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Detection of residual host cells after bone marrow transplantation using non-isotopic *in situ* hybridization and karyotype analysis

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Summary:

Karyotype analysis and interphase cytogenetics by means of non-radioactive *in situ* hybridization (NISH) with Y and X-specific probes were used to detect residual host cells in BM of 18 male patients who had received a BMT from their sisters. All patients but one had a malignant blood disease; 17 patients were clinically in continuous remission at the time of the investigations and throughout the follow-up period. No host cells were detected by karyotype analysis in patients who were in clinical remission. NISH with a biotinylated Y-specific probe showed residual host cells in 16 of the 17 patients in clinical remission. In samples from three patients the existence of host cells was further confirmed by revealing the XY-containing cells with two-color fluorescence *in situ* hybridization (FISH) using X- and Y-specific probes simultaneously. Hybridizations with a Y-specific probe on direct BM smears showed >3% residual host cells in 50% of the samples studied at or later than 2 months post-BMT. On conventional cytogenetic preparations <1% Y-specific cells were detected in all but two samples at ≥ 2 months post-BMT. There was no difference in the proportion of host cells between patients conditioned with total body irradiation and CY and those who received busulphan and CY. The recipient's stromal or epithelial cells in the aspirates probably account for most of the host cells detected. In conclusion, small numbers of residual host cells detected by interphase cytogenetics with a Y-specific probe do not indicate an imminent relapse. Conventional cytogenetic preparations are preferable to direct smears in studying the presence of residual host hematopoietic cells because of probably less contamination by stromal cells.

Allogeneic BMT following ablative chemo/radiotherapy for the treatment of malignant blood disorders is expected to result in a full chimerism after hematologic recovery. In recipients of sex-mismatch BM, karyotype analysis is often used to document the engraftment and to analyze the chimeric state of the

recipient after BMT.^{1,2} By karyotype analyses, however, only proliferating cells that reach mitosis can be studied. Furthermore, only a small number of cells is usually analyzed. Thus, if residual host cells are present in low numbers or if these cells do not proliferate, they cannot be detected by routine cytogenetic methods. By including interphase cells in the analyses more accurate information about the presence and number of residual cells and their correlation, e.g. with conditioning regimen can be obtained. We³ and others^{4,5} have recently demonstrated that interphase cytogenetics with a Y-specific probe is a sensitive method for documenting the presence of residual host cells in the BM after BMT. In this study we performed karyotype analysis and interphase cytogenetics with Y- and X-specific probes on 42 BM samples from 18 male patients treated with total body irradiation (TBI) and CY or busulphan (BU) and CY and a transplant from their sisters to study possible mixed chimerism.

Patients and methods

Patients

Eighteen male patients who received an allogeneic transplant from their HLA-identical MLC-negative sisters were studied (Table I). All patients but one had a malignant blood disease. Some of the results of patients 5–7 have been published earlier.²

The conditioning regimen of patients 1–4, 6–8, 10, 11, 13 and 18 consisted of CY 60 mg/kg on two consecutive days and TBI 2.0 Gy \times 6 (total 12 Gy) during five days. Patients 5, 12 and 14–17 received BU 4 mg/kg/day on 4 days and CY as above. Patient 9 (with aplastic anaemia) was conditioned with CY 50 mg/kg on four consecutive days.

A total of $2.3\text{--}3.9 \times 10^8$ nucleated BM cells/kg recipient body weight were transfused at the transplantation. No T cell depletion was done in any case. GVHD prophylaxis consisted of CYA combined with four doses of MTX.⁶ CYA was given as continuous iv infusion at a dose of 3 mg/kg/day for 2 weeks, and thereafter *per os* for 1 year 1–4 mg/kg/day depending on side-effects and blood concentrations. The engraftment was uncomplicated in all patients. Seven patients developed acute GVHD (five grade II, one grade III and one grade IV; Table I). Four of these seven

