Letters to the Editor

J Med Genet 1999;**36**:494–496

Simultaneous decrease of telomere length and telomerase activity with ageing of human amniotic fluid cells

EDITOR—Telomeres are specific chromatin structures that cap chromosome ends and protect against chromosome degradation and end to end fusion.[1] As conventional DNA polymerases cannot fully replicate the ends of linear DNA, a progressive loss of telomeric sequences occurs in each round of DNA replication. Telomerase adds telomere repeats onto chromosome ends to overcome this end replication problem.[1] Telomerase activity is detectable in human germ cells, most immortalised cell lines, and in 80-90% of human tumour samples, in which the telomere length is preserved.[2] However, telomerase activity is not detected in most normal human somatic cells, with the result that telomere loss occurs with each cell division. After extended doublings, these cells enter a period of slow growth called senescence or crisis and stop dividing. This process may depend on critical telomere loss in one or a few chromosomes. The shortest telomere in a cell may also play an important role in oncogenesis.[2] Recently, low level telomerase activity has been detected in several human somatic cells, for example, lymphocytes, endothelial cells, hair follicle cells, colonic crypt cells, and basal layer cells of the epidermis.[3] In spite of this activity, telomeres shorten after each successive round of DNA replication. We report here the analysis of telomere length and telomerase activity in two cultures of human amniotic fluid cells (AL1 and AL2), showing a progressive decrease in both with ageing.

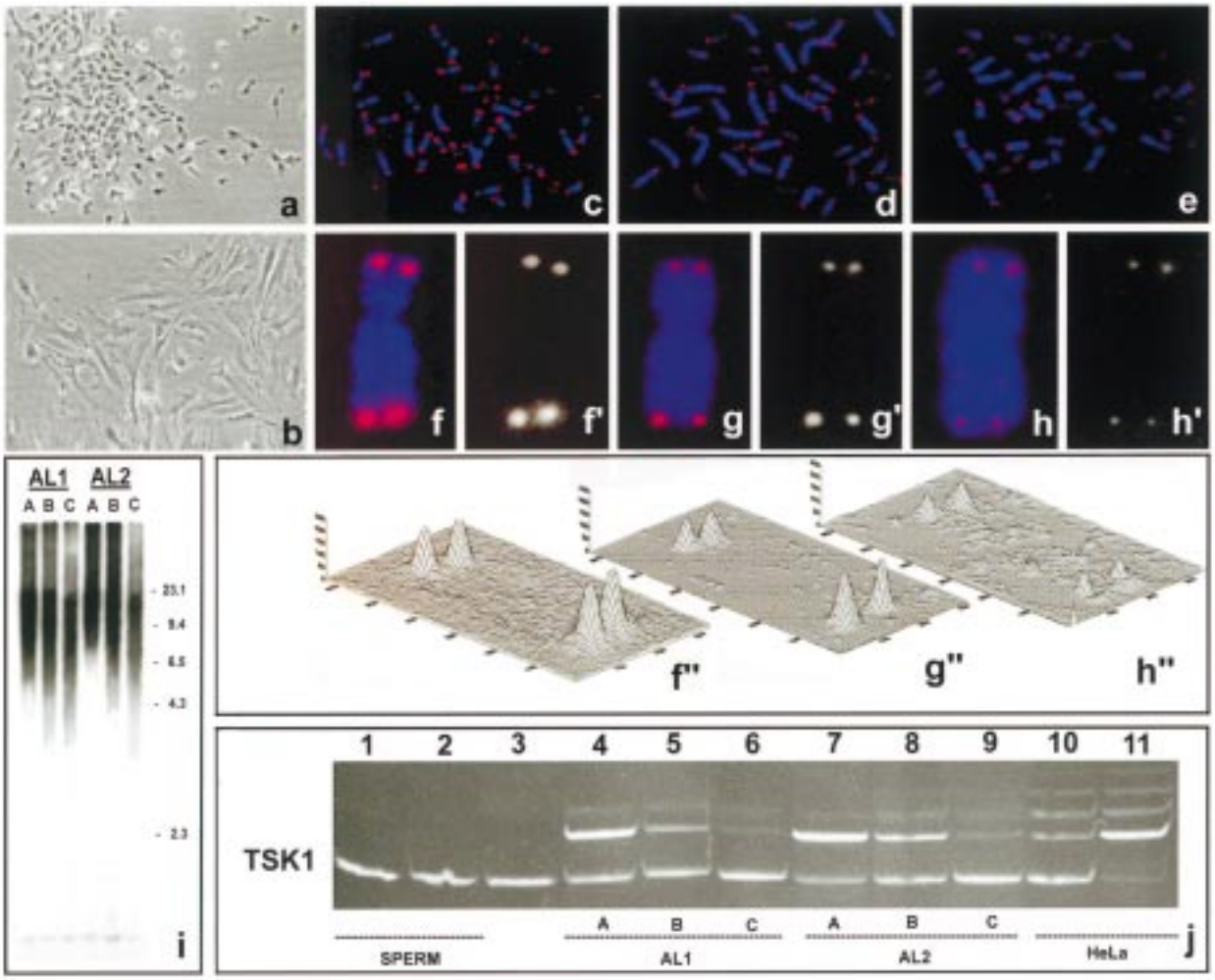

Figure 1 (Upper left) Cultured amniocytes at 14.7 MPD (a) and at 62.4 MPD (b) visualised under phase contrast microscopy. (Lower left) (i) Southern blot analysis of TRFs from amniocyte cultures AL1 and AL2 at MPD 14.7 (A), 42.7 (B), and 62.4 (C). (Upper right) FISH signals after hybridisation of a biotinylated telomeric probe detected using streptavidin-Cy3, with a round of signal amplification, from mitotic amniocytes at 14.7 (c), 42.7 (d), and 62.4 (e) MPD. Representative chromosomes with a pseudo-3D intensity profile showing strong (f, f', f"), medium (g, g', g"), and faint (h, h', h") telomeric FISH signals. (Lower right) (j) TRAP assay (Oncor) to assess telomerase activity. Lanes 1 and 2 correspond to extracts from 10 000 and 20 000 human sperm cells, respectively, used as a negative control. Lane 3 is a primer-dimer/PCR contamination control without cell extract. Lanes 4, 5, 6 and 7, 8, 9 correspond to amniocyte cultures AL1 and AL2 respectively at MPD 14.7 (A), 42.7 (B), and 62.4 (C). Extract from 10 000 cells was assayed for each experimental sample. Larger bands of the ladders appear very faint. Lanes 10 and 11 correspond to the activity of extract from 5000 and 10 000 cells, respectively, of a HeLa cell line used as a positive control. TSK1 is a template that amplifies a 36 bp internal control band semicompetitively with TRAP products.

Samples were obtained by amniocentesis at 14 weeks'gestation and subcultured over a period of 108 days. DNA extraction, chromosome spreads at metaphase, and cellular extracts were simultaneously performed in three stages. The first stage was 22 days after the initiation of culture (mean population doubling (MPD) 14.7), the second stage was established 64 days after the initiation of culture (MPD 42.7), and the final stage was 108 days after initiation of culture (MPD 62.4). The number of doublings between the first and second stage was 28, while between the second and third stage it was 19.7. The mean rate of cell division was 1.5 days, 1.5 days, and 6 days, at the first, second, and third stages, respectively.

