Risk of multisystem disease in isolated ocular angioma (haemangioblastoma)

EDITOR—Ocular angioma (haemangioblastoma) is the most common presenting feature of the multisystem familial cancer syndrome von Hippel-Lindau disease (VHL). Recognition of VHL is important because of the opportunity to reduce morbidity and mortality by early diagnosis of renal cell carcinoma, pheochromocytoma, and cerebellar, spinal, and ocular haemangioblastomas. Although the finding of typical and multiple ocular lesions indicates VHL, the risk of multisystem disease in those presenting with a single ocular lesion has not been determined. That such risk exists is shown by the presence of patients with solitary angiomas in families with VHL, and the identification of mutations in the VHL gene in affected subjects without a family history of disease owing to non-penetrance for VHL manifestations in relatives and a significant new mutation rate. Consequently, the management of patients with a solitary ocular lesion may be inappropriate, such that patients with VHL may be falsely reassured, and others without symptoms may be subjected to unnecessary surveillance. On the basis of previous estimates, we have used a Bayesian approach to calculate approximate risks for VHL disease in a patient presenting with a single ocular angioma in the context of other clinical and molecular information available.

The proportion of VHL patients who have a solitary ocular angioma after ophthalmic examination has been calculated in previous work on a cohort of VHL gene carriers, and this proportion, like all the following proportions, can be used as an estimate of a corresponding conditional probability. However, calculation of the reverse conditional probability, that is, the probability of VHL given a diagnosis of solitary ocular angioma, requires the use of Bayes’ theorem, as shown below:

\[
Pr(VHL|C) = \frac{Pr(C|VHL) \times Pr(VHL)}{Pr(C)}
\]

Here Pr(VHL) represents the probability of VHL disease in a patient presenting with a single ocular angioma. Pr(C|VHL) represents the probability of a solitary angioma given a diagnosis of VHL disease, which has been estimated in a recent survey of VHL gene carriers as 0.20. Pr(VHL) represents the population prevalence of VHL disease, which is taken as 18.9 \times 10^{-6}. Pr(C) represents the prevalence of patients who harbour a solitary ocular lesion, but who do not have underlying VHL disease. The existence of non-VHL ocular angioma has been confirmed recently in a British population using a joint molecular genetic and clinical approach, and its prevalence estimated as 9.0 \times 10^{-4}. The term Pr(VHL) refers to the probability of a person in the population not being affected with VHL disease and this was taken as unity. Substitution of these values gives a probability of underlying VHL disease in a person presenting with a solitary angioma of 29.6%.

However, in the clinic, further information about the patient and their relatives is usually available, including the results of screening for extraocular features of VHL, a parental history, and the results of DNA analysis for VHL gene mutations. The significance of the first two factors in determining the risk of underlying VHL depends on the age of the patient, such that negative findings in an older patient or older parents decreases the likelihood of them being non-penetrant VHL carriers. If DNA analysis were completely sensitive, a negative result would exclude the diagnosis. However, with current DNA screening methods used in most laboratories and the possibilities of mosaicism or non-coding region gene mutations, it is prudent to assume a significant false negative detection rate. To allow for such additional information, we modified the Bayes equation, using the multiplication rule of probability calculated, as follows:

\[
Pr(VHL|C_1 \text{ and any combination of } C_2, C_3 \text{ and } C_4) = \frac{Pr(C_1|VHL) \times Pr(VHL)}{Pr(C_1|VHL) \times Pr(VHL) + \prod\{Pr(C_i|VHL)\} \times Pr(VHL)}
\]

This equation represents the probability of underlying VHL given a solitary ocular angioma (C_1) and any combination of: C_2, a negative history in the parents for VHL complications; C_3, negative systemic screening; and C_4, negative DNA analysis, depending on the information available on the patient. Pr(C_i|VHL) represents the product of the probabilities of a single ocular angioma in a VHL patient and one or more of each of these negative investigations occurring in the presence of VHL disease. These were derived as follows. Pr(C_1|VHL) is the probability of a single ocular angioma given VHL is as above. Pr(C_i|VHL) is the probability of neither parent of a VHL patient having suffered VHL related complications. This was taken as the sum of the two mutually exclusive probabilities of new mutation and non-penetrance in a VHL patient 20 years older than the subject, taken from the studies of Maher et al and Maher et al, respectively. Pr(C_i|VHL) is the probability of negative systemic screening given VHL is equivalent to the probability of non-penetrance after systemic investigation. This decreases with increasing age of a VHL gene carrier and values were taken from age of onset data from the study of Maher et al. Pr(C_i|VHL) is the probability of negative DNA analysis for a germline VHL gene mutation in a known VHL carrier was conservatively assumed to be 27% (using standard PCR and Southern techniques) (unpublished observations and ref 6). These probabilities and their derivation are summarised in table 1. The probabilities Pr(C_i|VHL) represent i=1 the probability of a solitary angioma in a non-VHL subject (above) and i=2 to 4 each of the other negative states occurring in a non-VHL person. The latter three probabilities were taken as unity.

Given these calculations, the age related risk estimates for underlying VHL in a patient with a single ocular
angioma after careful ophthalmic examination, and a combination of other negative information, are summarised in table 2.

Although some caution should be exerted when extrapolating these results to other populations (for example, the mutation detection sensitivity will depend on the precise investigations performed and the prevalence of sporadic ocular angioma might vary), this analysis does, for the first time, provide clinicians with risk estimates for the likelihood of underlying systemic disease in patients with a solitary ocular angioma. This information will help determine the most appropriate investigation and management of such patients.

We gratefully acknowledge the Guide Dogs for the Blind Association and the TFC Frost Trust for support and Dr C Bunce for reading the manuscript.

Table 2 Risk estimates for the likelihood of VHL disease given a patient presenting with a single ocular angioma for differing combinations of clinical and molecular information

<table>
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<tr>
<th>Age group (y)</th>
<th>Other negative information</th>
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<th>21–40</th>
<th>41–60</th>
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<td>0.02</td>
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<tr>
<td>Parental history</td>
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<td>0.19</td>
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<td>0.09</td>
<td>0.09</td>
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<tr>
<td>Parental history + systemic screening</td>
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<td>0.17</td>
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<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>DNA + parental history</td>
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<tr>
<td>DNA + systemic screening</td>
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<td>DNA + systemic screening + parental history</td>
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<td>0.01</td>
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Confirmation of the assignment of the Sanjad-Sakati (congenital hypoparathyroidism) syndrome (OMIM 241410) locus to chromosome 1q42-43

Editor—Over the past 12 years, 26 patients with an unusual syndrome of congenital hypoparathyroidism associated with severe prenatal and postnatal growth retardation and a pattern of facial anomalies have been seen at the King Faisal Specialist Hospital and Research Centre, Saudi Arabia. The disorder has been listed by McKusick in OMIM as “hypoparathyroidism-retardation-dysmorphism syndrome; HRD” as entry 241410. Recently, Parvari et al. reported the assignment of the gene for this disorder to chromosome 1 at 1q42-43. Their report was based on a study of consanguineous Bedouin families from Israel and their linkage analysis was based on homozygosity by descent. This report describes a study of three consanguineous Saudi families, which yielded results consistent with the 1q42-43 location of the responsible gene. Blood samples were collected and DNA extracted from three Saudi families, consisting of first cousin parents and their 14 children, five of whom manifested the Sanjad-Sakati syndrome. DNA samples were pooled from the five affected children and a separate pooled sample prepared from the DNA of their nine unaffected sibs. The initial analysis included PCR amplified DNA markers linked to genes involved either in parathyroid structure or function. As no evidence of linkage was found, the analysis was expanded to the human genome screening set from Research Genetics (Huntsville, Alabama). The analysis proceeded from chromosome 22 to chromosome 1. A positive result was based on finding a single band in the pooled sample from the affected children indicating homozygosity, while the pooled sample from the unaffected sibs showed two or more bands. A positive result with marker DIS235 prompted analysis of all 20 samples separately with the additional markers D1S1656, D1S163, D1S179, D1S2712, D1S1540, D1S1680, D1S2678, D1S2680, D1S2850, D1S373, and D1S2670, all of which cluster around 1q42-43.

