

## Letters to the Editor

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### The CAG repeat within the androgen receptor gene in male breast cancer patients

EDITOR—Mutations of the *BRCA1* and *BRCA2* tumour suppressor genes have been identified in some cases of familial and early onset breast cancer.<sup>1,2</sup> Mutations of these genes, however, account for a relatively small proportion of the total cases of female breast cancer. Male breast cancer is a very rare disease, accounting for approximately 1% of all cases of breast cancer. Less is known about the genetic influences in its development. Male breast cancer has been linked to mutations of the *BRCA2* gene in some cases, with the frequency of mutations varying widely (from 4–40%) in those series studied.<sup>3,4</sup>

It has been suggested that there may be other genetic factors that confer a lower absolute risk to the person, but potentially could result in a substantial number of cases within a whole population.<sup>5</sup> We have already shown that a polymorphism in the *CYP17* gene is associated with an increased risk of male breast cancer.<sup>6</sup>

A region within exon 1 of the gene coding for the androgen receptor (located on chromosome Xq11–12) is highly polymorphic and contains a variable number of CAG repeats. The variability of the number of these repeats between different ethnic populations in the USA has been studied.<sup>7</sup> In vitro studies have shown that a relatively short CAG repeat sequence increases the level of transactivation of the androgen receptor.<sup>8</sup> The androgen receptor itself binds dihydrotestosterone and therefore is one factor in the regulation of the growth of prostate cells. This may account for the finding that short CAG repeat sequences have been associated with a higher risk of developing prostate cancer.<sup>9,10</sup> Abnormally long sequences of 40 repeats or more are found in patients with X linked spinal and bulbar muscular atrophy (Kennedy's disease).<sup>11</sup> This disease is associated with gynaecomastia and reduced fertility, suggestive of androgen insensitivity. Mutations of the androgen receptor gene may also result in reduced androgen receptor function and have been found in a few cases of male breast cancer.<sup>12,13</sup>

The aim of this study was to investigate whether increased length of the CAG repeat sequence in the androgen receptor gene is associated with the development of male breast cancer.

The selection of male breast cancer cases and controls has previously been described.<sup>6</sup> Ethical approval for the study was obtained through the Lothian Regional Ethics Committee.

DNA extraction was from whole blood by standard phenol/chloroform extraction. DNA extraction from wax embedded tissue was from 10 µm sections incubated at 55°C with a lysis buffer and proteinase K.

Using the published sequence,<sup>14</sup> the following primers were designed (Primer Designer v1.1 ©1990 Educational Software): ARG-F 5'-TGCGCGAAGTGATCCAGA ACC-3', ARG-R 5'-CTCATCCAGGACCAGGTAGCC-3'. These generate PCR fragments containing the CAG repeat sequence.

PCR reactions were performed in 50 µl aliquots, each containing 1 × PCR reaction buffer, 2 mmol/l MgCl<sub>2</sub>, 5 µl

dimethyl sulphoxide, 200 µmol/l deoxynucleoside triphosphates, 20 pmol of each primer, 1 unit of *Taq* polymerase (Life Technologies™), and approximately 100 ng DNA. The amplification was performed using an OmniGene thermal cyclers (Hybaid, UK) under the following conditions: initial denaturation at 94°C for three minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

The products were denatured and then run on 6% polyacrylamide gels with a 10 bp DNA ladder. The products were then ranked in order of length. Three representative products were sent for automated sequencing (DNASHEF, Department of Haematology, Royal Infirmary of Edinburgh) to confirm the number of CAG repeats, and these were used as size standards. The products were then run again, with those thought to be of equal length adjacent to each other in order to check the accuracy of the original estimation of length. A second re-run was then performed to confirm the accuracy of the results.

The lengths of the PCR products obtained varied between 224 bp and 272 bp (corresponding to 14 CAG repeats and 30 CAG repeats, respectively). PCR was unsuccessful with DNA extracted from eight of the archival wax embedded tissue sections.

The distribution of alleles among male breast cancer patients and controls is shown in fig 1. The median number of CAG repeats in both groups was 23. There were no statistically significant differences between the two groups (Mann-Whitney test, p=0.916).

Three patients showed evidence of two different alleles indicating the presence of two X chromosomes (fig 2). One of these (MBC8) was recorded on the Edinburgh Cytogenetics Register with a diagnosis of Klinefelter's syndrome. The other two patients (MBC42 and MBC 62) had died, but there was no record of clinical suspicion of Klinefelter's syndrome in their hospital case notes. Neither fathered any children. The data were reanalysed following exclusion of these three cases. The median number of CAG repeats for the remaining 53 male breast

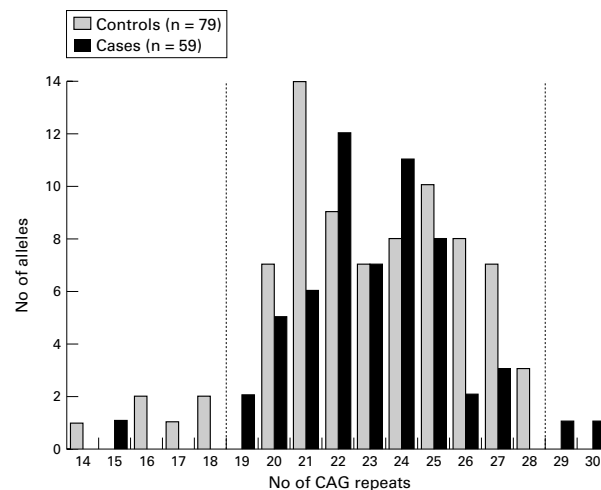


Figure 1 Distribution of CAG repeats in the androgen receptor gene among male breast cancer patients and controls.

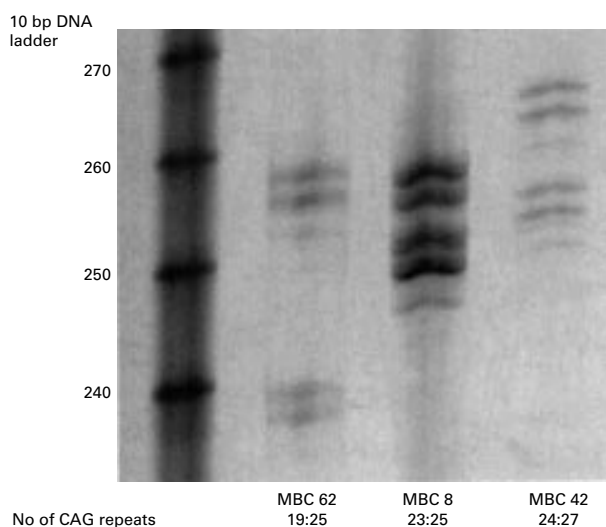


Figure 2 Genotyping of the three male breast cancer patients carrying two different alleles of the androgen receptor gene.

cancer patients was also 23. There was still no statistically significant difference between cases and controls ( $p=0.765$ ).

We have not observed any overall difference between the median CAG repeat length of male breast cancer patients and controls. However, no males in the control group had alleles containing more than 28 CAG repeats, whereas two of the male breast cancer patients had alleles with 29 and 30 repeats respectively. Only one of the male breast cancer patients had an allele containing 18 repeats or less, compared to six of the controls. To our knowledge, the length of this CAG repeat has only been studied in one group of male breast cancer patients previously.<sup>15</sup> There was found to be no significant difference between male breast cancer cases and controls. However, sequences of 30 repeats or more were found only among cases. Our results are consistent with these findings. In addition, it has been recently observed that women who are carriers of *BRCA1* mutations are at a significantly increased risk of breast cancer if they carry at least one androgen receptor gene allele with 28 or more CAG repeats.<sup>16</sup> We believe that a relatively long CAG repeat sequence within the androgen receptor gene may be implicated in a few cases of male breast cancer. Conversely, a short CAG repeat sequence might offer a degree of protection against male breast cancer.

It is well recognised that Klinefelter's syndrome is associated with an increased risk of male breast cancer.<sup>17</sup> One of the male breast cancer patients in our study was known to have had Klinefelter's syndrome. Our study of the androgen receptor gene has enabled us to identify a further two patients whom we suspect to have had Klinefelter's syndrome.

The findings presented in this study indicate that the CAG repeat sequence within the androgen receptor gene may, in some cases, be one useful molecular marker to identify males at increased risk of developing breast cancer.

Larger studies are required to define the importance of this CAG repeat in male breast cancer further. An international consortium has recently been set up and we have agreed to contribute our data to this.

There is also a GGC repeat sequence within exon 1 of the androgen receptor gene. This might be an interesting area for further study.

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## Deletion and duplication of the adenomatous polyposis coli gene resulting from an interchromosomal insertion involving 5(q22q23.3) in the father

EDITOR—Chromosomal rearrangements occur at a low frequency in the general population and chromosomal insertions occur at an estimated frequency of 1 in 5000 newborn infants.<sup>1</sup> Adjacent segregation of interchromosomal insertions results in a deletion or duplication of the inserted segment or more complicated imbalances through a recombination event at meiosis. In the case presented here, a balanced interchromosomal insertion between chromosomes 5 and 10, 46,XY,dir ins(10;5)(q25;q22q23.3), was carried by the father. Theoretically this insertion involves less than 1% of the haploid autosomal length and therefore a fetus with either a duplication or deletion is likely to be viable unless there are essential genes in this segment that are deleterious in an aneuploid conceptus. Generally deletions are more deleterious than duplications and there are few published cases where the clinical features of a duplication and deletion for the same chromosomal region have been described within the same family.<sup>2,3</sup>

In this paper we report four cases of a 5q22q23.3 deletion and one case of a duplication for the same region which includes the *APC* gene. All of the aneuploid offspring were within the same generation and the clinical features associated with 5q22q23.3 deletion with a similar genetic background will be compared with published cases.

Lymphocytes were cultured by standard methods including semi-synchronisation with thymidine and preparations were analysed using G banding.<sup>4</sup> Fluorescence in situ hybridisation (FISH) of the chromosome preparations involved YAC probe 37HG4 containing a 2.3 kb fragment of cDNA from the *APC* gene which recognises an *MspI* polymorphism.<sup>5</sup> The *APC* gene has been localised to the subband 5q22.1.<sup>5</sup> Standard FISH procedures were used and have been published elsewhere.<sup>6</sup> FISH images were viewed using computer enhanced image analysis systems (Vysis).

The family were investigated on the birth of the proband. The parents were first cousins of Asian origin and the mother did not speak any English. At the birth of the sec-

ond child the father was 34 and the mother 21 years of age. They have three clinically normal children (first, third, and eighth pregnancies, II.1, II.3, and II.8) although the chromosome constitution of one of them is unknown (II.1). However, the remaining five pregnancies resulted in chromosomally abnormal offspring (fig 1). The parents had genetic counselling after the birth of their second child (case I, II.2) and further follow up of family members was declined.

In case 1 (II.2) the pregnancy was uneventful and the infant was delivered at term. At birth his Apgar score was 3 at one minute, 6 at five minutes, and 9 at 10 minutes. Intubation failed as the vocal cords could not be visualised owing to the malformed head and neck. His birth weight was 1860 g and head circumference 34 cm. Physical examination showed cleft palate, micro-osmia, anteverted nostrils, micrognathia, downward slanting palpebral fissures, talipes equinovarus, polydactyly (nine toes on the right foot), low set ears, bilateral VII cranial nerve palsy, and ulnar deviation of the fingers (fig 2). He was also tachypnoeic from birth with chest wall recession and irregular respiratory effort. Chest x ray showed clear lungs. Septic and TORCH screen were normal. Arterial blood gases showed a mild metabolic acidosis. ECG was normal. The tachypnoea remained unexplained. The proband was initially nursed in oxygen but later tolerated air. The child showed restricted movement of all limbs. At 16 days an x ray showed a fractured right humerus with callous surrounding a fracture of the right clavicle. No pathological cause was found. He was extremely irritable, with a paucity of spontaneous movement and inability to feed without a nasogastric tube.

On day 23 he became deeply cyanosed with breathing even more laboured than previously and died suddenly the same day. Necropsy was declined on religious grounds.

The patient's karyotype showed a deleted chromosome 5, 46,XY,del(5)(q22q23.3). Investigation of the parents' blood indicated that the mother had a normal female karyotype. However, the father had an interchromosomal insertion of the 5q22q23.3 segment into chromosome 10, 46,XY,dir ins(10;5)(q25;q22q23.3) (fig 3).

The proband's karyotype was therefore 46,XY,der(5)dir ins(10;5)(q25;q22q23.3)pat.

