

Prenatal molecular cytogenetic diagnosis of partial tetrasomy 10p due to neocentromere formation in an inversion duplication analphoid marker chromosome

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Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. Neocentromeres are fully functional centromeres found on rearranged or marker chromosomes that have separated from endogenous centromeres. Neocentromeres often result in partial tri- or tetrasomy because their formation confers mitotic stability to acentric chromosome fragments that would normally be lost. We describe the prenatal identification and characterization of a de novo supernumerary marker chromosome (SMC) containing a neocentromere in a 20-wk fetus by the combined use of comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). GTG-banding of fetal metaphases revealed a 47,XY,+mar karyotype in 100% of cultured amniocytes; parental karyotypes were both normal. Although sequential tricolor FISH using chromosome-specific painting probes identified a chromosome 10 origin of the marker, a complete panel of chromosome-specific centromeric satellite DNA probes failed to hybridize to any portion of

the marker. The presence of a neocentromere on the marker chromosome was confirmed by the absence of hybridization of an all-human-centromere alpha-satellite DNA probe, which hybridizes to all normal centromeres, and the presence of centromere protein (CENP)-C, which is associated specifically with active kinetochores. Based on CGH analysis and FISH with a chromosome 10p subtelomeric probe, the marker was found to be an inversion duplication of the distal portion of chromosome 10p. Thus, the proband's karyotype was 47,XY,+inv dup(10)(pter→p14~15::p14~15→neo→pter), which is the first report of partial tetrasomy 10p resulting from an analphoid marker chromosome with a neocentromere. This study illustrates the use of several molecular strategies in distinguishing centric aliphoid markers from neocentric analphoid markers.

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Centromeres are functionally defined as the chromosomal regions responsible for ensuring the proper segregation of replicated sister chromatids during mitosis and meiosis. All normal human chromosomes contain alpha-satellite DNA at their cen-

tomeres (Murphy and Karpen, 1998). Neocentromeres are new centromeres that have formed on low- or single-copy DNA and do not contain alpha-satellite DNA, yet they have fully formed kinetochores containing all the normal functional kinetochore proteins thus far examined (Saffery et al., 2000). Neocentromeres provide mitotic stability to rearranged or marker chromosomes that have separated from endogenous centromeres and would normally be acentric and lost. Including this report, 42 neocentromeres have been described from 16 human chromosomes (Depinet et al., 1997; Warburton et al., 2000). Of these, the majority are found on small inversion duplication marker chromosomes, resulting in partial tri- or tetrasomy for the duplicated region (Blennow et al., 1994; Sacchi et al., 1996; Depinet et al., 1997; Warburton et al., 2000).

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Supernumerary marker chromosomes (SMCs), defined as chromosome fragments whose origin cannot be identified by standard cytogenetic banding, occur in approximately 1 per 2,500 livebirths, with an associated overall risk of an abnormal phenotype of about 13% (Buckton et al., 1985; Warburton, 1991). Recent surveys have demonstrated that a knowledge of the chromosomal origin of the SMC can provide additional prognostic information and associated risk estimates for use in genetic counseling (Crolla, 1998). SMCs that contain neocentromeres are derived from within chromosomal arms or telomeric regions, as opposed to the vast majority of SMCs that contain centromeric regions. Thus, neocentric SMCs could contain a significantly higher density of expressed euchromatic sequences than centric SMCs, presumably with a greater associated risk for an abnormal phenotype. Therefore, in order to provide the most complete prognostic information for genetic counseling, molecular cytogenetic characterization of SMCs should include determination of both the chromosomal identity and the relative position and size of the marker within the originating chromosome.

