Letters to the Editor

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Ulcerative colitis is not associated with differences in *MUC2* mucin allele length

EDITOR—The aetiology of inflammatory bowel disease (IBD) is complex and shows clear evidence of familial clustering,[1–3] and genetic linkage studies suggest a number of susceptibility genes.[4–12] Changes in mucin expression are a feature of IBD.[13–17] In ulcerative colitis there is a depletion of mucus, while the reverse is true for Crohn's disease.[18] Since the gene that encodes a major component of gel forming mucin of the large intestine, *MUC2,* located on 11p15.5, shows a high level of genetically determined polymorphism in length,[19–21] it has been considered as a potential risk factor in IBD.[22] We have previously shown that the allele lengths range in size between approximately 45 and 200 repeats as judged by cutting genomic DNA samples with the restriction enzyme *Hin*fI.[19] This corresponds to apomucin sizes ranging from M_r 400-760 000. It is our hypothesis that these differences have a functional significance by changing the properties of the mucins in terms of the amount of apomucin backbone available for glycosylation or altering the spacing between the cysteine rich domains which are involved in cross linking or both. Here we report the work of two groups which was aimed at testing the hypothesis that short alleles predispose to ulcerative colitis.

DNA was prepared from peripheral blood and digested with *Hin*fI. Agarose electrophoresis was used to separate the *Hin*fI fragments which were then transferred by passive blotting onto nylon membrane, and the membranes probed using the tandem repeat cDNA Smuc41 as probe.[23] Allele lengths were determined by comparison with molecular weight standards and samples containing alleles of known length. All the procedures were standard though the details differed in the two laboratories. Optimised conditions are described in Vinall *et al*[24] and a typical gel is shown in fig 1.

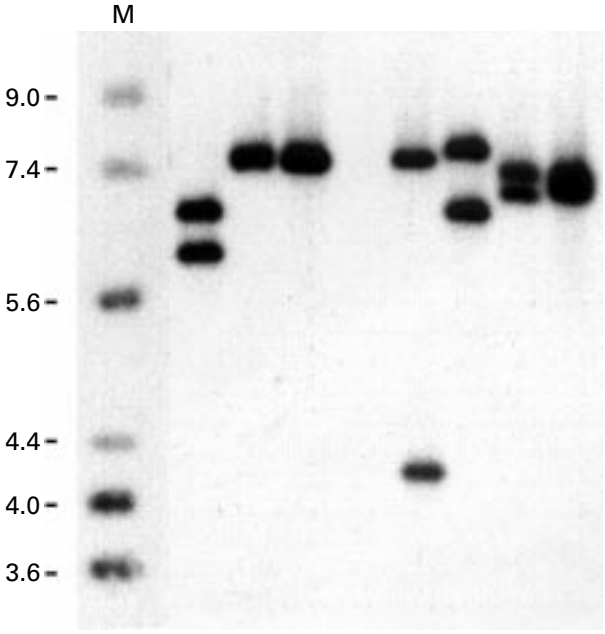

Figure 1 Photograph showing the MUC2 VNTR polymorphism detected using HinfI digested DNA in seven representative samples from the survey. The molecular weight markers (M) shown are the Raoul markers from Appligene.

We examined a total of 125 genetically unrelated patients with inflammatory bowel disease (68 UK, 57 USA). As controls we tested a similar number of spouses and healthy volunteers. All subjects were of European origin although the American survey contained people from both southern and northern Europe and a large number of Jewish people. The data from the two laboratories were kept separate and the results are shown in fig 2. As can be seen, there is a very similar distribution of allele lengths in both studies. The slight right shift in the American population as compared with the UK population probably reflects subtle differences

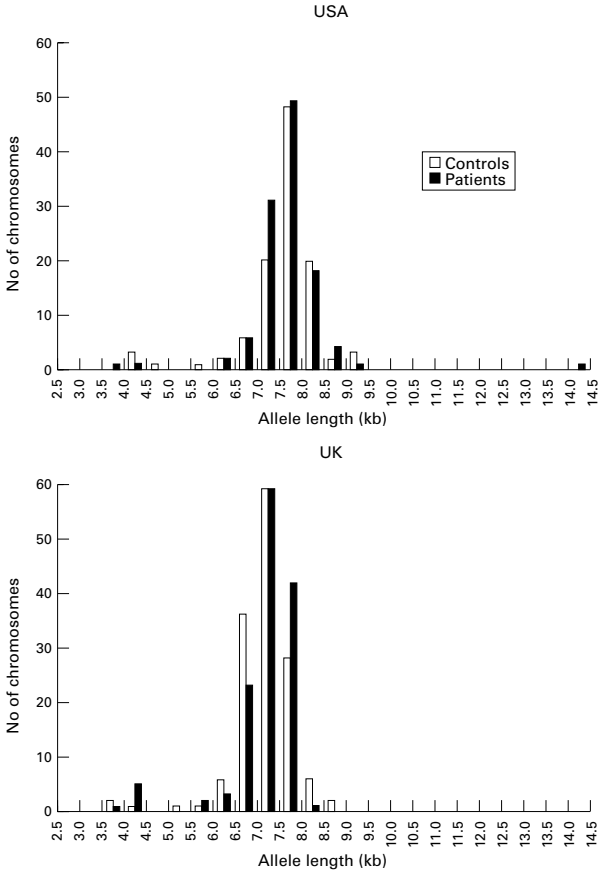

Figure 2 Histogram showing the distribution of allele length in patients with ulcerative colitis and healthy controls. Sizes of the HinfI fragments were determined by comparison of their mobility with that of controls of known size. Subjects showing a single band were assumed to be homozygous for two alleles of the same size. The distributions were similar when Jewish people were excluded. All of the UK samples were taken from people of northern European extraction. The patients comprised 30 females and 38 males and their mean ages were 53 and 43 respectively and included six who were Jewish. Forty eight of the UK cohort had extensive colitis, 12 left sided colitis, and eight distal colitis. The UK controls comprised 33 females and 38 males, mean ages 37 and 44 (one Jewish). The USA samples were all from people of European extraction. The patients comprised 31 females and 26 males, mean ages 40 and 39 respectively (25 Jewish). Fifty six of the USA cohort were classified as suffering from ulcerative colitis and one from proctitis. The USA controls comprised 24 females and 29 males with mean ages of 42 and 41 (13 Jewish). All subjects were unrelated to each other and 28% of the UK cohort and 40% of the USA cohort had a known family history of inflammatory bowel disease.

in the electrophoretic conditions used in each study. In both studies the short *MUC2* alleles are very rare in patients as well as controls (6/136 UK patients and 3/142 UK controls, 2/114 USA patients and 4/106 USA controls), and there is no significant difference in the numbers of "small" alleles (smaller than 5.0 kb) and "large" alleles (greater than 5.0 kb) between patients and controls (Fisher's exact test, UK data p=0.29, USA p=0.31). We also analysed the samples separately after exclusion of the Jewish subjects because of the suggestion of different genetic risk in Jewish and non-Jewish groups,[25 26] but there was no difference in distribution (not shown). One subject in the USA group had one unusually large allele. The significance of this rare allele is not known though an allele approaching this in size (12 kb) is found in one of the CEPH families. Careful scrutiny of the large allele distribution shows slight but statistically non-significant difference in distribution in the patients and controls (Mann-Whitney U test, UK data set p=0.11, USA data set p=0.36) which is in the opposite direction in both groups.

This negative evidence seems to rule out the idea that short *MUC2* allele length predisposes to ulcerative colitis, but does not exclude the possibility that other variations in the *MUC2* gene, such as "within repeat" sequence differences, or the final fully glycosylated *MUC2* mucin may play a role. Furthermore, in view of the recent results of Cho *et al*[12] it may be worth studying *MUC2* in Crohn's disease since some evidence has been obtained for linkage of Crohn's disease rather than ulcerative colitis to 11p.

