Chapter VI

Follow-up study of leukemia patients after chemotherapy or sex-mismatched bone marrow transplantation using fluorescence in situ hybridization with chromosome specific repetitive DNA probes

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6.1 Abstract

The purpose of this study was to explore the applicability of fluorescence in situ hybridization (FISH) with chromosome specific repetitive DNA probes for long term follow-up of leukemia patients and of patients that received a sex-mismatched bone marrow transplantation. Patients with myelodysplasia or acute leukemia whose leukemic cells carried numerical chromosomal aberrations were selected and followed with alpha satellite DNA probes that specifically hybridize to one chromosome type. The lower level of detection for aberrant cells is set by the naturally occurring background of cells with numerically aberrant appearance in normal populations of blood and bone marrow cells from healthy individuals. This background was found to range between 0.3-2 percent for the probes used. Although FISH data before and after chemotherapy were generally in agreement with morphological findings discrepancies were noticed in specific cases. These could be explained by the presence of cytogenetically distinct sub-clones that behave differently during treatment, the presence of fully differentiated leukemic cells, changes in the chromosomal constitution (clonal relapse) or misinterpretation of the initial karyotype.

Follow-up of patients that received a sex-mismatched allogeneic bone marrow transplantation, using simultaneous hybridizations with DNA probes specific for the X or for the Y chromosome, demonstrated that some patients, although in complete remission for several years, carried low numbers of recipient cells during the entire period of follow-up. In two cases a cytogenetic relapse was observed by the reappearance of high percentages of recipient type cells.

FISH was found to be a valuable tool for detection of leukemic cells during longer periods of time, with an approximate lower detection limit of 0.3-2 percent. It offers the possibility to rapidly monitor the efficacy of chemotherapy in leukemia and can be used for follow-up after treatment or to assess chimerism after sex-mismatched bone marrow transplantation.

6.2 Introduction

The ability to monitor leukemia patients during chemotherapy treatment with or without subsequent bone marrow transplantation for the presence of residual leukemic cells depends on the characteristics of the leukemic cells and the choice of the detection method. Parameters have to be chosen that allow discrimination of the leukemic cells from their normal counterparts. At the cytogenetic level a considerable fraction of the leukemia cases can be discriminated on the basis of numerical chromosomal aberrations (gain or loss of one or more chromosomes). Numerical aberrations occur in approximately 54% of the patients with acute myelocytic leukemia (AML). In 30% of children and 5% of adults with acute

lymphocytic leukemia (ALL) a hyperdiploid karyotype is found. A near-haploid karyotype is found in 1% of the ALL cases. In chronic myelocytic leukemia (CML) in blast crisis numerical chromosomal changes reach up to 70% (Heim and Mitelman 1987, Sandberg 1990). Although routine cytogenetic analysis provides detailed information on the complete karyogram, it only allows the analysis of a limited number of cells from the dividing fraction in a bone marrow sample and its use is therefore restricted to situations where high numbers of aberrant cells are present (e.g. at diagnosis or at relapse).

In situ hybridization (ISH) has become a powerful technique to localize chromosome specific nucleic acid sequences in the cell (Cremer et al. 1986, Pinkel et al. 1986, Trask 1991, Jenkins et al. 1992). Fluorescent detection of specific alpha satellite repetitive probes in combination with ISH (FISH) enables the recognition of the centromeres on the chromosomes as clearly localized and brightly fluorescent spots in metaphase spreads or in nuclei. This provides a means to enumerate the copy number of chromosomes. The number of centromeres present in the cell will be reflected by the number of fluorescent spots per nucleus. Numerical aberrations have been detected in this way in malignant cells(Cremer et al. 1986, Devilee et al. 1988b, Hopman et al. 1988, Anastasi et al. 1990, Van Dekken et al. 1990b, Jenkins et al. 1992, Pagliaro and Stanley 1993). The detection of small numbers of malignant cells bearing numerical aberrations using the FISH procedure might be hampered by the fact that in peripheral blood cells from healthy individuals small numbers of cells display an aberrant number of spots. This has been shown in normal cell populations from healthy volunteers (Poddighe et al. 1991).

