

Characterization of a neocentric supernumerary marker chromosome originating from the Xp distal region by FISH, CENP-C staining, and array CGH

S. Yu^a D. Barbouth^a P.J. Benke^b P.E. Warburton^c Y.-S. Fan^a

^aDr. John T. Macdonald Foundation Center for Medical Genetics, University of Miami Miller School of Medicine, Miami, FL; ^bJoe DiMaggio Children's Hospital, Hollywood, FL;

^cDepartment of Human Genetics, Mount Sinai School of Medicine, New York, NY (USA)

Manuscript received 1 May 2006; accepted in revised form for publication by M. Schmid, 12 June 2006.

Abstract. A small supernumerary marker chromosome (SMC) was observed in a girl with severe developmental delay. Her dysmorphism included prominent forehead, hypertelorism, down-slanting palpebral fissures, low-set/large ears, and flat nasal bridge with anteverted nares. This case also presented hypotonia, hypermobility of joints, congenital heart defect, umbilical hernia, failure to thrive, and seizures. The SMC originated from the distal region of Xp as identified by FISH with multiple DNA probes. Staining with antibodies to Centromere Protein C (CENP-C) demonstrated a neocentromere, while FISH with an α -satellite DNA probe showed no hybridization to the SMC. A karyo-

type was described as 47,XX,+neo(X)(pter→p22.31::p22.31→pter), indicating a partial tetrasomy of Xp22.31→pter. This karyotype represents a functional trisomy for Xp22.31→pter and a functional tetrasomy for the pseudo-autosomal region given that there is no X-inactivation center in the marker chromosome. The SMC was further characterized by microarray-based comparative genomic hybridization (array CGH) as a duplicated DNA fragment of approximately 13 megabase pairs containing about 100 genes. We have described here a new neocentromere with discussion of its clinical significance.

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Small supernumerary marker chromosomes (SMCs) are frequently reported in clinical cytogenetics studies. A neocentric SMC describes a small marker chromosome which does not have a normal centromere as characterized by α -satellite repeats. However, the neocentric SMCs do have a fully functional kinetochore and are mitotically stable. Over 70 neocentric SMCs have been reported, most of which were derived from distal chromosomal regions (Amor and Choo, 2002; Liehr et al., 2004; Warburton, 2004; Wong et al., 2006).

We report a neocentric SMC in a 4-year-old girl with multiple anomalies and severe developmental delay. Characterization of this SMC by FISH, Centromere Protein C (CENP-C) staining and CGH array analysis has identified a new human neocentromere that was derived from the Xp terminal region. This represents a location for human neocentromere formation that has not been described previously.

Materials and methods

Case report

The proband was a 4-year-old African American girl, the first child of a 27-year-old healthy mother and a 38-year-old healthy unrelated father. She was born after spontaneous vaginal delivery at 40 weeks gestation. There is no history of miscarriages, mental retardation, or congenital defects in the family. The pregnancy was normal. Her birth

Request reprints from Dr. Yao-Shan Fan

Dr. John T. Macdonald Foundation Center for Medical Genetics
P.O. Box 016820 (D-820), University of Miami Miller School of Medicine
Miami, FL 33136 (USA)
telephone: +1-305-243-6870; fax: +1-305-243-3805
e-mail: yfan@med.miami.edu

