

Sister chromatid differentiation and chromosomal in situ suppression hybridization: a combined methodology for analyzing cell proliferation and SCEs in individual chromosomes

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Abstract. A technique combining sister chromatid differentiation (SCD) with chromosomal in situ suppression (CISS) hybridization is described. This combined methodology allows simultaneous analysis of cell-proliferation kinetics and sister chromatid exchanges (SCEs) in chromosomes identified by probes. To demonstrate the usefulness of this approach, cultured fibroblasts from a patient with Pallister-Killian syndrome, *mos46/47,+i(12p)*, a chromosome mosaicism disorder, were studied. The fibroblasts were cultured in the presence of 5-bromodeoxyuridine (BrdU) for 72 h. Chromosome preparations

were stained by a modified fluorescence-plus-Giemsa method to obtain SCD. For identification of the normal chromosome 12 and the *i(12p)*, CISS hybridization with a biotin-labeled chromosome 12-specific library probe (LA 12NS01) was carried out after SCD. The hybridization was detected by an indirect immunofluorescence technique. For the analysis of cell kinetics and SCEs, the technique allows rapid, reliable identification of abnormal and normal cell populations. It also allows analysis of SCEs in individual chromosomes.

There are several techniques by which sister chromatids can be differentiated in order to detect exchanges between them (SCEs). Most of these methods are based on the incorporation of 5-bromodeoxyuridine (BrdU) for one or two DNA replication cycles *in vivo* or *in vitro*, followed by specific chromosome staining with fluorochromes or with fluorochromes plus Giemsa staining solution (for review, see Block, 1982). Regardless of the technique used, the value of the SCE bioassay lies in its proven ability to detect S-dependent clastogens (for review, see Littlefield, 1982, and references therein).

Sister chromatid differentiation (SCD) also permits analysis of cell-cycle kinetics and cell proliferation rates, as metaphase cells of different generations can be easily distinguished on the basis of their SCD pattern (Latt, 1974; Wolff and Perry, 1974; Tice et al., 1976; Morimoto and Wolff, 1980). In metaphases that have undergone only one cell cycle, both chromatids are darkly stained; in those that have completed two cell cycles, one chromatid is darkly stained and the other lightly stained, and in

those that have undergone three cycles, approximately half of the chromosomes are harlequin stained and the rest show a uniform light staining of both sister chromatids.

Thus far, the identification of individual chromosomes and the diagnosis of numerical and structural aberrations have been based mainly on chromosome banding analysis. Recently, more rapid and reliable recognition of chromosomal rearrangements has become possible through the introduction of library and satellite human DNA probes specific to individual chromosomes. While chromosomal in situ suppression (CISS) hybridization with DNA library probes from sorted chromosomes is used to recognize (or "paint") complete individual chromosomes, repetitive chromosome probes are used to detect heterochromatic regions with tandemly arranged repetitive DNA sequences (Moyzis et al., 1987; Cremer et al., 1988; Lichter et al., 1988; Pinkel et al., 1988; Greig et al., 1991; Pérez-Lozada et al., 1991; Tiainen et al., 1991).

In this report, we describe the combined use of SCD and CISS hybridization methodologies. For this study, skin fibroblasts from a Pallister-Killian (PK) syndrome patient were chosen since a numerical chromosome aberration, *+i(12p)*, has been found repeatedly in up to 90% of fibroblasts obtained from patients with this mosaic syndrome (Pauli et al., 1987; Warburton et al., 1987; Gorlin et al., 1990).

CISS hybridization with a biotinylated chromosome 12-specific library probe was used to assess the presence of *i(12p)* in the

Supported by grants from the Sigrid Juselius Foundation, Finnish Cancer Society, Finnish Academy of Sciences, University of Helsinki, and National Council of Scientific and Technological Research (CONICET) of Argentina.

Received 2 March 1992; accepted 2 April 1992.

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PK cells. By combining these two methods (SCD + CISS hybridization) in a dual-detection system, we were able to investigate cell proliferation and SCE formation in normal and abnormal cells grown simultaneously in culture.

