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ORIGINAL INVESTIGATION

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A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenM-FISH)

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Abstract Centromere-specific multi-color FISH (cenM-FISH) is a new multicolor FISH technique that allows the simultaneous characterization of all human centromeres by using labeled centromeric satellite DNA as probes. This approach allows the rapid identification of all human centromeres by their individual pseudo-coloring in one single step and is therefore a powerful tool in molecular cytogenetics. CenM-FISH fills a gap in multicolor karyotyping using WCP probes and distinguishes all centromeric regions apart from the evolutionary highly conserved regions on the chromosomes 13 and 21. The usefulness of the cenM-FISH technique for the characterization of small supernumerary marker chromosomes with no (or nearly no) euchromatin and restricted amounts of available sample material is demonstrated in prenatal, postnatal, and tumor cytogenetic cases. In addition, rarely described markers with the involvement of heterochromatic material inserted into homogeneously staining regions could be identified and characterized by using the cenM-FISH technique.

Introduction

According to ISCN 1995 (Mitelman 1995), marker chromosomes are structurally abnormal chromosomes of unknown origin. These marker chromosomes are frequently found in karyotypes of cancer patients and patients with constitutional genetic disorders (Mitelman 1995). Their characterization is of high clinical impact and is the requisite condition for further molecular investigations aimed at the identification of disease-related genes. The origin of marker chromosomes is nearly impossible to establish by conventional cytogenetics. The introduction of molecular cytogenetic techniques based on fluorescence in situ hybridization (FISH) has recently made it possible to throw light on this process (e.g. Langer et al. 1981; Pinkel et al. 1986). Indeed, several marker chromosomes have been characterized by using a panel of whole chromosome painting probes (WCPs), centromere-specific probes, or combined chromosome microdissection and FISH approaches (revFISH) (e.g. Rauch et al. 1992; Falzetti et al. 2000). These time- and material-consuming studies can now be performed in one single experiment by WCP-FISH with a variety of commercially available multi-probe devices (Joyce et al. 1999; Ogilvie et al. 2000), multicolor-FISH (M-FISH; Speicher et al. 1996), or multicolor spectral karyotyping (SKY) technology (Schröck et al. 1996). To obtain specific staining with these WCP techniques, chromosome in situ suppression (CISS; Lichter et al. 1988) of labeled repetitive sequences is required. Suppression of non-specific signals is achieved by pre-hybridizing the labeled probe in the presence of excess unlabeled COT1 repetitive DNA. Repetitive sequences present in centromeric regions of human chromosomes may also become suppressed by this technique and are therefore not painted or covered by these probes (Fig. 1C). Very small supernumerary marker chromosomes (SMC) found during pre- and postnatal cytogenetic analyses may often escape these 24-color FISH analyses. SMC normally include very small chromosome fragments with no (or nearly no) euchromatin or marker chromosomes with the involvement of heterochromatic material of unknown origin. For the rapid characterization of such markers, a new M-FISH technique that allows the unambiguous identification of all human centromeric regions, excluding chromosomes 13 and 21, by their individual coloring in one single step has been developed. This