In patients who underwent a sex-mismatched allogeneic bone marrow transplantation (SMM allo-BMT) the presence or absence of donor or host cells is reflected by the presence or absence of the corresponding sex chromosomes. With FISH, chimerism has been demonstrated using the sex chromosomes as markers (Durnam et al. 1989, Van Dekken et al. 1989, Arkesteijn et al. 1990, Przepiorka et al. 1990, Przepiorka et al. 1991, Bernasconi et al. 1993, Dewald et al. 1993a, Wessmann et al. 1993). The presence of one X chromosome is always correlated with a Y chromosome in a male cell and with a second X chromosome in a female cell. Therefore this group of patients is the ideal target for chimerism studies with double colour FISH in which two chromosomes can be detected simultaneously in one cell using two different probes and two different fluorescent dyes.

In this study we have examined the feasibility of the FISH procedure for the follow-up of leukemia patients. Firstly, we optimized the FISH procedure and determined in normal individuals the performance of the probes and the background frequencies of peripheral blood cells with aberrant numbers of spots. Secondly, for our follow-up study we selected patients with numerical chromosomal aberrations in their leukemic cells as judged by conventional cytogenetic analysis and followed them from the time of diagnosis, through the phase of complete remission

(detection of "minimal residual disease") till relapse, if this occurred. In a third study we followed patients who underwent sex-mismatched allogeneic BMT (SMM allo-BMT) with double colour FISH on the basis of the presence or absence of donor- or recipient sex chromosomes to determine the degree of chimerism. All patient studies were performed on bone marrow samples.

6.3 Materials and Methods

Peripheral blood and bone marrow samples

Peripheral blood from a healthy male volunteer was collected in a heparinized vacuum tube. Bone marrow from leukemia patients was collected in sterile heparinized flasks. After collection and during the process of cell separation the samples were kept at 4 C. The red cells were lysed by adding excess buffer, consisting of 155 mM NH_4Cl , 0.1 mM EDTA , and 11.9 mM $NaHCO_3$, to the blood or bone marrow samples. After centrifugation, the remaining nucleated cells were washed twice in Hanks HEPES Buffered Balanced Salt Solution.

Labeling of probe DNA

Complete plasmid with insert was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP using the nick-translation procedure (Sambrook et al. 1989). Probes used in this study are listed in Table 6.1 (Cooke 1979, Willard et al. 1983, Devilee et al. 1986, Donlon et al. 1986, McDermid et al. 1986, Waye and Willard 1986, Moyzis et al. 1987, Waye et al. 1987a, Waye et al. 1987b, Devilee et al. 1988a, Greig et al. 1989, Waye and Willard 1989, Looijenga et al. 1990). After nick translation the labeled products were tested for the amount of incorporated biotin using a direct spot-blot method and for the size of the probe fragments by alkaline gel electrophoresis and blotting. Average probe fragment sizes ranged between 200 and 400 base pairs.

Fluorescence in situ hybridization

Cell suspensions prepared as described above were treated with hypotonic buffer (75 mM KCl) for 10 minutes at room temperature. Cells were spun down and fixed with Carnoy s fixative under continuous vortexing. Fixation was repeated 3 times. Fixed cells were used either directly or were stored in fixative at -20 C for later analysis. After fixation cells were spotted onto cleaned microscope slides and the area was marked with a diamond tipped pen.

Directly before the hybridization procedure slides were placed in ethanol for at least 60 minutes at room temperature and air dried. Slides were then incubated in 0.1 M HCl/0.1% Tween-20 for 15 minutes to remove matrix and histon proteins and to improve accessibility of the nuclei for the DNA probe.

