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Detection of Residual Host Cells in Sex-mismatched Bone Marrow Transplantation in Various Hematological Diseases by Fluorescence *in situ* Hybridization

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Thirty-eight sex-mismatched bone marrow transplantation patients with various hematological diseases were followed-up using fluorescence *in situ* hybridization. Probes specific for various translocations, the X chromosome (DXZ1) and the whole Y chromosome (WCP Y), were used to assess successful engraftment and residual host cells. The combination of translocation and WCP Y probes enabled the identification of host and donor cells in addition to the identification of malignant vs. normal cells in the transplant recipient. Fifteen patients were sequentially followed up. The results obtained using the combination of translocation plus WCP Y probes were more reliable than those with DXZ1 plus WCP Y probes, or the translocation probe alone, especially when the percentage of residual leukemic cells detected by the translocation probe alone was around the cut-off level.

Key words: Residual host cells — Sex-mismatched BMT — FISH

Allogeneic BMT has become a popular means of treating hematological malignancies.[1] To confirm successful engraftment and minimum residual leukemic cells, the transplant must be monitored by means that allow early assessment of mixed chimeras after BMT. Several methods are currently employed to determine precisely the percentage of residual leukemic cells. Procedures that detect the blood cells of either recipient or donor origin are erythrocyte blood group (sub) typing, isoenzyme pattern, immunoglobulin allotyping, karyotyping, restriction fragment length polymorphism analysis, human lymphocyte antigen typing, polymerase chain reaction and FISH.[2–14] Conventional cytogenetics will detect engraftment and can analyze the chimeric state of the patient after sex-mismatched BMT.[11,12] However, this method is only effective for studying mitotic cells. Consequently, only a few cells are usually analyzed. Thus, if the residual host cells are present in low numbers or if these cells do not proliferate, they cannot be detected by routine cytogenetic methods. There are reports of detecting residual leukemic cells using a Y probe in sex-mismatched BMT.[10,13,14] By including interphase cells in the analyses, more qualitative information can be obtained in relation to the conditioning regimen of BMT.[15]

We compared the sensitivity of various combination probes using FISH to detect residual host cells and to identify the host cells as malignant or normal.

MATERIALS AND METHODS

Patients A total of 38 patients participated in this study. Group I (Table I) consisted of 19 leukemia patients with specific translocations [15 CML with t(9;22), one AML M2 with t(8;21), one AML M3 with t(15;17), one AML M5 with t(11q23) and one ALL with t(9;22)]. Group II (Table II) consisted of 19 patients without specific translocation but with either a distinct chromosomal aberration or a normal karyotype (four ALL, two AML M1, three AML M2, one AML M5b, two AML M7, one hypoplastic AML, three MDS, one ML and two aplastic anemia). All patients had sex-mismatched BMT without T-cell depletion. Bone marrow cells from three healthy people and lymphocytes from four healthy people were used as negative controls.

Chromosome analysis The karyotypes of unstimulated BM short-term cultures were analyzed as described.[14] Chromosomes were prepared by means of standard culture procedures and trypsin-Giemsa banding. Karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN 1991).[16]

Fluorescence *in situ* hybridization

Sample preparation: After repeated washing of the cells with Carnoy's fixative, air-dried smears were prepared

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Abbreviations: BMT, bone marrow transplantation; FISH, fluorescence *in situ* hybridization; WCP, whole chromosome painting; CML, chronic myelocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome, ML, malignant lymphoma; M- or m-BCR/ABL, major or minor breakpoint cluster region/Abelson gene; PML/RARA, promyelocytic leukemia/retinoic acid receptor alpha gene; RLC, residual leukemic cell; RHC, residual host cell; PBS, phosphate-buffered saline; SSC, standard saline citrate; RT-PCR, reverse transcriptase-polymerase chain reaction; CR, complete remission.

from each sample by dropping 10 μl aliquots of the cell suspension onto clean glass slides. After appropriate aging, the slides were treated with RNase (100 μg/ml in 2×SSC; 1 h; 37°C), washed twice with 2×SSC (5 min each), digested with pepsin (0.025% in 0.01 *N* HCl; 10 min; 37°C), washed with 1/20 *M* $MgCl_2$ in PBS (5 min; room temperature), fixed with 35% formamide and dehydrated in a graded ethanol series (70%, 80%, 100%; 3 min each). The DNA in the interphase/metaphase cells was denatured by immersing the slides in 70% formamide in 2×SSC (pH 7.0; 75°C), followed immediately by dehydration in an ice-cold ethanol series. Diluted and denatured probe (5.2 μl) was applied to a 9×9 millimeter square area on the slide and incubated at 37°C for 48–72 h in a moist chamber.