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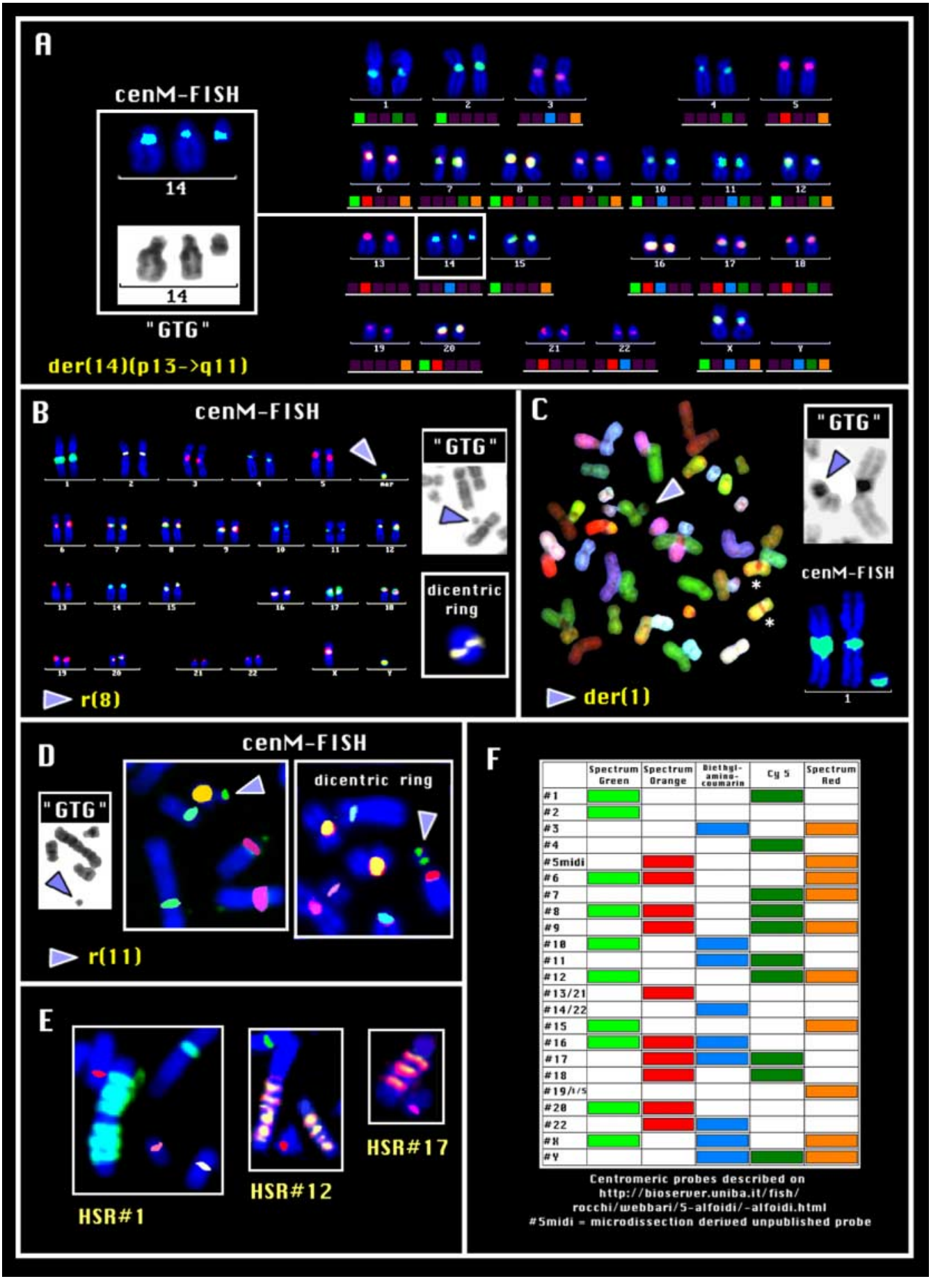
A
cenM-FISH
14
14
"GTG"
der(14)(p13->q11)
B
cenM-FISH
"GTG"
dicentric ring
r(8)
C
"GTG"
cenM-FISH
der(1)
D
cenM-FISH
"GTG"
dicentric ring
r(11)
E
HSR#1
HSR#12
HSR#17
F
Spectrum Green
Spectrum Orange
Diethyl-amino-coumarin
Cy 5
Spectrum Red
#1
#2
#3
#4
#5midi
#6
#7
#8
#9
#10
#11
#12
#13/21
#14/22
#15
#16
#17
#18
#19/15
#20
#22
#X
#Y
Centromeric probes described on
http://bioserver.uniba.it/fish/
rocchi/webbari/5-alfoidi/-alfoidi.html
#5midi = microdissection derived unpublished probe

