Rapid identification of multiple supernumerary ring chromosomes with a new FISH technique

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Abstract

Multiple supernumerary ring chromosomes are a rare cytogenetic finding which is poorly understood. With the introduction of FISH techniques, their chromosomal origin can now be defined clearly. The techniques described previously are complicated and time consuming. We report a new rapid technique which has been used to investigate two new cases. Multiple probes were hybridised to a single slide by means of marking the underside with a diamond pen to form a grid of squares, pipetting fixed cell suspension into the centre of each square, forming a rubber solution grid on the denatured, dehydrated slide following the lines on the underside, adding a mixture of probes into each square, and sealing the slide with a silicone rubber rim and a covering slide. The type of probe and the size, dimensions, and number of squares in the grid can be tailored to individual cases.

The two new cases examined here are mosaic for three (case 1) and four (case 2) supernumerary ring chromosomes derived from different chromosomes. Normal cell lines were also present. The karyotypes were established as 47,XY,+r(4)/47,XY,+r(17)/.../48,XY,+r(17),+r(20)/49,XY,+r(4),+r(17),+r(20)/46,XY for case 1 and 47,XX,+r(4)/47,XX,+r(8)/47,XX,+r(10)/48,XX,+r(X),+r(4)/.../49,XX,+r(X),+r(8),+r(10)/46,XX for case 2. Our findings suggest that the ring chromosomes were formed during meiosis, perhaps involving complex rearrangements, resulting in a germ cell containing all markers, with subsequent loss of markers during cell division. Our second case also shows that the outcome is not invariably mental or physical handicap.

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Numerous reports have been published on the presence of one supernumerary marker ring chromosome diagnosed pre- or postnatally.¹⁻³ In comparison, the presence of multiple supernumerary ring chromosomes is a rare cytogenetic finding which is poorly understood.³⁻⁵ Before the introduction of fluorescence in situ hybridisation (FISH) techniques, it was virtu-

ally impossible to ascertain their chromosomal origin. Since then, in situ hybridisation has revolutionised cytogenetics, and these small ring chromosomes can now be defined clearly. However, the techniques described previously are complicated and time consuming.^{2 6 7}

We report a new rapid technique which has been used to investigate two previously unreported cases of multiple supernumerary ring chromosomes.

Case reports

CASE 1 (FIG 1)

Fetal amniocytes were analysed from a 38 year old G4, P2 woman who was referred for amniocentesis because of raised maternal age. She had had a previous TOP for anencephaly. Initial cytogenetic analysis identified the karyotype as 47,XY,+r1/47,XY,+r2/47,XY,+r3/48,XY,+r1,+r2/48,XY,+r1,+r3/46,XY. All three rings were negative with X and Y centromere probes. C banding showed some euchromatin in r2 and probably also in r1 and r3. The pregnancy was terminated at 21 weeks and the fetus underwent a post mortem examination. The fetal pathology report describes minor dysmorphic features (slight brachycephaly, large anterior fontanelle, hypertelorism, nuchal thickening) but no major abnormality. The prenatal chromosome findings were confirmed on fetal gonadal fibroblasts. Parental chromosomes were normal.

CASE 2 (FIG 2)

Case 2 was born to a 34 year old G3, P2 woman at term. At birth, the female infant appeared hypotonic and minor dysmorphic features were noted (hypertelorism, micrognathia, full neck). Chromosome analysis on postnatal blood showed 47-50,XX,+1-4 mar/46,XX. The marker chromosomes varied in size from minute to larger than G chromosome size and appeared to have a ring shape in most cells. Apart from initial feeding problems, there were no medical concerns. Developmental milestones were reached at the appropriate age and Griffiths developmental assessments at 35 months and 3 years also showed age appropriate achievements except for gross motor delay caused by excessive joint laxity. When seen at the age of 4 years, she was learning to read and knew her numbers up to 20. She had a slim build and a slightly long face with epicanthic folds. Her joints remain very lax. Maternal chromosomes were normal, but paternal chromosomes were not available for testing.

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Figure 1 Fetal pathology photograph of case 1 showing hypertelorism, nuchal thickening, and large anterior fontanelle.

Figure 2 Clinical photographs of case 2 at 4 years 3 months showing (A) long face with epicanthic folds and (B) joint laxity. (Photographs reproduced with parental permission.)