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DALLAS M SWALLOW
LYNNE E VINALL
MRC Human Biochemical Genetics Unit, UCL Galton Laboratory, Wolfson House, 4 Stephenson Way, London, NW1 2HE, UK

JAMES R GUM
YOUNG S KIM
Department of Medicine, University of California Service, Gastrointestinal Research Laboratory (151M2), VA Medical Center, San Francisco California 94121, USA.

HUIYING YANG
JEROME I ROTTER
Division of Medical Genetics, Departments of Pediatrics and Medicine, Steven Spielberg Pediatric Research Center, Cedars-Sinai Research Institute and UCLA School of Medicine, Los Angeles, California, USA

MUDDASSAR MIRZA
Division of Medical & Molecular Genetics, GKT School of Medicine, 7th Floor, Guy's Tower, Guy's Hospital, London SE1 9RT, UK

JOHN C W LEE
JOHN E LENNARD-JONES
St Mark's Hospital, Harrow, Middlesex HA1 3UT, UK

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Mutation analysis of the *DKC1* gene in incontinentia pigmenti

EDITOR—There are a number of monogenic diseases with complex phenotypes which are clinically distinct but also overlap in phenotype with one or more other syndromes. If mutations in the same gene are responsible for causing the related syndromes, the diseases are allelic. Two diseases linked to Xq28, incontinentia pigmenti (IP, MIM 308310, Bloch-Sulzberger syndrome) and dyskeratosis congenita (DKC, MIM 305000, Zinsser-Cole-Engmann syndrome) show similarities in phenotype, although the modes of expression differ. Whereas IP is X linked dominant with embryonic lethality in males, the major form of DKC is X linked recessive. The gene responsible for causing DKC, *DKC1*, was recently identified[1] and maps about 20 kb proximal to the factor VIII gene, *F8C*.[2] Linkage analyses have provided evidence that the IP gene is located in the telomeric 2 Mb region of Xq28 distal to DXS52[3] and lod scores of highest significance were found around *F8C*.[4 5] The physical map position of *DKC1* and genetic linkage of the IP locus, together with the overlap in the DKC and IP phenotypes (table 1), raised the possibility that these two diseases could be allelic.

Table 1 Comparison of the IP and DKC phenotypes affecting ectodermal tissues and the haemopoietic system

	Incontinentia pigmenti (IP)	*Dyskeratosis congenita (DKC)*
Skin		
Reticulate hyperpigmentation	+ (late stage)	+ (early stage)
Hypopigmentation	+ (reticulate or linear)	+ (scattered macules)
Alopecia	+ (scarring)	+ (non-scarring)
Epidermal atrophy	+ (late stage)	+
Pigment incontinence	+	+
Teeth		
Hypodontia	+ (prominent)	+ (occasional)
Eyes		
Retinal involvement	+ (retinal detachment, vascular proliferation)	−
Epiphora	+ (rare, one case)	+ (frequent)
Haemopoietic system		
Pancytopenia	−	+
Bone marrow failure	−	+

The IP and DKC phenotypes share abnormalities in ectodermal derivatives, such as nail dystrophy, alopecia, hypodontia, and skin manifestations[6 7] (table 1). Both IP and DKC are characterised by the early appearance of reticulate skin pigmentation, although this manifests differently in the two diseases. In IP the clinical signs affecting the skin are initially apparent as an erythematous, inflammatory vesicular rash. The rash later becomes verrucous and streaks of hyperpigmentation follow. The pigmentation then fades in the second decade of life often leaving scarred and atrophic hypopigmented areas. In DKC patients the inflammatory and verrucous stages do not occur and the appearance of hyper- and hypopigmentation is progressive. The overlap in the skin abnormalities is confirmed by microscopic examination of skin biopsies from IP and DKC patients, which show common histological features such as epidermal atrophy and pigment migration.[8] In both disorders a defect in the immune system may be causing the skin manifestations. In IP the inflammatory vesicular rash points to an involvement of the immune system and is supported by observations that the rashes are associated with constitutional eosinophilia and may recur during feverish infections. Further, it has been suggested that the skin phenotype in IP resembles that observed in patients with graft versus host (GVH) disease.[9] A GVH-like pathogenesis suggestive of an involvement of the immune system in the skin also occurs in some DKC cases.[10 11]

DKC patients develop progressive pancytopenia of one or more cell lines and bone marrow failure is the main cause of death in the first or second decade of life in 90% of the cases.[12] This is accompanied by humoral and cellular disturbances of the immune system.[13] Pancytopenia and bone marrow failure are not associated with IP. There have been reports, however, of decreases in lymphocyte number and both neutrophil and lymphocyte dysfunction in IP.[14–16] Another abnormality of the peripheral blood system suggesting an involvement of the immune system is the occurrence of leucocytosis with eosinophilia in a substantial proportion of newborn females with IP in the absence of infection. A report on a male IP patient who died postnatally and showed excessive haemorrhaging and haemolysis at birth further indicates a defect in the haematological system.[17]

Extreme skewing of X chromosome inactivation has been observed in the blood cells of most DKC carrier females[18 19] as well as in the skin and haemopoietic cells of affected IP females.[20 21] The non-random inactivation of the X chromosome carrying the mutant allele in the skin cells of IP females is responsible for the disappearance of the clinical signs because of a positive selection for cells expressing the normal allele.[20 21] It is conceivable that a defect in the haemopoietic system leading to bone marrow failure as is observed in DKC males is not apparent in IP females because of a similar selective pressure favouring cells carrying the active normal X chromosome. The skewed X chromosome inactivation in IP females could in part explain the difference in female presentation and the more severe phenotype observed in hemizyous IP males.

The hypothesis that a different spectrum of mutations in the *DKC1* gene causes IP is compatible with the ubiquitous expression pattern of *DKC1*, its high degree of conservation, and the putative function of the peptide dyskerin in rRNA biogenesis.[1 22–24] Seventeen different mutations have been identified in DKC patients of which 82% are missense mutations.[25] To date no premature stop codon mutations, frameshifts, or whole gene deletions have been identified. Taken together, these observations strongly suggest an essential function for dyskerin and that complete loss of function mutations would not be viable. It appears likely that a null mutation in *DKC1* could explain the prenatal lethality observed in IP males and that the same mutation in an IP female might result in the clinical signs observed.

The genomic structure of the *DKC1* gene has been determined.[25] The coding sequence is split into 15 exons and the gene extends over 15 kb (accession numbers AJ0101395, AJ0101396). As intronic primers flanking each of the 15 exons had been designed for mutation screening of DKC patients, it was possible to screen the *DKC1* gene efficiently for mutations in IP patients. The analysis of a large number of IP patients of different nationalities was possible because of the collaborative efforts of five research groups. Thirteen of these families have been described previously.[4 5 21 26] All 15 exons of 23 female IP patients and one spontaneously aborted male fetus carrying the mutant allele[5] were subjected to SSCP analyses. SSCP protocols that had previously been shown to be efficient for mutation detection were used and the conditions for each exon were determined to allow good resolution of the two single strands.[25 27] No shifts were observed for any of the patients. To exclude point mutations which may have been missed by SSCP, all exons from two spontaneously aborted male patients were PCR amplified and sequenced, but no mutations were found. Furthermore, 18 of the 24 DNA samples analysed by SSCP plus 32 additional IP females and three additional IP males were analysed by Southern hybridisation using the full length *DKC1* cDNA as a probe. The following restriction enzyme digests were analysed: *Xba*I, *Bam*HI, *Eco*RI, *Pst*I, *Hin*dIII, *Sac*I, *Nco*I, *Bgl*II, and *Taq*I. No differences in dosage and no aberrant bands were detected when compared with DNA samples from normal males and females. The results from Southern hybridisations and the fact that all exons were amplifiable for two IP male patients indicate that a partial or whole gene deletion of *DKC1* as a general mechanism for causing IP is unlikely. Moreover, no mutations were identified in the coding region or at the exon-intron boundaries of the two IP male patients. Owing to the difficulty of obtaining sufficient cells with an active IP mutation bearing X chromosome from female patients and because very few IP male patients with a normal XY karyotype exist, no analyses were carried out at the RNA level. It therefore cannot entirely be ruled out that there may be mutations in the promoter region or in the 5' and 3' untranslated regions (UTR) which could alter the levels of *DKC1* mRNA directly or alter the stability of the transcript in IP patients. However, we consider this to be a very unlikely possibility and conclude that IP and DKC are not allelic.