◀ **Fig. 1** Centromere-specific multicolor-FISH (*cenM-FISH*) results of case 1 (**A**), case 2 (**B**), case 11 (**C**), case 14 (**D**), and cases 15–17 (**E**); labeling scheme of the centromere-specific probes used for cenM-FISH (**F**). The SMC are marked with *arrowheads* in **B–D** ("*GTG*" inverted DAPI banding). Images were captured on a Zeiss Axioplan microscope (Zeiss Jena, Germany) with the IKAROS and ISIS digital FISH imaging system (MetaSystems, Altlussheim, Germany) by using an XC77 CCD camera with on-chip integration (Sony). **A, B** Two prenatally detected SMC were characterized by cenM-FISH as being derived from chromosome 14 and 8, respectively. Case 1 (**A**) had a supernumerary derivative chromosome 14 [der(14)(p13→q11)] and case 2 (**B**) a minute ring chromosome 8 [r(8)] inherited from the mother (case 12). The ring presented with one centromere in 23 of 25 metaphase spreads analyzed by cenM-FISH and was dicentric in 2 of 25 metaphases [rdup(8)]. **C** A postnatally detected SMC could be characterized as a derivative chromosome 1 [der(1)] by M-FISH with WCP probes; this could be confirmed by cenM-FISH. Repetitive sequences present in centromeric regions of human chromosomes are not painted when the M-FISH technique is applied, as is readily apparent. Only small parts of the SMC (*arrowhead*) are painted specific by WCP1 (*green*) and its centromeric region is not stained. Moreover, any centromere of the human chromosomes is excluded from chromosome specific painting; this is most notable for chromosome 9 (*asterisks*). **D** An acquired SMC detected in bone marrow of a patient suffering from myeloproliferative syndrome could be characterized as a minute ring chromosome 11 [r(11)] by using cenM-FISH. A duplication of the material present in the ring chromosome [rdup(11)] could be detected in a small portion of analyzed metaphase spreads (5/30). **E** Involvement of heterochromatic material into homogeneously staining regions (*HSR*) has been established by cenM-FISH in a non-Hodgkin lymphoma case (case 15; *HSR#1*) and in two different osteogenic sarcoma cell lines (*HSR#12* in cell line SA1, case 16; *HSR#17* in cell line RH30, case 17)

technique, called centromere-specific multicolor-FISH (cenM-FISH), fills a gap in multicolor karyotyping and has been successfully applied in constitutional and cancer cytogenetics.

Materials and methods

Patients and cell lines

Cytogenetic and molecular cytogenetic studies were performed on chromosomes derived from amniotic fluid, peripheral blood, bone marrow, and cultured cell lines. Chromosome preparations were obtained according to standard techniques (Verma and Babu 1989). The cases studied included: three postnatal cases and nine prenatal cases with a single SMC, one prenatal case with two SMC, and a chronic myelogenous leukemia (CML) case with one SMC as the only acquired aberration. Routine cytogenetic analysis of the prenatal cases presented primarily mosaic karyotypes. These patients had been referred for amniocentesis mainly because of advanced maternal age or because of psychological reasons. Postnatal cases nos. 12 and 13 were inconspicuous. Case 11 presented with some facial and psychomotor abnormalities. One non-Hodgkin lymphoma (NHL) case with a homogeneously staining region (HSR)-marker chromosome and two osteogenic sarcoma cell lines RH30 and SA1 (Oliner et al. 1992) were also included in the study (see below).

24-color FISH

The 24-color FISH technique was performed by using microdissection-derived WCPs according to the method of Senger et al. (1998).

Table 1 Centromere-specific probes used for cenM-FISH; all probes (other than the microdissection derived probe: *Midi*) are available from Dr. M. Rocchi for research purposes (Resources for molecular cytogenetics: http://www.biologia.uniba.it/rmc/)

