A non-isotopic in situ hybridisation study of the chromosomal origin of 15 supernumerary marker chromosomes in man

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Abstract

Fifteen patients presenting with mosaic or non-mosaic karyotypes containing a distamycin-DAPI negative de novo or familial supernumerary marker chromosome were studied with non-isotopic in situ hybridisation using a library of alphoid centromere specific and satellite II/III probes. The in situ hybridisation studies showed that seven markers were derived from satellited autosomes (three chromosome 13/21, two chromosome 14, two chromosome 22), six from non-satellited autosomes (two chromosome 4, one chromosome 12, one chromosome 16, two chromosome 19), and one from the Y chromosome. One non-mosaic marker was negative for all the alphoid and satellite II/III probes used.

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Supernumerary marker chromosomes are found in approximately 2.0/1000 pregnancies studied for prenatal diagnosis¹ and in 0.3/1000 newborns.² The frequency of de novo markers increases with maternal age,³ thus accounting for their high incidence among amniocentesis specimens, most of which are referred for this reason. By excluding patients presenting with cytogenetically definable markers such as i(12p) and i(18p) (see Discussion), a recent multicentre study⁴ has calculated that the incidence of de novo supernumerary markers not identifiable using standard cytogenetic techniques is 1/2500 amniocenteses with satellited and non-satellited markers occurring with approximately equal frequencies.

The phenotypic risks, both intellectual and physical, conferred by the presence of a supernumerary marker have always been difficult to predict owing to the paucity of long term clinical follow up data from such cases. As a result, published risk estimates are given with large margins of error.¹⁴ This dilemma is at its most acute when a de novo marker is found prenatally.

A recent study by Warburton⁴ on a series of 377 353 amniocenteses carried out in the United States and Canada over a 10 year period calculated the risk of an abnormal phenotypic outcome in cases of de novo markers to be approximately 13% and, unlike previous studies,¹ found no significant difference in the risk conferred by a satellited compared with a non-satellited marker chromosome.

These estimates, however, do not take into account the chromosomal origin of the marker

which until recently has been impossible to determine. An exception has been those markers with distamycin-DAPI staining properties by which they are assumed to be derived from chromosome 15. The majority of de novo duplicated distamycin-DAPI staining markers formed from regions including 15pter→q12 are associated with mental impairment varying from mild to very severe.⁵⁻⁷

The isolation and cloning of centromere specific alphoid sequences,^{8,9} together with the development of non-isotopic in situ hybridisation techniques, have made it possible to identify the chromosomal origin of supernumerary marker chromosomes rapidly and systematically. The first reports used probes isolated from the alphoid sequences of the X and Y chromosomes to determine the origin of marker chromosomes in patients with Turner's syndrome.¹⁰⁻¹² More recently, studies using autosomal centromere specific probes have identified the chromosomal origin of 13 small ring chromosomes.^{13,14}

We have studied 15 supernumerary marker chromosomes, both familial and de novo, and have determined the chromosomal origin of 14. The phenotypes of 12 of the 15 patients are described in the light of the chromosomal origin of the markers.

Materials and methods

Non-isotopic in situ hybridisation was performed on conventional cytogenetic preparations, usually within 48 hours of making the slides. The in situ hybridisation method used is described in detail elsewhere.¹⁵ Briefly, biotinylated probe DNA (Biotin-16-dUTP, Boehringer Mannheim) was used at a final concentration of 0.5 to 2 ng DNA/μl of hybridisation mixture (50% formamide with 10% w/v dextran sulphate, 2 × SSC, and 1/20 vol of sonicated carrier herring sperm DNA). Hybridisation was carried out overnight at 42 to 45°C and after stringent washing (usually 0.2 × SSC/50% formamide at 37°C for 40 minutes) the sites of hybridisation were detected using either an indirect immunoperoxidase staining method, or fluorescent in situ hybridisation (FISH) following the protocol of Kievits *et al.*¹⁶

The library of probes used is listed in table 1, from which it can be seen that all centromeres could be individually identified with the exception of 5 and 19, and 13 and 21. In each experiment, the labelling of the normal homologues acted as an internal control and the marker was scored positive for a probe only if

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Table 1 Probes used.