Multipoint lod scores were generated using MAPMAKER/HOMOZ. Analysis of the data assumed equal frequencies of the alleles at each marker. The order of the markers was taken from the maps published by Brodie et al. The data showed that the affected sibs in the three families were homozygous for markers that clustered around the marker DIS235. A maximum lod score of 4.12 around DIS235 at 1q42-43 was obtained. Flanking markers D1S1656 and D1S2678 were consistent with those found by Parvari et al and suggest a candidate region maximally at 1 cM.

The initial report of Sanjad et al in 1988 and their definitive report in 1991 clearly established this as a distinct disorder with autosomal recessive inheritance. The consistency with which hypocalcaemic tetany or seizures or both occur in intrauterine growth retarded infants suggests that this is not a diagnosis likely to be missed. That this disorder has only been reported in consanguineous Arabic families suggests that a founder effect of a long standing mutation is responsible for this disorder.

Kenny-Caffey syndrome type 1 is clinically manifest as growth retardation, craniofacial anomalies, small hands and feet, hypocalcaemia, hypoparathyroidism, and radiological evidence of cortical thickening in the long bones with medullary stenosis and absent diploic space in the skull. The original reports of Caffey and Kenny and Linarelli suggested autosomal dominant inheritance and the condition is now referred to as Kenny-Caffey syndrome type 2. In 1997 Khan et al. reported on 16 affected children with Kenny-Caffey syndrome type 1 in six unrelated sibships born to healthy, consanguineous, Bedouin parents from Kuwait. From this group of patients, Diaz et al. in 1998 mapped the locus for this disorder to 1q42-43. All of this information taken together suggests
Molecular diagnosis is important to confirm suspected pseudoachondrodysplasia

EDITOR—Pseudoachondrodysplasia (PSACH) is an autosomal dominant chondrodysplasia. In the majority of clinically defined cases, mutations have been identified in the gene encoding cartilage oligomeric matrix protein (COMP). Mutations in the COMP gene have also been identified in some forms of multiple epiphyseal dysplasia (MED), a related skeletal dysplasia. All of the mutations associated with PSACH and MED have been found in exons encoding the type III repeat region or C-terminal domain of COMP.

Clinically, PSACH is characterised by short limbed dwarfism, which first becomes apparent in infancy, short fingers, ligamentous laxity, scoliosis, and early onset osteoarthritis (OA). Radiographic features include small irregular epiphyses with delayed ossification, flared metaphyses, anterior beaking of the vertebral bodies, and delayed maturation of the triradiate cartilage and acetabulum.

We report three patients who had previously been given erroneous diagnoses, in whom mutations in exon 13 of the COMP gene have been identified. This emphasises the utility of molecular diagnosis, particularly in adult patients where radiological diagnosis can be difficult.

All three affected subjects were born to unaffected parents. Each was of normal intelligence and normal facial appearance.

Case 1 presented at 5 years because of pain in both hips. Numerous diagnoses, including spondyloepiphysial dysplasia congenita with coxa vara and Morquio’s syndrome, were considered following x-ray examination. Extensive surgery over the following years included a left femoral osteotomy and bilateral Girdlestones operations to treat her osteoarthritis. She has had two unaffected children. Examination at 65 years showed her height at 136 cm (<3rd centile), reduced extension at the elbows, short, stubby fingers, and severe kyphosis. Radiological examination showed rhizomelic limb shortening, a prominent deltoid insertion, brachydactyly, metaphyseal broadening, extensive degenerative changes of the knee and elbow, femoral head destruction with formation of pseudoacetabula superiorly bilaterally, a thoracolumbar kyphosis with anterior wedging of the lower thoracic vertebral bodies, and a horizontal sacrum.

Case 2 first presented at 3 years with short stature (87.5 cm, <3rd centile) and bowed legs. Clinical and radiological examination suggested a diagnosis of spondyloepiphysial dysplasia type Kozlowski. Eight operations had been performed to effect tibial lengthening and straightening. Examination at 16 years showed a height of 124 cm (<3rd centile), genu varum, a waddling gait, and short stubby fingers. X-ray appearances showed ovoid vertebral bodies, epiphyseal involvement, hypoplasia of the iliac bone, splayed irregular metaphyses, and evidence of the multiple operations, with pins and a plate in situ.

Case 3, a 36 year old woman initially presented at 18 months with an intermittent limp. Radiological assessment at this time was normal. Referral at 9 years for investigation of bilateral hip and left knee pain confirmed short stature, 107 cm (<3rd centile), with rhizomelic limb shortening, short fingers, and a waddling gait. Radiological assessment showed abnormal epiphyses and metaphyses, but normal vertebral bodies. At this time diagnoses of multiple epiphyseal dysplasia, PSACH, and Morquio’s syndrome were all considered. At 21 years she had surgery to correct a subluxated left patella. She was recently referred to our department with a diagnosis of achondroplasia. She is awaiting bilateral total hip replacements for treatment of osteoarthritis. Examination showed a height of 125.5 cm (<3rd centile), short fingers, mild ligamentous laxity, and a waddling gait. X-ray appearances showed marked epiphyseal involvement of the knees, hips, and wrists bilaterally, anterior beaking of the vertebral bodies, and metaphyseal changes in the metacarpals.

All three cases presented during infancy, had height below the 3rd centile, and rhizomelic limb shortening, normal skulls, and short, stubby fingers. Cases 1 and 3 both had severe osteoarthritis affecting their hips bilaterally and necessitating surgery. Case 1 also had a severe dorsolumbar kyphosis and case 2 genu varum. The features are all within the recognised spectrum associated with PSACH and although radiological investigations were compatible with this diagnosis they were not definitive. Previous x-rays...
taken in childhood were not available from the three patients. In view of this, the clinical presentation, and the previous difficulty in diagnosis, molecular confirmation was sought.

Mutation screening in exons of the COMP gene using SSCP and sequence analysis has been described previously. In addition to mutation screening in genomic DNA from all three patients, we also screened for a COMP mutation in RNA isolated from a skin fibroblast cell line established on case 3. In this instance, cDNA was PCR amplified using primers flanking the type III repeat region and C-terminal domain of COMP. The oligonucleotide primers used were CaMF (5'-ggt gac act gtc cta gac-3') and CaMR (5'-ggt gag cgt gtc cta cag t-3'), which amplified a 789 bp fragment containing the entire type III repeat region, and CtF (5'-gaa gtc acg ctc acc gac-3') and CtR (5'-cta ggc ttg ccg cag tct cgg ctc cag ctt atg g-3'), which amplified a 702 bp fragment containing the entire C-terminal domain.