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NINA S HEISS
ANNEMARIE POUSTKA
Department of Molecular Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

STUART W KNIGHT
Department of Haematology, Imperial College School of Medicine, London, UK

SWAROOP ARADHYA
DAVID L NELSON
Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, Texas, USA

RICHARD A LEWIS
Departments of Ophthalmology, Medicine, Pediatrics, and Molecular and Human Genetics, Texas, Houston, USA

TERESA ESPOSITO
ALFREDO CICCODICOLA
MICHELE D'URSO
International Institute of Genetics and Biophysics (IIGB), Via G Marconi 10, 80125 Naples, Italy

ASMAE SMAHI
SOLANGE HEUERTZ
ARNOLD MUNNICH
Hopital des Enfants-Malades, Unité des Recherches sur les Handicaps Génétiques de l'Enfant, Paris, France

PIERRE VABRES
Service de Dermatologie, Centre Hospitalier Universitaire, Paris, France

HAYLEY WOFFENDIN
SUSAN KENWRICK
University of Cambridge, Department of Medicine, Cambridge Institute for Medical Research, Cambridge, UK

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A nonsense mutation in the retinal specific guanylate cyclase gene is the cause of Leber congenital amaurosis in a large inbred kindred from Jordan

EDITOR—Leber congenital amaurosis (LCA) (MIM 204000) has the earliest onset and is the most severe form of retinal dystrophy.[1–3] It is an autosomal recessive condition that is recognised within the first few months of life because of impaired vision and an extinguished electroretinogram.[4] Nystagmus, specifically pendular, and eye poking are frequently observed early on,[5] while hypermetropia and keratoconus may develop later during the course of the disease.[6 7] Genetic heterogeneity was confirmed when the first gene of LCA was mapped to chromosome 17p13.1 (*LCA1*) by homozygosity mapping in consanguineous Arab families.[8 9] Four different mutations in the retinal specific guanylate cyclase gene (*RETGC*) were found in four unrelated probands and thus *LCA1* was assumed to result from homozygous alterations in this gene.[10]

We report here a nonsense mutation in the *RETGC* gene, which in the homozygous state is responsible for LCA in a large inbred tribe from Jordan. We had already identified a large, highly inbred family from the Jordan valley consisting of about 2000 living subjects, in which affected members have LCA.[11] A 31 member subset of this family was investigated (fig 1). All members were examined by an ophthalmologist and a paediatrician. Four patients had ERG performed (Nos 3, 9, 13, 14). Blood samples were collected from 28 family members after obtaining informed consent from them or their legal guardian.

DNA was extracted from peripheral blood samples by standard procedures.[12] Seventeen different dinucleotide repeat markers reported to be linked to *LCA1* on chromosome 17 were used to test for linkage.[8 9] Amplification of these markers was performed according to the manufacturer's conditions (Research Genetics). Products

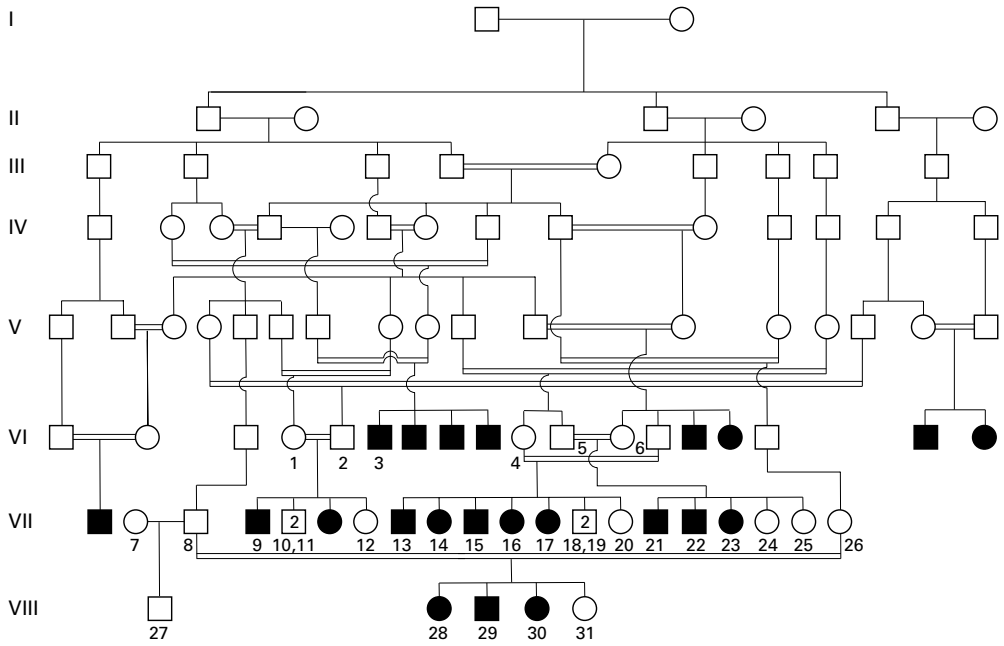

Figure 1 Extended partial pedigree of the clan. Note the extensive number of inbreeding loops in every generation. The 31 participating family members are marked in Arabic numerals.

were analysed on 6% denaturing polyacrylamide gels (7.7 mol/l urea). The polyacrylamide gels were silver stained using the protocol of Bassam *et al*.[13] Haplotype analysis was performed and the obligatory cross over events were noted. Since the family was highly inbred, identity by descent was enough to establish linkage.

The 20 exons of the *RETGC* gene were amplified using intronic primers flanking exon sequences using the previously reported conditions.[10] When necessary, the fragments were digested by one or more restriction endonucleases to yield fragments suitable for SSCP analysis. Amplified fragments were run on a fan cooled MDE gel for SSCP analysis at 6 W for 14 hours and then silver stained.

DNA from regions of the *RETGC* gene that showed unusual mobility of one allele in carriers and of the two alleles in affected subjects was sequenced using an automated ABI sequencer with dye terminator chemistry.

The reported extended family is a 2000 member tribe inhabiting a village in the Jordan valley, mostly depending on agricultural resources. The successive consanguineous marriages led to an extreme example of inbreeding and is reflected in the high prevalence of blindness which turned out to be LCA. The inbreeding coefficient in this tribe ranged from 0.037 to 0.09374 with an average of 0.0687.

The subset of the family included 13 affected subjects, their ages ranging from 10 to 45 years at the time of examination. All patients had poor vision noted at birth or shortly afterwards, as well as wandering eyes or pendular nystagmus. The visual acuity ranged from no light perception to 6/60. The majority had attenuated retinal blood vessels on fundus examination, some had pale optic discs, and about two thirds had keratoconus. Two patients had congenital cataracts (Nos 28 and 29) and one patient had bilateral microphthalmia and iris atrophy (No 16). One patient had bilateral macular abnormality similar to target macular lesions (No 17). Extinguished ERG was present in the four patients who underwent the test. The details of the clinical picture in the 13 affected subjects are summarised in table 1.

By analysing the haplotypes, it is quite obvious that the LCA in this family is linked to the *LCA1* locus previously described. All affected members were identical by descent for the disease haplotype (fig 2).

