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Mechanisms and Consequences of Small Supernumerary Marker Chromosomes: From Barbara McClintock to Modern Genetic-Counseling Issues

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Supernumerary marker chromosomes (SMCs) are common, but their molecular content and mechanism of origin are often not precisely characterized. We analyzed all centromere regions to identify the junction between the unique chromosome arm and the pericentromeric repeats. A molecular-ruler clone panel for each chromosome arm was developed and used for the design of a custom oligonucleotide array. Of 27 nonsatellited SMCs analyzed by array comparative genomic hybridization (aCGH) and/or fluorescence in situ hybridization (FISH), seven (approximately 26%) were shown to be unique sequence negative. Of the 20 unique-sequence-positive SMCs, the average unique DNA content was approximately 6.5 Mb (range 0.3–22.2 Mb) and 33 known genes (range 0–149). Of the 14 informative nonacrocentric SMCs, five (approximately 36%) contained unique DNA from both the p and q arms, whereas nine (approximately 64%) contained unique DNA from only one arm. The latter cases are consistent with ring-chromosome formation by centromere misdivision, as first described by McClintock in maize. In one case, a r(4) containing approximately 4.4 Mb of unique DNA from 4p was also present in the proband's mother. However, FISH revealed a cryptic deletion in one chromosome 4 and reduced alpha satellite in the del(4) and r(4), indicating that the mother was a balanced ring and deletion carrier. Our data, and recent reports in the literature, suggest that this "McClintock mechanism" of small-ring formation might be the predominant mechanism of origin. Comprehensive analysis of SMCs by aCGH and FISH can distinguish unique-negative from unique-positive cases, determine the precise gene content, and provide information on mechanism of origin, inheritance, and recurrence risk.

Introduction

Supernumerary marker chromosomes (SMCs) are extra, abnormal chromosomes whose origin cannot typically be determined by conventional chromosome-banding techniques. SMCs are common, occurring in four of every 10,000 newborns, but are approximately 7 times more prevalent in individuals with mental retardation.[1] The most common class of marker chromosomes are derived from acrocentric chromosomes and have a satellited or bisatellited structure, with chromosome 15 accounting for the highest percentage of this group.[2] Nonacrocentric-derived markers are somewhat less common and are often suspected to be small ring chromosomes on the basis of their morphological appearance and behavior (including mitotic instability leading to mosaicism).[3]

Certain marker chromosomes are large enough to be identified by G banding and have a well-established phenotype and prognosis. Examples include iso(12p), associated with Pallister-Killian syndrome[4] (PKS [MIM 601803]), and iso(18p), associated with mild-moderate mental retardation and a characteristic facial appearance.[5] For chromosome 15-derived marker chromosomes, referred to as inv dup(15), fluorescence in situ hybridization (FISH) analysis allows discrimination between large markers containing *SNRPN* [MIM 182279] that are tetrasomic for the Prader-Willi or Angelman Syndrome (PWS [MIM 176270] or AS [MIM 105830]) critical region and small markers that are negative for *SNRPN*. The former are associated with mental retardation, seizures, autistic features, and growth retardation, whereas the latter are usually associated with a normal phenotype.[6–8] FISH analysis of chromosome 22-derived markers can reveal whether the SMC contains the critical region for Cat-Eye syndrome (CES [MIM 115470]), which is characterized by ocular coloboma and other dysmorphic features.[9]

For the remainder of SMCs, empiric figures are used in a prenatal setting for the prediction of the risk of a phenotypic abnormality. These data were compiled in a classic paper published in this journal by D. Warburton in 1991[10] showing an overall risk for an abnormality for all marker chromosomes of 13%. Subdividing marker chromosomes into those containing satellites (derived from an acrocentric chromosome) compared to nonsatellited chromosomes showed a lower empiric risk of abnormality among satellited markers (11% versus 15%). Other studies have demonstrated the risk of abnormality for SMCs derived from nonacrocentrics to be is as high as 28%.[11] Consistent with this higher risk estimate, a recent study of 108 prenatally ascertained de novo SMCs found risks of 18% for satellited markers and 31% for nonsatellited markers.[12] The differences in risk estimates likely represent differences in the inclusion criteria among these studies.

Clearly, more precise knowledge of the size of the partial trisomy segment(s) and the gene content of the SMC would greatly improve our ability to predict phenotype and prognosis. Many groups have utilized various FISH techniques to identify a large number of marker chromosomes.[13–22] Recently, Liehr and colleagues established a SMC cell-line bank, such that these samples can be preserved for future

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characterization studies.[23] These authors also have begun to make genotype-phenotype correlations on approximately 400 cases of SMCs.[3] The majority of these cases consisted of previously reported SMCs that were mapped with a variety of FISH methods. Because most reports provided only the chromosomal band for the delineation of the breakpoints of the SMCs, precise determination of the gene content within the SMC that might be contributing to the phenotype is difficult.

Current molecular cytogenetic technologies make it possible for us to identify the specific gene content of SMCs at a high-resolution, and therefore to begin to define the genotype-phenotype correlations associated with SMCs. Over the past several years, array comparative genomic hybridization (aCGH) has proven to be more sensitive in the detection of small deletions and duplications as compared to standard cytogenetic techniques, such as G banding and FISH.[24–27] In addition, a single aCGH study has the capacity of generating information equivalent to many sequential or multiplex FISH assays, allowing for the rapid identification of unique DNA content. Recently, Shaffer and colleagues[28] utilized a microarray containing FISH-mapped bacterial artificial chromosome (BAC) clones covering approximately 5 Mb adjacent to each centromere to characterize SMCs. They fully characterized two-thirds of the SMC cases by aCGH, whereas the remaining SMCs contained euchromatic material that extended distal to the 5 Mb coverage on their array.[28] These results demonstrate the significant variability of DNA content contained within SMCs and emphasize the need for high-resolution characterization of marker chromosomes.

In cases in which the morphology of the SMC can be determined by cytogenetic analysis, almost 50% appear to represent ring chromosomes.[3] The mechanism by which these small ring chromosomes form in humans is poorly understood, but at least two major mechanisms can be considered.[29–31] In the first, one break occurs in the p arm and a second break occurs in the q arm, and the two broken ends of the centric fragment fuse together to form a ring (Model I). The resulting ring chromosome contains DNA from both the p and q arms. A second mechanism involves a break within the centromere, sometimes referred to as "centromere misdivision," along with a break in either the p or q arm, forming a small ring chromosome (Model II).

Comprehensive analysis of SMCs with aCGH and FISH analysis now has the capability to rapidly determine the degree of partial trisomy (gene content) present, as well as to provide information on the mechanism of formation and inheritance critical for the determination of accurate recurrence risks.