Each patient was found to have a mutation in exon 13 of COMP. Case 1 was heterozygous for an in frame 3 bp deletion of GAG between nucleotides 1394-1397, which is predicted to result in the deletion of glutamic acid at residue 457. Case 2 was found to be heterozygous for an in frame 3 bp deletion of GAC between nucleotides 1430-1444, which is predicted to result in the deletion of an aspartic acid residue from between residues 469-473. Case 3 was found to be heterozygous for a G to T transversion at nucleotide 1418, which is predicted to result in the substitution of glycine by serine at residue 465. All of the mutations affect residues within the seventh repeat of the type III repeat region and are predicted to result in a qualitative defect in the COMP protein. The in frame deletion of one or more residues from the type III repeats of COMP have been described previously. The G465S substitution affects a highly conserved glycine residue and the substitution of equivalent glycine residues in the second, fourth, and sixth type III repeats have previously been identified in patients with PSACH. Previous work has determined that in approximately 40% of cases of PSACH, deletion mutations in exon 13 of the COMP gene are responsible. Our work (present study and M D Briggs, unpublished data) suggests that approximately 50% of all PSACH results from various mutations, either deletions/duplications or point mutations, in exon 13 of the COMP gene and we propose that analysis of this exon would be an appropriate initial step in the evaluation of a patient with suspected PSACH. We have also shown that it is possible to screen for COMP gene mutations in mRNA isolated from skin fibroblast cell lines using RT-PCR. This approach will allow for the rapid screening of mutations and used in conjunction with genomic analysis of the COMP gene will help provide molecular diagnosis to confirm a clinically suspected diagnosis of PSACH.

Diagnosis of PSACH has previously been based on the clinical assessment, and the characteristic radiological features of people with short stature. These three cases show the difficulty in making a definitive diagnosis of PSACH in the absence of molecular confirmation. Accurate diagnosis is important, not only so that reproductive options may be considered in an informed context, but also so that accurate information about the prognosis of the condition may be provided. In case 1 the erroneous differential diagnoses had been made 50 years ago and had not been re-evaluated in the interim, despite the improved and expanded classification of the skeletal dysplasias. Molecular diagnosis can be especially helpful in adulthood when radiological diagnosis is more difficult owing to concomitant changes resulting from osteoarthritis.

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††MRC Tissue Bank at Hammersmith Hospital (L Wong).


Biallelic expression of IGFBP1 and IGFBP3, two candidate genes for the Silver-Russell syndrome

Silver-Russell syndrome (SRS) is a condition characterised by intrauterine and postnatal growth retardation with relative sparing of cranial growth, triangular facies, fifth finger clinodactyly, and facial, limb, or truncal asymmetry. The molecular basis of SRS remains elusive and seems likely to be heterogeneous. However, maternal uniparental disomy of chromosome 7 (mUPD7) has been found in approximately 10% of SRS patients, suggesting that at least one gene on chromosome 7 is imprinted and involved in the pathogenesis of this condition. Interest has surrounded the human chromosomal region 7p12-13, which is homologous to mouse proximal chromosome 11, since mUPD for this region in mice leads to prenatal growth failure. Within this region lie the genes for insulin-like growth factor binding proteins 1 and 3 (IGFBP1 and IGFBP3). Both are involved in regulation of fetal growth via the insulin-like growth factor axis. IGFBP1 and IGFBP3 are therefore obvious candidates for a role in the SRS phenotype associated with mUPD7.

In order to determine whether IGFBP1 and/or IGFBP3 are likely to be implicated in SRS, we have investigated their imprinting status in normal fetal tissues collected from termination of pregnancies. Samples were obtained from Queen Charlotte’s and Chelsea Hospital with the approval of the Research Ethics Committee of the Royal Postgraduate Medical School (96/495S) and from the MRC Tissue Bank at Hammersmith Hospital (L Wong). Genomic DNA and total RNA was extracted from these tissues and RT-PCR performed as previously described.

The primers used in this study are listed in table 1. PCR was carried out for 35 cycles for all reactions. Products


Letters
were sequenced on an ABI PRISM 377 automated DNA sequencer using the dRhodamine Ampli-Tag dye-terminator cycle sequencing kit, according to the manufacturer's instructions.

Since fetal IGFBP1 expression is highly tissue specific, its imprinting status was only investigated in fetal liver. Primers IGFBP1-BF and -ER were designed to span an expressed polymorphism at nucleotide 5772 within exon 4 (Genbank accession: M59316). These primers were used to screen genomic DNA from 16 first and second trimester fetuses. Five were identified as being heterozygous for the polymorphism and thus informative. Their gestational ages ranged from 5 to 17 weeks. Primers IGFBP1-EF and -ER, which span introns 2 and 3, were used to amplify liver cDNA derived from these informative fetuses. Control samples prepared from liver RNA without the addition of reverse transcriptase (RT) were also amplified. No genomic contamination was observed in any of the samples. RT-PCR products were sequenced using the reverse primer IGFBP1-ER and biallelic expression was seen in all five cases (fig 1).

An expressed polymorphism, consisting of an adenine insertion at nucleotide position 10049 (Genbank accession: M35878) within the 3' untranslated region of IGFBP3 (Dr I Morison, personal communication), was used to study the parental origin of expression in this gene. Primers IGFBP3-EF and -ER, from within exon 5, span this polymorphism and were used to screen fetal genomic DNA. The heterozygosity for this sequence change was 21%, with the adenine insertion being the most frequent allele in a UK based population. A total of 21 samples were screened and three heterozygous fetuses of 8-10 weeks' gestation were identified. Paired maternal DNA was homozygous in each of these fetuses. Sequence is shown in both forward (A) and reverse (B) directions. The polymorphic site is indicated by arrows.

Figure 1 Biallelic expression of IGFBP1 in liver from one 12 week heterozygous fetus. Sequence is shown in the reverse complement direction and the polymorphic site is indicated by arrows.

Table 1 Oligonucleotide primers used to investigate the imprinting status of IGFBP1 and 3

<table>
<thead>
<tr>
<th>Gene (Genbank No)</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temp (°C)</th>
<th>Nucleotide Nos</th>
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Figure 2 Biallelic expression of IGFBP3 in brain from one 10 week heterozygous fetus. Sequence is shown in both the forward (A) and reverse (B) directions. The polymorphic site is indicated by arrows.

4. Both parental alleles were detected in every tissue examined for all three fetuses (fig 2).

The expression of IGFBP3 was also investigated in fibroblasts and transformed lymphoblast cells from SRS patients. RNA was obtained from two normal subjects, two SRS patients with mUPD7, and four SRS patients without mUPD7. IGFBP3 mRNA expression was studied by RT-PCR using primers IGFBP3-GF and -ER which span intron 4. RT-PCR products were sequenced in both forward and reverse directions. Absence of genomic contamination was confirmed by using RT negative control samples and by sequencing RT-PCR products across the boundary of intron 66.

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<table>
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These results show that in fetal tissues both IGFBP1 and IGFBP3 are biallelically expressed. Expression of IGFBP3 in fibroblasts and lymphoblasts from patients with mUPD7 also rules out paternal specific expression of this gene in these cells in childhood. Although the expression of IGFBP1 could not be directly investigated in SRS patient cell lines, because of its tissue specificity, serum levels in mUPD7 patients were normal. We therefore found no evidence for imprinting of IGFBP1 or IGFBP3, making their involvement in SRS unlikely.