Chromosome	Region	Probe	Locus
1	Cen	pSD1.1	D1Z5
2/20	Cen	pBS4D	—
3	Cen	p23.5	D3Z1
4	Cen	ONCOR	D4Z1
5/1/19	Cen	ONCOR	D5/1/19Z1
6	Cen	ONCOR	D6Z1
7	Cen	p7t1	D7Z1
8	Cen	pJm128	D8Z2
9	Cen	pMR9A	—
9	qh	pHuR98	D9Z3
10	Cen	pα10RP8	D10Z1
11	Cen	pLC11A	D11Z1
12	Cen	pα12H8	—
13/21	Cen	LI.26	D13Z1
14/22	Cen	p14.1	—
15	Cen	pTRA20	—
16	Cen	pSE16	D16Z2
16	qh	pHuR195	D16Z3
17	Cen	p17H8	D17Z1
18	Cen	LI.84	D18Z1
20	Cen	p3.4	D20Z1
21/13	Cen	LI.26	D13Z1
22	Cen	p190.22	—
X	Cen	pSV2X5	DXZ2
Y	Cen	Y84	DYZ3
Y	p	Y-190	DYZ5

the normal homologues and the marker were labelled in at least three metaphases. This was particularly important with very small markers where slight increases of background labelling sometimes gave a false positive signal in single cells. One probe was used per slide and the library systematically applied until the chromosomal assignment was made. In cases 10 and 11, the marker chromosomes hybridised strongly with the probe D5/1/19Z1. Chromosome 1 was excluded by negative signal with D1Z1, and chromosome 19 was selected after a positive signal was obtained in both cases with D16Z2 which, at low stringency, cross hybridises with chromosomes 1, 4, 9, 16, and 19. As the chromosome 5 centromere did not stain with D16Z2 under these conditions, it was assumed that the markers were of 19 origin.

Analysis was carried out using a Carl Zeiss Axiophott microscope equipped with epifluorescence and FITC filter combination 09. Photographs were taken using Kodak P800/1600 push process transparency film.

Results

In 12 of the 15 cases examined, the supernumerary marker was de novo, two were inherited from the mother, and the status of the remaining marker could not be ascertained (table 2). The chromosomal origin in 14 of the 15 markers was resolved and in one case no signal was seen after hybridisation with all probes listed in table 1. Descriptions of the karyotypes and patients are given below.

CASE 1: 46,XX/47,XX, + MAR(4)MAT

Amniocentesis on a 38 year old woman showed a very small (<22) marker in 80% of amniocytes. The same marker was found in 70% of lymphocytes from the clinically normal mother. In situ diagnosis was performed on the fetal amniocytes, from which the marker was eventually lost in vitro. The pregnancy ended spontaneously at 32 weeks with a breech delivery of a phenotypically normal female. Nine months after birth, the baby was developing normally.

CASE 2: 46,XX/47,XX, + MAR(4) DE NOVO

Amniocentesis on a 37 year old woman showed a very small (<22) marker in 27% of amniocytes. The pregnancy was terminated and the presence of the marker confirmed in fetal tissues. No clinical details of the fetus were available.

CASE 3: 46,XX/47,XX, + MAR(12) DE NOVO

A very small marker (<22) was ascertained at amniocentesis performed on a 39 year old woman. The marker was found in all three primary cultures, but the frequency varied from 3% to 90% (figure A). The pregnancy continued and a normal female was delivered at term, when the marker was found with a frequency of 38% in the baby's lymphocytes. At 7 months the baby was developing normally.

Table 2 Ascertainment, chromosomal origin, and outcome in 15 cases with supernumerary marker chromosomes.