Continuous growth of human amniotic fluid cells leads to replicative senescence. After accruing a number of population doublings they enter the terminally non-dividing state. This is also reflected in cell morphology, as shown in fig 1a and b, whereby telomere length progressively decreased after each round of DNA replication, as shown by Southern blotting of terminal restriction fragment (TRF) length (fig 1i). Mean TRF lengths were similar in both AL1 and AL2. They were estimated as 14 kb (SD 0.26), 11 kb (SD 0.23), and 8.4 kb (SD 0.25) at 14.7, 42.7, and 62.4 MPD, respectively. These TRFs were obtained by *Alu*I digestion and may be longer than standard TRFs produced by *Hin*fI + *Rsa*I digestion. Nevertheless, this implies a regular rate of telomere loss of around 100 bp per cell duplication, which is in accordance with data from lymphocytes,[4] for example. Whole telomeric quantitative digital image analysis of FISH signals per mitosis also confirmed this progressive decrease (fig 1c-h"). Around 33-37 mitoses were analysed per experimental point in each AL1 and AL2. The heterogeneity in the whole telomeric signal among mitotic cells was maintained through successive cell doublings (Levine test for homogeneity of variances, $p<0.05$). In contrast to TRF analysis, FISH suggests a non-linear decrease of telomeres with successive cell doublings. Thus, a decrease per doubling of 0.9% (AL1) to 0.6% (AL2) was obtained from 14.7 to 42.7 MPD, while it was 1.9 (AL1) to 3.9(AL2) times greater from 42.7 to 62.4 MPD. Discordance of FISH data with TRF analysis may depend on technical factors, for example, efficiency of probe hybridisation in FISH. Furthermore, while TRF study includes the whole population, FISH is restricted to mitotic cells. This selection could bias the estimate for the whole cell population. Another possibility is that while TRFs consist of both TTAGGG and degenerate sequences and non-TTAGGG subtelomeric sequences, FISH shows only the former.[5] In the case of a relatively greater decrease in pure telomeric sequences at the last MPDs, this could not be discriminated in the TRF analysis owing to the adjacent long non-telomeric sequences. The coefficient of variation from the whole telomeric FISH signal was between 21.5% and 31.2% in the amniotic fluid cell samples. This variability is much greater than the 13-15.4% observed after quantifying the FISH signals of alphoid DXZ1 and classical DYZ1 satellite DNA loci from one of the amniotic fluid samples. Unlike telomeres, these sequences do not vary in length among cells, so these latter coefficients must correspond to the variability of FISH intensity owing to technical factors.

Recently, several human somatic cell types like lymphocytes or colonic crypt cells have been shown to present a low level of telomerase activity which does not prevent telomere shortening. Accordingly, the human telomerase catalytic subunit gene is expressed in these cell types.[3] Though in a previous report primary cultures of human amniocytes did not show telomerase activity,[6] we detected this in both uncultured and cultured amniocytes from 14 weeks' gestation (data not shown) using the TRAP assay (Oncor). Telomerase activity at the initial stage of evaluation (MPD 14.7) was 34% (AL1) to 48% (AL2) of that of a HeLa cell line. Then it progressively decreased, the decrease per doubling being 2.9% (AL1) to 2.27% (AL2) from 14.7 to 42.7 MPD, while it was 0.86% (AL1) to 1.66% (AL2) from 42.7 to 62.4 MPD, which is 3.4 (AL1)-1.36 (AL2) times less (fig 1j). It is interesting to note the remarkable decrease from the first to the second stage, despite the mean rate of cell division having been similar at both stages. This suggests that, in this case, reduction in telomerase activity is not related to the growth rate, but to cell ageing, that is, the previous number of cell duplications. Another possibility is the simultaneous presence of different cell populations, given the presumed heterogeneity of amniotic fluid cells. Subpopulations with higher levels of telomerase activity were identified in cultured keratinocytes,[7] as well as in T and B lymphocytes.[8 9] Depending on the stage of gestation, amniocytes belong mainly to the amnion, skin, and the urogenital, respiratory, and digestive systems. Therefore, the decrease in telomerase activity with ageing of amniocytes could be related to a progressive reduction in subpopulations with high telomerase activity.

Slight but detectable telomerase activity was evident at the final stage (fig 1j). It has been suggested that telomerase activity is proliferation rate regulated.[10] For example, in quiescent confluent cultures of immortal cell lines[11] telomerase activity is repressed. So, the residual telomerase activity of aged amniocyte cultures possibly depends on a very small subpopulation of cells with duplication potential. At 62.4 MPD, most cells are quiescent and previous assays have shown that only one or two mean doublings took place during a further month of culture. Therefore, though the initial drop in telomerase activity in amniocyte cultures seems to be related to cell ageing rather than to duplication rate, near crisis cultures where mean rate of division is prolonged could decrease telomerase activity as a result of both cell ageing and proliferation rate. As argued above, persistence of a low cell number of a presumed telomerase active subpopulation could also explain this residual activity.

Overall, proliferating human amniotic fluid cells in culture decreased the length of telomere sequences, as shown by Southern blotting and FISH. In spite of telomere shortening, telomerase activity was detected by the TRAP assay. Nevertheless, this activity decreased as cell cultures aged, mainly in the initial cell doublings.

We are grateful to Belén Ferreiro and Susana Dorado for their excellent technical assistance and to Dr Phil Mason for improving the English. This work was supported by the Consejo de Seguridad Nuclear and SAF(96/0033) (Spain) and the European Atomic Energy Community (Nuclear Fission Safety programme).

ALEJANDRO MOSQUERA
JOSÉ LUIS FERNÁNDEZ
Laboratorio de Genética Molecular y Radiobiología, Centro Oncológico de Galicia, Avda de Montserrat s/n, 15009 La Coruña, Spain

ASUNCIÓN CAMPOS
VICENTE J GOYANES
Sección de Genética, Hospital "Teresa Herrera", Instituto de Ciencias de la Salud, Universidad de la Coruña, Spain

JOSÉ RAMIRO-DÍAZ
JAIME GOSÁLVEZ
Unidad de Genética, Facultad de Biología, Universidad Autónoma de Madrid, Spain

1 Zakian VA. Telomeres: beginning to understand the end. *Science* 1995;**270**:1601-7.
2 Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* 1995;**5**:249-55.
3 Kilian A, Bowtell DDL, Abud HE, *et al*. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* 1997;**6**:2011-19.
4 Vaziri H, Schächter F, Uchida Y, *et al*. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet* 1993;**52**: 661-7.