Tissue and developmental stage specific imprinting is being reported in an increasing number of genes including KCN8 (KcLQT1), IGF2, CDKN1C (p57Kip2), UBE3A, and MEST. These make absolute exclusion of a gene as a candidate, on the basis of lack of observable monoallelic expression, difficult. Allelic expression of IGFBP1 and IGFBP3 was investigated during the fetal period and in tissues that were predicted to be biologically relevant. However, the possibility of imprinting of either gene in a particular cell type or restricted window of time could not
be ruled out. Although fetuses of up to 18 weeks' gestation were screened for IGFBP3 heterozygosity, the latest stage at which an informative fetus was identified was 10 weeks gestation. Demonstration of IGFBP3 biallelic expression was therefore limited to the first trimester.

Quantitative analysis was attempted using SSCP analysis but neither polymorphism could be detected by this method. As the relative level of transcripts from the two alleles was not determined, preferential expression from one parental allele in either gene was not excluded.

Contamination by maternal tissue could potentially obscure an imprinting effect in fetal samples. For tissues used to study IGFBP3 expression, paired maternal blood samples were available. All three maternal samples from heterozygous fetuses were found to be homozygous for the polymorphism studied. Maternal specific expression could therefore not have been missed. Although it is possible that maternal contamination of fetal tissue is obscuring paternal specific expression, the demonstration of biallelic expression in all samples studied makes this unlikely. Paired maternal tissue was not available for fetal liver tissues used in the study of IGFBP3. However, the consistent observation of both alleles in five different samples makes it unlikely that monoallelic expression is being masked by maternal contamination.

Several other candidates for SRS have already been excluded. The involvement of IGF1R in SRS is unlikely since no hemizygosity or mutations were found for this gene in a cohort of 33 SRS patients.11 Riesewijk et al.12 have also failed to find evidence for a role of MEST, an imprinted gene located at 7q32. Two other candidates lie close to IGFBP3 and IGFBP3 in the region 7p12-13. EGFR is biallelically expressed and therefore unlikely to play a role in SRS. Grb10 is homologous to mouse Meg1/Grb10, a gene recently identified in a systematic screen for maternally expressed imprinted genes by subtraction hybridisation.13 The imprinting status of Grb10 in man remains to be determined and its role in SRS is currently under investigation. Since imprinted genes tend to be located in clusters, it is likely that other imprinted genes lie within the regions surrounding MEST and probably also, Grb10. These regions remain of interest in the search for candidate genes for SRS.

**Mutational analysis of the human pancreatic secretory trypsin inhibitor (PSTI) gene in hereditary and sporadic chronic pancreatitis**

**Editor—**Hereditary pancreatitis (HP) is an autosomal dominant disease with about 80% penetrance that mainly affects white families. Although pancreatitis was hypothesised to result from inappropriate activation of pancreatic zymogens by Chiarà in 1896, and the genetic nature of HP was identified by Comfort et al.1 in 1952, the precise mechanism underlying the pathogenesis of HP has remained a mystery until recently. By familial linkage analysis, a genetic defect was mapped to chromosome 7q35 by Le Bodi et al., and independently confirmed by two other groups in 1996. Soon after, a single G to A mutation resulting in an arginine (R) to histidine (H) substitution (R117H) in the third exon of the cationic trypsinogen gene was identified as being associated with HP by Whitcomb et al.2

Trypsinogen is synthesised in the acinar cells of the pancreas and is activated into trypsin upon cleavage of the activation peptide by enterokinase. Trypsin plays a central role in pancreatic exocrine physiology by acting as the trigger enzyme which leads to the activation of all the pancreatic digestive proenzymes as well as trypsinogen itself. When the R117H mutation was identified, Whitcomb et al.2 concluded that this mutation did not affect the tertiary structure of trypsin, nor alter its catalytic activity or interfere with trypsin inhibitor binding, since the three dimensional position of R117 was located on the opposite surface of the trypsin molecule to the catalytic and trypsin inhibitor binding sites. They hypothesised instead that the R117H mutation eliminated a “fail safe” mechanism for the inactivation of trypsin by abolishing an important autolytic site. Thus, the stabilised mutant enzyme would disrupt the trypsin activation/inhibition balance and trigger the pancreatic autodigestion process which results in pancreatitis under certain conditions. This model coincided...
with Chiara's pancreatitis hypothesis and has been supported by in vitro mutagenesis data. When the R117H mutation in rat trypsin was replaced by other amino acids, the rate of autolysis of certain mutant enzymes was significantly slower than that of the wild type protein. While the R117H mutation has been shown to be a common mutation in HP by several laboratories worldwide, further mutations in the cationic trypsinogen gene have been reported recently. These mutations are also presumed to facilitate the trypsin autodigestion process by altering either the tertiary structure of the protein or the binding of the pancreatic secretory trypsin inhibitor (PSTI).

However, mutations in the cationic trypsinogen gene do not appear to be the whole story. When 14 HP families from different regions of France were scanned for mutations in the cationic trypsinogen gene by denaturing gradient gel electrophoresis (DGGE) analysis, no mutations were detected in either part of the promoter region, in the intron/exon junctions, or in the gene coding sequence of six families. Furthermore, segregation analysis of one family with microsatellite markers (D7S640, D7S495, D7S684, D7S661, D7S676, D7S688) showed that the affected subjects had inherited two different haplotypes. Locus heterogeneity in HP was also suggested by the negative linkage and absence of the R117H mutation in two out of eight families studied by Dasouki et al. These findings, along with the incomplete penetrance of HP, indicated that another gene, or maybe even more than one, is involved in the pathogenesis of HP.

Human PSTI, a single chain polypeptide consisting of 56 amino acids, is also synthesised in the acinar cells of the pancreas. Its main physiological function is believed to be the prevention of the trypsin driven digestive enzyme activation cascade and of pancreatic autodigestion. Because of this central role of PSTI as a negative regulator of trypsin activity, it has been speculated that mutations in this gene may contribute to the development of pancreatic cancer. To date, no mutations have been reported in the human PSTI gene, which is located on chromosome 5. We therefore sought to investigate the possibility of mutations in the PSTI gene in a cohort of hereditary and sporadic chronic pancreatitis patients, as part of a continuing effort to gain further insight into the molecular basis of this disorder.

The human PSTI gene is approximately 7.5 kb long and is separated into 4 exons. By designing three exonic primer pairs (sequence not shown), we first successfully amplified the three introns of the PSTI gene from genomic DNA samples. The sizes of them were 1.7 kb, 1.5 kb, and 3.5 kb respectively, with a total length of ~6.7 kb, which is within the range of 7.5 kb. The three PCR fragments were then cloned into the pGEM®-T vector (Promega) and the inserts partially sequenced using T7 and SP6 promoter primers. Their identity was confirmed by comparing the resulting sequence with the published corresponding exon/intron boundary sequence. With the availability of the intronic sequence of ~100 bp immediately flanking each exon, combined with the published sequence of the 5' regulatory region and the 3' untranslated region of the PSTI gene, five DGGE primer pairs were designed to allow for a complete scanning of the 334 bp DNA sequence upstream from the translation start point, as well as of all four exons and corresponding exon/intron junctions of the gene. Detailed information about gel preparation, buffer system, and electrophoresis apparatus for DGGE analysis has been described in our previous paper. Specifically, the primer sequence, annealing temperature, optimal linear gradient range, and migration time for each amplicon are set out in table 1. The presence of a DNA variant, which was indicated by an altered pattern in the DGGE analysis, was first confirmed by independent PCR/DGGE analysis. Then a second PCR was performed under the same conditions as for DGGE analysis and the resulting PCR product was cloned and sequenced on an ABI 310. For identifying heterozygous mutations, at least three colonies were sequenced using the T7 and SP6 promoter primers. Each DNA variant has been confirmed by reamplifying and resequencing from both strands in order to avoid artefacts introduced by PCR or sequencing errors.

