A supernumerary marker chromosome with a neocentromere derived from 5p14→pter

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EDITOR—Supernumerary marker chromosomes (SMCs) comprise a heterogeneous group of structurally arranged chromosomes. SMCs are found in approximately 0.14-0.72/1000 newborns[1–3] and they may be associated with developmental abnormalities and malformations.[4] The great variability of clinical symptoms in patients with SMCs is the result of the difference in the genetic content of the marker. The phenotypic consequences of SMCs are difficult to predict, especially if a de novo marker is detected prenatally. Therefore, the precise identification of a marker chromosome is of essential importance in genetic counselling. Earlier figures based on the results of a large prenatal multicentre study using conventional methods suggested a risk for an abnormal phenotype of 13% for SMCs.[5] Combining FISH data and conventional analyses, the estimated risk for an abnormal phenotype turned out to be twice as high, approximately 28% for non-acrocentric autosomal SMCs and about 7% for acrocentric autosomal SMCs.[6] An even more accurate method of identification of the chromosomal origin of supernumerary marker chromosomes is FISH with microdissection probes and reverse painting, allowing a definite delineation of phenotype-karyotype correlations.[7]

In recent years, a novel class of mitotically stable human marker chromosomes that are devoid of alpha satellite DNA has been identified.[8 9] These analphoid markers have been shown to contain functional centromeres outside the normal centromere domain, which are called neocentromeres. Marker chromosomes derived from chromosome 5 are rare and a marker chromosome 5 with a neocentromere has not been reported so far.

We describe a patient with an inverted duplication of the distal part of the short arm of chromosome 5 and the formation of a neocentromere leading to a supernumerary marker chromosome. The comparison of the clinical findings of this patient with a tetrasomy of distal 5p with similar cases previously described suggests a gene dosage effect of this chromosome segment. Absence of centromere specific sequences in the marker and a weak reaction with anticentromere antibodies indicates the formation of a neocentromere between bands 5p14 and 5p15 and therefore the first analphoid SMC described that originates from chromosome 5.

Case report

This girl is the first child of healthy, non-consanguineous parents. The mother was 21 and the father 25 years old at her birth. Prenatal ultrasound showed intrauterine growth retardation with an abdominal circumference below the mean. The fetus showed severe microretrognathia (fig 1) and unilateral foot deformity on ultrasound. A transabdominal chorionic villous biopsy, performed at 25 weeks of gestation, showed an aberrant 47,XX,+?C karyotype in three metaphases analysed from direct villi preparations. G banded chromosome preparations from an amniocyte sample showed an unbalanced female karyotype in the fetus with 47,XX,+mar. The supernumerary marker was detected in all 19 metaphases analysed. Normal parental karyotypes indicated a de novo origin of the marker in the fetus.

The parents decided to continue the pregnancy. Delivery was induced at gestational week 37 (+5) because of cessation of intrauterine growth. The girl weighed 1890 g (<3rd centile) and was 43 cm long (3rd centile). The head circumference was 31.5 cm (3rd centile). The Apgar score was 1/6/7. She had respiratory difficulties and was ventilated for four days. She had microretrognathia and a cleft hard and soft palate (Pierre-Robin anomaly). Spontaneous motor activity was markedly reduced with generalised muscular hypotonia initially. Muscle tone, however, turned out to be unstable and progressed gradually to hypertonia. She developed contractures of her fingers, elbows, and feet.