5 Henderson S, Allsopp R, Spector D, *et al*. In situ analysis of changes in telomere size during replicative ageing and cell transformation. *J Cell Biol* 1996;**134**:1-12.
6 Wright WE, Piatyszek MA, Rainey WE, *et al*. Telomerase activity in human germline and embryonic tissue and cells. *Dev Genet* 1996;**18**:173-9.
7 Yasumoto S, Kunimura C, Kikuchi K, *et al*. Telomerase activity in normal epithelial cells. *Oncogene* 1996;**13**:433-9.
8 Weng NP, Levine BL, June CH, Hodes RJ. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc Natl Acad Sci USA* 1995;**92**:11091-4.
9 Weng NP, Granger L, Hodes RJ. Telomere lengthening and telomerase activation during human B cell differentiation. *Proc Natl Acad Sci USA* 1997; **94**:10827-32.
10 Broccoli D, Godley LA, Donehower LA, Varmus HE, de Lange T. Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol Cell Biol* 1996;**16**:3765-72.
11 Holt SE, Aisner DL, Shay JW, Wright WE. Lack of cell cycle regulation of telomerase activity in human cells. *Proc Natl Acad Sci USA* 1997;**44**:10687-9.

J Med Genet 1999;**36**:496–498

45,X/47,XX,+18 constitutional mosaicism: clinical presentation and evidence for a somatic origin of the aneuploid cell lines

EDITOR—Constitutional mosaicism for two distinct chromosome aneuploidies is a rare cytogenetic abnormality. Usually in such cases, an autosomal aneuploidy is associated with a gonosomal aneuploidy. Little is known about the sequence of errors leading to such complex conditions. The only available studies addressing this issue concern three mosaic autosomal/gonosomal cases involving chromosome 8 (two cases) and chromosome 21 (one case), in all of which chimerism could be ruled out.[1 2] A mitotic origin was inferred for both mosaic trisomy 8 cases,[1] whereas the initial error in the trisomy 21 mosaic most likely occurred at meiosis.[2]

So far, trisomy 18 combined with monosomy X has been observed in three cases.[3–5] We have recently observed a fourth patient with mosaic monosomy X/trisomy 18. We report the clinical and cytogenetic characteristics and the results of molecular analysis, which was undertaken in order to determine the origin of the aneuploid cell lines.

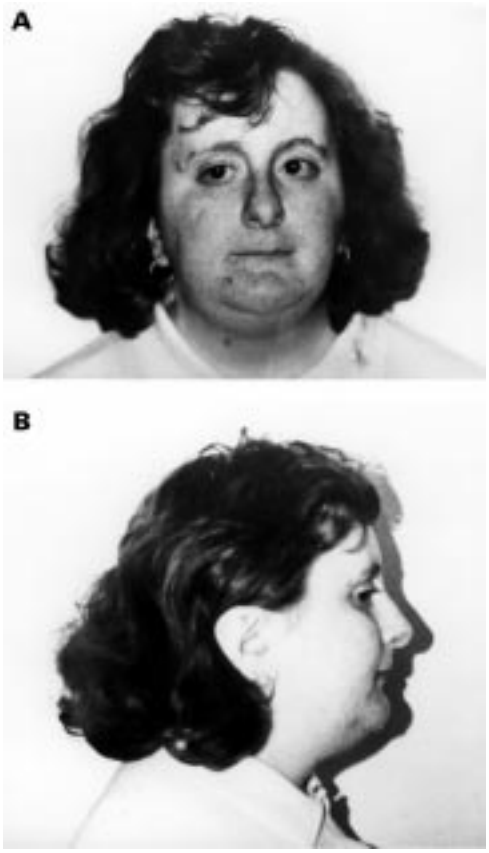

Figure 1 (A) Front and (B) lateral view of the proband aged 23 years 2 months. (Photographs reproduced with permission.)

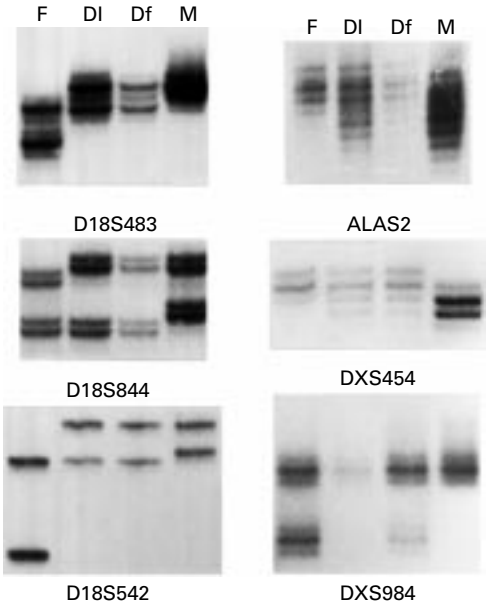

Figure 2 Typing of X chromosome and chromosome 18 microsatellite polymorphisms. The locus investigated is indicated below each autoradiogram. F=father, Dl=daughter, lymphocyte DNA, Df=daughter, skin fibroblast DNA; M=mother.

The female proband was the second child of healthy, unrelated parents. There was no family history of congenital anomalies or chromosome disorders. The father was 32 and the mother 34 years old at the time of her birth. She was born at term after an uneventful pregnancy and her birth weight was 3600 g. Psychomotor development was slightly delayed and she attended school up to the age of 14 years. Menarche occurred at 11 years, with regular menses up to 15 years. Thereafter, menses became progressively less frequent, until secondary amenorrhoea developed at 18 years of age.

The patient was referred for clinical and cytogenetic evaluation at the age of 23 years 2 months because of secondary amenorrhoea, mild mental retardation, and minor congenital anomalies. On clinical examination, height was 150.5 cm (3rd centile), weight 76 kg (>97th centile), and occipitofrontal circumference (OFC) 56 cm (50th centile). The face was asymmetrical, with a narrow nose and prominent nasal root and columella (fig 1). The ears were large and low set, with folded helices and large lobes (fig 1). The mouth was small, with a thin vermilion border and highly arched palate. There was bilateral cubitus valgus, brachydactyly, left Dubois sign, and abundance of white lines on both palms. Dermatoglyphics were (right) *t*, *abcd*, $L^{U}WWWW$ and (left) *t*, *abcd*, $L^{U}AAWW$. There were bilat-

Table 1 Clinical and cytogenetic findings in X0/+18 mosaicism

Reference	Clinical findings	Lymphocyte chromosomes		Fibroblast chromosomes	
		X0	+18	X0	+18
4	Short neck with pterigium, shield chest, streak gonads, normal intelligence	56%	37%	93%	7%
5	Growth retardation, pectus carinatum, cubitus valgus, buphthalmos, mesocephaly, severe psychomotor delay, ovarian agenesis	53%	47%	100%	—
3	Short stature, facial asymmetry, normal intelligence (gonadal status unknown)	43%	57%	83%	17%
Present case	Secondary amenhorroea, facial asymmetry, mild dysmorphism, mild mental retardation, short stature	25%	75%	77%	23%

eral genua valga, flat feet, and asymmetry of the lower limbs, the right being 2 cm shorter than the left. Several naevi were present on the face, trunk, and limbs. The patient had been treated for dorsal scoliosis. Pelvic ultrasound showed hypoplasia of the uterus and gonads, with normal kidneys. Brain magnetic resonance imaging showed a partially empty sella with pituitary hypoplasia.