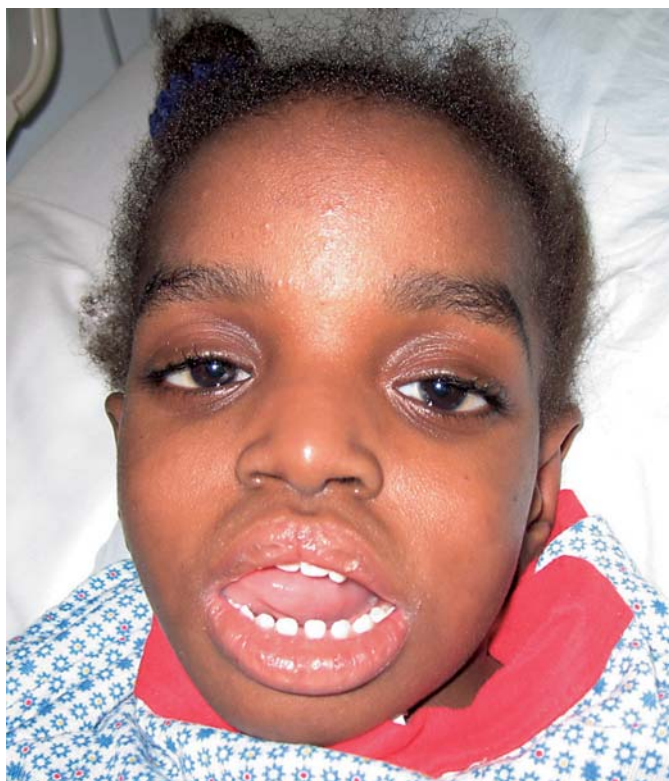


Fig. 1. Patient at 4 years of age showing features of prominent forehead, hypertelorism, down-slanting palpebral fissures, low-set/large ears, flat nasal bridge with anteverted nares and macroglossia.

weight was 2.84 kg. A physical examination revealed dysmorphic features including prominent forehead, hypertelorism, down-slanting palpebral fissures, low-set/large ears, flat nasal bridge with anteverted nares and macroglossia (Fig. 1). She also had umbilical hernia, hypotonia, hypermobility of joints, and congenital heart defect including ventricular septal defects, patent foramen ovale with left to right shunt, and persistent left superior vena cava draining into the coronary sinus. She developed poor weight gain, gastroesophageal reflux, seizures and had severe developmental delay. At 7 months of age, her height and weight were under the 5th percentile. At 4 years of age, she was only babbling and was unable to roll over.

Chromosome analysis

Metaphase chromosome preparations were obtained from PHA stimulated lymphocyte cultures from the patient and her mother according to standard procedures. Chromosomes were analyzed with G-banding at a level of resolution of 550 bands. The father was not available for karyotyping.

Fluorescence in situ hybridization (FISH)

FISH analyses were carried out according to the protocols recommended by the manufacturer of the DNA probes. This study used α -satellite DNA probes for all chromosome centromeres (ALL CEN, Oncor, Bethesda, MD), a probe for all-centromere α -satellite repeats derived from a CENP-A chromatin immunoprecipitation (Alonso et al., 2003), a probe for whole X chromosome painting (WCPX, Oncor, Bethesda, MD), multiple probes for the subtelomere regions (Chromoprobe Multiprobe T system, Cytocell, Cambridge, UK), and a locus-specific probe for the Kallmann chromosome region at Xp22.3 (LSI KAL SpectrumOrange/CEP X SpectrumGreen, Vysis, Downers Grove, IL).

CENP-C staining

Immunofluorescence staining with antibodies to centromere protein-C (CENP-C) was performed on metaphase chromosomes and rabbit anti-CENP-C was detected with goat anti-rabbit IgG as described (Warburton et al., 2000).

Array CGH analyses

Array CGH analysis was performed using a Spectral Chip™ 2600 kit (Spectral Genomics, Houston, TX) as recommended by the manufacturer with some modifications. The array chip covers the whole human genome with 2,600 BAC clones spaced with an average interval of approximately 1 megabase DNA. Briefly, patient genomic DNA was extracted from peripheral blood using the Pure Gene kit (Gentra Systems, Minneapolis, MN). Both reference DNA (Promega, Madison, WI) and test DNA were digested using *DpnII* (New England Biolabs, Ipswich, MA), then were purified using Zymo Research's Clean and Concentrator™ (Orange, CA). DNA was labeled by BioPrime random labeling kit (Invitrogen, Carlsbad, CA) using either Cy3- or Cy5-labeled dCTPs (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). In this study, a dye swap labeling, i.e., Cy3-labeled test/Cy5-labeled reference DNA for one chip set and Cy3-labeled reference/Cy5-labeled test DNA for a second set, was performed. The labeled test and reference DNA samples were combined, precipitated, and then hybridized to array chip slides. After hybridization and washing, the slides were scanned with a GenePix 4200A scanner (Molecular Devices, Sunnyvale, CA) and the 16-bit TIFF images were captured using GenePix Pro 6.0 software. The image was analyzed using Spectralware™ 3.0 software (SpectralGenomics™, Houston, TX).