At the age of 4½ months, the girl was 58 cm long, weighed 3700 g, and OFC was 37.5 cm. All values were below the 3rd centile. The girl was severely dystrophic and continued to be so at a recent follow up at 8 months. Psychomotor development was delayed. She smiled reactively, but had no head control. She required tube feeding because of dysphagia owing to her

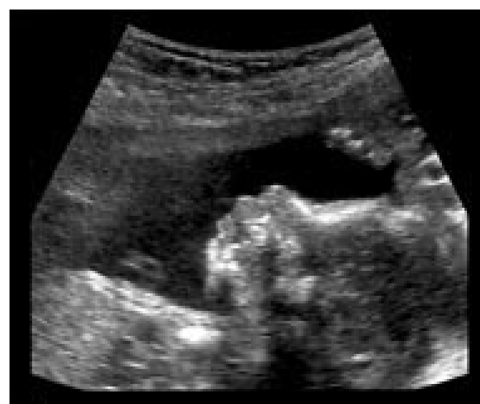

Figure 1 Prenatal ultrasound in the 25th gestational week showing the profile of the fetus with severe retromicrognathia.

cleft palate and generalised muscular hypotonia. Craniofacial features included microcephaly, prominent forehead with narrow temples, telecanthus, slight protrusion of the bulbi, upward slanting, narrow palpebral fissures, a short nose with a depressed nasal bridge, prominent philtrum, small mouth with a thin upper lip, cleft hard and soft palate, severe microretrognathia, and large, thin ears (fig 2). There were no signs of auditory or visual impairment. Radiography showed thoracic asymmetry, a high diaphragm, and a left convex scoliosis of the thoracic spine. Echocardiography identified an atrial septal defect and a hypoplastic right pulmonary artery. Ultrasound examination of the brain showed enlarged lateral ventricles and gyral flattening. She had had two epileptic fits previously (see Note added in proof). Abdominal ultrasound investigations were normal. There was no family history of congenital malformations.

Methods

Prenatal cytogenetic analysis was performed on short term chorionic villi cultures and amniocyte cultures. Chromosomes from peripheral blood were examined at birth and at the age of 6 months. Metaphase chromosomes were analysed by standard trypsin-Giemsa banding (GTG), quinacrine banding (QFQ), C banding (CGB), and NOR staining. In both parents, chromosomes from lymphocyte cultures were examined. To determine the origin of the marker chromosome microdissection, DOP amplification, biotin labelling of the probe, and reverse painting were performed according to the protocol of Friedrich *et al.*[10] To evaluate the construction of the supernumerary marker chromosome, FISH studies were applied using the pan alpha satellite probe ("all human centromere", Oncor Inc, Gaithersburg, MD), a chromosome 1/5/19 specific alpha satellite probe (D1Z7/D5Z2/D19Z3, Oncor), a telomere probe identifying the consensus telomeric sequence (T_2AG_3 "all human telomeres", Oncor) and a cosmid probe specific for the locus D5S23 mapped to subband 5p15.3 (Oncor). Primed in situ labelling (PRINS) with a satellite III DNA probe was carried out following the protocol of Koch *et al.*[11]

The immunofluorescence procedure was essentially the "solvent staining" method developed by Earnshaw *et al.*[12] Before immunostaining, the lymphocyte culture was blocked in colcemid for two hours. The primary antibody was an autoimmune anti-kinetochore antibody (CREST)[13] followed by a FITC labelled goat anti-human antibody (Vector). Results of FISH and immunostaining of the kinetochores were visualised in a Zeiss Axiophot epifluorescence microscope and documented using the digital image capture system ISIS (Metasystems, Altlussheim) connected to a CCD camera.

To disclose the parental origin of the supernumerary marker chromosome, eight polymorphic microsatellites localised to 5pter-5p13 (D5S392, D5S406, D5S1953, D5S208, D5S486, D5S1473, D5S419, D5S426) were analysed by PCR amplification of genomic DNA from the parents and DNA from the patient's placenta. PCR primers were obtained from MWG-Biotech AG (Ebersberg, Germany). DNA was amplified in a total volume of 25 µl using standard conditions and a "high touch down protocol" with annealing temperatures of 63°C.[14] The products were separated on 8% polyacrylamide gels and visualised by silver staining. Dosage differences between alleles indicated the involvement of the respective locus in the triplication.