The SSCP assay of exon 13 showed a homozygous pattern in affected subjects and a heterozygous pattern in obligate carriers. This pattern was not present in 10 unrelated controls. The DNA sequencing in all affected members showed a homozygous Gln to stop mutation in exon 13 at nucleotide position 2646 (cDNA) (CAG→TAG) (fig 3). Obligate carriers were heterozygous for this nonsense mutation.

Despite being homozygous for the same mutation, affected family members showed clinical heterogeneity for

Table 1 The main clinical manifestations of Leber congenital amaurosis in the 13 affected subjects

ID	Age/sex	Visual acuity		Keratoconus	Retinal vessels	Optic discs	Remarks
		Right	Left				
3	45 y/M	No LP	No LP	Yes	Attenuated	Pale	Corneal hydrops
9	22 y/M	HM	HM	Yes	Attenuated	Pale	
13	14 y/M	No LP	No LP	Yes	Attenuated	Normal	Corneal hydrops
14	15 y/F	HM	HM	Yes	Attenuated	Normal	
15	19 y/M	6/60	5/60	Yes	Attenuated	Normal	
16	20 y/F	LP	LP		Microphthalmia	Not seen	Iris atrophy
17	23 y/F	HM	HM	Yes	Attenuated	Pale	Macular lesions
21	15 y/M	HM	HM	No	Attenuated	Normal	
22	13 y/M	HM	HM	Yes	Attenuated	Normal	
23	10 y/F	CF	CF	No	Attenuated	Normal	
28	11 y/F	No LP	LP	No	Not seen	Not seen	Cataracts
29	13 y/M	LP	LP	Yes	Not seen	Not seen	Cataracts
30	16 y/F	LP	LP	No	Attenuated	Normal	

M: male; F: female; LP: light perception; HM: hand movement; CF: counting fingers.

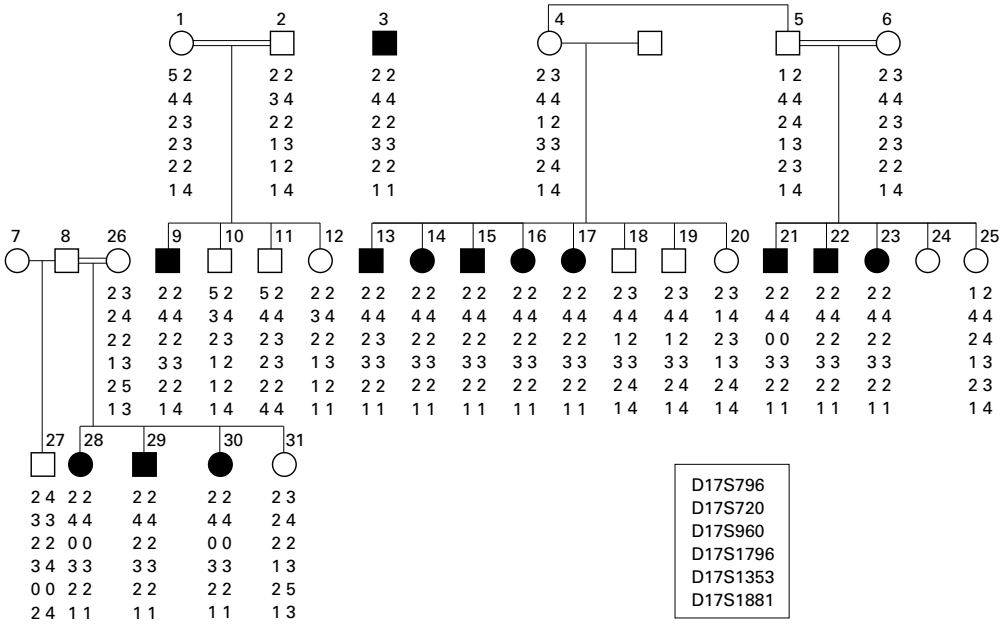

Figure 2 Haplotypes of six selected dinucleotide markers within the region of linkage on 17p13.1. The markers are arranged from telomere to centromere. The marker names are shown in the closed box. The affected subjects are homozygous for the extent of the haplotype.

symptoms and signs other than the impaired visual acuity and nystagmus. This suggests that other factors, possibly environmental, as well as genetic play a role in the variability in clinical expression of this monogenic disorder.

Since the mutations detected so far denote either profound instability of the protein or premature translation termination, it strongly suggested that LCA is the result of abolished production in cGMP in photoreceptor cells.[10] The mutation in our family produces premature termination in translation, which strengthens this suggestion. The presence of congenital cataracts and congenital microphthalmia in this family suggest that the *RETGC* gene may play a role in eye development in utero as well.

Linkage analysis in this family followed by the detection of the mutation provides us with a potent set of tools for carrier identification. This can be applied to premarital testing and counselling, which provides a socially acceptable solution to this problem in a large family with widely practised intermarriage. Prenatal diagnosis can also be provided to married couples who are known carriers.

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HATEM EL-SHANTI
Departments of Paediatrics and Medical Laboratory Sciences, School of Medicine, Jordan University of Science and Technology, Irbid, Jordan and National Center for Diabetes, Endocrinology and Genetics, Amman, Jordan

MAHMOUD AL-SALEM
Department of Ophthalmology, School of Medicine, Jordan University of Science and Technology, Irbid, Jordan

MAHMOUD EL-NAJJAR
Department of Physical Anthropology, Yarmouk University, Irbid, Jordan

KAMEL AJLOUNI
Department of Internal Medicine, School of Medicine, Jordan University, Amman, Jordan and National Center for Diabetes, Endocrinology and Genetics, Amman, Jordan

JOHN BECK
VAL C SHEFFIELD
Howard Hughes Medical Institute and Department of Pediatrics, University of Iowa Hospitals and Clinics, Iowa City, Iowa, USA

EDWIN M STONE
Department of Ophthalmology, University of Iowa Hospitals and Clinics, Iowa City, Iowa, USA

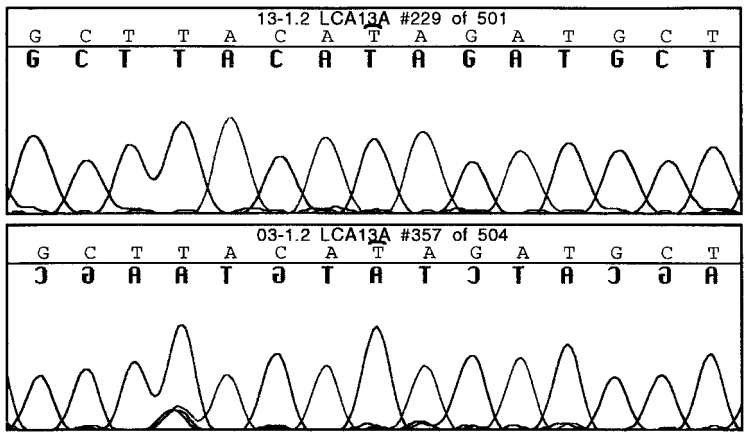

Figure 3 Sequence from forward (above) and reverse (below) directions. The mutation is homozygous and lies within exon 13. The change is a T (TAG=stop) in place of C (CAG=Gln). The T is flanked by As and is marked with a cap.

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Skewed X chromosome inactivation in a female with haemophilia B and in her non-carrier daughter: a genetic influence on X chromosome inactivation?

EDITOR—Phenotypic expression of X linked disorders in females may be the result of an X chromosome anomaly or homozygosity for the mutated gene, but is probably most frequently the result of skewed X chromosome inactivation. Skewed X inactivation may be the result of a chance event, but may also be because of genetic factors.[1] We report here the results of X inactivation analysis in a family with haemophilia B, which showed extremely skewed X inactivation both in a female haemophilia patient and in her non-carrier daughter, indicating a possible genetic influence on X chromosome inactivation in this family. Familial skewed X inactivation may interfere with carrier detection, since skewed X inactivation with the mutant gene on the inactive X chromosome may lead to a normal phenotype in a carrier.