Table I Patient and control characteristics and outcome of the BMT

Patients	Diagnosis	Karyotype in BM at diagnosis	Status at BMT	Age/sex	Acute GVHD grade	Chronic GVHD	Outcome (months)	Cause of death
1	AML	46XY,9q-/46,XY	CRI	36/M	II	+	Alive, CR(75+)	-
2	ALL	51,XY,+X,+4,+4,+14,+15/46XY	CRI	24/M	-	-	Alive, CR(58+)	-
3	CML	46XY,t(9;22)(q34;q11)	CP	32/M	-	+	Alive, CR(44+)	-
4	MM	46,XY	-	38/M	-	+	Died, CR(41)	Obstructive pulmonary disease
5	AML	46,XY,t(5;11)(q35;p11)	CRI	21/M	II	+	Alive, CR(43+)	-
6	AML	46,XY,t(8;21)(q22;q22)	CRI	16/M	-	-	Alive, CR(43+)	-
7	ALL	NS	CRII	25/M	-	+	Alive, CR(42+)	-
8	CML	46,XY,t(9;22)(q34;q11)	CP	25/M	II	-	Alive, CR(38+)	-
9	AA	46,XY	-	24/M	III	+	Died, CR(7)	Cardiac death?
10	AML	46,XY	CRI	19/M	-	-	Alive, CR(30+)	-
11	ALL	46,XY	CRI	25/M	II	+	Alive, CR(29+)	-
12	AML	46,XY,8p-	Prim ref	29/M	IV	-	Died, CR(7)	Acute GVHD
13	MM	46,XY	-	45/M	-	-	Alive, CR(25+)	-
14	AML	46,XY,t(15;17)(q22;q21)/46,XY	CRI	34/M	-	+	Alive, CR(22+)	-
15	MDS-AML	45,X,-Y/42,X,-Y,4p-,5q-,6p-, -7,11p+,-16,17p+,10q+,-19, 20q-,+mar,+mar/44,+r,inc/46,XY	Prim ref	45/M	-	-	Died (4)	Relapse
16	AML	46,XY	CRI	48/M	-	-	Alive, CR(10+)	-
17	ALL	46,XY	CRI	45/M	-	-	Alive, CR(9+)	-
18	CML	46,XY,t(9;22)(q34;q11)	CP	42/M	II	-	Alive, CR(7+)	-
<i>Controls</i>								
19-28	Healthy	46,XY		33-68/M				
29-39	Healthy	46,XX		20-78/F				
40	ALL	48-50,XX,+4,+8,+F,+G/46,XX		29/F				
41	Myeloma	46,XX		40/F				
42	CML	46,XX,t(9;22)(q34;q11)		27/F				

AA = aplastic anaemia; CP = chronic phase; F = female; inc = incomplete karyotype; M = male; MDS-AML = AML transformed from myelodysplastic syndrome; mar = marker chromosome; MM = multiple myeloma; NS = not studied; prim ref = primarily refractory; r = ring chromosome

patients progressed to chronic GVHD. Four other patients developed chronic GVHD without any clinical signs of preceding acute GVHD. Four patients died during the study (Table I).

Karyotype analysis

Karyotype analyses were performed either on unstimulated BM cells from direct cultures or cells cultured for 24-48 h as described earlier.³ The chromosome preparations were made using standard techniques and examined after trypsin-Giemsa banding.⁷ Approximately 20 metaphases were analysed per sample.

Interphase cytogenetic analysis

Interphase cytogenetic studies with Y- and X-specific probes were performed on direct BM smears or conventional cytogenetic slides prepared as described above.

DNA probes. For single-target NISH and double-target two-colour FISH, human Y- and X-specific DNA probes were used. The probes recognizing the heterochromatic region of the long arm of the Y chromosome were pY431 (a gift from K. Smith, School of Medicine, The Johns Hopkins University, Baltimore, MD, USA), and pHY2.1.⁸ The X chromosome was identified by using the pXBR probe⁹ which

identifies the centromeric region of the X chromosome. The probes were labelled by standard nick-translation with biotin-11-dUTP (Sigma) or with digoxigenin-11-dUTP (Boehringer Mannheim, Germany).

NISH. BM smears or conventional cytogenetic BM preparations from the 18 patients were investigated after the BMT by NISH with the biotinylated Y-specific probe, pY431. Six patients were also analyzed before the BMT.

Before hybridization the previously stained smears were refixed for 30 min in acetic acid-methanol (1/3). If hybridization were detected by employing peroxidase enzyme, all BM preparations were incubated for 30 min in methanol containing 3% (v/v) hydrogen peroxide before hybridization.¹⁰ The smears were further incubated in ethanol and xylene.¹⁰ The conventional preparations were treated with pepsin¹¹ (0.01-0.1 mg/ml in 0.01 N HCl; Sigma, St. Louis, MO, USA) to remove the cytoplasm. A post-fixation step with 1% formaldehyde in PBS containing 50 mM MgCl₂ was included in some of the experiments to improve the morphology of the cells.¹² The NISH was carried out as described earlier.¹⁰ The signals were detected by indirect immunoperoxidase detection system using diaminobenzidine (DAB; Sigma) as substrate.¹⁴ Cells were evaluated with a phase-contrast microscope.

