or higher (χ^2 with 2 df=1.0, $p=0.61$).

A novel polymorphism (IVS4 -31insT) was found in intron 4 in one patient, 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 used, 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 cases⁶ 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 10q23.3 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.*,²⁵ who found very few *PTEN* mutations in primary prostate cancer tumours, and the work of Cairns *et al.*,¹⁷ who found a positive correlation between high prostate tumour grade and presence of *PTEN* mutation.

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- Office of Population Censuses and Surveys. *Cancer Statistics Registrations 1991*. London: 1996.
- Carter BS, Beatty TH, Steinberg GD, Childs B, Walsh PC. Mendelian inheritance of familial prostate cancer. *Proc Natl Acad Sci USA* 1992;**89**:3367-71.
- Smith JR, Freije D, Carpten JD, *et al*. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. *Science* 1996;**274**:1371-4.
- Cooney KA, McCarthy JD, Lange E, *et al*. Prostate cancer susceptibility locus on chromosome 1q: a confirmatory study. *J Natl Cancer Inst* 1997;**89**:955-9.
- Gronberg H, Xu J, Smith JR, *et al*. Early age at diagnosis in families providing evidence of linkage to the hereditary prostate cancer locus (HPC1) on chromosome 1. *Cancer Res* 1997;**57**:4707-9.
- Eeles RA, Durocher F, Edwards S, *et al*. Linkage analysis of chromosome 1q markers in 136 prostate cancer families. The Cancer Research Campaign/British Prostate Group UK Familial Prostate Cancer Study Collaborators. *Am J Hum Genet* 1998;**62**:653-8.
- Berthon P, Valeri A, Cohen-Akenine A, *et al*. Predisposing gene for early-onset prostate cancer, localized on chromosome 1q42.2-43. *Am J Hum Genet* 1998;**62**:1416-24.
- Xu J, Meyers D, Freije D, *et al*. Evidence for a prostate cancer susceptibility locus on the X chromosome. *Nat Genet* 1998;**20**:175-9.
- Eeles R. The genetics of prostate cancer in cancer biology and medicine. In: Waring M, Ponder B, eds. *The genetics of cancer*. Vol 4. Amsterdam: Kluwer Academic Press, 1995:69-70.
- Gray IC, Phillips SM, Lee SJ, Neoptolemos JP, Weissenbach J, Spurr NK. Loss of the chromosomal region 10q23-25 in prostate cancer. *Cancer Res* 1995;**55**:4800-3.
- Komiya A, Suzuki H, Ueda T, *et al*. Allelic losses at loci on chromosome 10 are associated with metastasis and progression of human prostate cancer. *Genes Chrom Cancer* 1996;**17**:245-53.
- Trybus TM, Burgess AC, Wojno KJ, Glover TW, Macoska JA. Distinct areas of allelic loss on chromosomal regions 10p and 10q in human prostate cancer. *Cancer Res* 1996;**56**:2263-7.
- Edwards SM, Dearnaley DP, Arden-Jones A, *et al*. No germline mutations in the dimerization domain of MXI1 in prostate cancer clusters. The CRC/BPG UK Familial Prostate Cancer Study Collaborators. *Cancer Research Campaign/British Prostate Group*. *Br J Cancer* 1997;**76**:992-1000.
- Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* 1997;**57**:2124-9.
- Li J, Yen C, Liaw D, *et al*. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997;**275**:1943-7.
- Steck PA, Pershouse MA, Jasser SA, *et al*. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997;**15**:356-62.
- Cairns P, Okami K, Halachmi S, *et al*. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res* 1997;**57**:4997-5000.
- Wang SI, Parsons R, Ittmann M. Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas. *Clin Cancer Res* 1998;**4**:811-15.
- Liaw D, Marsh DJ, Li J, *et al*. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 1997;**16**:64-7.

- 20 Nelen MR, van Staveren WC, Peeters EA, *et al.* Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum Mol Genet* 1997;6:1383-7.
- 21 Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. *Nat Genet* 1998;19:348-55.
- 22 Suzuki A, de la Pompa JL, Stambolic V, *et al.* High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol* 1998;8:1169-78.
- 23 Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES. Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet* 1996;58:1347-63.
- 24 Wang SI, Puc J, Li J *et al.* Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res* 1997;57:4183-6.
- 25 Feilottter HE, Nagai MA, Boag AH, Eng C, Mulligan LM. Analysis of PTEN and the 10q23 region in primary prostate carcinomas. *Oncogene* 1998;16:1743-8.

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Mutation analysis of *H19* and *NAP1L4* (*hNAP2*) candidate genes and *IGF2* DMR2 in Beckwith-Wiedemann syndrome

EDITOR—Beckwith-Wiedemann syndrome (BWS) is a human overgrowth disorder with a variable phenotype and genetic heterogeneity. Recent data indicate that the BWS locus is subject to genomic imprinting and current evidence shows that in many patients the disease is associated with epigenetic lesions of genes on 11p15.5. BWS is characterised by pre- and postnatal overgrowth, macroglossia, and anterior abdominal wall defects. Additional, but variable complications include organomegaly, hypoglycaemia, hemihypertrophy, genitourinary abnormalities, and a predisposition to embryonal tumours in about 5% of patients.¹ The genetics of BWS are complex, but parent of origin effects, suggesting genomic imprinting, have been implicated in the pathogenesis of three major groups of patients²: (1) for patients (~2%) with chromosome 11p15.5 abnormalities, duplications are of paternal origin and balanced translocations or inversion breakpoints of maternal origin; (2) in familial cases (~15% of all cases) which exhibit more complete penetrance with maternal transmission; and (3) approximately 20% of sporadic cases have uniparental disomy (paternal isodisomy) for chromosome 11p15.5. Cloning of genes in the vicinity of BWSCR1, the most distal breakpoint cluster associated with BWS balanced cytogenetic anomalies, and within the area of minimal disomy present in cases of paternal isodisomy, has led to the identification of a group of genes as potential candidates in the aetiopathology of BWS. Thus, multiple imprinted genes bounded centromerically by *NAP1L4* and telomerically by *L23mrp* (*RPL23L*) have been identified.³⁻⁶

For a gene to be a good candidate to account for a significant number of BWS cases, it should map to this region and constitute either a paternally expressed growth promoter or a maternally expressed growth inhibitor. However, because factors involved in maintaining or modifying genomic imprints may affect the epigenotype and therefore expression status of imprinted genes, it is possible that an underlying lesion in a non-imprinted gene may manifest itself as an imprinted trait. Extensive characterisation of *IGF2*, *CDKN1C*, and *KVLQT1* in BWS patients has been undertaken already. With the exception of the involvement of *KVLQT1* in some balanced translocations and inversions,⁷ and *IGF2* in paternal duplications,⁸ the only mutations within the coding sequence of a candidate gene described are for *CDKN1C*.⁹⁻¹⁴ In our series, germline *CDKN1C* mutations accounted for ~40% of familial cases and 5% of sporadic cases.¹⁴ This suggests that further BWS genes remain to be identified. Most sporadic BWS patients show LOI of *IGF2*^{15,16} and a candidate BWS gene might cause BWS by influencing *IGF2* imprinting status.

The *H19* gene maps approximately 200 kb telomeric of *IGF2* in humans; synteny is conserved in the mouse.¹⁷ 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.¹⁸ 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.^{19,20} However, in other cases with biallelic *IGF2* expression, *H19* and *IGF2* allelic methylation is normal.¹⁶ 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.²¹

The most centromeric imprinted gene in the 11p15.5 imprinted region is the candidate tumour suppressor gene *TSSC3*.^{22,23} The *NAP1L4* 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.^{24,25} Although *NAP1L4* 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. *NAP1L4* 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 strong²⁶ and it is possible that mutations in *NAP1L4* might appear to have an allele specific effect even if *NAP1L4* 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.*,²⁷ a BWSCR1 breakpoint in the region of *NAP1L4* was associated with *IGF2* LOI and normal *H19* expression. Importantly, *NAP1L4* is expressed in normal kidney and some Wilms tumours (WT) lacking *NAP1L4* expression show *IGF2* LOI (Munroe *et al.*, unpublished observations). While mutations in *NAP1L4* have not been found in sporadic Wilms tumours,²⁵ those associated with a genetic predisposition have not yet been examined, leaving open the possibility that *NAP1L4* 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,²⁸ 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

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

Site	Nucleotide position	Correct sequence	Polymorphism
Exon 1	1531		C→T
Exon 1	1569		T→A
Exon 1	1737	G→A	
Intron 2	2418	+CG	
Intron 2	2441	+CGG	
Intron 2	2461		G→C
Intron 3	2599	+G	
Intron 3	2653	-A	
Exon 4	2791		G→C
Exon 5	2894	G→A	
Exon 5	2976		T→C
Exon 5	2992		T→C
Exon 5	3238		A→G
Exon 5	3281		T→C

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.¹ Peripheral blood samples were obtained from all patients and high molecular weight genomic DNA was extracted as described previously.²⁰

The *H19* genomic sequence was numbered as in Brannan *et al.*²⁹ 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.^{19,20} 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).¹⁶ Southern analysis (*PstI* and *SmaI* digest) did not show evidence of a genomic rearrangement in any case.^{19,20}

Comparison of the *H19* sequences in BWS cases and controls to the sequence published by Brannan *et al.*²⁹ (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),^{34,35} 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 previously²⁵ 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.¹⁶ 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' CCC TCT GCC CGT GGA CAT TAG 3' and reverse: 5' GGC GGG GTC TTG GGT GGG TAG 3'. The PCR conditions were as for SSCP of *NAP1L4* with an annealing

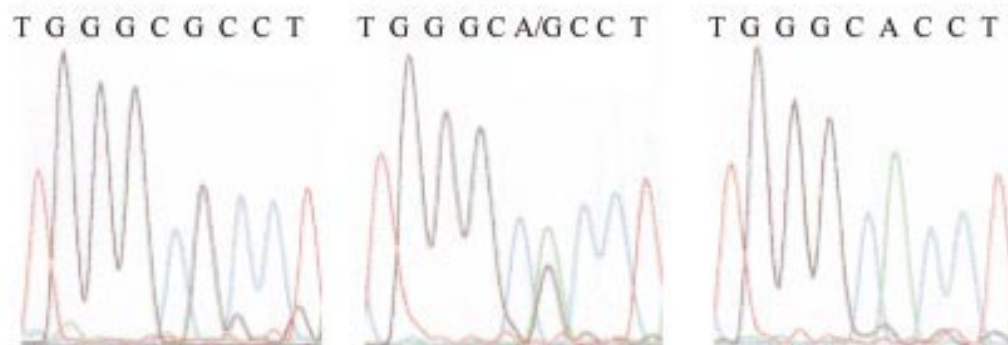


Figure 1 *H19* gene A/G polymorphism at nt 3238.

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. Thirteen 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*.^{13–15, 19} Manipulation of the mouse *H19* gene shows that mutations in this locus may result in LOI of *IGF2*,³⁰ 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.²¹ Similarly, deletion of sequences upstream and downstream of *H19*, including an endoderm specific enhancer, which affect 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 inactivation³¹ 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,³² deletion of the mouse *H19* gene does not affect the imprinting status of *Mash2*, *Cdkn1c*, or *Kcnq1*,³³ 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 BWSCR1 breakpoint²⁷ is compatible with an *H19* independent pathway of *IGF2* imprinting control. BWSCR1 rearrangements associated with LOI *IGF2* may show loss of a parental allele specific methylation pattern at *KVLQT1*, but not more distally at *IGF2/H19*.³⁶ 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 BWSCR1. *NAP1L4* (*hNAP2*) lies at the centromeric boundary of the imprinted gene cluster on 11p15^{24, 25} and maps centromeric to the BWSCR1 region in the candidate region for a cis acting regulator of *IGF2* imprinting. To date, evidence of imprinting has not been described 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 downregulator. 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 candidature 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.

