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Molecular Cytogenetic Analysis of Eight Inversion Duplications of Human Chromosome 13q That Each Contain a Neocentromere

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Neocentromeres are fully functional centromeres that have arisen in previously noncentromeric chromosomal locations on rearranged chromosomes. The formation of neocentromeres results in the mitotic stability of chromosomal fragments that do not contain endogenous centromeres and that would normally be lost. Here we describe a unique collection of eight independent patient-derived cell lines, each of which contains a neocentromere on a supernumerary inversion duplication of a portion of human chromosome 13q. Findings in these patients reveal insight into the clinical manifestations associated with polysomy for portions of chromosome 13q. The results of FISH and immunofluorescent analysis of the neocentromeres in these chromosomes confirm the lack of α-satellite DNA and the presence of CENtromere proteins (CENP)-C, -E, and hMAD2. The positions of the inversion breakpoints in these chromosomes have been placed onto the physical map of chromosome 13, by means of FISH mapping with cosmid probes. These cell lines define, within chromosome 13q, at least three distinct locations where neocentromeres have formed, with five independent neocentromeres in band 13q32, two in band 13q21, and one in band 13q31. The results of examination of the set of 40 neocentromere-containing chromosomes that have thus far been described, including the 8 neocentromere-containing chromosomes from chromosome 13q that are described in the present study, suggest that chromosome 13q has an increased propensity for neocentromere formation, relative to some other human chromosomes. These neocentromeres will provide the means for testing hypotheses about sequence requirements for human centromere formation.

Introduction

The centromere is the chromosomal component responsible for the proper segregation of replicated chromatids during mitosis and meiosis. A better understanding of the requirements for centromere formation and function is required before the promise of artificial human chromosomes used as autonomous gene-delivery vectors can be fully realized (Warburton 1999).

Normal human centromeres all contain up to several million base pairs of tandemly repeated α-satellite DNA. Introduction of α-satellite DNA into human fibrosarcoma HT1080 cells can result in de novo centromere formation (Harrington et al. 1997; Ikeno et al. 1998; Henning et al. 1999). However, the results of examination of inactive centromeres in dicentric chromosomes and of neocentromeres demonstrate that unknown epigenetic modifications are required for centromere formation (Warburton et al. 1997).

Stable dicentric chromosomes have an inactive centromere containing a large array of α-satellite DNA that does not form a centromere, suggesting a reversibility of the centromeric epigenetic mark (Sullivan and Schwartz 1995). Conversely, neocentromeres, which are found on mitotically stable chromosomes separated from endogenous centromeres, do not contain α-satellite DNA yet have fully functional centromeres, sug-

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gesting acquisition of the centromeric epigenetic mark by previously noncentromeric genomic DNA (Choo 1997). Thus, studies of the requirements for human-centromere formation are no longer done simply to seek the particular primary sequence that determines centromere formation but, rather, are also done to investigate the nature of the associated epigenetic modifications required for centromere formation. Possible mechanisms for this epigenetic modification include the presence and propagation of a centromere-specific nucleosome containing the histone H3–like CENP-A (Warburton et al. 1997), a differential replication timing of centromeric DNA, or covalent modifications such as DNA methylation or histone H4 deacetylation (Murphy and Karpen 1998; Wiens and Sorger 1998).

Neocentromeres provide an opportunity to examine and compare single- or low-copy centromeric DNA in the absence of large arrays of repetitive DNA, thereby permitting further assessment of the role of the primary DNA sequence in centromere formation. Approximately 40 neocentromeres have been described on derivatives of 15 different human chromosomes. As a result of the increasingly routine clinical application of FISH with chromosome-specific α-satellite DNA probes to identify the origin of marker chromosomes, analphoid neocentromeres are identified and are reported at an increasing rate.

The first neocentromere to be molecularly characterized—one that was derived from a pericentric deletion of chromosome 10 and that was found in human chromosome 10q25—was shown to have sequence-tagged site (STS) and restriction maps that were identical to those of the corresponding regions in normal chromosomes 10 (Voullaire et al. 1993; du Sart et al. 1997). Remarkably, the DNA sequence of the underlying 80 kb of neocentromeric DNA (ncDNA) had few, if any, sequence characteristics that would suggest a reason for its centromere formation (Barry et al. 1999). However, given that there were few a priori expectations for what these centromeric sequence characteristics might be, it may not be surprising that the characteristics were not apparent from this first example. Additional examples of ncDNA will be critical for sequence comparisons, to identify and to evaluate conserved primary or secondary sequence characteristics that may be important for centromere formation (Koch 2000).

In the present report, we will describe the initial molecular cytogenetic analysis of a novel collection of eight independent neocentromeres that have all formed in chromosome 13q. The patients in whom these chromosomes were identified have permitted us to evaluate the clinical features associated with chromosomally pure partial tetrasomy or trisomy 13q without aneuploidy for any other chromosomes. According to the results of FISH and immunofluorescence analysis, these 13q neocentromeres are all negative for α-satellite DNA, but they are positive for kinetochore proteins. FISH analysis of the inversion breakpoints, done with physically mapped ordered cosmids from chromosome 13q used as FISH probes, has permitted delineation of the relative sizes of these chromosomes and placement of the breakpoints onto the physical map of chromosome 13 (Cayanis et al. 1998). At least three distinct locations within chromosome 13q have formed neocentromeres, with multiple independent examples in bands 13q32 and 13q21. Of the total of 40 human neocentromeres that have thus far been described, 8 occur in chromosome 13q, suggesting nonrandom chromosome involvement. This might indicate either selection for fetal survival or differences in the propensity for neocentromere formation in different parts of the genome. The implications of this unique set of neocentromeres in the analysis of the requirements for human centromere formation are discussed.

Material and Methods

Patient Ascertainment

Patients with chromosome 13q neocentromeres were identified through a survey of the literature, including meeting abstracts, and authors were invited to submit Epstein-Barr virus–transformed lymphoblast or fibroblast cell lines for further analysis. All patients were originally ascertained as a result of developmental delay and congenital malformations, and diagnosis was made cytogenetically by means of G-banding, spectral karyotyping (SKY) FISH (Huang et al. 1998), whole-chromosome painting probes, and/or comparative genomic hybridization (CGH) (du Manoir et al. 1993). Detailed clinical features were noted for each patient (table 1). The overall spectrum of affected systems was identified. To ensure completeness, specific follow-up inquiries were made with regard to each system not mentioned in the original report.