A cytogenetic investigation performed on peripheral blood lymphocytes showed the presence of a mosaic chromosome constitution, 45,X/47,XX,+18. Out of a total of 200 metaphases scored, 25% were 45,X and 75% were 47,XX,+18. The distribution of the two cell lines was found to be reversed in skin fibroblasts; the 45,X and 47,XX,+18 karyotypes were found in 77% and 23% of the cells, respectively. Trisomy 18 was confirmed by fluorescence in situ hybridisation with a chromosome 18 specific painting probe (Oncor, Gaithesburg, MD). Parental chromosomes were normal.

In order to determine the mechanisms underlying mosaic aneuploidy, genomic DNA extracted from peripheral blood leucocytes and skin fibroblasts was subjected to 30 rounds of PCR amplification in the presence of α^{32}P-dCTP, followed by electrophoresis on denaturing polyacrylamide gels. The average genetic distance between the informative chromosome 18 loci investigated was 13.5 cM according to the integrated Genethon and CHLC maps. The parental origin of the missing X chromosome was determined by visual comparison of band intensities within and between individual electrophoretic lanes.

The proband's DNA displayed both a paternal and a maternal allele at X chromosome loci DXS1003, ALAS2, DXS983, DXS984, and DXS454 (fig 2). However, the latter showed consistently reduced intensity compared to the former on visual inspection of the autoradiograms. This difference was more pronounced in DNA extracted from skin fibroblasts, where the majority of the cells were monosomic for the X chromosome, than in leucocyte DNA, where the XX hyperdiploid line predominated. Based upon these data, it was concluded that the zygote initially possessed both a paternally and a maternally derived X chromosome, and subsequently lost the maternal copy at an early stage of development.

A single paternal and a single maternal allele were observed in the proband's DNA at the following informative loci on chromosome 18: D18S59, D18S476, D18S63, D18S464, D18S542, D18S453, D18S56, D18S468, D18S450, D18S483, D18S543, and D18S844 (fig 2). Although double crossovers between two adjacent markers might have occurred, the likelihood of such events is very low, in view of the spacing of the loci investigated. Therefore, it was concluded that the trisomic cell line most likely originated in a somatic cell at the postzygotic stage.

The clinical manifestations observed in our proband can be attributed to the combined effects of mosaic monosomy X and trisomy 18. The phenotype of the three previously reported cases with a similar chromosome constitution is highly variable, ranging from multiple malformations with early mortality[5] to normal intelligence with predominance of Turner stigmata.[3 4] In our proband clinical manifestations attributable to trisomy 18, such as mild mental retardation, small mouth with thin lips, arches on the fingertips, and partially empty sella, were more pronounced compared to two of the previous patients who had a lower proportion of trisomic cells both in blood and in skin fibroblasts[3 4] (table 1).

However, it must be noted that, owing to lack of information on the distribution of the aneuploid cell lines in additional tissues and at different stages of development, clear cut correlations between phenotypic expression and cytogenetic findings cannot always be established. Thus, the severe trisomy 18 manifestations observed in a 45,X/47,XY,+18 patient who had a relatively low proportion of trisomic cells in both lymphocytes and fibroblasts[5] (table 1) may be related to the presence of a higher fraction of trisomic cells in other tissues. Also, the percentage of trisomic cells in peripheral blood is not always associated with more severe manifestations in mosaic trisomy 18 cases, as shown by the finding of trisomy 18 in 100% of lymphocytes and 8% of skin fibroblasts in a girl with normal intelligence and mild dysmorphism.[6]

The results of microsatellite typing suggest that both aneuploidies originated as independent somatic events at an early developmental stage. Since no euploid mitoses were found, the first error, involving chromosome 18, might have occurred at the first zygotic division, giving rise to a trisomic and to a non-viable monosomic cell. Thereafter, the trisomic line should have lost chromosomes X and 18, either simultaneously or at subsequent stages. Alternatively, two independent errors, involving chromosome 18 and the X chromosome, might have occurred separately in the two cells after normal completion of the first zygotic division.

Theoretically, mixoploid chromosome constitutions may arise by combined meiotic/mitotic errors or by a series of mitotic errors. The latter sequence of events seems to be more common, since it has been reported in three out of four cases, including the present one, in which DNA studies were performed.[1 2] It remains to be established whether the concomitance of several independent mitotic errors is determined by chance events or by an underlying chromosome instability, related to disturbance of the mechanisms which control correct chromosome segregation at mitosis. The latter hypothesis might also explain why the observed frequencies of double aneuploidies seem to be higher than those expected based on the prevalences of the single aberrations.[7]

The authors are indebted to Laura Alesi and Maurizio Graziosi for expert technical assistance.

MAURIZIO GENUARDI
M GRAZIA POMPONI
LOREDANA TORRISI
GIOVANNI NERI
Istituto di Genetica Medica, Facoltà di Medicina e Chirurgia "A Gemelli", Università Cattolica del S Cuore, Largo Francesco Vito 1, 00168 Rome, Italy

M LETIZIA STAGNI
CARLA TOZZI
Laboratorio di Citogenetica, Servizio di Anatomia ed Istologia Patologica, Azienda Ospedaliera S Giovanni-Addolorata, Rome, Italy

1 DeBrasi D, Genuardi M, D'Agostino A, *et al*. Double autosomal/gonosomal aneuploidy: study of nondisjunction in two cases with trisomy of chromosome 8. *Hum Genet* 1995;**95**:519-25.
2 Harada N, Abe K, Nishimura T, *et al*. Origin and mechanism of formation of 45,X/47,XX,+21 mosaicism in a fetus. *Am J Med Genet* 1998;**75**:432-7.
3 Franceschini P, Guala A, Camerano P, Franceschini D, Varden MP, Signorile F. Ulrich-Turner phenotype with unusual manifestation in a patient with mosaicism 45,X/47,XX,+18. *Am J Med Genet* 1996;**62**:26-8.
4 Schinzel AA, Schmid W, Prader A. Turner phenotype: mosaic 45,X/47,XY,+18. *J Med Genet* 1974;**11**:101-4.
5 Serville F, Fontan D, Laurent C, Cazauran JM, Verger P. Mosaic 45,X/47,XY,+18. *Hum Genet* 1977;**36**:351-3.
6 Graham DA, Jewitt MM, Fitzgerald PH. Trisomy 18 mosaicism with complete peripheral lymphocyte trisomy and normal intelligence. *Clin Genet* 1991;**41**:36-8.
7 McCorquodale MM, Cummins T, Furlong JJ. Prenatal diagnosis of trisomy 21 and X/XX sex chromosome mosaicism. *Prenat Diagn* 1977;**5**:295-8.