The chromosomal origins of SMCs can be identified using fluorescence in situ hybridization (FISH) with either chromosome-specific centromeric probes or whole chromosome paints (Callen et al., 1992), although this approach requires multiple probing to identify the source chromosome. Recent advances using multicolor FISH techniques (Schröck et al., 1996; Speicher et al., 1996) can more easily identify the chromosomal origin, but not the originating position, of the fragment within the chromosome. Reverse FISH (Ohta et al., 1993; Thangavelu et al., 1994; Viersbach et al., 1994), where the marker is microdissected, DOP-PCR amplified, and hybridized back to normal chromosomes, reveals both the origin and position of the marker, but it is a highly specialized, labor-intensive procedure not amenable to routine clinical application. Comparative genomic hybridization (CGH), where patient and normal reference genomic DNA are differentially labeled and hybridized to normal metaphase chromosomes, has been particularly useful in clinical cytogenetics and reveals both the origin and position of extra chromosomal material (Bryndorf et al., 1995; Levy et al., 1997; Levy et al., 1998).

In this report, we describe the molecular cytogenetic characterization of an unusual marker chromosome, found to be an inversion duplication of 10p (pter→p14~15) with a neocentromere, giving rise to partial tetrasomy for chromosome 10p. The identity and originating position of the SMC from chromosome 10p required several sequential FISH procedures, whereas more detailed information was obtained from a single CGH procedure.

Materials and methods

Cytogenetic studies

Cultures of peripheral blood lymphocytes, amniocytes, and fibroblasts from the products of conception were set up and analyzed using standard techniques (Brown and Lawce, 1997; Priest, 1997).

CGH

CGH probes were prepared and washed as previously described (Kallioniemi et al., 1994; Levy et al., 1998). CGH images were captured with an ImagePoint cooled CCD video camera (Photometrics) through a Labophot-2A fluorescence microscope (Nikon). Chromosome identification and karyotyping were facilitated by counterstaining with 0.5 µg/ml DAPI in Vectashield (Vector Laboratories) (Florijn et al., 1995). CGH analysis was performed as described by Levy et al. (1998). The fluorescence ratios (green/red) for at least 10 of each autosome and 7 of each sex chromosome were obtained per slide. An upper threshold of >1.20 was used to define a gain of chromosome material, whereas a lower threshold of <0.80 was used to interpret a loss (deletion) of chromosome material (du Manoir et al., 1995). Gains and losses were only considered relevant if the 95% confidence intervals (data not shown), derived from the combination of each single chromosome profile, met the threshold criteria. Digital image analysis was performed using a Cytovision Probe system and CGH software (Applied Imaging Corporation).

FISH

FISH using a complete set of chromosome-specific pericentromeric and satellite sequences for chromosome identification was performed using the chromoprobe multiprobe I system (Cytocell Ltd.), which consists of a 3 × 8 array of raised bosses, onto each of which a different fluorescein-labeled probe is reversibly bound. FISH using all 24 whole-chromosome paints was performed using the chromoprobe multiprobe OctoChrome system (Cytocell Ltd.), consisting of an eight-square device wherein each square contains three different chromosomes in three independent fluorophores, Ruby Red, Emerald Green, and Sapphire Blue. The biotinylated all-human-centromere (alpha-satellite) probe was acquired from Oncor and detected with streptavidin-FITC (Vector Laboratories). The 10p (GenBank accession number Z96139) and 10q (D10S2490) subtelomeric probes were acquired from Vysis. All FISH studies were performed according to the manufacturers' instructions. Metaphase spreads were counterstained with 0.5 µg/ml DAPI in Vectashield (Vector Laboratories) (Florijn et al., 1995).

Immunofluorescence

Immunofluorescence with rabbit anti-CENP-C (kindly provided by William Earnshaw, University of Edinburgh) and simultaneous FISH with a chromosome 10 paint (Oncor) was performed on 3:1 methanol:acetic acid-fixed cultured amniocytes, essentially as described (Alonso et al., in press). The biotinylated 10 paint was detected with streptavidin-FITC (Vector Laboratories), and rabbit anti-CENP-C was detected with goat anti-rabbit IgG-TRITC (Sigma).