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- 1 Elliott M, Maher ER. Beckwith-Wiedemann syndrome. *J Med Genet* 1994;31:560-4.
- 2 Reik W, Maher ER. Imprinting in clusters: lessons from Beckwith-Wiedemann syndrome. *Trends Genet* 1997;13:330-5.
- 3 Hu RJ, Lee MP, Connors TD, et al. A 2.5-Mb transcript map of a tumor-suppressing subchromosomal transferable fragment from 11p15.5, and isolation and sequence analysis of three novel genes. *Genomics* 1997;46:9-17.
- 4 Crider-Miller SJ, Reid LH, Higgins MJ, et al. Novel transcribed sequences within the BWS/WT2 region in 11p15.5: tissue specific expression correlates with cancer type. *Genomics* 1997;46:355-63.
- 5 Reid, LH, Davies C, Cooper PR, et al. A 1-MB physical map and PAC contig of the imprinted domain in 11p15.5 that contains TAPAI and the BWS/WT2 region. *Genomics* 1997;43:366-75.
- 6 Tsang P, Gilles F, Yuan L, et al. A novel L23 related gene 40kb downstream of the imprinted H19 gene is biallelically expressed in mid adult and fetal tissues. *Hum Mol Genet* 1995;4:1499-507.
- 7 Lee MP, Hu RJ, Johnson LA, Feinberg AP. Human KVLQT1 gene shows tissue-specific imprinting and encompasses Beckwith-Wiedemann syndrome chromosomal rearrangements. *Nat Genet* 1997;15:181-5.
- 8 Slavotinek A, Gaunt L, Donnai D. Paternally inherited duplications of 11p15.5 and Beckwith-Wiedemann syndrome. *J Med Genet* 1997;34:819-26.
- 9 Hatada I, Ohashi H, Fukushima Y, et al. An imprinted gene p57Kip2 is mutated in Beckwith-Wiedemann syndrome. *Nat Genet* 1996;14:171-4.
- 10 Lee MP, DeBaun M, Randhawa G, Reichard BA, Elledge SJ, Feinberg AP. Low frequency of p57KIP2 mutation in BWS. *Am J Hum Genet* 1997;61:304-9.
- 11 O'Keefe D, Dao D, Zhao L, et al. Coding mutations in p57 KIP2 are present in some cases of Beckwith-Wiedemann syndrome but are rare or absent in Wilms tumors. *Am J Hum Genet* 1997;61:295-303.
- 12 Okamoto K, Morison IM, Reeve AE, Tommerup N, Wiedemann HR, Friedrich U. Is p57KIP2 mutation a common mechanism for Beckwith-Wiedemann syndrome or somatic overgrowth? *J Med Genet* 1998;35:86.
- 13 Hatada I, Nabetani A, Morisaki H, et al. New p57 KIP2 mutations in Beckwith-Wiedemann syndrome. *Hum Genet* 1998;100:681-3.
- 14 Lam WWK, Hatada I, Ihishi S, et al. Analysis of germline CDKN1C (p57^{KIP2}) mutations in familial and sporadic Beckwith-Wiedemann syndrome (BWS) provides a novel genotype-phenotype correlation. *J Med Genet* 1999;36:518-23.
- 15 Weksberg R, Shen DR, Fei YL, Song QL, Squire J. Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat Genet* 1993;5:143-50.
- 16 Joyce JA, Lam WK, Catchpoole DJ, et al. Imprinting of IGF2 and H19: lack of reciprocity in sporadic Beckwith-Wiedemann syndrome. *Hum Mol Genet* 1997;6:1543-8.
- 17 Paulsen M, Davies KR, Bowden LM, et al. Syntenic organisation of the mouse distal chromosome 7 imprinting cluster and the BWS syndrome region in chromosome 11p15.5. *Hum Mol Genet* 1998;7:1149-59.
- 18 Forne T, Oswald J, Dean WL, et al. Loss of the maternal H19 gene induces changes in Igf2 methylation in both cis and trans. *Proc Natl Acad Sci USA* 1997;94:10243-8.
- 19 Reik W, Brown K, Schneid H, Le Bouc Y, Bickmore W, Maher ER. Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by an altered imprinting pattern in the IGF2-H19 domain. *Hum Mol Genet* 1995;4:2379-85.
- 20 Catchpoole DR, Lam W, Valler D, et al. Epigenetic modification and uniparental disomy of H19 in Beckwith-Wiedemann syndrome. *J Med Genet* 1997;34:353-9.
- 21 Ripoché MA, Kress C, Poirier F, Dandolo L. Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element. *Genes Dev* 1997;11:1596-604.
- 22 Qian N, Frank D, O'Keefe D, et al. The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51 implicated in fat expression and apoptosis. *Hum Mol Genet* 1997;6:2021-9.
- 23 Lee MP, Feinberg AP. Genomic imprinting of a human apoptosis gene homologue TSSC3. *Cancer Res* 1998;58:1052-6.
- 24 Hu RJ, Lee MP, Johnson LA, Feinberg AP. A novel human homologue of yeast nucleosome assembly protein, 65 kb centromeric to the p57KIP2 gene, is biallelically expressed in fetal and adult tissues. *Hum Mol Genet* 1996;5:1743-8.
- 25 Rodriguez P, Munroe D, Prawitt D, et al. Functional characterisation of human nucleosome assembly protein-2 (NAP1L4) suggests a role as a histone chaperone. *Genomics* 1997;44:253-65.
- 26 Feil R, Kelsey G. Genomic imprinting: a chromatin connection. *Am J Hum Genet* 1998;61:1213-19.
- 27 Brown, KW, Villar AJ, Bickmore W, et al. Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic IGF2 expression through an H19 independent pathway. *Hum Mol Genet* 1996;5:2027-32.
- 28 Sun FL, Dean WL, Kelsey G, Allen ND, Reik W. Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. *Nature* 1997;389:809-15.
- 29 Brannan CI, Dees EC, Ingram RS, Tilghman SM. The product of the mouse H19 gene may function as an RNA. *Mol Cell Biol* 1990;10:28-36.
- 30 Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 1995;375:34-9.
- 31 Plenge RM, Hendrich BD, Schwartz C, et al. A promoter mutation in the XIST gene in two unrelated families with skewed X-chromosome inactivation. *Nat Genet* 1997;17:353-6.
- 32 Lee JT, Jaenisch R. Long range cis effects of ectopic X-inactivation centres on a mouse autosome. *Nature* 1997;386:275-9.
- 33 Caspari T, Cleary MA, Baker CC, Guan XJ, Tilghman SM. Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol Cell Biol* 1998;18:3466-74.
- 34 Redeker E, Van Moorsel CJA, Feinberg A, Mannens M. TaqI and RsaI polymorphism in the H19 gene (D11S813). *Hum Mol Genet* 1993;2:823.
- 35 Van Gurp RJHLM, Oosterhuis JW, Kalscher K, Mariman CM, Looijenga HJ. Biallelic expression of the H19 and IGF2 genes in human testicular germ cell tumours. *J Natl Cancer Inst* 1994;86:1070-5.
- 36 Smilnich NJ, Day CD, Fitzpatrick GV, et al. A maternally methylated CpG-island in *KoLQTI* is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *Proc Natl Acad Sci USA* (in press).

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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.¹ Over 800 *CFTR* mutations have been identified.² Five mutations in Ashkenazi Jews,^{3,4} 24 in whites,⁵ and 15 in African-Americans⁶ 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.⁷ 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.⁸ That of ΔF508 is 70% in northern Europeans but it is less than 50% in Spanish and Hispanics.⁹ 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 south western United States showed that only 58% of Hispanic CF alleles were detected by screening 23 recurrent mutations.⁹ Thus, there is a need 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.¹⁰ Although the structure and function of ΔF508 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

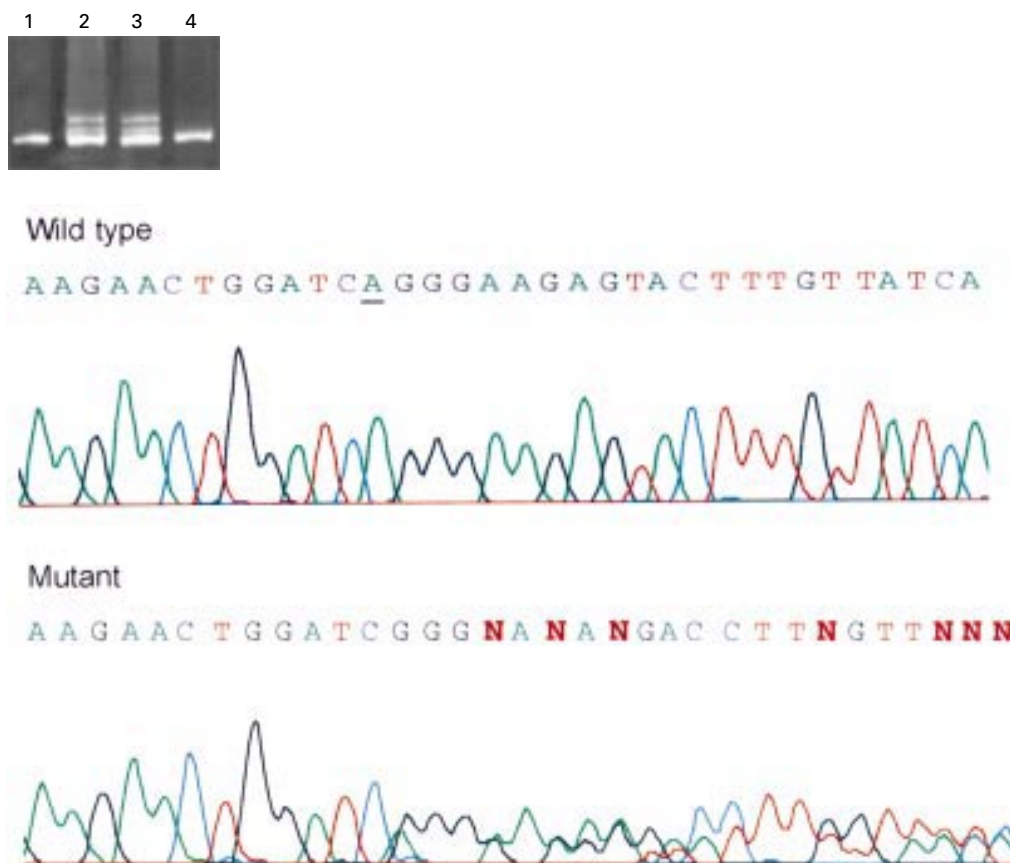


Figure 1 (Above) TTGE analysis of the DNA fragment containing *CFTR* exon 20. Lanes 1 and 4, fragments containing wild type exon 20. Lanes 3 and 4, exon 20 containing fragments amplified from patients 5 and 6. (Below) The upper panel shows the wild type DNA sequence surrounding the nucleotide position 3876 in the *CFTR* gene. The underlined "A" is deleted in heterozygous mutant DNA (lower panel).

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.⁹ We recently described the clinical features of a patient homozygous for R1066C and a group of Hispanic patients with the 3849+10kbC>T mutation.^{11,12} 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 America¹³ 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.¹⁴ Exon 20, which contains the 3876delA mutation and its flanking intron regions were PCR amplified by using prim-

ers 5'GGTCACCATTGAAAGTGT3' (forward) and 5'ATGAGAAAACCTGCACTGGA3' (reverse). The PCR reactions were performed under standard conditions as described previously.¹⁵ 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.¹⁵ 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))

Table 1 Clinical presentations of Hispanic cystic fibrosis patients with 3876delA frameshift mutation in the CFTR gene

	Patient No						
	1	2	3	4	5	6	7
Gender	F	M	F	F	F	F	M
Genotype	3876delA/1949del84	ΔF508/3876delA	ΔF508/3876delA	ΔF508/3876delA	3876delA?	ΔF508/3876delA	ΔF508/3876delA
Ethnicity	Hispanic/Hispanic	Hispanic/Hispanic	Hispanic/Hispanic	Hispanic/Hispanic	Hispanic/Hispanic	Hispanic/Hispanic	Hispanic/Hispanic
Age/age at diag*	(19)/2 mth	19/3 wk	10/4 mth	4/6 mth	(20)/5m	17/16m	26/7m
Sweat Cl ⁻ /age†	142/2 mth	84/1 mth	84/4 mth	99/4 mth	90/5m	96/10y	133/7m
Sweat Cl ⁻ (date)	142 (3/9/78)	84 (9/19/79)	84 (2/28/89)	99 (3/24/95)	90 (8/15/78)	96 (12/4/91)	133 (1/4/74)
FEV ₁ ‡/age	101/15, 37/18	103/14, 114/18	79/7.5, 113/10	N/A	102/15, 20/19	105/12, 33/16	34/20, 30/23
FVC§/age	96/15, 20/18	96/14, 97/18	72/7.5, 137/10	N/A	96/15, 30/19	103/12, 22/16	64/20, 63/23
Pancreatic status¶	PI	PI	PI	PI	PI	PI	PI
Ht/wt/age**	5/5/15, 5/5/18	5/22/14, 5/6/19	10/50/1.5, 95/95/10	7/5/0.5, 16/10/4	5/6.5/15, 5/5/18	5/5/12, 5/5/17	5/5/20, 5/5/25
Bacterial colonisation	<i>P aeruginosa</i> <i>S aureus</i>	<i>P aeruginosa</i> <i>S aureus</i>	<i>P aeruginosa</i>	<i>P aeruginosa</i> <i>S aureus</i>	<i>P aeruginosa</i> <i>S aureus</i>	<i>P aeruginosa</i> <i>S aureus</i>	<i>P aeruginosa</i> <i>S aureus</i>
Respiratory	ABPA††	ABPA	Distress as newborn	Gastro-oesophageal reflux	ABPA	ABPA, persistent cough	ABPA
Gastrointestinal status			Gastro-oesophageal reflux	Gastro-oesophageal reflux	Gastro-oesophageal reflux		
Other complications	Death	Pneumonia Bronchoscopy at 11 Hyponatraemia	Liver disease at 6, portal hypertension at 7, malabsorption, hyponatraemia	Liver disease at 10 months, malnutrition, requiring G tube at 11 months of age	Cor pulmonale, ovarian teratoma, death	Insulin dependent diabetes	

*Age at diagnosis in years; y, year; mth, month; wk, week. The number in parentheses denotes the age at death.

†Concentration of sweat chloride is in mmol/l; y, year; mth, month.

‡FEV₁ denotes forced expiratory volume in one second, percentage predicted/age at testing in years.

§FVC is a measurement of forced vital capacity, percentage predicted/age at testing in years.

¶PI, Pancreatic insufficiency, as determined by the patients' dependence on pancreatic enzyme supplements.

**Ht, height, and wt, weight, are both in percentage of normal. Age is in years.

††Allergic bronchopulmonary aspergillosis.

Patients 3 and 4 are sibs.