Results

Prenatal chromosome analysis of amniocyte cultures and postnatal studies in peripheral blood lymphocyte cultures showed 47,XX chromosomes and a non-satellited, C band negative marker of unknown origin in 19 and 100 cells studied respectively. The slightly asymmetrical marker was approximately the size of a chromosome 20. Chromosome analysis of the parents showed normal karyotypes, which indicated a de novo origin of the additional material in the fetus.

In order to determine the origin of the marker, reverse painting was performed (fig 3A). Hybridisation with the microdissection library onto the patient's metaphases gave signals covering the whole marker chromosome and the distal short arm of chromosomes 5

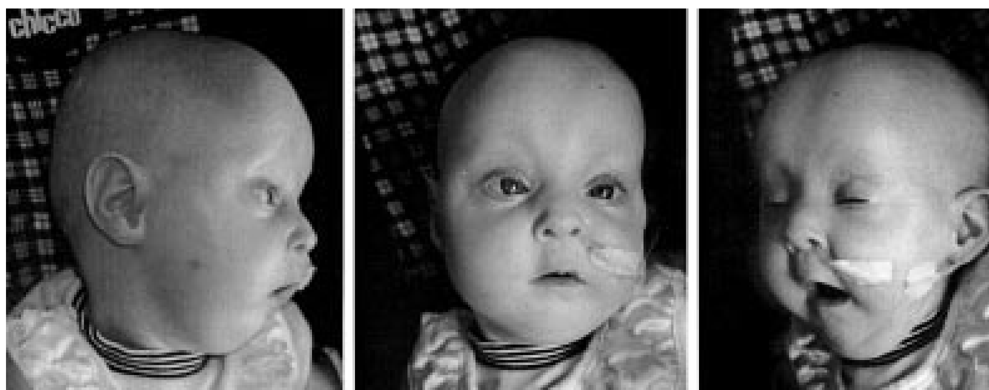

Figure 2 Patient at the age of 8 months with distinct craniofacial dysmorphic features.