Two-color FISH. BM samples from three patients (16-18) were also studied by two-colour FISH using X-

and Y-specific DNA probes simultaneously. Hybridization conditions were as described earlier.¹⁵ The biotinylated X-specific and digoxigenin-labelled Y-specific probes were detected simultaneously by the indirect immunofluorescence method. Three subsequent steps were performed: the first layer contained monoclonal mouse anti-digoxin antibody (1:500; Sigma) and TRITC-conjugated avidin (1:1000; Vector) in $4 \times \text{SSC}$, 0.05% Tween-20; the second layer, FITC-conjugated sheep anti-mouse antibody (1:200; Sigma) and goat anti-avidin-biotin (1:200; Vector Laboratories, Burlingame, CA, USA), and the third layer, FITC-conjugated donkey anti-sheep antibody (1:200; Sigma) and TRITC-conjugated avidin (1:1000; Vector). Cells were evaluated with a Zeiss Axiophot equipped with epifluorescence.

Controls. Direct BM smears and conventional BM preparations from 10 healthy males, eight healthy females and three female patients (40–42) who had received a marrow graft from a female donor were pre-treated and hybridized simultaneously with the patient's slides. Also conventional chromosome preparations prepared from blood samples from the healthy donors were included in interphase cytogenetic studies as controls.

Evaluation of the signals. Hybridization studies were carried out in a blind fashion. Only non-overlapping interphase cells with good morphology were analysed.

Results

Karyotype analysis

A total of 42 patient samples from the 18 patients post-BMT were analyzed by karyotype and interphase cytogenetic analyses. Karyotype analyses showed no residual male host cells at any time point studied in patients who remained in clinical remission (17 of 18) (Table II). Only female cells with a karyotype of 46,XX were seen. One patient relapsed during the investigation period (patient 15). In this patient, at the onset of

relapse, karyotype analysis showed host cells with one of the same clonal abnormalities (45, X, -Y; 2 out of 20 metaphases studied) which had been detected at the time of diagnosis (Table I).

Interphase cytogenetic analysis

Controls. Hybridizations of the BM smears from five healthy male donors showed that, on the average, 96% (range 94–98%) of the interphase cells showed a Y-specific signal (Figure 1A). Approximately 1000 cells per sample were scored. In BM smears from three healthy females 0.1% of the cells in one smear showed a Y-specific signal but no Y-specific cells were found in the other two. In BM smears from the three female BMT recipients there were no Y chromosome-positive cells. Approximately 800 cells were analyzed per sample. Analyses of conventional cytogenetic preparations from five healthy male donors showed that, on the average, 97% (range 95–99%) of the approximately 1000 cells per sample analyzed were Y-specific. Analyses of conventional cytogenetic preparations from five female donors showed one Y chromosome-positive cell in one sample. Approximately 800 cells were analyzed per sample. Two-colour FISH with the biotinylated X-specific probe and digoxigenin-labelled Y-specific probe on two male BM and blood samples studied revealed that 84–92% of the cells (approximately 200 cells analyzed per sample) showed an X- and Y-specific signal simultaneously (Figure 1B). XY-specific cells were not detected in the two control female blood samples analyzed.

BM samples from two female donors showed that approximately 97% (range 96–97%) of the interphase cells showed two signals after FISH with the X-specific probe pXBR325.200 interphase cells per sample were scored.

Patients. In six patients interphase cytogenetic analysis of BM aspirate with the biotinylated Y-specific probe pY431 was performed before BMT. Hybridizations on BM smears from patients 12, 13, 16–18 with a normal sex chromosome constitution (Table I), showed a clear hybridization signal in approximately 95%

Table II Proportion of residual male host cells in BM samples of the patients in continuous remission post-BMT^a