J Med Genet 1999;**36**:498

Prevalence of Prader-Willi and Angelman syndromes among mentally retarded boys in Brazil

EDITOR—Prader-Willi (PWS) and Angelman syndromes (AS) are caused by paternal or maternal deletions in 15q11-q13 respectively. Although diagnosis of these diseases may occasionally be made by high resolution chromosomal studies, it is best achieved using FISH or parent of origin analysis on Southern blots. To establish the prevalence of PWS and AS in the general population or among mentally retarded subjects, we need easy, rapid, and low cost screening procedures. In the July 1998 issue of the *Journal of Medical Genetics*, Jacobsen *et al*[1] described a screening procedure based on loss of heterozygosity at five microsatellite loci studied by multiplex PCR. For those subjects who were homozygous for the five loci, they conducted further testing with 12 additional microsatellite loci and a methylation sensitive PCR assay (M-PCR) at the SNRPN locus. With this screening approach they examined 285 profoundly retarded adults and detected four cases of AS, a prevalence of 1.4%.

We have recently completed a study searching for PWS and AS in a group of moderately to severely mentally retarded boys in Brazil. Our screening procedure was based on two tests, the methylation sensitive PCR assay for the α exon in the 5' region of the SNRPN gene (M-PCR)[2 3] and a multiplex PCR involving three of the polymorphic microsatellites in the critical PW/AS region.[4] For the former, PCR products were separated on non-denaturing polyacrylamide gels stained by silver nitrate,[5] while for the latter we used Cy5 labelled primers followed by resolution in an automatic fluorescent DNA sequencer (ALF Express, Pharmacia Biotech). Based on frequencies estimated on normal Brazilian subjects, we would expect that less than 1% of the population would be homozygous for all three microsatellites by chance alone. This double screening procedure was capable of diagnosing with 100% accuracy five patients (three PWS and two AS) known to have chromosomal deletions by FISH (Coriell Cell Repository, Camden, New Jersey, USA) when studied together with DNA samples from 20 normal Brazilians.

Among mentally retarded boys attending schools for the mentally handicapped in the city of Belo Horizonte, Brazil, 285 without Down syndrome were randomly ascertained. These 256 boys (127 severely retarded and 129 with mild-moderate mental retardation), whose parents gave written permission, were included in the study. School professionals assessed the severity of the mental retardation by applying the Raven scale. All children had been clinically examined by one of us (MJBA). Our results showed that all the boys displayed amplification of both maternal and paternal bands on the M-PCR. Four boys showed a single band at all three microsatellite loci but were considered to be true homozygotes because of a normal M-PCR result and also absence of clinical evidence of PWS or AS on clinical examination. We concluded that none of the 256 patients had PWS or AS.

Jacobsen *et al*[1] found a minimum prevalence of 1.4% for AS among severely mentally retarded adults. However, their finding of four affected out of 285 tested is not significantly different from zero by any statistical procedure. Moreover, their study population consisted of severely retarded adults while we studied children with varying degrees of involvement.

In conclusion, our data are at variance with the claim made by Jacobsen *et al*[1] of a high prevalence of AS among the mentally retarded. Further studies will have to be undertaken in an effort to assess the true prevalence of PWS and AS among mentally retarded subjects.

ANDRÉIA M L VERCESI
Department of Biochemistry, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

MARIA RAQUEL S CARVALHO
Department of General Biology, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

MARCOS J B AGUIAR
Department of Paediatrics, Universidade Federal de Minas Gerais, and GENE - Núcleo de Genética Médica, Belo Horizonte, MG, Brazil

SÉRGIO D J PENA
Departmento de Bioquímica e Imunologia, Instituto de Ciências Biológicas-UFMG, Av Antônio Carlos 6627, 31.270-901 Belo Horizonte, MG, Brazil, and GENE - Núcleo de Genética Médica, Belo Horizonte, MG, Brazil

1 Jacobsen J, King BH, Leventhal BL, Christian SL, Ledbetter DH, Cook EHJ. Molecular screening for proximal 15q abnormalities in a mentally retarded population. *J Med Genet* 1998;**35**:534-8.
2 Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;**93**:9821-6.
3 Kubota T, Das S, Christian SL, Baylin SB, Herman JG, Ledbetter DH. Methylation-specific PCR simplifies imprinting analysis. *Nat Genet* 1997;**16**:16-17.
4 Mutirangura A, Greenberg F, Butler M, *et al*. Multiplex PCR of three dinucleotide repeats in the Prader-Willi/Angelman critical region (15q11-q13): molecular diagnosis and mechanism of uniparental disomy. *Hum Mol Genet* 1993;**2**:143-51.
5 Santos FR, Pena SDJ, Epplen JT. Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. *Hum Genet* 1993;**90**:655-6.

J Med Genet 1999;**36**:499–500

Mutation of FGFR2 (cys278phe) in craniolacunia and pansynostosis

EDITOR—Craniolacunia ("Lückenschädel") describes rounded or finger-like defects of the inner table of the membranous portion of the skull that are surrounded by strips of normal bone.[1] In most cases, craniolacunia is associated with spina bifida and a resulting myelo- or myelomeningocele. Additional associated anomalies include hydrocephalus, Arnold-Chiari malformation (congenital hindbrain hernia), and Klippel-Feil sequence (fusion of cervical vertebrae/hemivertebrae).[2] The pathological mechanism resulting in craniolacunia is not known. Since depressions in the inner table of the calvarium are particularly deep in cases of oxycephaly, scaphocephaly, and brain tumour, increased intracranial pressure was invoked as an aetiological factor.[1] Increased intracranial pressure, however, can give rise to impressiones digitatae (impressions of cerebral gyri) but does not in itself result in craniolacunia. Thus, craniolacunia has not been reported in any patient with FGFR associated autosomal dominant craniosynostosis. A more recent hypothesis assumes inter-