Denaturation of the target DNA was accomplished by placing the slides in 70% formamide/2 x SSC pH 7 at 70°C for 2 minutes followed by dehydration in an ice-cold ethanol series of 70%, 85% and 100%. The hybridization mixture consisted of 1 μg/ml probe DNA, 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide at pH 7. The probe was denatured in the hybridization mixture for 10 minutes at 90°C and quenched on ice immediately. Approximately 12 μl of this denatured hybridization mixture was pipetted onto the marked area of the microscope slide. The area was covered with a plastic coverslip and the slide was placed in a humid atmosphere at 37°C. Hybridization was performed for 4 hours. After hybridization slides were washed 3 times for 2 minutes in 2 x SSC followed by a stringent washing in 50% formamide, 2 x SSC. Five minutes at 45°C was used routinely but with probes known for high non-specific binding, stringency was increased by either increase of the temperature, formamide concentration or time of washing. Slides were washed in 2 x SSC and finally placed in 4 x SSC/ 0.05% Triton-x-100(SSC-T).

Visualization of the biotin-labeled probe binding was accomplished by incubation of the slides with avidin-FITC (av-FITC) conjugate; 5 μg/ml (Vector, Burlingame, CA, USA) in 5% non-fat dry milk in SSC-T with 0.002% sodium-azide, for 20 minutes at 37°C. After this period cells were washed 3 times for 2 minutes each with SSC-T buffer. In those cases where amplification of the signal was required, slides were incubated with biotin-labeled goat-anti-avidin; 5 μg/ml (Vector, Burlingame, Ca, USA) in 5% non-fat dry milk, for 20 minutes at 37°C. After washing the av-FITC incubation step was repeated. When single hybridizations were performed, nuclear DNA was counter stained with propidium-iodide (1 μg/ml) in Slowfade (Molecular Probes, Eugene, OR, USA). When double hybridizations were performed the biotin-labeled probes were detected using Avidin-Texas red (Vector, Burlingame, CA, USA) and the digoxigenin labeled probes were detected using anti-digoxigenin-FITC (green fluorescence) while DNA was counter stained with DAPI (1μg/ml).

Scoring of fluorescent spots.

A Zeiss Axioskop-20 microscope was used. Screening of the slides was performed with 63 x objective lens. Every nucleus in the field of view was taken into account. Per slide 300 to 500 nuclei were scored. When the fluorescent signals did not allow a classification to a group with a discrete number of spots the nuclei were scored as "1 or 2", or "split spots" (i.e. fluorescent spots were seen that seemed to be split in two, thus appearing as two smaller spots close together each with half the fluorescence intensity of a single spot). In Table 6.1 the scoring is given in this detailed way. In the following Tables only the percentage of cells with clear-cut fluorescent signals in leukemia or donor/host type of cells is shown. This explains why in Table 6.4 the sum of the percentage host and donor cells is not exactly 100. Per patient each subsequent sample was scored by the same observer.

6.4 Results

Probe performance.

In Table 6.1 the results of a number of probes that were used for FISH are listed. Analysis of peripheral blood cells from a healthy male volunteer revealed that with the FISH method a small percentage of cells appeared to have aberrant numbers of spots. This was the case for all probes tested. For 7 out of 13 autosome specific probes and for the 2 sex chromosome specific probes the frequency of expected numbers of spots per cell exceeds 95%, 3 probes score in-between 94% and 95% and the remaining 3 probes score lower. The probe for chromosome 20 performs slightly lower than 90%. Rarely, nuclei were observed with no spots at all. Observations that are difficult to interpret (i.e. 1 or 2 spots or split spots) are listed in columns 7 and 8 respectively. The percentage of cells missing one fluorescent spot ranges from 5.6 (probe 20) to 0.2 (Yprobe) and the percentage of cells that had one additional fluorescent spot ranges from 2.1 (probe 8) to 0.3 (probes 10 and 12). The presence of nuclei with an aberrant number of fluorescent spots in normal cell populations implies a lower detection level for the analysis of leukemia samples.