With case 2 (II.3, fig 1), the mother had an amniocentesis at 17 weeks' gestation; a normal male chromosome constitution was reported and the pregnancy continued to term. The baby had no dysmorphic features at birth and examination at 5 years of age showed a unilateral simian

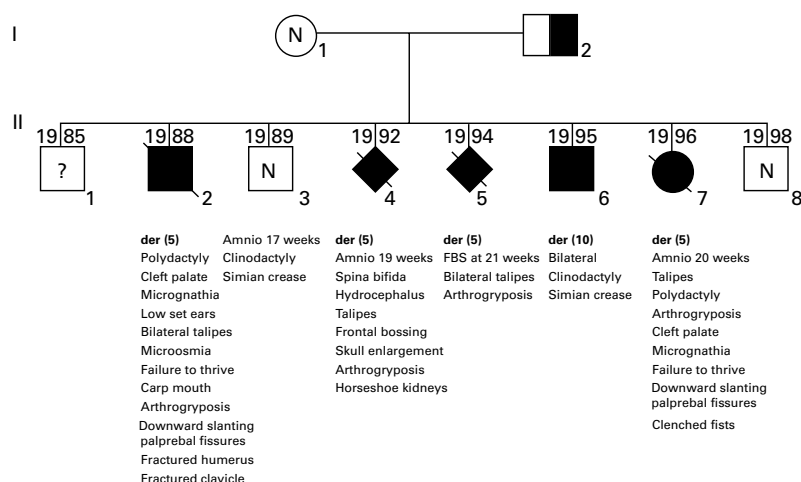


Figure 1 Diagrammatic summary of clinical features in the family. The father (I.2) carries the interchromosomal insertion. N=normal karyotype.

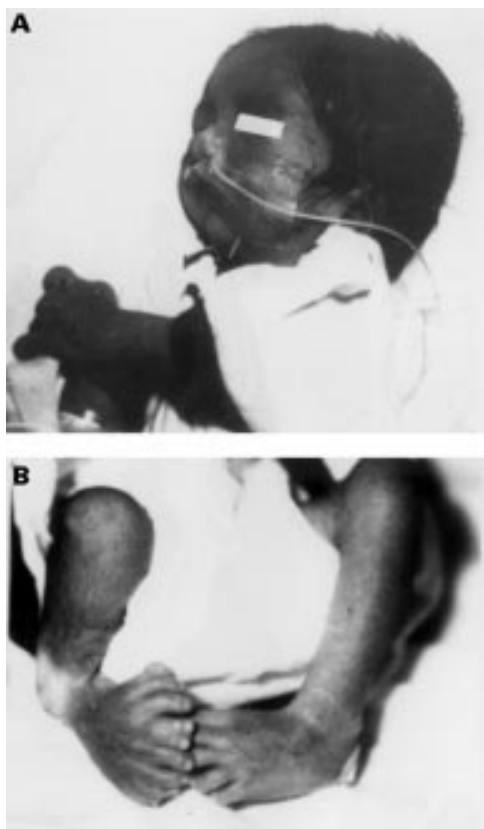


Figure 2 (A) Case II.2 at birth and (B) polydactyly and talipes of the right foot. (All photographs reproduced with permission.)

crease, clinodactyly, and a right undescended testis. An orchidopexy was later performed.

With case 3 (II.4, fig 1), the parents initially declined prenatal diagnosis but ultrasound scan at 19 weeks' gestation showed hydrocephalus and lumbosacral spina bifida. The pregnancy was terminated at 22 weeks' gestation after cytogenetic analysis of amniotic cells showed an abnormal karyotype, 46,XY,der(5)dir ins(10;5)(q25;q22q23.3)pat. At necropsy the fetus weighed 280 g, crown-rump length was 17.0 cm, and head circumference 24.5 cm. External examination showed frontal bossing and a large head. The lumbar meningocele was confirmed but the fetus also had borderline hydrocephalus, a horseshoe kidney, and bilateral talipes. No heart, lung, or brain abnormalities were evident, but the lung, adrenal, and thymus showed congestion.

With case 4 (II.5, fig 1), the mother presented in the second trimester and ultrasound scan at 21+4 weeks' gestation showed a normal sized fetus for gestation. The head circumference was 18.6 cm, BPD 53 cm, and the heart, spine, and diaphragm appeared normal. However, polydactyly (six toes on each foot), bilateral talipes, and arthrogryposis of the elbows and fingers were evident, and a pterygium at each elbow was suspected on ultrasound. A fetal blood sample was taken for cytogenetic studies and analysis showed a male fetus with the der(5). FISH studies using the APC probe showed only one signal in all divisions examined, 46,XY,der(5)dir ins(10;5)(q25;q22q23.3).ish der(5)dir ins(q25;q22q23.3)(37HG4-) (fig 4). The parents elected to terminate the pregnancy.

With case 5 (II.6, fig 1), the mother presented in the second trimester and an ultrasound scan at 19 weeks' gestation was normal. Prenatal diagnosis was declined. The male child, born at term weighing 2700 g, was clinically normal on initial assessment. Blood was sent for karyotyping at birth by the paediatricians because of the previous family history.

Neonatal blood showed a male child with a duplication of 5q22q23.3, 46,XY,der(10)dir ins(10;5)(q25;q22q23.3)pat (fig 5). FISH using the APC probe showed three signals on blood interphase cells, nuc ish 5q22.1(37HG4x3). Detailed examination of the child after cytogenetic investigation showed a third fontanelle, bilateral clinodactyly, a unilateral simian crease, and an undescended left testis. No other dysmorphic features were evident. The child was developmentally normal at 2 years of age.

In the pregnancy of case 6 (II.7, fig 1), the mother underwent initial ultrasound examination at 20 weeks' gestation. This showed talipes, polydactyly, and arthrogryposis. Cytogenetic analysis of the amniotic fluid showed a female fetus with a der(5), 46,XX,der(5)dir ins(10;5)(q25;q22q23.3)pat. The parents elected to continue the pregnancy and a dysmorphic child was born at term. The child had a cleft palate, downward slanting palpebral fissures, clenched fists, restricted hip movement, polydactyly of the right foot (six toes), hirsutism, talipes, micrognathia, and low set ears (fig 6). She also had similar respiratory difficulties to case 1. Examination at 3 months showed further dysmorphism including developmental delay, glossoptosis, barrel shaped chest, abnormal lumbosacral spine, increased tone, simple left ear, unilateral accessory auricle, and epicanthic folds. The child had to be fed by nasal tube as she was unable to feed orally. The child died at 11 months of age and necropsy was declined.

With case 7 (II.8, fig 1), the mother declined prenatal diagnosis but serial ultrasound appeared normal and growth was satisfactory. The pregnancy and birth were uncomplicated. A male child was born at term and the Apgar scores were 8 at one minute and 9 at five minutes. There were no dysmorphic features at 8 weeks of age except unilateral clinodactyly which was also present in the mother. The karyotype was normal, 46,XY.

The oldest male child (II.1) has not been karyotyped but his growth and development are normal.

Interchromosomal insertions are individually rare events and in this family both a deletion and duplication of the inserted region were viable. Interchromosomal insertions involve three break rearrangements and theoretically such insertions can lead to aneusomy via two mechanisms, segregation or recombination. However, observed aneusomies have mostly resulted from segregation<sup>7</sup> and only exceptionally from recombination.<sup>8</sup> In this family adjacent 1 segregation of the insertion has provided a unique study of four monosomy 5q22q23.3 offspring with a similar genetic background and a clinically normal child with a duplication of 5q22q23.1. There has been one other reported case of a (10;5) interchromosomal insertion and this gave rise to an infant with duplication of a more proximal region of 5q(q13q22).<sup>9</sup>

Of the previous 27 cases where a deletion of 5q13-31 has been published, 24 have arisen de novo<sup>11-13,31</sup> and three were recombinants from an intrachromosomal insertion.<sup>10,12</sup> As far as we are aware this is the first reported case of deletion 5q resulting from segregation of an interchromosomal insertion in the parent.

Earlier publications have described either a distal 5q22 or proximal deletion of 5q13 because of the similarity of the bands in 5q. Consistent clinical features associated with deletion of distal 5q22-q31 include developmental delay, low birth weight, failure to thrive, decreased fetal movement, polyhydramnios, camptodactyly, small mouth, high arched palate, micrognathia, hypertelorism, downward slanting palpebral fissures, short stature, head circumference above 35 cm, frontal bossing, epicanthic folds, depressed nasal bridge, anteverted nostrils, low set ears, short neck, cleft palate (50% cases), head and heart abnormalities (50% cases), talipes, simian crease, and

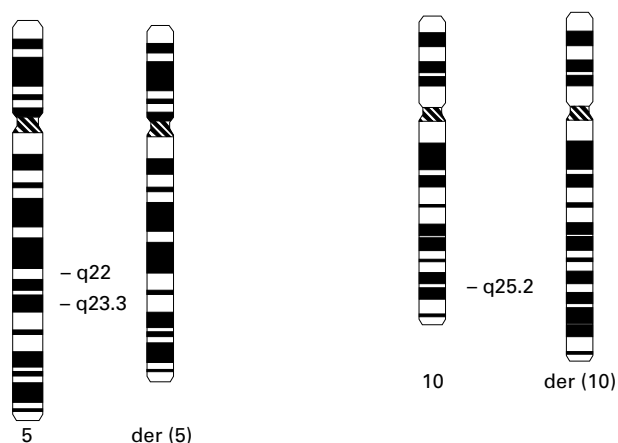


Figure 3 A diagrammatic representation of the interchromosomal direct insertion between chromosomes 5 and 10 in the father.

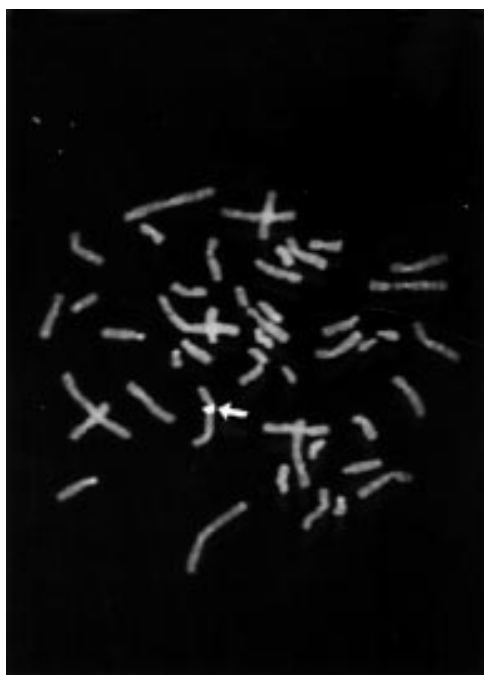


Figure 4 FISH using YAC probe 37HG4 showing a single signal (arrowed) in a metaphase from II.2.

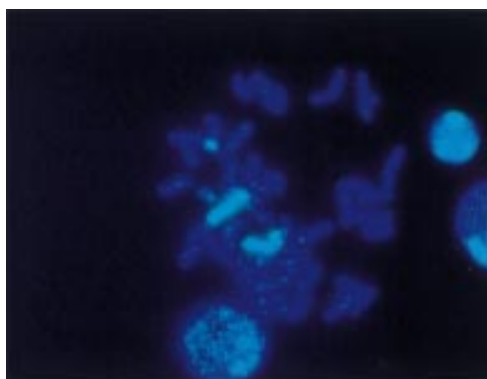


Figure 5 FISH using chromosome 5 paint showing a duplication of chromosome 5 material inserted into chromosome 10.

mental and motor retardation. Other clinical features less frequently observed were dislocation of the hips (28%), thin upper lip (28%), carp shaped mouth (28%), and repeated respiratory and urinary infections (43%).<sup>32</sup> The four cases described here all had talipes and arthrogyposis while case 1 also had low birth weight,

failure to thrive, depressed nasal bridge, micro-osmia, anteverted nostrils, low set ears, micrognathia, carp shaped mouth, cleft palate, downward slanting palpebral fissures, single simian crease, short neck, mental retardation and recurrent respiratory infections. Case 3 had skull enlargement, frontal bossing, and low set ears while case 6 had cleft palate, downward slanting palpebral fissures, low set ears, and respiratory problems. Only three published cases have a similar breakpoint (5q22q23.3) to the deletion cases described here and two had mental retardation, multiple epidermoid cysts, long midface, and APC.<sup>12</sup> A third case<sup>19</sup> described an adult with a bossed, high forehead, long philtrum, high arched palate, joint laxity, long fingers, and APC. Interstitial deletions of 5q22 have been reported in adults with polyposis coli but as the cases described here have a larger deletion involving 5q23.3 they are unlikely to survive to adulthood when APC would be evident.

Several consistent features between the cases presented in this paper and previously published cases of a deletion of 5q22q23.3 include arthrogyposis and talipes, suggesting that some limb development genes as well as *APC* are localised to this chromosomal region. One of the malformations displayed by cases 1, 4, and 6 was polydactyly. In view of the proposed role of *APC* in some forms of apoptosis and the function of the *Drosophila* homologues of *APC*,  $\beta$ -catenin, and *GSK3 $\beta$*  in segment polarity and other aspects of cell fate,<sup>33</sup> the finding of polydactyly in this family is of interest. Mice carrying the *Apc* mutation have been seen to display digital abnormalities, but polydactyly is not a frequent finding in patients with deletions of the *APC* locus and may therefore be a consequence of genes other than *APC* in the family presented here.<sup>34</sup>

Reports of duplication for the distal 5q segment are rare, as many are a result of an unbalanced segregation of a translocation and are also deleted for another chromosome. Case 5 and that of Gilgenkrantz *et al*<sup>6</sup> are unique in that they are both the result of familial insertion. Three patients with a duplication of 5q22q23.3 have been reported and were all clinically dysmorphic with developmental delay. Features included microcephaly, psychomotor and mental retardation, small ears, strabismus, sloping, enlarged forehead, prominent nasal bridge, small chin, disproportionately long arms, esotropia, spasticity, and episodes of self mutilation.<sup>32</sup> Patients with a more distal duplication have features which include low birth weight, brachycephaly, clinodactyly, facial anomalies with protruding nose, and enlarged forehead.<sup>32</sup> Case 5 is unusual in that duplication of a segment of 5q22q23.3 incorporating a known gene has occurred without obvious



Figure 6 (A) Case II.7 at birth and (B) polydactyly and talipes of the feet.

phenotypic effect at 4 years of age. The child had a simian crease, clinodactyly, and undescended testis, but these features are also found in normal subjects; this is supported by the presence of the same phenotype in one of the sibs (case 2) who had a normal karyotype. The presence of an extra copy of *APC* could have a longer term effect especially in the colorectum. This child is effectively a natural experiment and if no phenotypic abnormalities of the colorectum occur then this would have implications for the safety of gene therapy for FAP in that an extra copy of *APC* in a colonocyte is not harmful.