FISH probes: M-BCR/ABL, m-BCR/ABL and PML/

Table I. Clinical Findings and Details of FISH Analysis of 19 Patients with Leukemia-specific Translocation after Sex-mismatched BMT

Patient No.	Sex	Diagnosis	Months after BMT	% of residual leukemic cells with		% of RHC with DXZ1+WCP Y probes	Follow-up status
				translocation probe alone	translocation probe+WCP Y		
1	F	CML	2	8.5	0.3	0.8	CR
			15	1.9	0	1.4	CR
2	F	CML	12	7.4	0	0.3	CR
			24	5.5	0	0	CR
			57	3.6	0	0	CR
3	M	CML	4	1.6	0.2	1.2	CR
			5	6.2	0.2	0.6	CR
			9	6.4	0	0.3	CR
4	M	CML	1	7.1	0.3	1.8	CR
			12	5.9	0.3	0.6	CR
			15	4.1	0.2	0.3	CR
5	M	CML	2	5.3	0.2	0.6	CR
			50	6.2	0.5	0.7	CR
6	F	CML	1	7.2	2.9	3.2	R
			3	12.9	6.6	8.0	R
			5	19.9	12.1	10.4	R
7	F	CML	1	7.2	0.5	3.9	PR
			3	13.9	2.5	5.2	R
			4	29.0	19.1	19.2	R
8	F	CML	4	57.2	46.8	45.0	R
			5	52.4	40.5	41.0	R
9	M	CML	1	7.3	1.8	4.1	R
			4	6.7	3.6	5.4	R
			6	8.5	4.3	5.7	R
10	M	M2	2	8.2	0.3	2.5	CR
			8	7.8	0.2	0.7	CR
			12	1.4	0.2	0.9	CR
11	M	M3	1	7.5	0.2	0.8	CR
			10	4.5	0.2	0.6	CR
12	M	M5	1	1.4	0.1	0.2	CR
			3	1.6	0.5	1.3	CR
			9	8.4	5.1	2.2	R
13	M	ALL	1	19.9	14.4	17.3	R
			3	18.5	19.6	18.0	R
14	F	CML	1	5.8	0.1	0.6	CR
15	F	CML	1	15.5	0.1	1.6	CR
16	F	CML	7	8.1	0.2	1.0	CR
17	F	CML	26	5.2	0.1	1.0	CR
18	F	CML	3	7.4	0.8	3.9	CR
19	F	CML	38	5.4	4.6	5.1	R

CML, chronic myelocytic leukemia; ALL, acute lymphoblastic leukemia; M2, acute myeloid leukemia M2 subtype; M3, acute myeloid leukemia M3 subtype; M5, acute myeloid leukemia M5 subtype; RHC, residual host cells; CR, complete remission; PR, partial remission; R, relapse.

RARA probes (Oncor, Gaithersburg, MD) were used to detect BCR/ABL and PML/RARA chimeric genes, respectively. Other probes were AML-1 (provided by Dr. M. Ohki, Japan National Cancer Institute), CD3 YAC (provided by Dr. M. Seto, Aichi Cancer Center, Aichi), biotin-labeled chromosome X centromere (DXZ1; Oncor) and spectrum orange WCP Y, which paints the whole Y chromosome (GIBCO BRL, Gaithersburg, MD). The *AML-1* gene cosmid and CD3 YAC probes were labeled with biotin-14-dATP using a nick translation kit (GIBCO BRL). The *AML-1* gene cosmid probe located on chromosome 21q22 consists of cY3 and cY8, which span the breakpoint region in intron 5 of the *AML-1* gene. The CD3 YAC probe containing the *MLL* gene from 11q23 was used to detect 11q23 translocations by interphase FISH.

We used three sets of probes. The first was a specific translocation probe alone, the second was a combination of the probes for DXZ1 and WCP Y and the third was the specific translocation probe plus the Y chromosome probe (WCP Y). The DXZ1 and WCP Y probes were diluted and denatured according to the manufacturer's recommendations. The AML-1 and WCP Y probes were mixed with human Cot I DNA (Oncor) prior to denaturation. Thereafter, the probes were annealed for at least 1 h. The probes were mixed just before hybridization. Patients in group II were studied using only the combination of DXZ1 and WCP Y probes.