Table 6.1 Probe performance on healthy male volunteer blood cells as observed by fluorescence in situ hybridization (in % of cells)

			Frequency of fluorescent spots per nucleus					
	probe							
chromosome	name	reference*	0	1	2	3	1 or 2	split
1	PUC1.77	22		1.4	95.1	0.8	1.9	0.8
3	pa3.5	23	0.8	1.9	96.1	1.2		
7	p7t1	24	0.3	0.3	96.7	0.6	0.9	1.2
8	D8Z2	25		0.9	93.5	2.1	1.5	2.0
9	pHUR98	26	0.3	1.8	94.3	0.9	1.5	1.2
10	D10Z1	27		0.3	97.0	0.3	0.9	1.5
11	pLC11A	28		1.1	96.0	0.8	0.8	1.3
12	pα12H8	29	0.3	0.8	95.4	0.3	1.6	1.6
16	pSE16	30		2.3	92.2	0.6	2.3	2.6
17	p17H8	31		2.1	95.2	0.6	1.5	0.6
18	L1.84	32		1.4	94.9	0.6	1.1	2.0
20	p3.4	33		5.6	89.7	1.3	1.3	2.1
22	p22/1:2.1	23		0.6	95.3	0.9	1.6	1.6
X	pBAMX5	34	1.0	98.6	0.4			
Y	pY2.45	Amersham	0.2	99.4	0.4			

Split spots are defined as two smaller spots close together, each with approximately half the fluorescence intensity of a single spot.

350 nuclei were scored per probe.

*: refers to the article in which the probe is described.

The findings on the peripheral blood samples were taken as a guideline for probe performance but not used to determine the ultimate lower detection level for each probe in bone marrow samples.

Leukemia follow-up

In Table 6.2 the results are given from 8 patients with acute leukemia under therapy. For each patient, the diagnosis and the time point after diagnosis at which bone marrow samples were taken are indicated in the second and third column. In the fourth column the results from conventional cytogenetic analysis are indicated of which only numerical aberrations are given that were found at the specific time points. When no aberrant cells were found, the normal karyotype was listed. The fifth column shows the percentage of blast cells in the bone marrow smear found by cytology. Column 6 indicates the probe that is used. Column 7 shows the percentage of aberrant cells as judged by the number of fluorescent spots per nucleus.

In general, FISH data as well as morphology data show a sharp decrease in the percentage of aberrant cells or blasts after the first course of chemotherapy. Once in remission, in 4 cases (patients 1, 3, 5 and 8), values of aberrant cells established by FISH remained around the levels of those observed in the normal cells from the healthy volunteer (Table 6.1). In three cases (patients 2, 6 and 7) a relapse was observed as judged by the increasing percentage of nuclei with numerical aberrations. Conventional cytogenetic analysis was performed only occasionally at the follow-up time points. In two cases (patient 2: 36 months after diagnosis and patient 3: 5 months after diagnosis) patients were morphologically close to a complete remission. In both cases 100% normal karyotypes were found in the bone marrow whereas FISH showed 3.3% and 0.9% of aberrant cells respectively. Despite the high percentage of blasts in the bone marrow sample from patient 6 at 12 months after diagnosis, the conventional cytogenetic analysis does not indicate the presence of a -Y.

In several cases the percentage of aberrant cells as observed by FISH did not correspond with the percentage of blasts as found by morphology. In patient 4 the percentage of aberrant cells that was found by FISH was much higher than the percentage of blasts found by morphology.