Results

Observation of SMC

Chromosome analysis of cultured lymphocytes by G-banding showed a karyotype, 47,XX,+mar in all 50 cells examined (Fig. 2a). The SMC was smaller than any other chromosomes in the karyotype. However, the origin of the SMC could not be identified. Her mother had a normal female karyotype, 46,XX. This SMC is likely to be de novo, although a chromosome analysis for the father is not possible.

Identification of the SMC

Initial FISH study with a probe for all centromeric repeats did not show hybridization signals (data not shown). With multi-telomere FISH, the SMC showed at its two ends strong signals of hybridization with the probe for the Xpter and Ypter regions indicating an isochromosome structure (Fig. 2b). Further study with a FISH probe for the Kallmann syndrome region at Xp22.3 clarified that the SMC was derived from the distal region of Xp (data not shown). FISH with a whole chromosome X painting probe showed that the marker was composed of only the X chromosome material (data not shown). With a combined result of G-banding and FISH, the karyotype was described as 47,XX,+der(X) (pter → p22.31::p22.31 → pter).

Confirmation of a neocentric SMC

A combined centromere FISH and simultaneous immuno-fluorescent staining using anti-CENP-C antibody showed both FISH and CENP-C signals around centromeric regions in 46 normal chromosomes. As expected, the

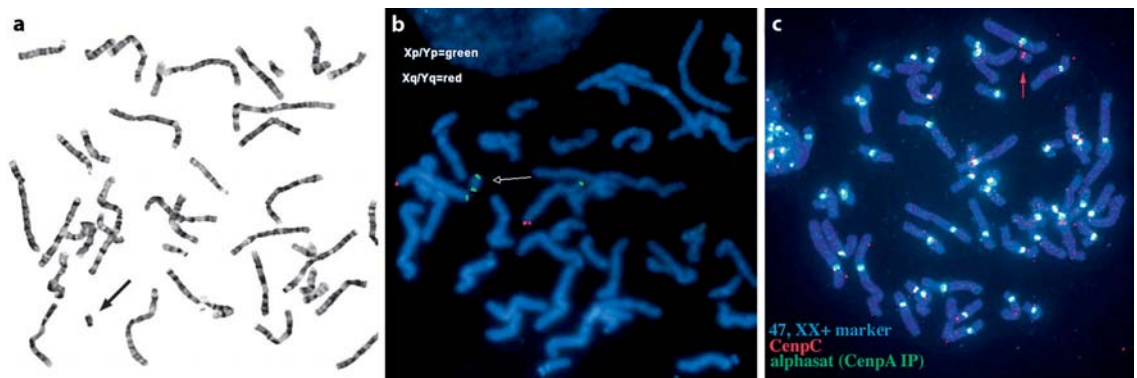


Fig. 2. (a) A partial G-banded metaphase showing the SMC; (b) FISH using probes for the subtelomeric regions of Xp (green) and Xq (red) showed symmetric green hybridization signals on the SMC; (c) A combined CENP-C staining (red) and FISH using α -satellite DNA probe (green) showed positive CENP-C staining and negative FISH signal on the SMC.

SMC exhibited no FISH signals but showed a single large CENP-C staining (Fig. 2c). This confirmed the neocentromere and the karyotype was revised to be 47,XX,+neo(X) (pter \rightarrow p22.31::p22.31 \rightarrow pter).