The patient was a 40 year old female who belonged to a family with moderate haemophilia B[2] (fig 1). Plasma factor IX (FIX) activity was 0.02-0.03 units/ml, which was identical to the FIX activity in the affected male relatives. She had suffered from bleeding episodes after tooth extraction as a child and heavy bleeding from the episiotomy after her first delivery. A C→T transition, causing mutation S360L in exon 8 of the factor IX gene, was found in the male relative with haemophilia using single strand conformation analysis (fig 1, II.4). Both the female haemophilia patient and her mother (III.1 and II.3) were heterozygous for the mutation, whereas her two daughters (IV.1 and IV.2) did not have the mutation.

The X chromosome inactivation pattern (XIP) was determined by PCR analysis of a polymorphic trinucleotide repeat in the first exon of the androgen receptor (AR) gene which, after digestion with the methylation sensitive enzyme *Hpa*II, gives a PCR product from the inactive X chromosome only.[3] XIP was measured as the ratio between the PCR products from the two X chromosomes and scored as random (ratios 50:50-80:20) or skewed (ratios >80:20-95:5). When one band only was visible, the pattern was scored as extremely skewed (ratios >95:5). DNA from a girl with a deletion within chromosome Xp22 who had previously been shown to have a skewed XIP was used as a control. Samples containing mixtures of DNA from two males with well separated bands were run as quantitative controls. The most extreme mixture that gave two visible bands was 95:5. The results of X inactivation analysis are shown in fig 2. Each allele at the AR locus gives two equally strong bands on a denaturing polyacrylamide gel owing to the two complementary DNA strands migrating slightly differently during electrophoresis. After digestion of the patient's DNA with *Hpa*II one double band only, the band inherited from the father, was visible. The patient therefore had an extremely skewed XIP with the maternally inherited X chromosome as the active X chromosome in the majority of the cells. Identical results were obtained with DNA from

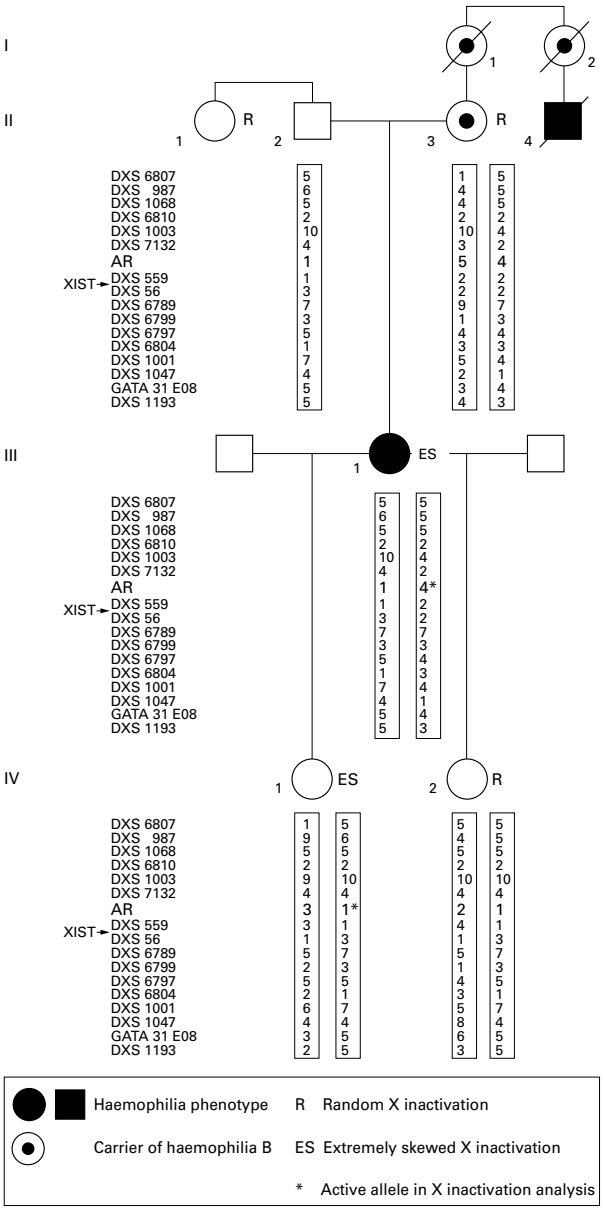

Figure 1 Pedigree of haemophilia family modified from Ørstavik et al.[2] The mutation 360L was identified in II.4, II.3, and III.1, but not in IV.1 or IV.2.

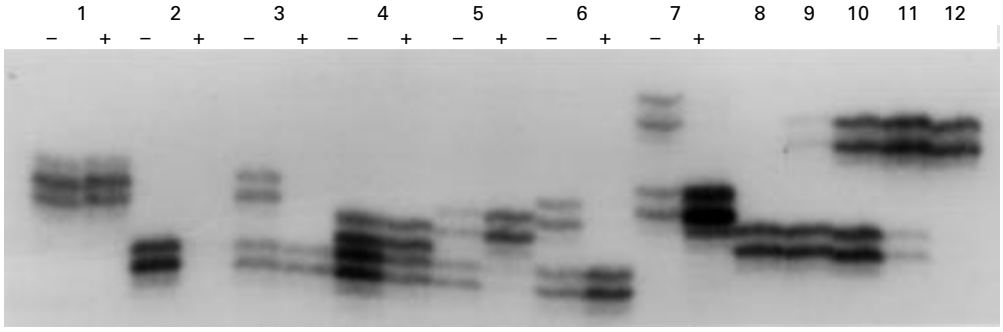

Figure 2 X chromosome inactivation analysis at the androgen receptor locus. −=undigested DNA, +=HpaII digested DNA. Lane 1: patient's mother (II.3, random XIP). Lane 2: patient's father (II.2). Lane 3: patient (III.1, extremely skewed XIP). Lane 4: patient's daughter (IV.2, random XIP). Lane 5: patient's daughter (IV.1, extremely skewed XIP). Lane 6: patient, fibroblast DNA (extremely skewed XIP). Lane 7: control sample, girl with a deletion within chromosome Xp22 and a known skewed XIP. Lanes 8-12: mixture of DNA from two males in ratios 5:95, 20:80, 50:50, 80:20, 95:5.

cultured fibroblasts. Thus, extremely skewed X inactivation is most probably the cause of haemophilia in this female. The patient's oldest daughter also had an extremely skewed pattern, since after *Hpa*II digestion the paternally inherited double band was the only visible band. The second daughter, the patient's mother, and a paternal aunt all had a random pattern. No other females of the family were available for X inactivation analysis.

The finding of two members of a family with an extremely skewed XIP could be a chance occurrence. However, an extremely skewed pattern is rare since it was not found in any of 148 blood donors.[4] A skewed XIP may be caused by selection against an unidentified deleterious gene on the X chromosome, which has been claimed to be an important cause of familial skewed X inactivation.[5] A greater opportunity for selection exists in blood cells with more cell divisions than in most other tissues. Since extreme skewing was also found in fibroblast DNA from our patient, a selection mechanism in this family does not seem likely, but cannot be excluded.

A skewed pattern may also be the result of a genetic influence on X inactivation, which is in agreement with a previous report of more than one affected female in a family with haemophilia B.[6] The *XIST* (X inactivation specific transcript) gene, located at Xq13, is expressed from the inactive X only and is a candidate gene for mutations giving rise to skewed X inactivation.[7] Marker analysis along the X chromosome of DNA from the patient, her parents, and two daughters showed that the daughters had inherited identical alleles from their mother, which were the mother's paternally inherited alleles, including the markers DXS559 located between *AR* and *XIST,* and DXS56 located distally to *XIST* (fig 1). An exception was the marker DXS987, located on the distal part of Xp, where the two sisters had inherited different alleles from their mother. No information was available distally to this marker, since the more distal marker DXS6807 was uninformative. Thus, the extremely skewed X inactivation in our family does not cosegregate with *XIST*. Furthermore, the *AR* allele on the active X chromosome in the haemophilia patient was her maternally inherited allele, whereas the *AR* allele on the active X chromosome in her daughter was the allele inherited from her healthy maternal grandfather.