FISH detection: Post hybridization washes were performed with 50% formamide (43°C; 10 min) and with 2 ×SSC (37°C; 10 min). The probes were detected with avidin fluorescein (1 : 200, Vector Laboratories, Burlingame, CA) and anti-digoxigenin rhodamine (1 : 200, Boehringer Mannheim, Mannheim) then counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (5 ng/ml, Sigma, St. Louis). At least 1000 nuclei were scored under a fluorescence microscope fitted with appropriate absorption and excitation filters (Olympus Optical Co., Tokyo).

Only those cells in which the signals were of regular size and intensity were included in the study. Overlapping nuclei were not scored. Nuclei with overlapping green and red signals were classified as 9;22 or 15;17 translocations. When three signals were observed using

Table II. Clinical Findings and Details of FISH Analysis after Sex-mismatched BMT in 19 Patients with Hematological Disorders without Specific Chromosomal Translocations

Patient No.	Sex	Diagnosis	Months after BMT	% of RHC cells with DXZ1+WCP Y	Follow-up status
20	M	ALL	3	1.4	CR
			5	0.2	CR
21	F	AA	1	0	CR
			5	0.6	CR
			8	0.1	CR
22	F	ALL	1	2.1	CR
23	M	ALL	2	1.7	CR
24	F	ALL	30	1.6	CR
25	M	M2	1	1.7	CR
26	F	AML (hypo)	3	0	CR
27	F	M2	1	0	CR
28	M	M2	1	0.3	CR
29	F	M1	1	0.2	CR
30	M	AA	11	0.2	CR
31	M	ML	2	34.8	R
32	F	M5b	1	0.3	CR
33	M	M1	3	3	CR
34	M	MDS	15	0.2	CR
35	F	MDS	72	1.2	CR
36	F	M7	1	1.9	CR
37	M	M7	39	1.5	CR
38	F	MDS	2	7.3	R

ALL, acute lymphoblastic leukemia; M1, acute myeloid leukemia M1 subtype; M2, acute myeloid leukemia M2 subtype; AA, aplastic anemia; M5b, acute myeloid leukemia M5b subtype; M7, acute myeloid leukemia M7 subtype; MDS, myelodysplastic syndrome; ML, malignant lymphoma; CR, complete remission; R, relapse; RHC, residual host cell.

the AML-1 or CD3 YAC probe, this was considered a positive detection of 8;21 or 11q23 translocation, respectively. Using the WCP Y probe in combination with specific translocation or DXZ1 probes allowed us to determine whether the cells were of host or donor origin.

RESULTS

Chimeras are considered to be cytogenetically complete when the genetic markers detect only the donor type of hematopoietic cells after BMT.[15] All but two patients (nos. 8 and 31) had complete chimerism according to conventional cytogenetics (data not shown).

The cut-off levels for detection of specific translocations were M-BCR/ABL, 5.2%; m-BCR/ABL, 5.1%; PML/RARA, 4.9%; AML-1, 6.5% and CD3 YAC, 7.1% (mean+2SD). The sensitivity of the probe combination (translocation probe+WCP Y) was 0.1%. Fifteen patients could be analyzed sequentially by FISH (Table I and Table II).