Characterization of the DNA fragment of the SMC

The array CGH analysis showed additional copies of 18 BAC clones which cover approximately 13 Mb of DNA in size starting from Xp22.31 to the Xpter region (Fig. 3). It is known that about 100 genes have been mapped to this region.

Discussion

Laboratory findings

The majority of cases reported with an SMC have a mosaic karyotype. This is also true for the cases with a neocentric SMC. However, our case showed an SMC in 50 of 50 (100%) G-banded metaphases derived from a peripheral blood cell culture, indicating the stability of the marker chromosome.

Identification of an SMC is important for genetic counseling. However, this can be difficult in some cases. It is known that the vast majority of SMCs have chromosome specific α -satellite repeats and that 60% of them are derived from acrocentric chromosomes. Different FISH techniques have been used to determine the origin and chromatin content of SMCs (Liehr et al., 2006). In our case, the initial FISH analysis with DNA probes for the α -satellite repeats failed to show any hybridization. The negative result prompted us to perform FISH with multiple probes for the subtelomeric regions given that most of the neocentric SMCs are derived from subtelomeric regions (Warburton, 2004). As expected, the origin of the SMC was identified by multi-telomere FISH and was confirmed by a locus specific probe. The FISH analysis clearly showed an inverted duplication of the Xp22.31 \rightarrow pter region. This marker chromosome was considered to be a neocentric SMC and was confirmed by posi-

tive anti-CENP C antibody staining. The size of the duplicated DNA sequence was approximately 13 Mb as determined by array CGH study. Thus, we fully identified this abnormal karyotype and characterized the marker chromosome as a neocentric SMC.

Formation of neocentric SMCs

Over 70 neocentric SMCs have been reported and they are distributed non-randomly across the human genome. The neocentric SMCs originating from chromosome 3q, 13q, and 15q are more common, collectively accounting for approximately half of all cases (Amor and Choo, 2002; Warburton, 2004). To our current knowledge, only one case was described as having a possible neocentromere site in Xq involved in a recombinant X chromosome in a patient with Turner syndrome (Kaiser-Rogers et al., 1995). Our case represents a first report of a neocentromere site derived from the Xp distal region. The neocentric SMC in our case is an inverted duplication which is the most common structure of SMCs. In general, inverted duplications are thought to be formed during meiosis by a U-type exchange of the two sister chromatids resulting in mirror-image chromosomes (Warburton et al., 2000). The observation of fully assembled kinetochores with functional kinetochore proteins at the neocentromeres supports the hypothesis of an epigenetic mechanism for neocentromere formation (Saffery et al., 2000; Amor and Choo, 2002; Warburton, 2004; Wong et al., 2006). Most of the reported neocentromeres are de novo constitutional events with several exceptions (Wandall et al., 1998; Tyler-Smith et al., 1998). In our case, the mother had a normal karyotype, but the father was not accessible for chromosome analysis. Most likely, this neocentric SMC is de novo, although an inherited marker chromosome cannot be ruled out.

The mechanism by which neocentric sites are non-randomly distributed across the human genome is unknown. One hypothesis is that there are genomic 'hotspots' which are favorable to neocentromere formation in certain regions of the genome (Amor and Choo, 2002; Warburton, 2004).