Ultrasonography is becoming an increasingly more useful tool for detecting pregnancies at risk for a chromosome abnormality or genetic disorders. However, for couples with a known chromosome rearrangement, prenatal diagnosis is essential if a viable offspring with an abnormal clinical outcome is likely. It is also recommended that cases of clinically normal subjects with unbalanced karyotypes are published so that an informed decision can be made by parents prenatally when a similar rearrangement is identified. This paper presents such an example, as duplication of 5q22q23.3 may not result in clinical abnormality.

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## Hypoparathyroidism, retarded growth and development, and dysmorphism or Sanjad-Sakati syndrome: an Arab disease reminiscent of Kenny-Caffey syndrome

EDITOR—In the December 1998 issue of *Journal of Medical Genetics*, four letters discussed whether or not Kenny-Caffey syndrome (KCS) is a part of CATCH 22.<sup>1-4</sup> These were based on a report of four Bedouin sibs with an unusual form of KCS that includes the additional features of marked IUGR, severe psychomotor retardation, and microcephaly. Two of the affected sibs and their phenotypically normal mother were found to have microdeletion 22q11.<sup>5</sup> The same authors subsequently suggested that this entity represents the Arab variant of KCS and because of some clinical resemblance to DiGeorge syndrome (DGS) they suggested that the phenotype is the result of 22q11 microdeletion or some abnormality of chromosome 10p where a second locus for DGS lies.<sup>6</sup>

This entity was originally described by Sanjad *et al*<sup>7</sup> in 1988 and 1991 as a new syndrome consisting of congenital hypoparathyroidism, seizures, growth and developmental retardation, and dysmorphic features in a group of Arab children of consanguineous parents. Several other reports followed.<sup>9-14</sup> All were Arabs, particularly Bedouin, with several sets of multiple affected sibs. The children have recognisable identical facies with deep set eyes, depressed nasal bridge with a beaked nose, long philtrum, thin upper lip, micrognathia, and large, floppy ear lobes. Medullary stenosis and other skeletal defects were found in most of them. This, together with the hypocalcaemia, hyperphosphataemia, and low concentration of immunoreactive parathyroid hormone in some of them, makes the phenotype similar to KCS. Recently the syndrome was localised to 1q42-43 by three independent groups.<sup>15-17</sup> Although they have a similar phenotype, different locus names have been given, namely HRD for hypoparathyroidism, retarded growth and development, and dysmorphism,<sup>14</sup> KCS for autosomal recessive KCS,<sup>15</sup> and SSS for Sanjad-Sakati syndrome.<sup>17</sup> The name of the syndrome in OMIM is hypoparathyroidism-retardation-dysmorphism and is given the number 241410 with autosomal recessive inheritance considered certain. The acronym Sanjad-Sakati syndrome is shown as an alternative.

It is also listed among the Arab diseases in our text book *Genetic disorders among Arab populations*.<sup>18</sup>

With regard to the family with 22q11 microdeletion, the association is probably fortuitous or as a remote possibility it might be the cause in a subset of patients with this Arab disease. This issue should be easily resolved at the molecular level.

I believe that it is important for us (practitioners and scientists) to decide on one term for this disorder. For convenience and in order to credit the original authors, I suggest the acronym "Sanjad-Sakati" for the syndrome and HRD for the locus, an abbreviation which refers to the components of the syndrome.

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## Tandem duplication within the neurofibromatosis type 1 gene (*NF1*) and reciprocal t(15;16)(q26.3;q12.1) translocation in familial association of *NF1* with intestinal neuronal dysplasia type B (IND B)

**EDITOR**—Neurofibromatosis type 1 (*NF1*) is a common human disorder (1/3500 live births) with neuroectodermal involvement resulting in dermatological manifestations of café au lait spots, cutaneous/subcutaneous neurofibromas, and freckling of major folds.<sup>1</sup> Hamartomas of the irides (Lisch nodules), well observed on slit lamp examination, are helpful phenotypic markers. Owing to diagnostic uncertainties, especially in young patients, an international scoring system has been discussed and agreed upon.<sup>2</sup> Half of the cases result from fresh mutations, others show an autosomal dominant mode of inheritance. The gene mutated in *NF1* maps to 17q11.2, is composed of 57 plus at least three alternatively spliced exons,<sup>3</sup> and is of ubiquitous expression. The encoded product, referred to as neurofibromin, is a member of the so called GTPase activating proteins (GAPs), and is an upstream downregulator of the p21Ras/Raf/MAPkinase signalling pathway.<sup>4</sup> Though genetic homogeneity is a hallmark of this condition, phenotypic heterogeneity has been exemplified by an extreme spectrum of diversity ranging from malformation or malignant variants to virtually benign dermatological changes.<sup>1</sup>

In particular, and among the many causes for gastrointestinal involvement in *NF1* patients, the association with intrinsic intestinal dysmotility (IID), resulting from intestinal neuronal dysplasia type B (IND B)<sup>5,6</sup> or aganglionic

megacolon (Hirschsprung's disease, HSCR)<sup>7</sup> has been documented and is now well established.

We report here a family showing aggregation of *NF1* and IID in two sibs, in one of whom congenital megacolon necessitated a Duhamel abdominoperineal pull through with tired suction biopsies of the colon and analysis of the whole excised specimen indicating IND B. This kindred provided a unique opportunity to unravel the genetic bases for the association between two such disorders of neural crest cell development.

The proband was seen at the age of 27 months for investigation of a multiple congenital anomaly/mental retardation (MCA/MR) complex. This young female was born to unrelated white parents. A familial component of both neurofibromatosis type 1 and severe intestinal dysmotility was shown. According to the history, intrauterine growth retardation had been evident from 6 months of gestation. Recurrent caesarean section was performed at 38 weeks of gestation. Birth weight was 2360 g, crown-heel length 46 cm, and OFC 32 cm. The Apgar scores were 8 and 10 at one and five minutes. Congenital heart disease was then diagnosed owing to heart failure with evidence of ventricular septal defect (membranous), persistent ductus arteriosus, and coarctation of the aorta. A two step surgical procedure including tissue grafting led to complete recovery. However, unresolved growth retardation ranging between -3 and -4 SD, cognitive impairment, the presence of multiple cutaneous café au lait spots, and persistent severe constipation indicated a possible MCA/MR syndrome.

On examination, the child was of short stature (76 cm, -3.5 SD), low weight (8.22 kg, -3.25 SD), and had relative macrocephaly (48 cm, ~mean). A distended abdomen contrasted with a generally wasted appearance (BC/OFC=0.26, normal  $\geq$ 0.30). Minor facial anomalies were also noted including frontal bossing, temporal narrowing, depressed nasal root, small, tapered chin, and hypoplastic ear lobes. There were downward slanting palpebral fissures but no true hypertelorism (ICD=25 cm, OCD=67 cm) (fig 1). High vaulted (uncleft) palate as well as hypoplastic and widely spaced teeth were also observed. There was apparent anterior displacement of the anus. The extremities were normal. Dermatological scrutiny showed >10 large (>5 mm) café au lait spots over her trunk and several achromic patches on the four limbs. There were no cutaneous/subcutaneous neurofibromas and axillary freckling was not a feature. The child could not walk unaided but rather crawled on all fours, and speech consisted of

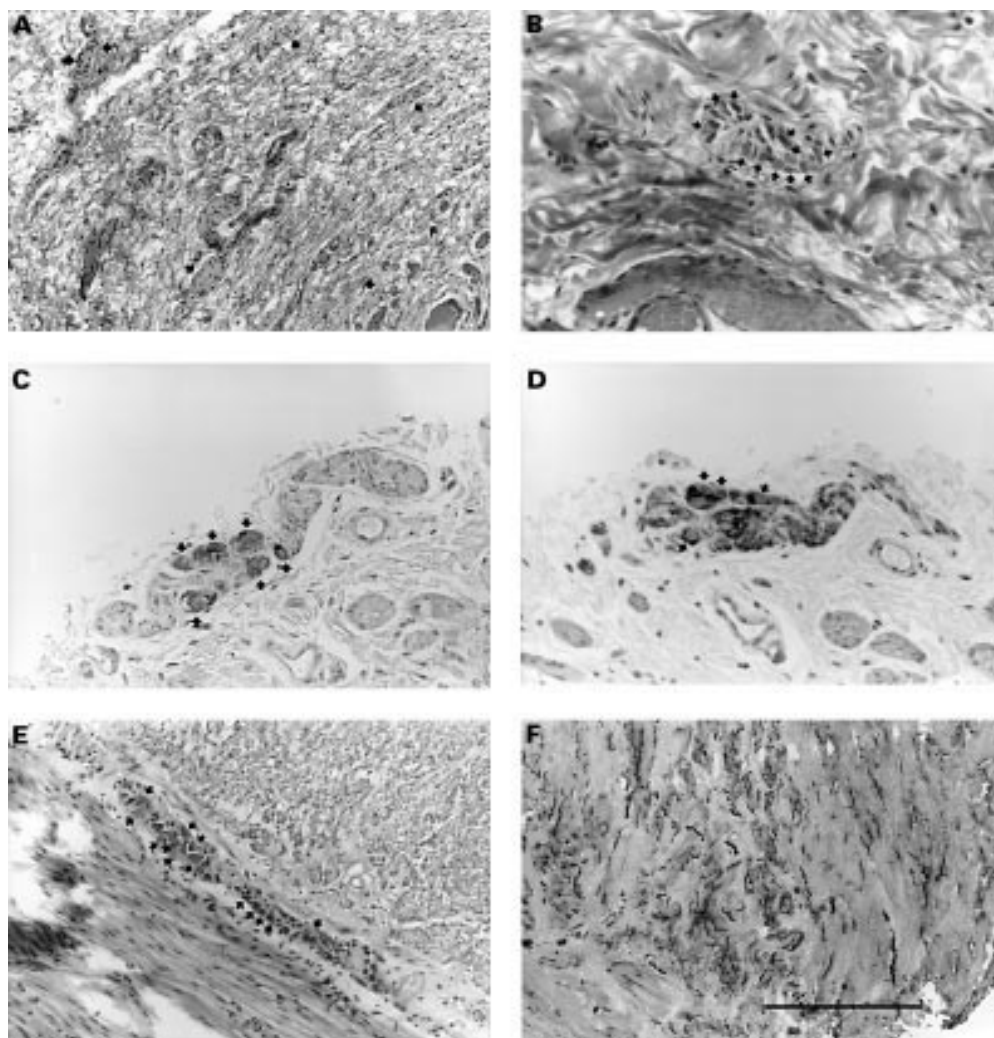


**Figure 1** The proband aged 27 months. Note downward slanting palpebral fissures, apparent hypertelorism, depressed nasal root, small, tapered chin, and scar from sternotomy.



**Figure 2** Contrast enema. Distended intestines are consistent with the diagnosis of congenital megacolon.





**Figure 3** Intestinal neuronal dysplasia. Suction biopsies of the rectum, sigmoid, and left colon, and the subsequent colectomy specimen were routinely processed for histology and immunostained for neurone specific enolase (NSE), a marker of neurones, and protein S100, a marker of Schwann cells, in order to identify intrinsic nervous structures better. In addition, acetylcholinesterase activity could be ascertained from an archival frozen sample. (A) Abnormally increased density of the nervous structures (arrows) and Schwann cell hyperplasia are consistent with hyperplasia of the rectal submucosal plexuses; ganglion cells (double arrow) are occasionally visible at this magnification (haematoxylin, eosin, and saffron, scale=400  $\mu$ m). (B) "Giant" submucosal sigmoid ganglion, that is, containing more than 10 neurones (arrows) with typical, large, amphophilic cytoplasm (haematoxylin, eosin, and saffron, scale=150  $\mu$ m). Neurones of a myenteric plexus are visible after immunostaining with anti-NSE antibody (C, arrows) and are negative for antiprotein S100 antibody, which specifically stains the Schwann cells (D, arrows) (serial sections, immunoperoxidase, scale=150  $\mu$ m). (E) A myenteric sigmoid plexus showing numerous neurones (arrows, haematoxylin, eosin, and saffron, scale=400  $\mu$ m). (F) Acetylcholinesterase staining of the rectal muscular layer showing numerous, coarse, and undulating fibres (dark) (frozen section, scale=750  $\mu$ m).