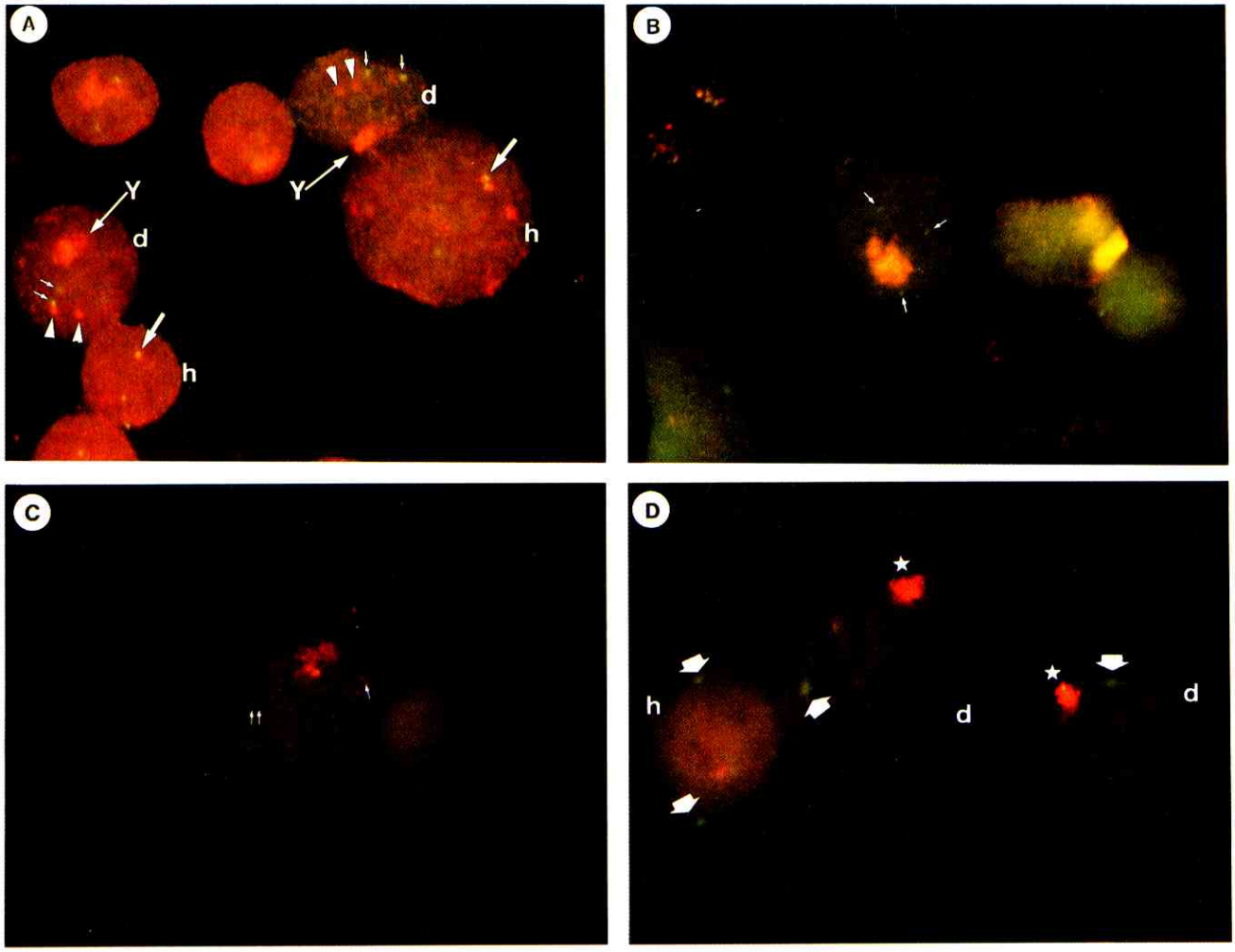

Fig. 1. A, Representative FISH analysis using a combination of M-BCR/ABL and WCP Y probes in a female patient showing donor (d) and residual leukemic cells (h). Chimeric M-BCR/ABL signals (yellow) are indicated by large arrows, BCR signals (red) by arrowheads and ABL signals (green) by small arrows. B, A representative residual leukemic cell from patient 10 (AML M2) showing translocation of the *AML-1* gene (three green signals; arrows) and a Y signal (red). C, A representative residual leukemic cell from patient 12 (AML M5) showing translocation of the *MLL* gene (three green signals of CD3 YAC probe; arrows) and a Y signal (red). D, Representative interphase nuclei hybridized with the DXZ1 and WCP Y probes showing mixed chimerism of both female (host; h) and male cells (donor; d). X and Y signals are indicated by arrows and asterisks, respectively.

Patients in group I (Table I) were analyzed using three detection systems: specific translocation alone, DXZ1 plus WCP Y and specific translocation plus WCP Y probes. Fifteen CML patients had residual leukemic cells in the range of 1.6–57.2% when analyzed by M-BCR/ABL probe and 0–46.8% when analyzed by M-BCR/ABL plus WCP Y. An M2 patient, analyzed two months after BMT (patient 10 in Table I), had 8.2% of residual leukemic cells detected by the *AML-1* gene probe and 0.3% by AML-1 plus WCP Y probes. An M3 patient, analyzed one month after BMT (patient 11), had 7.5% of RLC detected by the PML/RARA probe alone and 0.2% by PML/RARA plus WCP Y probes. An M5 patient, analyzed 1 month after BMT (patient 12), had 1.4% residual leukemic cells detected by CD3 YAC probe alone and 0.1% by the CD3 YAC plus WCP Y probes. A patient with Ph^1 ALL had 19.9% of RHC detected by m-BCR/ABL and 14.4% by m-BCR/ABL plus WCP Y. Fig. 1 shows representatives of the cells analyzed with various combination probes.