Results

Case report

A 37-yr-old G3, P1, TOP1 mother presented for amniocentesis at 17 wk gestation because of advanced maternal age. Cytogenetic studies performed on cultured amniocytes revealed a male fetus with an extra marker chromosome, 47,XY,+mar. A level II high-resolution ultrasound, performed at 19 wk, did not reveal any fetal anatomical anomalies and indicated the fetal growth to be within normal parameters. The fetal heart appeared normal on a fetal echocardiogram done at 20 wk. The pregnancy was terminated at 22 wk, and the fetus was examined by a pediatric pathologist.

The disrupted fetus was received as the product of a dilatation and evacuation procedure. By foot length, the fetal growth was appropriate for 22 wk gestation. External genitalia were those of a normal immature male. Fragmentation of the upper portion of the head hindered recognition of craniofacial abnormalities; however, there was no evidence of cleft lip or palate. Palmar creases were unremarkable. There was no foot deformity. With the exception of partial disruption of the liver and

spleen, the abdominal organs, including the genitourinary tract and retroperitoneal testes, were unremarkable. The thoracic organs were evulsed from the thoracic cavity; the fragmented heart could not be evaluated for congenital defects. Histological examination of the viscera was within normal limits. Postmortem radiographic examination of the spine, pelvis and extremities was unremarkable. The 176 g placenta exhibited focal disproportionate villous immaturity and edema and mildly increased perivillous fibrin. The umbilical cord contained three vessels and was unremarkable.

Cytogenetic studies

GTG-banding of metaphases from cultured amniocytes and fibroblast cultures from the products of conception showed an unbalanced male karyotype in all cells with an extra unidentified marker chromosome; 47,XY,+mar (Fig. 1). Parental karyotypes from peripheral blood lymphocytes were both normal, indicating a *de novo* origin of the marker chromosome in the fetus. The *de novo* marker was C-band negative (data not shown).

FISH with a complete panel of chromosome-specific centromeric probes (Cytocell Ltd.) did not show any signal on the marker chromosome, suggesting the presence of a neocentric marker chromosome. FISH results for the chromosome 10 centromeric probe are shown in Fig. 2a. Sequential tricolor chromosome painting (Cytocell Ltd.) indicated the marker to be derived from chromosome 10. FISH results with the tricolor paint probes for chromosomes 5, 7, and 10 are shown in Fig. 2b. The absence of alpha-satellite DNA on the marker was confirmed by using an all-human-centromere alpha-satellite probe (Oncor), which hybridized to all normal human centromeres but did not hybridize to the marker chromosome (Fig. 3a). The presence of a centromere on the marker chromosome was confirmed by immunofluorescence with antibodies to CENP-C, which showed the characteristic double-dot CENP-C kinetochore pattern at the centromeres of all chromosomes, including the marker (Fig. 3b).