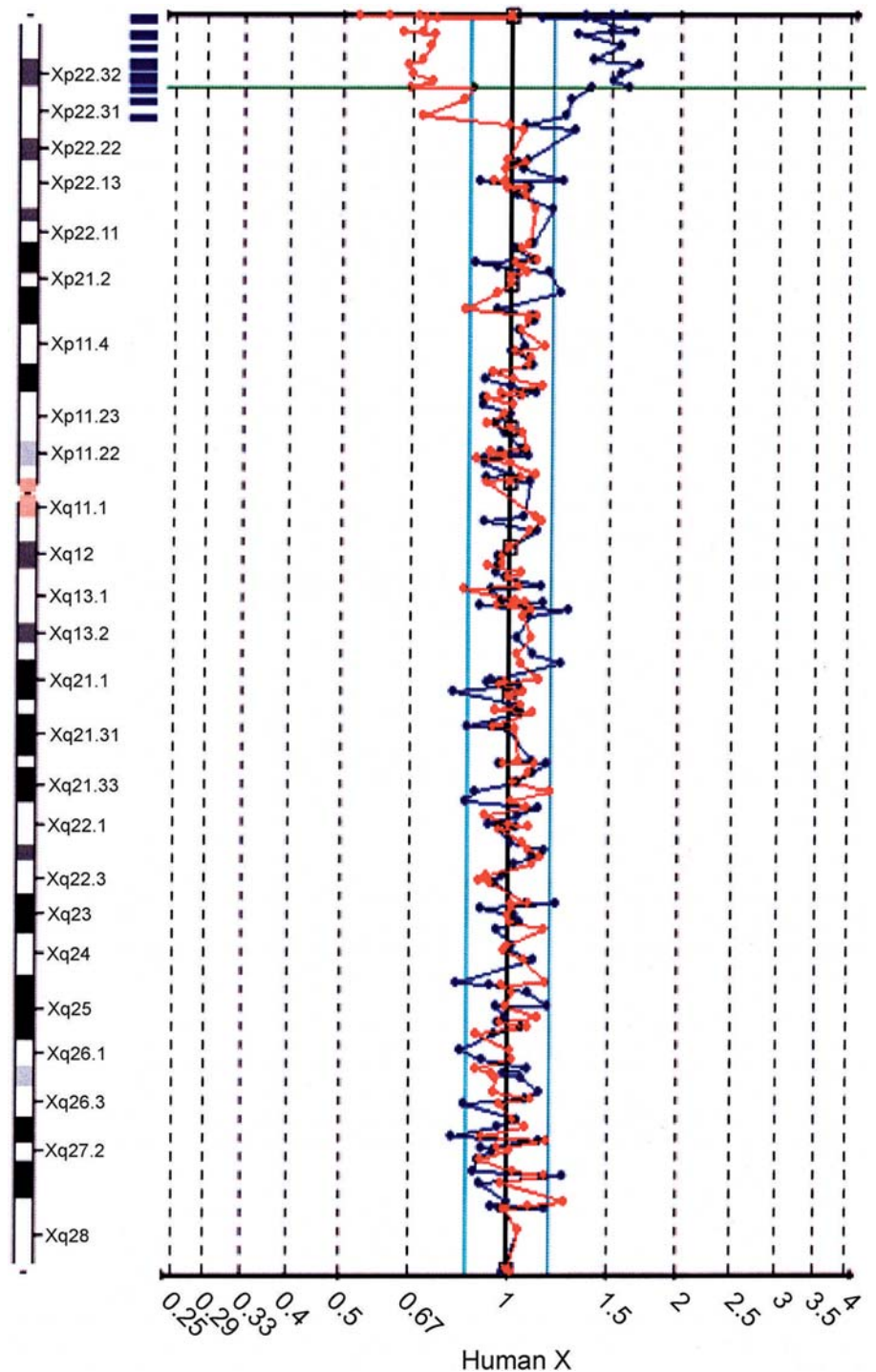


Fig. 3. Array CGH shows additional copies of the Xp distal region, as indicated by the blue bars on the right of the ideogram of the X chromosome and a mirror image of green and red dots outside the normal range on the graph. The array CGH was performed with a dye swamp labeling and therefore each BAC probe is shown with two dots, one green and one red. The two aqua vertical lines define the normal ratio range of hybridization signals. The DNA fragment with additional copies is approximately 13 Mb in size as shown by the hybridization signals of 18 BAC clones (LLNOYCO3M11D2, LLNOYCO3M15D10, LLNOYCO3M34F5, RP11-839D20, GS839 D20, 509O10, RP1-98C4, RP5-1123N13, RP4-617A9, AC079264.23, RP11-366M24, RP11-294K6, RP11-143E20, CTB-9P2, RP11-383I22, RP11-451G24, RP11-89B5, and AC003658.1).