Material and Methods

BAC-Clone Selection and Validation

Previous efforts by the BAC Resource Consortium have placed numerous BAC clones on the human genome sequence.[32] To enhance this dataset for the pericentromeric regions, our laboratory developed a molecular ruler clone panel for each of the 43 chromosome arms. A schematic of this design is shown in Figure 1. The junction between the pericentromeric repeats and the unique chromosome arm was identified, and BAC clones were selected from this point to at least 5 Mb distal on each arm. Clones were initially chosen from the National Center for Biotechnology Information (NCBI) Build 33 with the University of California, Santa Cruz (UCSC) genome browser database, whereas subsequent clones were chosen on the basis of the most current NCBI build.[33–35] A contig of BAC clones were selected to cover the first megabase of DNA adjacent to the pericentromeric-unique DNA junction. Extending from this contig, one BAC clone was placed every 500 kb up to at least 5 Mb into the p and q arms of each chromosome arm. Initial criteria for unique clone selection required the clones to be fully sequenced and to contain less than 10% duplicated sequence using the segmental duplication track.[36] For each chromosome arm, the most proximal, intermediate, and distal FISH-validated clones meeting these criteria are listed in Table 1. These clones contain less than 10% segmental duplications, and their chromosomal localization was confirmed by FISH analysis. For cases in which a SMC was identified that contained unique DNA greater than 5 Mb, additional clones were selected up to 15 Mb from the centromere gap. All FISH-tested clones (n = 540) are listed in Table S1 available online.

In addition to the clones within the unique chromosome arm, clones were selected and analyzed within the pericentromeric repeat regions for each chromosome arm. These clones were utilized in FISH analyses for the delineation of the pericentromere-unique DNA junctions and for the characterization of SMCs that did not contain unique DNA. By definition, clones in this region have significant repetitive DNA or segmental duplications; thus, all of these clones contained more than 10% segmental duplications. If sequenced clones were not available commercially, or if a clone did not map correctly after two attempts, a corresponding clone from the BAC end track or the Human 32K BAC Re-Array set was chosen.

Bacterial stabs were obtained from The BACPAC Resource Center (BPRC) at Children's Hospital Oakland Research Institute (Oakland, CA) or from Invitrogen (Carlsbad, CA) and streaked onto Luria-Bertani (LB) plates with the appropriate antibiotic. For each chromosome arm, at least four clones were mapped: the most proximal unique clone, as well as the clones located approximately 1, 3, and 5 Mb from the most proximal clone. Any additional clones that were selected for SMC characterization were also analyzed by FISH.

DNA was isolated from overnight cultures with the appropriate antibiotic via an alkaline lysis procedure with an automated extraction system (Autogen, Holliston, MA). For FISH assays, fluorescently labeled nucleotides (Spectrum Orange-dUTP, Spectrum Green-dUTP [Abbott Molecular, Des Plaines, IL] or Diethylaminocoumarin-5-dUTP [PerkinElmer Life Sciences, Boston, MA]) were incorporated into the clone DNA with a standard nick-translation or random priming reaction. Slides were baked at 65°C for proper aging, washed in 2× saline sodium citrate (SSC) at 37°C for 30 min, and hydrated sequentially in 70%, 80%, and 95% ice-cold ethanol. Chromosomes were denatured in 70% formamide and 2× SSC at 73°C for 55 s and then hydrated as before. Prior to hybridization, probes were denatured at 73°C for 7 min and reannealed at 45°C for 2 min. Chromosome spreads were hybridized overnight at 37°C. Slides were washed in 0.4× SSC and 0.3% NP-40 at 73°C for 2 min, washed in 0.2× SSC and

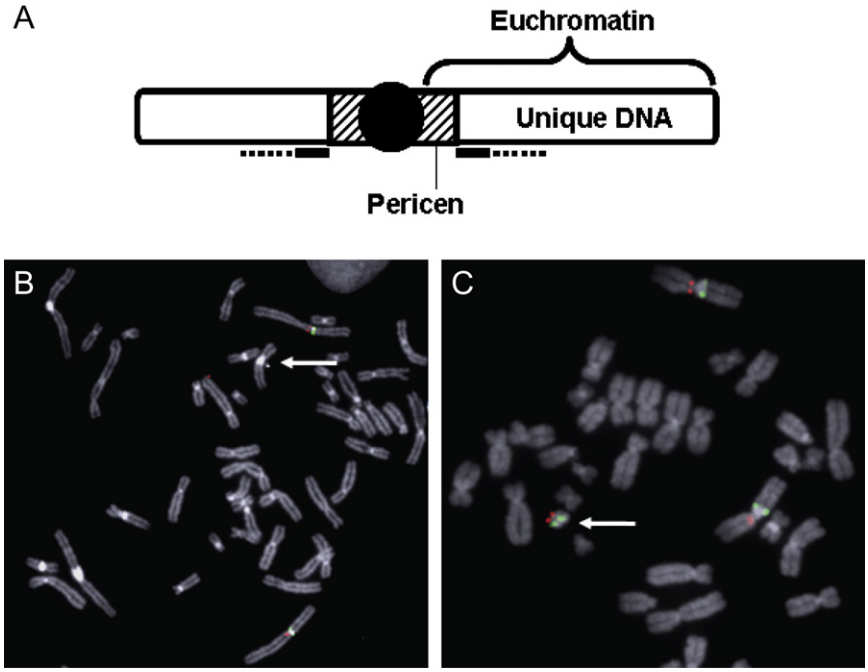

Figure 1. Probe Design Strategy and FISH Analysis for SMCs

(A) The black circle represents the large array of alpha-satellite DNA comprising each human centromere region. Because these sequences are highly repeated and difficult to sequence, they appear as the centromere gaps on physical maps of the genome. Adjacent to the alpha-satellite DNA on each arm is a pericentromeric region (diagonal lines), which is usually comprised of a complex arrangement of segmental duplications and is polymorphic in size. The junction of the unique DNA with this pericentromeric zone was identified for each chromosome arm, and a 1 Mb contig of BAC clones was developed (solid black line). The dotted black lines represent clones spaced every 500 kb up to a minimum of 5 Mb for each unique chromosome arm. Euchromatin refers to the unique DNA and the pericentromeric repeats of each chromosome arm, in contrast to heterochromatin, which is comprised of highly repeated satellite DNAs, including alpha-satellite arrays.