Table 6.2 Follow up of bone marrow samples from leukemia patients with numerical aberrations employing FISH with chromosome specific probes

Patient	Diagnosis (FAB)	time point (months)	Cytogenetic aberration[1]	% Blasts (cytology)	Probe used for FISH	% Numerical aberration (FISH)[2]	
1	AML-M1	0	+10 (100%)	90.0	10	59.3	
		1	-	15.8		1.3	
		2.5	-	14.4		0.6	
		4	-	4.2		0.6	
		5	-	3.0		0.6	
		5.5	-	3.6		0.6	
		9	-	19.6		0.6	
		9.5	-	8.0		0.6	
2	AML-M1	0	+11 (100%)	62.0	11	-	
		36	46, XY (100%)	10.4		3.3	
		38	-	2.6		3.1	
		40.5	-	-		2.9	
		49	-	62.4		25.2	
3	AML-M2	0	+8 (100%)	54.0	8	-	
		5	46, XX (100%)	6.2		0.9	
		6	-	5.0		0.6	
		11	-	4.6		0.9	
		16	-	0.8		0.6	
		21	-	3.2		0.3	
4	AML-M2	0		43.6	Y	97.5	
		1.5	-	4.2		10.8	
		3	-	4.6		1.0	
		4	-	3.8		5.3	
		4.5	-	3.0		3.4	
5	AML-M2	0	minus Y (100%)	81.5	Y	-	
		4	-	78.8		88.8	
		5	-	5.0		3.3	
		6.5	-	3.8		0.9	
		8.5	-	0.6		1.1	
		9.5	-	1.8		0	
		13.5	-	2.8		0.2	
		15	-	1.4		0.6	
6	AML-M4	0	minus Y (14%)	42.0	Y	-	
		3	-	2.6		0.2	
		5	-	4.0		0.3	
		12	46, XY 100%	80.0		95.3	
						#7	#17
7	RAEB	0	(64%), -17(76%	8.3	7 and 17	-	-
		17	-	17.7		67.8	4.9
		18.5	-	0.4		3.5	5.4

Table 6.2 continued

		19	-	dry tap		17.3	2.5
		20	-	>> 5		16.7	1.8
8	AML-M2	0	+8 (100%)	35.4	8	37.3	
		1.5	-	4.6		1.1	
		3	-	2.8		0.0	

1: diagnosed by conventional cytogenetics
2: 300-500 nuclei were scored per sample
3: bone marrow biopsy
-: not determined

The percentage of cells characterized by -Y at 4 and 4.5 months after diagnosis was 5.3 and 3.4 respectively, which is well above the lower detection level of this probe. Blast cell frequencies smaller than 5% indicate morphological complete remission at these timepoints. Proof of imminent relapse was not obtained since this patient died because of a pneumomitis infection shortly after the last bone marrow sample was taken. In patient 7, 67.8% of the cells carry a monosomy for chromosome 7 at 17 months after initial diagnosis while at this stage 17.7% of blast cells are found in the bone marrow. Chemotherapy was given and after an initial decrease in numerically aberrant cell numbers, an increase is observed at 19-20 months that concurs with the rise of the percentage of blast cells in the bone marrow as judged by bone marrow biopsy. In contrast to the observations with the chromosome 7 specific probe, FISH in this patient with a probe specific for chromosome 17, which was reported to be a second numerical aberration, showed low numbers of aberrant cells at any time point and no increase at the time of relapse.

In patient 1, a 90% blast cell count was observed in the bone marrow at diagnosis. This was much higher than the percentage of cells with a trisomy for chromosome 10 found by FISH (59.3%). After the first course of chemotherapy the percentage of cells with a trisomy 10 as observed by FISH dropped to a level close to that of the threshold that can be expected for the chromosome 10 probe. Yet, only a partial remission was achieved (15.8% blasts by morphology). From this time point on the low number of aberrant cells as determined by FISH remained unaltered. The second treatment course did not further reduce the number of blasts in the bone marrow (14.4%). A complete remission was achieved after the third course of chemotherapy. Nine months after diagnosis an increase in blasts was observed (19.6%) while the analysis with FISH remained negative.

Sex-mismatched allo-BMT.