Molecular Cytogenetic Analysis

FISH and immunofluorescence were performed essentially as described elsewhere (Alonso et al., in press). FISH done with commercially available probes—for example, with WCP 13, CEP 13/21, LSI 13 RB1, and LSI 13 (all from Vysis), and an "all-centromere" probe (Oncor)—was performed according to the manufacturers' directions. Cosmids from chromosome 13q were derived from the chromosome 13 physical map available from The Human Genome Project at Columbia University (Cayanis et al. 1998). An additional chromosome 13–specific painting probe was prepared by means of *Alu* PCR of DNA from a rodent human somatic-cell hybrid containing chromosome 13 as its only human

Table 1

Clinical Features of Patients with Partial Trisomy or Tetrasomy 13q

	Trisomy 13q14-qter, Patient 13a	Trisomy 13q21-qter, Patient 13b	Tetrasomy 13q21-qter		Tetrasomy 13q31-qter, Patient 13e	Tetrasomy 13q32-qter		
			Patient 13c	Patient 13d		Patient 13f	Patient 13g	Patient 13h
Age[a]	17 mo	10 mo	8 years	Died at 13 d	4 years	Died at 3 years	Died at 10 years	11 years
Growth	Normal birth weight	Low birth weight	Normal	AGA at 7 mo	Normal	Normal birth weight	Normal birth weight; normal growth at 5 years and below normal growth at 8 years	Normal birth weight; normal growth at 7 years, obese
Head	Left plagiocephaly	Brachycephaly and microcephaly	Normal	Normal CT scan	Normal	Microcephaly and scaphocephaly	Normocephalic; normal CT scan at birth	Normal
Facies	Flat philtrum	Thick eyebrows and curly eyelashes	Submucosal cleft palate and high nasal bridge	Hypertelorism and left cleft lip/gum	Thick eyebrows and hypertelorism	Bulbous nose, short neck, and high-arched palate	Bifid nose, anteverted nostrils, and hypertelorism	Bulbous nose, bifid tip, anteverted nostrils, high-arched palate, and epicanthus
Eyes	Bil. glaucoma, nystagmus, and bil. retinoblastoma	Bil. microphthalmia	Strabismus	Normal	Strabismus	Agenesis in the right eye and left microphthalmia	Iris and choroidal colobomata	Right microphthalmia, coloboma, and tumor of the right optic nerve
Ears	Right total deafness and left conductive diseases	Normal	Conductive deafness and earlobe anomaly	Low set and posteriorly rotated	Deformed lobes	Low set and posteriorly rotated, with right hearing loss	Low set	Right low set, posteriorly rotated, right preauriculoar pit, and right hearing loss
CNS	Hypertonicity and abnormal brain density on CT	Developmental delay	Mild mental retardation	Hypotonia	Mild mental retardation and seizures	Profound mental retardation and agenesis of corpus callosum	Intractable seizures, loss of function, and hypotonia; MRI at age 10 years shows swollen gray matter	Intractable seizures, moderate mental retardation, early hypotonia, later ataxia, and hemiparesis
Skin/teeth	Hemangioma of the arm and facial vitiligo	Normal	Patchy pigmentation, multiple caries, and left accessory nipple	Normal	Excess teeth (three in lower jaw)	Hemangioma of the arm, head, and neck; scalp lesion	Hemangioma of the head and back	Hemangioma of the head and neck; poorly implanted and delayed teeth
Heart/lung	Normal	Normal	Normal	ASD, VSD, PDA, and pulmonary hypoplasia	PDA, diaphragmatic hernia, and bronchial anomalies	PDA, ventricular outflow obstruction, and subaortic stenosis	Normal	PDA at birth but now normal
Gastrointestinal	Normal	Normal	Normal	Normal	Intestinal malrotation	Normal	Normal	Normal
Extremities	Normal	Bil. postaxial polydactyly	Right camptodactyly and toe hypoplasia	Left postaxial polydactyly	Normal	Normal	Tapering fingers	Small hands and feet, clinodactyly of the hands, and syndactyly 2/3 of feet
Skeletal	Normal	Normal	Normal	11 ribs and hemivertebrae	Scoliosis	11 ribs	Normal	Right hemiatrophy, asymmetrical chest, and wide-spaced nipples
Urogenital	Hypospadius, cryptorchidism, and normal renal USG	Normal female	Normal female	Normal male and left renal agenesis	Hypospadius and hydronephrosis	Normal female	Normal male	Subseptated uterus, lower septated vaginal canal, and duplicated left ureter

Note.—AGA = average for gestational age; ASD = atrial septal defect; MRI = magnetic-resonance imaging; PDA = patent ductus arteriosus; USG = ultrasonogram; VSD = ventricular septal defect; bil. = bilateral.

[a] At last examination.

complement (Liu et al. 1993). DNA used for FISH probes was labeled with either biotin or digoxygenin and was detected with either fluor-conjugated streptavidin or antidigoxygenin, by use of standard protocols.

Immunofluorescence with antibodies to CENP-C was performed on 3:1 methanol:acetic acid–fixed chromosomes. Immunofluorescence with antibodies to CENP-A, CENP-E, and hMAD2 was performed on aqueous cytospin preparations. Rabbit anti–CENP-C and rabbit anti-hMAD2 were detected with the use of goat anti-rabbit immunoglobulin G. Chromosomes were counterstained with 4,6 diamidino-2-phenylindole (DAPI) (blue). Photomicrographs were obtained on a Nikon Eclipse 800 fluorescent/phase microscope equipped with a Sony DKC 5000 camera; each image was collected separately, by use of single-pass filter sets (Chroma) and by merging of separate color channels with the use of Adobe Photoshop.

Results

Molecular Cytogenetic Analysis of Neocentromere-Containing Inversion Duplication 13q Chromosomes

In each patient presented here, the results of karyotype analysis revealed the presence of a supernumerary chromosome, the origins of which were identified by use of FISH with chromosome-specific painting probes, multicolor SKY FISH, or CGH. The chromosome 13 origin was confirmed by use of chromosome 13–specific whole-chromosome paint. G-banding confirmed the symmetrical inversion duplication and localized the breakpoint for each chromosome. Each chromosome contained a primary constriction that was localized to a specific G-band. However, the results of FISH done with the use of an α-satellite DNA probe specific for both chromosome 13 and chromosome 21, as well as an "all-centromere" probe, showed no signal on these chromosomes, thereby confirming the presence of an analphoid neocentromere. Representative examples of the cell lines of patients 13b and 13a are shown in figure 1*A* and figure 1*B,* respectively.