Among the 14 French HP families we previously studied, the R117H mutation was detected in four families, a K23R mutation in one family, a N29I in two families, and a 28delTCC in one family. None of these cationic trypsinogen mutations was detected in the remaining six families. Considering the fact that a certain fraction of HP families do not carry the trypsinogen mutations, and ~20% of the subjects carrying these mutations are non-symptomatic, we decided to analyse all the 14 families in order to screen for a possible disease causing mutation and also for a possible second mutation which may have an effect on phenotype in the PSTI gene. DGGE analysis and subsequent sequencing showed three DNA variants. They were ~253T>C, IVS1-37T>C, and a missense mutation c.101A>G (resulting in N11S) respectively, named according to the recommendations for a nomenclature system for human gene mutations.

<table>
<thead>
<tr>
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<th>Exon 3</th>
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</table>

Table 1 Primers, conditions for DGGE analysis, and DNA variants detected in the human PSTI gene
clearly a natural polymorphism. The IVS1-37>T and c.101A>G (N11S) variants occurred together in one family without trypsinogen mutations and were present in the same haplotype. These have been classified as neutral polymorphisms based primarily on the fact that they did not segregate with the disease and that they were present in control chromosomes. Moreover, the IVS1-37>T variant did not appear to affect the splice recognition sites and the c.101A>G variant did not replace the asparagine (N) at position 11 of the protein with an amino acid of different physical characteristics, although N11 is conserved in the human and two types of rat PSTI proteins.

Owing to the similar clinical, laboratory, and pathological features of hereditary and sporadic chronic pancreatitis, we also undertook DGGE analysis of sporadic chronic pancreatitis. An additional three heterozygous DNA variants were separately identified in one out of 30 patients with sporadic chronic pancreatitis. The first was a C to T transition at position 163 of the PSTI cDNA, resulting in a proline (P) to serine (S) change at position 32 of the protein (c.163C>T (P32S)). This variant did not change a conserved amino acid and it was present in control chromosomes, indicating a harmless effect on phenotype. The second was a G to A substitution at position 41 upstream from the translation start site (−41G>A). This −41G is not conserved in the human and the two types of rat PSTI genes and is located −20 bp downstream from the main transcription start site. This suggests that the −41G→A substitution could not have a significant effect on the transcriptional or translational activity of the PSTI gene. We believe it to be a rare polymorphism as it was not detected in 400 control chromosomes. The third variant was a C to T transition at position 174 of the cDNA resulting in a silent mutation at position 35 of the protein (c.174C>T (C35C)). In addition, a heterozygous C to T substitution at position 22 upstream from the translation start site (−22C>T) was detected in two out of 200 control subjects (representing 400 chromosomes). All of the DNA variants detected in this study as well as their frequency evaluated in control chromosomes are described in table 1.

DGGE analysis is one of the most sensitive and efficient established mutation scanning techniques to date. It can allow for the discrimination of DNA molecules differing by as little as only one base change. Using this technique, we identified nearly 100% of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in a certain population. In this study, we detected up to seven different DNA variants. Given this high sensitivity of DGGE analysis, although we cannot exclude the possibility of mutations in the more upstream 5′ regulatory region or in the remaining intronic sequences, our results strongly suggested that the PSTI gene could be neither a cause nor a cofactor in the development of HP. Future research into this disease may be directed towards other pancreatic digestive proenzyme genes such as the anionic trypsinogen and mesotrypsinogen genes.

When mutations in the cationic trypsinogen gene were identified as the molecular basis of HP, it was questioned whether they could predispose patients to develop sporadic pancreatitis. Until now, these cationic trypsinogen mutations have not been detected in sporadic chronic pancreatitis. In this study, although seven different DNA variants in the human PSTI gene were identified in sporadic chronic pancreatitis, none of them seems to have a functional effect on phenotype. Recently, mutations in the CFTR gene have been reported to be closely associated with this disorder and it would be interesting to look at whether CFTR also plays a role in the hereditary form of pancreatitis.

In conclusion, this study is the first comprehensive search for possible mutations in the human PSTI gene that may be linked to pancreatitis, and represents the first identification of seven DNA variants of the gene. Furthermore, PSTI has been excluded from involvement in the pathogenesis of hereditary and sporadic chronic pancreatitis.

We thank Isabelle Quere and Caroline Jacques for help with sequencing, Claudine Verluay for help with DGGE analysis, and Marguerie Corse for reading the manuscript. This work was supported by the INSERM (CRI No 96-07). JMC is a postdoctoral scientist receiving a grant from the INSERM.

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13 Tacht D, Mossner J, Keim V. Mutations of the cationic trypsinogen gene have been reported to be closely associated with this disorder and it would be interesting to look at whether CFTR also plays a role in the hereditary form of pancreatitis.
A case of inv dup(8p) with early onset breast cancer

EDITOR—More than 50 cases have been described with inv dup(8p) which can be either di- or monocentric.1–4 A rough estimate of the prevalence of both is 1/22 000-30 000 of the white population. Concurrently with the 8p duplication, markers at the tip of chromosome 8 are consistently deleted. All the cases described are associated with mental retardation, facial dysmorphism, brain defects and/or developmental delay. Allele loss and amplifications of regions of chromosome 8p are commonly reported in sporadic breast cancer,5–7 and two recent papers have suggested linkage to 8p11-12 in familial breast cancer.5–8 We report a case of 8p duplication and inversion in a woman who developed breast cancer at the age of 36, with a personal history of developmental abnormality and a family history of breast and other cancers. Because of the possible link between this chromosomal abnormality and a breast cancer predisposing gene on chromosome 8p, we analysed the chromosome in more detail. Our analysis suggests, however, that the cancers and the chromosomal abnormality are unrelated.

The patient (DD003-1EW) was born in 1951. She was considered to have had a birth injury resulting in hypoxic encephalopathy and cerebral palsy. At the age of 25 she had a left breast biopsy which was diagnosed as benign, and at the age of 36 (in 1987) she had an infiltrating ductal carcinoma of the right breast. In 1988 chromosome analysis was undertaken by G banding because of a suspected “developmental disorder of the brain”. This showed an inversion and duplication of chromosome 8p. In her family, her mother is well, her maternal grandfather was reported to have had colon cancer, his sister (the patient’s great aunt) to have had breast and colon cancers and his mother breast and pancreatic cancer, a son and grandson of the great aunt to have had leukaemia of unspecified type, and a granddaughter to have had breast cancer in her 30s. None of these family members could, however, be contacted.

An 8p+ karyotype was reported suggesting an inv dup(8p)(p11.2 → p23.1) after routine G banding chromosome analysis. Metaphase chromosomes from EBV immortalised lymphoblasts from the patient were prepared after synchronisation with thymidine and incubation with colcemid by standard techniques. Fluorescence in situ hybridisation (FISH) studies performed with a whole chromosome 8 paint (Cambio) showed that the additional material present in the short arm of the rearranged chromosome 8 is derived from chromosome 8 (data not shown). To define the breakpoints of the rearrangement more accurately, dual colour FISH experiments were performed using total yeast DNA from YAC clones from chromosome 8 (HGMP Resource Centre, UK) together with a chromosome 8 specific centromeric probe (Boehringer Mannheim). By analysis of 20 metaphases each it could be shown that the short arm of the rearranged chromosome 8p is dicentric with most of the short arm duplicated (cen → sp23.1) and inverted (fig 1). The telomeric region distal to p23.1 is deleted. The proximal breakpoint seems to be the centromere as the dicentric chromosome 8 shows a second centromere at the very end of the tip of the short arm.