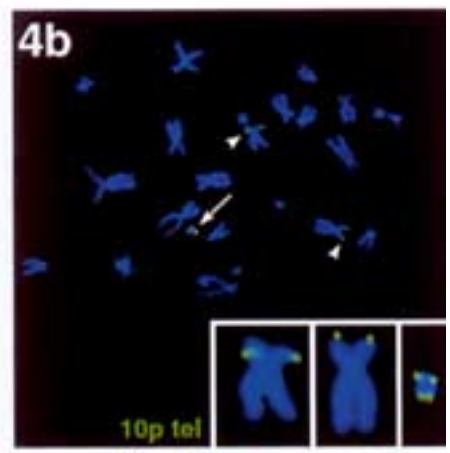
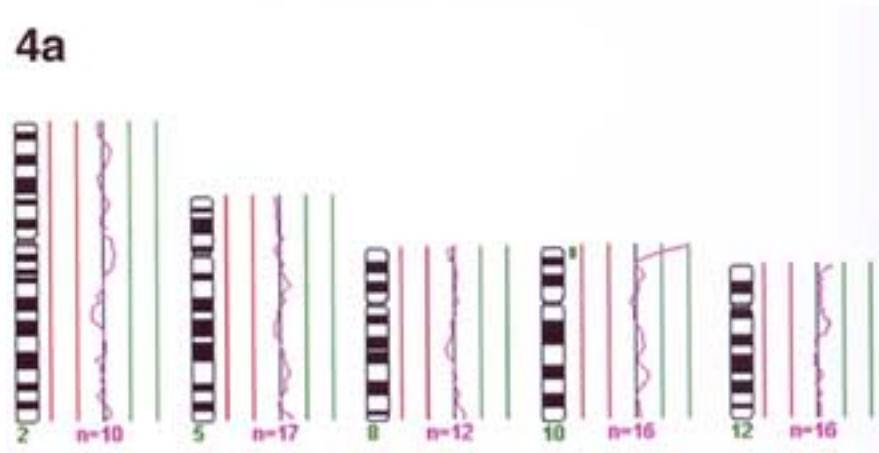
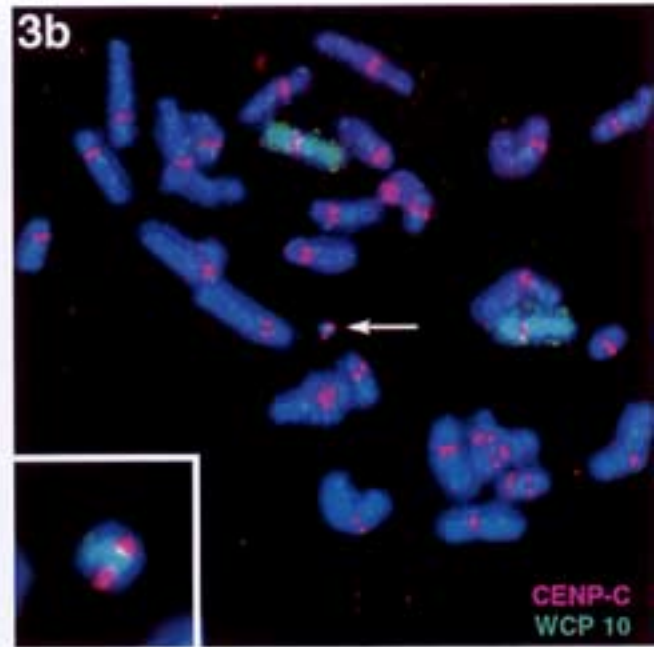
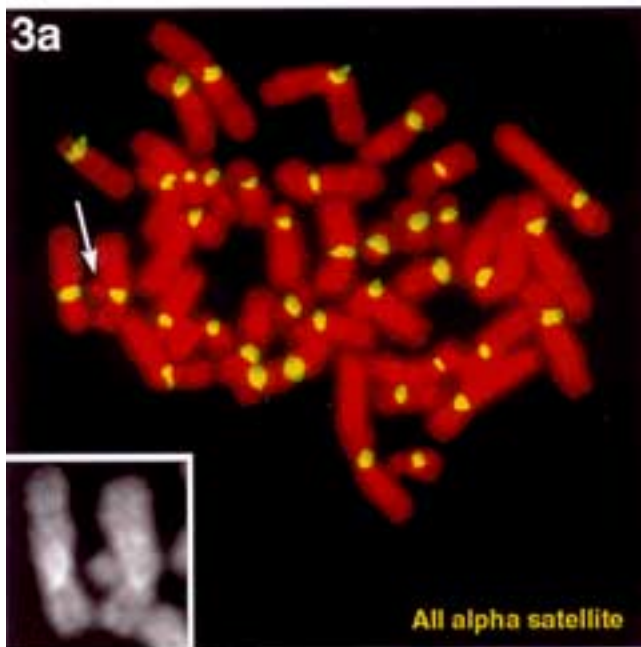
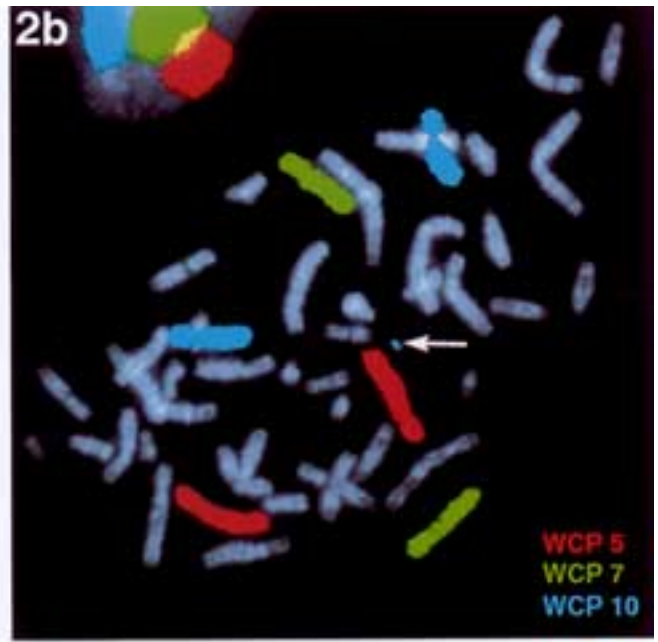
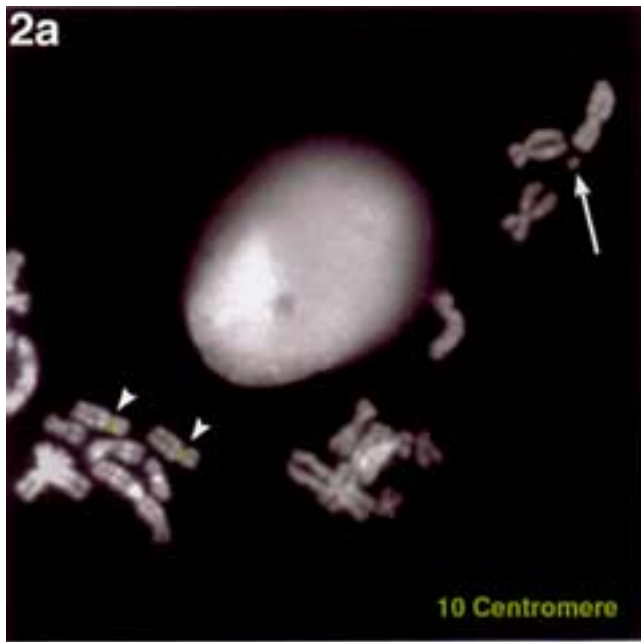
CGH analysis was performed by co-hybridizing fluorescein-labeled fetal DNA and Texas Red-labeled control DNA (46,XY) to normal male metaphase spreads. The CGH profile of chromosome 10 (Fig. 4a) showed a gain from 10pter to the border of 10p15 and 10p14 (10p14 ~ 15), demonstrating that the additional chromosomal material on the marker was derived from this region. CGH results are displayed with representative chromosome profiles (Fig. 4a). All other chromosomes (data not shown) had normal CGH profiles, with no significant deviation from a value of 1.0. The CGH ratio profile observed for chromosome 10 appeared significantly over-represented in the 10pter → p14 ~ 15 region, which suggested the marker is disomic for this region, resulting in tetrasomy. FISH with subtelomeric 10p and 10q probes (Vysis) confirmed that the marker was derived from distal 10p (data not shown). FISH using a 10p probe (Vysis) revealed two distinct pairs of signals at each end of the marker, consistent with an inversion duplication of the distal 10p sequences (Fig. 4b). The 10p probe also hybridized to the telomeric region of each normal chromosome 10, confirming partial tetrasomy for 10p (Fig. 4b).