Months post-BMT	Mean proportion of Y chromosome-specific cells (range)					
	Interphase cytogenetics				Karyotype analysis	
	Smears	No. of patients studied	Conventional cytogenetic preparations	No. of patients studied	Host cells	No. of patients studied
1	5.3 (1.2–13.6)	4	3.2 (1.4–6.3)	3	0	7
2	4.1 (0.7–5.4)	5	1.1 (0–2.4)	5	0	9
4	1.4 (0–3.3)	4	0.6 (0.1–2.0)	7	0	8
8	6.2 (5.5–7.2)	3	0.4 (0.3–0.5)	2	0	3
12	5.4 (3.1–9.4)	3	0.5 (0–0.9)	3	0	5
24	1.4 (0.5–3.3)	5	0.3 (0–0.6)	2	0	6
36	2.3 (0.8–3.8)	2	0	1	0	2
48	0	1	–	–	0	1
60	1.3	1	0	1	0	1

^aData do not include patient 15 who relapsed at 2 months post-BMT

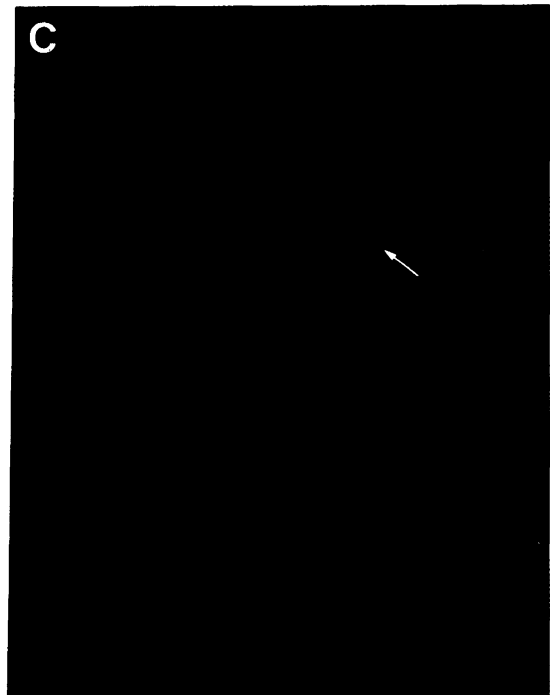
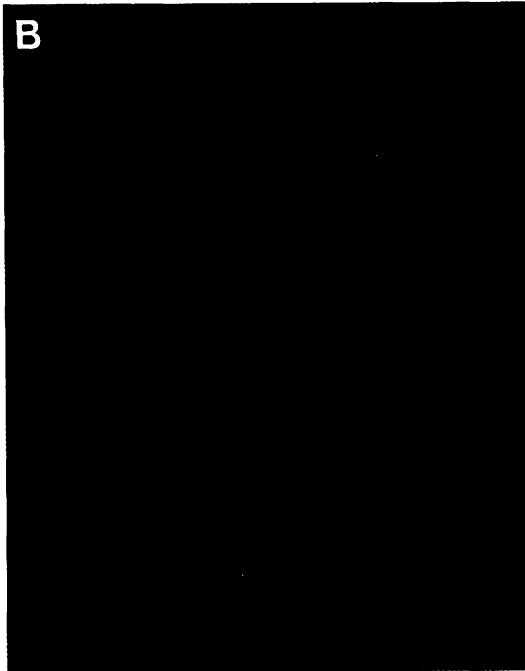
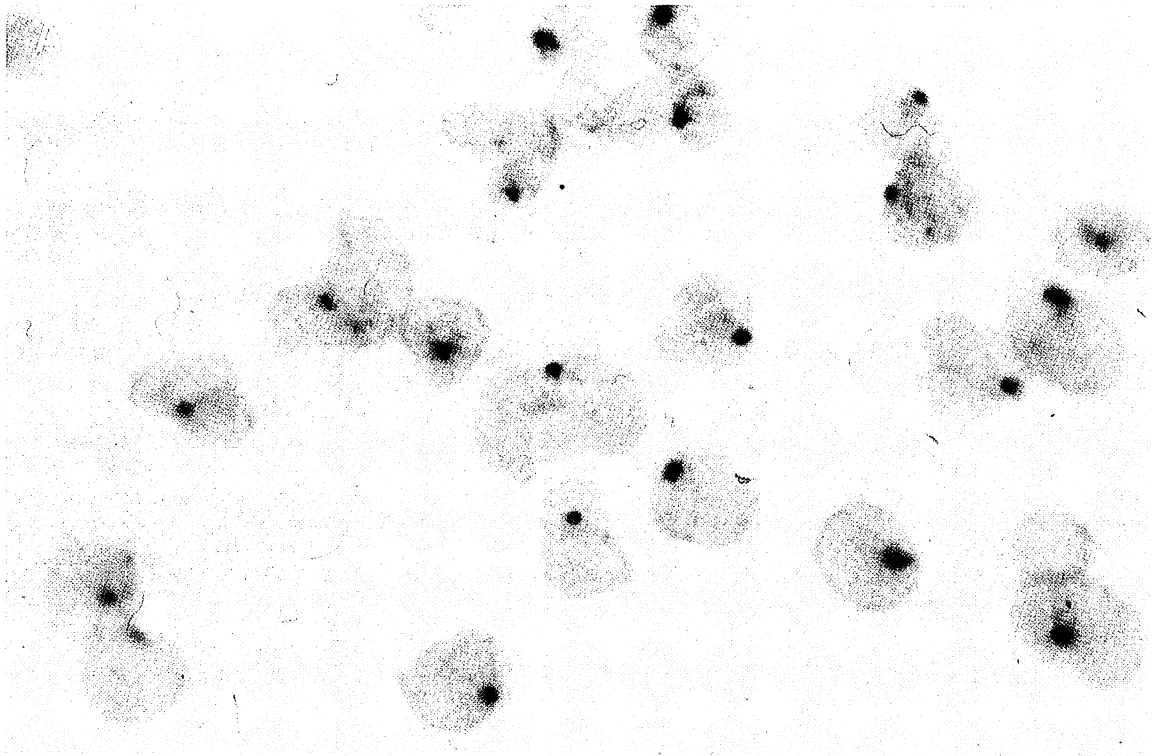