To confirm formation of a kinetochore at these neocentromeres, immunofluorescence was performed, for each cell line, with antibodies to proteins found to be associated with active kinetochores—for example, CENP-C and/or CENP-E. Figure 1*C* provides a representative example of this immunofluorescent analysis, showing the presence of CENP-C at the neocentromere, as well as all other centromeres, in the cell line from patient 13g. In a further investigation of the functional spectrum of neocentromeres, immunofluorescence showed that hMAD2, a component of the spindle-assembly checkpoint (Li and Benezra 1996), is also present at all neocentromeres examined in colcemid-treated cells, as is shown (see figure 1*D*) for the cell line of patient 13e.

Ideograms of the abnormal chromosomes in these patients are shown in figure 2*A;* they reveal the cytogenetically determined positions of the neocentromere and the inversion breakpoints, relative to normal chromosome 13. Six (13c–h) of the eight patients (fig. 2*A;* table 2) have 23 normal chromosome pairs, including two normal chromosomes 13, plus the inversion duplication of chromosome 13q, resulting in partial tetrasomy for the duplicated portion of chromosome 13q. The remaining two patients—patients 13a and 13b (figure 2*A;* table 2)—have one normal chromosome 13, an inversion duplication chromosome, and a complementary deletion of one of the normal chromosomes 13, resulting in partial trisomy for the duplicated/deleted region.

No patient had a chromosomal duplication for the most-proximal region of chromosome 13q (q11.2-q13). Patient 13a had trisomy for a region extending from q14-qter, distal to the retinoblastoma gene, plus an apparently complementary ring chromosome containing q14-pter (Dowhanick et al. 1998). Patients 13c and 13d had tetrasomy for q21-qter, whereas patient 13b had a complementary terminal-deletion chromosome that contained 13pter-13q21 and that resulted in trisomy for q21-qter. Patient 13e had tetrasomy for q31-qter, and patients 13f–13h had tetrasomy for q32-qter. Figure 2*A* also shows the cytogenetic position of a panel of four ordered cosmids from chromosome 13q that were used as FISH probes to confirm the morphology of these chromosomes and that were used as reference cosmids for further analysis in this report.

Clinical Findings in Patients with Partial Trisomy or Tetrasomy of Chromosome 13q

Each of these patients has tetrasomy or trisomy for discrete segments of chromosome 13q uncomplicated by aneusomy for other chromosomal segments. This provides a unique opportunity to define the clinical features associated with duplication for particular regions of chromosome 13q (table 1). The features of trisomy 13 that were most frequently seen in these patients were microphthalmia or other major eye defects (five of eight patients), hemangiomata (four of eight patients), and deafness (four of eight patients). All of these defects were found in patients with the smallest duplications, indicating that distal duplication is sufficient for development of these anomalies. Patients 13g and 13h, who had the most-distal tetrasomy (q32-qter), showed a similar developmental pattern of early seizures that first responded to medication and then became intractable, with subsequent severe neurological deterioration occurring (Lozzio et al. 1997; Govaerts et al. 1999). Polydactyly and cleft lip or palate, which are features seen