Case	Chromosomal origin	Parental	Ascertainment	Frequency of marker (%)	Morphology	Size	Outcome	HGM*
1	4	Maternal	PND for maternal age	80	V small	<22	Normal	DD46
2	4	De novo	PND for maternal age	27	V small	<22	TOP†	DD68‡
3	12	De novo	PND for maternal age	38	V small	<22	Normal	DD121
4	13/21	Maternal	Developmental delay	100	Bisatellited	≤22	Abnormal	—
5	13/21	De novo	Mental retardation	10	Bisatellited	=22	Abnormal	DD135
6	13/21	De novo	PND for maternal age	100	Satellited	≤22	Pregnancy continuing	DD503
7	14	De novo	Speech and developmental delay	90	Small	≤22	Abnormal	DD227
8	14	De novo	PND for maternal age	60	Small	≤22	TOP†	DD357
9	16	De novo	PND for maternal age	90	Small	≤22	Normal	DD104
10	19	Not known	Floppy baby	50	Small	~21	Abnormal	—
11	19	De novo	PND for maternal age	60	Variable	<22	Abnormal	DD549
12	22	De novo	PND for maternal age	100	Bisatellited	& ~21 ≤22	Normal	DD347
13	22	De novo	PND for maternal age	100	Bisatellited	≤22	TOP§	DD87
14	Y	De novo	PND for maternal age	42	Small	≤22	TOP	DD107
15	NK	De novo	PND for maternal age	100	Small	≤22	TOP§	DD46

* Depositor number at the European Cell & Culture Collection, Porton Down, Salisbury SP4 0JG, UK.

† Termination of pregnancy: phenotype not known.

‡ Marker lost in vitro.

§ Termination of pregnancy: normal fetus.

|| Termination of pregnancy: hermaphrodite fetus.

PND = prenatal diagnosis.

(A) Immunoperoxidase staining of a metaphase from case 3 (mar 12) after in situ hybridisation with pα12H8 shows the very small marker (arrowhead) and both normal chromosome 12 centromeres (arrows) labelled. (B) An example of the dicentric marker seen in 13% of the metaphases examined from case 11 (mar 19—partial metaphase). The marker (arrowhead) shows two discrete areas of diaminobenzidine precipitation after immunoperoxidase detection after in situ hybridisation with the D5/1/19Z1 probe. Normal 1, 5, and 19 centromeres (arrows) show distinct brown staining in contrast to the magenta/blue C banding observed on some other chromosomes.

CASE 4: 47,XY, + MAR(13/21)MAT

The proband was referred at the age of 4 years for marked developmental and speech delay and was found to have a bisatellited marker approximately the size of a chromosome 22. His mother had the same marker in 44% of her lymphocytes and had required special schooling, particularly with reading and writing. Both the proband and his mother had strikingly similar round facial features with brachydactyly. The maternal grandmother also carried the marker in 1% of lymphocytes but was intellectually and phenotypically normal. The in situ hybridisation diagnosis of the marker's origin was performed on the proband's lymphocytes.

CASE 5: 46,XY/47,XY, + MAR(13/21) DE NOVO

The patient was referred at the age of 41 for mild mental retardation. A bisatellited marker, the size of a chromosome 22, was found in 10% of peripheral blood lymphocytes. The patient had attended a school for children with special educational needs and on examination was noted to have a rounded face, short stubby fingers, and slightly lax joints but was otherwise normal in appearance.

CASE 6: 47,XX, + MAR(13/21) DE NOVO

Amniocentesis on a 41 year old woman showed a small (≤ 22) satellited marker chromosome in all cells examined. The pregnancy is continuing to term.

CASE 7: 46,XY/47,XY, + MAR(14) DE NOVO

This patient was first examined cytogenetically at 3 years of age because of delayed speech, and on re-examination eight months later his overall development was around 3 years, but still below this for speech. At birth he was noted to have a left undescended testicle, bilateral preauricular sinuses, bifid tongue, wide set and

slightly downward sloping eyes, and hypermobile joints. A marker, smaller than a chromosome 22, was found in 90% of lymphocytes studied.

CASE 8: 46,XY/47,XY, + MAR(14) DE NOVO

Chorionic villus sampling from a 41 year old woman showed a small (≤ 22) marker chromosome in 60% of cells derived from long term cultures. The pregnancy was terminated at the end of the first trimester and therefore no morphological evaluation was possible.