Materials and methods

Cell culture

Cultured fibroblasts from a patient with PK syndrome were selected for the study. The cells were grown *in vitro* in complete culture medium [80% RPMI 1640 (GIBCO, Grand Island, NY, USA), 20% fetal calf serum (GIBCO), 0.29 mg/ml L-glutamine (GIBCO), 100 U/ml penicillin (GIBCO), and 100 µg/ml streptomycin (GIBCO)] for 5–10 passages before SCD analysis. The cells were plated at a density of 1.0×10^6 cells in TC75 Falcon flasks, and BrdU (Sigma Chemical Co., St. Louis, MO, USA) was added to the cultures at a final concentration of 10 µg/ml 2 h after seeding. The subcultures were then wrapped in aluminum foil to protect them from light and incubated at 37 °C in a 5% CO₂ atmosphere for 72 h.

Before harvest, Colcemid (0.1 µg/ml, GIBCO) was added for the last 16 h of culture. The cells were rinsed with warm Hank's balanced salt solution and then collected by trypsinization and centrifugation. Chromosome spreads were obtained using hypotonic treatment (0.075 M KCl, 15 min at 37 °C), fixation with methanol:acetic acid (3:1), and air drying.

After air drying for at least 16–18 h, the slides were processed according to the combined methodology described below. The main steps of the procedure are summarized in Fig. 1.

Sister chromatid differentiation (SCD)

After air drying, the slides were processed for SCD according to the combined fluorescence-plus-Giemsa method of Korenberg and Freedlander (1974) and Perry and Wolff (1974), with some modifications. The chromosome spreads were treated with 1 µg/ml of Hoechst 33258 (Sigma) in 0.1 M phosphate buffer (pH 6.8) for 20 min. The slides were then mounted in Hoechst-free 0.1 M phosphate buffer and exposed to UV light emanating from a Philips TUV 15-W tube (Philips, The Netherlands) for 1 h. After UV exposure, the slides were rinsed in deionized water, dried, and treated using the procedure of Korenberg and Freedlander (1974). The slides were incubated for 5–10 min at 88 °C in 1 M Na₂PO₄ (pH 8.2–8.4), rinsed in deionized water, and air dried for at least 16–18 h before *in situ* hybridization.

When SCEs were to be analyzed by conventional light microscopy, the slides were stained with 5% Giemsa aqueous solution, and well-spread metaphases were located, analyzed, and photographed using a Zeiss III photomicroscope with dry objectives on Kodak Ektachrome 64T color slide film or Kodak Imagelink HQ black-and-white film. The slides were then washed for 3 h with methanol:acetic acid (3:1) and air dried for at least 16–18 h before *in situ* hybridization.

Chromosomal *in situ* suppression (CISS) hybridization

Hybridizations were carried out with DNA from a bacteriophage library probe established from sorted human chromosomes 12 (Library LA 12NS01, American Type Culture Collection, Rockville, MD, USA). The probe was labeled by nick translation using biotin-11-dUTP (Sigma Chemical Co., St. Louis, MO, USA) according to the instructions of the supplier (Nick-translation Kit, Bethesda Research Laboratories, Bethesda, MD, USA). CISS hybridization was performed as described in detail elsewhere (Lichter et al., 1988).

After hybridization, the biotin-labeled probe was detected by an indirect immunofluorescence technique using fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA, USA) and biotin-conjugated goat anti-avidin (Vector Laboratories). The hybridization signals were amplified as described by Pinkel et al. (1986). Cells were counterstained with 1 µg/ml of propidium iodide (Sigma) and 0.2 µg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) and then mounted in fluorescence antifading buffer (Johnson and de Nogueira-Araujo, 1981).

Fluorescence microscope analysis was performed using a Leitz Laborlux D fluorescence photomicroscope fitted with appropriate filter combinations (Leitz I2/3, A, and N2 filters). When necessary, metaphases previously identified in the SCD study were relocated and photographed on Kodak Ektachrome 400 ASA color slide film.