The sex-mismatched bone marrow transplanted patients have been followed using double hybridization with the sex chromosomes as indicators for the presence of host cells in the bone marrow. In control experiments, in which 400 nuclei were scored using double hybridization on normal peripheral blood cells from healthy volunteers, nuclei with a pattern of fluorescent spots that would suggest the opposite

sex were not found, implying a higher sensitivity due to the absence of double false signals (Table 6.3).

Table 6.3 Double hybridization with X and Y chromosome specific probes on healthy donor blood cells (male; % of cells)

	spots per nucleus X probe			
Y probe	0	1	2	split
0	-	0.6	-	-
1	0.3	96.1	0.3	1.4
2	-	-	-	-
split	-	0.6	-	0.8

400 nuclei were scored

The results are given in Table 6.4. The first column shows the diagnosis and the sex-mismatch (male to female or female to male). Column 2 shows the time after transplantation at which bone marrow samples were taken. Columns 3 and 4 show the conventional cytogenetic data, column 5 shows the percentage of host cells and column 6 shows the percentage of donor cells found by FISH. In the double hybridization experiments recipient derived cells were found in almost every sample.

Table 6.4 Bone marrow follow up of sex-mismatched allo-BMT patients with FISH using double hybridization with X and Y probes

	months after	conventional cytogenetics		FISH	
patient	allo BMT	% host cells	% donor cells	% host cells	% donor cells
patient 1	12	0	100	0.7	98.1
AML-M2	15.5	0	100	0.4	98.9
female to male	18.5	-	-	0.0	97.4
patient 2	32	-	-	0.0	98.6
ALL	37	-	-	0.0	97.4
female to male	45.5	0	100	0.0	98.0
	58	0	100	0.0	98.0
patient 3	1	0	100	0.4	97.8
AML	7	0	100	0.7	98.1
female to male	9.5	0	100	0.2	98.0
	12.5	0	100	1.2	97.9
	20	0	100	0.0	97.6
	31	15	85	24.4	75.6

Table 6. 4 continued

patient 4	-1.3	100	0	99.4	0.0
CML-Ph+	25	0	100	2.1	95.8
male to female	38	65.5	34.5	55.1	43.9
	51	91	9	97.0	2.4
patient 5	1.5	0	100	0.0	100
AML-M3	6	0	100	0.0	100
male to female	9	0	100	0.0	99.3
	12	0	100	0.0	100
	18	0	100	0.2	98.8
	24	0	100	0.0	99.2
patient 6	6	0	100	0.0	97.4
CML-Ph+	13	0	100	3.3	95.0
male to female	18	0	100	3.0	95.4
	24	0	100	2.9	96.6
patient 7	-1	100	0	96.2	0.0
CML-Ph+	4	47	53	33.5	62.0
male to female	5	-	-	38.1	60.2
	5.5	-	-	43.9	51.6
	6	-	-	47.3	48.5
	6.5	50	50	48.1	46.9

T-cell depletion of the graft was performed in all patients

Patients 4 and 7: first samples were taken shortly before BMT

400-500 nuclei were scored

- : not determined

Two cases (patients 2 and 5) were completely reconstituted by hematopoietic cells from the donor (full chimerism). In patient 2 no host cells were observed in the bone marrow up to 58 months after transplantation while in patient 5 only one host derived cell was found 18 months after BMT. The other cases showed the continuous presence of host cells in their bone marrow. In two of those a leukemia relapse was observed after transplantation (patients 3 and 4). Patient 4 clearly showed an increase in host derived cells in the bone marrow detected by FISH, while at the same time conventional cytogenetic analysis showed a recurrence of Philadelphia chromosome positive cells. Though apparently in cytogenetic relapse, to date the morphological findings do not indicate a hematological relapse. In patient 7 who received a matched unrelated donor transplantation, high percentages of host cells were present 4 months after transplantation. So far, Philadelphia chromosome positive cells have not reappeared.