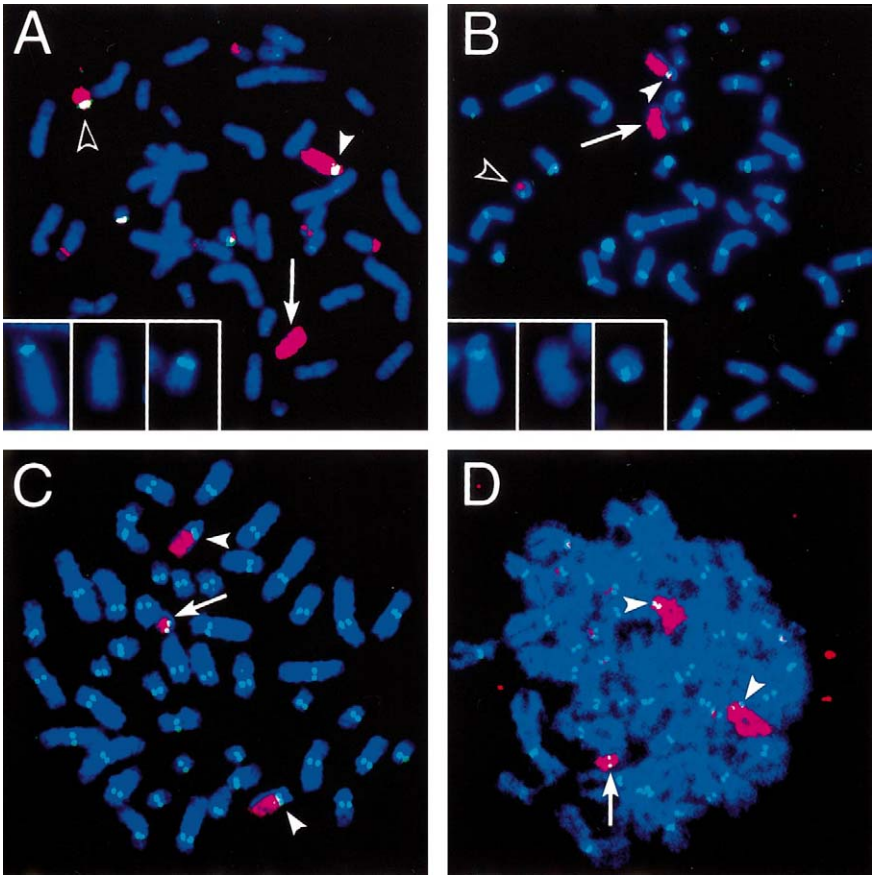

Figure 1 FISH and immunofluorescent analysis of neocentromeres. *A,* FISH analysis of the cell line of patient 13b. Whole-chromosome 13 paint (Wcp 13, *red*) hybridized to the inversion duplication 13(q21-qter) chromosome (*white arrow*) as well as to the normal chromosome 13 (*white arrowhead*) and the deleted 13 (pter-q21) (*unwhitened arrowhead*). Chromosome 13 and chromosome 21–specific α-satellite DNA (*green*) did not hybridize to the inversion duplication chromosome 13 (*middle inset panel*), but it did hybridize to the centromeres of the normal chromosome 13 (*left inset panel*), the deleted 13 (*right inset panel*), and the two normal chromosomes 21. *B,* FISH analysis of the cell line in patient 13a. Wcp13 (*red*) hybridized to the inversion duplication 13(q14-qter) (*white arrow*) as well as to the normal chromosome 13 (*white arrowhead*) and the ring 13 (pter-q14) (*unwhitened arrowhead*). An "all centromere" probe (*green*) did not hybridize to the inversion duplication 13 (*center inset panel*), but it did hybridize to all normal centromeres, including the normal 13 (*left inset panel*) and ring 13 (*right inset panel*). Insets show enlarged chromosomes from the same metaphase spread seen in the main figure, which was rotated, when necessary, with the wcp13 signal (*red*) removed for clarity. *C,* Combined FISH and immunofluorescent analysis of the cell line of patient 13g. Wcp13 (*red*) hybridizes to the inversion duplication 13(q32-qter) (*white arrow*) and to the two normal chromosomes 13 (*unwhitened arrowheads*). CENP-C (*green*) was seen at the neocentromere on the inversion duplication 13 as well as at all normal centromeres. *D,* Combined FISH and immunofluorescence analysis of the cell line from patient 13e. Wcp13 (*red*) hybridizes to the inversion duplication 13(q31-qter) (*white arrow*) as well as to the two normal chromosomes 13 (*unwhitened arrowheads*). hMAD2 (*green*) is seen at the neocentromere on the inversion duplication 13 as well as at all normal centromeres. In some cases, Wcp13 faintly detects the short arm of additional acrocentric chromosomes. *A–C,* 3:1::methanol:acetic acid–fixed chromosome spread; *D,* aqueous cytospin chromosome spread.

in >50% of patients with full trisomy 13 (Chu et al. 1994), were each found in only two of the patients with the most-extensive duplications (table 1). With the exception of holoprosencephaly, all of the major features of trisomy 13 were found in at least two of the eight patients. Thus, trisomy for the proximal bands 13q11-13q13 does not appear to be necessary for the development of most of the more-common features of trisomy 13 (e.g., facial clefting, polydactyly, seizures, eye anomalies, deafness, hemangioma, and heart defects). However, it is striking that none of the eight patients had holoprosencephaly or other midbrain anomalies, which are hallmarks of full trisomy 13. This feature may thus be associated with duplication of the most-proximal region of the chromosome. Notably, gene expression may be inactivated in the vicinity of the neocentromere on these chromosomes, potentially influencing the clinical features observed.

FISH Mapping of Inversion Breakpoints

These inversion duplication chromosomes have been characterized by means of FISH mapping, to further lo-

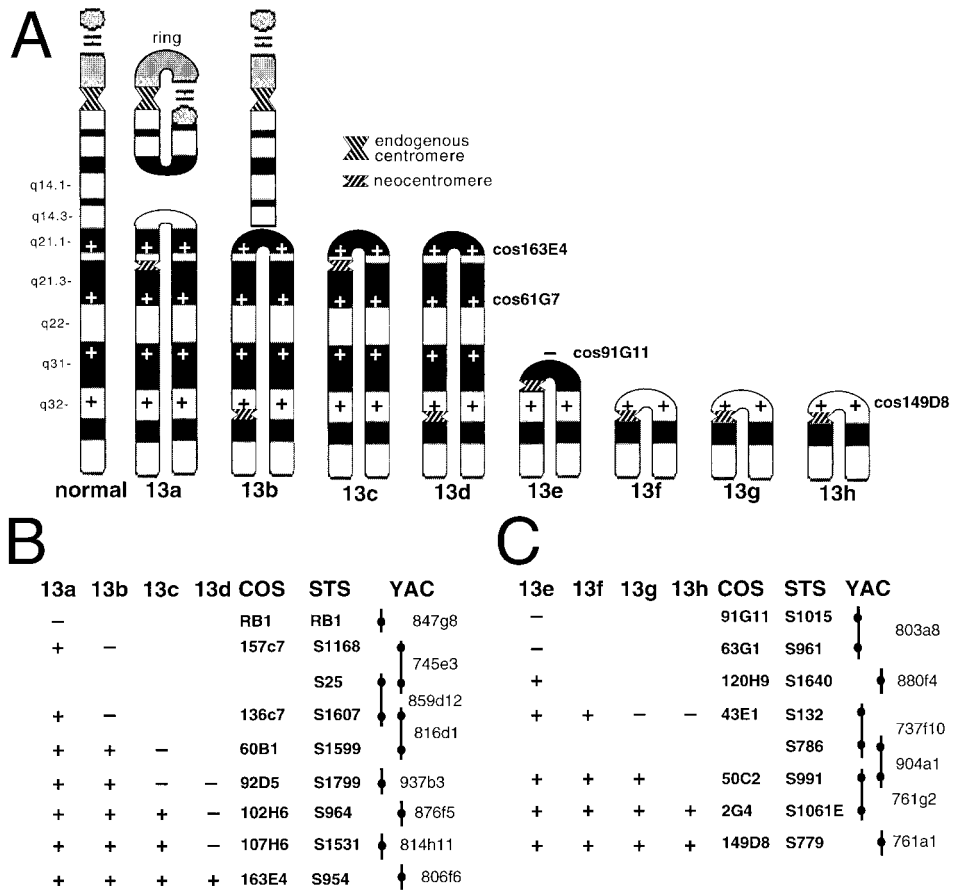

Figure 2 Characterization of inversion duplication distal 13q neocentromere chromosomes. *A*, Ideograms of all abnormal derivative chromosomes 13 found in the cell lines are shown in a comparison with a normal chromosome 13 (*left*). The cytogenetically determined position of the neocentromere and the inversion breakpoint is shown for each chromosome. The cell lines of patients 13a and 13b both contain deletion chromosomes that are complementary to the inversion duplication chromosomes, resulting in trisomy for the duplicated region. Cell lines from patients 13c – 13h each have two normal chromosomes 13 and, thus, are tetrasomic for the duplicated region. Reference cosmids used as FISH probes on cell lines are indicated; a plus sign (+) indicates that such cosmids are present on chromosomes, whereas a minus sign (−) indicates that they are absent from the chromosome. The relative order of 13q32 neocentromeres and cosmid 149D8 is not implied. The relative order of the 13q21 neocentromeres and cosmid 163E4 is not implied. *B*, FISH mapping of the 13q14 and 13q21 breakpoints. *C*, FISH mapping of the 13q31 and 13q32 breakpoints. The cosmid used as the FISH probe as well as the corresponding STSs and YAC are indicated. Contiguous YACs are indicated when applicable. Note the inclusion of cosmids, shown in panel *A*, for reference in panels *B* and *C*. Breakpoints have been placed onto the existing physical map of ordered cosmids and YACs from chromosome 13 (Cayanis et al. 1998).