Fig. 1. Metaphase prepared from cultured amniocytes indicating the additional marker chromosome (arrow).

Discussion

This report has shown the prenatal molecular cytogenetic characterization of an unusual SMC consisting of an inversion duplication of chromosome 10p with a neocentromere, resulting in tetrasomy for 10pter → p14 ~ 15. Although more than 60 cases of trisomy 10p have been reported (Wiktor et al., 1994; Hon et al., 1995; Clement et al., 1996; Fryns et al., 1997; Benzacken et al., 1998; Berend et al., 1999; Granata et al., 2000), this case represents not only the first report of a 10p neocentromere but also the first account of partial tetrasomy for the short arm of chromosome 10. The clinical features of the 10p syndrome include distinct craniofacial anomalies, various organ malformations, skeletal abnormalities, bilateral foot deformities, flexion abnormalities (especially talipes equinovarus), severe mental and psychomotor retardation, developmental delay, seizures, and hypotonia (Stengel-Rutkowski et al., 1977; Goodman and Gorlin, 1983; De Grouchy and Turleau, 1984). In the present case, none of the major structural abnormalities was apparent on a high-resolution ultrasound or on a fetal echocardiogram. In addition, a detailed examination of the products of conception revealed a relatively normal phenotype with development appropriate for that stage of gestation. The absence of any major anatomical anomalies suggests that many of the clinical findings of trisomy 10p may be due to partial aneuploidy of regions more proximal than 10p14 ~ 15. It is also possible that partial aneuploidy, in the form of an anaphoid SMC with a neocentromere, may give rise to an entirely different phenotype, due to the chromatin remodeling of endogenous chromosome material in order to facilitate the formation of the



neocentromere. In this case, the creation of a neocentromere may have effectively inactivated certain genes which, in the aneuploid state, contribute to the features observed in the trisomy 10p syndrome. In any event, the absence of major structural anomalies does not eliminate the risk of other features, such as mental/psychomotor retardation, developmental delay, and seizures.

Recent clinical surveys of phenotypes associated with marker chromosomes include enough cases to distinguish different risks for different chromosomal origins, e.g., acrocentric versus non-acrocentric autosomes (Crolla, 1998). However, these surveys primarily considered SMCs containing endogenous centromeres, which presumably do not contain large amounts of euchromatic sequences. In contrast, neocentromere-containing marker chromosomes can, in theory, originate from any part of the genome, and therefore may contain a higher percentage of transcribed euchromatic sequences than centromeric SMCs, potentially leading to relatively more severe phenotypes. However, the relatively mild phenotype of the present case suggests the possibility that formation of the neocentromere may be concomitant with chromatin remodeling, which may then lead to inactivation of the surrounding genes. On a relatively small neocentric marker, the inactivated genes could represent a significant portion of the marker chromosome, potentially resulting in a phenotype not consistent with aneuploidy for that region.

Over 42 neocentromeres have been described derived from 16 human chromosomes. Of these, 31 are found on inversion duplication chromosomes, some of sufficient size to allow identification by cytogenetic banding (Barbi et al., 2000; Warburton et al., 2000). Twenty-two of these, including the case presented here have otherwise normal karyotypes, giving rise to partial tetrasomy for the duplicated region. Eight cases have apparently formed from duplicated excised segments, giving rise to trisomy for the duplicated region. Seven neocentromere chromo-

somes have resulted from either para- or pericentric deletions, usually found within balanced karyotypes which presumably had a fetal survival advantage over unbalanced cases. This case represents the fourth neocentromere reported on chromosome 10. The first neocentromere described in humans was located at 10q25.2 on a chromosome formed from p and q fusion in association with an excised centric ring (Voullaire et al., 1993; du Sart et al., 1997). The other two reports include a mitotically stable ring chromosome 10(q11→q23) which resulted from an excision within a single arm (Depinet et al., 1997) and a large inversion duplication 10(q11.2→qter), which remains the only case of a somatically derived neocentromere, found in leukemic bone marrow (Abelovich et al., 1996). These cases formally demonstrate at least three distinct neocentromere locations within chromosome 10.