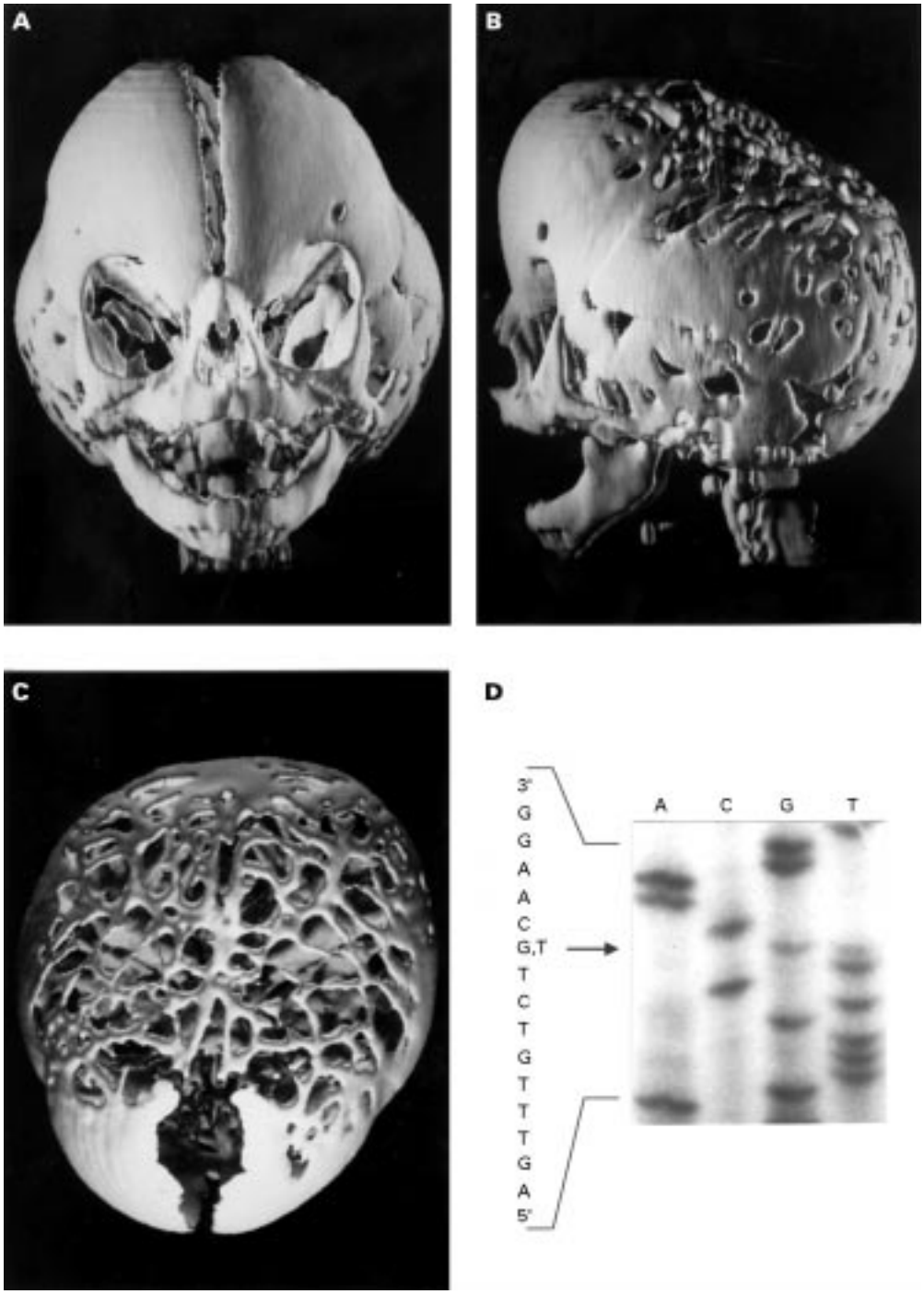

Figure 1 3D computed tomography of the patient's skull at 3 months (A-C). (D) DNA sequence of exon 7 showing a G→T transversion at nucleotide position 833 (counted from the first base of the start codon ATG). This results in the exchange of a cysteine for a phenylalanine at amino acid position 278.

nal decompression and concomitant external compression of the calvarium as the cause of craniolacunia.[2] Accordingly, an abnormal contact between brain and membranous calvarium may cause abnormal radial progression of ossification during early embryogenesis (8-12 weeks' gestation). Based on the frequent finding of an association of craniolacunia and spina bifida, some authors have suggested that the continued escape of fluid from a myelocele or into an encephalocele might cause microcephaly and craniolacunia.[3] However, none of these mechanistic explanations is entirely convincing. As a first step towards an understanding of craniolacunia at the molecular level, we report on a mutation in the fibroblast growth factor receptor 2 (FGFR2) gene in a girl with severe craniolacunia.

The patient was born at term to a 38 year old mother and a 46 year old father after an uneventful pregnancy. At birth, she presented with cloverleaf skull, microcephaly owing to pansynostosis of the calvarial sutures, and proptosis. Magnetic resonance imaging (MRI) showed an Arnold-Chiari malformation. The thumbs and big toes were slightly broadened. No other limb abnormalties were observed. 3D computed tomography of the skull at 3 months showed characteristic craniolacunia (fig 1A-C). She underwent craniectomy at 3 months and resection of the cerebellar tonsils six weeks later. MRI investigation of the vertebral column at 12 months showed normal vertebrae without evidence of spina bifida. At 1 year of age she is developing normally and is starting to walk and talk.

EDTA blood from the patient and her clinically normal parents was obtained and DNA was extracted according to standard procedures. Exon 7[6] (exon U according to Miki *et al*,[5] exon 5 according to Johnson *et al*[4]) of the FGFR2 gene was amplified by PCR using primers 5'GTCTCTCATTCTCCCATCCC3' (forward) and 5'GAAGGAGACCCCAGTTGTG3' (reverse) (30 cycles at 94°C, 61°C, 72°C for 30 seconds each preceded by three minutes at 94°C and followed by a final extension at 72°C for seven minutes). In addition, paternity was confirmed using the STRP markers HUMFIBRA, HUMVWFA31/A, D18S51, HUMTHO1, D19S253, and D21S11 of Urquhart *et al*.[7]

The amplification product of exon 7 was sequenced and a G→T transversion was observed at nucleotide position 833 (counted from the first base of the start codon ATG) resulting in the exchange of a cysteine for a phenylalanine at amino acid position 278 in the patient. This cys278phe mutation was not found in either parent. Paternity was confirmed using six STRPs, all of which were informative (not shown), thus indicating that the mutation had occurred de novo. Changes of the cysteine at position 278 have been detected in other cases of craniosynostosis,[8–10] but craniolacunia was not reported in any of these. Alteration of the cysteine disrupts a central disulphide bond within immunoglobulin-like loop III of the FGF receptor. Consequently, its structural integrity is destroyed and the receptor might become constitutively activated,[11] as suggested by experiments in a *Xenopus* oocyte system. The formation of novel intermolecular disulphide bonds might cause this activation,[12] which appears to trigger premature closure of the calvarial sutures. Although a role of the defective receptor in the origin of craniolacunia can at present not be proven, the physiological function of FGFs in osteogenesis[13] makes such a function likely. Given that identical mutations in FGFR can result in completely different phenotypes,[14 15] additional genes ("modifier genes") are thought to play a major role in the resulting phenotype of FGFR associated craniosynostoses. In the present case this phenotype includes craniolacunia. The absence of spina bifida in our patient precludes a mechanistic model involving abnormal flux of cerebrospinal fluid from a myelocele.[2]

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to DS and UM (STE770/1-2).