Studies have revealed that the sequence of neocentromeres is not similar to the known centromeric sequences except that it is also TA rich (Lo et al., 2001; Satinover et al., 2001; Alonso et al., 2003). It is possible that increased AT content may provide a more favorable disposition for neocentromere formation (Lo et al., 2001). Recent data demonstrated an overall hypermethylation of the neocentric DNA at non-conventional CpG islands in mammals (Wong et al., 2006).

Meanwhile, it was noted that transcription and 'chromatin-boundary activity' were permissible at a neocentromere through selective hypomethylation of pockets of sequences without compromising its overall silent chromatin state and function (Wong et al., 2006).

Table 1. Comparison of clinical features in cases with functional extra copies of the Xp distal region

Common features described ^a	Features observed in our case
Mental retardation	+
Intrauterine growth retardation	-
Reduced birth weight	-
Feeding difficulties	-
Heart defect	+
Seizures	+
Hypotonia	+
Trigonocephaly	+
Prominent cranial sutures	+
Asymmetric skull	-
High forehead	-
Prominent metopic suture	+
Flat occiput	-
Broad face	-
Slight upward palpebral fissures	-
Convergent strabismus	-
Hypertelorism	+
Drooping cheeks	+
Sparse eyebrows	-
Prominent nose root	+
Flat nose tip	-
Thin nasal wings	-
Small nares	-
Low septum insertion	-
Prominent philtrum columns	-
High and narrow palate	+
Malocclusion	+
Everted lower lip	+
Prominent mucuous lower lip	+
Short integumental lower lip	-
Receding maxilla	-
Low-set/large ears	+
Narrow thorax	+
Scoliosis	+
Sacral pit	-
Overlapping fingers	-
Clinodactyly 5	-

^a Features were commonly seen in cases of Panasiuk et al. (2004), Melaragno et al. (1998) and Gustashaw et al. (1994).

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Genotype/phenotype correlation

Several cases with ‘pure’ extra copies of partial Xp containing Xp21.2→pter (Gustashaw et al., 1994), Xp22→pter (Melaragno et al., 1998) and Xp22.1→pter (Panasiuk et al., 2004) have been reported. In these cases, the extra copy of the partial Xp segment was translocated onto the short arm of an acrocentric chromosome, leading to physical separation of the Xp segment from the X inactivation center. Analysis of X inactivation status by observing replication banding patterns indicated that the rearranged extra copy of the Xp segment remained active, i.e., a functional disomy. In our case, the neocentric SMC was derived from the distal region of Xp resulting in a partial tetrasomy for the Xp22.31→pter. Our case represents a functional trisomy for the Xp distal region in general and a functional tetrasomy for the pseudoautosomal region in particular given that there is no X-inactivation center in this neocentric SMC.

We have compared the clinical features observed in our case with those commonly seen in the several known cases with a functional disomy of Xp (Gustashaw et al., 1994; Melaragno et al., 1998; Panasiuk et al., 2004) (Table 1). Our case has shared many dysmorphic features with the above cases. It should be noted that the DNA segment with additional copies in our case is much smaller than those described in other cases. Likely, these shared phenotypes are determined by functional extra copies of the genes mapped to the very distal region of Xp. Our case also has some other features which were not commonly observed in the above cases, such as hypertelorism, down-slanting palpebral fissures, macroglossia, and umbilical hernia. It is likely that a functional trisomy may have additional phenotypic impact in comparison with a functional disomy.

Acknowledgements

We thank Hongbo Zhu, Carmen E. Casas, and Fanny Cheung for their technical assistance in the analysis of this case.