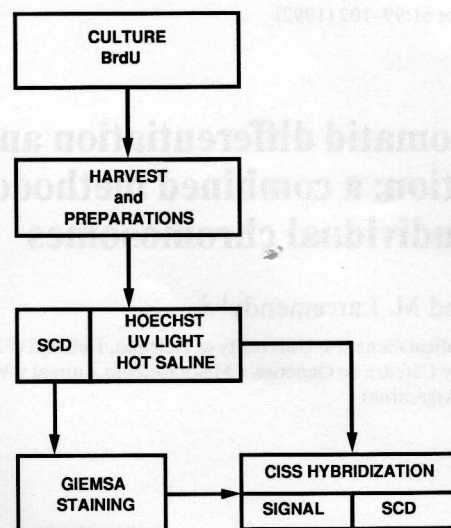


Fig. 1. Main steps in the combined SCD-CISS methodology.

Fig. 2. Pallister-Killian syndrome fibroblast undergoing its second mitosis after BrdU incorporation. (A) Sister chromatid differentiation observed after Giemsa staining. (B, C) Same metaphase as in A, after removal of Giemsa stain and after CISS hybridization with a biotinylated chromosome 12-specific library DNA probe (LA 12NOS1) detected with avidin-FITC and propidium iodide (B) or DAPI (C) counterstaining. The sister chromatid differentiation seen in all the chromosomes after DAPI counterstaining (C) or Giemsa staining (A) indicates that the cell performed two cell cycles in the presence of BrdU. Arrows indicate chromosomes 12, and arrowhead shows the location of i(12p). Note that the hybridization signals are restricted to the brightly stained chromatid of chromosomes 12 and i(12p) (B), corresponding to the darkly stained chromatid after Giemsa staining (A).

Fig. 3. Pallister-Killian syndrome fibroblasts undergoing their first mitosis after BrdU incorporation. CISS hybridization was performed with a biotinylated chromosome 12-specific library DNA probe (LA 12NOS1) detected with avidin-FITC and propidium iodide (A, C) or DAPI (B, D) counterstaining. The lack of sister chromatid differentiation in all the chromosomes after DAPI counterstaining (B, D) indicates that the cells have performed one cell cycle in the presence of BrdU. Arrows indicate chromosomes 12, and arrowhead shows the location of i(12p). (A, B) Diploid metaphase; (C, D) aneuploid metaphase carrying i(12p).

Fig. 4. Pallister-Killian syndrome fibroblast undergoing its second mitosis after BrdU incorporation. CISS hybridization was performed with a biotinylated chromosome 12-specific library DNA probe (LA 12NOS1) detected with avidin-FITC and propidium iodide (A, B) or DAPI (C) counterstaining. The differentiation of the sister chromatids in all the chromosomes after DAPI counterstaining (C) indicates that the cell has performed two cell cycles in the presence of BrdU. Arrows indicate chromosomes 12. Since no i(12p) was observed after CISS hybridization, the metaphase belongs to the normal diploid cell population.

Fig. 5. Pallister-Killian syndrome fibroblasts undergoing their third or subsequent mitosis after BrdU incorporation. CISS hybridization was performed with a biotinylated chromosome 12-specific library DNA probe (LA 12NOS1) detected with avidin-FITC and propidium iodide (A, C) or DAPI (B, D) counterstaining. The differentiation of sister chromatids in some of the chromosomes after DAPI counterstaining (B, D) indicates that the cells have performed at least three cell cycles in the presence of BrdU. Arrows indicate chromosomes 12, and arrowhead shows the location of i(12p). (A, B) Diploid metaphase; (C, D) aneuploid metaphase carrying i(12p).