(B) A representative metaphase from case 6 shows positive hybridization to a centromere alpha-satellite probe (aqua) on the two normal chromosome 2 homologs and the SMC (arrow). The normal homologs also show positive hybridization for a 2p clone (green) and 2q clone (red), but no hybridization is observed on the SMC. The 2p clone (RP11-349C16) is located 2.9 Mb from the centromere gap and the 2q clone (RP11-708D7) is 0.3 Mb from the centromere gap.

(C) A representative metaphase from case 3 shows positive hybridization on both normal chromosome 1 homologs and the SMC (arrow) for unique clones on 1p (red) and 1q (green). The 1p clone (RP11-22F13) is located 1.1 Mb from the heterochromatin gap on the short arm, while the 1q clone (RP4-679C16) is located 1.5 Mb from the heterochromatin gap on the long arm.

0.1% NP-40 at room temperature for 30 s, and stained with DAPI for 3 min. Slides were mounted in VectaShield antifade solution (Vector Laboratories, Burlingame, CA) and analyzed via digital imaging with a CCD camera and software (SmartCapture 2, Digital Scientific, Cambridge, UK).

Patient Samples

Informed consent was obtained from participants according to a protocol approved by the Institutional Review Board at Emory University. Once a patient was entered into the research study, blood or amniocytes were sent to the Emory Genetics Laboratory for culture for FISH analysis and for DNA isolation.

Samples from 26 patients with nonsatellited SMCs were recruited from several clinical diagnostic laboratories (cases of satellited marker chromosomes were excluded). Of these, eight cases were ascertained prenatally and 18 cases were ascertained postnatally. Case 23 contained two SMCs that were derived from two different chromosomes; therefore, these SMCs were counted separately. The clinical indications for cytogenetic analysis and salient clinical features are listed in Table S2. Case 11 has been reported separately in more detail.[37]

Array CGH

Two microarray designs were utilized for these experiments: a commercially available array (Agilent Human Genome CGH Microarray Kit 44b) and a custom designed 4x44k CGH array.[38] In the custom array design, the most proximal unique BAC clone for each centromere region was represented with approximately ten probes, which then transitioned into the whole genome backbone coverage of one probe every 75 kb.

The experimental procedures followed the manufacturer's recommended protocol (Agilent Technologies, Santa Clara, CA). In brief, genomic DNA (1–3 μg) was digested with AluI and RsaI (Promega, Madison, WI) for 2 hr. The DNA was labeled for 2 hr with random primers, Cy-3- and Cy-5-dUTP dyes, and Exo-Klenow fragment (Agilent Technologies, Santa Clara, CA). Patient DNA (labeled with Cy-3) was combined with a normal control DNA sample (labeled with Cy-5) of the opposite sex (Promega, Madison, WI) and hybridized to the array in the presence of Cot-1 DNA (Invitrogen, Carlsbad, CA). After a 24 hr hybridization at 65°C, the slides were washed and scanned with the GenePix Autoloader 4200AL (Molecular Devices, Sunnyvale, CA).

Array Analysis and FISH Confirmation

BlueFuse software (BlueGnome, Cambridge, UK) was utilized for the examination of the data. Normalization of the data was performed with Block Lowess, which corrects for intensity-related variation within images. Regions of copy-number alterations were detected with set thresholds for the channel ratios based on two or three standard deviations from the median of the autosomes. Channel 1 (Ch1) represented the patient sample and channel 2 (Ch2) represented the normal control DNA. The thresholds for the $\log_2$ ratios were set at 0.26 for amplifications and −0.32 for deletions. In order for the software to call an abnormality, the

Table 1. Proximal, Intermediate, and Distal FISH-Tested Centromere Clones

Chr	Proximal Clone	Distance from Gap (Mb)	Intermediate Clone	Distance from Gap (Mb)	Distal Clone	Distance from Gap (Mb)
1p	RP11-22F13	1.1	RP11-39H13	3.5	RP11-350E19	5.9
1q	RP4-679C16	1.5	RP11-314N2	3.1	RP11-126K1	6.7
2p	RP11-349C16	2.9	RP11-269K22	4.9	RP11-147C20	7.4
2q	RP11-708D7	0.3	RP11-173M4	3.0	RP11-609J13	5.0
3p	RP11-714D16	0.3	RP11-695J20	1.4	RP11-14B7	5.0
3q	RP13-503K1	0.2	RP11-8N23	2.3	RP11-319J24	4.9
4p	RP11-317G22	0.6	RP11-381K8	3.1	RP11-542I3	5.3
4q	RP11-365H22	0	RP11-231C18	2.5	RP11-533F5	4.9
5p	CTC-339C6	0.5	CTD-2282F8	3.0	CTD-2005E22	5.0
5q	CTD-2276O24	0.6	RP11-94D20	3.1	RP11-175M2	5.6
6p	RP11-513F12	2.1	RP11-360D14	3.6	RP3-357H1	6.9
6q	RP11-396K16	0.4	RP11-59D5	2.5	RP11-24C14	5.5
7p	RP11-792I5	2.5	RP11-636L15	3.8	RP11-95E2	7.4
7q	RP11-144H20	0.5	RP11-416N13	2.7	RP13-936H15	5.4
8p	RP11-643N23	0.2	RP11-465K16	3.0	RP11-479P21	4.7
8q	RP11-1134I14	1.1	RP11-22C8	3.0	RP11-759A9	4.4
9p	RP11-113O24	7.6	RP11-297B17	9.0	RP11-366F19	12.7
9q	RP11-274B18	4.3	RP11-366I5	7.0	RP11-404E6	9.1
10p	RP11-365P10	2.3	RP11-382K22	3.0	RP11-479G22	5.8
10q	RP11-351D16	1.1	RP11-67C2	3.5	RP11-463P17	6.2
11p	RP11-709C9	3.2	CTD-2532J14	4.9	RP11-513I7	7.9
11q	RP11-131J4	0.4	RP11-659P15	2.9	RP11-736I10	5.0
12p	RP11-1035D8	0.8	RP11-414A12	3.3	RP11-1110J8	5.1
12q	RP11-804F13	1.0	RP11-242C24	2.0	RP11-328C8	4.9
13q	RP11-61K9	1.4	RP11-569O4	2.5	RP11-165I9	6.6
14q	CTD-2292M16	1.6	RP11-903H12	2.0	RP11-66N24	5.0
15q	RP11-289D12	2.1	RP11-73C9	3.1	RP13-911E13	6.3
16p	RP11-43P5	0.1	RP11-1378C7	2.0	RP11-297C4	4.7
16q	RP11-46D6	0.1	RP11-189E14	3.0	RP11-397C9	5.0
17p	RP11-64J19	1.0	RP11-28B23	3.3	RP11-367G9	5.2
17q	RP11-173M1	0.3	RP11-1148O4	3.0	RP11-640N20	5.0
18p	RP11-681N23	1.4	RP11-78A19	3.5	RP11-243E13	5.0
18q	RP11-254G11	0.1	RP11-403A21	3.0	RP11-203G13	5.0
19p	RP11-771C12	0.2	CTB-135N1	2.3	CTC-251H24	6.0
19q	CTC-452L11	0.1	CTC-525D6	2.2	CTC-442E9	4.5
20p	RP4-694B14	0.6	RP11-526K17	1.7	RP5-1049G11	5.6
20q	RP5-1018D12	1.3	RP11-60H7	2.9	RP5-977B1	6.5
21q	RP11-625C23	1.2	RP11-1L19	3.5	RP1-238P10	5.5
22q	RP11-419G2	1.4	RP11-488D20	3.4	RP11-432M13	6.6
Xp	RP3-344I7	0.5	RP11-382E3	3.2	RP1-290F12	5.5
Xq	RP1-267M5	0.4	RP11-262B12	2.6	RP11-747I9	3.9
Yp	RP11-375P13	2.6	-	-	RP11-115H13	4.4
Yq	RP11-350I10	0.9	RP11-283B24	3.5	RP11-442J5	4.6