The occurrence of extremely skewed XIP in a mother and her daughter in this family may be because of a genetic influence on skewing of X inactivation, but a chance event cannot be excluded.

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KAREN HELENE ØRSTAVIK
RAGNHILD ELISE ØRSTAVIK*
Department of Medical Genetics, Ullevål University Hospital, Oslo, Norway

MARIANNE SCHWARTZ
Department of Clinical Genetics, University Hospital Rigshospitalet, Copenhagen, Denmark
**Present address: Department of Rheumatology, Diakonhjemmets Hospital, Oslo, Norway.*

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46,XX/46,XY at amniocentesis in a fetus with true hermaphroditism

EDITOR—The finding of a mixture of both 46,XX and 46,XY cells in amniotic fluid culture has been frequently described. In the great majority of cases, the finding is followed by the birth of a normal male infant, leading to a consensus that the finding is the result of contamination with maternal cells in a normal male fetus. There are, however, several other possible explanations for a 46,XX/46,XY karyotype at amniocentesis. These include the presence of cells from an undiagnosed twin pregnancy, cross contamination in the laboratory, the presence of cells from a "vanished" male twin, and true fetal chimerism.[1] Reassuringly, all previously reported cases where 46,XX/46,XY has been found at amniocentesis have resulted in the birth of a child with a normal genital phenotype.

We present a case where a 46,XX/46,XY karyotype was found on amniocentesis. This resulted in the birth of an infant with true chimerism and an abnormal genital phenotype. We describe the possible outcomes when a 46,XX/46,XY karyotype is found at amniocentesis and discuss the implications for counselling.

A couple were seen for genetic counselling after an amniocentesis, performed because of advanced maternal age, showed a karyotype 46,XX[30]/46,XY[4]. Thirty clones from four independent cultures showed 46,XX cells, and four clones from two of these cultures showed 46,XY cells. In a fifth dispersed culture, of 60 cells examined, 58 were 46,XX and two were 46,XY. Laboratory error was very unlikely because the karyotype was found in two independently handled samples. An ultrasound examination showed male genitalia with normal appearance.

The couple were counselled regarding the possible clinical outcomes. It was explained that the 46,XX cells might be of maternal origin, in which case the child would be a normal male. The possibility of true chimerism was also raised. Severe genital ambiguity was considered unlikely because of the ultrasound findings, but the possibility of either normal or abnormal genitalia was discussed. It was explained that genital surgery may be necessary, and that there were risks of infertility and gonadoblastoma associated with true chimerism. It was also emphasised that because of the normal autosome and sex chromosome content in both cell lines, there was no reason to expect intellectual compromise. The couple decided against further investigation.

The infant was examined after delivery and had minor genital abnormalities. There was a well developed penis with a short ventral length and a penoscrotal web. The left gonad was present in the scrotum, measuring 2-3 ml, and the right gonad was not palpable. Ultrasound examination failed to locate the right gonad. A micturating cystourethrogram showed grade II vesicoureteric reflux on the right without obstruction. An HCG stimulation test showed normal testosterone production. A peripheral blood karyotype showed 46,XX[25]/46,XY[5].

At the age of 6 months the child underwent exploratory laparoscopy. A normal testis and vas was present on the left, with an ovary and uterus present on the right. The ovary was prophylactically removed.

Cases of true 46,XX/46,XY chimerism are usually ascertained in early childhood during investigation of the abnormal appearance of external genitalia. 46,XX/46,XY chimerism is known to have a variety of presentations, including ambiguous genitalia, hypospadias, gynaecomastia, and inguinal herniae,[2] and is responsible for about 13% of cases of true hermaphroditism.[3] The true frequency of the genotype, however, is unknown because of the bias in ascertainment. Rare cases with normal male and normal female phenotype have also been ascertained as an incidental finding.[2 4 5]

The mechanism for chimerism is uncertain, but possibilities include double fertilisation of an ovum and its first or second polar body, or the secondary fusion of two independently fertilised ova. Two recent studies[6 7] using DNA fingerprinting techniques have shown the tetragametic origin of cases of XX/XY hermaphroditism, proving that these cases of chimerism originated by the fusion of two fertilised gametes.

The finding of a mixture of 46,XX and 46,XY cells at amniocentesis has been reported to occur at a frequency of about 1.5 per 1000.[8–10] The vast majority of these cases are the result of contamination by maternal cells in an otherwise normal male fetus.[1 10] Given that maternal contamination would only be detected if the fetus were male, maternal cell contamination is expected to occur at double this rate, that is, at about 3 cases per 1000. Maternal cell contamination is usually detected by the finding of a 46,XX/46,XY karyotype, although cases where 100% of cells were 46,XX have been discovered only after the unexpected delivery of a normal male.[8] The maternal cells are thought to arise from the outgrowth of cells from small fragments of maternal tissue removed by the amniocentesis needle. Maternal cell contamination is more likely to occur where the first few millilitres of fluid are not discarded, when a large needle is used for the procedure, and when the fluid is blood stained.[8]

This case serves as a reminder that not all cases of 46,XX/46,XY karyotype at amniocentesis can be explained by maternal cell contamination where the fetus is a normal male. We have identified six other published cases where there has been an alternative explanation for the presence of the dual cell line at prenatal diagnosis.

The first two cases involved twin pregnancies.[10 11] In both cases a 46,XX/46,XY karyotype was found at amniocentesis of the female twin. The XY cells were considered to have arisen from cross contamination from the other (male) twin. This situation would not present a diagnostic dilemma at prenatal diagnosis unless the twin pregnancy was previously undiagnosed.

Three cases have been reported where a 46,XX/46,XY result on amniocentesis has been followed by the delivery of a normal XX female[1 10] The source of the XY cell line in these cases has not been explained. It has been hypothesised that the XY cells may have arisen from a male twin which had since died and been resorbed. The so called "vanishing twin" has been proposed in other situations as an explanation for discordance between prenatal and postnatal cytogenetic analysis.[12–14] Another possible explanation is that the 46,XY cell line was actually derived from the fetus, but could not be found in the tissues that were sampled after birth.

In the sixth case, an amniocentesis performed at 16 weeks' gestation showed 46,XX and 46,XY cells in a 4:1 ratio.[2] All samples from all flasks showed a similar ratio of 46,XX and 46,XY cells. Maternal contamination was considered unlikely and the couple was counselled that there was a risk of abnormal sexual development. The infant was born with male genitalia that were normal in external appearance. A testosterone level and pelvic ultrasound were also normal. Postnatal karyotype on blood confirmed the 46,XX/46,XY chimerism, with 46,XY cells predominating. The authors believed this to be the first documented prospectively ascertained human chimera.

Our case represents the second report of the prenatal diagnosis of a true XX/XY chimera and the first case with true hermaphroditism.

When a 46,XX/46,XY karyotype is found at amniocentesis, the result can be confusion and anxiety for both parents and physicians. The amniocentesis will usually have been performed for other reasons, such as advanced maternal age, and the result is unexpected. Fortunately, the vast majority of cases have been shown to result from the contamination of the specimen by maternal cells. These pregnancies would be expected to result in the birth of a genetically and phenotypically normal male baby. Including our case, there are only seven reported cases where a 46,XX/46,XY karyotype at amniocentesis has resulted from a cause other than maternal contamination. It is possible that some other cases of true fetal chimerism have been wrongly attributed to maternal contamination. Nonetheless, the fact that no phenotypically abnormal infants have previously been reported suggests that true fetal chimerism at prenatal diagnosis is a rare event.