D STEINBERGER
U MÜLLER

Institut für Humangenetik, Schlangenzahl 14, 35392 Giessen, Germany

T H JÜNGER
H P HOWALDT

Zentrum für Zahn-, Mund-, und Kieferheilkunde, Mund-, Kiefer- und Gesichtschirurgie, 35392 Giessen, Germany

P CHRISTOPHIS

Zentrum für Neurologie und Neurochirurgie, Neurochirurgische Klinik, 35392 Giessen, Germany

1 Vogt EC, Wyatt GM. Craniolacunia (Lückenschädel). *Radiology* 1941;**36**:147-53.
2 Wilson WG, Alford BA, Schnatterly PT. Craniolacunia as the result of compression and decompression of the fetal skull. *Am J Med Genet* 1987;**27**:729-30.
3 Gardner WJ, Poolos PN. Craniolacunia: the result of embryonal hydrocephalus? *Teratology* 1976;**13**:189-90.
4 Johnson DE, Lu J, Chen H, Werner S, Williams LT. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol Cell Biol* 1991;**11**:4627-34.
5 Miki T, Bottaro DP, Fleming TP, *et al*. Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc Natl Acad Sci USA* 1992;**89**:246-50.
6 Givol D, Yayon A. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J* 1992;**6**:3362-9.
7 Urquhart A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. *BioTechniques* 1995;**18**:116-21.
8 Oldridge M, Wilkie AOM, Slaney SF, *et al*. Mutations in the third immunoglobulin domain of the fibroblast growth factor receptor-2 gene in Crouzon syndrome. *Hum Mol Genet* 1995;**4**:1077-82.
9 Meyers GA, Day D, Goldberg R, *et al*. FGFR2 exon IIIa and IIIc mutations in Crouzon, Jackson-Weiss, and Pfeiffer syndromes: evidence for missense changes, insertions, and a deletion due to alternative RNA splicing. *Am J Hum Genet* 1996;**58**:491-8.
10 Steinberger D, Vriend G, Mulliken JB, Müller U. The mutations in FGFR2-associated craniosynostoses are clustered in five structural elements of immunoglobulin-like domain III of the receptor. *Hum Genet* 1998;**102**:145-50.
11 Neilson KM, Friesel R. Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane and kinase domains. *J Biol Chem* 1996;**271**:25049-57.
12 Robertson SC, Meyer AN, Hart KC, Galvin BD, Webster MK, Donoghue DJ. Activating mutations in the extracellular domain of the fibroblast growth factor receptor 2 function by disruption of the disulfide bond in the third immunoglobulin-like domain. *Proc Natl Acad Sci USA* 1998;**95**:4567-72.
13 Wilkie AOM. Craniosynostosis: genes and mechanisms. *Hum Mol Genet* 1997;**6**:1647-56.
14 Steinberger D, Reinhartz T, Unsöld R, Müller U. FGFR2 mutation in clinically nonclassifiable autosomal dominant craniosynostosis with pronounced phenotypic variation. *Am J Med Genet* 1996;**66**:81-6.
15 Pulleyn LJ, Reardon W, Wilkes D, *et al*. Spectrum of craniosynostosis phenotypes associated with novel mutations at the fibroblast growth factor receptor 2 locus. *Eur J Hum Genet* 1996;**4**:283-91.

J Med Genet 1999;36:501–502

Smith-Magenis syndrome and tetralogy of Fallot

EDITOR—The interstitial deletion of the short arm of chromosome 17 was first reported in 1982 by Smith *et al*[1] in two unrelated patients presenting with congenital heart defects and facial clefts. Since then a clinically recognisable phenotype has been described,[2–8] including brachycephaly, midfacial hypoplasia, a broad, flat nasal bridge, prominent lower jaw, inverted V shaped upper lip with a prominent cupid's bow, hoarse voice, speech delay, psychomotor and growth retardation, and behavioural problems which are often characteristic. Behavioural problems are present in most patients and include self-injurious behaviour such as hand biting, head banging, onychotillomania (pulling out finger and toenails), and polyembolokiolamania (the insertion of foreign bodies into body orifices).[9] A characteristic self hugging behaviour has also been described.[10] Hearing loss, ocular problems,[11 12] scoliosis, and sleep disturbance[13] also occur.

The estimated frequency of cardiac lesions is 37%[4] and those which have previously been described include atrial septal defects, ventricular septal defects, tricuspid and mitral valve stenoses or regurgitation, mitral valve prolapse, subvalvular aortic stenosis, and supravalvular pulmonary stenosis.[2 4]

The estimated frequency of SMS is 1 in 25 000 but this may be an underestimate, as if the deletion is small it may be missed on routine cytogenetic analysis and may only be detectable by fluorescence in situ hybridisation (FISH).

Diagnosis of SMS is often delayed until the characteristic physical and behavioural phenotypes become evident and diagnosis in an infant can be difficult, particularly for a non-geneticist. We report two cases who presented with similar cardiac defects and in whom the diagnosis was made on the basis of routine cytogenetic investigation before the phenotype had fully evolved.

Patient 1 was born at 38 weeks' gestation after an uneventful pregnancy weighing 2100 g (<3rd centile). His head circumference was 31.5 cm (<3rd centile). He was admitted to the neonatal unit as he was small for gestational age and hypothermic. He became cyanotic at 12 hours of age and an echocardiogram showed features of TOF with a patent ductus arteriosus.

Subtle dysmorphic features were noted in the patient. He had a round face, brachycephaly, hypertelorism, low set ears, a flat nasal bridge, a prominent cupid's bow, and a short philtrum (fig 1). He had deep creases on the palms of his hands and soles of his feet, and his finger tips were square. He had bilateral inguinal herniae.

Cytogenetic analysis was requested by the cardiologists to exclude DiGeorge syndrome, but identified a microscopically visible deletion of the proximal short arm of chromosome 17. In situ hybridisation studies were consistent with a deletion within 17p11.2, confirming a diagnosis of SMS. Parental chromosomes were normal.

His rate of growth is normal although he remains below the 3rd centile for weight. He has shown some signs of developmental delay. At 14 months he is sitting unsupported but not pulling to stand. He is alert, sociable, and vocalising but has no recognisable words. He has not yet encountered any problems with behaviour and has had no disruption of sleep pattern.

The patient had a surgical correction of his congenital heart defect at the age of 9 months and is at present awaiting stenting of his left lower lobe pulmonary artery. Pulmonary valve replacement is planned to follow this.

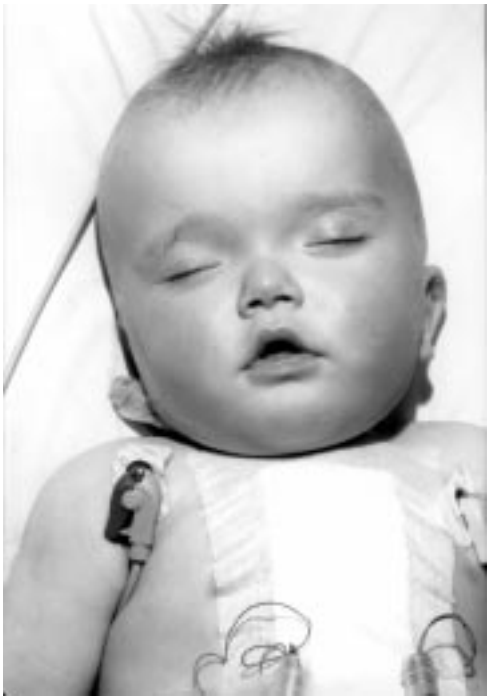

Figure 1 Patient 1.

Patient 2 was born by elective caesarian section for breech presentation at 39 weeks' gestation weighing 2730 g (25th centile). Her head circumference was 32 cm (9th centile) and length was 47.5 cm (9th centile). She was in good condition at birth and required no resuscitation. A systolic murmur was noted on her first day check and subsequently an echocardiogram confirmed TOF.