The proximal and intermediate clones mapped uniquely, whereas three of the distal clones displayed crosshybridization. All 540 FISH-mapped clones are listed in the Supplemental Data.

minimum number of probes included in a region of deletion or amplification was set to five oligonucleotides.[38]

For confirmation of the array results, FISH analyses were performed with the centromere clone panel. For SMCs containing unique DNA, at least three clones were tested: the most proximal unique clones for each chromosome arm and the most distal clone from the chromosome arm that contained unique DNA. For SMCs that did not contain unique DNA, the most proximal unique clones for each chromosome arm were tested. Other abnormalities in addition to the SMC detected by aCGH (cases 8 and 12) were also confirmed by FISH.

Bioinformatics Resources

The UCSC genome browser (May 2004) was utilized for the assessment of the genomic architecture of the SMCs (Segmental Duplications track) and for the assessment of the number of known genes (UCSC Known Genes track) contained within the SMCs.[36,39,40] The genes were displayed in four colors representing the level of supporting data: black, entry in the Protein Databank (PDB); dark blue, either a corresponding RefSeq messenger RNA (mRNA) that is reviewed or validated or a corresponding Swiss-Prot protein; medium blue, corresponding RefSeq mRNA that is not reviewed nor validated; and light blue, no corresponding PDB entry, RefSeq mRNA, or Swiss-Prot protein. For this study, the number of known genes included the genes that have an entry in the PDB (black) or a validated RefSeq mRNA or Swiss-Prot protein entry (dark blue). Noncoding genes and splice variants were not included. In addition, genes with at least five family members were counted as one gene family, such as the histone H2 family and the S100 protein family.

Results

Similar to our previous development of molecular-ruler clones for all human telomeres,[41] a molecular-ruler clone panel for all human centromeres was developed (Figure 1A). The most proximal unique BAC clone for each centromere was identified, and a panel of clones was developed up to approximately 5 Mb from the centromere or heterochromatin gap. Table 1 displays three FISH-mapped clones for each chromosome arm. The centromere clone panel contains 963 clones, including 756 clones in the unique regions of the chromosome arms and 207 clones in the pericentromeric regions.

Clone Validation

Approximately 50% (n = 382) of the clones located in the unique chromosomal arms were analyzed by FISH (Table S1). From this subset, only 85% of the clones mapped uniquely and to the correct location in the genome. The remaining 15% of the clones mapped incorrectly (7.6%) or displayed crosshybridization to other sites (7.4%), despite being predicted to be unique in the databases. This is consistent with results from Ballif et al. that demonstrated that approximately 2% of clones in the centromere regions mapped incorrectly and approximately 22% of clones displayed crosshybridization.[28] Subtelomeric regions have also been shown to be problematic in the mapping of the human genome,[33,42] and together, these results provide a cautionary note regarding utilization of data and clones from public genomic databases without independent experimental confirmation of location and uniqueness.

Approximately 76% (n = 158) of the clones located within the pericentromeric repeat region were analyzed by FISH (Table S1). As expected for regions containing substantial repeats and segmental duplications, only approximately 27% of clones showed a unique signal to the correct location, whereas approximately 5% mapped to the wrong location and approximately 68% displayed crosshybridization to additional sites.

Determination of Unique DNA Content of SMCs

The centromere-specific molecular-ruler clone panel was utilized for the characterization of SMCs obtained from multiple cytogenetic laboratories. To determine whether a SMC contained unique sequence DNA, we tested the most proximal unique BAC clones from the molecular-ruler clone set (see Table 1) on the samples. The location of the clone is indicated according to the distance from the centromere gap into the p or q arm.

The mapping results, unique DNA content, and gene content of all 27 SMCs are shown in Table 2. The total euchromatin column indicates the size of the SMC including the pericentromeric repeat DNA and unique DNA, whereas the total unique column shows only the amount of unique DNA (as determined by the most proximal unique clone). The marker chromosomes derived from nonacrocentrics were presumed to be rings, on the basis of their appearance and mitotic instability. Seven of the markers (26%) did not contain unique DNA and were thus comprised solely of pericentromeric repeats and/or centromeric alpha-satellite DNA. An example is shown for case 6 (Figure 1B), which contained a SMC known to be derived from chromosome 2 from studies performed in the referring laboratory. The most proximal unique clone on 2p (RP11-349C16) was not present on the SMC, and the most proximal unique clone on the long arm (RP11-708D7) was also negative. Because this SMC was detected prenatally, the determination that it is unique negative and less likely to be associated with an abnormal phenotype was valuable information for genetic counseling.

The remaining 20 SMCs were unique positive and contained varying amounts of unique DNA, ranging in size from 0.3 Mb to 22.2 Mb (Table 2). Case 3 is an example of a marker chromosome that contains unique material derived from both the p and q arms of chromosome 1 (Figure 1C). The proband presented with developmental delay, and the SMC was determined to be derived from chromosome 1 by the referring laboratory. In this case, the most proximal unique clones on 1p (RP11-22F13) and on 1q (RP4-679C16) were both positive on the SMC (Figure 1C). These results indicated that the SMC contained unique material derived from both chromosome arms, and further FISH mapping was necessary for the delineation of the breakpoints of the SMC (Table 2; data not shown).