Materials and methods

For hybridisation of multiple probes to a single slide, the slide was first marked with a diamond pen on the underside to form a grid of 12 squares of approximately 0.8 cm. Then 1.5 μ l of fixed cell suspension was pipetted onto the top surface of the slide into the centre of each square; this typically gave 15–40 metaphase spreads per square, depending on the thickness of the original preparation. The slide was pretreated in $2 \times$ SSC (pH 7.0) at 37°C for 30 minutes, then dehydrated through an ethanol series (70%, 90%, 100%, two minutes each); denaturation was at 70°C in 70% deionised formamide/ $2 \times$ SSC (pH 7.0) for two minutes, followed by dehydration in an ice cold ethanol series (concentrations as above). Biotinylated and FITC labelled whole chromosome paints were obtained from Cambio. Two μ l aliquots of each of the 24 paints were combined in pairs (one biotinylated with one FITC labelled) in 12 0.5 ml Eppendorf tubes. After mixing, the paints were denatured by incubation at 70°C for two minutes, followed by 30–60 minutes prehybridisation at 37°C. Biotinylated and digoxigenin labelled centromeric probes were obtained from Oncor. Probes were mixed in pairs (one biotinylated with one digoxigenin labelled), 1 μ l of each probe with 18 μ l hybridisation mixture (Hybrisol VI, Oncor). Denaturation was at 70°C for five minutes; probe mixtures were then kept on ice until required. A rubber solution grid was formed on the denatured, dehydrated slide by the extrusion of Cow Gum (rubber solution) through a micropipette tip using a 1 ml disposable syringe. The lines marked on the underside of the slide were followed to ensure that the squares created by the grid contained the fixed chromosome material. Each mixture of paints or centromeric probes was carefully pipetted into the individual squares created by the rubber solution grid. A silicone rubber rim of the same dimensions as the clear glass area of the slide and width 0.2 cm was applied to the slide with Cow Gum and another slide laid on the top. This provided a seal but ensured that the covering slide did not touch the surface of the paints and thus cause mixing of the paints across the top of the grid (fig 3). Hybridisation was at 37°C overnight in a humidified chamber. The covering slide, silicone rubber rim, and rubber solution grid were removed as one piece and stringent washes and detection were according to standard protocols, that is, stringent washes: 65% formamide/ $2 \times$ SSC (pH 7.0) at 43°C for 15 minutes, $2 \times$ SSC (pH 7.0) at 37°C for eight minutes, detection using the Cambio protocol which entails TR/avidin, α -avidin, TR/avidin for the biotinylated probes, in conjunction with either a two layer antibody system for the FITC labelled paints or one layer of FITC- α -digoxigenin for the digoxigenin labelled centromere probes. The chromosomes were counterstained with DAPI in Vectashield and viewed using a fluorescence microscope attached to a PSI MacProbe Poweregene system.

Figure 3 Stages in the setting up of multiprobe hybridisation: (A) standard microscope slide with grid scored on under surface; (B) rubber solution grid laid down; (C) individual probe mixtures contained within the wells; (D) second slide on top of silicon rubber gasket.

Figure 4 In situ hybridisation of whole chromosome paints to metaphase spreads from case 2. Spreads from two of the 12 squares are shown. Arrows indicate marker chromosomes. (A) Biotinylated chromosome 8 paint (red) and FITC-X chromosome paint (green), showing one marker of chromosome 8 origin and one unlabelled marker. (B) Biotinylated chromosome 16 paint (red) and FITC chromosome 4 paint (green), showing one marker of chromosome 4 origin and one unlabelled marker. These images were acquired using a PSI Powergene system.

Results

CASE 1

In situ hybridisation was carried out with chromosome specific centromere probes (117 cells examined) and whole chromosome paints (70 cells examined). The ring chromosomes hybridised to three different centromeric probes. The smallest ring (in approximately 55% of cells) hybridised to a chromosome 17 specific probe, but not to whole chromosome paints. The next largest ring (in approximately 30% of cells) hybridised to a chromosome 20 specific centromeric probe and also to chromosome 20 paint, indicating the possibility of the presence of euchromatin. The largest ring (in approximately 53% of cells) hybridised to a chromosome 4 specific centromere probe, with an indication that this was a dicentric ring. No hybridisation was observed with whole chromosome paints. There was one cell which con-

tained all three markers, suggesting the presence of such a cell line. The final karyotype designation is 47,XY,+r(4)/47,XY,+r(17)/.../48,XY,+r(17),+r(20)/49,XY,+r(4),+r(17),+r(20)/46,XY.