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 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 different ages. Patients 1, 5, and 6 showed a drastic drop in both FEV₁ and FVC values within four years, indicating a rapid deterioration in pulmonary function. Patient 4 was too young to be tested for FEV₁ and FVC. Pulmonary function of patients 2 and 3 has remained normal (>90% predicted values). All patients had infections of *Pseudomonas* and *Staphylococcus* (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,^{5,10} 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.⁵ 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 Hispanic 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/ΔF508, 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.^{8,16} 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.¹⁷ This patient had a severe clinical course as well: a sweat chloride concentration of 80 mmol/l, PI, and both respiratory and digestive problems.¹⁷

It is intriguing that after extensive search for CF mutations in Spanish,⁷ European,⁵ Ashkenazi Jewish,³ and African-American⁶ populations, the 3876delA mutation was not discovered. Since most of our Hispanic CF patients are descendants of people from Mexico and South

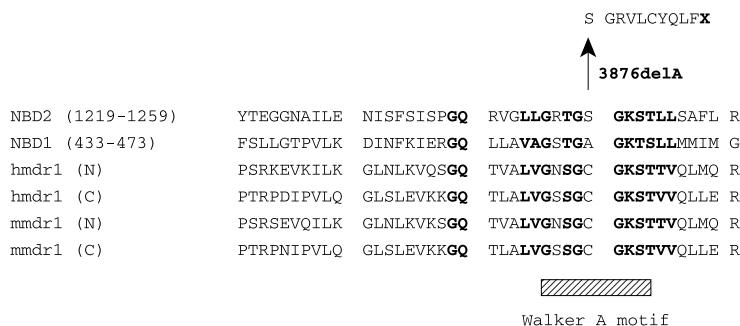


Figure 2 3876delA changes the conserved sequences in Walker A motif of the NBD2 domain of CFTR.¹ The arrow indicates the corresponding position of the single adenylic acid deletion. Conserved nucleotides are in bold. X, translation termination codon; hmdr, human multidrug resistance protein; mmdr, mouse multidrug resistance protein. N and C indicate the N-terminal and C-terminal domains of the gene where the Walker A motif is located.

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 assignment 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.²¹

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.²² 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.^{5, 23} 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.¹⁵ The 3876delA mutation offers a good opportunity for understanding the molecular mechanisms of CF pathogenesis.

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- Riordan JR, Rommens JM, Kerem BS, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 1989;245:1066-73.
- Cystic Fibrosis Consortium. CFTR Mutation Table. 1999. Web site: <http://www.gent.sickkids.on.ca/cftr/cgi-bin/FullTable>
- Abeliovich D, Lavon IP, Lerer I, et al. Screening for five mutations detects 97% of cystic fibrosis chromosomes and predicts carrier frequency of 1:29 in the Jewish Ashkenazi population. *Am J Hum Genet* 1992;51:951-6.
- DeMarchi JM, Caskey CT, Richards CS. Population-specific screening by mutation analysis for diseases frequent in Ashkenazi Jews. *Hum Mutat* 1996;8:116-25.
- Welsh MJ, Tsui LC, Boat TF, Beaudet AL. Cystic fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic and molecular bases of inherited disease*. Vol III. New York: McGraw-Hill, 1995.
- Macek M, Mackova A, Hamosh A, et al. Identification of common cystic fibrosis mutations in African Americans with cystic fibrosis increases the detection rate to 75%. *Am J Hum Genet* 1997;60:1122-7.
- Casals T, Ramos MD, Gimenez J, Larriba S, Nunes V, Estivill X. High heterogeneity for cystic fibrosis in Spanish families: 75 mutations account for 90% of chromosomes. *Hum Genet* 1997;101:365-70.
- Shoshani T, Augarten A, Gazit E, et al. Association of a nonsense mutation (W1282X), the most common mutation in the Ashkenazi Jewish cystic fibrosis patients in Israel, with presentation of severe disease. *Am J Hum Genet* 1992;50:222-8.
- Grebe TA, Seltzer WK, DeMarchi J, et al. Genetic analysis of Hispanic individuals with cystic fibrosis. *Am J Hum Genet* 1994;54:443-6.
- Stern RC. The diagnosis of cystic fibrosis. *N Engl J Med* 1997;336:487-91.
- Liang M, Wong LJC, Klein D, Shapiro B, Bowman CM, Hsu E. Cystic fibrosis in a Puerto Rican female homozygous for the R1066C mutation. *J Med Genet* 1998;35:84-5.
- Liang M, Wertz KK, Bowman CM, Hsu E, Shapiro B, Wong LJC. 3849 + 10kb C>T splicing mutation in Hispanic CF patients. *Mol Genet Metab* 1998;64:1-4.
- Arzimanoglou I, Tuchman A, Li Z, et al. Cystic fibrosis carrier screening in Hispanics. *Am J Hum Genet* 1995;56:544-7.
- Wang J, Bowman CM, Wong LJC. Improvement of mutation detection in Hispanic cystic fibrosis patients with temporal temperature gradient gel electrophoresis. *Proceedings of the 13th North American CF Conference* 1999 (in press).
- Chen T, Boles RG, Wong LJC. Detection of mitochondrial DNA mutations by temporal temperature gradient gel electrophoresis. *Clin Chem* 1999;45:1162-7.
- The Cystic Fibrosis Genotype-Phenotype Consortium. Correlation between genotype and phenotype in patients with cystic fibrosis. *N Engl J Med* 1993;329:1208-13.
- Granell R, Solera J, Carrasco S, Molano J. Identification of a nonframeshift 84-bp deletion in exon 13 of the cystic fibrosis gene. *Am J Hum Genet* 1992;50:1022-6.
- Walker HE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the α and β subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1982;1:945-51.
- Hwang TC, Nagel G, Nairn AC, Gadsby DC. Regulation of the gating of the cystic fibrosis transmembrane conductance regulator Cl channels by phosphorylation and ATP hydrolysis. *Proc Natl Acad Sci USA* 1994;91:4698-702.
- Bianchet MA, Ko YH, Amzel LM, Pedersen PL. Modeling of nucleotide binding domains of ABC transporter proteins based on a F₁-ATPase topology: structural model of the nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator (CFTR). *J Bioenerg Biomembr* 1997;29:503-24.
- Carson MR, Travis SM, Welsh MJ. The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *J Biol Chem* 1995;270:1711-17.
- Cotton JF, Welsh MJ. Covalent modification of the nucleotide binding domains of cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 1998;273:31873-9.
- Cutting GR, Kasch LM, Rosenstein BJ, Tsui LC, Kakazian HH, Antonarakis SE. Two patients with cystic fibrosis nonsense mutations in each cystic fibrosis gene, and mild pulmonary disease. *N Engl J Med* 1990;323:1685-9.

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.¹ 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.²

The clinical features of Alström syndrome are well illustrated in our families.³ 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.¹ Sensorineural deafness presented late in the first decade. Renal failure frequently develops during the third decade,³ 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 criti-

cal region of 10 cM in our families. D2S136 lies 4 cM telomeric to the minimal critical region reported by Macari *et al.*,⁵ while our centromeric boundary (D2S286) is coincident with that of Collin *et al.*⁴ Using MLINK,⁶ a maximum two point lod score of +4.62 was obtained for the marker D2S2113 at $\theta=0$. Genetic linkage analysis thus confirmed our clinical diagnosis and the recent reports of an Alström syndrome gene location at chromosome 2p13.^{4,5}

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://mmenu.hgmp.ac.uk/OMIM/203800>
- 2 Russell-Eggitt IM, Clayton PT, Coffey R, *et al.* Alström syndrome: report of 22 cases and a literature review. *Ophthalmology* 1998;105:1274-80.
- 3 Marshall JD, Ludman MD, Shea SE, *et al.* Genealogy, natural history and phenotype of Alström syndrome in a large Acadian kindred and three additional families. *Am J Med Genet* 1997;73:105-61.
- 4 Collin GB, Marshall JD, Cardon LR, *et al.* Homozygosity mapping of Alström syndrome to chromosome 2p. *Hum Mol Genet* 1997;6:213-20.
- 5 Macari F, Lautier C, Girardet A, *et al.* Refinement of genetic localisation of Alström syndrome on chromosome 2p12-13 by linkage analysis in a North African family. *Hum Genet* 1998;103:658-61.
- 6 Cottingham RW Jr, Idury RM, Schaffer AA. Faster sequential genetic linkage computations. *Am J Hum Genet* 1993;53:252-63.

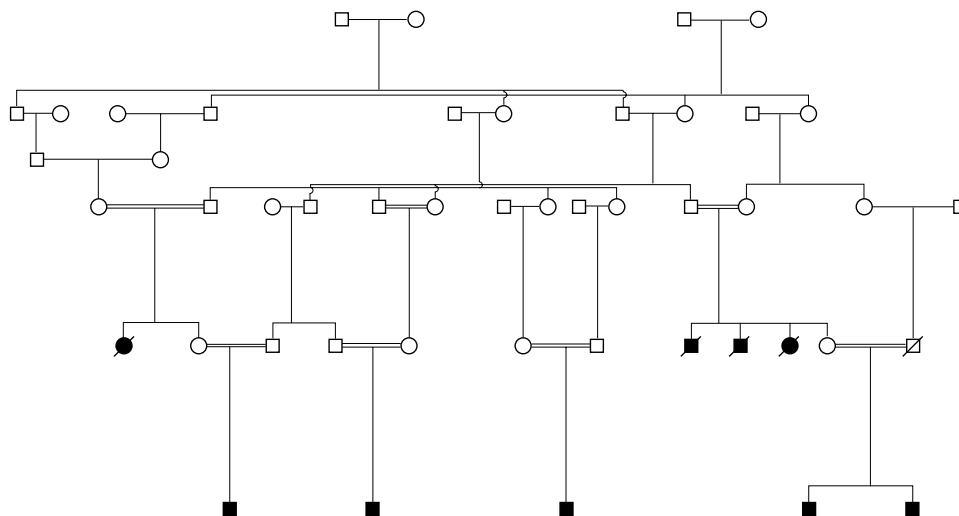


Figure 1 The simplest genealogical links between affected family members of family 1.

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.⁴ 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.²⁻⁴ 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,



Figure 1 The proband at 8 years 9 months of age. Front and side views illustrate prominent forehead, low nasal bridge, anteroposteriorly flattened thorax, and lumbar hyperlordosis.

mutation analysis of the *FGFR3* gene, located in the 4p16.3 region, showed a recurrent mutation (N540K) in several unrelated hypochondroplasia patients.^{7,8} 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.^{9,10} 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.¹¹⁻¹⁴

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-

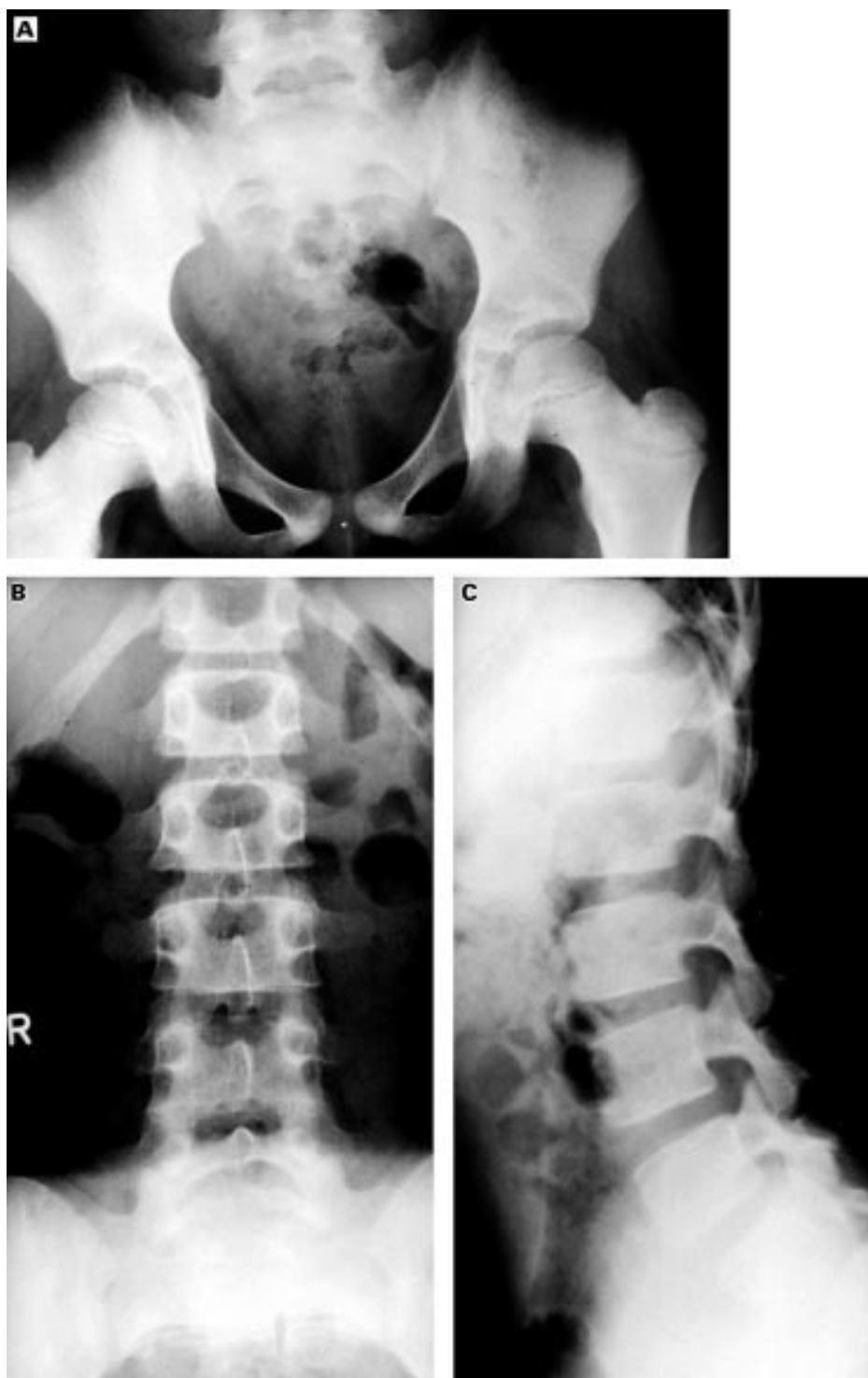


Figure 2 Radiographs of the spine and pelvis in the proband at the age of 8 years 9 months. (A) The pelvis is normal. (B) Anteroposterior view of the lumbar spine shows minimal increase in interpedicular distance from the first to fifth lumbar vertebra. (C) Lateral view of the lumbosacral spine illustrates mild anteroposterior shortening of the lumbar pedicles and accentuated lumbar lordosis with the sacrum tilted more horizontally.