Chromosome	Probe	Chromosome	Probe	Chromosome	Probe
1	D1Z5	9	pZ4.1	17	D17Z1
2	D2Z1	10	D10Z1	18	D18Z1
3	D3Z1	11	D11Z1	19+1+5	D1Z7
4	p4n1/4	12	D12Z3	20	D20Z2
5	Midi	13+21	D13/21Z1	22	D22Z4
6	D6Z1	14+21	D14/22Z1	X	DXZ1
7	D7Z2	15	D15Z3	Y	DYZ3
8	D8Z1	16	D16Z2		

cenM-FISH

Plasmid DNA (see Table 1), with centromere-specific DNA inserts, and the DNA of a microdissection library for the centromere of chromosome 5 were amplified by the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR; 50 µl volume) according to Senger et al. (1998). Each probe was labeled separately by using a secondary DOP-PCR (20 µl volume). Labeling was performed according to the labeling scheme in Fig. 1F, with d-UTPs carrying biotin, diethylaminocoumarine, SpectrumRed, SpectrumOrange, or SpectrumGreen as ligands. All labeled probes were mixed, aliquoted into 25 equal portions, and each precipitated together with 25 µg t-RNA and 2 µg COT1-DNA. A small amount of COT1-DNA was necessary to avoid undesirable cross-hybridization of the various centromere-specific probes (this amount of COT1-DNA represents only a small fraction of the amount usually necessary in WCP-specific 24-color FISH). The pellets were vacuum-dried and stored at –20°C until used. Prior to use, the DNA pellet was resuspended in 12 µl hybridization buffer, consisting of 2 g dextransulfate in 10 ml 50% deionized formamide, 2× SSC (1× SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50 mM phosphate buffer. This yielded a sufficient volume of probe mix for covering a 24×24 mm slide surface. The probe solution was denatured for 5 min at 75°C and prehybridized for 10 min at 37°C.

The RNase and pepsin pretreatments, denaturation, and addition of the probe to the sample were performed according to standard FISH protocols (Liehr et al. 1995). Slides were incubated overnight at 37°C in a humidified chamber and postwashed (3×5 min in 50% formamide solution at 42°C, followed by 3×5 min in 2× SSC at 42°C) in a 100 ml Coplin jar. Biotin was detected with a Cy5-avidin/biotinylated anti-avidin system. The slides were counterstained with DAPI (4,6-diamidino-2-phenylindole) solution and covered with an anti-fade medium.

The results were evaluated on a fluorescence microscope equipped with a charge-coupled device camera and an image-analysis system (MetaSystems, Altlussheim, Germany). Integration times were between less than 1 s and 5 s for the six different fluorochromes (including DAPI). The used probes were highly chromosome-specific, and possible cross-hybridization was blocked by small amounts of COT1 DNA; the achieved signals were thus unambiguous in all tested cases. Imaging analysis was necessary as the Cy5 spectrum was not visible to the human eye. In addition, false colors had to be created by the image-analyzing system for a reliable evaluation, and the system provided information with regard to which fluorochromes lit up in which centromeric region.

The cenM-FISH results were verified by using single-color FISH with commercially available centromeric probes and a standard FISH protocol (data not presented).

Results

The cenM-FISH technique is similar to the 24-color-FISH technique with WCPs (Speicher et al. 1996; Schröck et al. 1996). It differs from 24-color-FISH in that repetitive sequences located at the centromeric regions of human chromosomes are its target. CenM-FISH is based on all available centromere-specific DNA probes (Willard and Waye 1987; see also Table 1), which are labeled with five different fluorochromes (see Fig. 1F), mixed, and then hybridized simultaneously. Since the centromeres for chromosomes 1, 5, and 19 (D1Z7), 4 and 9 (pZ4.1), 13 and 21 (D13/21Z1), and 14 and 22 (D14/22D1) share nearly identical alphoid subfamilies, they normally cannot be distinguished by standard molecular cytogenetic techniques. To overcome the problems with chromosomes 4/9 and 14/22, additional centromere 4-specific (p4n1/4) and centromere 22-specific (D22Z4) probes in different color combinations were applied. For chromosome 5, a microdissection-derived specific probe (unpublished data) was used, and for chromosome 1, a probe specific for the heterochromatic subcentromeric region in 1q12 (D1Z5) was employed. Interestingly, after DOP-PCR amplification of probe D1Z7, strong signals on chromosome 19 and (virtually) none on chromosomes 1 and 5 were obtained. The alpha satellite DNA of the chromosomes 13 and 21 is 99.7% sequence-identical (Maratou et al. 1999), and no specific repetitive peri-centromeric DNA has been identified for either of these two chromosomes so far. Thus, apart from chromosomes 13 and 21, all centromeres were covered by a specific probe or probe combination in the presented cenM-FISH approach.