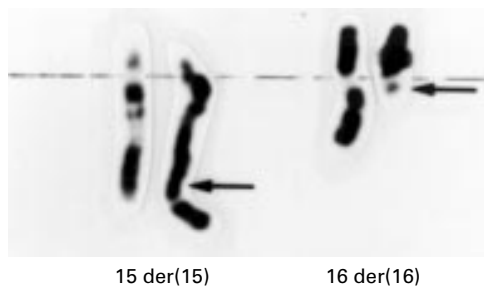
only a few words. The developmental quotient score was 57, as ascertained by the Binet test.

Cholesterol was slightly raised (6 mmol/l, normal <5.3), but triglycerides were within normal limits. Metabolic screening was negative. Skeletal x ray showed evidence of delayed bone age (18 months), and soft tissue abdominal x ray showed faecal impaction with stercoliths. Distended intestines on contrast enema indicated megacolon (fig 2). Ophthalmological evaluation, including slit lamp examination of the irides and funduscopy, was normal. MRI showed enlargement of the right rear aspect of the myelencephalon and a bright signal with contrast enhancement consistent with the diagnosis of hamartoma or low grade glioma. Bright signals were also evident at the medial cerebellar peduncle and in the left semioval centre on T2 weighted sequences, consistent with so called unidentified bright objects (UBOs).

Because of the congenital megacolon, surgical biopsies of the colon and rectum were performed and were found to be consistent with aganglionosis with Schwann cell hyperplasia in the Auerbach plexuses and hypoganglionosis with Schwann cell hyperplasia in the Meissner plexuses. A Duhamel abdominoperineal pull through was thus per-

formed. Analysis of suction biopsies of the rectum, sigmoid, and left colon and of the whole subsequent colectomy specimen pointed to intestinal neuronal dysplasia type B (IND B), based on the presence of abnormal submucosal plexuses showing focal hyperplasia (in terms of density and sizes), occasional giant ganglia harbouring >10 neurones, and nerve cell buds along afferent nerves. The presence of giant ganglia, two to three times as large as their normal counterparts, was set forth as the only reliable, age independent diagnostic criterion for IND B,<sup>8</sup> since hyperplasia of the submucosal plexuses, an increase in acetylcholinesterase (AChE) activity in the nerve fibres of the lamina propria, and low SDH activity in nerve cells were shown to normalise with the ongoing maturation of the enteric nervous system. In addition, myenteric plexuses were either normal or hypertrophied, with numerous mature neurones (fig 3).

In order to address the possibility of a cytogenetic aberration in relation to a MCA/MR syndrome in the proband, her chromosome complement was established. From analysis of high resolution RBG banded chromosomes, it was apparent that she had a cytogenetically bal-



**Figure 4** Reciprocal balanced translocation. Wild type as well as derivative chromosomes 15 and 16 from the proband are shown prepared from whole blood lymphocytes with RBG labelling at the 550 band level, following the guidelines of the International System for Human Cytogenetic Nomenclature (ISCN 1995). Arrows point to breakpoints on der(15), in 15q26.3, and on der(16), in 16q12.1.

anced reciprocal translocation, her specific chromosomal constitution being 46,XX, t(15;16)(q26.3;q12.1) (fig 4). This abnormality was inherited from her father (I.1, fig 5A), whose lymphocytes harboured the same translocated chromosomes and was also shared by her older sister (II.1) and healthy brother (II.2) (data not shown; see below for additional family data).

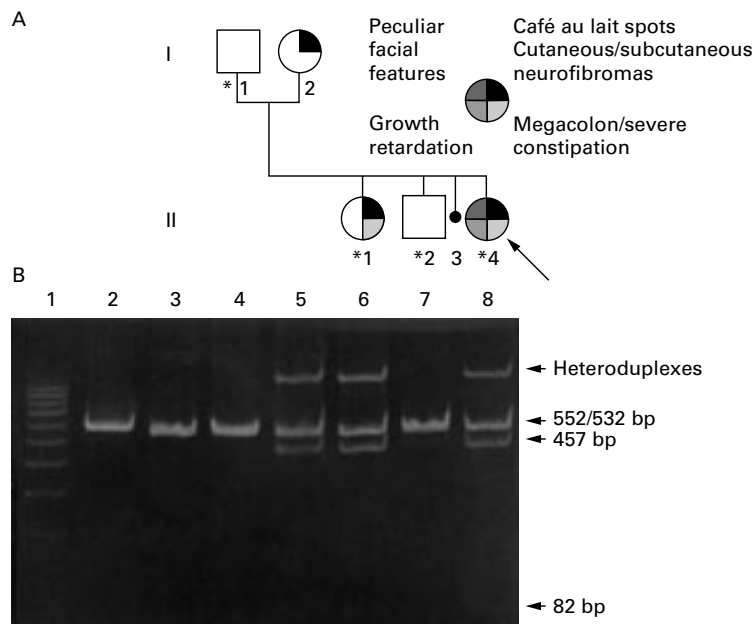
The mother of the proband (I.2, fig 5A) presented with classical neurofibromatosis type 1 with multiple large café au lait spots and cutaneous/subcutaneous neurofibromas. Her condition was seemingly inherited through the maternal grandmaternal lineage. The father of the proband (I.1) had no apparent dermatological ailment, malformation, or cognitive alteration. The older daughter (II.1) presented with café au lait spots and had some degree of problem with school performance and cognitive impairment (VIQ=76, PIQ=83, TIQ=77, according to WISC-R). She had severe constipation with megacolon and a clinical course typical of HSCR until a favourable outcome followed successful medical treatment. The sibship also included an older brother (II.2) who had not, to our knowledge, been referred for dermatological, morphologi-

cal, or cognitive problems. A spontaneous abortion, occurring at 2 months of gestation (II.3), was also reported, without further details.

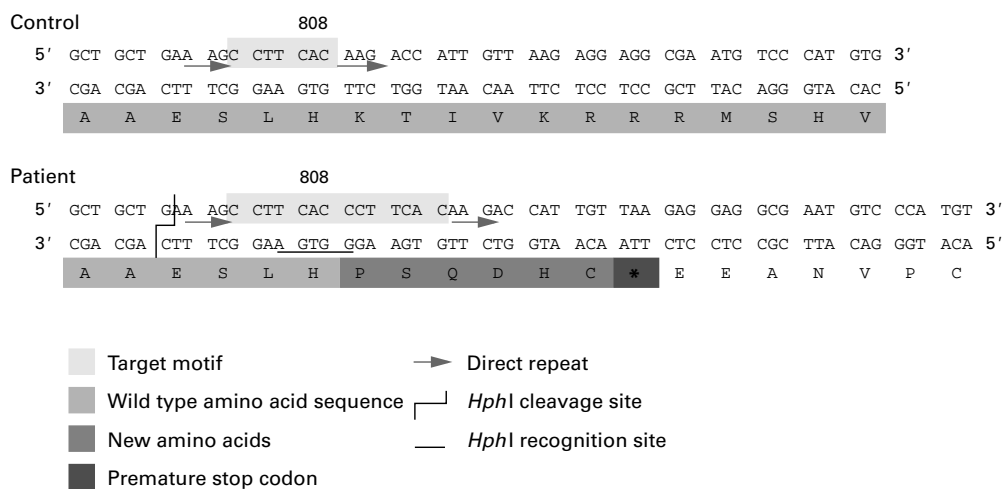
This familial association of NF1 and IND B provided a unique opportunity to test for alternative causal mechanisms, namely the random aggregation of two common monogenic disorders versus a discrete neurocristopathy with possible genotype-phenotype correlation at the *NF1* locus or modifying loci lying elsewhere in the genome.

Indeed, although no specific gene for IND B has been found, the latter condition may segregate with typical monogenic HSCR.<sup>9</sup> Consistently, and following strict diagnostic guidelines, ~30% of IND B patients have accompanying aganglionosis, that is, HSCR.<sup>10</sup> Therefore, screening for a mutation in the genes found to be mutated in monogenic non-syndromic HSCR, that is, the *RET* proto-oncogene,<sup>11-13</sup> the genes encoding endothelin receptor B (*EDNRB*),<sup>14 15</sup> or its ligand endothelin 3 (*EDN3*),<sup>16</sup> was a prerequisite to dissecting this familial association further. Molecular analysis of the *RET* proto-oncogene was carried out using DGGE based on psoralen modified primers for exons 3, 5, 9-13, 16, 18, 20, and SSCP for exons 1, 2, 4, 6-8, 14, 15, 17 and 19, as previously described.<sup>15</sup> SSCP in conditions previously determined was similarly carried out for the seven *EDNRB*<sup>9</sup> and the five *EDN3* exons.<sup>16</sup> Following these procedures, no mobility shift was observed. We assume that no mutation of the relevant genes was present in the proband's DNA.

Since none of these genes was faulty in the proband, which favoured a discrete neurocristopathy, it was of particular interest to identify the *NF1* lesion. The latter was detected as a result of routine screening for the previously reported recurrent R816X mutation using previously described PCR amplification of *NF1* exon 16 followed by restriction with endonuclease *HphI*.<sup>17</sup> Sequence analysis, carried out in conditions previously specified,<sup>17</sup> showed a heterozygous tandem duplication of the 7 bp motif (5' CCTTCAC 3'), nt 2418-2424, inclusive, lying towards



**Figure 5** (A) Family pedigree. Arrow indicates the proband. II.3 was a miscarriage. Complex phenotypic traits are specified as shown in the key. The presence of the reciprocal t(15;16)(q26.3;q12.1) translocation in I.1, II.1, II.2, and II.4 is indicated by an asterisk. The proband and her older sister (II.1) both have NF1 skin symptoms, have severe constipation/megacolon, and harbour the familial cytogenetically balanced reciprocal translocation. (B) Restriction with *HphI*. The *NF1* exon 16 amplicon is shown restricted for I.1 (lane 4), I.2 (lane 5), II.1 (lane 6), II.2 (lane 7), and II.4 (lane 8), co-electrophoresed with molecular weight marker GenLadder™ 100 (Genaxis Biotechnology, Montigny-le-Bretonneux, France; lane 1). Arrowheads indicate theoretical fragment sizes in base pairs (bp) as compared to a healthy control, unrestricted (552 bp fragment, lane 2) and restricted (532 bp fragment, lane 3). Healthy subjects I.1 and II.2 display the expected wild type 532 bp fragment, whereas I.2, II.1 and II.4, diagnosed with NF1, have wild type 532 bp as well as mutant 457 bp and 82 bp fragments. Note the presence of heteroduplexes in lanes 5, 6, and 8.



**Figure 6** *NF1* tandem duplication. Double stranded sequence of *NF1* exon 16, 5' extremity, is represented in control and patient with translation into protein. The tandem duplication of a 7 bp target motif flanked by two short direct repeats in the patient results in a frameshift mutation and leads to a putative truncated protein as shown by the insertion of six new amino acids, immediately followed by a premature translation termination codon (TAA). This tandem duplication also generates a recognition site for type II endonuclease *HphI*, as indicated above.

the 5' extremity of the exon and flanked by two short direct tandem repeats (5' AAG 3'). This DNA lesion (2424-2425insCCTTCAC) caused a frameshift 3' to codon 808, unchanged, with the addition of six new codons immediately followed by a translation termination signal (TAA), and generated a bona fide recognition site for *HphI* (5' GGTGA 3') (fig 6). This specific lesion featured as a rare mutational event (one case identified among >200 independent *NF1* chromosomes tested; data not shown) and was therefore not amenable to genotype-phenotype correlation analysis. However, this out of frame duplication, lying 5' to the GAP related domain (GRD) specifying region of the gene, was most probably a null mutation and therefore probably did not cause variation in the phenotype.

Using the aforementioned screening method, the status of the rest of the family with regard to the mutation was eventually determined. Predictably, the mother and sister, diagnosed with *NF1*, were heterozygotes for the 2424-2425insCCTTCAC lesion, whereas the father and older brother were both wild type homozygotes (fig 5B).

Alleles that modify the severity of the phenotype (or penetrance) of a given, well established monogenic factor, that is, mutation of *RET*, *EDN3*, or *EDNRB* in HSCR, with or without coexisting IND B, have been postulated and some of the relevant genes or loci subsequently identified. The first such example was illustrated by a large inbred Mennonite HSCR pedigree that segregated a missense mutation in *EDNRB*<sup>18</sup> and otherwise showed linkage disequilibrium with marker alleles mapped to 21q22. This finding was highly suggestive of a HSCR genetic modifier linked to this chromosomal region,<sup>19</sup> which might elsewhere account for high HSCR prevalence among trisomy 21 patients.<sup>20</sup> More compelling evidence for a HSCR modifier is exemplified by the genes encoding glial cell line derived neurotrophic factor (*GDNF*)<sup>21-23</sup> and, more recently, neurturin (*NRTN*),<sup>24</sup> two highly homologous natural ligands of the *RET* tyrosine kinase receptor protein. Indeed, since *GDNF* and *NRTN* were found to be mutated in families also segregating well characterised *RET* alleles, it was postulated that alterations of these genes were not sufficient in themselves to cause HSCR, but that they probably contributed to the severity of the phenotype, or to higher penetrance of the *RET* mutations.