calize the positions of the breakpoints and to permit determination of the relative sizes of the chromosomes. The breakpoint in the cell line of patient 13a is located in chromosome band q14, which can be distinguished cytogenetically as the most proximal of all the cell lines examined (see fig. 2*A*). The breakpoints in the cell lines of patients 13b–13d all occur in chromosome band 13q21 and thus require higher-resolution FISH mapping for determination of their relative sizes. In patient 13e, the breakpoint in the cell line can be distinguished cytogenetically in chromosome band 13q31. In patients 13f–13h, the breakpoints in the cell lines all occur in band 13q32 and require higher-resolution FISH mapping. FISH done with a panel of ordered cosmids from chromosome 13q (Cayanis et al. 1998) has been used to determine the position of the inversion breakpoints, where probes absent from the inversion duplication chromosome are proximal to the breakpoint and where probes present on the inversion duplication chromosome are distal to the breakpoint, relative to positions in a normal chromosome 13. Chromosome 13–specific painting probes were used to identify all normal and derivative chromosomes 13 in the cells, which acted as hybridization controls.

Figure 2*B* and *C* shows the placement of the breakpoints on the published physical map of chromosome 13 (Cayanis et al. 1998); the presence or absence of the cosmid on the inversion duplication chromosome in each cell line as well as the corresponding STSs and yeast-artificial chromosomes (YACs) are indicated. For clarity,

Table 2

Chromosomal Rearrangements with Neocentromeres

Chromosome	Chromosomal Abnormalities[a]	Rearrangement	Complement[b]	(%) Mosaicism[c]	Reference
1	Del (pter→p36.1::p32→qter), +ring (p32→neo→p36.1)	Paracentric deletion	Balanced	100 (fib), 97 (lym)	Slater et al. (1999)
2	Del (pter→p21::p11→qter), +der (p11→neo→p21)	Paracentric deletion	Balanced	100 (fib)	Petit and Fryns (1997)
3a	Del (pter→neo→p23::q25→qter), +ring (p23→q25)	Paracentric deletion	Balanced	100 (fib), 100 (lym)	Maraschio et al. (1996)
3b	Der (p11→q11), +del (pter→p11::q11→q26neo→qter)	Paracentric deletion	Balanced[d]	100 (fib), 100 (lym)	Wandall et al. (1998)
3c	+Invdup (qter→3q27.2::3q27.2→neo→qter)	Inversion duplication	Tetrasomy q27.2→qter	Mosaic	Teshima et al. (1998)[e]
3d	+Invdup (qter→3q27.2::3q27.2→neo→qter)	Inversion duplication	Tetrasomy q27.2→qter	Mosaic	Teshima et al. (1998)[e]
3e	+Invdup (qter→3q27.1::3q27.1→neo→qter)	Inversion duplication	Tetrasomy q27.1→qter	30 (lym), 6 (fib)	Portnoi et al. (1999)
3f	+Invdup (qter→3q26.2::3q26.2→neo→qter)	Inversion duplication	Tetrasomy q26.2→qter	57 (fib)	Cockwell et al., in press
4	Ring (q21→q21neo), +del (pter →q21::q21→qter)	Paracentric deletion	Balanced	75 (fib)	Grimbacher et al. (1999)
8a	+Invdup (pter→p23.1::p23.1→neo→pter)	Inversion duplication	Tetrasomy pter→p23	100 (lym)	Ohashi et al. (1994)
8b	+Invdup (qter→q23::q23→neo→qter)	Inversion duplication	Tetrasomy qter-q23	75 (lym)	Sulcova et al. (1999)[e]
9a	Del (p12→qter), +invdup (pter→p12::p12→p23neo→pter)	Inversion duplication	Trisomy pter→p12	100 (lym)	Vance et al. (1997)
9b	+Invdup (pter→p21.2::p21.2→p23neo→pter)	Inversion duplication	Tetrasomy pter→p21.2	100 (lym)	Depinet et al. (1997)
10a	Del (pter→p11.2::q23.2→q25.2neo→qter), +r(p11.2→q23.2)	Paracentric deletion	Balanced	100 (fib), 100 (lym)	Voullaire et al. (1993)
10b	+Invdup (qter→q11.2::q11.2→neo→qter)	Inversion duplication	Tetrasomy qter→q11.2	90 (lym)	Abeliovich et al. (1996)
10c	Del (pter-q11::q23→qter), +ring (q11→neo→q23)	Paracentric deletion	Balanced	80 (fib), 62 (lym)	Depinet et al. (1997)
11	Del (pter→q22), +invdup (qter→q22::q22→neo→qter)	Inversion duplication	Trisomy qter→q22	100 (fib),100 (lym)	Depinet et al. (1997)
13a	Ring (pter→q14), +invdup (qter→q14::q14→q21neo→qter)	Inversion duplication	Trisomy q14-qter	100 (lym)	Dowhanick et al. (1998)[e]
13b	Del (pter→q21), +invdup (qter→q21::q21→q32neo→qter)	Inversion duplication	Trisomy q21-qter	100 (lym)	Rivera et al. (1999)
13c	+Invdup (qter→q21::q21neo→qter)	Inversion duplication	Tetrasomy q21-qter	49 (lym)	Tohma et al. (1998)[e]
13d	+Invdup (qter→q21::q21→q32neo→qter)	Inversion duplication	Tetrasomy q21-qter	88 (fib)	Warburton et al. (1997)
13e	+Invdup (qter→q31::q31neo→qter)	Inversion duplication	Tetrasomy q31-qter	60 (lym)	Tohma et al. (1998)[e]
13f	+Invdup (qter→q32::q32neo→qter)	Inversion duplication	Tetrasomy q32-qter	98 (lym), 8 (fib)	Depinet et al. (1997)
13g	+Invdup (qter→q32::q32neo→qter)	Inversion duplication	Tetrasomy q32-qter	100 (lym), 100 (fib)	Govaerts et al. (1999)[e]
13h	+Invdup (qter→q32::q32neo→qter)	Inversion duplication	Tetrasomy q32-qter	74 (lym), 25 (fib)[f]	Lozzio et al. (1997)[e]
14	Del (pter→q32.1), +invdup (qter→q32.1::q32.1→neo→qter)	Inversion duplication	Trisomy q32-qter	100 (lym)	Sacchi et al. (1996)
15a	+Invdup (qter→q23::q23neo→qter)	Inversion duplication	Tetrasomy q23-qter	70 (lym)	Blennow et al. (1994)
15b	+Invdup (qter→q24::q24neo→qter)	Inversion duplication	Tetrasomy q24-qter	80 (lym)	Blennow et al. (1994)
15c	+Invdup (qter→q25.3::q25.3→neo→qter)	Inversion duplication	Tetrasomy q25.3-qter	82 (lym)	Depinet et al. (1997)
15d	+Invdup (qter→q25.3::q25.3→neo→qter)	Inversion duplication	Tetrasomy q25.3-qter	74 (lym)	Depinet et al. (1997)
15e	+Invdup (qter→q26.1::q26.1→neo→qter)	Inversion duplication	Tetrasomy q26.1-qter	86 (lym)	Depinet et al. (1997)
15f	+Invdup (qter→q25::q25→neo→qter)	Inversion duplication	Tetrasomy q25-qter	100 (lym)	Huang et al. (1998)
15g	+Invdup (qter→q25::q25→neo→qter)	Inversion duplication	Tetrasomy q25-qter	79 (lym)	van den Enden et al. (1996)
17	Del (pter→q22:q23→qter), +invdup (q22→q23::q23neo→q22)	Inversion duplication[g]	Trisomy q22→q23	100 (lym)	Ravnan et al. (1999)[e]
20	Del (qter→p11.2), +invdup (pter→p11.2::p11.2→p21neo→pter)	Inversion duplication	Trisomy p11.2-pter	100 (fib), 100 (lym)	Voullaire et al. (1999)
X	Rec (qter→q12neo→q13.1 or q11.2::q23 or q24→qter)	Inversion duplication[h]	X, deletion X	82 (lym)	Kaiser-Rogers et al. (1995)[e]
Ya	Morphologically normal Y, inactive cen + q12neo	None	Normal	5 (lym)	Bukvic et al. (1996)
Yb	Inverted Y, inactive cen + neo	Inversion	Recombinant Y	Unstable	Rivera et al. (1996)
Yc	Morphologically normal Y, inactive cen + q12neo	None	Normal	94 (lym)	Tyler-Smith et al. (1999)
Yd	Inversion Y (qter→q11.2::q11.2neo→qter)	Inversion duplication	Deletion Y	70 (fib)	Warburton et al. (1997)