The formation of human neocentromeres at many diverse locations within human chromosomes suggests that centromere formation may not depend on specific primary DNA sequence but, instead, may be an epigenetic process (Murphy and Karpfen, 1998). This point is well illustrated in the present case, where a neocentromere has formed only on the marker chromosome, despite the fact that both normal chromosomes 10 contain homologous 10p sequences. Analysis of the 80-kb DNA sequence underlying the 10q25.2 neocentromere, the only molecularly characterized neocentromere thus far, revealed an unremarkable primary sequence composition (Barry et al., 1998), which was 100% identical to the progenitor (father's) sequence, thus ruling out the possibility that *de novo* mutations were responsible for the formation of the neocentromere (Barry et al., 2000). Nevertheless, the possibility that certain chromosomal regions have an increased propensity for neocentromere formation is suggested by their higher-than-proportional frequency in chromosome 13q (N = 8), 15q (N = 7), and 3q (N = 5) (Warburton et al., 2000). A conserved secondary sequence structural feature consisting of a double dyad symmetry was observed between the 10q25.2 neocentromere sequence and alpha-satellite DNA (Koch, 2000). Additional examples of molecularly characterized neocentromeres will permit further evaluation of the importance of conserved primary or secondary DNA sequence in neocentromere formation.

Whole chromosome paints and chromosome-specific alpha-satellite DNA probes have been very useful in delineating the origins of marker chromosomes (Callen et al., 1992). However, such an approach is expensive and laborious, as numerous probes may be required until the source chromosome is identified. While reverse FISH reveals both the chromosomal identity and originating position on the source chromosome, this technique requires specialized micromanipulation equipment to microdissect and prepare probes from the region of interest. Newer FISH methodologies (utilized in this report) allow for complete screening of all human chromosomes by using either multiple centromeric, telomeric, or whole-chromosome painting probes on a single slide. However, even sequential- and multicolor-FISH cannot identify the exact chromosome regions from which the marker originated. In contrast, CGH is able to reveal the identity of the source chromosome as well as map the regions comprising the marker to specific bands, all in a single hybridization. Characterization of an SMC with respect to its

Fig. 2. (a) FISH with a fluorescein-labeled chromosome 10 centromeric probe showing the two normal chromosomes 10 (arrowheads) and the lack of signal on the marker chromosome (arrow). **(b)** Tricolor FISH indicating the marker to be of chromosome 10 origin. Whole-chromosome paints are directly labeled with Ruby Red (chromosome 5), Emerald Green (chromosome 7), and Sapphire Blue (chromosome 10).

Fig. 3. (a) FISH with a biotinylated all-human-centromere alpha-satellite probe shows signals on all chromosomes examined except for the marker chromosome (arrow). The inset panel shows an enlargement of the marker. **(b)** Combined FISH and immunofluorescence analysis demonstrating the presence of CENP-C (pink) on all chromosomes including the neocentric marker (arrow). The biotinylated whole chromosome 10 paint (wcp 10) was detected with streptavidin-FITC (green) and is observed on both normal chromosomes 10 as well as on the marker. The inset panel shows an enlargement of the chromosome 10-derived neocentric marker.

Fig. 4. (a) Partial ideograms and CGH profiles showing a gain on chromosome 10 from 10pter to the border of 10p15 and 10p14 (10p14 ~ 15). The lines in the CGH profiles represent ratios of (from left to right) 0.5, 0.75, 1.0, 1.25, and 1.5. A ratio of 1.0 represents the balanced state of the chromosome copy number. **(b)** FISH with a fluorescein-labeled (green) 10p telomeric probe (10ptel) shows normal hybridization patterns on the two normal chromosomes 10 (arrowheads) and two distinct pairs of signals at each end of the marker (arrow). The inset panel shows an enlargement of chromosomes 10 and demonstrates the inversion duplication marker.

size and origin is important for karyotype/phenotype correlations, which, in turn, provide prognostic information for genetic counseling.

As the capabilities of molecular cytogenetics expand, more detailed characterization of the chromosomal origins of SMCs

will permit improved correlation of clinical presentation with specific chromosomal regions. Such information would directly benefit prenatally ascertained cases of marker chromosomes, providing couples with a means to make rational and informed decisions concerning the pregnancy.

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