The average amount of unique DNA for these 20 SMCs was approximately 6.5 Mb, with an average of approximately 33 known genes. The average size of the total euchromatic DNA content, including the pericentromeric repeats, was approximately 7.6 Mb. Half of these 20 SMCs contained less than 5 Mb of unique DNA, five SMCs contained 5–10 Mb of unique DNA, and the remaining five SMCs contained greater than 10 Mb of unique DNA.

Identification of a Cryptic Deletion Associated with a Small Ring Chromosome Formed by the "McClintock Mechanism"

One of our cases (case 7) involved a mosaic familial r(4) that was ascertained prenatally in another laboratory. The mother, who is intellectually normal but has unilateral ear anomalies and minor visual deficiencies, was also found to carry the r(4) in approximately 66% of her peripheral lymphocytes. The parents were counseled that the marker chromosome in the fetus and mother appeared to be identical, and the pregnancy was continued. At 3 years of age, the proband was referred to a genetics clinic for mild speech delay. Re-analysis of the proband's chromosomes confirmed the presence of the r(4) and showed two normal chromosome 4 homologs with positive hybridization for unique BAC clones on the p arm and q arm, located 1.08 Mb and 0.55 Mb from the centromere gap, respectively (Figure 2A). The ring chromosome was positive for the p arm clone but negative for the q arm clone, indicating the presence of unique DNA from the short arm but not the

Table 2. Summary of Mapping Results of SMCs with FISH and aCGH

Case	Chr	Ascertainment	p arm Unique (Mb)	q arm Unique (Mb)	Total Unique[a] (Mb)	Total Euchromatin[b] (Mb)	Number of Genes[c]	Origin	Percent of Cells with SMC	Array type; Result[d]
1	1	Prenatal	13.7	0	13.7	14.8	>50	de novo	57%	Not tested
2	1	Postnatal	>13.9	0	>13.9	>15	>50	de novo	25%	Not tested
3	1	Postnatal	0.2	11.4–15	11.6–15.2	14.2–17.8	>50	de novo	10%	Custom; Not detected: Mosaic
4	1	Postnatal	0[e]	0	0	0.1	0–5	de novo	66%	Custom; Not detected: No unique
5	2	Postnatal	0[e]	8.5	8.5	9.5	11–50	de novo	41% +1; 50% +2	44b; Detected
6	2	Prenatal	0[e]	0	0	0.2	0–5	de novo	100%	Not tested
7	4	Prenatal	4.4	0	4.4	5.0	11–50	Mat	33%	44b; Detected
8	4	Postnatal	4.2	18.0	22.2	22.8	11–50	Pat	60%	Custom; Detected[d]
9	5	Postnatal	0	10.9–12.6	10.9–12.6	11.5–13.2	11–50	de novo	25%	Not tested
10	5	Prenatal	1.1	1	2.1*	3.2	0–5	de novo	88% +2; 12% +1	Not tested
11	8	Postnatal	3.1	0.8	3.9	5.2	11–50	de novo	96%	44b; Detected
12	8	Postnatal	4.5	2.2	6.7	8	11–50	de novo	45% +1; 45% +2	44b; Detected[d]
13	11	Postnatal	0.2	2.3	2.5	6.1	11–50	de novo	14%	44b; Not detected: Mosaic
14	13	Prenatal	N/A	8.6–13.5	8.6–13.5	10–14.9	11–50	de novo	100%	Not tested
15	15	Postnatal	N/A	0[e]	0	0.3	0–5	de novo	70%	Not tested
16	16	Postnatal	0.7	0	0.7	0.8	0–5	de novo	85%	44b; Detected
17	17	Prenatal	7.3	0	7.3	8.3	>50	de novo	60%	44b; Detected
18	18	Postnatal	0[e]	0	0	1	0–5	Pat	80%	Custom; Not detected: No unique
19	18	Prenatal	1.2	0	1.2	2.6	6–10	de novo	100%	Not tested
20	19	Postnatal	0	0.3	0.3	0.4	0–5	de novo	100%	Custom; Detected
21	20	Postnatal	6.6	0[e]	6.6	8.5	11–50	Mat	68%	44b; Detected
22	20	Postnatal	0[e]	0.3	0.3	1.9	6–10	de novo	75%	Custom; Detected
23a	20	Prenatal	0	0	0	0	0–5	de novo	6%	Custom; Not detected: Mosaic and No unique
23b	14/22	Prenatal	N/A	0	0	0	0–5	de novo	6%	Custom; Not detected: Mosaic and No unique
24	21	Postnatal	N/A	0.4	0.4	1.6	6–10	de novo	50%	Not tested
25	21	Postnatal	N/A	4.7	4.7	5.9	11–50	Unk	75%	44b; Detected
26	13/21	Postnatal	N/A	0	0	0	0–5	de novo	24%	Not tested

* indicates that case 10 contains two SMCs: one that contains only p arm DNA and a second that contains only q arm DNA. N/A indicates that the p arms of the acrocentric chromosomes were not tested.

[a] Total unique DNA indicates the amount of unique DNA sequences present on the SMC.

[b] Total euchromatin indicates the amount of pericentromeric and unique DNA sequences present on the SMC.

[c] See Material and Methods for definition of known genes. Genes are grouped as: 0–5, 6–10, 11–50, and > 50.

[d] aCGH results detected an additional abnormality.

[e] SMC contains pericentromeric DNA (but no unique DNA) for this chromosome arm.

long arm of chromosome 4. The proband has an approximately 4.4 Mb partial trisomy of unique DNA (approximately 5 Mb including the pericentromeric repeats) in about 33% of her peripheral lymphocytes. This proximal region of chromosome 4p includes approximately 16 known genes.

FISH studies of the mother showed the same pattern of positive hybridization for unique BAC clones from 4p on her ring chromosome and a small positive signal for the chromosome 4 alpha-satellite probe (Figure 2B). However, unlike the proband, the mother had only one normal chromosome 4 homolog with positive hybridization to the 4p unique clones and centromeric alpha-satellite probe. The second chromosome 4 homolog showed no hybridization to the 4p unique clones and had a slightly reduced intensity of the alpha-satellite signal, and therefore was deleted for approximately 4.4 Mb of unique DNA from proximal 4p. This result indicates that the mother is a balanced carrier for a cryptic pericentromeric deletion and a complementary ring chromosome, as depicted in Figure 2C. Because the r(4) in present in only approximately 66% of the mother's peripheral lymphocytes, she is monosmic for 4p in 33% of cells in this tissue. The level of mosaicism might vary substantially in other tissues, and given her normal intelligence, one would predict a higher percentage of balanced cells might be present in the brain.