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).

surements 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*.¹⁵ 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 asparagine by threonine, a neutral and

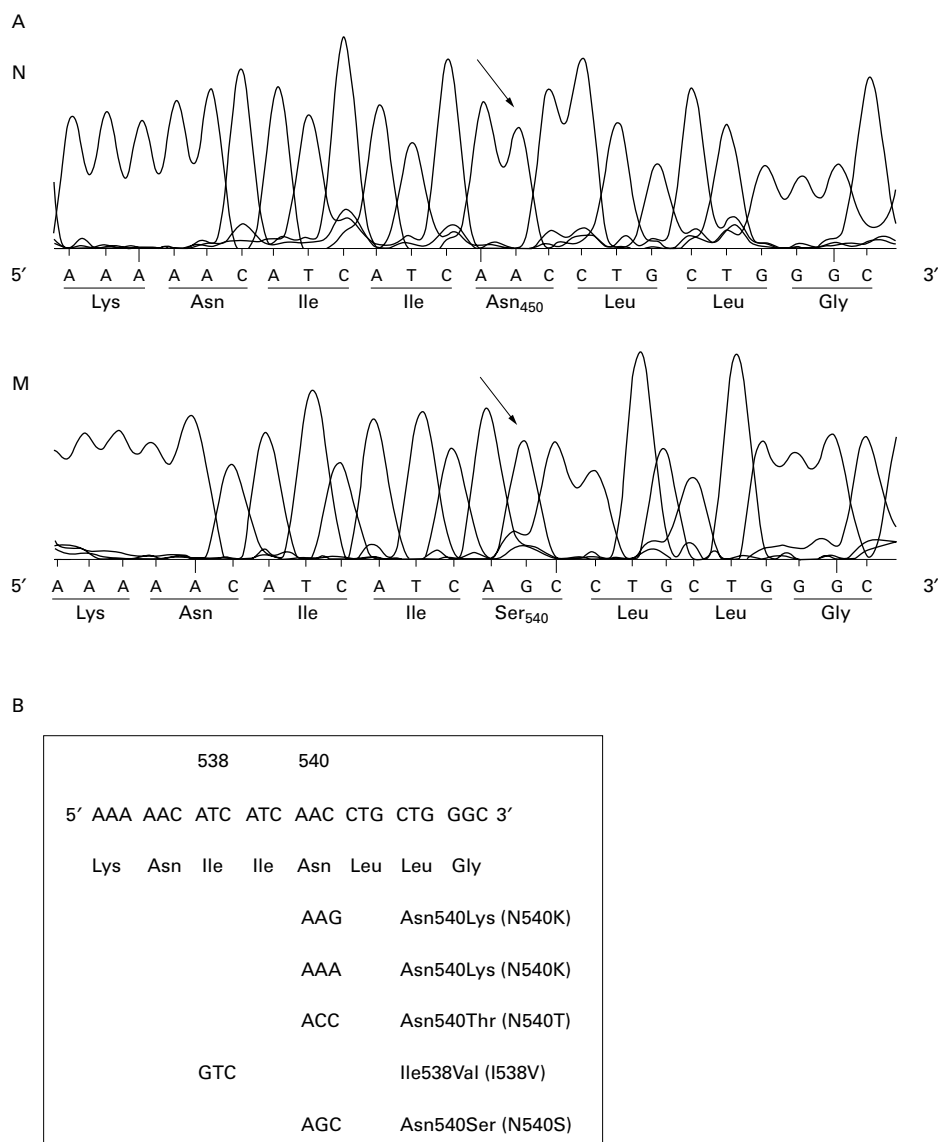


Figure 4 (A) Adenine to guanine transition in codon 540 of the FGFR3 gene in the proband. Partial nucleotide sequence of the normal (N) and mutant (M) allele is shown. The nucleotide change was found in three of seven clones. (B) Partial nucleotide sequence of the tyrosine kinase I domain of the FGFR3 gene related to the Asn540Ser and other mutations known to cause hypochondroplasia.

polar amino acid similar to serine, has been reported in hypochondroplasia.⁹

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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- Walker BA, Murdoch JL, McKusick VA, Langer LO, Beals RK. Hypochondroplasia. *Am J Dis Child* 1971;122:95-104.
- Hall BD, Spranger J. Hypochondroplasia: clinical and radiological aspects in 39 cases. *Radiology* 1979;133:95-100.
- Maroteaux P, Falzon P. Hypochondroplasia: revue de 80 cas. *Arch Fr Pediatr* 1988;45:105-9.
- Rimoin DL, Lachman RS. Genetic disorders of the osseous skeleton. In: Beighton P, ed. *McKusick's heritable disorders of connective tissue*. St Louis: Mosby-Year Book, 1993:557-689.
- Le Merrer M, Rousseau F, Legeai-Mallet L, et al. A gene for achondroplasia-hypochondroplasia maps to chromosome 4p. *Nat Genet* 1994;6:318-21.
- Hecht JT, Herrera CA, Greenhaw GA, Francomano CA, Bellus GA, Blanton SH. Confirmatory linkage of hypochondroplasia to chromosome arm 4p. *Am J Med Genet* 1995;57:505-6.
- Bellus GA, McIntosh I, Smith EA, et al. A recurrent mutation in the tyrosine kinase domain of fibroblast growth factor receptor 3 causes hypochondroplasia. *Nat Genet* 1995;10:357-9.
- Prinos P, Costa T, Sommer A, Kilpatrick MW, Tsiouras P. A common FGFR3 gene mutation in hypochondroplasia. *Hum Mol Genet* 1995;4:2097-101.
- Deutz-Terlouw PP, Losekoot M, Aalfs CM, Hennekam RCM, Bakker E. Asn540Thr substitution in the fibroblast growth factor receptor 3 tyrosine kinase domain causing hypochondroplasia. *Hum Mutat Suppl* 1998;1:S62-5.
- Grigelioniene G, Hagenäs L, Eklöf O, Neumeyer L, Haerid PE, Anvret M. A novel missense mutation Ile538Val in the fibroblast growth factor receptor 3 in hypochondroplasia. *Hum Mutat* 1998;11:333.
- Rousseau F, Bonaventure J, Legeai-Mallet L, et al. Clinical and genetic heterogeneity of hypochondroplasia. *J Med Genet* 1996;33:749-52.
- Hilbert M, Hilbert K, Spranger J, et al. Hypochondroplasia, Achondroplasia und thanatophore Dysplasie als Folge von Mutationen des

- Fibroblastenwachstumsfaktorrezeptor-3-Gens (FGFR3). *Monatsschr Kinderheilkd* 1998;146:687-91.
- 13 Prinster C, Carrera P, Del Maschio M, *et al.* Comparison of clinical-radiological and molecular findings in hypochondroplasia. *Am J Med Genet* 1998;75:109-12.

- 14 Ramaswami U, Rumsby G, Hindmarsh PC, Brook CGD. Genotype and phenotype in hypochondroplasia. *J Pediatr* 1998;133:99-102.
- 15 Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* 1993;60:1-41.

J Med Genet 2000;37:224-225

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.¹ 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,^{2,3} but families with many of the features of CADASIL had been described by Worster-Drought *et al*⁴ in the 1930s as familial presenile dementia with spastic paralysis, by Sourander and Walinder⁵ as hereditary multi-infarct dementia, and by Stevens *et al*⁶ as chronic familial vascular encephalopathy. In 1996, the responsible gene was identified as *NOTCH3*,⁸ a member of the Notch family of signalling proteins originally identified in *Drosophila*.⁹

Notch and Notch homologues control the ability of non-terminally differentiated cells to respond to differentiation/proliferation signals through local cell interactions.¹⁰ 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.¹¹ This is thought to release the intracellular domain which translocates to the nucleus to regulate the transcription of genes that ultimately determine cell fate.^{12,13}

To date, 26 separate mutations have been found in *NOTCH3*, 24 as described by Joutel *et al*¹⁴ in the French families and two additional mutations from American families, as reported by Meeks *et al*.¹⁵ 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,¹⁶ German,¹⁷

Swiss,¹⁸ Italian,^{19,20} American,^{21,22} and Japanese families.²³ 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, 608544, 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 19.²⁴ 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 Centricon™ 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

Diagnosis	No of cases	Neuropathology	Clinical symptoms	MRI	Family history	Linkage analysis	Mutations		
							Nucleotide	Amino acid	
Family 1	Definite	19	Definite	1, 2, 3, 4	Typical	Yes	Pos	C583T	R171C
Family 2	Definite	21	Definite	1, 2, 3, 4	Typical	Yes	Pos	C475T	R135C
Family 3	Probable	2	NA	1, 3, 4	Typical	Yes	NA	C499T	R143C
Family 4	Probable	4	NA	1, 2, 3	Typical	Yes	NA	C499T	R143C
Family 5	Probable	3	NA	1, 3, 4	Typical	Yes	NA	C622T	R184C
Proband family 6	Probable	1	NA	1, 2	Typical	Yes	NA	C622T	R184C

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 earlier¹⁴ (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 reported polymorphisms.¹⁴ For example, the G684A polymorphism in exon 4 was found with a frequency that matches the 0.17 described by Joutel *et al.*¹⁴

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.

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- 1 Ruchoux MM, Maurage CA. CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *J Neuropathol* 1997;56:947-64.
- 2 Mas JL, Dilouya A, De Recondo J. A familial disorder with subcortical ischemic strokes, dementia, and leukoencephalopathy. *Neurology* 1992;42:1015-19.

- 3 Tournier-Lasserre E, Joutel A, Melki J, *et al.* Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy maps to chromosome 19q12. *Nat Genet* 1993;3:256-9.
- 4 Worster-Drought C, Hill TR, McMenemy WH. Familial presenile dementia with spastic paralysis. *J Neurol Psychopathol* 1933;14:27.
- 5 Worster-Drought C, Greenfield JG, McMenemy WH. A form of familial presenile dementia with spastic paralysis. *Brain* 1940;63:237-61.
- 6 Sourander P, Walinder J. Hereditary multi-infarct dementia. Morphological and clinical studies of a new disease. *Acta Neuropathol* 1977;39:247-54.
- 7 Stevens DL, Hewlett RH, Brownell B. Chronic familial vascular encephalopathy. *Lancet* 1977;i:1365.
- 8 Joutel A, Corpechot C, Ducros A, *et al.* Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 1996;383:707-10.
- 9 Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* 1985;43:567-81.
- 10 Fleming RJ, Purcell K, Artavanis-Tsakonas S. The NOTCH receptor and its ligands. *Trends Cell Biol* 1997;7:437-41.
- 11 Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* 1991;67:687-99.
- 12 Hardy J, Israel A. In search of γ -secretase. *Nature* 1999;398:466-7.
- 13 Bray S. A notch affair. *Cell* 1998;93:499-503.
- 14 Joutel A, Vahedi K, Corpechot C, *et al.* Strong clustering and stereotyped nature of notch3 mutations in cadasil patients. *Lancet* 1997;350:1511-15.
- 15 Meeks JJ, Deng HX, Biller J, Cohen B, Siddique T. Two novel mutations in Notch3 in North American CADASIL patients. *Stroke* 1999;30:250.
- 16 Wieleaard R, Bornebroek M, Ophoff RA, *et al.* A four-generation Dutch family with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), linked to chromosome 19p13. *Clin Neurol Neurosurg* 1995;97:307-13.
- 17 Mellies JK, Baumer T, Muller JA, *et al.* SPECT study of a German CADASIL family: a phenotype with migraine and progressive dementia only. *Neurology* 1998;50:1715-21.
- 18 Jung HH, Bassetti C, Tournier-Lasserre E, *et al.* Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: a clinicopathological and genetic study of a Swiss family. *J Neurol Neurosurg* 1995;59:138-43.
- 19 Malandrini A, Carrera P, Palmeri S, *et al.* Clinicopathological and genetic studies of 2 further Italian families with cerebral autosomal dominant arteriopathy. *Acta Neuropathol* 1996;92:115-22.
- 20 Sabbadini G, Francia A, Calandriello L, *et al.* Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Clinical, neuroimaging, pathological and genetic study of a large Italian family. *Brain* 1995;118:207-15.
- 21 Bonthuis DJ, Mathews KD, Adams HP. CADASIL in a North American family: Linkage to chromosome 19. *Neurology* 1996;46:10003.
- 22 Desmond DW, Moroney JT, Lynch T, *et al.* CADASIL in a North American family - clinical, pathologic, and radiologic findings. *Neurology* 1998;51:844-9.
- 23 Uyama E, Kamimura K, Takahashi K, Tokunaga M, Tabira T, Uchino M. Notch3 gene mutation in the first Japanese CADASIL family. *Ann Neurol* 1998;44:M11.
- 24 St Clair D, Bolt J, Morris S, Doyle D. Hereditary multi-infarct dementia unlinked to chromosome 19q12 in a large Scottish pedigree: evidence of probable locus heterogeneity. *J Med Genet* 1995;32:57-60.