To compensate for weak signals on certain chromosomes (i.e., chromosomes 2, 4, 11, 20, and 22) attributable to insufficient repetitive sequence copy number, the amount of probe used initially in the DOP-PCR amplification was doubled.

The probes thus obtained were highly chromosome-specific. Possible cross-hybridization was blocked by the addition of very small amounts of COT1 DNA. The signals achieved by this process were unambiguous in all tested cases. Moreover, the results were confirmed by subsequent single-color FISH experiments.

The utility of the cenM-FISH technique in the characterization of very small SMC with no (or nearly no) euchromatin was demonstrated in one leukemia case and ten prenatal and three postnatal cases (see Fig. 1). The leukemia case presented with an acquired small ring chromosome 11 (for details, see Table 2). WCP-specific 24-color FISH was able to provide informative results as to the origin of the corresponding SMC in only four of the 14 analyzed cases. The SMC were characterized by cenM-FISH as derivative chromosomes 1, 2, 8 (three cases), 14 (two cases), 15

Table 2 Overview of the results of the cases analyzed by using cenM-FISH; chromosomal breakpoints were determined by amalgamating the results of cenM-FISH, M-FISH, and GTG-banding. The osteogenic sarcoma cell lines are described in Oliner et al. (1992)

Case	Cytogenetics	M-FISH	cenM-FISH
Prenatal cases			
1	47,XX,+mar[9]/46,XX[5]	No result	+der(14)(p13→q11)
2	47,XX,+mar[43]/46,XX[7]	+der(8)	+r(8)(p11;q12)
3	47,XX,+mar[16]/46,XX[7]	No result	+der(8)(p11→q11).
4	47,XY,+mar[5]/46,XY[5]	No result	+inv dup(14)(q11)
5	47,XX,+mar[20]	No result	+inv dup(21)(q11)
6	48,XX,+2mar[16]/46,XX[4]	No result	+2 inv dup (15)(pter→q11.1::p11.1 or q11.1→p13)
7	47,XX,+mar[6]/46,XX[44]	No result	+inv dup(15)(q11)
8	47,XX,+mar[20]	+der(15)	+der(15)(p13→q?13::q?13 or q?→q? or q?13)
9	47,XX,+mar[10]/46,XX[5]	No result	+der(X)(p11.1→q11)
10	47,XX,+mar[20]	No result	+der(20)(p11.1→q11.1)
Postnatal cases			
11	47,XX,+mar[15]	+der(1)	+der(1)(p?12→q12)
12	47,XX,+mar[15]	+der(8)	+r(8)(p11;q12)
13	47,XY,+mar[15]	No result	+der(2)(p11.1→q11.1)
Tumorcytogenetic cases			
14	47,XY,+mar[20] (Bone marrow: CML)	No result	+r(11)
15	Hyperdiploid with HSR marker (bone marrow: NHL)	der(1)t(18;1;18)	6-fold amplification of centromere 1
16	Hyperdiploid with HSR marker (osteogenic sarcoma cell line: RH30)	Not determined	3-fold amplification of centromere 17
17	Hyperdiploid with HSR marker (osteogenic sarcoma cell line: SA1)	Not determined	6- and 4-fold amplification of centromere 12 on 2 markers

(three cases), 20, 21, and chromosome X in the 13 constitutional cytogenetic cases studied.

Three cases with marker chromosomes involving heterochromatic material inserted into HSR were characterized by applying cenM-FISH. Juxtacentromeric material of chromosome 1 was shown to be amplified in the NHL case. Alphoid material derived from chromosomes 12 and 17 was amplified in two different osteogenic sarcoma cell lines (Table 2), possibly by being co-amplified.

Discussion

The utility of the cenM-FISH technique in the characterization of very small SMC with no (or nearly no) euchromatin has been demonstrated in prenatal, postnatal, and leukemia cases (for details, see Table 2).