Mechanism of Formation of SMCs

Seventeen nonacrocentric SMCs contained unique sequence DNA from at least one chromosome arm. To assess mechanism of formation, we excluded the subset of cases that

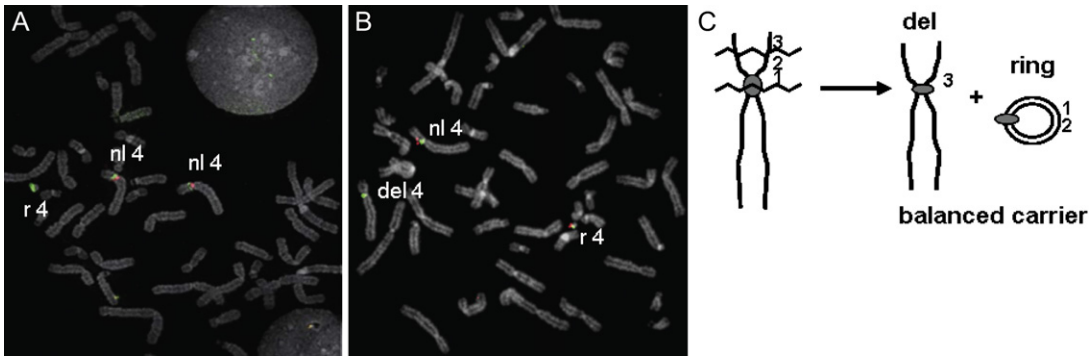

Figure 2. FISH Analysis of the ring(4) in Case 7

(A) FISH analysis with unique pericentromeric BAC clones for chromosome 4. The proband shows two normal chromosome 4 homologs with positive hybridization for a p arm-specific probe RP11-191J2 (green) and a q arm-specific probe RP11-724F22 (red), located 1.1 Mb and 0.6 Mb from the centromere, respectively. On the supernumerary ring chromosome, only the p arm probe shows positive hybridization, excluding the presence of unique DNA from the q arm in ring formation.

(B) The mother of case 7 showed the same pattern of positive hybridization for unique BAC clones from 4p on her ring marker (red signal corresponding to clone RP11-500G9 at 5.02 Mb from the centromere gap) and a small positive signal for the chromosome 4 alpha-satellite probe (green). The mother shows one normal chromosome 4 homolog with positive hybridization to 4p unique clones and centromeric alpha satellite, but also a deleted chromosome 4 homolog negative for 4p unique clones and a slightly reduced intensity alpha-satellite signal.

(C) Model of small-ring-chromosome formation by centromere misdivision is shown. One chromosomal break occurs within the centromeric alpha satellite array, and a second break occurs in either the p or q arm of the chromosome. This mechanism produces two functional centromeres and two viable chromosome products. The resulting balanced carrier state comprises a deleted chromosome and a complementary ring chromosome. This schematic figure is virtually identical to that drawn by B. McClintock in 1938 on the basis of her observations in maize.[61]

contained only pericentromeric repeats for one chromosome arm (cases 5, 21, and 22). Of the remaining 14 fully informative cases, five SMCs (36%) contained unique sequence DNA derived from both the p and q arms of the chromosome, consistent with Model I (see Introduction). Nine cases (64%) contained unique DNA derived from either the p or q arm of the chromosome and are consistent with Model II. Other recent studies[15,16,28] have assessed the unique DNA content of marker chromosomes by using FISH, providing data on whether the SMCs contain DNA derived from one or both chromosome arms. Combining our 14 cases with these studies, 41 of 50 cases (82%) of ring marker chromosomes are consistent with a mechanism of centromere misdivision (Model II).

Comprehensive Analysis of SMCs via aCGH

After the development of a custom oligonucleotide array containing centromere molecular ruler coverage, aCGH was determined to be the most efficient method for the rapid determination of the DNA content of SMCs. Targeted FISH analysis can then be performed for the confirmation of the aCGH results and for the studying of parents or additional family members. Several additional cases illustrate the varying amounts of unique DNA present in SMCs and the precision of aCGH in identifying breakpoints and determining unique DNA and gene content. Case 21 is a mosaic (68%) SMC derived from chromosome 20 (Figure 3A). A gain of approximately 7 Mb in the region of the p arm was observed, indicating that the SMC contains unique DNA from the p arm of chromosome 20 but not the q arm. FISH analysis with the molecular-ruler clone panel verified these array results.

Case 22 is a small, mosaic SMC derived from chromosome 20. A gain of approximately 0.3 Mb of unique DNA in the region of the q arm of chromosome 20 can be seen by aCGH (Figure 3B). FISH results with the unique BAC clones in the centromeric region of chromosome 20 confirmed the aCGH data (data not shown).

As shown in Table 2, the chromosome origin and size of the SMC were determined by aCGH for 11 cases. These results were consistent with FISH analyses in all cases, with breakpoints within 1 Mb. The breakpoints determined by aCGH were more precise than those determined by FISH, because the average probe spacing was at least 75 kb in the microarray studies. Six SMCs were undetectable by aCGH (Table 2) because of low-level mosaicism of the SMCs (<15%) (cases 3, 13, 23a, and 23b) or no unique DNA was present on the SMCs as determined by FISH (cases 4, 18, 23a, and 23b).

The combination of FISH along with a microarray containing genome-wide coverage, rather than a pericentromere-targeted array, is advantageous in the characterization of marker chromosomes. Microarray data revealed additional abnormalities in two cases (cases 8 and 12). For case 8, a paternally inherited SMC derived from chromosome 4 was detected by aCGH and confirmed by FISH. The aCGH results also revealed an approximately 5.7 Mb interstitial deletion of chromosome 6. This deletion at

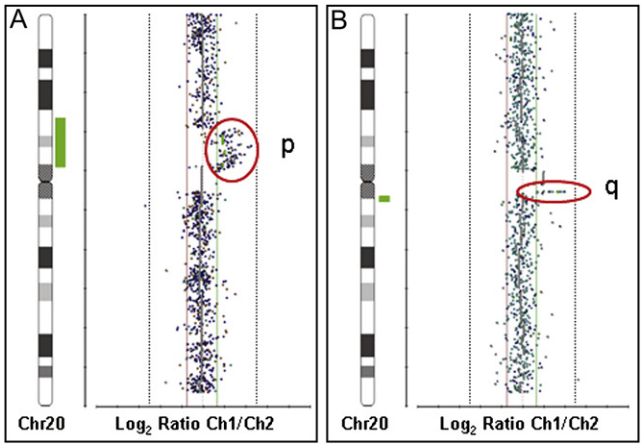

Figure 3. Array CGH Analysis of Two SMCs Containing Unique DNA from Only One Chromosome Arm

The x axis displays the $\log_2$ ratios of the patient sample (Ch1) versus a normal control sample (Ch2).