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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.¹ 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.²

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.³ Three mutations account for 94.4% of the CF chromosomes; these are the $\Delta F508$ (64.1%), 621+1G→T (22.6%), and A455E (7.7%) mutations.

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.⁴

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

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

	0-10 years		10.1-20 years		Over 20 years		All ages	
	No	%	No	%	No	%	No	%
<i>(A) Genotype</i>								
$\Delta F508/\Delta F508$	15 (1)	40.5	21 (2)	36.2	18 (3)	42.9	54 (6)	39.4
$\Delta F508/621+1G \rightarrow T$	12 (1)	32.4	16 (1)	27.6	10 (1*)	23.8	38 (3*)	27.7
$\Delta F508/A455E$	1	2.7	6	10.3	5	11.9	12	8.8
$\Delta F508/I148T$	1	2.7	1	1.7			2	1.5
$\Delta F508/Y1092X$			3 (1)	5.2	1	2.4	4 (1)	2.9
$\Delta F508/Q890X$					1	2.4	1	0.7
$\Delta F508/R1158X$					1	2.4	1	0.7
$621+1G \rightarrow T/621+1G \rightarrow T$	2 (1)	5.4	4	6.9	1	2.4	7 (1)	5.1
$621+1G \rightarrow T/A455E$	1	2.7	4	6.9	3	7.1	8	5.8
$621+1G \rightarrow T/711+1G \rightarrow T$	2 (1)	5.4	2 (1)	3.4			4 (2)	2.9
$621+1G \rightarrow T/Y1092X$	1	2.7					1	0.7
$621+1G \rightarrow T/S489X$	1	2.7					1	0.7
$621+1G \rightarrow T/G85E$			1 (1)	1.7	1 (1)	2.4	2 (2)	1.5
$A455E/R117C$	1	2.7					1	0.7
$N1303K/I148T$					1	2.4	1	0.7
Total	37		58		42		137	
Death	(4)	10.8	(6)	10.3	(5*)	11.9	(15*)	10.9
<i>(B) Mutation</i>								
$\Delta F508$	16 (1)	43.2	25 (3)	43.1	21 (3)	51.2	62 (7)	45.6
$621+1G \rightarrow T$	18 (3)	48.6	23 (3)	39.7	12 (2*)	29.3	53 (8*)	39.0
$A455E$	3	8.1	10	17.2	8	19.5	21	15.4
Total	37		58		41		136	
Death	(4)	10.8	(6)	10.3	(5*)	(12.2)	(15*)	(11.0)

(): Number of deaths.

*Including one accidental death.

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. It does not include 10 CF patients who died between 1973 and 1989 and were not genotyped. The proportion of patients homozygous for $\Delta F508$ remains quite constant between the three age groups, whereas there is a decline in that of the patients carrying the $621+1G \rightarrow T/\Delta F508$ genotype and an increase of those having a $\Delta F508/A455E$ or $621+1G \rightarrow T/A455E$ 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 $621+1G \rightarrow T$ mutation, but not A455E, were included in a unique group. Patients homozygous or compound heterozygous for the $\Delta F508$ mutation, without the $621+1G \rightarrow T$ or the A455E mutation, were grouped together. We observed an increase of A455E and a depletion of $621+1G \rightarrow T$ at older age groups, whereas the frequency of the $\Delta F508$ 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 dead patients in the A455E group. No significant difference was found between the $\Delta F508$ and the $621+1G \rightarrow T$ 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 age⁵⁻⁷ 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 $\Delta F508$ and $621+1G \rightarrow T$ alleles are known to be severe mutations conferring pancreatic insufficiency when

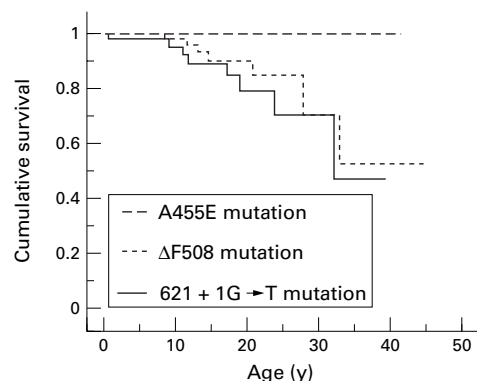


Figure 1 Survival analysis (Kaplan-Meier cumulative) for three *CFTR* mutations in a French Canadian population.

they are combined with another severe mutation.^{4 8 9} 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.² 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.

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- 1 Boat TF, Welsh MJ, Beaudet AL. Cystic fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*. New York: McGraw-Hill, 1989:2649-80.
- 2 De Braekeleer M, Allard C, Leblanc JP, Simard F, Aubin G. Genotype-phenotype correlation in cystic fibrosis patients compound heterozygous for the A455E mutation. *Hum Genet* 1997;101:208-11.
- 3 De Braekeleer M, Verlingue MC, Allard C, et al. Complete identification of cystic fibrosis transmembrane conductance regulator mutations in the CF

population of Saguenay Lac-Saint-Jean (Quebec, Canada). *Clin Genet* 1998;53:44-6.

- 4 De Braekeleer M, Simard F, Aubin G. Little phenotypic variability in three CF sibs compound heterozygous for the 621+1G→T and the 711+1G→T mutation. *Clin Genet* 1997;51:214-15.
- 5 Gan KH, Geus WP, Lamers CBHW, Heijerman HGM. Genetic and clinical features of patients with cystic fibrosis diagnosed after the age 16 years. *Thorax* 1995;50:1301-4.
- 6 Glavac MR, Glavac D, Chernick M, di Sant'Agnese P, Dean M. Screening for CF mutations in adult cystic fibrosis patients with a directed and optimized SSCP strategy. *Hum Mutat* 1994;3:231-8.
- 7 Férec C, Verlingue C, Guillermit H, et al. Genotype analysis of adult cystic fibrosis patients. *Hum Mol Genet* 1993;10:1557-60.
- 8 De Braekeleer M, Allard C, Leblanc JC, Simard F, Aubin G. Genotype-phenotype correlation in five cystic fibrosis patients homozygous for the 621+1G→T mutation. *J Med Genet* 1997;34:788-9.
- 9 The Cystic Fibrosis Genotype-Phenotype Consortium. Correlation between genotype and phenotype in patients with cystic fibrosis. *N Engl J Med* 1993;329:1308-13.

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The heritability of high myopia: a reanalysis of Goldschmidt's data

EDITOR—The prevalence of myopia varies widely.¹⁻³ 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.⁴⁻⁹ High myopia (usually defined as a refractive error ≥ -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.^{2 10 11} 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.¹² An influential series of population studies by Young *et al*^{13 14} 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.^{15 16} 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.¹⁷⁻²⁰

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.^{12 21} 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 Bear¹² and Goss *et al*²¹ suggest that myopia is likely to be aetiologically heterogeneous and that, in the case of high myopia, both polygenic and various monogenic 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 the rare connective tissue diseases Marfan syndrome and Stickler syndrome types 1 and 2, tracing the conditions to defects in the fibrillin, *COL2A1*, and *COL11A1* genes, respectively.²²⁻²⁴ 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.^{25 26} In the case of simple myopia (myopia that

Table 1 Heritability estimates for myopia from family studies

Study	Heritability	Relationship	Sample No	Comments
Sorsby <i>et al</i> ¹⁵	$h^2 = 0.45$ $h^2 = 0.49$ $h^2 = 0.72$	Parent-offspring Midparent-offspring Sib-sib	28 families 106 subjects	UK population
Nakajima <i>et al</i> ¹⁶	$h^2 = 0.16$ $h^2 = 0.42$	Parent-offspring Parent-offspring	162 pairs of subjects 35 pairs of subjects	Subset chosen as having been "measured more accurately"
Young <i>et al</i> ¹³	$h^2 = 0.10$ $h^2 = 0.98$	Parent-offspring Sib-sib	197 subjects	Eskimo population. Environment related shift in myopia prevalence with age. Only analysed a subset of population
Young and Leary ¹⁴	$h^2 = 0.46$	Midparent-offspring	1083 subjects	Eskimo population. Environment related shift in myopia prevalence with age
Keller ¹⁷	$h^2 = 0.37$	Parent-offspring	289 pairs of subjects	US population
Hegmann <i>et al</i> ¹⁸	$h^2 = 0.24$	Midparent-offspring	163 families 866 subjects	US population. Randomly selected families
Alsbirk ¹⁵	$h^2 = 0.14$ $h^2 = 0.04$ $h^2 = 0.50$	Parent-offspring Midparent-offspring Sib-sib	483 subjects	Eskimo population. Environment related shift in myopia prevalence with age
Johnson <i>et al</i> ¹⁹	$h^2 = -0.03$	Parent-offspring	76 families	Mixed Eskimo and white population. Environment related shift in myopia prevalence with age. Only analysed a subset of population
Ashton ³⁶	$h^2 = 0.49$ $h^2 = 0.74$	Midparent-offspring Sib-sib	377 families	Significant shift in refraction with age

h^2 = heritability, US = United States, UK = United Kingdom.

Table 2 Heritability estimates for myopia from twin studies

Study	Heritability	No of twin pairs	Age	Calculation	Comments
Sorsby <i>et al</i> ⁶⁰	$h^2 = 0.87$	MZ = 78 DZ = 40 (ls)	4–14	$h^2 = \frac{r_{mz} - r_{dz}}{1 - r_{dz}}$	Calculation of heritability by Goss <i>et al</i> ²¹
Nakajima ⁵¹	$h^2 = 0.83$	MZ = 39	12–17	$h^2 = \frac{\Delta_{DZ} - \Delta_{MZ}}{\Delta_{DZ}}$	Same subject group and calculation in 2 studies. Only used part of sample for analysis
Nakajima <i>et al</i> ⁶⁶	$h^2 = 0.73$	DZ = 10 (ls)			
Kimura ⁵²	$h^2 = 0.80$	MZ = 33 DZ = 16 (nls)	15–20	$h^2 = \frac{\Delta_{DZ} - \Delta_{MZ}}{\Delta_{DZ}}$	
Hu ⁵³	$h^2 = 0.61$	MZ = 49 DZ = 37 (nls)	7–19	$h^2 = \frac{r_{mz} - r_{dz}}{1 - r_{dz}}$	
Lin and Chen ⁵⁴	$h^2 = 0.25$	MZ = 90 DZ = 36 (ls)	7–23	$h^2 = 2(r_{MZ} - r_{DZ})$	
Teikari <i>et al</i> ⁵⁵	$h^2 = 0.58$	MZ = 54 DZ = 55 (ls)	30–31	$h^2 = 2(r_{MZ} - r_{DZ})$	
Angi <i>et al</i> ⁶⁶	$h^2 = 0.11$	MZ = 19 DZ = 20 (ls)	3–7	$h^2 = 2(r_{MZ} - r_{DZ})$	Unusually high prevalence of form deprivation myopia

h^2 = heritability, MZ = monozygotic, DZ = dizygotic, ls = like sexed, nls = non-like sexed, r_{mz} = correlation coefficient of MZ twins, r_{dz} = correlation coefficient of DZ twins, r_{MZ} = intrapair correlation coefficient in MZ twins (correlation in liability), r_{DZ} = intrapair correlation coefficient in DZ twins, Δ_{DZ} and Δ_{MZ} not defined by the authors.

occurs in the absence of other abnormalities), Olmedo *et al*²⁷ and Pintado *et al*²⁸ 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.^{29–30} The latter work has confirmed the aetiologically heterologous nature 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 aetiological 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,²¹ we also calculated a risk ratio for sibs (λ_s) for high myopia. In both cases, these calculations were based on a reanalysis of the data of Goldschmidt.⁵

Methods. The 1968 study by the Danish geneticist Ernst Goldschmidt⁵ 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 affected eyes rather than the number of affected people. Since the latter figure is required for calculations of a risk ratio for high myopia (see below), we have reanalysed Goldschmidt’s data in order to estimate the prevalence of highly myopic subjects, as follows. Of the 9243 children screened by Goldschmidt, 877 were myopic. In a subset of 815 of these myopes (see above), 39 were myopic by ≤ -6.00 D in

at least one eye. Thus the prevalence of high myopia was calculated as $(39/9243) \times (877/815) = 0.45\%$.

The second population group studied by Goldschmidt⁵ 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 $(2 \times 12/9243) \times (877/815) = 0.28\%$. 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,⁵ using the method described by Falconer³¹ and Vogel and Motulsky,³² where heritability is defined as the ratio of additive genetic variance (V_A) to phenotypic variance (V_P). Briefly, midparent-offspring heritability was calculated as $h = (r/0.5)$ where r was the correlation between midparent versus offspring ocular refractions. As suggested,³¹ offspring data were averaged for each family analysed. Sib-sib heritability was calculated as $h^2 = 2r$ 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.⁵

The risk ratio for sibs (λ_s) for high myopia was calculated for Goldschmidt’s child population according to the formula $\lambda_s = \text{risk to sibs of affected probands/population prevalence}$, as described.³⁴ 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 (λ_s) 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*.³⁵ This contained refractive data for a representative cross section of North American families. Unfortunately, however, the population group studied by Sperduto *et al*³⁵ was separated both in time (one and a half generations) and geography (Denmark versus the United States) from the population studied by Goldschmidt.⁵ The population prevalence of myopia in the sample of Sperduto *et al*³⁵ 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.*,³⁵ 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 (λ_s) for low myopia was therefore ≈ 1.5 .