Chromosome analysis was requested by the cardiologists, again to exclude DiGeorge syndrome. This patient also had a microscopically visible interstitial deletion of chromosome 17p11.2. FISH studies using the probe D17S258 confirmed a deletion in the region 17p11.2 known to be responsible for Smith-Magenis syndrome. Parental chromosomes were normal.

This patient had dysmorphic features such as brachycephaly, a flat, wide nasal bridge, epicanthic folds, and a low set, prominent left ear. Her weight and head circumference have continued to follow the 10th centile. At the age of 1 year she began to manifest signs of developmental delay. She was still quite floppy, being unable to sit without support. Her hearing was normal on testing and she had two syllable babble. She smiled, was sociable and cooperative, and has not as yet had any problems with behaviour or sleep disturbance. An ophthalmology assessment has been normal apart from finding slight myopia. At the age of 18 months she was crawling but not yet walking. From the cardiac point of view, she has had her TOF repaired with an excellent result.

There is a well known association between congenital heart disease and some interstitial chromosomal deletions, and chromosomal analysis is often requested by cardiologists to rule out deletions particularly of chromosome 22q11, characteristic of DiGeorge syndrome. This is the case with both of the patients we report. SMS is a syndrome which may not be well known to cardiologists and dysmorphic features may be subtle, making a clinical diagnosis difficult in an infant. It is fortuitous that the deletions in our patients were visible microscopically, as in

some patients with SMS the deletion is not easily visible and the diagnosis would first have to be suspected and FISH for 17p11.2 be specifically requested. We suggest that SMS is a diagnosis worth considering in the differential diagnosis of children with congenital heart disease and appropriate dysmorphic features, even if initial routine cytogenetic analysis has been unfruitful. This may be particularly relevant to cardiologists who may not be familiar with the syndrome.

It is also of interest that the heart lesion in our patients is not one that has previously been reported in association with SMS and we suggest that TOF should be considered as part of the spectrum of features which can be found in patients with SMS.

It is not yet known whether children with deletions detectable only by FISH can be expected to have fewer problems than those with a cytogenetically visible deletion. It could be argued that a larger, visible deletion may involve more than just the genes in the critical region involved in the development of the classical SMS phenotype. If it was found that children with deletions visible only by FISH did have a milder phenotype, this would be useful in counselling parents.

Our patients were diagnosed early, before the phenotype had fully evolved. If diagnosis is delayed parents may feel that they are in some way to blame for the behavioural problems encountered by their child. Early diagnosis would enable explanation of the condition so that parents could be aware of potential problems before they occur, although behavioural problems may be difficult to discuss with parents of a young baby. Early diagnosis would also enable discussion of recurrence risks and prenatal diagnosis as, even if the deletion appears de novo, the small risk of germline mosaicism remains.

The authors would like to thank Drs Alan Fryer and Ian Ellis for their help and advice.

ELIZABETH SWEENEY

Mersey Regional Clinical Genetics Service, Royal Liverpool Children's Hospital, Alder Hey, Eaton Road, Liverpool L12 2AP, UK

IAN PEART
MAGDI TOFEIG

Department of Paediatric Cardiology, Royal Liverpool Children's Hospital, Alder Hey, Eaton Road, Liverpool 12 2AP, UK

BRONWYN KERR

Department of Genetics, Royal Manchester Children's Hospital, Pendelbury, Manchester M27 1HA, UK

1 Smith ACM, McGavran L, Waldstein G, Robinson J. Deletion of the 17 short arm in two patients with facial clefts. *Am J Hum Genet* 1982;**34**:410A.
2 Smith ACM, McGavran L, Robinson J, *et al.* Interstital deletion of (17)(p11.2p11.2) in nine patients. *Am J Hum Genet* 1986;**24**:393-414.
3 Stratton RF, Dobyns WB, Greenberg F, *et al.* Report of six additional patients with a new chromosome deletion syndrome. *Am J Med Genet* 1986;**24**:421-32.
4 Greenberg F, Lewis RA, Potocki L, *et al*. Multi-disciplinary clinical study of Smith-Magenis syndrome (deletion 17p11.2). *Am J Med Genet* 1996;**62**: 247-54.
5 Lockwood D, Hecht F, Dowman C, *et al.* Chromosome subband 17p11.2 deletion: a minute deletion syndrome. *J Med Genet* 1988;**25**:732-7.
6 Moncla A, Livet MO, Auger M, Mattei MG, Giraud F. Smith-Magenis syndrome: a new contiguous gene syndrome. Report of three new cases. *J Med Genet* 1991;**28**:627-32.
7 Colley AF, Leversha MA, Voullaire LE, Rogers JG. Five cases demonstrating the distinctive behavioural features of chromosome deletion 17(p11.2p11.2) (Smith-Magenis syndrome). *J Paediatr Child Health* 1990; **26**:17-21.
8 Greenberg F, Guzzetta V, Montes de Oca-Luna R, *et al.* Molecular analysis of the Smith-Magenis syndrome: a possible contiguous-gene syndrome associated with del(17)(p11.2). *Am J Hum Genet* 1991;**49**:1207-18.
9 Smith ACM, Dykens E, Greenberg F. Behavioural phenotype of Smith-Magenis syndrome (del 17p11.2). *Am J Med Genet* 1998;**81**:179-85.
10 Finucane B, Konar D, Haas-Givler B, Kurtz MB, Scott CI Jr. The spasmodic upper body squeeze: a characteristic behaviour in Smith-Magenis syndrome. *Dev Med Child Neurol* 1994;**36**:78-83.
11 Finucane BM, Jaeger ER, Kurtz MB, Weinstein M, Scott CI Jr. Eye abnormalities in the Smith-Magenis contiguous gene deletion syndrome. *Am J Med Genet* 1993;**45**:443-6.
12 Barnicoat AJ, Moller HU, Palmer RW, Russell-Eggitt I, Winter RM. An unusual presentation of Smith-Magenis syndrome with iris dysgenesis. *Clin Dysmorphol* 1996;**5**:153-8.
13 Smith ACM, Dykens E, Greenberg F. Sleep disturbance in Smith-Magenis syndrome (del 17 p11.2). *Am J Med Genet* 1998;**81**:186-91.

NOTICE

British Human Genetics Conference

The British Human Genetics Conference will be held on 27-29 September 1999 at the University of York, England. There will be special sessions on: Animal models: opportunities and limitations; Telomeres and telomerase; Cross cultural counselling; Joint symposium with the Cancer Family Study Group "How do faulty genes cause cancer"; Workshops including "CF - 10 years on" and "DNA breakage and recombination at meiosis", as well as plenary sessions. The Carter Lecture will be given by Professor John Todd on "Genetics, aetiology, apoptosis, and natural selection in type 1 diabetes". Further information from the Conference Office, British Society for Human Genetics, Clinical Genetics Unit, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK. Tel/fax: 0121 627 2634, email: bshg@bham.ac.uk, website: http://www. bham.ac.uk/bshg