[a] All deletions and rearrangements are written inclusive of the chromosomal material present. Del = deletion; Invdup = inversion duplication.

[b] Chromosome complement is approximated cytogenetically and considers possible microdeletions or insertions.

[c] Mosaicism for neocentromere-containing chromosome. The percentage of mosaicism for fibroblasts (fib) and lymphcytes (lym) is given when ascertained. Mosaic (cell lines 3c and 3d) = patient reported as mosaic, but percentage of mosaicism and cell type are not given.

[d] Father balanced; dysmorphic proband mosaic (90% fib, 70% lym) for centric fragment der (p11→q11).

[e] American Society of Human Genetics Annual Meeting abstract.

[f] An additional 15% had two markers per cell, and 11% of cells had no markers analyzed on lymphocytes at birth.

[g] Complex rearrangement with both paracentric deletion and inversion duplication of neocentric deleted fragment.

[h] Asymmetrical inversion duplication.

the reference cosmids used to characterize the chromosomes (see fig. 2*A*) are included in this analysis. Figure 2*B* shows mapping proximal to cosmid 163E4 in chromosome band q21, and figure 2*C* shows mapping proximal to cosmid 149D8 and distal to cosmid 91G11. This FISH mapping has permitted the ordering of the chromosomes by size (see fig. 2*A*), with chromosomes presented from largest to smallest, from left to right. It would be possible to further molecularly characterize each breakpoint to high resolution, by means of additional cosmid and YAC mapping, if it were determined to be of interest for neocentromere characterization.

Three Distinct Neocentromere Locations in Chromosome 13q

The results of cytogenetic analysis showed at least three distinct neocentromere locations within chromo-

some 13q, with five independent neocentromeres in band 13q32, two in band 13q21, and one in band 13q31 (fig. 2*A*). Simultaneous FISH with selected reference cosmids (fig. 2*A*) and immunofluorescence with CENP-C have been used for molecular cytogenetic characterization of these different neocentromere positions in the cell lines of patients 13a, 13d, and 13e (fig. 3). In patient 13a, findings from FISH analysis of the cell line with cosmid 61G7 located in 13q21.3/13q22 shows hybridization to either side of the inversion breakpoint. Simultaneous CENP-C immunofluorescence showed the neocentromere to be between these FISH signals, consistent with its cytogenetic localization to the more-proximal chromosome band 13q21. In patient 13d, the results of FISH analysis of the cell line with cosmid 91G11 located in 13q31 again shows hybridization to either side of the inversion breakpoint. In this patient, however, CENP-C immunofluorescence showed the neocentromere to be distal to both FISH signals near one end of the mirror-image chromosome, consistent with its cytogenetic localization in chromosome band 13q32. Finally, in patient 13e, the results both of FISH analysis of the cell line with cosmid 149D8 located in 13q32 and of CENP-C immunofluorescence showed the neocentromere to be near the inversion breakpoint between the FISH signals, consistent with its localization to chromosome band 13q31. The positions of the neocentromeres have not been localized within chromosome subbands (e.g., the relative positions of the 13q32 neocentromeres and cosmid 149D8 and of the 13q21 neocentromeres and cosmid 163E4 have not been determined by simultaneous immunofluorescence and FISH (see fig. 2*A*).