As to *NF1*, analysis of several quantitative or binary traits among a large sample of familial cases concluded that the number of café au lait spots and the number of cutaneous neurofibromas, showing high concordance among MZ

twin pairs but weaker concordance in more distant relatives, were determined by non-allelic, "modifying" loci.<sup>25</sup> Absence of correlation between the characters analysed suggested that these loci were also trait specific. The presence or absence of intestinal neuronal dysplasia, or bona fide HSCR, is a rare configuration that could not have been assessed. However, modifying loci remain a strong possibility, especially since *NF1* and HSCR/IND B are both characterised by disruption or maldevelopment of neural crest cell derivatives. In other words, alleles of genes whose products interfere with the determination, migration, or differentiation of ganglion cells of the enteric plexuses might, in the presence of a perturbed Ras signalling pathway, lead to IID. In this respect, it is noteworthy that the proband's *NF1* phenotype is consistent with the involvement of a broad spectrum of neural crest cell derivatives, that is, also including motor neurones of the enteric nervous system, conotruncus, and cranial crest mesectoderm.<sup>26</sup>

Finally, the most salient aspect of this report is the cosegregation, in two sisters affected with *NF1* and megacolon, of an *NF1* lesion inherited from the mother, in whom it results in a classical presentation of the condition, and of a cytogenetically balanced reciprocal translocation inherited from the father and shared by an older brother, in whom this chromosomal aberration has no apparent pathogenicity. None of the translocation breakpoints (15q26.3 and 16q12.1) has yet been involved in HSCR or IND B, alone or in a more complex phenotype (see the review by Passarge<sup>27</sup> and a recently recognised entity by Mowat *et al*.<sup>28</sup>). Therefore, these chromosomal regions provide obvious candidate locations for additional *NF1* or HSCR/IND B modifiers or both.

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## Identification of novel alleles at a polymorphic microsatellite repeat region in the human NRAMP1 gene promoter: analysis of allele frequencies in primary biliary cirrhosis

EDITOR—Primary biliary cirrhosis (PBC) is a chronic, slowly progressive cholestatic liver disease believed to result from autoimmune mechanisms. The initiation of the disease is likely to be multifactorial with genetic, infectious, and environmental factors contributing. A familial predisposition to PBC has been reported, but studies to investigate an association between PBC and polymorphisms at a number of genetic loci have not been conclusive. The aetiology of the disease remains unknown but it has been suggested that R forms of *E coli*<sup>1</sup> and *Mycobacterium gordonae*<sup>2</sup> may play a potentially pathogenic role in PBC, though this has not been established. A common characteristic feature of PBC is the presence of granulomas and it is interesting to note that these tend to disappear as the lesions progress and fibrosis and cholestasis appear, that is, secondary effects of tissue damage.

NRAMP1 (natural resistance associated macrophage protein 1) was isolated as the human homologue of the

mouse *nramp1* gene (previously designated *Ity/Lsh/Bcg*) which, when mutated, is responsible for susceptibility to a number of macrophage trophic intracellular pathogens including *Mycobacterium bovis*, *Salmonella typhimurium*, and *Leishmania donovani*.<sup>4</sup> Expression of the gene is restricted to cells of the mononuclear phagocytic system (macrophages and granulocytes)<sup>5</sup> and it plays an important role in the activation of macrophages and innate immunity. When *nramp1* is mutated, mice fail to control pathogen growth in the early stages of infection. Sequence analysis of *nramp1* and recent functional studies suggest that the gene encodes a multispinning transmembrane transporter protein<sup>6</sup> with specificity for divalent metal cations,<sup>7</sup> but its physiological role in relation to macrophage function is still poorly understood. In the human gene at least 10 polymorphic sites have been described,<sup>8-10</sup> one of which spans a microsatellite repeat region in the 5' untranslated promoter region of the gene. This is a functional polymorphism affecting levels of NRAMP1 expression.<sup>11</sup> The alleles at this site have been inconsistently reported; Blackwell *et al*<sup>8</sup> initially reported identification of four alleles where alleles 1, 2, 3, and 4 had 11, 10, nine, and four repeats of the final dinucleotide repeat in the sequence t(gt)<sub>5</sub>ac(gt)<sub>5</sub>ac(gt)<sub>n</sub>g. In subsequent reports they have designated the sequence t(gt)<sub>5</sub>ac(gt)<sub>10</sub>g as allele 4<sup>11-13</sup> and have most recently reported allele 4 to have the sequence t(gt)<sub>5</sub>ac(gt)<sub>9</sub>g<sup>14</sup>. Liu *et al*<sup>8</sup> identified three alleles at this site which they allocated alleles 1, 2, and 3 with 2 bp

**MnII**

ggacatgaagactcgattag | gccaacgaggggtcttggactccagatcaaagag  
cctgtacttctgagcgtaat | cgggttgctccccagaaccttgaggtctagtttctc

**RsaI**                      **RsaI**

aataagaagacctgactctgtgtgtgtgt | acgtgtgtgtgt | acgtgtgtgtgtgtg  
ttattcttctggactgagacacacacaca | tgcacacacaca | tgcacacacacac

**MnII**

**tgtgt** | gtggcagaggggggtgtggtcatggggatgacatgaatacgcaagggg  
**acac** | **aca**cgctctccccacaccagtacccataactgtacttatgcttccccg

aggaagcatctgaaatcagagctaa  
tccttcgtagacttttagtctcgatt

**Figure 1** Sequence of the promoter region of the human *NRAMP1* (Accession number X82016, 59–240 bp) amplified by PCR to genotype the polymorphic microsatellite region shown in bold. The *RsaI* and *MnII* restriction enzyme sites used to digest the amplified DNA are shown. The enzymes recognise the following sites and cut at the position shown by the vertical slash. *n* represents any base. *MnII*(*n*)<sub>gagg</sub>(*n*)<sub>ctcc</sub> *RsaI*gac. The sequence for allele 3 is used in this figure. Sequences of the other alleles identified in this study and those previously published are shown in table 1.

increments of increasing size and allele 1 having the sequence t(gt)<sub>7</sub>ac(gt)<sub>5</sub>ac(gt)<sub>5</sub>g.<sup>8</sup> We adopted and added to the allele naming system used by Blackwell *et al*<sup>9 11–14</sup> as we have shown evidence from restriction enzyme digestion and sequencing that three of the alleles we have identified are consistent with their reported alleles 1, 2, and 3.

Associations with *NRAMP1* and susceptibility to a number of diseases including Crohn's disease,<sup>15</sup> tuberculosis,<sup>16</sup> leprosy,<sup>17</sup> and rheumatoid arthritis (RA)<sup>12 13</sup> have been reported. The reported *NRAMP1* and RA association is with the allele driving highest levels of *NRAMP1* expression (allele 3) of the microsatellite repeat region polymorphism discussed above. Allele 3 was found to be transmitted to RA affected children in preference to allele 2 in a study using identity by descent sib pair analysis.<sup>12 13</sup> We investigated this polymorphic site in patients with PBC.

Using restriction fragment length polymorphism (RFLP) analysis we genotyped 46 PBC patients, 76 alcoholic liver disease (ALD) patients, 39 hepatitis C patients, and 78 normal, healthy, non-cirrhotic subjects for the microsatellite repeat polymorphism in the promoter region of the *NRAMP1* gene. PCR products of approximately 194 bp, generated using NRAMPFpol GGACATGAAGACTCGCATTAGG (59–70 bp Genbank Accession number X82016) and NRAMPRpol TTAGCTCTGATTTTCAGATGCTTCC (240–217 bp Genbank Accession number X82016) PCR primers, were digested separately with *RsaI* and *MnII*, resolved on 6% denaturing polyacrylamide gel by electrophoresis, and silver stained.<sup>18</sup> We found three alleles as previously reported,<sup>9 11–14</sup> but in addition two new alleles which we named allele 5 and allele 6.

The PCR products from cases with these novel genotypes were cloned into the vector pCR2.1 using Invitrogen TA Cloning System Version 2.0 and the plasmids used to transform competent XL-2 blue cells. The cloned DNA inserts from successful transformants showing the appropriate RFLP pattern were then sequenced. Allele 5 was found to have the sequence t(gt)<sub>4</sub>ac(gt)<sub>5</sub>ac(gt)<sub>10</sub>g with

a gt deletion in the first (gt)<sub>5</sub> dinucleotide repeat identified in the other alleles. Allele 6 had the sequence t(gt)<sub>5</sub>ac(gt)<sub>5</sub>ac(gt)<sub>5</sub>at(gt)<sub>5</sub>ggcaga(g)<sub>7</sub> which had an extra guanidine inserted in a hexanucleotide g repeat 7 bp 3' of the final gt of the polymorphic site in addition to the final polymorphic gt repeat being interrupted. Despite finding these novel alleles we have not identified a pattern representing any of the sequences published as allele 4<sup>9 11–14</sup> in any of the 246 cases analysed.

The sequence of the five alleles we have detected in our study (alleles 1, 2, 3, 5, and 6) and allele 4 reported by Blackwell *et al*<sup>9 11–14</sup> are shown in table 1. In the PBC population we genotyped, allele 5 was significantly more frequent (Fisher's exact test) in the PBC patients (8/53) we studied than in normal controls (3/78) (p<0.024), ALD (2/76) (p<0.012), or hepatitis C patients (0/39) (p<0.012) but was still uncommon.

The repetitive nature of the sequence we analysed requires a stringent, sensitive, and reproducible detection method, owing to the possibility of slippage or infidelity of the *Taq* polymerase enzyme during PCR amplification. The method we adopted gave consistent results when we analysed DNA extracted from the same blood sample at different times, DNA extracted from blood taken from the same person at different times, and DNA extracted from blood and paraffin embedded material from the same person, and we are confident that our findings are genuine. A number of alleles at this site have previously been reported by sizing this polymorphic region as a single fragment. Our strategy used restriction enzyme digests designed to size both the whole polymorphic site as a single fragment (*MnII*) and the size of fragments containing the 5' dinucleotide and 3' dinucleotide repeats of the region as distinct entities (*RsaI*) as shown in fig 1. It was the combined results of these digests which allowed detection of alleles 5 and 6 and confirmation of alleles 1, 2, and 3 reported by Blackwell *et al*.<sup>9 11–14</sup> It is important to note that had we been relying solely on results from a single analysis of the size of the microsatellite repeat as a whole, as previous reports have done, allele 5 would have been mistaken for allele 3, the allele which is at the highest frequency in all previously published reports.

Our finding of two new alleles at this functional polymorphic site warrants further analysis in other populations and investigation into the effect of allele 5 on levels of *NRAMP1* expression. Though we may not have identified a biologically significant association with PBC and this site, it is interesting to consider whether previous studies of this polymorphic site may have contained cases with unrecognised allele 5.

**Table 1** Summary of alleles of human *NRAMP1* 5' promoter polymorphic site. Sequence of the alleles identified in previous studies (alleles 1–4) and in this study (alleles 1, 2, 3, 5, and 6)

Allele 1 <sup>9 11–14</sup>	t(gt) <sub>5</sub> ac(gt) <sub>5</sub> ac(gt) <sub>11</sub> ggcaga(g) <sub>6</sub>
Allele 2 <sup>9 11–14</sup>	t(gt) <sub>5</sub> ac(gt) <sub>5</sub> ac(gt) <sub>10</sub> ggcaga(g) <sub>6</sub>
Allele 3 <sup>9 11–14</sup>	t(gt) <sub>5</sub> ac(gt) <sub>5</sub> ac(gt) <sub>9</sub> ggcaga(g) <sub>6</sub>
Allele 4 <sup>11–13</sup>	t(gt) <sub>5</sub> ac(gt) <sub>10</sub> ggcaga(g) <sub>6</sub>
Allele 4 <sup>9</sup>	t(gt) <sub>5</sub> ac(gt) <sub>5</sub> ac(gt) <sub>8</sub> ggcaga(g) <sub>6</sub>
Allele 4 <sup>14</sup>	t(gt) <sub>5</sub> ac(gt) <sub>9</sub> ggcaga(g) <sub>6</sub>
Allele 5	t(gt) <sub>4</sub> ac(gt) <sub>5</sub> ac(gt) <sub>10</sub> ggcaga(g) <sub>6</sub>
Allele 6	t(gt) <sub>5</sub> ac(gt) <sub>5</sub> ac(gt) <sub>4</sub> at(gt) <sub>5</sub> ggcaga(g) <sub>7</sub>

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## No evidence for imprinting in distal 18q

EDITOR—Partial loss of chromosome 18q (MIM 601808) results in characteristic clinical features including mental retardation, short stature, developmental delay, CNS defects, dysmorphic facies, and hearing loss. By phenotype mapping in 26 patients, Strathdee *et al*<sup>1</sup> showed that a region critical for many of the 18q- features lies in 18q22-23. Later, they were able to refine this region to an approximate 6 Mb segment within 18q23.<sup>2</sup> However, the clinical picture of 18q- patients is extremely variable, rendering a precise prediction of the clinical outcome impossible, even when the extent of the deletion is determined.<sup>1-4</sup> Among the factors contributing to this phenotypic variability, the genetic background of affected patients, environmental factors, and possibly genomic imprinting of genes in 18q may play a role. The selective expression of the paternal or maternal allele of a gene responsible for a phenotypic feature<sup>5-7</sup> might influence the phenotype of 18q- patients, depending on whether the mutation arose in the maternal or paternal germline. Most of the 18q- patients have a paternal deletion.<sup>8</sup> Assuming deletions originate with the same frequency in the maternal and paternal germline, imprinting of maternal genes could explain a more severe phenotype in patients with a paternal deletion, leading to a higher detection rate.