CASE 9: 46,XY/47,XY, + MAR(16) DE NOVO

Amniocentesis on a 43 year old woman showed a small (≤ 22) marker chromosome in 90% of cells examined. The pregnancy continued to term and at 17 months the baby was developing normally.

CASE 10: 46,XX/47,XX, + MAR(19) NOT KNOWN

This patient was referred at the age of 3 weeks for failure to thrive, severe floppiness, and pneumonia. A small (≤ 22) marker seen in half of the peripheral lymphocytes examined was positive for the D5/1/19Z1 probe, and by a series of other hybridisations all centromeres except 19 were excluded as the origin.

CASE 11: 46,XY/47,XY, + MAR₁(19)/47,XY, + MAR₂(19)/47,XY, + MAR₃(?) DE NOVO

This patient's marker chromosome was originally discovered in 60% of the cells examined from long term cultures after a CVS biopsy from his 36 year old mother. At term the marker was found with the same frequency in the proband's peripheral blood. He was referred to this laboratory for in situ studies at the age of 14 months at which stage he was found to have a large head (on the 98th centile), frontal bossing, epicanthic folds, hypotonia, and developmental delay. In situ hybridisation studies showed four distinguishable cell populations. Forty percent of cells were normal (46,XY); 27% contained a small (≤ 22) marker with a single D5/1/19Z1 centromeric signal; 13% had a dicentric marker approximately the size of a chromosome 21 and positive for two copies of D5/1/19Z1 (figure B); and the remaining 20% of cells contained a small marker (≤ 22) which failed to show a signal with any of the probes in the library. Thirty cells were examined from each hybridisation in order to exclude the possibility that the negative signal in the unlabelled markers was a technical artefact.

CASE 12: 46,XY/47,XY, + MAR(22) DE NOVO

Amniocentesis on a 39 year old woman showed a small (≤ 22) bisatellited marker in all amniocyte cells examined. At birth, the presence of the marker was confirmed in the baby's lymphocytes but with a frequency of 80%. The in situ studies were carried out on the proband's lymphocyte chromosomes. At the age of 5

years he was developing normally except for slight high frequency hearing loss on the right side.

CASE 13: 47,XX, + MAR(22) DE NOVO

Amniocentesis at the age of 35 showed a small (≤ 22) bisatellited marker chromosome in all cells. The pregnancy was terminated, and the presence of the marker was confirmed in the fetus. At necropsy the fetus appeared normal.

CASE 14: 45,X/46,X, + MAR(Y) DE NOVO

Amniocentesis on a 39 year old woman showed a mosaic karyotype with a small (≤ 22) marker in 42% of amniocytes examined. The mar(Y) was ring like in appearance and gave two distinct signals with both the DYZ3 (centromere) and DYZ5 (Yp heterochromatin) probes. The pregnancy was terminated and at necropsy the fetus was found to be a hermaphrodite with a short penis, no uterus, and with an ovary on the left side and a testis on the right.

CASE 15: 47,XX, + MAR(?ORIGIN) DE NOVO

Amniocentesis on a 36 year old woman showed the presence of a small (≤ 22) marker in all cells examined. In situ hybridisation was performed with all the probes in the library but no signal was seen from the marker in the 10 cells examined after each hybridisation. The pregnancy was terminated and the necropsy report indicated an apparently normal fetus with no major congenital abnormalities.

Discussion

The phenotypic consequences of a supernumerary marker chromosome remain difficult to evaluate despite a number of conventional cytogenetic staining methods. Some markers, for example i(12p) and i(18p), can be characterised with conventional cytogenetic methods and these have well defined abnormal phenotypic consequences.^{17,18} Furthermore, distamycin-DAPI staining techniques are used to identify chromosome 15 derived markers. Markers involving duplications of 15pter→q21 are associated with varying degrees of mental handicap.⁵⁻⁷ However, the use of distamycin-DAPI alone for marker diagnostic purposes should be interpreted with caution in the light of two recent studies. In the first, Smeets *et al*¹⁹ showed that approximately 6% of normal subjects have three copies of 15p (that is, the region defined by the probe D15Z1 and distamycin-DAPI). In these cases, two signals were seen on the normal chromosomes 15 and a third on either a 13p or 14p. A recent study by Stergianou *et al*²⁰ repeated these experiments but in addition used an alphoid chromosome 15 centromere specific probe (pTRA-25).²¹ In all cases with three 15p distamycin-DAPI signals only two 15 centromere signals were observed, always from the normal 15 homologues. These results suggest

that a distamycin-DAPI staining pseudodacentric marker chromosome may not necessarily be dicentric for 15, but could be a heterozygous 13/15 or 14/15 marker with significantly altered risks of phenotypic abnormality.