Figure 1 Single-target NISH (A) and double-target two-color FISH (B and C) on interphase nuclei from BM aspirates. (A) Biotinylated Y chromosome-specific DNA probe pY431 hybridized to BM cells of a control male. Hybridization was detected by indirect immunocytochemical procedure using peroxidase enzyme/DAB. Brown precipitate reveals the Y chromosome in the nuclei. The cells were counterstained lightly with Hematoxylin. (B and C) Double-target FISH with the biotinylated X chromosome centromere-specific probe pXBR (avidin-TRITC; red signal) and with digoxigenin-labelled Y chromosome-specific probe pHY2.1 (FITC-conjugated antibodies; green signal) to BM cells of a male control (B) and of a male patient (C; post-BMT). Arrow points to a residual male cell among donor female cells. Microphotographs (B and C) were taken by single exposure through a double band-pass filter from Omega (Brattleboro, VT, USA).

(range 88–98%) of the interphase cells studied. Hybridizations on cultured BM preparations from patients 9, 13 and 17 showed a clear signal in approximately 94% (range 93–96%) of the cells analyzed. Hybridizations on BM smear from patient 15 showed a Y-specific signal in 69% of the cells studied, while 49% of the cells on conventional slides were Y-positive. Karyotype analysis of this patient had revealed two clones with a missing Y chromosome (Table I).

Interphase cytogenetic analysis of the samples from BMT patients who were in clinical remission during the investigation period (17 of 18) revealed the presence of host cells in 16 patients (94%). In eight patients host cells were detected at or later than 2 years, up to 5 years post-BMT. The proportion of residual male host cells analyzed from BM chromosome preparations decreased rapidly, reaching the average of 0.7% at 2 months post-BMT (Table II). Thereafter, the proportion of host cells found was <1% in all but one patient (in whom the detection was done with double-target two-color NISH). On the smears the proportion of residual host cells was higher and more variable in comparison with the results recorded from conventional BM chromosome preparations (Table II). Of 12 cases where BM smear and conventional chromosome preparation from the same aspirate were studied, in 10 the proportion of host cells was higher in the smear. At least 400 cells per patient sample were analyzed in these studies.

There are 13 of the 16 patients, who were in remission at 2 months post-BMT and had host cells detected at this time point or later, alive with no evidence of disease at 7–78 months after the transplantation. There were no relapses among these patients.

One of the patients (patient 15) relapsed during the study period. At the onset of relapse (at 2 months post-BMT) this patient had a Y-specific signal in 28% (conventional BM chromosome preparation) to 39% (BM smear) of the cells analyzed.