(A) Array CGH analysis with a commercial oligonucleotide array (Agilent 44b) showed a gain of copy number on chromosome 20 for case 21. A group of probes in the p arm adjacent to the centromere gap exceeds the threshold for duplication.

(B) Array CGH analysis with a custom oligonucleotide array showed a gain of probes on chromosome 20 for case 22. A group of probes in the q arm of chromosome 20 exceeds the threshold for duplication.

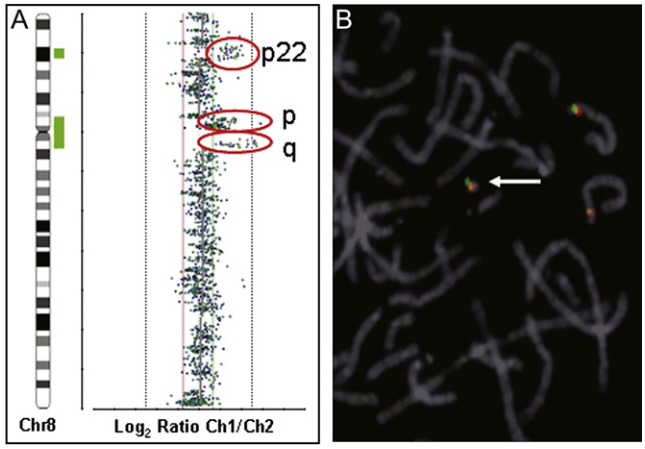

Figure 4. Array CGH and FISH Results of a SMC with a Complex Rearrangement—Case 12

(A) Array CGH analysis with a commercial oligonucleotide array (Agilent 44b) shows the gain of probes on the p and q arms adjacent to the centromere gap of chromosome 8, as well as an additional gain much more distally at 8p22.

(B) A duplication of 8p22 was confirmed by FISH analysis with unique BAC clones from 8p22 (red; RP11-10C8, green; RP11-433L7), which showed positive hybridization to the marker chromosome (arrow).

6q22.31–q22.32 was confirmed by FISH and was determined to be inherited from the proband's mother (data not shown). A recent paper reported a larger deletion of this region (9.9–11.6 Mb in q22.31q23.1) in a phenotypically normal individual.[43]

The results of case 12 which contains two de novo SMCs derived from chromosome 8 are shown in Figure 4. The mosaic markers (45% +2mar, 45% +mar) were ascertained in a proband with learning disabilities and obesity. The aCGH results reveal a gain of approximately 4 Mb of the p arm and approximately 3 Mb of the q arm material adjacent to the centromere gap of chromosome 8 (Figure 4A). FISH-mapping studies using the molecular-ruler clones confirmed the array results. In addition, FISH studies demonstrated that the second marker chromosome contained only approximately 0.5 Mb of DNA from the p arm and approximately 3 Mb of euchromatic DNA from the q arm (data not shown). The array results also identified an additional gain of approximately 3.8 Mb in the 8p22 region (Figure 4A). This result alone cannot determine whether the gain was the result of a complex rearrangement in the SMC or an additional duplication of 8p22 elsewhere in the genome. BAC clones specific to the amplified region of 8p22 (RP11-10C8 and RP11-433L7) were utilized in FISH assays for confirmation that the additional material was contained within the larger marker chromosome. Both clones were present on the marker chromosome, confirming that a complex rearrangement involving this region occurred in the formation of the SMC (Figure 4B). In this case, the combination of genome-wide aCGH and FISH technologies allowed for an accurate assessment of the size and chromosomal origin of the DNA contained within the two SMCs.

Discussion

Despite the significance of centromeres in the stability and segregation of human chromosomes, these regions remain a challenge to the final completion of mapping and sequencing the human genome.[33,44] The main obstacle is the correct assembly of the DNA sequences because these regions contain complex repetitive sequences in the transition zone from centromeric alpha-satellite DNA into the pericentromeric repeat regions and unique chromosome-specific sequences.[36,45,46]

In this study, targeted analysis of all 43 human pericentromeric regions was performed for the identification of the junction of the unique DNA with the pericentromeric repeats. The most proximal unique BAC clone for each chromosome arm was identified and validated by FISH assays, and "molecular rulers" of validated BAC clones from the centromere gap to approximately 5 Mb of each chromosome arm were developed (see Table 1 and Table S1). Recently, Ballif and colleagues developed a panel of 974 FISH-mapped clones covering approximately 5 Mb of the unique centromere regions and utilized these clones on a BAC-based microarray.[28] Although the clone selection for the most proximal unique clones varied for several chromosome arms, the independent analysis by both groups yielded similar average distances of the most proximal clone to the centromere gap (1.2 Mb in this study and 1.6 Mb in Ballif et al.[28]).

Identification of Unique-Negative versus Unique-Positive SMCs

Improved physical maps of each human chromosome and the development of BAC clones precisely mapped in each centromeric region now allows molecular characterization of SMCs in a research or clinical setting by FISH or aCGH. Of particular clinical importance might be the ability to rapidly distinguish unique sequence-negative from unique sequence-positive SMCs, because the former are less likely to be associated with abnormal outcomes if identified prenatally.

Approximately 26% (7 of 27) of the SMCs in our study were unique negative. One of these (case 23) provides an example of prenatal ascertainment, in which the fetus was found to have two SMCs derived from different chromosomes. Our analysis showed no unique DNA present on either of these two SMCs. At 9 months of age, the patient is healthy with normal developmental milestones. Larger, prospective studies are needed to confirm the predicted low risk associated with unique-negative SMCs, but certainly this precise determination of DNA content should be more predictive than empiric data based simply on morphology (e.g., satellited versus nonsatellited) or banding characteristics (e.g., presence or absence of C band-negative material).

Of the 27 SMCs reported here, approximately 74% (20 of 27) were unique-positive SMCs, containing an average of approximately 6.5 Mb unique sequence and approximately 33 known genes. These represent significant partial trisomies and are more likely to be causative of abnormal clinical features. Two of our three most severely affected cases (cases 3 and 14) were found to have marker chromosomes that contained at least 8.6 Mb and 11.6 Mb of unique DNA sequences, consistent with the notion that larger marker chromosomes are more likely to be associated with a severe phenotype.