Results. Goldschmidt⁵ 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 Goldschmidt⁵ 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 (λ_s) for high myopia, calculated from Goldschmidt's data, is 20.0 (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.*³⁵ is the only one containing sufficient detail to enable a risk ratio for low myopia to be calculated. This study yields a $\lambda_s \approx 1.5$ for low myopia. Importantly, because the population prevalence of myopia in the unselected sample studied by Sperduto *et al.*³⁵ averaged 35%, the risk ratio could never have exceeded the theoretical maximum of 2.9 (numerator in λ_s equation must be ≤ 1 , denominator = 0.35).

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 paper⁵ 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 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,^{6,35} 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,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 inaccuracy in both heritability and risk ratio estimations.

It could be argued that the λ_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 λ_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 gene effects in determining ocular refraction.^{13,31} Falconer³¹ 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 (λ_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.*^{29,30} 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, 50% 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 λ_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 analysis³⁶ is consistent with this view, although a recent study of the inheritance of corneal astigmatism³⁷ 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.^{4,38-40} However, a reanalysis of Goldschmidt's sam-

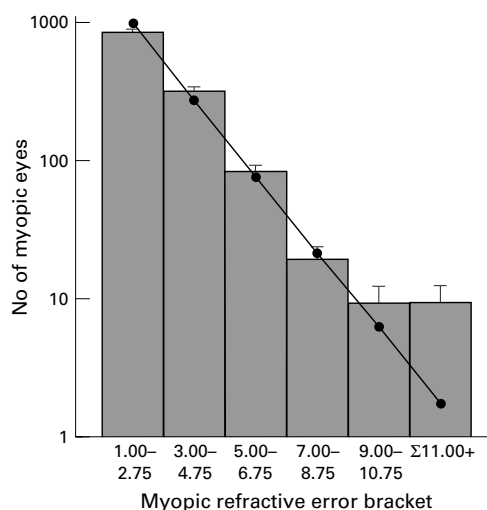


Figure 1 Frequency distribution of myopic ocular refractions in 13-14 year olds (data from Goldschmidt). Goldschmidt's data was found to fit a single inverse exponential function (Number of eyes = $3528 e^{0.6594 \times \text{ocular refraction}}$, $r^2 = 0.99$) when myopia ≥ -9.00 D was not included in the analysis (shown as individual data points and line of regression). (Note that in estimating best spheres to correct refractive errors in subjects, the spheres available to the clinician are quantised in units of 0.25 diopters. Consequently, in the groupings shown in the figure there are inevitable systematic errors of classification in a (presumably) continuous refractive error distribution, at the margins of 2.75 to 3 D, for example. However, this small error is unlikely to have a significant effect on the observed distribution of refractive errors.) Error bars show \sqrt{n} , where n is the number of observations (that is, number of eyes). The data point plotted for $\geq 11.00+$ was calculated using the above equation, for myopia extending 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.

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 this bimodal (or multimodal) distribution reflects two or more aetiologically distinct types of myopia. Betsch,³⁹ Scheerer,⁴⁰ Tron,⁴² and Hirsch⁴³ all reached similar conclusions, albeit with a variety of optimal "cut off" levels at which point the bi- or multimodality became evident. However, none of these authors based their analyses on an unselected population group such as that provided by Goldschmidt. The differences in heritability and risk ratio between high and low myopia are consistent with this idea of aetiological bimodality, although this is clearly an oversimplification.^{43 44}

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 aetiological distinct, major forms of myopia. Taken together, these results suggest that genetic factors play a significant role in the development of high myopia.

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- Working Group on Myopia Prevalence and Progression, Committee on Vision, Commission on Behavioral and Social Sciences and Education. *Myopia: prevalence and progression*. National Research Council. Washington DC: National Academy Press, 1989.
- Curtin BJ. *The myopias: basic science and clinical management*. Philadelphia: Harper & Row, 1985.
- Saw SM, Katz J, Schein OD, Chew SJ, Chan TK. Epidemiology of myopia. *Epidemiol Rev* 1996;**18**:175-87.
- Sorsby A, Sheridan M, Leary GA, Benjamin B. Vision, visual acuity, and ocular refraction of young men. *BMJ* 1960;**i**:1394-8.
- Goldschmidt E. On the etiology of myopia. *Acta Ophthalmol* 1968;**suppl** 98.
- Sperduto RD, Seigel D, Roberts J, Rowland M. Prevalence of myopia in the United States. *Arch Ophthalmol* 1983;**101**:405-7.
- Zadnik K, Mutti DO. Prevalence of myopia. In: Rosenfield M, Gilmartin B, eds. *Myopia and nearwork*. Oxford: Butterworth-Heinemann, 1998.
- Yap M, Wu M, Liu ZM, Lee FL, Wang SH. Role of heredity in the genesis of myopia. *Ophthalmic Physiol Opt* 1993;**13**:316-19.
- National Advisory Eye Council. *Vision research. A national plan: 1999-2003*. London: National Eye Institute, 1999.
- Sorsby A. Refraction. In: *Ophthalmic genetics*. 2nd ed. London: Butterworths, 1970.
- Weymouth FW, Hirsch MJ. Theories, definitions and classifications of refractive errors. In: Grosvenor T, Flom MC, eds. *Refractive anomalies: research and clinical applications*. Stoneham, MA: Butterworth-Heinemann, 1991.
- Bear JC. Epidemiology and genetics of refractive anomalies. In: Grosvenor T, Flom MC, eds. *Refractive anomalies: research and clinical applications*. Stoneham, MA: Butterworth-Heinemann, 1991.
- Young FA, Baldwin WR, Box RA, Harris E, Johnson C. The transmission of refractive error within eskimo families. *Am J Optom* 1969;**46**:676-85.
- Young FA, Leary GA. The inheritance of ocular components. *Am J Optom* 1972;**49**:546-55.
- Alsbrink PH. Refraction in adult West Greenland Eskimos. *Acta Ophthalmol* 1979;**57**:84-95.
- Bear JC, Richler A, Burke G. Nearwork and familial resemblances in ocular refraction: a population study in Newfoundland. *Clin Genet* 1981;**19**:462-72.
- Hoyt CS, Stone RD, Fromer C, Billson FA. Monocular axial myopia associated with neonatal eyelid closure in human infants. *Am J Ophthalmol* 1981;**91**:197-200.
- Raviola E, Wiesel TN. An animal model of myopia. *N Eng J Med* 1985;**312**:1609-15.
- Robb RM. Refractive errors associated with haemangiomas of the eye-lids and orbit in infancy. *Am J Ophthalmol* 1977;**83**:52-8.
- Wallman J, Turkel J, Trachtman J. Extreme myopia produced by modest changes in early visual experience. *Science* 1978;**201**:1249-51.
- Goss DA, Hampton MJ, Wickham MG. Selected review on genetic factors in myopia. *J Am Optom Assoc* 1988;**59**:875-84.
- Ahmad NN, Ala-Kokko L, Knowlton RG, et al. Stop codon in the procollagen II gene (COL2A1) in a family with Stickler syndrome (arthrochondrodysplasia). *Proc Natl Acad Sci USA* 1991;**88**:6624-7.
- Maumenee IH. The Marfan syndrome is caused by a point mutation in the fibrillin gene. *Arch Ophthalmol* 1992;**110**:472-3.
- Richards AJ, Yates JRW, Williams R, et al. A family with Stickler syndrome type 2 has a mutation in the COL11A1 gene resulting in the substitution of glycine 97 by valine in alpha 1(XI) collagen. *Hum Mol Genet* 1996;**5**:1339-43.
- Glass IA, Good P, Coleman MP, et al. Genetic mapping of a cone and rod dysfunction (Åland Island eye disease) to the proximal short arm of the human X chromosome. *J Med Genet* 1993;**30**:1044-50.
- Sertie AL, Quimby M, Moreira ES, et al. A gene which causes severe ocular alterations and occipital encephalocele (Knobloch syndrome) is mapped to 21q22.3. *Hum Mol Genet* 1996;**5**:843-7.
- Olmedo MV, Munoz JJ, Carracedo A, Gomez-Ulla F, Rodriguez MJ, Salorio MS. Myopia: a new genetic approach. *Invest Ophthalmol Vis Sci* 1992;**33**:712.
- Pintado MH, Olmedo MV, Munoz JJ, et al. Genetic differences between low and high myopia. *Invest Ophthalmol Vis Sci* 1992;**33**:712.
- Young TL, Ronan SM, Drahozal LA, et al. Evidence that a locus for familial high myopia maps to chromosome 18p. *Am J Hum Genet* 1998;**63**:109-19.
- Young TL, Ronan SM, Alvear AB, et al. A second locus for familial high myopia maps to chromosome 12q. *Am J Hum Genet* 1998;**63**:1419-24.
- Falconer DS. Heritability. In: *Introduction to quantitative genetics*. 3rd ed. Harlow, UK: Longman, 1989.
- Vogel F, Motulsky AG. *Human genetics. Problems and approaches*. Heidelberg: Springer-Verlag, 1996.
- Altman DG. *Practical statistics for medical research*. London: Chapman and Hall, 1991.
- Weeks DE, Lathrop MG. Polygenic disease: methods for mapping common disease traits. *Trends Genet* 1995;**11**:513-19.
- Sperduto RD, Hiller R, Podgor MJ, et al. Familial aggregation and prevalence of myopia in the Framington offspring eye study. *Arch Ophthalmol* 1996;**114**:326-32.
- Ashton GC. Segregation analysis of ocular refraction and myopia. *Hum Hered* 1985;**35**:232-9.
- Clementi M, Angi M, Forabosco P, Di Gianantonio E, Tenconi R. Inheritance of astigmatism: evidence for a major autosomal dominant locus. *Am J Hum Genet* 1998;**63**:825-30.
- Tscherning M. *Studier over myopiens aetologi*. København: 1882.
- Betsch A. Über die menschliche refraktionskurve. *Klin Monatsbl Augenheilkd* 1929;**82**:365-79.
- Scheerer R. Zur entwicklungsgeschichtlichen auffassung der brechzustände des auges. *Ber Dtsch Ophthal Ges* 1929;**47**:118-26.
- Stenström S. Untersuchungen über die variation und kovariation der optischen elemente des menschlichen auges. *Acta Ophthalmol* 1946;**suppl** 26.
- Tron EJ. The optical elements of the refractive power of the eye. In: Ridley F, Sorsby A, eds. *Modern trends in ophthalmology*. New York: Paul B Hoeber, 1940.
- Hirsch MJ. An analysis of inhomogeneity of myopia in adults. *Am J Optom* 1950;**27**:562-71.
- Hirsch MJ, Ditmars DL. Refraction of young myopes and their parents: a re-analysis. *Am J Optom* 1969;**46**:30-2.

- 45 Sorsby A, Leary GA, Fraser GR. Family studies on ocular refraction and its components. *J Med Genet* 1966;3:269-73.
- 46 Nakajima A, Kimura T, Kitamura K, Uesugi M, Handa Y. Studies on the heritability of some metric traits of the eye and body. *Jpn J Med Genet* 1968;13:20-39.
- 47 Keller JT. A comparison of the refractive status of myopic children and their parents. *Am J Optom* 1973;50:206-11.
- 48 Hegmann JP, Mash AJ, Spivey BE. Genetic analysis of human visual parameters in populations with varying incidences of strabismus. *Am J Hum Genet* 1974;26:549-62.
- 49 Johnson GJ, Matthews A, Perkins ES. Survey of ophthalmic conditions in a Labrador community. I. Refractive errors. *Br J Ophthalmol* 1979;63:440-8.
- 50 Sorsby A, Sheridan M, Leary GA. *Refraction and its components in twins*. MRC Report No 303. London: HMSO, 1962.
- 51 Nakajima A. The heritability estimates of the optical components of the eye and their mutual relationship by a new method of measurement on twins. *Proc 2nd Int Congr Hum Genet* 1963;1:280-7.
- 52 Kimura T. Developmental change of the optical components in twins. *Acta Soc Ophthalmol Jpn* 1965;69:963-9.
- 53 Hu D. Twin study on myopia. *Chin Med J* 1981;94:51-5.
- 54 Lin LLK, Chen CJ. Twin study on myopia. *Acta Genet Med Gemellol* 1987;36:535-40.
- 55 Teikari JM, O'Donnell J, Kaprio J, Koskenvuo M. Impact of heredity in myopia. *Hum Hered* 1991;41:151-6.
- 56 Angi MR, Clementi M, Sardei C, Piattelli E, Bisantis C. Heritability of myopic refractive errors in identical and fraternal twins. *Graefes Arch Clin Exp Ophthalmol* 1993;231:580-5.

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Progressive neurological deterioration in a child with distal arthrogyposis 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.¹ Burian rediscovered the entity and called it the “whistling face syndrome”.² There is genetic heterogeneity.³⁻⁶ Both autosomal dominant^{7,8} and recessive inheritance⁹⁻¹² have been described.

The psychomotor development of affected children is usually normal in the autosomal dominant forms,^{6,13,14} although mild motor delay attributable to joint anomalies has been reported occasionally.^{3,15-18} A few patients with severe developmental retardation have been described, all with autosomal recessive inheritance.¹⁹⁻²²

We describe a child who presented from birth with distal arthrogyposis, 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.*²³ 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, phytanic, 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 2nd centile, and weight was on the 50th centile for 12 months (-3 SD for his age). He was very thin, with an expressionless face and bilateral hand contractures with camptodactyly and ulnar deviation. His chest was flat with decreased anterior-posterior diameter (fig 2). 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



Figure 1 The patient in the neonatal period with puckered mouth and H shaped cutaneous dimpling of the chin.



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.