6.5 Discussion

FISH has several advantages that makes it an attractive method in addition to conventional cytogenetic analysis. No short term cultures are required, analysis of the metaphases and recognition of the banded chromosomes is not necessary and due to the fact that interphase nuclei which are derived directly from the patient are studied, selective outgrowth of subpopulations during short term culture is prevented and a representative percentage of aberrant cells in the patient is obtained. The number of nuclei that can be analyzed routinely with FISH is at least tenfold higher than the number of metaphases that is routinely analyzed with conventional cytogenetic analysis. Therefore FISH allows rapid quantitative analysis and follow up of patient material from diagnosis through complete remission.

As demonstrated in this study, in the blood from the healthy volunteer a small number of cells can be found with an aberrant number of chromosomes when analyzed with FISH. For some probes low numbers of such cells are found (e.g. probes specific for chromosomes 10, X, Y) For other probes this frequency is higher (e.g. the probe that is specific for chromosome 20). When the number of spots per nucleus is lower than expected, this might be due to poor penetration of the probe, incomplete denaturation or loss of target DNA during the hybridization procedure. Furthermore, overlapping signals that cannot be distinguished in two-dimensional microscopic analysis might contribute to this phenomenon. A higher number of spots per nucleus might be due to non-specific probe binding to other centromeres and subsequent incomplete stringent washing. On the other hand some cells actually might contain aberrant copy numbers for particular chromosomes. Incomplete or rearranged karyotypes in healthy individuals have been observed (Prieur et al. 1988, Kuffel et al. 1991) and the older age of the individual seems to play a role in this phenomenon. Especially in fully differentiated cells the presence or absence of chromosomes will not be detrimental for vital cellular functions provided that relevant genes for those particular functions are not involved. For the detection of leukemia, the percentage of cells with aberrant numbers of spots in bone marrow from patients has to be significantly different from the control values. Different tests have been evaluated (Kibbelaar et al. 1993) in which controls as well as artificial aberrant cell mixtures were made up of the same cell sample. Since a proper control for studies on bone marrow from hematological malignancies is not available (i.e. normal bone marrow cells from the same individual), we considered the values found in nuclei from peripheral blood of a healthy volunteer as a guideline (Table 6.1). With respect to these values the background level for the detection of aberrant cells is reached at a range of 0.3% to 2% (probe 20 not included) depending on the probe that is used and the type of aberration that has to be detected. In this respect our control studies do not greatly deviate from what others have published (Poddighe et al. 1991, Chen et al. 1992) indicating an appropriate FISH methodology.

In the patient studies, the data obtained by FISH were compared to the morphological criteria that are routinely used to determine the remission status of the leukemia patient. In general the FISH data were in agreement with data obtained by cytology. Nevertheless, some patients had higher (patients 1 and 4) others lower (patient 7) numbers of aberrant cells as determined by FISH. The presence of a higher number of aberrant cells can only be explained by the fact that differentiated, non-blast like cells, carry the numerical aberration that is detected by FISH. A lower number of aberrant cells can occur when the conventional cytogenetic analysis indicates a specific numerical aberration while the target region for the probe is not involved and therefore still present in the nucleus as a diploid set. Also subclones with specific numerical aberrations can selectively be eliminated during chemotherapy when they are more sensitive for the treatment. The following cases from our studies further illustrate these points.

In patient 1 the percentage of cells carrying a trisomy for chromosome 10 as determined by FISH is almost twice as low as the percentage of blasts found in the diagnostic marrow aspiration. This observation strongly suggests the presence of at least two sub-clones in the patient, one of which carrying a trisomy for chromosome 10. Nevertheless conventional cytogenetic analysis resulted in a +10 in all the analyzed metaphases. Since conventional cytogenetic analysis allows only the analysis of a small number of cells and since only the dividing fraction can be studied, the non-trisomy containing subpopulation of the leukemic cells might have remained unnoticed. In subsequent marrow studies the percentage of cells with a trisomy 10 as detemined with FISH dropped and remained at a level close to the background values while the morphological data revealed presence of blast cells at several stages during the follow-up period. Apparently the trisomy 10 subpopulation was more sensitive to the chemotherapy employed and was therefore eradicated.