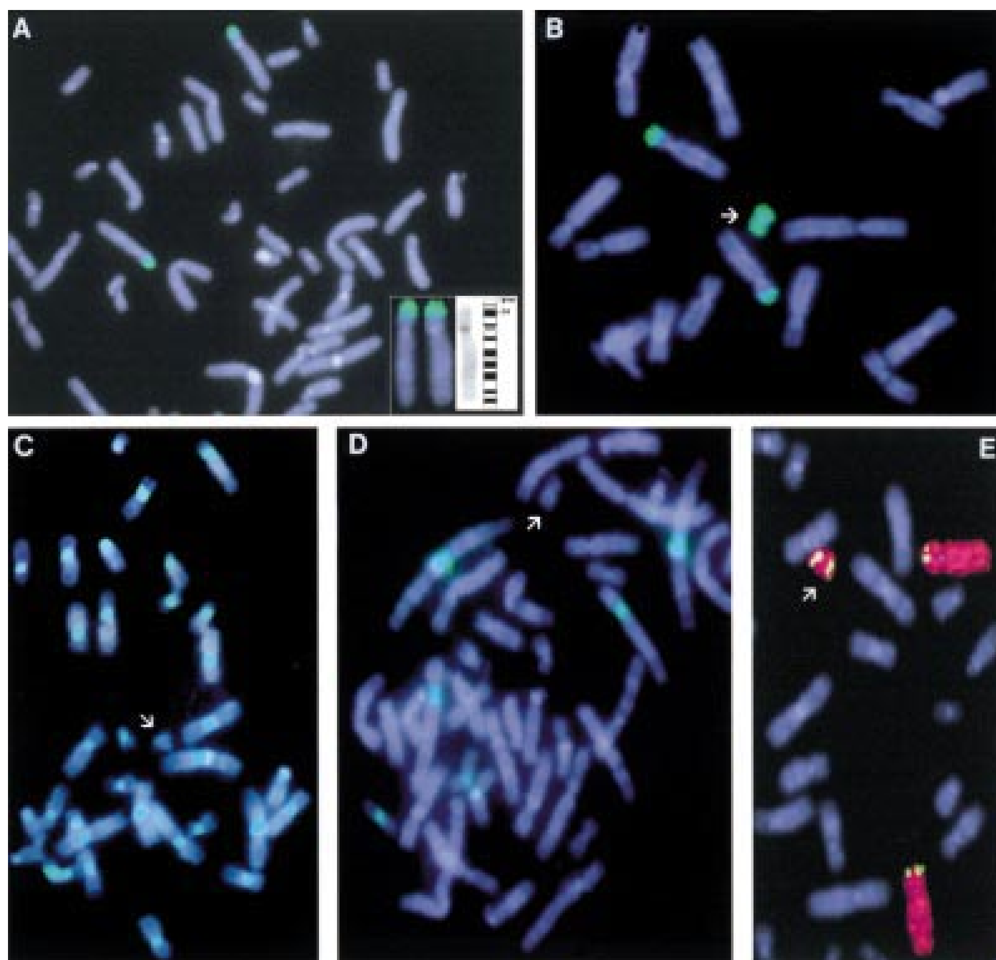

Figure 3 FISH analyses of the patient's chromosomes delineating the structure of the mirror image duplication of the marker chromosome. (A) Reverse chromosome painting using a microdissection library generated from the marker chromosome. Chromosomal painting to a normal lymphocyte metaphase shows a distinct hybridisation signal in distal 5p. The signal position in comparison with the enhanced DAPI banding pattern shows hybridisation signals in 5pter→5p14. (B) The same probe hybridised to the patient's metaphase chromosomes. The entire marker as well as the most distal portion of both chromosomes 5p are painted. No other chromosome is labelled. (C) FISH with a pancentromeric probe failing to detect the centromere of the marker identified by GTG banding. (D) An alphoid probe specific for the chromosomes 1, 5, and 19 alpha satellite does not produce any signal on the marker (arrow). (E) Dual colour FISH with a unique sequence probe localised in 5p15.3 (green signals) and wcp 5 (red signal) on a metaphase of the patient showing two areas of hybridisation on the marker (arrow) indicating an inverted duplication as well as hybridisation on both normal chromosomes.

(p14→pter). No centromeric region was labelled with this probe and no hybridisation signals were seen on other chromosomes (fig 3B). The mitotic stability of the marker chromosome in the patient's cells pointed to the presence of a functional centromere. Hybridisation with a pan alpha satellite centromere probe at low stringency conditions showed signals at the centromeres of all chromosomes except the marker (fig 3C). Neither FISH with a probe specific to the centromeres of chromosomes 1, 5, and 19 (fig 3D) nor PRINS with a pericentromeric satellite III probe yielded detectable centromeric signals (not shown). Telomere and subtelomere specific sequences could be found at both ends of the marker chromosome (fig 3E). There was, however, a weak reaction with antibodies directed against centromere specific proteins (fig 4). Thus, the mitotic stability of the C band negative marker chromosome is the result of the formation of a neocentromere. FISH and G banding studies thus characterised the marker as an inverted duplication of 5p14→pter. The appearance of the marker was not totally symmetrical. A primary constriction at the presumed neocentromere was seen on the border of band 5p14 to 5p15.1 (fig 5). The complementary deletion of chromosome 5 has not been recovered. The patient has, therefore, partial tetrasomy of the distal part of chromosome 5p, and the karyotype is 47,XX,+inv dup (5)(pter→p14::p14[neocen]→pter).

For determination of the parental origin of the de novo marker, eight different DNA polymorphisms were tested on genomic DNA. While most of the markers were uninformative, loci D5S1473 and D5S426 showed evidence of an increase in paternal dosage indicating paternal origin of the marker chromosome (fig 6).