Survey of Reported Neocentromeres

The presence of a neocentromere results in the mitotic rescue of what would have been acentric unstable chromosomes that would normally have been lost. Forty neocentromeres, including the eight 13q neocentromeres presented here (table 2), have been described in derivatives of 15 human chromosomes. Seven examples have been seen on chromosomes where neocentromere formation has permitted recovery of both complementary products of an interstitial deletion event. Of these seven examples, three—3a, 3b, and 10a (table 2)—resulted from neocentromere formation on a fusion chromosome consisting of two acentric terminal fragments, with recovery of the complementary pericentric deletion fragment. The other four examples—1, 2, 4, and 10c (table 2)—resulted from neocentromere formation on a paracentric deletion fragment, with recovery of the complementary fusion of the centric and acentric terminal fragments. In both of these types of rearrangements, the resulting karyotype appears to be balanced, resulting in minimal loss of genetic material and a relatively mild phenotype that can be exacerbated by mosaicism for either derivative chromosome (Wandall et al. 1998).

Twenty-eight cases of neocentromere formation, including each of the chromosome 13q neocentromeres presented here (table 2), have permitted recovery of supernumerary inversion duplications of distal chromosomal regions (Rivera et al. 1999). In 21 cases, the karyotype is normal except for the supernumerary chromosome, giving rise to partial tetrasomy for the duplicated portion. In seven cases, a complementary terminal deletion results in a partial trisomy for the duplicated region. In general, only cases with duplication of a relatively small distal subfragment were seen, with the notable exception of a somatic mutation resulting in inversion duplication of chromosome 10qter-q11.2, as seen in leukemic bone marrow (Abeliovich et al. 1996). A chromosome 17–derived neocentromere resulted from a paracentric deletion and inversion duplication of the deleted region, giving rise to an inversion duplication 17q22-q23::q23-q22 neocentromere-containing chromosome (Ravnan et al. 1999).

Neocentromere formation on the sex chromosomes appears to involve unusual chromosomal rearrangements, compared with neocentromere formation on the autosomal neocentromeres. The single reported example of an X chromosome neocentromere is seen on an asymmetrical inversion duplication fragment (Kaiser-Rogers et al. 1995). Four neocentromeres have been described on derivative Y chromosomes. Whereas Yd (table 2) is an inversion duplication where the endogenous Y centromere has been deleted, the other three Y-chromosome derivatives all contain both an inactivated endogenous Y centromere and a neocentromere in the heterochromatic long arm.

The results of examination of the 40 neocentromeres reported to date reveal that some chromosomal regions have a higher-than-expected proportion of observed neocentromeres; these regions include chromosome 13q ($n = 8$), which includes 13q32 ($n = 5$), as well as distal chromosome 15q ($n = 7$) and distal chromosome 3q ($n = 5$) (table 2). These high proportions of neocentromere formation might be attributed to the compatibility of fetal survival for trisomies of these chromosomal regions. However, neocentromeres have thus far not been observed on derivatives of chromosomes 18 or 21, despite a high tolerance of trisomies for these chromosomes. The combined length (mean percent of the haploid genome) for chromosome 18 (2.93%) and chromosome 21 (1.9%) is 4.83%, which is longer than the length of chromosome 13 (3.74%). Thus, observation of 8/40 chromosome 13q–derived neocentromeres and of 0/40 chromosome 18– or chromosome 21–derived neocentromeres permits rejection of the hypothesis that there is an equal propensity, per unit of genome length, for neocentromere formation on chromosomes 13, 18,

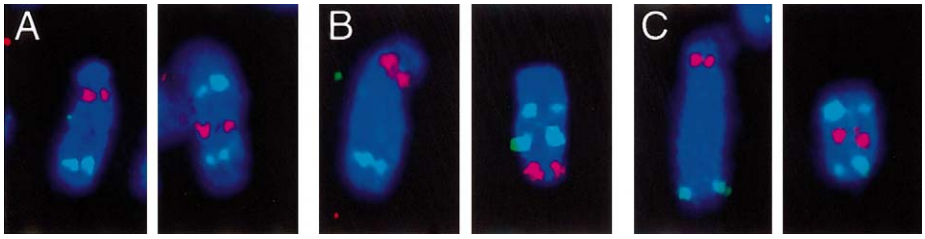

Figure 3 Molecular cytogenetic analysis of neocentromere position. For each panel, a normal chromosome 13 (*left*) and the inversion duplication 13 chromosome (*right*) from the same metaphase spread are shown. The results of FISH done with the use of reference cosmid probes from chromosome 13q (*green*; see fig. 2*A*) and of immunofluorescence done with the use of antibodies to CENP-C (*red*) are shown. *A*, Patient 13a's cell line, hybridized with cosmid 61G7 from chromosome band 13q21.3. *B*, Patient 13d's cell line, hybridized with cosmid 91G11 from chromosome band 13q31. *C*, Patient 13e's cell line, hybridized with cosmid 149D8 from chromosome band 13q32.

and 21 ($P < .001$; Fisher exact test). Similar statistical arguments can be made with use of the observed frequency of neocentromeres observed on the X chromosome ($n = 1$), which is easily tolerated in multiple inactive copies. Similar observations would result if chromosomes 18, 21, and X had a disproportionately lower propensity for neocentromere formation. Thus, the existing set of described neocentromeres provides statistical support for the idea that the propensity for neocentromere formation can vary in different chromosomal regions.

Discussion

We have obtained and have performed molecular cytogenetic characterization of eight independent inversion duplication 13q chromosomes with neocentromeres. These 13q neocentromeres do not contain α-satellite DNA that can be detected by FISH (fig. 1*A* and *B*), and they are forming centromeres in previously noncentromeric chromosomal locations. Thus, neocentromeres provide an opportunity to examine the requirements for human centromere formation in the absence of the large amounts of tandemly repeated DNA found at normal centromeres.

Neocentromeres have thus far been shown to contain all active kinetochore proteins examined, including constitutive structural proteins—for example, CENP-A and CENP-C (Depinet et al. 1997; Warburton et al. 1997) (fig. 1*C*)—and microtubule-binding proteins—for example, CENP-E. We have also confirmed the presence of the spindle-assembly checkpoint protein hMAD2 (fig. 1*D*) (Li and Benezra 1996) suggesting that neocentromeres are competent to activate this checkpoint. The results of a recent study have shown identical distribution patterns for >20 kinetochore proteins in normal centromeres and two other neocentromeres (examples 10a and 20; see table 2) (Saffery et al. 2000). As new kinetochore proteins—for example, CENP-G (He et al. 1998) and CENP-H (Sugata et al. 1999)—are identified, it will be important to test for their presence at neocentromeres as well as at inactive centromeres in dicentrics, to assess the centromere activity of the proteins and to find any differences between neocentromeres and normal centromeres (Warburton et al. 1997; Aagaard et al. 2000).