Five of the patients summarised in table 2 presented with an abnormal phenotype. With so few cases in each chromosomal subcategory it is not possible to detect any striking pattern of phenotypes associated with markers of different chromosomal origins. Nevertheless, the phenotypic effect attributable to the presence of a supernumerary marker may arise through a number of mechanisms. In this context, it is interesting that case 7 in this study and a case recently reported by Temple *et al*²² both had undescended testes. Temple *et al*²² showed that their proband was uniparentally disomic for the maternal chromosomes 14. The supernumerary marker(14) in case 7 of this study may also be maternal in origin resulting in an excess of maternally derived genes for the pericentric region of chromosome 14. Marker chromosomes may, therefore, cause an abnormal phenotype not only by dosage effects, but also because of chromosome specific imprinting resulting in an imbalance between the normal ratio of maternal and paternal genes. Indeed, such an effect is well established in some patients with markers derived from chromosome 15 who have the Prader-Willi syndrome.⁷

Six of the remaining 10 cases ascertained through prenatal diagnosis had normal phenotypes. In case 3, the chromosomal assignment of the marker(12) was made postnatally. With the known association of Pallister-Killian syndrome and the tetrasomy 12p marker chromosome,¹⁷ it is difficult to assess retrospectively how the chromosomal assignment of the marker(12) would have affected the risk estimates if the in situ results had been known prenatally. The marker(12) in this case suggests that triplication (or quadruplication) of the pericentromeric region of chromosome 12 alone is not sufficient to produce the Pallister-Killian phenotype.

It is interesting to note that cases 12 and 13, both of which had karyotypes with non-mosaic bisatellited and dicentric marker(22) chromosomes, were phenotypically normal. Bisatellited markers thought to be derived from chromosome 22pter→p11 are associated with the 'cat eye syndrome',²³ which has a wide spectrum of associated malformations varying from mild to very severe. However, because of the markers' small size and indistinct staining characteristics, their derivation from chromosome 22 remains conjectural.²³ We have recently used in situ hybridisation with the chromosome 22 centromere specific probe p190.22 to show that the marker in a case presenting with several of the classical features of cat eye syndrome, that is, anal atresia, accessory auricles, preauricular sinuses, and slight dysmorphic features, was indeed a dicentric marker(22) (Howard and Crolla, unpublished results). Cases 12 and 13 in this study may, therefore, represent extremely

mild examples of cat eye syndrome. Alternatively, the parental origin of an additional chromosome 22 marker may affect the phenotype. It is known that persons who have maternal uniparental disomy for chromosome 22 are phenotypically normal.^{24,25} However, the phenotype of persons with paternal uniparental disomy 22 is unknown. It is conceivable that subjects with a maternally derived marker 22 may be asymptomatic but those with a paternally derived marker 22 will manifest the cat eye syndrome.

The stable retention, even as low grade mosaic cell lines, of markers suggests that they have functional kinetochores, but whether they invariably retain intact alphoid sequences is open to question in the light of the cases described in the present paper (cases 11 and 15) and that studied by Callen *et al.*¹⁴ (case 2). Although these markers were negative for all the centromere and pericentromeric probes, they were stable within their respective karyotypes.

Knowledge of the chromosomal origin of a supernumerary marker chromosome may provide significant prognostic information once larger numbers of patient/karyotype correlations have been described. However, other factors such as imprinting and dosage effects may also have to be known before an accurate risk estimate for any given *de novo* marker can be given.²⁶ A first step towards unravelling these complex events remains the chromosomal origin of the supernumerary markers.

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