Discussion

A partial tetrasomy of 5p14→pter was found in a newborn girl with congenital anomalies. It was the result of a presumably mitotically stable, supernumerary marker chromosome. The identification of the marker was achieved using chromosomal microdissection, generating a painting probe via DOP-PCR and reverse

Figure 4 Immunostaining of kinetochores using anticentromere antibodies showing weak reaction on the marker chromosome. Partial metaphase showing immunostaining with CREST (left), DAPI staining (middle), FISH with a chromosome (5p) microdissection derived probe (right) identifying the marker chromosome.

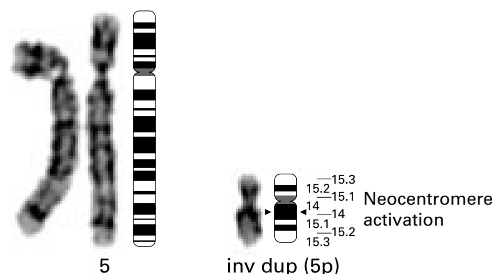

Figure 5 Partial GTG banded karyotype of the patient with the normal chromosomes and the marker chromosome. Ideogram of the marker chromosome showing the chromosomal rearrangement based on the FISH results. Arrowheads indicate the breakpoint at 5p14.

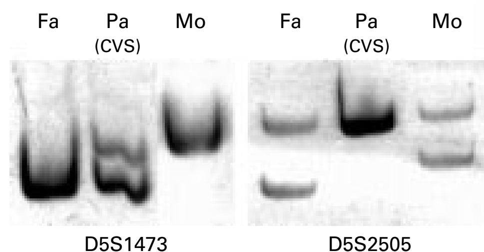

Figure 6 Polyacrylamide gel electrophoresis (PAGE) with PCR products of highly polymorphic microsatellites (D5S1473 and D5S2505) showing paternal origin of the marker chromosome.

painting. FISH performed with a probe from the most distal 5p region showed that the marker was an inverted duplication. So far, there has been only one report of tetrasomy 5p of almost the same region (5p14→p15.3) as in our patient.[15] This patient had a triplication on one chromosome 5 and showed comparable clinical manifestations. In these two patients the most prominent symptoms were severe hypotrophy of the newborn, hypertonia, failure to thrive, brain abnormalities and seizures, a distinct facial appearance (in particular, significant microretrognathia), cardiovascular malformations, and flexion contractures of different joints.

Tetrasomy of the whole short arm of chromosome 5 has been described previously in three patients. These patients, however, were mosaics for an isochromosome 5p.[16–18] With the exception of similar facial dysmorphic signs, they did not appear to be severely affected, although larger regions of chromosome 5 were included in the tetrasomy (table 1). This observation could be explained by the presence of mosaicism.

Duplications leading to complete trisomy of 5p14→pter cause mild clinical symptoms. The main characteristics are short stature and psychomotor retardation indicating that the phenotypic severity might depend on specific regions of the duplicated material.[19] Duplications of the whole of 5p (p11→pter) as well as partial proximal duplications affecting at least band 5p13 are associated with a more severe phenotype including macro- and dolichocephaly, facial anomalies with microretrognathia and large, simple ears, additional eye anomalies, abnormalities of the central nervous system, congenital heart defects, and renal and intestinal malformations. Newborn infants who survived showed moderate to severe mental retardation and nearly half of the patients developed infantile seizures. The critical region of 5p associated with a severe phenotype was, therefore, proposed to be proximal to band p14.[20]