Neither the breakpoints nor the telomeric deletion lay in regions associated with breast carcinomas, which frequently show allelic deletions in regions 8p11-p12 and 8p21-p22 in sporadic cases.6 8p12 is also found to be amplified in 10-15% of breast tumours.7 For the NEFL marker (8p11-p12), a lod score of 2.5 was obtained by linkage analysis using families unlinked to BRCA1 or BRCA2 indicating the presence of a putative BRCA3 gene.8 Because samples from other family members of the index case were not available for linkage analysis, mutational analysis of BRCA1 and BRCA2 was performed in the proband but did not detect any mutation (data not shown).

In conclusion, the presented case of inv dup(8p) shows the genotype of other reported cases associated with developmental delay and/or mental retardation. The occurrence of breast cancer is probably coincidental and unrelated to the chromosome 8p rearrangement.

We thank Ian Roberts for providing help with the image analysis system. This work was supported by grant D96/03248 to MS from the Deutsche Krebsforscher.

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Appendiceal carcinoma complicating adenomatous polyposis in a young woman with a de novo constitutional reciprocal translocation t(5;8)(q22;p23.1)

EDITOR—Familial adenomatous polyposis (FAP) is an autosomal dominant condition characterised by the presence of more than 100 adenomatous polyps in the colon and rectum. Polyps generally first appear in the second or third decade of life and are usually most numerous distally. Left untreated, colorectal cancer is virtually inevitable and generally arises in the fourth or fifth decade.1 Adenocarcinoma of the appendix is an uncommon neoplasm and has only rarely been reported in association with FAP.2

The gene responsible for FAP, APC, was initially localised to the long arm of chromosome five (5q) by linkage.3,4 This followed a case report describing carcinomas of the rectum and ascending colon, adenomatous polypsis, mental retardation, and various dysmorphic features in a 42 year old man with a constitutional deletion of 5q.5 Most patients with FAP have normal karyotypes.6 Mental retardation and dysmorphic features are unusual in such people but characterise those rare patients with cytogenetically visible 5q deletions and FAP.5–12 The few reports detailing the clinical findings in patients with submicroscopic deletions of APC suggest that such people may be mentally normal.13,14

In this report we describe a patient with adenomatous polyposis, mental retardation, and an apparently balanced translocation t(5;8)(q22;p23.1) causing submicroscopic deletion of APC and MCC.

Clinical data were obtained by review of medical records. In addition, the patient was interviewed and examined by two of the authors (JF and AS) before her death. Cytogenetic studies were performed using standard techniques on a 72 hour peripheral blood culture with GTG banding, as previously reported.6 Slides for fluorescence in situ hybridisation (FISH) were obtained using the cell suspension retained after routine cytogenetic harvest. RNAse treatment, probe and chromosomal denaturation, and hybridisation conditions were as previously described15 with the stringencies adjusted after assessment of the optimal conditions for each probe combination. The biotinylated probes were detected with...
avidin-FITC (Calbiochem) followed by biotinylated anti-avidin (Vector) and finally avidin-FITC. The digoxigenin labelled probes were detected with mouse anti-digoxigenin followed by sheep anti-mouse Ig-rhodamine conjugated antibody (Boehringer Mannheim). Chromosomes, counterstained with DAPI (Sigma) and visualised by fluorescence microscopy (Zeiss), were captured using a computer image analysis system (Cytovision).

The following probes were used in FISH studies: MCC 40CI (partial MCC cDNA nucleotides 1634-3969), FB70B (partial APC cDNA nucleotides 2877-6452), and APC (full length APC cDNA) each kindly provided by Dr Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore); D5S23 (chromosome 5p probe, Vysis); EGR-1 (chromosome 5q31 probe, Vysis); CHR8B/wcp8 (chromosome 8 library probe, Cambio); D8Z1 (chromosome 8 centromere probe, Oncor).

Genomic DNA was extracted from blood samples using the Instagene purification matrix (Biorad) according to the manufacturer's instructions. Each polymerase chain reaction (PCR) used 50-100 ng genomic DNA, 50 pmol/l of each oligonucleotide primer, 0.2 mmol/l of each dNTP (Pharmacia), 1.25 U AmpliTaq DNA polymerase (Perkin Elmer) in AmpliTaq reaction buffer (10 mmol/l Tris-HCl, 50 mmol/l KCl, MgCl optimised for each primer pair, pH 8.3) to a final volume of 25 µl. Thirty five amplification cycles were performed using an FTS-320 Thermal Sequencer (Corbett Research).

An RsaI polymorphism in exon 11 of APC was analysed by amplification of exon 11 and digestion of the PCR product with RsaI (Boehringer). Reaction products were separated by electrophoresis in a 10% non-denaturing polyacrylamide minigel (Biorad) and visualised by ethidium bromide staining. The 255 bp amplification product yielded digestion fragments of 155 bp and 100 bp. A CA repeat polymorphism within MCC (CAMBC) and a CA repeat polymorphism between APC and MCC (LNS-CA) were labelled by [35S]dATP incorporation during PCR and characterised by electrophoresis in a 6% denaturing polyacrylamide gel and autoradiography as previously reported.

The patient (fig 1) was the second child of a 32 year old mother and a 33 year old father. Her older brother was well and mentally normal. She was born prematurely at 29 weeks' gestation after an uncomplicated pregnancy. There were no major problems in the neonatal period but she was slow to speak and did not walk until the age of 2 years. At that time she began to have generalised convulsions. These were only partially controlled by medication and continued until the age of 18. Her performance at school was poor. Her hospital file notes an estimated IQ between 70 and 80 at the age of 10 although the method of assessment was not recorded. Physical examination at this time showed crowded dentition and she later required extensive dental work. The posterior hairline was noted to be low. The head circumference was normal (75th centile) while height was on the 10th centile. The third and fourth toes were short with the fifth toe longer than the fourth. Bilateral genu recurvatum was evident and the patient was mildly ataxic. There were no skin lesions and the fundi were normal.

![Figure 2](http://jmg.bmj.com/) (A) GTG banded karyotype showing the translocation t(5;8)(q22;p23.1) in the patient. The arrows indicate the sites of the breakpoints.

(B) Normal and derivative chromosomes 5 and 8.
Visual acuity and hearing were unimpaired. An EEG at the age of 4 was abnormal with excessive generalised slow wave activity. A cerebral CT scan at 9 years showed mild ventricular dilatation and localised atrophy in the frontal areas and bordering the interhemispheric commissure. She attended a special school until the age of 17 and was actively involved in sports. Subsequently she worked successfully in a sheltered workshop while living independently with a group of mildly handicapped people.

She presented acutely at 26 years of age with a 24 hour history of right iliac fossa pain. She was febrile and had guarding and rebound tenderness at the site of her pain. A diagnosis of acute appendicitis was made and an appendicectomy performed. The appendix contained a mucin secreting carcinoma arising in a dysplastic villous adenoma and invading through the full thickness of the muscle wall. A right hemicolectomy was performed. The appendiceal stump had foci of adenomatous change but there was no residual carcinoma. Numerous small adenomatous polyps were noted throughout the right colon but the exact number was not recorded. Sigmoidoscopy subsequently showed left sided polyps but these were less numerous than had been found proximally. Eight months after hemicolectomy she presented again with a painful right iliac fossa mass. Laparotomy confirmed local tumour recurrence which could be only partially excised. She was treated with 5-fluorouracil and folinic acid but died approximately 12 months later. A post mortem examination was not performed.