Evidence for imprinting in 18q came from linkage studies of bipolar affective disorder (BPAD). While several reports have shown linkage to 18q,<sup>9-15</sup> in some studies most of the linkage evidence derived from families with affected phenotypes in only the paternal lineage and from marker alleles in 18q11 and 18q21 transmitted on the paternal chromosome.<sup>9-10,13-16</sup> Genomic imprinting might explain the uniparental linkage, if critical maternal genes are imprinted (inactivated) and thus inheritance of a BPAD gene predisposes to the illness only if it is inherited from the father. Hence, depending on the exact location, imprinted genes in 18q would be strong candidates for the 18q- syndrome and for BPAD.

Encouraged by these observations, we started a PCR based screen in distal 18q to test for imprinting by analysing 22 expressed sequence tagged sites (ESTs) within 18q22-23. The ESTs consisted of unidentified as well as identified transcripts, including the myelin basic protein (MBP, MIM 159430), the galanin receptor (GALNR, MIM 600377), cerebellin 2 (CBLN2, MIM 600433), cytochrome b5, and the nuclear factor of activated T cells (NF-ATc, MIM 600489). The ESTs were chosen mainly because of their location within the 18q- critical region and their expression in brain which is severely affected in the 18q- syndrome.<sup>17-19</sup> The strategy is similar to the one described by Wevrick and Francke,<sup>20</sup> who showed that transcription of the small nuclear ribonucleoprotein associated polypeptide N (SNRPN) in peripheral blood lymphocytes and Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines of Prader-Willi syndrome patients is abolished. We tested for expression by PCR analysis of cDNA derived from lymphoblastoid cell lines of 18q- patients using specific EST primers that are only present in one copy in these patients.

EBV transformed cell lines derived from three patients with 18q deletions (patients 11, 17, and 18) have been previously described. The breakpoint of sample 11 is located proximal to D18S214 and D18S269 at approximately 80 cM, the breakpoint of sample 18 is located proximal to D18S165 and D18S252 at approximately 100 cM, and the breakpoint of sample 17 is located proximal to D18S299 between the two other breakpoints.<sup>1</sup> An EBV transformed cell line from a subject with a normal karyotype was used as a control (C). A lymphoblastoid cell line derived from a Prader-Willi syndrome patient (GM09189) who has a deletion involving the SNRPN gene, del(15)(pter→q11.2::q13→qter), was obtained from the Coriell Cell Repository.

Cytoplasmic RNA was prepared using a kit (RNeasy, Qiagen, Hilden) according to the manufacturer's instructions. Subsequently, RNA was treated with DNase I (AGS, Heidelberg) to remove any residual genomic DNA. cDNA synthesis was performed by using the MMLV reverse transcriptase (Promega, Madison) according to the manufacturer's protocol.

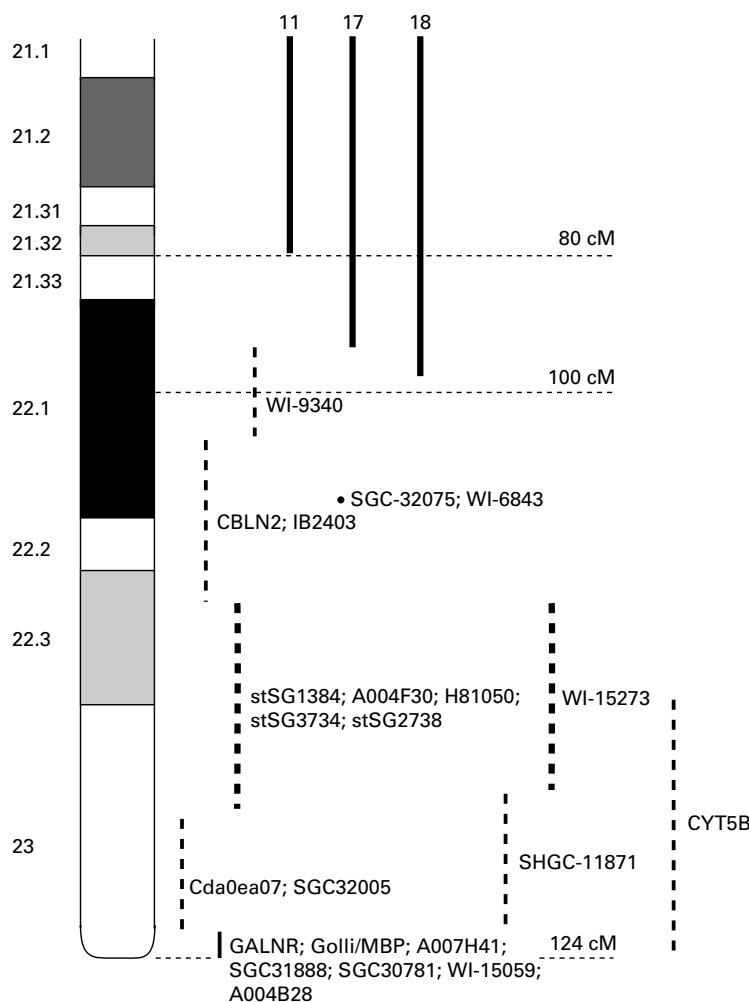


Figure 1 Schematic drawing of distal human chromosome 18q. The truncated chromosomes of the patients in this study are shown at the top; the approximate map positions of the ESTs tested are indicated by dashed bars on the right.

ESTs that mapped in 18q22.2-qter were identified from the gene map/Unigene map database of the National Center for Biotechnology Information (NCBI), National Institutes of Health (<http://www.ncbi.nlm.nih.gov/SCIENCE96/> and <http://www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html>). EST primer sequences were obtained from the websites of the Radiation Hybrid Database, European Bioinformatics Institute, Hinxton, UK (<http://www.ebi.ac.uk/RHdb/index.html>), and of the Whitehead Institute Center for Genome Research, Boston, MA, USA ([www.genome.wi.mit.edu/](http://www.genome.wi.mit.edu/)). Three additional sets of oligonucleotides were used for controls. MBP-PCR1/2 (5'-GGA CCT CGT GAA TTA CAA TC-3' and 5'-ATT TAC CTA CCT GTT CAT CC-3') amplifies a polymorphic region 5' of the *MBP* gene that is not transcribed. SNRPN-A/B (5'-AGA TGG CCG AAT CTT CAT TG-3' and 5'-AGC AAC ACC AGA CCC AAA AC-3') amplifies a 150 bp segment of the *SNRPN* gene that is known to be maternally imprinted.<sup>20,21</sup> Finally  $\beta$ -actin-A/B (5'-TCG TGC GTG ACA TTA AGG AG-3' and 5'-AGC ACT GTG TTG GCG TAC AG-3') are primers derived from exon 4 and exon 5 of the  $\beta$ -actin gene, respectively, and will amplify a 274 bp product only in RNA samples.

PCR analyses were performed in 1  $\times$  Q buffer, 1  $\times$  Q solution (Qiagen, Hilden), 0.1 mmol/l dNTPs (Pharmacia, Uppsala), 0.02 U/ $\mu$ l Platinum *Taq* DNA polymerase (Life Technologies, Rockville) in a total volume of 25  $\mu$ l in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Norwalk). Optimal primer concentrations and annealing tempera-

tures were established before testing. Cycling was performed for 30 seconds at 95°C, 30 seconds at the appropriate annealing temperature, and 30 seconds at 72°C for 34 cycles. Amplification products were analysed on a 3% agarose gel.

Three cell lines containing 18q deletions were used for imprinting analysis (fig 1). The origin of the deletion had been previously determined using polymorphic markers.<sup>1</sup> Samples 11 and 18 contain paternally derived rearrangements while sample 17 contains a maternally derived rearrangement. The hemizygous deletion in all three cell lines includes the region 18q22.1-qter.<sup>1</sup> cDNA was prepared from RNA for each of the three cell lines as well as a cell line containing a normal karyotype. To ensure that the RNA samples were free of genomic DNA contamination, PCR analysis was first performed using primers for a region that is located 5' of the *MBP* gene. Amplification was not observed in any of the samples but was observed in genomic DNA controls (data not shown), indicating the lack of DNA contamination in the RNA samples. To ensure that cDNA samples were not degraded, PCR was performed using primers derived from two exons of the  $\beta$ -actin gene. Amplification of the appropriate size product was seen with all cDNA samples (data not shown).

Twenty two ESTs that mapped to 18q22.2-qter were analysed for imprinting. Table 1 lists the ESTs that were tested as well as the composite results. Nine ESTs did not produce amplification products in the sample derived from a subject with a normal karyotype. Fig 2A shows a

Table 1 ESTs tested in this study and results of the PCR screening

EST	Name	Map position	Forward primer	Reverse primer	GM09189							
					11	17	18	C	(PWS) A	AM		
WI-9340	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform1	98-102 cM	TGAGAAACGAAATCTCTATCCG	AGGCAGCAAGTTTTTATAAAGGC	+	+	+	+	+	+	+	+
siSG4756	Highly similar to cerebellin precursor	102-109 cM	TCCTTTTGTGCTTTGGG	AGAAGCCACCTGAAAAGTCA	+	+	+	+	+	+	+	+
siSG1384	Hypothetical 52.9 kDa protein in CDC26-YMR31 intergenic region	109-118 cM	CCAGTAGTGAGGTTTGAGGG	GGCTCCTCTGATCTCTCG	+	+	+	+	+	+	+	+
A004F30	Similar to probable E1-E2 ATPase	109-118 cM	CTTGACTGTTTACTGAAAAG	CTGAGAATTAGACTGAAAAGTTTA	-	-	-	-	-	-	-	-
SHGC-11871	Nuclear factor of activated T cells (NF-ATc)	117-123 cM	ATGCCGGTTAGTGAGACCG	AAGCAGCTTAGGGTGCAAA	+	+	+	+	+	+	+	+
hGR1/2	Galanin receptor 1	>123 cM	CTGGCTACCTGCTCTCTG	GAAAGGCCCTGGTGGTAG	-	-	-	-	-	-	-	-
WI-9286	Golli/myelin basic protein	18q23	AGTCTCGTCCGCTGCTG	GCCACCATCCCTTGTGAG	+	+	+	+	+	+	+	+
CYT5B.A/B	Cytochrome b-5	18q23	TCCATCCAGATGACAGACCA	CTGCCATGTTATAGGCCGAT	+	+	+	+	+	+	+	+
SGC32075	Unidentified transcript	109 cM	TATTTATTTACCAGCTGTGTTTC	CCTGTGTCAAGAGAGCACAA	+	+	+	+	+	+	+	+
WI-6843	Unidentified transcript	109 cM	ACATGGTGAACATTTCCAATAACA	TGGACTCGGTACTCCGTTCT	+	+	+	+	+	+	+	+
IB2403	Unidentified transcript	102-109 cM	TGGCATAGAAAAGGGTAAA	GGCAGCTCTCCAGAAGTAAT	+	+	+	+	+	+	+	+
WI-15273	Unidentified transcript	109-117 cM	TCCTGTGCTCCAGTCC	TGGAGATCAAGATCCTCCG	-	-	-	-	-	-	-	-
H81050	Unidentified transcript	109-118 cM	TTTGACACTACAGGTTCTATGAC	GGCCACACTACTCAGAGACATT	+	+	+	+	+	+	+	+
siSG3734	Unidentified transcript	109-118 cM	TCAACTTAIGTGTGCTGCTGG	TACCCAAACCGTGTGGTGA	-	-	-	-	-	-	-	-
siSG2738	Unidentified transcript	109-118 cM	CGTCGACCCAGTITTTG	AGTGCCTGCTTTGTCACATC	-	-	-	-	-	-	-	-
SGC32005	Unidentified transcript	118-123 cM	GCAATCGCCGACAGTTAAAT	TGAATGCTCAGTGAGGGTCC	+	+	+	+	+	+	+	+
Cda0e07	Unidentified transcript	118-123 cM	CTGTCTTGGTCTGTTAAACTG	TGATGGTAAITGGCTGTGGTG	+	+	+	+	+	+	+	+
A004B28	Unidentified transcript	123-124 cM	CCTAAATATGAATAGCACAC	GAAAGGAGGTTAATTTCTTATC	-	-	-	-	-	-	-	-
A007H41	Unidentified transcript	123-124 cM	CTCTCAGCTTCAGTTTTC	TCTTCCCAAAAATGACAG	-	-	-	-	-	-	-	-
SGC31888	Unidentified transcript	123-124 cM	GAGTTAAITGAGCAAGAATGATT	GCTGTTTGGAGGCCCTCAC	+	+	+	+	+	+	+	+
SGC30781	Unidentified transcript	123-124 cM	AAAGTTGTCGCACTGAATAAGAG	GACAAAGTGAACAAGATCAATTTTTT	-	-	-	-	-	-	-	-
WI-15059	Unidentified transcript	123-124 cM	CTCTCCTAGGGGGTGTGATGC	GCGAGCCCGTTAGTGTGATG	-	-	-	-	-	-	-	-

11 = cDNA derived from a lymphoblastoid cell line of patient 11 with a paternal deletion. 17 and 18 = cDNAs derived from lymphoblastoid cell lines of patients 17 and 18 with a maternal and a paternal deletion, respectively. C = control cDNA derived from a lymphoblastoid cell line of a person with a normal chromosome 18. A and AM = genomic DNA of normal subjects. NT = not tested.