The distinct phenotypes of the two patients with distal tetrasomy, however, seem to be more compatible with the phenotypes of patients with complete trisomy of the whole short arm of chromosome 5 (table 1).[19 21 22] Symptoms shared by both groups of patients include distinct facial anomalies with severe microretrognathia and large ears, eye anomalies, abnormalities of the central nervous system, congenital heart defects, and respiratory difficulties. Discordant findings were microcephaly, hypertonia, and contractures in the two patients with tetrasomy 5p14→pter. In these patients, the most proximal breakpoint of the marker chromosome was estimated to be at 5p14. It may be that for the clinical picture a gene dosage effect is of more significance than the breakpoint involved leading to trisomy or tetrasomy.[15] Deregulated expression of genes can be caused by amplification of regulatory DNA regions. Interestingly, a gene for topoisomerase related function protein 4 (*TRF-4*) required for sister chromatid cohesion and mitotic chromosome condensation resides in 5p14, which is frequently amplified in various tumours.[23 24] Reviewing known genes in 5pter→5p14 shows that the expression pattern of different genes like cadherin 10 or adenylcyclase 2 is largely brain specific and they may be involved in human brain disorders.

Mitotically stable, analphoid markers were first described by Callan *et al*[25] and Crolla *et al*[26] and at least 40 such markers have been registered.[8 9] Chromosomal rearrangements with neocentromeres derived from chromosome 5 have not been reported so far. However,

the characteristics of other analphoid markers are similar to our patient's marker. Inversion duplications are the most common type in that a duplication of a relatively small distal subfragment is seen. As in 21 of 40 cases, the karyotype in our patient is normal except for the supernumerary chromosome giving rise to partial tetrasomy for the duplicated portion. The neocentromere does not occur at the inversion breakpoint, making the appearance of the marker asymmetrical. When examined with molecular techniques, no neocentromere has been shown to localise to the inversion breakpoint.[9] In most cases with tetrasomy, the marker is present in the mosaic state. In our patient the marker was identified in two different tissues, in all 19 amniocytes and in 100 metaphases from peripheral blood. However, the question arises whether the marker will be lost over time. Focusing on markers with neocentromeres, Reddy *et al*[8] reported the development of mosaicism as a result of age, thus suggesting mitotic instability of these markers. In vitro results indicated that markers with neocentromeres are stable in short term lymphocyte cultures, while they are less stable in long term lymphoblast and fibroblast cultures.[27] Mitotic instability has long been known from alphoid markers and even inherited markers may appear as mosaics. In a follow up of children with supernumerary marker chromosomes, Gravholt and Friedrich[28] pointed to the fact that mosaics change constantly under the assumption that markers containing ribosomal DNA proliferate, whereas those lacking ribosomal DNA are selected against and disappear.

DNA polymorphism studies disclosed the paternal origin of the marker and duplication of identical paternal alleles at polymorphic loci suggested a mitotic origin, as already described for other examples of analphoid marker chromosomes.[27 29 30] Deriving from a single parental chromosome, the resulting marker chromosome would, therefore, be identical in their primary DNA sequences in both chromosome arms. This suggests a priori that both arms carry the same putative latent centromeric site. The mechanism of activation or inactivation of a neocentromeric site remains unknown, but the situation parallels that found in dicentric chromosomes, in which only one centromere remains active. Spreading of an epigenetic state *in cis* might be responsible.[31] Generally, the interaction between centromeric DNA and proteins seems to be more complex than previously thought. As shown by Gimelli *et al*[32] recently, the same alpha satellite DNA sequence could either organise an active centromere or in other situations bind many