In samples from patients 16–18 the presence of host cells was further confirmed by revealing the XY-containing cells with double-target two-color FISH with a biotinylated X- and a digoxigenin-labelled Y-specific probe simultaneously. Hybridizations revealed that at 1 month post-BMT 1.4%, at 4 months post-BMT 2% and at 8 months post-BMT 5.5% of the cells in samples from patients 18, 16 and 17, respectively, were residual host cells containing both X and Y chromosomes. Hybridizations on samples from patients 16 and 18 were performed on conventional chromosome slides and in the case of patient 17 on direct BM smear.

There was no difference in the proportion of host cells between patients conditioned with TBI and CY and those who received BU and CY.

Discussion

In this study we found that 16 of 17 male recipients of a BMT from a female sibling donor who were in

continuous remission had residual host cells in the BM up to several years post-BMT when studied by interphase cytogenetics using a Y-specific DNA probe. This finding was confirmed in three patients by demonstrating XY-containing cells with double-target two-color FISH using X- and Y-specific probes simultaneously. These findings are in accordance with the findings of other studies using NISH with a Y chromosome-specific probe.^{4,5} Karyotype analysis showed no host cells.

The use of interphase nuclei allows rapid examination of a large number of cells from different cell populations. This is an obvious advantage in comparison with karyotype analysis as cells that are not proliferating can be analyzed. By using simultaneously X- and Y-specific probes in follow-up studies of sex-mismatched BMT cases, the host origin of the cells studied is more reliably revealed than by using Y-specific probes alone. However, by using these probes we were not able to identify the cancer specificity of the host cells found.

The finding of persisting residual host cells was without doubt real and not due to technical problems, as the sensitivity of our interphase cytogenetic analysis with the Y-specific probe was quite high (approximately 97%), and false positive results were rare ($\leq 0.02\%$). The fact that no host cells were found in karyotype analysis might be due to the low number of metaphases studied (usually 20). A more probable reason is that the host cells were not dividing or their mitotic index was very low.

Hybridizations on patient samples revealed differences between conventional cytogenetic preparations and direct BM smears. On conventional cytogenetic preparations the proportion of host cells later than 2 months post-BMT was <1% in all but two samples. These findings agree with the interphase cytogenetic results of Durnam *et al.*⁴ and Przepiorcka *et al.*⁵ who studied 87 and 10 BMT patients, respectively. Three out of 87 patients studied by Durnam *et al.*⁴ and all 10 patients studied by Przepiorcka *et al.*⁵ were treated by T-cell depleted BMT. On BM smears the proportion of host cells was >3% in about 50% of the smears studied at or after 2 months post-BMT. Even at ≥ 2 years after the transplantation four of nine patients studied had >1% host cells in the smear preparations. As to the nature of the residual host cells, it is probable that many or all of them are stromal cells. It has been demonstrated that host stromal cells persist in the marrow irrespective of the time post-transplant.^{16,17} It is likely that the proportion of stromal cells is higher in direct BM smears than in the conventional cytogenetic preparations. This probably accounts for the differences between the results obtained with these two kinds of samples. Studies combining immunophenotyping with double-target NISH would reveal the nature of the residual recipient cells.

In our study there were no differences in the proportion of host cells in the marrow between patients conditioned with TBI and CY and those who received BU and CY. In three previous studies employing

restriction fragment length polymorphism (RFLP), 15–35% of the recipients of non-T-cell-depleted marrow have shown mixed chimerism, and the results have been similar in patients conditioned with TBI and those who have received BU.^{18–20} Thus our results agree with these previous results although the sensitivity of our NISH method is far greater.

In conclusion, residual host cells are found in the BM of BMT recipients in continuous clinical remission even years after transplantation. Small numbers of residual host cells do not indicate imminent relapse. Most or all of the host cells may be of stromal origin. For studying the presence of a possible residual host hematopoietic cell population with a Y-specific probe, conventional cytogenetic preparations are preferable to direct BM smears because of probably less contamination by stromal cells. However, the presence of stromal cells always invalidates to some extent the interpretation of the results when using Y-specific probes. Therefore, probes which identify cancer-specific abnormalities are preferable for studying residual hematopoietic host cells.

Acknowledgements

This work was supported by grants from the Finnish Cancer Society, the Emil Aaltonen Foundation, the Sigrid Jusélius Foundation, the University of Helsinki, and the Deutsche Krebshilfe (Mildred Scheel Stiftung). Kristiina Heinonen, MSc, is acknowledged for providing karyotypic data of one of the patients. Drs Lore Zech and Seppo Pakkala are acknowledged for their comments on the manuscript.

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