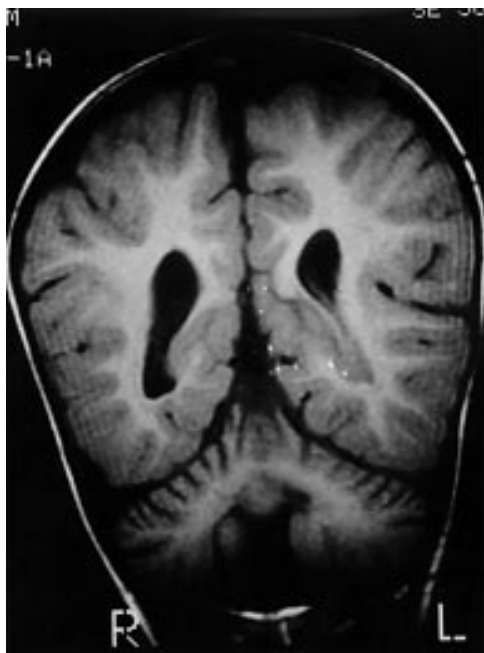


Figure 3 MRI at the age of 3 years. T1 weighted coronal image. Prominent cerebral and cerebellar atrophy.

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.¹⁴ However, EMG and structural abnormalities have not always been detected in the muscles involved.^{14 24-26} 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.³

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.²⁰ 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*²³ 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^{6 13 14} and some of the cases with autosomal recessive inheritance.¹²

Recently, a variant of FSS with dominant inheritance has been mapped to chromosome 11p15.5-pter.⁴ A small group of patients with severe central nervous system involvement and presumed autosomal recessive inheritance has been described (table 1).

Illum *et al*⁹ 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.⁹ Schrandner-Stumpel *et al*,²⁰ Di Rocco *et al*,²¹ Zampino *et al*,²² and Hageman *et al*²⁷ reported other similar cases. Schrandner-Stumpel *et al*²⁰ reported three unrelated patients who had distal arthrogryposis, severe developmental retardation, and a “whistling face” associated with the Pierre-Robin sequence. Zampino *et al*²² 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

Table 1 Comparison of the present patient to those previously reported

Clinical symptoms	Illum <i>et al</i> ⁹	Schrandner-Stumpel <i>et al</i> ²⁰	Di Rocco <i>et al</i> ²¹	Zampino <i>et al</i> ²²	Present patient
Polyhydramnios	2/3	3/3	–	–	–
Postnatal growth retardation	1/1	2/3	Died 6 mth	+	+
Hypotonia	3/3	3/3	+	–	+
Distal contractures	3/3	3/3	+	+	+
Whistling face	3/3	3/3	+	+	+
High palate	1/3	–	+	+	+
Seizures	2/3	3/3	+	–	Single
Severe mental retardation	1/1	3/3	+	+	+
Early death	3/3	1/3	At 6 mth	At 5 mth	
Neuroimaging abnormalities		2/3		+	+

spectrum rather than syndrome because of the different pathogenic mechanisms (muscular, skeletal, and neurological), the wide range of clinical manifestations, and the genetic heterogeneity.

Our patient manifests characteristic features of whistling face with profound mental retardation and central hypotonia (normal muscle biopsy). An MRI obtained at the age of 6 months was normal, but a repeat study at the age of 3 years showed progressive cerebral, cerebellar, and brain stem atrophy (fig 3). Schrandner-Stumpel *et al*²⁰ described a slightly enlarged cisterna magna and interhemispheric fissure in one patient and ventricular enlargement in the other, and Zampino *et al*²² found atrophy of the brain stem and the cerebellum, both on CT scans.

We suggest that there may be several distinct genetic syndromes with whistling face and distal contractures, one autosomal dominant with no neurological involvement, fitting the definition of the distal arthrogyroses and designated DA2, and the others possibly autosomal recessive with variable central nervous system involvement. The syndromes with normal intelligence may be caused by a primary abnormality of tendon growth and development,²³ while the others may be attributed to a primary or secondary brain anomaly as suggested by several authors.¹⁹⁻²² The progressive atrophy seen on our patient's MRI may suggest that features of whistling face with severe neurological involvement may not be the result of a primary brain malformation but rather an as yet unknown metabolic neurodegenerative disorder, starting in utero and causing fetal hypokinesia and prominent hypotonia from birth. The lack of any developmental milestones, the acquired microcephaly, failure to thrive, and early death are manifestations of the postnatal downhill course in this disorder. The early death seen in most of the patients (table 1) may be attributed to brain stem involvement with progressive feeding and respiratory difficulties. Our patient underwent an extensive metabolic evaluation. We have ruled out organic and amino acidurias, peroxisomal and mitochondrial disorders, and recently also disorders of glycoconjugates, which have been implicated in patients with developmental delay and cerebellar atrophy.²⁸ Deficiency of a factor important in both pre- and postnatal brain development may be the primary abnormality in this syndrome. More cases are needed to understand and delineate this syndrome further and to facilitate mapping.

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- Freeman EA, Sheldon JH. Cranio-carpotarsal dystrophy: an undescribed congenital malformation. *Arch Dis Child* 1938;13:277-83.
- Burian F. The "whistling face" characteristic in a compound cranio-facio-carpal syndrome. *Br J Plast Surg* 1963;16:140-63.
- Fitzsimmons JS, Zaldua V, Chrispin AR. Genetic heterogeneity in the Freeman-Sheldon syndrome: two adults with probable autosomal recessive inheritance. *J Med Genet* 1984;21:364-8.
- Krakowiak PA, O'Quinn JR, Bohnsack JF, *et al*. Variant of Freeman-Sheldon syndrome maps to 11p15.5-pter. *Am J Hum Genet* 1997;60:426-32.
- Sanchez M, Kaminker CP. New evidence for genetic heterogeneity of the Freeman-Sheldon syndrome. *Am J Med Genet* 1986;25:507-11.
- Wang TR, Lin SJ. Further evidence for genetic heterogeneity of whistling face or Freeman-Sheldon syndrome in a Chinese family. *Am J Med Genet* 1987;28:471-6.
- Fraser FC, Pashayan H, Kandish ME. Cranio-carpo-tarsal dysplasia: report of a case in father and son. *JAMA* 1970;211:1374-6.
- Wetstein A, Buchinger G, Braun A, von Bazn UB. A family with whistling-face-syndrome. *Hum Genet* 1980;55:177-89.
- Hashemi G. The whistling face syndrome: report of a case with a renal anomaly. *Indian J Pediatr* 1973;40:23-4.
- Alves AFP, Azevedo ES. Recessive form of Freeman-Sheldon's syndrome or "whistling face". *J Med Genet* 1977;14:139-41.
- Kousseff BG, McConnachie P, Hadro TA. Autosomal recessive type of whistling face syndrome in twins. *Pediatrics* 1982;69:328-31.
- Dallapiccola B, Giannotti A, Lembo A, Sagui L. Autosomal recessive form of whistling face syndrome in sibs. *Am J Med Genet* 1989;33:542-4.
- Weinstein S, Gorlin RJ. Cranio-carpo-tarsal dysplasia or whistling face syndrome: clinical considerations. *Am J Dis Child* 1969;117:427-33.
- Vanek J, Janda J, Amblerova V, Losan F. Freeman-Sheldon syndrome: a disorder of congenital myopathic origin? *J Med Genet* 1986;23:231-6.
- Rintala AE. Freeman-Sheldon syndrome, cranio-carpo-tarsal dystrophy. *Acta Paediatr Scand* 1968;57:553-6.
- Emery AE, Nelson MM. A familial syndrome of short stature deformities of the hands and feet, and an unusual facies. *J Med Genet* 1970;7:379-82.
- Antley RM, Uga N, Burzynski NJ, Baum RS, Bixler D. Diagnostic criteria for the whistling face syndrome. *BDOS* 1975;XI(5):161-8.
- O'Connell DJ, Hall CM. Cranio-carpo-tarsal dysplasia: a report of seven cases. *Pediatr Radiol* 1977;123:719-22.
- Illum N, Reske-Nielsen E, Skovby F, Askjaer SA, Bernsen A. Lethal autosomal recessive arthrogyrosis multiplex congenita with whistling face and calcifications of the nervous system. *Neuropediatrics* 1988;19:186-92.
- Schrandner-Stumpel C, Fryns JP, Beemer FA, Rive FA. Association of distal arthrogyrosis, mental retardation, whistling face, and Pierre Robin sequence: evidence for nosologic heterogeneity. *Am J Med Genet* 1991;38:557-61.
- Di Rocco M, Erriu MI, Lignana E. Distal arthrogyrosis, mental retardation, whistling face and Pierre Robin sequence: another case. *Am J Med Genet* 1992;44:391.
- Zampino G, Conti G, Balducci F, Moschini M, Macchiaiolo M, Mastroiacovo P. Severe form of Freeman-Sheldon syndrome associated with brain anomalies and hearing loss. *Am J Med Genet* 1996;62:293-6.
- Bamshad M, Bohnsack JF, Jorde LB, Carey JC. Distal arthrogyrosis type 1: clinical analysis of a large kindred. *Am J Med Genet* 1996;65:282-5.
- Sharma RN, Tandon SN. "Whistling face" deformity in a compound cranio-facio-carpal syndrome. *BMJ* 1970;4:33.
- Sauk JJ, Delaney JR, Reaume C, Bradjord R, Witkop C. Electromyography of oral-facial musculature in cranio-carpal disease. *Clin Genet* 1974;6:132-7.
- Vaitiekaitis AS, Hornstien L, Neal HW. A new surgical procedure for correction of lip deformity in cranio-carpo-tarsal dysplasia (whistling face syndrome). *J Oral Surg* 1979;37:669-72.
- Hageman G, Willemse J, van Ketel BA, Verdonck AFMM. The pathogenesis of fetal hypokinesia. A neurological study of 75 cases of congenital contractures with emphasis on cerebral lesions. *Neuropediatrics* 1988;18:22-33.
- Hagberg BA, Blennow G, Kristiansson B, Stibler H. Carbohydrate-deficient glycoprotein syndromes: peculiar group of new disorders. *Pediatr Neurol* 1993;9:255-62.

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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-q21.1,¹ 6p23-pter,² 6q22-q24,³ 7p12-p13,⁴ 8p23.1,⁵ 9p22-p24,⁶ 14q13-q22,⁷ 15q12,⁸ and 18p.⁹ Some of these duplications are without apparent phenotypic effect,^{5,8} while in other cases there are mild phenotypic abnormalities.^{1,2} Genomic imprinting has been shown to have an effect on the phenotypic expression of dup(15)(q12)⁸ and also dup(6)(q24),³ and is a point for consideration in other small duplications.

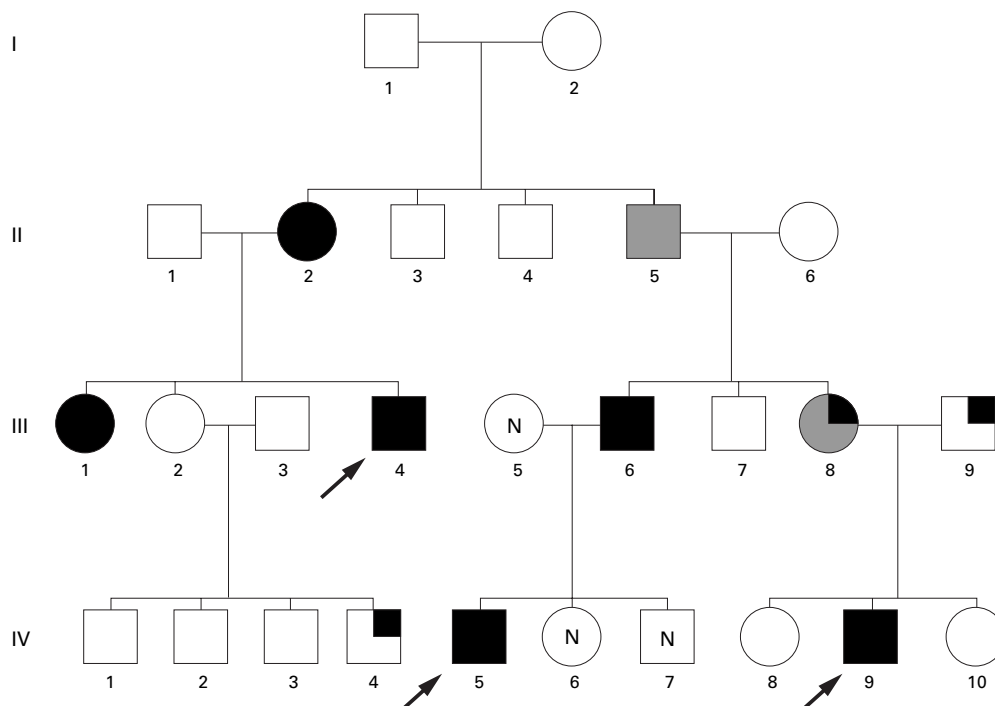


Figure 1 Family pedigree. Subjects with the 10p duplication are shown as filled symbols, obligate heterozygotes as grey symbols, and subjects with mental defect of unknown cause who have not yet been cytogenetically studied are shown quarter filled. III.5, IV.6, and IV.7 have normal karyotypes. We understand III.7, IV.8, and IV.10 to be of normal intelligence.

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,^{2,9} 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.^{10,11} Tandem duplication is generally *de novo* with rare cases reported of direct inheritance.^{3-6,8,9} 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, flat midface, 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



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

10p14 (46,XY,dup(10)(p13p15)) (fig 4A, B, D). 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)¹² 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 protocol¹³ of the procedure described by Kallioniemi *et al.*¹⁴ 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



Figure 3 II.2 and III.4 at ages 16 and 47, respectively, showing only subtle dysmorphism. The clearest observation is of maxillary flatness.