Table 1 Clinical features in patients with complete trisomy 5p and tetrasomy 5p

		Mosaic tetrasomy (5)(p11→pter)			*Tetrasomy (5)(p14→pter)*	
	Complete trisomy 5p (p11→pter) (n=8)*	*Stanley et al*[16]	*Sijmons et al*[17]	*Lorda-Sanchez et al*[18]	*Harrison et al*[15]	*Present case*
Maternal age	28–47 (mean 29.5)	29	40	31	28	21
Paternal age	26–35 (mean 29.8)	29	40	30		25
Gestational age (weeks)	At term 6	40	34	39	41	38
Sex	4F / 4M	F	M	F	F	F
Birth weight (g)	1275–3000	2670	2550	3040	2900	1890
Birth length (cm)	44–55			46	52.5	43
OFC at birth (cm)	32.5–39			36.5	32.5	31.5
Macro/microcephaly	8/8 macro		Macro	Macro	Micro	Micro
Ventriculomegaly	5/8		+	+	+	+
Supraorbital ridges	1/8 depressed			Depressed	Depressed	Depressed
Hypertelorism	5/8		+			−
Epicanthus	5/8	+				+
Upward slanting palpebral fissures	5/8	+	+			+
Eye abnormalities	1/8				Coloboma	−
Depressed nasal bridge/short nose	7/8		+	+		+
Midface hypoplasia	4/8			+	+	+
Philtrum	5/8 long	Long		Long	Short	Short
Macroglossia	4/8					−
Palate	2/8 high	High				Cleft
Microretrognathia	6/8		+	+	+	+
Dysplastic ears	8/8		+	+	+	+
Preauricular pits	0/8		+	+		−
Short neck/redundant skin	7/8			+	+	−
Clinodactyly	1/8	+		+	+	+
Proximally implanted toes	1/8			+	+	+
Club feet	5/8			+		−
Congenital heart defect	6/8			+	+	+
High diaphragm	1/8				+	+ (right side)
Respiratory difficulties	6/8		+	+		+
Recurrent infections	2/8			+		+
Failure to thrive	4/8			+	+	+
Postnatal growth failure	3/8					+
Muscle tone	8/8 hypo	Hypo	Hypo	Hypo	Hyper	Hypo <-> hyper (alternating)
Flexions contractures	0/8				+	+
Psychomotor retardation	5/8	+	+	+		+
Seizures/abnormal EEG	4/8	+	+	+	+	+
Early death	4/8			+	+	
Others	Genital anomalies, larynx anomaly, bronchomalacia, generalised hyperpigmentation				Gut malrotation, dysplastic kidneys	Thoracospinal scoliosis

*Only cases with complete trisomy 5p without involving another chromosome aberration were considered, six cases reviewed by Lorda-Sanchez *et al*,[19] one by Reichenbach *et al*,[21] and one by Velagaleti *et al*.[22]

fewer proteins, thus forming an inactive centromere.

In our patient, FISH analyses with a chromosome 5 specific alpha satellite probe, a pan alpha satellite probe, and a satellite III probe failed to detect any common centromeric or pericentromeric sequences on the inv dup(5) marker, thus indicating the presence of a neocentromere. Immunofluorescence with antibodies against centromere proteins confirmed the presence of a functional centromere at the constricted arm of the inv dup(5p) chromosome. A neocentromere is a newly derived functional centromere formed outside the normal centromere domain.[33] Neocentromeres have been observed most often in chromosomes 13 and 15, but, so far, they have not been detected in chromosomes 5, 6, 7, 12, 16, 18, and 19.[9] Many theories have been put forward to explain their origin. The most convincing one is the presence of many different centromere competent sites within the human genome, which by epigenetic modification can be modified into functional centromeres.[34] Most probably, the crucial point is not the DNA base sequence as such but rather the conformation assumed by the DNA. Based on ideas from previous publications, Koch[35] has elaborated the hypothesis that double dyad symmetries of a particular size as well as short, conserved base motifs adjacent to the dyad symmetries are common in alpha and non-alpha satellite DNA and may define the mitotically functional human centromere. Further studies will disclose which mechanisms involved in the formation of a neocentromere are valid. This type of marker may therefore be useful in delineating the key components of the functioning centromere.

- We report on a 9 month old girl with congenital anomalies, dysmorphic features, seizures, and psychomotor retardation who had a prenatally diagnosed supernumerary marker chromosome. Chromosomal microdissection and reverse painting disclosed the 5p origin of the marker.
- The marker chromosome reacted weakly with anticentromere antibodies but it was C band negative and devoid of detectable alpha satellite or pericentromeric satellite III sequences suggesting the formation of a neocentromere in a previously undescribed chromosomal region at 5p14.
- The karyotype of the patient was 47,XX,+inv dup(5)(pter→ p14::p14→ [neocen]→pter), resulting in tetrasomy of distal 5p. Paternal origin of the de novo marker was shown by microsatellite analysis.
- The patient had some but not all manifestations of the dup(5p) syndrome including severe microretrognathia and large ears, eye anomalies, abnormalities of the central nervous system, congenital heart defects, and respiratory difficulties.