CASE 2

In situ hybridisation with whole chromosome specific paints (459 cells examined) and centromere specific probes (217 cells examined) confirmed the presence of four small ring chromosomes and showed that they were derived from chromosomes 4, 8, 10, and X, in order of increasing size. All four markers hybridised to chromosome paints as well as centromere probes (fig 4). The marker frequencies were: r(4) 77% of cells, r(8) 46% of cells, r(10) 13.5% of cells, r(X) 13.5% of cells. These markers were present in 12 different combinations. A normal cell line was also detected. Consequently, the karyotype was given as 47,XX,+r(4)/47,XX,+r(8)/47,XX,+r(10)/48,XX,+r(X),+r(4)/.../49,XX,+r(X),+r(8),+r(10)/46,XX.

Discussion

The identification of the origin of small regions of chromosome material by FISH has in the past been either time consuming and tedious, requiring the commitment of relatively large amounts of chromosome material (such as sequential or batch testing of probes⁸), or has required the use of sophisticated and expensive apparatus (such as that required for flow sorting^{9,10} or microdissecting¹¹ chromosomes before reverse painting, or computer systems required for the production of multicolour images following combinatorial or ratio labelling). We are only aware of one previous publication of a method for the hybridisation of a complete set of paints to one slide¹²; we found this method to be

unworkable, as it was not possible to remove the vacuum grease (used to attach the punched "gasket" which provided wells for the chromosome paints) from the slide. A commercially available product ("Chromosome Multiprobe System" (Cytocell)) consists of a kit comprising a coverslip with individual paints dried onto 24 raised "bosses" which is inverted onto a slide flooded with chromosome material. It is our understanding that this product is not straightforward to use. A recent publication¹³ describes a method for the detection of marker chromosomes using primed in situ labelling (PRINS). Only eight chromosomes can be tested on one slide; the number is presumably limited by the size of the coverslip used.

The method described in this paper is technically straightforward, extremely economical, and uses only materials and equipment available in most routine cytogenetic laboratories. It is also fast (results can be obtained 48 hours following the harvest of chromosomes) and flexible; the type of probe and the size, dimensions, and number of squares in the grid can be "tailored" to individual cases. In our two cases, the origin of the supernumerary ring chromosomes was rapidly and accurately identified with this technique.

Although there are many published reports of cases with two supernumerary rings originating from different chromosomes,^{3 14 15} we are only aware of three previously published cases with more than two supernumerary ring chromosomes. Mascarello *et al*⁴ reported one patient with a mosaic karyotype where all cells examined contained one to five supernumerary ring shaped markers. Tozzi *et al*⁵ presented a patient who was mosaic for one to five such ring chromosomes in nearly all cells studied. Both patients had several clinical features in common (brachycephaly, hypoplastic supraorbital ridges, webbed neck with low posterior hairline, pectus excavatum, and mental retardation). At the time, it was impossible to trace the origin of these ring markers which had occurred de novo in both cases, but Hoo and Lin¹⁶ suggested that they were derived from one chromosome. Plattner *et al*⁷ contributed a third patient with mosaicism for one to four additional marker ring chromosomes present in every cell. In situ hybridisation could clarify their origin to some degree and showed that they were derived from different chromosomes. The karyotype was given as 47,XX,+r(X)/48,XX,+r(X),+r(8)/49,XX,+r(X),+r(8),+r(14 or 22)/50,XX,+r(X),+r(8),+r(14 or 22),+r(15). This patient was born with multiple congenital abnormalities (microcephaly, facial asymmetry, scoliosis, congenital hip dislocation) and subsequently suffered from seizures. Developmental aspects were not mentioned and the parental chromosomes were not examined.

Callen *et al*⁶ pointed out that mosaicism was a common feature in patients with supernumerary ring chromosomes, consistent with either the postzygotic formation of these ring markers or a postzygotic instability resulting in

loss during cell division. All five cases with multiple rings discussed here display mosaicism.

Different mechanisms of ring formation have been suggested, including chromosome breakage with fusion of the proximal and loss of the distal fragments with or without misdivision, telomere to telomere fusion of a rod without loss of genetic material, and more complex models with additional chromosome rearrangements such as duplication or translocation preceding ring formation. This may occur either at meiosis or postzygotically. The presence of multiple rings of different origin indicates that it is more likely that the ring chromosomes were formed during meiosis, perhaps involving more complex chromosome rearrangements, resulting in a germ cell containing all markers, with subsequent postzygotic loss of markers during cell division. This would explain why in two out of the five cases, no normal cell lines were found, for if the ring formation happened postzygotically, one would expect the presence of a normal cell line.