Table 1 Mechanism and outcome of 46,XX/46,XY at amniocentesis

	Mechanism	Fetal karyotype	Origin of X chromosomes	Pregnancy outcome (phenotype)	Estimated frequency
1	Maternal contamination	46,XY	All maternal	Normal male	1–2/1000
2	Twin pregnancy	46,XX and 46,XY	Maternal and paternal	Normal female and normal male twins	Rare (2 cases reported)
3	Male cells of unknown origin (possible "vanishing twin")	46,XX*	Maternal and paternal	Normal female	Rare (3 cases reported)
4	Cross contamination in laboratory	46,XX or 46,XY	Non-parental	Normal male or normal female	Unknown
5	True fetal chimerism	46,XX/46,XY	Maternal and paternal	True hermaphrodite/ normal male/ normal female	Rare (2 cases reported)
6	Parthenogenetic chimera	46,XX/46,XY	All maternal	Phenotypic uncertain: possible physical/intellectual compromise	Never reported at prenatal diagnosis

*The presence of male cells in the fetus that were not detectable after delivery cannot be excluded.

In assessing the potential outcomes of a pregnancy where 46,XX/46,XY has been discovered, six possible explanations for the occurrence should all be considered (table 1). The sixth possibility, that of a human parthogenetic chimera (composed of a biparental XY cell line and an XX cell line containing only maternally derived chromosomes) has never been described at amniocentesis, but has been reported in a child[15] and potentially could be encountered prenatally. All possibilities other than maternal cell contamination are rare.

We suggest the following approach to the further assessment of a 46,XX/46,XY karyotype at amniocentesis. Firstly, the possibility of a twin pregnancy should have already been excluded by ultrasound at the time of the amniocentesis. It may also be possible to exclude laboratory cross contamination as a cause, depending on the method used by the laboratory in processing the specimen. The level of mosaicism present should also be assessed; the presence of 46,XX cells in single culture or clone (level I or II "mosaicism") is almost certain to represent maternal cell contamination. An ultrasound examination showing male genitalia or a history of a traumatic procedure would add weight to this conclusion.

In cases where there are two or more 46,XX cells present, distributed over two or more flasks (level III "mosaicism"), the initial investigation should be a detailed ultrasound with particular attention to the genitalia. The appearance of male genitalia is consistent with maternal cell contamination as the cause of the 46,XX cells, although a true chimera could also have this appearance. If the genitalia appear female or ambiguous, careful consideration should be given to alternative explanations.

The choice of subsequent investigation will depend on the facilities available. The situation might be clarified by repeating the amniocentesis, which would be expected to show 46,XY unless true chimerism was present. The disadvantages of this approach are, firstly, the small risk involved in repeating the procedure and, secondly, the likelihood that the risk of maternal contamination complicating the second procedure is greater than would be expected by chance alone.[16] An alternative approach is to use polymorphic microsatellite markers to determine the parental origin of the X chromosomes.[17] Comparison of chromosome polymorphisms as visualised on banded karyotypes of maternal blood and amniotic fluid may also yield distinct patterns.[18] If all three X chromosomes are shown to be maternal in origin, then maternal cell contamination is almost certainly present, notwithstanding the remote possibility that the fetus is a parthogenetic chimera. The presence of a non-parental X chromosome strongly suggests that laboratory cross contamination has occurred. If both maternal and paternal X chromosomes are found, the possibilities are, firstly, a female fetus with the presence of male cells of unknown origin (the so called "vanishing twin") and, secondly, true fetal chimerism. Fetal blood sampling may be of benefit in confirming true fetal chimerism, although a finding of 46,XX in blood will still not exclude chimerism in other tissues.

The difficulty in differentiating true chimerism from the situation of a female with male cells of unknown origin may relate to the hypothesis that these two phenomena have identical genetic origins. It is possible that both conditions originate from dizygotic male and female twins. In the case of true fetal chimerism, the two embryos fuse to become one embryo, whereas in the second case, the male twin dies leaving residual cells in the amnion. There would therefore be no genetic way of differentiating the two situations.

If true chimerism cannot easily be excluded, the possibility of true hermaphroditism should be discussed with the couple. Relevant details include the issues of sexual ambiguity, genital surgery, possible infertility and gonadal tumours. The expectation of normal intelligence is an important component of counselling. It must be emphasised, however, that hermaphroditism is an unlikely outcome. Further invasive prenatal testing should be used judiciously according to the ultrasound findings, the availability of DNA and chromosome polymorphism studies, and the wishes of the couple. Where the possibility of true hermaphroditism cannot be excluded, consultation with an endocrinologist may be of benefit.

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DAVID AMOR
MARTIN B DELATYCKI
Victorian Clinical Genetics Service, Royal Children's Hospital, Flemington Road, Parkville, Melbourne, Victoria 3052, Australia

MARLENE SUSMAN
ELLEN CASEY
TANIA NASH
Cytogenetic Services Victoria, 165 Burwood Road, Hawthorn, Victoria 3122, Australia

GARRY WARNE
Department of Endocrinology, Royal Children's Hospital, Melbourne, Australia

JOHN HUTSON
Department of Surgery, Royal Children's Hospital, Melbourne, Australia

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A dominant relationship between the *ACE* D allele and serum ACE levels in a Ghanaian population

EDITOR—The *ACE* gene has a 287 bp Alu insertion in intron 16.[1] The presence (I) or absence (D) of this insertion produces three population genotypes, II, ID, and DD. The D allele has been proposed as an indicator of cardiovascular risk in several studies,[2–4] although this was not supported in a large study on US physicians.[5] There is a codominant relationship between *ACE* ID genotype and serum ACE levels in white populations, with the D allele associated with increased levels.[1] It is not clear whether a similar relationship exists in black populations. One report showed no difference in serum ACE levels between the different I/D genotypic groups in American blacks.[6] Two others, both on the Jamaican population,[7 8] suggested an important impact of the D allele. It is possible that the black populations reported could have a genetic contribution from other ethnic groups. We therefore examined a Ghanaian population, where African descent was known back to the grandparental generation, to see if any relationship existed between ACE polymorphism status and circulating serum ACE levels.

There were 97 subjects, 70 males and 27 females. The mean age (SD, range) was 26.2 (4.9, 22-45) for males and 28 (7.2, 22-51) for females. None were on ACE inhibitors or any other medication, and all were normotensive, healthy volunteers from the Kumasi area and could be considered representative of that region. All subjects had Ghanaian parents and grandparents. No females were pregnant.

Table 1 Serum ACE in U/l for each ACE genotype

	ACE genotype							
	II	*ID*				*DD*		
	24	26	48	82	60	50	21	23
	27	48	62	42	56	20	43	35
	42	31	47	74	57	37	33	8
	42	15	35	45	57	24	46	60
	19	31	25	25	24	53	89	31
	36	43	55	98	62	83	70	
	25	44	28	62	27	50	125	
	26	61	52	82	21	20	69	
	33	52	56	71		21	65	
	74	62	68	33		35	77	
	32	38	97	34		90	65	
	44	37	32	37		117	82	
	30	39	36	35		100	38	
	29	132	103	77		41	38	
Mean (SD)	34.5 (13.6)	51.3 (23.8)				53.3 (29.5)		
Median (range)	31 (19–74)	47.5 (15–132)				46 (8–125)		

Blood for ACE levels was collected in serum tubes. These were centrifuged and the serum transferred to sterile plastic tubes and frozen at −20°C. For DNA extraction, blood taken into EDTA tubes was kept at −20°C. All specimens were transported frozen on dry ice and were only thawed before analysis.