Note added in proof

At 17 months, the girl had developed recurrent seizures, the EEG being severely abnormal, like the chaotic picture of a hypsarrhythmia.

The microdissection method was established during a guest professorship of Dr Friedrich at the University of Marburg. We are gratefully to Evelyn Winkler for excellent technical assistance. This work was supported by P E Kempkes Stiftung Marburg, We are particularly grateful to the parents of our patient for their cooperation.

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A case of Roberts syndrome described in 1737

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EDITOR—In 1735 Johanna Sophia Schmied, from the village of Taucha near Leipzig, gave birth to a stillborn child with multiple abnormalities, described at the time as a "very rare" monster. The case was reported by a local physician, Gottlieb Friderici, in a tract, *Monstrum humanum rarissimum*, published in Leipzig two years later.[1] Friderici performed a necropsy and published his findings along with a case history of the pregnancy and two detailed plates engraved by a local draughtsman "from life". The mother was aged 28 years, of short stature and slender, with a "choleric-melancholic" temperament. She had been married to a "hunchback" for 10 years, and they had three other children, all "free of imperfections"; the fourth child is that described by Friderici. "Halfway" though the pregnancy, the fetal movements were felt very faintly and the uterus was not thereafter seen to increase in size, whereas her husband recalled that in the previous pregnancies her belly had grown normally. The baby was stillborn after a labour of seven hours.

A large anterior encephalocele was present. Friderici remarked that, although the appearance resembled hydrocephalus, the protuberance contained cerebral matter. The frontal bone was very abnormal to the bridge of the nose. The nose was "vestigial", but the nostrils were patent, and a probe inserted into the oral cavity passed through a fissure in the palatal bone. The mouth was "lipless", the eyes protruded, and the orbits were shallow. A tiny external auditory meatus was found, but the pinnae were absent. The legs, like the forearms, were "simple", composed of only one bone. There were pterygia in the popliteum (M in fig 1), the groin, and running from the mouth to the upper thorax. The digits of the feet were distorted but all digits were present. The fingernails "resembled those of an animal". All ribs were present. In the engraving the thorax appears deformed, though this may be because the illustration was made from the reconstructed body after necropsy. The pleural cavities and pericardium contained "thin fluid". The liver appeared unusually large and the kidneys were unequal in size. Two small intra-abdominal testes were located. No external genitalia were identified. Quantities of meconium were passed via the anus.

The specimen was brought to Friderici and examined within a few hours of delivery. Fig 1 was printed life sized and was hand coloured. The crown-rump length of the figure is 20 cm and the foot length 4.5 cm; these dimensions correspond to a gestational age of some 24 weeks, compatible with the history, and suggesting that it was drawn in the correct proportions. The head appears microcephalic, though this may be because of the encephalocele. The upper limbs show marked shortening, and were likened by Friderici to the wing of a chicken without the feathers. The combination of anterior encephalocele, microcephaly, shallow orbits, cleft palate, marked micrognathia, hypomelia of the upper limbs, single forearm and leg bones (most probably absence of the radius and fibula, though fusion is possible), and flexion contractures is consistent with a severe lethal form of Roberts-SC phocomelia syndrome (MIM 268300). The inheritance of this condition is autosomal recessive with great variability of expression; the largest review has shown that it is more often sporadic than hereditary.[2] Consanguinity is not discussed in the account but the physical descriptions of the parents as of short stature and a "hunchback" do not rule out their having had minor dysmorphic features. It is too early in gestation to assess cryptorchidism, and growth retardation cannot be assessed owing to probable intrauterine death