Epigenetic Determination of Neocentromeres

The cell lines presented here demonstrate at least three distinct locations for neocentromere formation in chromosome 13q, with multiple independent examples in chromosome bands 13q32 ($n = 5$) and 13q21 ($n = 2$) as well as one example in chromosome band 13q31. The inversion duplication chromosome from the cell line in patient 13d, for example, contains a neocentromere in band 13q32 (fig. 2; fig. 3*B*). However, although homologous sequences from 13q32 are also found at the opposite end of this chromosome as well as on both copies of the normal chromosome 13 in these cells, only one of the four homologous regions is forming a neocentromere. Thus, the formation of the neocentromere is indicative of the epigenetic modification that is required ancillary to the primary sequence for centromere function. The presence of homologous sequences in the same cell, in both a centromeric state and a noncentromeric state, will permit investigation of the nature of these epigenetic modifications.

Despite a requirement for unknown epigenetic modification at centromeres, primary DNA sequence may nonetheless contribute to centromere formation. For example, formation of de novo centromeres has been shown to occur on transfected α-satellite DNA (Harrington et al. 1997; Ikeno et al. 1998), albeit at relatively low frequencies, possibly as the result of an increased propensity for α-satellite DNA to acquire centromere-specific epigenetic modifications, such as an increased affinity for CENP-A (Shelby et al. 1997). In this light, neocentromere sequences may share some sequence

characteristics with α-satellite DNA that increase their propensity for centromere formation. Although no conserved primary-sequence characteristics were observed in a comparison of α-satellite DNA and the sequenced 80 kb of 10q25.2 neocentromere DNA (Barry et al. 1999), an interesting conserved structural feature consisting of a double-dyad symmetry was observed (Koch 2000). The presence of this or other conserved sequence-based structures at additional neocentromeres will provide further evidence for a role for DNA sequence in centromere formation.

The analysis of multiple neocentromeres in the same chromosomal location, represented by the 13q32 and 13q21 neocentromere cell lines presented here (table 2), provides a powerful means with which to address the importance of primary DNA sequence in neocentromere formation. If multiple independent neocentromeres from the same chromosomal location are shown to be forming on the same underlying genomic clone, this will strongly suggest a role for that primary DNA sequence in neocentromere formation, without the requirement that investigators recognize conserved sequence characteristics. The neocentromere DNA from these chromosomes will be cloned with the use of techniques such as colocalization of physically mapped cosmids and kinetochore proteins at high resolution on extended chromosome preparations (du Sart et al. 1997). An alternative approach is to immunoprecipitate neocentromere-containing chromatin, with the use of either polyclonal antibodies to endogenous CENP-A or CENP-C, or anti-HA monoclonal antibodies from neocentromere lines expressing HA-tagged CENP-A (Vafa and Sullivan 1997).

Positions of Inversion Breakpoints and Neocentromeres

A reasonable hypothesis would be that neocentromeres might form at the inversion or deletion breakpoints in chromosomal-rearrangement events, perhaps as a result of the random acquisition of centromeric epigenetic modifications during the disruption of chromatin structure. However, only 2/40 neocentromeres (15a, 15b; table 2) have been reported to occur either at or near inversion breakpoints, on the basis of cytogenetic characterization, and no neocentromere has been shown to localize to the inversion breakpoint, when examined closely with the use of molecular techniques. Furthermore, none of the neocentromeres resulting from interstitial deletion events has occurred at breakpoints. In cell lines from patients 13a, 13b, 13d, and 13f, cytogenetic analysis is sufficient to rule out the possibility of neocentromere formation at the inversion breakpoint. Nevertheless, in the cell lines of patients 13c, 13e, 13g, and 13h as well as in many of the inversion duplications from other chromosomes (table 2), it remains a formal possibility that the neocentromere colocalizes with the inversion breakpoint. Cell lines from patients 13g and 13h may share a common breakpoint, suggesting a possible relationship to the neocentromere formation. In *Drosophila,* neocentromere formation has been proposed to result from *cis*-spreading of centromeric epigenetic modifications to neighboring noncentromeric DNA during chromosome rearrangements (Williams et al. 1998).

Neocentromere Formation: Selection versus Propensity

The high proportion of neocentromeres on chromosome 13q and the lack of neocentromeres on chromosomes 18 and 21 suggest that chromosome 13 has a higher propensity for neocentromere formation that is distinct from an ascertainment bias for the compatibility of trisomy 13 with fetal survival. This increased propensity may be a function of properties such as gene density, amount of heterochromatin, frequency of chromosomal rearrangements, or even number of endogenous centromeres per unit of DNA length. The possibility remains that chromosome 13q simply contains a stochastically higher number of primary DNA sequence motifs that have a high propensity for centromere formation, consistent with the three distinct locations shown in the present study. Other chromosomal regions with a high proportion of neocentromere formation include chromosomes 15q and 3q, for which seven and five cases were reported, respectively (table 2).

Despite the possibility for a higher propensity of neocentromere formation in particular chromosomal regions, the karyotype of neocentromere-containing cell lines obtained will nonetheless be constrained by an ascertainment bias for compatibility with fetal survival (table 2). Instances of interstitial deletion would have a positive selection for the neocentromere, to keep the karyotype balanced, which may help to stabilize neocentromere formation. Cases of partial tetrasomy would have strong negative selection for the neocentromere, whereas, in cases of partial trisomy, the selection would depend on whether either monosomy or trisomy for the duplicated region was more compatible with fetal survival. Somatic rearrangements involving activation of neocentromeres would not be subject to selection for fetal survival and might emerge as mechanisms for neoplasia (e.g., Abeliovich et al. 1996).

Thus, we have presented a collection of eight independent neocentromeres found in chromosome 13q. These lines will provide a unique and valuable resource for investigation of the process of neocentromere formation. Further understanding of neocentromere activation may suggest strategies to increase the reliability of providing centromere function to human artificial chromosomes.

Note added in proof.—After acceptance of this manuscript, a single example of a chromosome 21–derived neocentromere has been described on an invdup 21(qter→q21.1::q21.1→neo→qter) (Barbi et al. 2000). This case is unique in that the complementary centric terminal deletion chromosome 21(pter-q21.1) has not been recovered, resulting in trisomy 21(q21.1-qter) and monosomy 21(pter-q21.1).

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