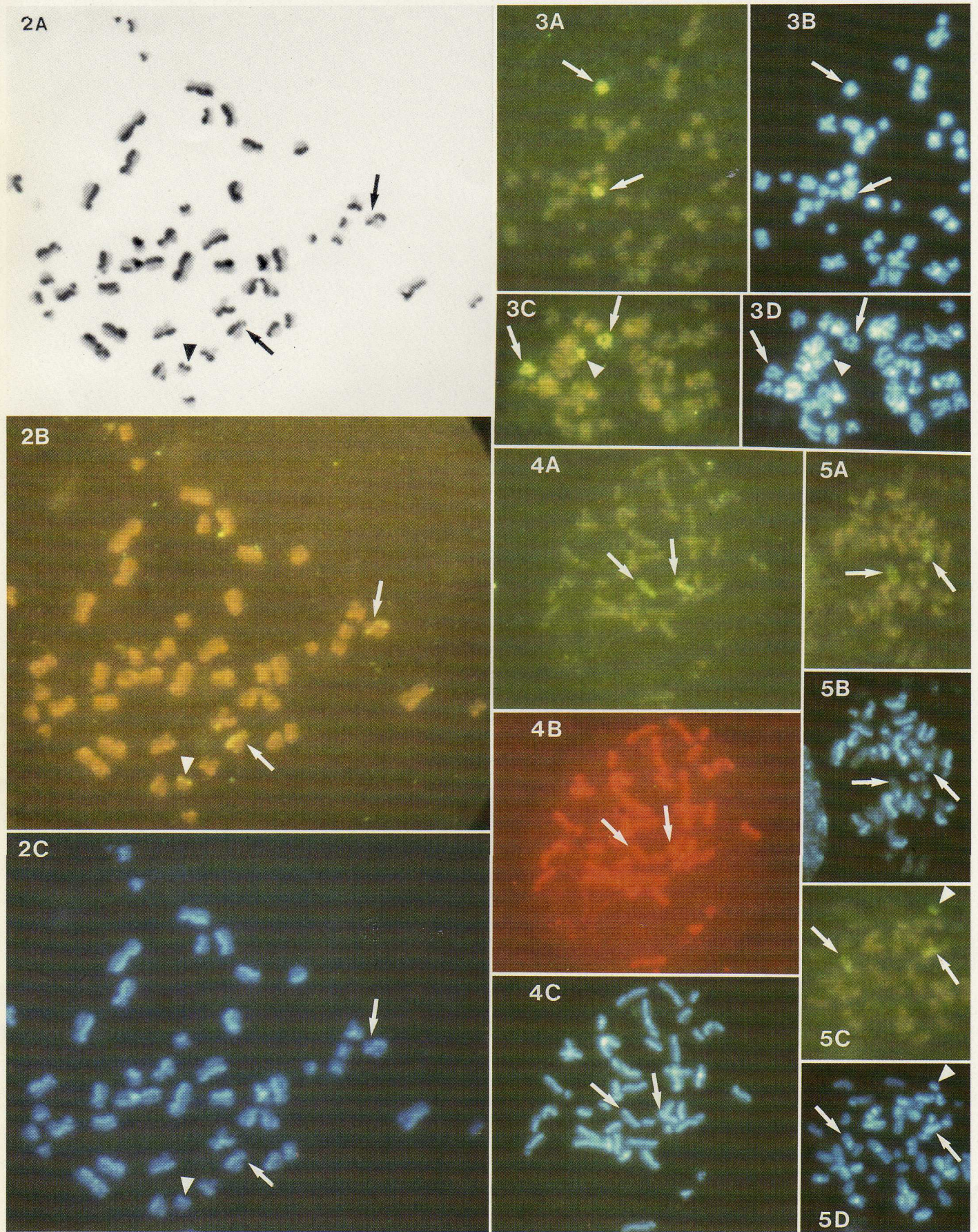


Table I. Different stains and microscope filters used in the combined SCD-CISS methodology and appearance of the chromosomes at metaphase

	Type of staining				Giemsa
	Fluorochrome				
	FITC	Propidium iodide (PI)	FITC + PI	DAPI	
Filter	I 2/3	N2	I 2/3	A	-
Excitation wavelength (nm)	450-490	530-560	450-490	340-380	-
Chromosome color	-	Red	Red	Blue	Violet
Hybridized probe color	Yellow	-	Yellow	-	-
SCD					
BrdU monosubstituted chromatid color	-	Bright red	-	Bright blue	Dark violet
BrdU bisubstituted chromatid color	-	Dull red	-	Dull blue	Pale violet

Results and discussion

The procedure presented above allows the detection of SCD, after chromosome counterstaining with fluorochromes such as DAPI and propidium iodide (Figs. 2C, 3B, 3D, 4B, 5B, and 5D), simultaneously with the identification of individual chromosomes on the same metaphase by chromosome painting, using CISS hybridization with a chromosome 12-specific sorted DNA library probe (Figs. 2B, 3A, 3C, 4A, 5A, and 5C).