PCR was performed exactly as described previously.[9] Owing to the possibility of mistyping ID subjects as DD,[10] all DD genotypes were confirmed with insertion specific primers, again as previously described.[8] ACE serum levels were measured using "ACE Reagent" kit from Sigma in the diagnostic Chemical Pathology laboratory where the test is used routinely and has been quality control checked. Serum samples are known to dilute as expected in this test, but this was not performed with this set of samples.

The Kruskal-Wallis one way analysis of variance was used as the data were not normal even after log transformation.

The genotypes and serum ACE levels are given in table 1. The data are not normal and positively skewed and with greater variability in the ID and DD groups than in the II group (fig 1). Using the Kruskal-Wallis one way analysis of variance by ranks gives a p value of 0.03 for a tendency for members of some groups to exceed members of others. Table 1 gives the median and range for each group, from which it is evident that the members of the II group tend to have lower serum ACE levels than the ID or DD groups. There is a considerable spread of ACE enzyme activity in all groups, but more especially in the ID and DD groups. The ages in each genotypic group were similar at 26.8, 26.6, and 26.4 (means) years for II, ID, and DD respectively. There were more females in the II group than in the ID or DD groups, at 42%, 24%, and 27% respectively, but the serum ACE levels for males and females in the II group were 35.8 and 32.8 U/l, suggesting

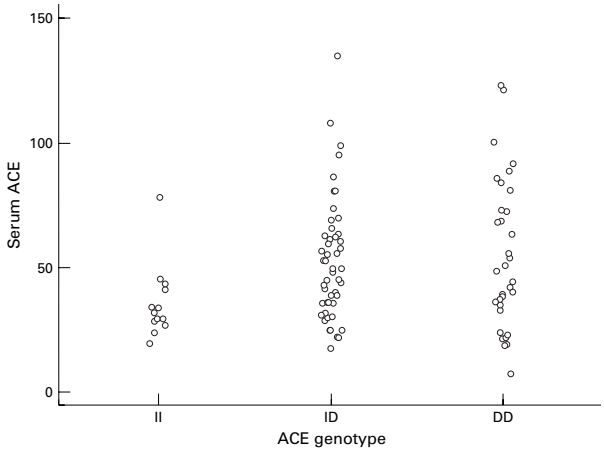

Figure 1 Serum ACE in U/l plotted against ACE genotype.

that gender was not an important confounder. The prevalence of the I allele in the population was 0.4, similar to that reported in the Jamaican black population.[7 8] The sample population is in Hardy-Weinberg equilibrium.

[1]Three reports on black populations have been published, one on American blacks which showed an absence of any association between genotype and ACE serum levels,[6] while the others showed a significant effect of the D allele.[7 8] These last two studies, both on Jamaican populations, suggested the same general trend but with slightly differing results. Forrester *et al*[7] showed a significant difference between serum ACE in all three genotypic groups with the same codominant effect seen in whites. McKenzie *et al*[8] also showed significance between all groups, but this was less pronounced between II and ID than for any of the other cross comparisons. The ACE levels reported by McKenzie *et al*[8] showed considerable overlap between groups, and this was especially prominent between the ID and DD groups. We also found considerable variation in the ACE serum levels within genotypic groups, with the greatest scatter in the ID and DD groups. There were significantly lower serum ACE levels in the II group compared to the ID or DD groups in the Ghanaian population, but no difference between the ID or DD groups. This trend is seen in the data of McKenzie *et al*,[8] but not at all in that of Forrester *et al*.[7] Since these two reports studied the same population, it is possible that the difference is simply a statistical artefact.

Our data show that in a black African population the trend in McKenzie *et al*[8] is increased to produce a dominant effect of the D allele on ACE serum levels rather than codominant. It may be that there has been genetic input from white gene pools in the Jamaican population which has produced a less dominant relationship between the *ACE* D allele and serum ACE levels than we have shown. The fact that Forrester *et al*[7] attempted to show black ethnicity by having "three or four grandparents of predominantly African origin" shows the problems with such a population. Unlike Blom *et al*,[6] we do find a relationship between ACE I/D polymorphism and ACE serum levels in the black population, but one where the D allele shows dominance rather that codominance. The numbers in this study are not large and the data could be influenced by this, but the sample is larger than that used by Rigat *et al*[1] to show the codominant influence of the I and D alleles in whites. Nonetheless, a much larger study in this or another black African population would be useful to confirm these data, with a matched white population as a comparison.

S JEFFERY
A K SAGGAR MALIK
A CROSBY
Medical Genetics Unit, St George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, UK

M BLAND
Department of Public Health Sciences, St George's Hospital Medical School, London SW17 0RE, UK

J B EASTWOOD
Department of Renal Medicine, St George's Hospital Medical School, London SW17 0RE, UK

J AMOAH-DANQUAH
J W ACHEAMPONG
J PLANGE-RHULE
Department of Medicine, University of Science and Technology, School of Medical Sciences, Kumasi, Ghana

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Coexistence of Gaucher disease type 1 and Joubert syndrome

EDITOR—Van Royen-Kerkhof *et al*[1] reported two boys with Gaucher disease type 1 and Joubert syndrome (JS). Their case 1 had, in addition to mental retardation, choreoretinal colobomas, cerebellar vermis agenesis, and abnormal breathing, agenesis of the corpus callosum, hydrocephalus (no further details given), and generalised seizures. Their case 2 had prenatal hydrocephalus and "fulfilled the diagnostic criteria for JS". Information about brain anatomy and retinal findings in case 2 is lacking.

We question the diagnosis of JS in these two patients. The authors cite a 1992 paper[2] but fail to reference 1997 and 1998 publications that better define the phenotype and characteristic neuroimaging of JS.[3–5] In these most recent publications, the "molar tooth sign" is defined as well as a number of distinct posterior fossa abnormalities not discussed by Van Royen-Kerkhof *et al*.[1] This is a significant omission because vermis hypoplasia alone is not pathognomonic for JS and can be seen in mimicking conditions that produce congenital oculomotor apraxia and ataxia.[6 7] In addition, to the best of our knowledge, corpus callosum agenesis, hydrocephalus, and generalised seizures are not associated with JS, as these features were not encountered in our combined series of more than 60 JS patients. At a 1998 Child Neurology Society symposium on JS sponsored by the National Institutes of Health, there was general agreement about the common and occasional abnormalities in JS that did not include the clinical or radiological features reported by Van Royen-Kerkhof *et al*[1] in the two children with Gaucher disease type 1. The proceedings of the symposium will appear in the *Journal of Child Neurology* in the autumn of 1999.

EUGEN J BOLTSHAUSER
Department of Paediatrics, Children's University Hospital, CH-8032 Zurich, Switzerland

BERNARD L MARIA
Division of Pediatric Neurology, University of Florida, College of Medicine, Gainesville, FL 32610, USA

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This letter was shown to Drs van Royen-Kerkhof *et al*, who reply as follows.

We thank Drs Boltshauser and Maria for their comments on our Letter to the Editor.[1] The diagnosis of Joubert syndrome (JS) in case 1 was made 17 years ago on clinical grounds including a CT scan; MRI scanning was not yet available in those days. We were not aware of the paper of 1997.[3] The other papers the authors mention had not been published at the time our paper was submitted. As a consequence the "molar tooth sign" was not discussed. We think that corpus callosum agenesis and hydrocephalus are rather non-specific developmental defects whose presence or absence do not argue significantly for or against JS. In our opinion the presence in both cases 1 and 4 of episodic hyperpnoea/apnoea remains an argument in favour of JS.

A VAN ROYEN-KERKHOF
B T POLL-THE
Department of Metabolic Diseases, University Medical Centre, PO Box 85090, 3508 AB Utrecht, The Netherlands

F A BEEMER
Department of Medical Genetics, University Medical Centre, PO Box 85090, 3508 AB Utrecht, The Netherlands