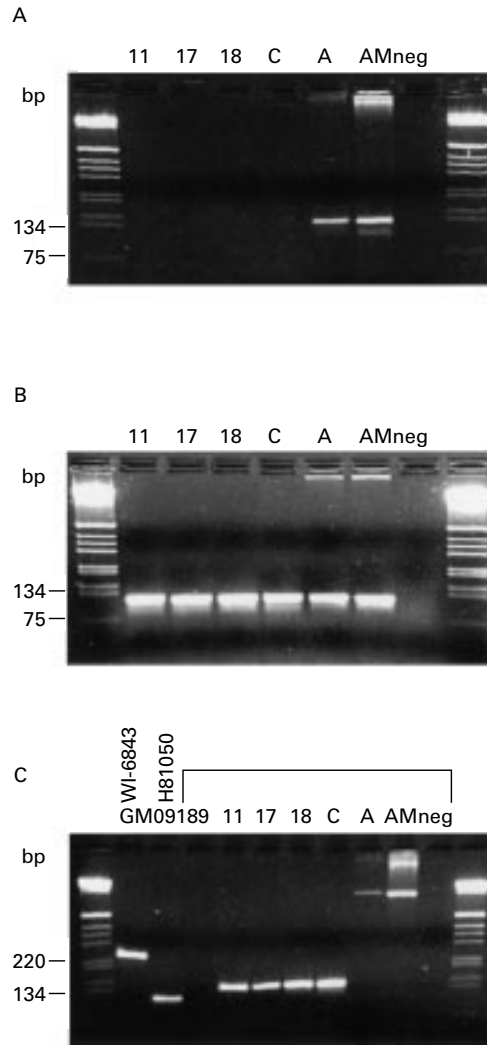


Figure 2 Imprinting analysis of 18q genes. PCR analysis of cDNA derived from patients with maternal 18q deletions (patient 17), paternal 18q deletions (patient 11 and 18), or normal control (C) is shown. Amplification using genomic DNA from two normal subjects (A and AM) as well as a sample lacking any DNA input (neg) are shown. Size markers are included in each gel. (A) EST A007H41. (B) EST WI-9340. (C) Amplification using a cDNA derived from a patient with Prader-Willi syndrome (GM09189) and a paternal 15q deletion is shown. Amplification with two 18q ESTs (WI-6843 and H81050) are shown as well as amplification using SNRPN primers, a known maternally imprinted gene.

representative result from EST A007H41. This showed that the genes from which these ESTs were derived were not expressed in lymphoblastoid cells. Appropriate amplification was observed when genomic DNA was used, showing that the lack of amplification was not the result of inappropriate PCR conditions.

The remaining 13 ESTs all produced an amplification product in the sample derived from a subject with a normal karyotype. For all 13 ESTs, amplification was observed in the samples that contained either maternally or paternally derived deletions. A representative result from EST WI-9340 is shown in fig 2B. This showed that imprinting did not occur in lymphoblastoid cells for the 13 genes that were tested.

To ensure that imprinting could be detected, PCR analysis was performed using primers derived from the SNRPN gene which is known to be maternally imprinted. As shown in fig 2C, no amplification was observed in GM09189, a cell line derived from a Prader-Willi syndrome patient, which contains a paternally deleted



chromosome 15. Since the remaining homologue is maternally imprinted, *SNRPN* is not expressed and therefore no amplification was observed. ESTs that mapped to 18q (WI-6843 and H81050) did amplify, showing that amplification could be obtained from the RNA sample.

In this study, 13 genes that mapped to the distal region of chromosome 18 were investigated to determine whether they might show an imprinting effect. All 13 genes were expressed from the single remaining allele, regardless of whether it was maternally or paternally derived. The basis for the experiments was that there is significant clinical variability in patients with 18q deletions. It is possible that imprinting may play a role in this variability.

A clear phenotypic distinction between subjects bearing a paternal and a maternal deletion was not previously possible.<sup>1</sup> In addition, translocation mouse models did not show any evidence for imprinting in distal MMU18,<sup>22-23</sup> which represents the syntenic region of 18q23. However, in the study by Strathdee *et al.*,<sup>1</sup> only very limited numbers of patients were available and the extent of the deletion differed in almost all of them. Also, imprinting is not always conserved between mouse and man<sup>24</sup> and there are at least three human genes that have been shown to be imprinted despite their location within a region excluded from imprinting by translocation studies in mice.<sup>23-25-27</sup> This is probably because of difficulties in detecting more subtle phenotypic alterations in animal models.

The lack of detection of an imprinting effect can be interpreted in several ways. First, imprinting of certain genes in 18q does exist, but we did not test these genes. We do not know whether any of the genes tested are involved in the phenotype of the 18q- syndrome, although the galanin receptor, the myelin basic protein, cytochrome 5b, and NF-ATc have been proposed to be candidates.<sup>19-28-29</sup> In our study, 13 of the 22 ESTs distal to 100 cM were informative compared to about 36 genes or so found in this region, according to the NCBI Unigene map. Thus, we cannot exclude other imprinted genes in 18q22-23, though in many cases clusters of genes are imprinted and might therefore be easier to detect when testing a set of genes that are distributed over a chromosomal region. Second, imprinting of genes in 18q may be restricted to certain tissues or developmental stages. Since only RNA from lymphoblastoid cell lines was tested, we may have missed imprinting that occurred in other tissues. While local and temporal restrictions of imprinting are well known,<sup>30-36</sup> EBV transformation of lymphocytes does not seem to modify imprinting mechanisms, as has been shown for *SNRPN*,<sup>20-37-38</sup> *PAR-1* and *PAR-5*,<sup>38</sup> and *IPW*.<sup>39</sup> Thus, the method described here should at least be able to detect ubiquitous imprinting of genes and all imprinting phenomena that affect lymphocytes. Finally, it is possible that there is simply no imprinting in the region investigated. If this holds true, we have to conclude that mechanisms such as modifying genes outside 18q and environmental factors influence the phenotypic picture of 18q- patients. In this case, we may have to consider that the parent of origin effects in bipolar disorder linked to 18q represent statistical artefacts, an assumption consistent with observations by Durner and Abreu<sup>40</sup> and McMahon *et al.*,<sup>41</sup> who did not observe a consistently paternal parent of origin effect.

Clinical heterogeneity is well known in chromosomal syndromes. The parental origin of the rearrangement has been reported to influence the phenotype in a number of cases. Among them are the paternal duplication of 11pter-p15.4 which results in Beckwith-Wiedemann syndrome (MIM 130650),<sup>42</sup> and monosomy 15q11-q13 which results in Prader-Willi syndrome (MIM 176270) if the paternal chromosome is affected and in the clinically different

Angelman syndrome (MIM 105830) if the maternal chromosome is affected.<sup>43</sup> More subtle influences of imprinted genes on the phenotype might be involved in other chromosomal rearrangements<sup>44</sup> like the deletion associated retinoblastoma, which displays slower tumour progression if the maternal allele is deleted,<sup>45</sup> suggesting that imprinted genes close to *RB* might influence tumour growth. Kato *et al.*<sup>25</sup> showed that the serotonin receptor 2 (*HTR2*) gene, which is located within the rearranged chromosomal region in 13q14, is paternally imprinted. The authors speculate that *HTR2* is a gene promoting the growth of retinoblastoma and that tumour progression depends on whether its active or inactive copy is retained. Other examples are Williams syndrome patients with a deletion of 7q11.23 who display significantly more severe growth retardation and microcephaly if the arrangement is maternally derived,<sup>46</sup> and Turner syndrome patients (45,X) who show significantly poorer verbal and higher order executive function skills when the retained X chromosome is of maternal origin.<sup>47-48</sup>

This study presents the initial analysis for identifying imprinted genes in 18q. Although this study did not identify any imprinted genes, the approach can easily be expanded to investigate additional genes. The ability to detect imprinted genes located on other chromosomes in lymphoblastoid cells shows that the approach used here is a viable one and should be continued as a method for investigating whether imprinting effects might be involved in the clinical variability of the 18q- syndrome.

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## Renal angiomyolipomata and learning difficulty in tuberous sclerosis complex

EDITOR—Tuberous sclerosis complex (TSC) is a dominantly inherited disease of high penetrance, characterised pathologically by the presence of hamartomata in multiple organ systems. Well known clinical manifestations include epilepsy, learning difficulties, behavioural problems, and skin lesions. Many patients have renal lesions, usually angiomyolipomata (AML), which can cause clinical problems secondary to haemorrhage or by compression and replacement of healthy renal tissue, which rarely causes end stage renal failure.<sup>1</sup> Cysts, polycystic renal disease, and renal carcinoma can also occur. Polycystic disease has an early onset clinically and is the result of large contiguous deletions on chromosome 16 affecting both the *TSC2* gene and the gene for adult onset polycystic kidney disease.<sup>2</sup> Tuberous sclerosis complex exhibits genetic heterogeneity.<sup>3</sup> Mutations in two recently identified genes, *TSC1* at 9q34 and *TSC2* at 16p13, each result in an apparently similar phenotype, although recent work has suggested that mutations in *TSC2* may be associated with more severe disease.<sup>4</sup> Both genes are tumour suppressor genes, the strongest evidence for this being the loss of heterozygosity around the normal gene at 9q34 or 16p13 in hamartomata from tuberous sclerosis patients.<sup>5,6</sup> There is evidence that the severity of learning difficulties in tuberous sclerosis complex is related to the number of hamartomata in the brain.<sup>7</sup> Until now, no one has reported on a correlation between the severity of the phenotype in two or more organs. We report on a correlation between renal hamartomata and learning difficulties in a population based sample of tuberous sclerosis complex patients (table 1).

As part of a larger prevalence study that began in 1985, patients identified with tuberous sclerosis complex and living in the Bath Health District have been followed longitudinally. All patients have undergone at least one abdominal ultrasound examination, performed by the authors, during the last two years. We investigated the association between angiomyolipomata and intellectual impairment because of an apparent association we had noticed in our clinical work with TSC patients (table 1). We made no attempt to explore any other associations. The presence of learning difficulty in this population was ascertained as previously described.<sup>8</sup> The correlation between renal angiomyolipomata and learning difficulty was analysed using a two sided Fisher's exact test (table 2). Of 22 patients known to be alive and living in the Bath Health District in August 1998, nine had learning difficulties and all had angiomyolipomata. Thirteen patients were of normal intellect and five of these had angiomyolipomata ( $p=0.006$ ).

This apparent association between renal angiomyolipomata in tuberous sclerosis complex and learning difficulties has not previously been noted. The association reaches statistical significance despite the small numbers

Table 1 Angiomyolipomata and learning difficulties in TSC patients

AML + / LD +	AML + / LD -	AML - / LD -	AML - / LD +
M 24 y	F 19 y	M 86 y	
M 29 y	F 62 y	M 45 y	
M 42 y	F 13 y	F 22 y	
F 38 y	F 16 y	F 59 y	
M 10 y	M 26 y	F 33 y	
M 11 y		M 6 y	
M 6 y		M 24 y	
M 9 y		F 46 y	
F 10 y			

AML = angiomyolipoma.  
LD = learning difficulty.  
M = male.  
F = female.  
y = years.

Table 2 Two sided Fisher's exact test

	Learning difficulties	
	+	-
Renal angiomyolipomata		
+	9	5
-	0	8

p=0.006

involved in the study and remains significant even when two patients of normal intellect and isolated renal cysts are transferred from the unaffected to affected groups (p=0.046); it is possible that renal cysts may form in TSC because renal tubules are blocked by small renal angiomyolipomata. We do not think there is an absolute correlation between learning difficulty and renal angiomyolipomata; we have patients outside the Bath district with learning difficulties and no renal pathology.

The age range in our population is 6-86 years (median 24 years). Using Wilcoxon rank sum tests we found no significant association between age and learning difficulty (p=0.09) or between age and the presence of AMLs (p=0.09) in this population. Similarly there is no evidence of a significant relationship between gender and either learning difficulty (p=0.1) or AML presence (p=1.0) when the relationships are independently investigated using Fisher's exact tests. There is no reason to suppose, therefore, that the association described between intellectual impairment and renal angiomyolipomata is confounded significantly by either gender or age in this sample.

One explanation for the observed correlation would be that certain patients with tuberous sclerosis complex have an increased propensity to the formation of hamartomata resulting both in more cerebral tubers (and therefore a higher risk of learning difficulties) and in a greater likelihood of renal angiomyolipoma formation. Previously,

patients with tuberous sclerosis complex and learning difficulty appear to have had a reduced life expectancy; epidemiological surveys have consistently shown lower than expected numbers of elderly tuberous sclerosis patients with learning difficulties.<sup>8</sup> We believe this is because of an increased death rate among this group from epilepsy, brain tumours, and intercurrent illness. However, with changing attitudes to the management of patients with learning difficulties, improved management of epilepsy, and more vigilant surveillance, more of these patients survive into adulthood. One implication of our finding is that we will see an increase in complications from renal hamartomata as more tuberous sclerosis patients with intellectual difficulties survive for longer.