Cytogenetic analysis showed a female karyotype with a translocation involving chromosomes 5 and 8 at breakpoints q22 and p23.1 respectively (fig 2). The translocation was cytogenetically balanced. Both parents had normal karyotypes. The patient’s brother refused testing.

FISH studies were performed using several probe combinations: APC, D5S23, and EGR-1; FB70B and D5S23; FB70B and D8Z1; MCC 40CI and CHR8B/wcp8 (fig 3); and MCC 40CI and D8Z1. These studies confirmed the translocation and showed submicroscopic deletion of both APC and MCC. The APC and MCC probes hybridised only to the normal chromosome 5 and not to the der(5) or der(8) chromosomes.

To confirm that the translocation identified had resulted in deletion of the APC and MCC genes, intragenic polymorphisms were examined in DNA from the patient and her parents. The RsaI polymorphism in exon 11 of APC was informative in the family. The patient’s mother was homozygous for absence of the restriction site whereas her father was homozygous for presence of the restriction site. The patient had only a maternally derived allele, sug-
Table 1  Clinical features in patients with cytogenetic abnormalities and adenomatous polyposis

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A: ascending colon; App: appendix; C: caecum; D: descending colon; Del: deletion; Den: dental abnormalities; Des: desmoid tumour; Dis: disruption; DP: duodenal polyp; ECMs: extracolonic manifestations of Gardner's syndrome; NR: not reported; O: osteoma; Prox: proximal; R: rectum; Ret: congenital hypertrophy of the retinal pigment epithelium; S: sigmoid colon; SC: skin cysts; T: transverse colon.

There are 10 previously described cases in eight families of cytogenetically visible deletions encompassing the APC gene from exon 11 to the 3' untranslated region. In the current report, a submicroscopic deletion of the 5q translocation and polyposis, including the APC gene from exon 11 to the 3' untranslated region, has been either deleted or disrupted. In the family described by Mandl et al., the deletion spanned a smaller region of the APC gene, resulting in a chromosomal deletion of the APC gene and polyps. In this family, mental retardation and some dysmorphic features were noted. The APC gene has been involved in the development of familial adenomatous polyposis and carcinomas. The APC gene is a promising target for genetic testing and may produce null alleles although the explanation for this negative effect type (FAP or familial adenomatous polyposis [FAP]) is not fully understood. The reverse is true of more classic FAP [AAPC], which has been associated with mental retardation and a range of dysmorphic features.
NF2 gene deletion in a family with a mild phenotype

EDITOR—Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder that predisposes to bilateral vestibular schwannomas and other nervous system tumours. Two clinical subtypes have been proposed. The severe type (Wishart) has an onset before 25 years of age, a rapid course, and multiple nervous system tumours. The mild type (Gardner) has a later onset with a more benign course, often restricted to bilateral vestibular schwannomas.1

The NF2 tumour suppressor gene is localised on chromosome 22q12 and encodes a protein called merlin or schwannomin, which is related to a family of cytoskeleton associated proteins.2 Since the identification of NF2, various germline mutations have been identified,3,4 as well as somatic mutations. In general, germline mutations associated with a mild phenotype include missense mutations and small in frame deletions or insertions.5

In our study, the proband (III.2) is an 18 year old boy with a mild facial palsy. Slight enlargement of both vestibular branches of the eighth cranial nerve was observed after MRI scanning, but no other clinical features have been observed. His mother (II.4) was operated on at the age of 28 for bilateral vestibular schwannomas; she died at the age of 45 from breast cancer but with stable vestibular schwannomas. The grandmother (I.2) of the proband had progressive deafness followed by surgery for a right sided vestibular schwannoma at the age of 48 after which she died owing to postoperative complications. At necropsy a right and left sided vestibular schwannoma were found.

DNA from the proband and the available family members was prepared from peripheral blood according to standard procedures. Microsatellites were amplified from genomic DNA by the polymerase chain reaction (PCR). Eight polymorphic markers in the NF2 gene region were studied in the family: n2GAI (intron 1), D22S929 (intron 1), n2CT31 (intron 1), n2CAII (intron 3), n2CAIV (intron 8), n2CAV (intron 10), n2CAVI (intron 13), and n2GAI.6

We thank the patient and her parents for their cooperation, the patient's surgeon, Dr John Neophytou, and general practitioner, Dr John Cameron, for providing clinical details, Dr Dorothy Painter for reviewing the histopathology, Cathy Abbott for assistance in preparing DNA probes, and Nicole Chia for assistance in the cytogenetic studies.

Cytogenetic studies with G banding were normal. PCR fragments from the \( NF2 \) gene were used as FISH probes, including a pool of exon 1 and intron 1 fragments (2 and 0.7 kb), a pool of intron 1 fragments (2-2.5 and 3.5 kb), and a pool of intron 15 fragments (2-2.8 and 3.6 kb)\(^9\) (for primer sequences see http://www.cepbh.fr/nf2deletion/). The different PCR fragment pools were used separately and combined as FISH probes. Normal controls were studied in parallel.

In the family we studied, five polymorphic markers were informative. Using these markers we identified the proband (III.2) as a carrier of a deletion in the \( NF2 \) region. He had not inherited any allele from his mother (fig 1). The deleted region extends at least from intron 1 to intron 10 of the \( NF2 \) gene, as measured by the microsatellites.

The FISH experiment on peripheral lymphocytes from II.4 confirmed these results (fig 2). The mother of the proband has only one copy of the \( NF2 \) gene, suggesting that the deletion extends at least from exon 1 to intron 15. The \( NF2 \) gene contains only 17 exons.\(^2\) This method has been shown to be a very efficient way to detect the large deletion in the \( NF2 \) gene.

Affected subjects in this family are heterozygous for a deletion in the \( NF2 \) region, which cosegregates with a relatively mild phenotype. Mild phenotypes have recently been reported in \( NF2 \) families\(^10\)\(^\text{–}\)\(^11\) and in five isolated cases with a complete gene deletion.\(^9\)

The \( NF2 \) genotype-phenotype correlation is not completely clear yet; however, frameshift and stop mutations which produce a truncated protein are usually associated with severe phenotypes.\(^7\) On the other hand, large deletions with complete inactivation of the \( NF2 \) gene have been observed in patients with a milder phenotype.\(^7\)

In contrast, large \( NF1 \) gene deletions are usually associated with a more severe phenotype than point mutations or small deletions or insertions in the \( NF1 \) gene. In \( NF1 \), however, the deletions are substantially larger than the \( NF1 \) gene, indicating the presence of a possible contiguous gene deletion syndrome and thus explaining the more severe phenotype in these patients.\(^12\)\(^\text{–}\)\(^13\) In the case of \( NF2 \) gene deletions, the nature and the exact size of the deletions remains to be determined. Hemizygosity for the \( NF2 \) gene seems to have less severe consequences for the phenotype than the presence of a truncated protein. It has been shown that merlin function depends on the formation of an intra- and intermolecular complex (homo- and heterodimers).\(^14\) Truncated proteins may block these molecular interactions and might have an effect on the growth of schwann cells even before the normal \( NF2 \) allele has been inactivated.