Although the small number of cases in this study is not sufficient to contribute to chromosome-specific SMC genotype-phenotype correlations, an international database of SMCs with clinical descriptions has been established (see Web Resources below).[3] The current molecular cytogenetic techniques that allow for detailed molecular data on the gene content and size of SMCs will significantly improve on such correlations in the future. As more information is obtained on the size and gene content among markers derived from the same chromosome, risk estimates might be refined. In addition to the chromosome origin and unique DNA content, the level of mosaicism might also alter the risk associated with an abnormal phenotype. A recent study of 137 marker cases demonstrated that 41% were mosaic, whereas the remaining SMCs were present in every cell.[2] For mosaic SMCs, the levels of mosaicism have been shown to vary among different tissues.[47] Taking all of these factors into consideration, including the levels of mosaicism, unique DNA, and gene content of SMCs, we would expect that the ability to predict the clinical significance in a prenatal setting and the determination of prognosis in a young child will be greatly improved.

Rediscovery of the McClintock Mechanism of Small-Ring Formation and Implications for Genetic Counseling

The relative frequency of two major mechanisms of small-ring-chromosome formation was assessed in this study and demonstrated that more than half of the SMCs are consistent with a mechanism of centromere misdivision (Model II). One of these cases (case 7) involved a patient with a maternally inherited mosaic small r(4) initially identified prenatally. Analysis of the mother's chromosomes revealed a cryptic deletion present in one of her chromosome 4 homologs, creating a mosaic balanced carrier state for a del(4) and complementary r(4).

At least 11 additional cases of deletion associated with a complementary ring chromosome have been reported in the literature.[31,48–57] These cases all involved visible deletions in one homolog, whereas case 7 in this study is the first reported cryptic deletion associated with ring-chromosome formation. Three of the previously reported cases[31,55,57] involve the more rare class of marker chromosomes that do not contain detectable alpha-satellite sequences and are referred to as neocentric markers.[58,59]

Identification of such cryptic balanced carriers is obviously essential for accurate genetic counseling about recurrence risks, as carriers are at high risk for two different unbalanced offspring by transmission of only the deleted homolog or the ring chromosome along with a normal homolog. Examples of both of these transmissions have been reported in the literature,[48,51–53,56,57,60] and in at least one family, both unbalanced products were identified in affected children with different phenotypes.[51]

This mechanism of breakage within the centromere creating a pericentromeric deletion and complementary ring chromosome was first described in 1938 by B. McClintock[61] in one of her classic papers on maize cytogenetics. In this work, she noted that "the size of the ring-shaped chromosome and the extent of the deficiency in the rod-chromosome were comparable." She also noted that "the deficient rod and its compensating ring chromosome arose as the result of two breaks in the normal chromosome V, one break passing through the spindle fiber attachment region," referring to the centromere of the chromosome. Because this description matches perfectly with the model here being suggested as the major mechanism for human ring marker formation, we propose that this mechanism be referred to as the McClintock mechanism.

It is currently standard practice in clinical laboratories for cytogeneticists to identify the chromosome of origin of a marker chromosome by using probes only from the centromeric alpha-satellite regions. By this approach, only cytogenetically visible deletions in the surrounding euchromatic DNA have previously been noted. It is not uncommon for them to identify a small marker chromosome in a child with developmental delay and/or mental retardation but then find the same marker in a normal parent and perhaps other normal family members. In this situation, the marker chromosome in the proband is

usually considered coincidental to her/his delay or abnormal phenotype. This approach should now be considered insufficient, given the possibility of cryptic pericentromeric deletions and a balanced del and ring state in a normal parent and other relatives. It is imperative to perform molecular cytogenetic investigations to rule out a cryptic pericentromeric deletion producing a balanced del and ring carrier state in the parent carrying a marker chromosome. These follow-up studies are best performed by FISH for the detection of the cryptic deletion because aCGH and other quantitative molecular methods could demonstrate apparently normal dosage results.

Comprehensive SMC Analysis by aCGH and FISH

Different aCGH platforms, using either BAC clones or oligonucleotides, have become readily available for the detection of copy-number imbalances, and several studies have recently used this technology to characterize SMCs.[28,62–66] High-resolution genome-wide analysis of SMCs via aCGH has proven to be advantageous in the detection of complex rearrangements that might result in the formation of a marker chromosome. The genome-wide coverage of the custom oligonucleotide array, with enhanced probe density in the unique pericentromere regions, provides the opportunity for us to determine the size and precise content of SMCs in one assay. Even with the development of the pericentromeric molecular ruler clones covering up to 5 Mb of unique DNA, additional clones had to be selected for the completion of the FISH-mapping studies of nearly 35% of the cases presented in this paper. It has recently been suggested that a pericentromeric BAC-based microarray including coverage of the most proximal unique 10 Mb of DNA would be valuable in the sizing of SMCs[28] so that these larger marker chromosomes could be accurately sized. Our data suggest that the coverage would need to extend to at least 15 Mb as approximately 19% of the SMCs in this study contained at least 10 Mb of unique DNA derived from a single chromosome arm. Furthermore, the complex rearrangement of the 8p22 region involved in the SMC (case 12) would not have been detected with typical FISH-mapping studies or a targeted pericentromeric array. These results support the use of a genome-wide microarray in the characterization of SMCs. Such a microarray allows for the sizing and characterization of SMCs in an efficient manner, as well as the identification of other potential imbalances elsewhere in the genome.

Our studies also demonstrate that FISH analysis is often required in conjunction with aCGH studies. In this study, four SMCs (cases 3, 13, 23a, and 23b) were undetectable by aCGH because of a low level of mosaicism. As determined by metaphase FISH analysis, the SMCs were present in less than 14% of the cells. However, we easily detected another marker chromosome that was present in only 33% of the cells (case 7), suggesting that minimum detection range is between 14%–33%. This range is similar to a previous report where a mosaic trisomy 21 sample was easily detected by aCGH when present in only 20% of cells, but not in 10% of cells.[64] In addition, some SMCs might not appear as a gain by aCGH because of a lack of unique DNA content. In this study, four marker chromosomes did not contain unique DNA, as determined by aCGH and FISH studies (cases 4, 18, 23a, and 23b). For these samples, FISH was required for the identification of the chromosomal origin of the SMC with alpha-satellite and pericentromeric-repeat-containing probes.

Tremendous progress has been made since the landmark study by Warburton in 1991 describing the empiric risk figures for small supernumerary marker chromosomes detected prenatally.[10] Analysis by aCGH and FISH is now feasible on a timely clinical basis and can accurately determine the size and gene content of such markers. The development of genotype-phenotype databases for the determination of clinical significance and prognosis will be extremely useful for prenatal and pediatric settings.

Supplemental Data

Two tables are available at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Database of SMCs, http://www.med.uni-jena.de/fish/sSMC/00START.htm
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
UCSC genome browser, http://genome.ucsc.edu/

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