Thirteen patients from group I (Table I) were sequentially analyzed using these 3 sets of probes. Patient 1, who was in remission, showed an increase of the residual host cells with DXZ1 plus WCP Y, whereas cells with specific translocation plus WCP Y showed a decrease. Similarly, in patient 3 DXZ1 plus WCP Y probes showed an increase, while cells with the specific translocation plus WCP Y probes showed a decrease when the patient was in CR.

Since patients in group II did not have a leukemia-specific translocation, those samples were analyzed using DXZ1 plus WCP Y probes following sex-mismatched BMT. Only two patients (31 and 38 in Table II) showed a relapse, while the others were in CR. Patients 20 and 21 were analyzed sequentially and they were in CR.

DISCUSSION

With the exception of patients 2, 26 and 27, the remaining patients had at least some RHC in the bone marrow for several months when studied by interphase FISH (using DXZ1 plus WCP Y probes), regardless of whether they were in CR or not. This finding is in accordance with the findings of other studies using FISH.[17, 18]

Three patients (nos. 1–3) showed no chimerism of RLC and donor cells at the end of follow-up using M-BCR/ABL plus WCP Y probes. Patients 1 and 3 showed chimerism only when DXZ1 plus WCP Y probes were used. The finding of a difference between the larger number of RHC (as identified by the DXZ1 plus WCP Y combination) and the smaller number of RLC (as identified by the specific translocation plus WCP Y probe combination) indicates the presence of surviving normal host cells. Residual recipient progenitors may survive even after intensive conditioning regimens.[18]

The three different sets of probes used in sex-mismatched BMT provided different information. The specific translocation probes alone could only detect whether cells were normal and abnormal, not whether the cells were of host or donor origin. Moreover they showed a higher percentage of abnormal cells (due to overlapping signals, which yield false-positive results) than the probe combinations. The combination of DXZ1 plus WCP Y could detect chimeras between host and donor cells, but could not detect abnormal host cells. The combination of specific translocation and WCP Y could differentiate the normal and abnormal host cells, as well as estimating the degree of chimerism.

A high incidence of chimerism is a common finding in patients grafted with a T-cell-depleted marrow.[19, 20] Our study showed that in unmanipulated bone marrow transplants, some host cells are retained. The relationship between mixed chimerism and leukemia relapse has been a matter of controversy.[21–25] Although mixed chimerism is not necessarily associated with a poor prognosis, several groups have suggested that mixed chimerism is associated with an increased risk of leukemia relapse.[26–29] During the follow-up, 9 of 38 patients relapsed. These patients had 0.5 to 46.8% residual leukemic cells. Patients 7 and 12 were in partial and complete remission, respectively, but they later developed features of relapse. We suggest that instead of estimating RLC, leukemic cell doubling time will be more important for predicting the clinical outcome. However, the number of relapses among the sequentially analyzed patients is too small to allow correlation with the biological activity of the cells.

Several groups, using a nested RT-PCR technique capable of detecting from one copy/10^6 cells up to one copy/10^7 cells,[30–32] showed the persistence of BCR-ABL expression in blood and/or bone marrow of some CML patients after BMT. However, healthy persons frequently show the presence of BCR-ABL[33] and BCL-2 rearrangements[34] by PCR, raising a question as to the usefulness of RT-PCR to detect relapse in such cases. Using FISH to detect RLC in such cases is more appropriate.

FISH using the combination of specific translocation and WCP Y probes has its own limitations. This method cannot be used for patients recieving BMT from donors of the same sex. It is suggested that the combination of FISH marker with either biochemical[4, 5, 9] or other polymorphic genetic markers[8] would be helpful in these cases.

The observation of persistent recipient cells which may later disappear or contribute to eventual relapse raises some additional questions. Do these cells persist because they are a part of a long-lived cell population, such as lymphocytes, or do they represent a less committed pro-

genitor cell pool, capable of self-renewal? Lymphocytes survive for several years in normal individuals, and viable, potentially functional T-lymphocytes may survive intensive pre-transplant conditioning regimens and can proliferate after incubation with interleukin-2.[35] In contrast, polymorphonuclear cells have a very short life in peripheral blood (2–3 days). Among the patients in our study, recipient cells persisted in patients who relapsed as well as those who remained in continuous CR. Some patients (nos. 2, 26 and 27) also showed disappearance of recipient cells during their course. This made it difficult to distinguish with certainty between long-lived recipient lymphocytes and stem cells with self-renewal capacity. Studies of chimerism following BMT need to be done in conjunction with an examination of cell morphology to answer these questions. A possible approach would be to perform FISH on cell populations which have been sorted on the basis of cell-surface antigens.

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