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Absence of fragile X syndrome in Nova Scotia

EDITOR—Fragile X syndrome is the leading cause of inherited mental retardation, with an incidence that is generally estimated to be about 1/1250 to 1/4000 in males and 1/2000 to 1/8000 in females.1,2 An extremely high estimate was reported by Rousseau et al.,3 who indicated that as many as 1/259 females from Quebec, Canada, are premutation carriers. In contrast, Tranebjaerg et al.4 reported a prevalence of 0.04/1000 males in Funen, Denmark. The syndrome is characterised clinically by the triad of (1) long, narrow face with protruding chin and large ears, (2) macro-orchidism, and (3) mental retardation.5 The molecular basis for the disease is usually an expanded triplet (CGG) repeat located in the 5' region of the fragile X mental retardation (FMR1) gene.6 In normal subjects, there are fewer than 60 copies of this CGG repeat; carrier females and transmitting males have a premutation that usually ranges from 60-200 repeats and in affected subjects the number is expanded to >200 copies.7 The mutations are associated with absence or reduction in FMR1 gene expression. “Fully expanded” alleles are heavily methylated, in contrast to normal alleles, which are unmethylated.8

Contrary to what might be expected of a very common disorder that confers a selective disadvantage, normal alleles appear to have a low mutation rate. The conversion of a normal allele to a premutation, or to a full mutation, has not been reported.9 Indirect evidence of a low mutation rate is provided by the finding of founder chromosomes.10

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**Figure 1** FMR-1 (CGG)n allele size distribution in (A) 167 institutionalised subjects with mental retardation and (B) 1226 random alleles from Guthrie spots.
Studies of populations from the United States,12 France and Spain,13 Belgium/The Netherlands,14 northern Europe and the United States,15 Italy,16 United Kingdom,17 Sweden,18 and Finland19–21 have shown that FMR1 mutations are in apparent linkage disequilibrium with specific alleles at microsatellite loci FRAXAC1 and DXS548, which are located 7 kb and 150 kb proximal to the CGG repeat region, respectively. About two thirds of full mutation FMR1 alleles are associated with a few specific haplotypes. In some reports, the 204 bp allele at the DXS548 locus was associated with approximately 25% of fragile X and 8% of normal chromosomes. The Swedish and Finnish studies found linkage disequilibrium between FMR1 and other DXS548 alleles.

Given the high prevalence of fragile X syndrome reported elsewhere,1–3 a population the size of Nova Scotia (one million) is expected to include approximately 200 to 550 cases, with two to six newly identified patients each year. However, despite testing of patients presenting with mental handicap by cyto genetic analysis since 1980 (n=423) and mutation analysis since 1991 (n=650), only a single case has been identified. This family had recently moved here from elsewhere in Canada. This disorder may be extremely rare in Nova Scotia; however, patients might be overlooked, misdiagnosed, or not referred for laboratory testing. It has been estimated that more than 50% of fragile X cases are undiagnosed in the Netherlands.22 Because the implications to extended family members who may be at high risk for having affected children are significant, we screened subjects with mental retardation and no known diagnosis from seven regions of the province of Nova Scotia for CGG expansion. We also investigated the FMR1 allele size distribution in a random sample of Nova Scotians.

Molecular analyses of DNA samples from 177 males with significant mental handicap, using PCR methods previously described by Fu et al23 and Miller et al,24 identified 30 different FMR1 alleles with repeat sizes ranging from 19 to 59 triplets (fig 1A). Although three subjects had repeat sizes in the upper normal range (54, 57, 59), no premutations or full mutations were found. The absence of mutation in the samples with these large normal alleles was confirmed by Southern blotting using probe pE 5.1 and restriction enzymes EcoRI and BstHII.24

The FMR1 CGG repeat size distribution in 1226 random alleles (470 males and 378 females) from Guthrie newborn screening samples (dried blood spots taken at birth) from the general Nova Scotia population was determined according to the methods of Carducci et al25 and Fu et al.7 They contained 36 different allele sizes ranging from seven to 55 repeats (fig 1B). No premutations or fully expanded alleles were identified using this PCR based method. Thirty eight females showed only one allele size. Some of these subjects could be premutation or mutation carriers; however, assuming Hardy-Weinberg equilibrium and allele frequencies observed in male samples, we expected 45 homozygotes, which is more than we observed. The FMR1 allele size distribution differed between the patient and general population sample (χ²=12.34, 42 df, p=0.015). However, this result is apparently because of differences in regional representation in the two samples; chi-square analysis that takes geographical origin of the subjects into account showed no significance differences (analysis not shown).

Table 1 shows the allele distribution at locus DXS548 (determined as described by Zhong et al27) within our institutionalised population compared to that in published groups of fragile X patients and normal subjects.12–21 25 26

A comparison of the DXS548 allele distribution between the previously reported fragile X populations22–27 showed that these differed from one another (p<0.00001) to a greater extent than did normal groups (p=0.053) from the same geographical regions. The greater diversity among fragile X populations was still apparent, even when the relatively isolated Scandinavian populations18–21 were excluded from the calculation (p=0.00006).

Table 1 DXS548 allele size distribution in subjects with mental retardation in Nova Scotia compared to published data from normal and fragile X populations

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<th>Allele size (bp)</th>
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<tr>
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* p values were generated from χ² tests of independence that were used to compare the DXS548 allele frequency in subjects with mental retardation from Nova Scotia to that of other reported normal and fragile X populations.
Studies from elsewhere have shown that approximately 2-10% of developmentally delayed patients are positive for the fragile X mutation by cytogenetic or molecular analysis. For example, 16 of 219 male and 15 of 128 female mentally retarded children studied from Birmingham, UK, had mutations in the FMR1 gene.27 Similarly, a study of children with special educational needs from Salisbury, UK, found an expanded allele in four out of 180 male and 0 of 74 female cases. 28 We have analysed a similar number of males with mental handicaps and found none. This patient population and our screen of 1226 random alleles showed bimodal size distributions similar to those reported elsewhere, 29 30 but identified no allele expansions into the premutation or full mutation ranges. Fragile X syndrome, as suspected from our previous lack of detection through cytogenetic and molecular testing, is therefore rare in Nova Scotia.

The conclusion that fragile X syndrome is rare in Nova Scotia is also supported by molecular analysis of the microsatellite locus DXS548 in the same “high risk” patient population with mental retardation. Alleles at this locus have been shown to be in linkage disequilibrium with FMR1 expansions in multiple populations. 29 30 We have shown that the allele distribution in our high risk group resembles that of other normal populations, with a dearth of those alleles commonly associated with fragile X.

It is noteworthy that the DXS548 allele distribution in previously studied fragile X populations is more heterogeneous than those in the corresponding normal populations. 31 32 This observation suggests the possibility of multiple origins of the fragile X mutations from a limited number of pre-premutation alleles, 31 33 with additional diversity generated by recombination and mutation of tightly linked microsatellite marker loci. Nova Scotia has long been noted for having a high prevalence of specific rare genetic disorders in various regions, for example, Niemann-Pick type D disease, 34 Huntington disease, 35 Charcot-Marie-Tooth disease, 36 acute intermittent porphyria, 37 Fabry disease, 38 and nephrogenic diabetes insipidus. 39 Common ancestry of affected subjects in each of these cases has been documented, and molecular analysis supports the conclusion of a founder effect. Given the low rate of mutation, the absence of fragile X syndrome in Nova Scotia can be seen as an example of an “absence of founder effect”. Considering that our population (approximately one million) has tens of thousands of founders, from multiple founding groups (chiefly English, Scottish, Irish, French, and German immigrants in the 18th and 19th centuries), this phenomenon appears remarkable.

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20 Oudert C, Morret E, Serre JL, et al. Linkage equilibrium between the fragile X mutation and two closely linked CA repeats suggests that fragile X chromosomes are derived from a small number of founder chromosomes.