Characteristics of the fluorescent microscope filters, excitation wavelengths, banding, and fluorochromes used are summarized in Table I.

SCD and CISS hybridization with the aforementioned fluorochromes was also achieved following the use of Giemsa staining for differentiating sister chromatids, according to Korenberg and Freedlander (1974) and Perry and Wolff (1974). However, chromosome painting by CISS hybridization prior to use of the SCD procedure yielded no differential staining of the sister chromatids.

In second-generation metaphases, in which the sister chromatids of all chromosomes can be clearly differentiated, the lightly staining chromatid after Giemsa staining (Fig. 2A) or the dully fluorescent one after DAPI staining (Figs. 2C and 4C) is always lighter after CISS hybridization than its sister chromatid (Figs. 2B and 4A). In extreme cases ($\leq 5\%$ of the metaphases examined), the hybridization signal induced after immunological detection was restricted to the dark (Giemsa staining) or bright (DAPI staining) chromatid (Fig. 2). Consequently, in third-generation metaphases, the hybridization signals were very weakly painted (Fig. 5). Whether the BrdU-monosubstituted chromatid yields a lighter hybridization signal than the unsubstituted one was not assessed in this study. The molecular mechanism by which BrdU weakens the hybridization signal of the probe also warrants further study. We are not aware of any previous reports of this phenomenon.

Table II shows the results of SCE analysis of the whole complement, chromosomes 12, and the i(12p) chromosome in normal and aneuploid PK fibroblasts. (This analysis took approximately 4 working hours, showing that this methodology does not require more microscopy time than does the scoring of SCEs

Table II. Percentage of metaphases at the first (1st), second (2nd), and third or subsequent (≥ 3 rd) cell division, and SCE frequency in fibroblasts of Pallister-Killian syndrome patients^a

	Type of chromosome complement	
	46,XY	47,XY,+i(12p)
Percentage of cells in metaphase		
1st	14	40
2nd	77	59
≥ 3 rd	9	1
Number of cells analyzed	231	134
SCE ^b		
Whole complement	3.5 \pm 0.2	3.8 \pm 0.2
Chromosomes 12	0.3 \pm 0.1	0.2 \pm 0.1
i(12p)	-	0

^a Harvest was performed 72 h after seeding, including 3 h of Colcemid treatment; BrdU was added 2 h after seeding and was present until harvesting.

^b The results are expressed as the mean \pm standard error of the mean of the number of SCEs/metaphase or chromosome; 50 second-generation mitoses were analyzed.

in conventional Giemsa harlequin-stained preparations.) No differences in baseline SCE frequency were observed between normal and abnormal cells for either the entire complement or chromosome 12 (Table II). No SCEs were observed in the i(12p) chromosome of aneuploid cells (Table II).

Table II also illustrates the potential of the technique for studies of cell-cycle kinetics. In PK fibroblasts, the proportion of first or second metaphases was higher in aneuploid cells than in normal cells. Thus, the proliferation of aneuploid cells was slower than that of normal fibroblasts, under the prevailing culture conditions.

The present study is aimed at demonstrating the versatility of this combined SCD-CISS methodology in measuring the kinetics of cell division and the incidence of SCEs in two different types of cells growing simultaneously in a mosaic tissue. Accordingly, the results should not be construed as an analysis of SCEs and cell proliferation in PK syndrome per se.

Leukemias, lymphomas, and, indeed, most neoplasms are known to contain both normal and abnormal cells. At present,

chromosome abnormalities are regarded as the best tumor-specific and diagnostic markers (Mitelman, 1985, and references therein). Specific probes are available for many of these consistent chromosome abnormalities, allowing malignant cells to be accurately discriminated from normal cells in cancerous tissue. Researchers in the biology of cancer urgently need information about the effect of various lymphokines, growth factors, and cytostatics on the proliferation of different normal and abnor-

mal cells in neoplasms. The combined methodology reported here appears to be a powerful direct technique for this type of study.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Mrs. Paula Kvik.

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