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## Clinical geneticists' attitudes and practice towards testing for breast cancer susceptibility genes

EDITOR—Cancer genetics, and in particular breast cancer genetics, is the fastest expanding discipline within clinical genetics. Cancer referrals now constitute a third of all referrals to most clinical genetics centres. Currently there are no national guidelines on predictive testing for *BRCA1* and *BRCA2*. Several members of the same family may be seen in different centres and offered different clinical management. Such differences may in part be attributable to differences in funding of genetic services and testing at the service or research level, but it is clear that this area also involves various ethical dilemmas that may well be viewed differently by different practitioners. In order to investigate the nature and degree of variation that exists in practice and attitudes among clinical geneticists, we have undertaken a survey of all clinical geneticists in the United Kingdom who deal with cancer genetics.

Four clinical case scenarios were devised from the authors' own clinical experience to assess attitudes and practice towards breast cancer gene testing. Questionnaires were sent to 57 geneticists in the United Kingdom, representing all specialist registrar and consultants involved in

cancer genetics. Each was asked to respond to questions relating to each scenario and to state the reasons for their decisions. Forty seven completed questionnaires were received (83% compliance). All clinical genetics centres in the UK were represented by at least one response. In three instances a joint response involving more than one geneticist from a centre was returned. The four clinical cases are given below. For each case the salient points raised by selected respondents for arriving at their decision are given.

Case 1. A woman has been shown to carry a pathogenic mutation in the *BRCA1* gene. She is 9 weeks pregnant and requests a prenatal test to see whether the fetus also carries this mutation. Participants were asked whether they would be prepared to offer prenatal testing after appropriate counselling.

Twenty four (51%) respondents stated that they would be prepared to offer prenatal testing to the woman after counselling. Fifteen (32%) said they would not and 17% did not know what their action would be. Most of the respondents who would offer such testing indicated that if the counsellors had given all the relevant information, it was up to the woman to make a decision. Most commented that the woman's experience of cancer in the family was likely to be a strong motivating factor in the decision to request prenatal diagnosis and that counsellors were not in a position to deny this experience. Many also commented that a pregnancy could be terminated anyway for "social"

reasons and that refusal to offer predictive testing would therefore not necessarily prevent a termination. The following quotations illustrate some of the opinions given.

"I would find the idea difficult but after counselling and with full information it is the couple's decision. Any other stance in a country accepting social termination is difficult." "If we are not prepared to offer prenatal diagnosis we cannot engage in counselling." "In some families the cancer burden is enormous. The child may not only have to grow up without a mother but also with the possibility of developing the disease herself." "It is within the patient's rights." "Prenatal tests are offered for other potentially treatable conditions of late onset."

Most of the respondents who did not know what their action would be raised the issue that it depended on why she wished to have prenatal testing. They felt that testing might be appropriate if the woman would definitely proceed to termination should the mutation be detected in the fetus. If there was doubt about this, however, or the test was done for information only, then respondents would be reluctant to offer testing, since continuation of the pregnancy would be interfering with the autonomy of the child and future adult. In effect, such testing would equate to doing a predictive test on a child who might not want to know its own genetic status.

"I would attempt to dissuade her but if she was still insistent then I would feel obliged to offer a termination. There are many other disorders where I would not be entirely happy with offering a prenatal diagnosis/termination but if after careful counselling the patient still wants it then I would. After all a woman can have a termination for essentially social reasons anyway."

Those who felt it would be inappropriate to offer prenatal *BRCA* testing focused on the uncertainties that still lie with such information. These are summarised by the following quotations.

"Not a lethal disorder with no treatment. If testing and no termination takes place the child could face insurance problems during life." "Even if positive, penetrance is only 80-90% maximum. There are screening options and treatment options." "The reasons for not doing the test are: (1) incomplete penetrance and uncertainties of penetrance in some cases, and (2) the availability of measures for early cancer detection and prophylaxis which although experimental will almost certainly improve over the next 30 years." "Future child should have autonomy." "Adult onset disease treatable if detected early. Prophylactic surgery a possibility." "Effects of mutations uncertain and too far in the future."

Some responses were more directive: "Would try to talk her out of it."

Many respondents also felt that legally they could not offer a termination if the result showed that the fetus carried the pathogenic mutation as summarised in the following quotation.

"The 1967 Abortion Act does not apply here, therefore we have to rely on case law to do a termination for fetal abnormality. I would be concerned about the legal position here as I don't think the fetus would necessarily be classed as severely handicapped."

*Case 2.* The mother of a 15 year old girl had undergone *BRCA1* testing because of a very strong family history of breast and ovarian cancer and a pathogenic *BRCA1* mutation had been identified. The 15 year old who was quite mature for her age and well informed about her risks was very keen to have a predictive test so that she could "plan her life accordingly". Participants were asked whether they would offer this 15 year old girl a predictive test.

Thirty four percent (16/47) of the respondents were prepared to offer predictive testing. However, 23 (49%)

were not prepared to do so. The remaining seven (15%) did not know what action they would take. Those who were prepared to offer predictive testing gave the following reasons.

"At the age of 15 a young person can be considered 'Gillick' competent and able to give consent to treatment or investigation. If the girl has thought it through I would be prepared to do the test." "I believe there is benefit for her knowing at the age of 15, potential reassurance and potential lifestyle changes, usage of the contraceptive pill and planning etc if she is a gene carrier." "After exploring her reasons for doing it now."

For those who would not be prepared to offer predictive testing the reasons given included the following.

"Risk is not imminent. She is likely to have 10-20 years before it does become imminent and circumstances may have changed dramatically by then. It is hard to see how any immediate decisions, for example, life plans, would be influenced by knowledge of carrier status at 15." "Thin end of wedge. Why not a mature 12 year old?" "I would advise more time for reflection. It could well affect her self image if she has a positive result and relationships with mother, and she is at a critical stage in her education which could be disrupted by a positive gene test." "I would encourage her to wait. When life has been planned, that is, with regard to jobs, mortgages, life insurance etc." "Her risk is negligible at this age." "Would try very hard to dissuade her, explaining it wouldn't make any difference to her management." "There is little information about the effects of predictive testing in young people and the psychological sequelae." "If she thinks this information will help plan her life she needs more explanation."

*Case 3.* A woman who does not fit the local criteria for genetic testing for *BRCA1* or *BRCA2* is insistent that she wants testing even though the limitations of the test have been explained. She asks if the test can be done privately? Respondents were asked whether they would be prepared to give her the name of a commercial company providing testing.

Seventy percent (32/47) questioned were prepared to give her this information. Most respondents felt that they could not deny knowledge on principle and it was her decision to spend her own money. Furthermore, the information is relatively easily accessible and withholding such information could heighten any anxiety. Some of the particular comments made were as follows.

"If the company find the mutation other family members have their risk altered/increased and then would probably be referred to the genetics service." "This is already done for paternity testing. I see it as an obligation to tell the client of any other resources."

Twenty five percent (12/47) stated they would not be prepared to give the name of a commercial company to the woman. Some felt that providing this information might be seen as an endorsement of the company and that a commercial company does not provide any form of counselling.

"I would be concerned about equity of access to the service and queue jumping by using private labs if people could afford it." "I am prejudiced against a commercial company that does not provide general counselling support for such testing. I would point out the disadvantages of a commercial company for whose quality control operating standards and lack of counselling support I could not comment." "We are not agents for private companies." "If she does not fit the local criteria, the chances of finding a mutation are low. Her anxiety will not be resolved by an inappropriate and almost certainly uninformative test."

Only two respondents (4%) gave don't know as a response and no comments were received in respect of this action.

*Case 4.* It was stated that a 27 year old woman attended the clinic because her identical twin sister had just developed carcinoma of the breast. Their mother also had breast cancer at the age of 45. She died at the age of 50. There was no blood or tissue available from the mother. Respondents were asked what risk would they give the consultand of developing breast cancer. They were also asked whether they would be prepared to offer any genetic testing and if so which test or tests they would offer and why?

The majority (31/47, 66%) stated that the woman's lifetime breast cancer risk was between 70 and 80%, which from published evidence seems to be the most accurate figure.<sup>1</sup> However, some estimates were as low as 30% and others as high as 90%. Respondents were not asked how they arrived at these figures. Most of the respondents (34/47, 72%) would offer genetic testing. Their management of the case in respect of gene testing showed differences.

"Can only offer diagnostic testing, not predictive testing." "Would do diagnostic test in sister and then predictive test in patient, because there is a chance they are not actually identical." "Confirm they are identical and then treat as a diagnostic test." "I would counsel the two sisters in parallel but genetically the risks are identical, the only difference is the penetrance issue of this gene in identical sibs." "We could not give any good news, we could only give bad news."

Only two of the respondents commented on the difficulty of interpreting a negative test.

We carried out this survey to assess variability among cancer geneticists within the United Kingdom in attitudes and practice towards breast cancer gene testing. The results of our survey clearly show that differences do exist. The differences seen in response to the clinical scenarios were, perhaps not surprisingly, most pronounced in relation to situations having the greatest ethical component.

Prenatal testing is offered for a number of genetic disorders. With the notable exception of disorders such as Huntington's disease, for which there is no disease modifying treatment, interest in prenatal testing has centred on severe diseases presenting early in life. Attitudes to prenatal testing for adult disease for which some form of treatment exists varied considerably. The issue of testing for breast cancer susceptibility genes is further complicated by the fact that such genes are not fully penetrant and that sporadic disease is common in the population.

One of the central themes of genetic counselling laid down in Peter Harper's seminal text is that counselling should be non-directive.<sup>2,3</sup> The aim of genetic counselling is to ensure that people have the necessary facts to enable them to arrive at their own decisions. If one adopts this as the central tenet of the genetic counselling services, it is the person's decision as to whether to have prenatal testing. It may be naive to believe that all genetic counselling is truly non-directive; genetic issues are complex and counsellors' personal opinions may be apparent or inferred from the manner in which the explanation is made. We found it interesting that a large percentage of those surveyed would be reluctant to engage in prenatal counselling for breast cancer susceptibility genes and furthermore that a proportion of clinicians would actively dissuade a woman from pursuing prenatal testing if she was a *BRCA1* mutation carrier. This is clear evidence against non-directive counselling.

Informed consent is clearly a central part of any predictive testing programme. The age at which a person can give consent for any medical procedure has been the subject of considerable debate in recent years. In case 2 we presented the hypothetical situation of a girl of 15 requesting a predictive test for *BRCA1*. Clearly a woman's risk of breast cancer before the age of 25 is small, and therefore it can be deemed that there is no immediate urgency for testing from the perspective of risk. Many of the respondents made this point, some giving it as a reason for not agreeing to offering a predictive test. However, many other reasons contribute to a person's decision to undertake genetic testing and ultimately the issue centres on at what age a person is able to make this type of decision. Some respondents felt that such testing would be illegal at this age; however, both case law (Gillick *v* West Norfolk and Wisbech AHA, 1986) and statute law (the Children's Act, 1989) allow children under the age of 18 to make independent decisions about themselves if they are deemed "sufficiently mature". One respondent pointed to anecdotal evidence that presymptomatic testing experience in Huntington's disease suggests that all those under the age of 25 who were found to be carriers had major psychological problems subsequently. The only published evidence seems to point to a greater difficulty in coping with the disease when it is first learnt of during adolescence rather than adulthood.<sup>4</sup> While deferring testing may ensure that the test is not done in haste, any directive counselling in terms of trying to dissuade her clearly runs counter to the Harper ideal of counselling.

Our next scenario concerned private laboratories. Unlike the United States, Britain has little in the way of private genetic services and both counselling and genetic laboratories are largely confined to the National Health Service. It is conceivable that things may change and there may be an increase in private genetic services. Any growth in private genetics is likely to be confined to laboratory tests and a concern here is that it may be unaccompanied by any form of counselling. Furthermore, the motivation by such enterprises is financial remuneration and hence many of those offered tests may be at a low probability of being gene carriers and at a risk not significantly different from that of the general population. It is therefore unlikely that an expansion of the activities of private laboratories will be greeted enthusiastically by clinical geneticists. Whether one should provide the address of such a laboratory if requested to do so is perhaps a different point. The responses to this question indicated a number of views relating to this issue. Some felt that giving such information could be construed as an endorsement of a private company. Alternatively, a failure to convey the address could be seen as a paternalistic attitude and erosion of free choice.

In a recent article Rosser *et al*<sup>5</sup> highlighted that there are differences in the estimation of risk made by geneticists for identical family histories of breast cancer. We also found evidence of this from the responses to case 4. While most risks were given as between 70 and 80%, estimates ranged from 30-90%. Case 4 concerned gene testing in a family with an identical twin. This case was chosen to illustrate the fact that the distinction between diagnostic and predictive testing becomes rather blurred in the context of one affected and one unaffected identical twin. Several respondents (13%, 6/47) did not comment on this and said they would proceed with predictive testing once a mutation had been detected in the identical twin. Clearly, if the twins are really identical, then only diagnostic testing is possible even in the unaffected twin. It is perhaps not surprising that there were considerable differences in participants' responses.

The field of cancer genetics is rapidly evolving and clinical practice is developing to meet the challenge of this changing field. It is perhaps not surprising that differences in clinical practices exist; however, there will be pressure for these to coalesce to similar policy. We hope that this article will draw to attention to some areas in breast cancer genetics where there are considerable differences in opinion and serve as a discussion for helping to devising guidelines for those in the field.

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