The origin of small supernumerary ring chromosomes which previously defied identification can therefore now be accurately defined; evaluation of the significance of these abnormalities remains, however, problematical. The five cases mentioned here, although similar in some respects, are all unique in their chromosome make up and euchromatin content (both of which are likely to differ in different tissues) and the outcome, surprisingly, as our second case shows, is not invariably mental or physical handicap or both.

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- Kosztolányi G. Does "ring syndrome" exist? An analysis of 207 case reports on patients with a ring autosome. *Hum Genet* 1987;75:174-9.
- Callen DF, Eyre HJ, Ringenbergs ML, Freemantle CJ, Woodroffe P, Haan EA. Chromosomal origin of small ring marker chromosomes in man: characterization by molecular genetics. *Am J Hum Genet* 1991;48:769-82.
- Plattner R, Heerema NA, Howard-Peebles PN, Miles JH, Soukup S, Palmer CG. Clinical findings in patients with marker chromosomes identified by fluorescence in situ hybridization. *Hum Genet* 1993;91:589-98.
- Mascarello JT, Jones MC, Chambers SR. A patient with extreme variation in number and size of small marker chromosomes. *Hum Genet* 1987;75:191-4.
- Tozzi C, Calvieri F, Alesi L, Neri G. Multiple "marker" chromosomes: a novel cytogenetic finding in a patient with mental retardation and congenital anomalies. *Am J Med Genet* 1988;29:353-9.
- Rauch A, Pfeiffer RA, Trautmann U, Liehr T, Rott HD, Ulmer R. A study of ten small supernumerary (marker) chromosomes identified by fluorescence in situ hybridization (FISH). *Clin Genet* 1992;42:84-90.
- Plattner R, Heerema NA, Yurov YB, Palmer CG. Efficient identification of marker chromosomes in 27 patients by stepwise hybridization with alpha-satellite DNA probes. *Hum Genet* 1993;91:131-40.
- Blennow E, Annerén G, Bui TH, Berggren E, Asadi E, Nordenskjöld M. Characterization of supernumerary ring marker chromosomes by fluorescence in situ hybridization (FISH). *Am J Hum Genet* 1993;53:433-42.
- Carter NP, Ferguson-Smith MA, Perryman MT, *et al*. Reverse chromosome painting: a method for the rapid analysis of aberrant chromosomes in clinical cytogenetics. *J Med Genet* 1992;29:299-307.
- Rack KA, Harris PC, MacCarthy AB, *et al*. Characterization of three de novo derivative chromosomes 16 by "reverse chromosome painting" and molecular analysis. *Am J Hum Genet* 1993;52:987-97.

- 11 Ohta T, Tohma T, Soejima H, *et al.* The origin of cytologically unidentifiable chromosome abnormalities: six cases ascertained by targeted chromosome-band painting. *Hum Genet* 1993;92:1-5.
- 12 Larin Z, Fricker MD, Maher E, Ishikawa-Brush Y, Southern EM. Fluorescence in situ hybridization of multiple probes on a single microscope slide. *Nucleic Acids Res* 1994;22:3689-92.
- 13 Hindkjær J, Brandt CA, Strømkjær H, Koch J, Kølvræ S, Bolund L. Primed in situ labelling (PRINS) as a rational procedure for identification of marker chromosomes using a panel of primers differentially tagging the human chromosomes. *Clin Genet* 1996;50:437-41.
- 14 Aalfs CM, Jacobs ME, Nieste-Otter MA, Hennekam RCM, Hoovers JMN. Two supernumerary marker chromosomes, derived from chromosomes 6 and 9, in a boy with mild developmental delay. *Clin Genet* 1996;49:42-5.
- 15 Copeland KC, Hansen K, Moore CM. Multiple congenital anomalies, mental retardation and hypogonadotropic hypogonadism in a boy with small marker chromosomes. *Am J Med Genet* 1985;20:607-12.
- 16 Hoo JJ, Lin CC. Invited editorial comment. Multiple, variable-sized, minute marker chromosomes. *Am J Med Genet* 1988;29:361-3.