Only four out of the 14 cases analyzed revealed an informative result using 24-color FISH with WCPs. The origin of the other 10 markers could have been clarified by step-by-step hybridization of all human centromeres (Starke et al. 1999) or by microdissection and reverse painting (revFISH). The latter is a well-established procedure for the characterization of markers (Senger et al. 1990). However, revFISH requires sophisticated equipment not generally available in every laboratory. Both M-FISH and revFISH techniques are restricted by their requirement for sufficient patient material. One of the advantages of the cenM-FISH technique is that it can be performed on a previously GTG-banded slide.

Small SMC have been reported in 0.01%–0.05% of all fetuses (Crolla 1998). The limitation of available sample material is commonly a problem in prenatal diagnosis. Of the ten cases in this study, seven presented mosaicism in their karyotype. This further reduces the amount of informative metaphase spreads by 20%–67%. As there are large variations in small SMC with respect to their origin, the possibility of imprinting and isodisomic effects, and in the corresponding clinical outcome (Crolla 1998), the characterization of prenatally detected small SMC is of the greatest interest for the further medical care and genetic counseling of the parents. Postnatal detection and characterization of small SMC can help to explain the clinical phenotype of a patient (e.g. Blennow et al. 1995). If there are no manifestations of the genotype, the characterization of SMC can provide important information about regions in the human genome that are genetically inactive. Characterization of one or more heterochromatic small SMC in a patient can be followed by testing for uniparental disomy. This strategy has been used to explain phenotypic abnormalities (Chudoba et al. 1999a).

Acquired small SMC, like that characterized in case 14 (see Table 2) as being derived from chromosome 11 with no (or virtually no) euchromatic material, have not previously been described and/or characterized frequently (Meloni-Ehrig et al. 1999), perhaps because of the lack of suitable molecular cytogenetic techniques. The possibility of the rapid characterization of small SMC, consisting mainly of heterochromatic material, within 24 h after detection by GTG-banding indicates that the cenM-FISH technique could become a key molecular cytogenetic tool in the field of prenatal diagnosis.

Tumorcytogenetic markers with the involvement of heterochromatic material in HSR have not been frequently described (Liehr et al. 1997; Sirvent et al. 2000). By the application of cenM-FISH, juxtacentromeric material derived from chromosome 1 has been shown here to be amplified in a non-Hodgkin lymphoma case. Alphoid material of chromosomes 12 and 17 has also been shown (co?-) amplified in two different osteogenic sarcoma cell lines (see Table 2). Up to now, little is known about such markers, and their clinical significance is not yet certain. CenM-FISH could be the method of choice for identifying and characterizing additional such amplicons. Conclusions about the adjacent chromosomal material with genetically active regions could then also be made. Moeover, studies of the mechanism of centromere inactivation in SMC chromosomes could be performed in these cases, as investigated by Sirvent and coworkers (2000). The cenM-FISH technique applied to cancer cytogenetics may therefore resolve, in a relatively short time, a variety of karyotypic aberrations that involve heterochromatin material and that until now have resisted WCPs-based 24 colors-FISH analysis. CenM-FISH results may be helpful in the identification of new cancer-related genes located in or near the pericentromeric regions.

Finally, it must be stated that the use of the cenM-FISH method is restricted to chromosomes with “normal” human centromeres and cannot identify mitotically stable chromosomes with neocentromeres (Koch 2000) or mitotically instable chromosomes without (peri-)centromeric material.

In summary, we report a new multicolor-FISH technique for the one step identification of all human chromosomes by available centromere-specific probes. CenM-FISH technique offers a valuable complementation to other multicolor-FISH procedures, such as WCP-FISH with commercial multi-probe devices (Joyce et al. 1999; Ogilvie et al. 2000), M-FISH (Speicher et al. 1996), SKY (Schröck et al. 1996), RX-FISH (Müller et al. 1997), and the multicolor-banding technique (Chudoba et al. 1999b), none of which cover the heterochromatic material of human chromosomes. The positive identification of marker chromosomes in prenatal and postnatal cases and in acquired malignancy in cancer validate the usefulness of this powerful technique.

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