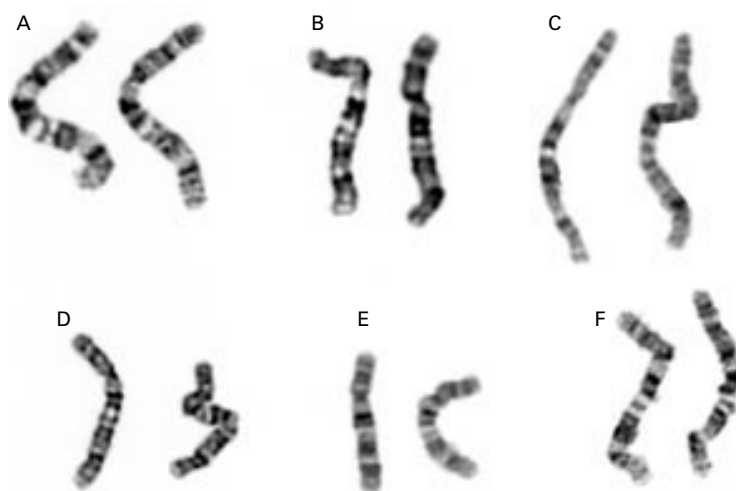


Figure 4 GTL banded partial karyotypes showing the normal chromosome 10 and the duplicated chromosome 10 (on the right) from (A) IV.9, (B) IV.5, (C) III.6, (D) III.4, (E) II.2, and (F) III.1.

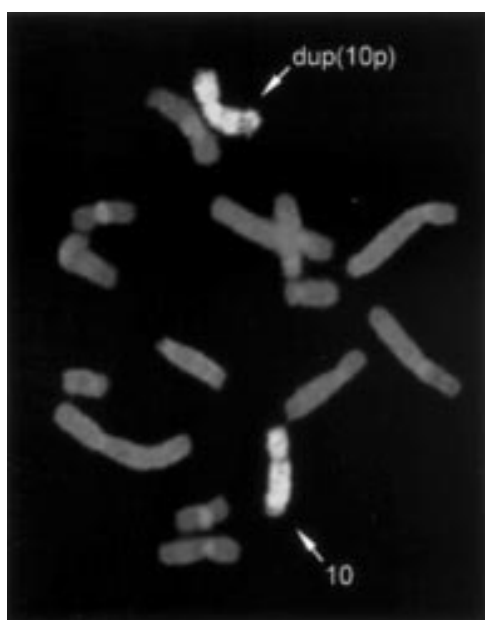


Figure 5 Chromosome painting using a chromosome 10 specific paint showing uniform hybridisation to both the normal and duplicated chromosomes 10.

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 dysmorphism, 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,¹⁵ but to our knowledge only two show duplication for a very similar region. Stone *et al*¹⁶ described $\text{dup}(10\text{p}14 \rightarrow 10\text{pter})$ in a father and two daughters, and Benzacken *et al*¹⁵ reported $\text{dup}(10)(\text{p}14 \rightarrow \text{pter})$ 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*.¹⁶ Optic nerve defects, subtle or major, were described in the three affected subjects in the family of Stone *et al*¹⁶ and agenesis of the corpus callosum was shown in the patient of Benzacken *et al*.¹⁵ 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

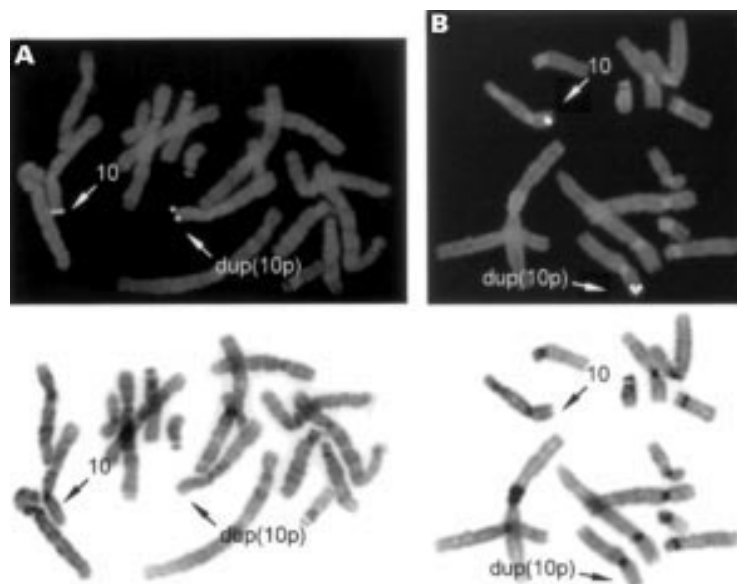


Figure 6 FISH studies on metaphase cells with duplication 10p14 using (A) probe Jc2216 which maps to 10p15, showing hybridisation of equal intensity to both the normal and the duplicated chromosomes 10, and (B) probe Jc2080 which maps to 10p14, showing increased hybridisation on the duplicated chromosome compared with the normal chromosome 10.

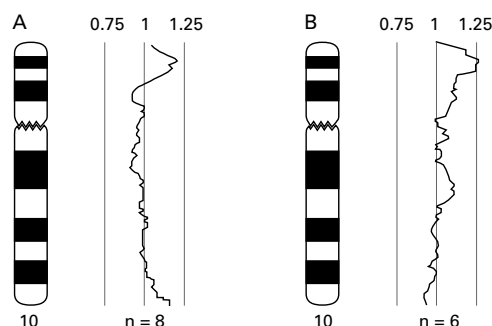


Figure 7 CGH profile for chromosome 10 obtained from hybridisation of labelled genomic test DNA hybridised together with normal female genomic DNA. (A) Test DNA obtained from IV.9. (B) Test DNA obtained from IV.5 (n =number of chromosomes included in the profile). Deviation of the profile towards the 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.

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 I J Skelton, patient III.4 by Dr S Anderson, and patient IV.9 by Dr H Zehnwrith.

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- Glass IA, Stormer P, Oei PTSP, Hacking E, Cotter PD. Trisomy 2q11.2→q21.1 resulting from an unbalanced insertion in two generations. *J Med Genet* 1999;35:319-22.
- Delatycki MB, Voullaire L, Francis D, *et al*. Directly inherited partial trisomy of chromosome 6p identified in a father and daughter by chromosome microdissection. *J Med Genet* 1999;36:335-8.
- Gardner RJ, Mungall AJ, Dunham I, *et al*. Localisation of gene for transient neonatal diabetes mellitus to an 18.72 cR₃₀₀₀ (~5.4 Mb) interval on chromosome 6q. *J Med Genet* 1999;36:192-6.
- Schaefer GB, Novak K, Steele D, *et al*. Familial inverted duplication 7p. *Am J Med Genet* 1995;56:184-7.
- Barber JCK, Joyce CA, Collinson MN, *et al*. Duplication of 8p23.1: a cytogenetic anomaly with no established clinical significance. *J Med Genet* 1998;35:491-6.
- Haddad BR, Lin AE, Wyandt H, Milunsky A. Molecular cytogenetic characterisation of the first familial case of partial 9p duplication (p22p24). *J Med Genet* 1996;33:1045-7.
- Pot MLH, Giltay JC, van Wilsen A, Breslau-Siderius EJ. Unbalanced karyotype, dup 14(q13-q22), in a mother and her two children. *Clin Genet* 1996;50:398-402.
- Cook EH, Lindgren V, Leventhal BL, *et al*. Autism or atypical autism in maternally but not paternally derived proximal 15q duplication. *Am J Hum Genet* 1997;60:928-34.
- Wolff DJ, Raffel LJ, Ferré MM, Schwartz S. Prenatal ascertainment of an inherited dup(18p) associated with an apparently normal phenotype. *Am J Med Genet* 1991;41:319-21.
- Mazzarella R, Schlessinger D. Pathological consequences of sequence duplications in the human genome. *Genome Res* 1998;8:1007-21.
- Lupski JR. Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *Trends Genet* 1998;14:417-22.
- Zheng CJ, Ma NSF, Dorman TE, *et al*. Development of 124 sequence-tagged sites and cytogenetic localization of 217 cosmids for human chromosome 10. *Genomics* 1994;22:55-67.
- Voullaire L, Wilton L, Slater H, Williamson R. Detection of aneuploidy in single cells using comparative genomic hybridization. *Prenat Diagn* 1999;19:846-51.
- Kallioniemi OP, Kallioniemi A, Piper J, *et al*. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumours. *Genes Chrom Cancer* 1994;10:231-43.
- Benzacken B, Lapierre JM, Siffroi JP, Chalvon A, Tachdjian G. Identification and characterization of a *de novo* partial trisomy 10p by comparative genomic hybridization (CGH). *Clin Genet* 1998;54:334-40.
- Stone D, Ning Y, Guan XY, Kaiser-Kupfer M, Wynshaw-Boris A, Biesecker L. Characterization of familial partial 10p trisomy by chromosomal microdissection, FISH, and microsatellite dosage analysis. *Hum Genet* 1996;98:396-402.

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 lenticonus¹). 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.² 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 protein³ and has been the subject of several large mutation studies.^{4–6} 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.⁷ 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.⁷ 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 47.⁸ 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

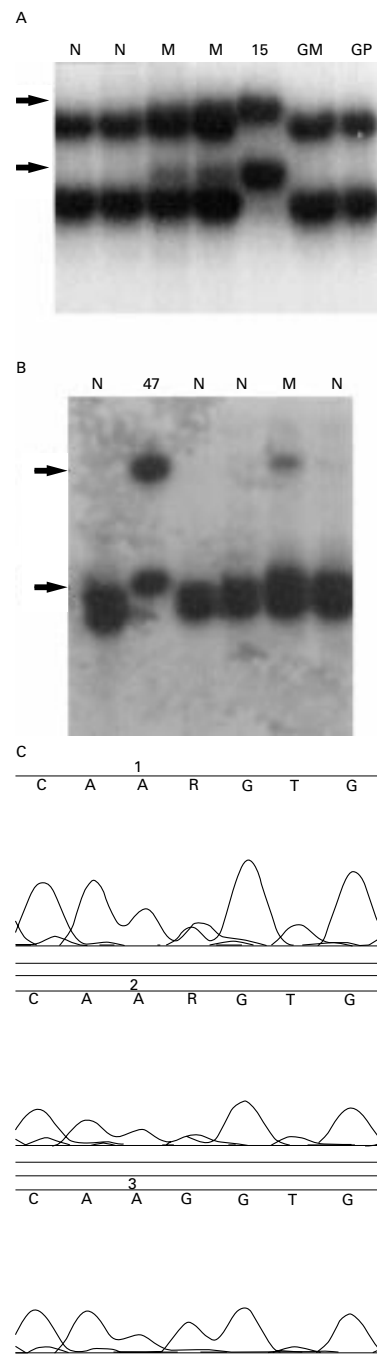


Figure 1 Somatic mosaicism in AS. Mosaicism is shown by SSCP analysis in (A) the mother of patient 15 using oligonucleotides flanking exon 26 (taccattgattactcttgc and aataaattctcactac) and (B) the mother of patient 47 using oligonucleotides flanking exons 11 and 12 (tgtctctctctcttagg and gctctctctctctctac). SSCP analysis has been described previously.⁷ Briefly, genomic DNA was extracted from peripheral blood samples and internally labelled PCR products were generated and separated on an 8% polyacrylamide/5% glycerol gel. Proband samples are numbered, M indicates maternal samples, and N indicates normal controls. In the case of patient 15, the maternal grandmother (GM) and grandfather (GP) were also analysed. (C) In the father of patient 11, mosaicism was confirmed by direct sequencing of exon 25 using an ABI 377 automated sequencer. Top panel shows sequence from patient 11 (female heterozygote for 2114G→A, labelled R); middle panel shows father, presumed mosaic; bottom panel is a normal control.

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*.⁷ 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 $\alpha 5$ 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.⁹

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- 1 Flinter FA, Chantler C, Cameron JS, Houston I, Bobrow M. Genetics of classic Alports-syndrome *Lancet* 1988;ii:1005-7.
- 2 Hostikka SL, Eddy RL, Byers MG, Hoyhtya M, Shows TB, Tryggvason K. Identification of a distinct type-IV collagen alpha-chain with restricted kidney distribution and assignment of its gene to the locus of X-chromosome-linked Alport syndrome. *Proc Natl Acad Sci USA* 1990;87:1606-10.
- 3 Zhou J, Leinonen A, Tryggvason K. Structure of the human type-IV collagen COL4A5 gene. *J Biol Chem* 1994;269:6608-14.
- 4 Kawai S, Nomura S, Harano T, et al. The COL4A5 gene in Japanese Alport syndrome patients. Spectrum of mutations of all exons. *Kidney Int* 1996;49:814-22.
- 5 Renieri A, Bruttini M, Galli L, et al. X-linked Alport syndrome: an SSCP-based mutation survey over all 51 exons of the COL4A5 gene. *Am J Hum Genet* 1996;58:1192-204.
- 6 Knebelmann B, Breillat C, Forestier L, et al. Spectrum of mutations in the COL4A5 collagen gene in X-linked Alport syndrome. *Am J Hum Genet* 1996;59:1221-32.
- 7 Plant KE, Green PM, Vetrie D, Flinter FA. Detection of mutations in COL4A5 in patients with Alport syndrome. *Hum Mutat* 1999;13:124-32.
- 8 Boye E, Flinter F, Zhou J, Tryggvason K, Bobrow M, Harris A. Detection of 12 novel mutations in the collagenous domain of the COL4A5 gene in Alport-syndrome patients. *Hum Mutat* 1995;5:197-204.
- 9 Zlotogora J. Germ line mosaicism. *Hum Genet* 1998;102:381-6.