The diagnosis sample of patient 4, with a loss of the Y chromosome in all leukemic cells at diagnosis, shows a twofold higher amount of aberrant cells according to the FISH method as compared to cytology. Also at 1.5 months after diagnosis, when the patient had reached complete remission (4.2% blasts in the bone marrow), 10.8% of the bone marrow cells showed a loss of the Y chromosome. The only explanation for the discrepancy between the number of -Y cells by FISH and the number of blasts in the bone marrow is that differentiated leukemic cells are present. The question whether these cells are fully differentiated or still maintain leukemic potential remains unsolved.

Patient 7, characterized by a monosomy 7 and 17, showed a similar phenomenon as observed in patient 4. When a probe specific for chromosome 7 was used 17 months after diagnosis, the number of aberrant cells found with FISH was higher than the amount of blasts in the bone marrow at that timepoint. It is striking that the fluctuation in the frequency of cells carrying a monosomy 17 neither correlated with

the fluctuations in the percentage of blast cells nor with the percentage of cells with a -7 found by FISH at the same time points. In the leukemic cells of this particular patient a complex karyotype was found including a marker chromosome and the loss of one chromosome 17. Apparently, the centromere region of chromosome 17 was present in the marker and therefore in situ hybridization on interphase nuclei, applying the probe for the alpha satellite DNA from chromosome 17, resulted in two fluorescent spots.

Since in SMM allo-BMT patients the chromosomal constitution of each individual bone marrow cell is either donor or recipient type this offers the possibility to investigate in much greater detail the presence of recipient cells in these patients using the sex chromosomes as markers. Theoretically, after double hybridization with two different probes, the threshold level for double false hybridizations (i.e. cells appearing to be from the opposite sex) is reduced to the product of the chance of false positive or negative hybridizations for each of the individual probes. Threshold detection levels of 0.2% for the Y probe and 0.4% for the X probe results in a chance of 1/500 x 1/250 = 1/125,000 that both probes give a false signal in the same nucleus. In healthy donor material the presence of cells showing a hybridization pattern of the opposite sex for both probes was not found (Table 6.3). The method of scoring by microscope allows at the most the scoring of 1000 cells on a routine basis. For methods with thresholds reaching 1/125,000 other techniques are required. For this purpose the combination of FISH with flow cytometry employing intact nuclei is currently under investigation. This will allow the analysis of higher cell numbers provided that clear-cut discrimination can be achieved between the positive and negative populations.

The SMM allo-BMT patients that were followed could be divided into two categories. Those that had a complete hematopoietic reconstitution of donor bone marrow and those in which recipient type cells could be found. Two SMM allo-BMT patients showed a hematopoietic reconstitution that was (almost) completely of the donor type (patients 2 and 5). To date those patients are in complete remission. Four patients showed long lasting persistence of low levels of recipient cells in the bone marrow (patients 1, 3, 4 and 6) in percentages up to 3.3%. Two of these patients (patients 3 and 4) have developed a relapse. Although relatively high levels of recipient cells were continuously present in patient 7 no hematological signs of relapse were observed.

In the literature controversy exists with regards to the relationship between mixed chimerism and relapse (Singer et al. 1983, Schattenberg et al. 1989, Bernasconi et al. 1993, Roux et al. 1993, Schattenberg et al. 1993). That there is a correlation between mixed chimerism and relapse rate is suggestive but remains unproven by this study. Low numbers of host cells may indicate the presence of leukemic cells that are in a dormant state and therefore do not lead immediately to leukemia. On the other hand the host cells

might be derived from a small population of the normal hematopoietic system that survived marrow ablative therapy (mixed chimerism). At longer time intervals further normal host hematopoietic reconstitution must be considered. Finally the host cells may not be from the hematopoietic system at all but may have concurrently been collected together with the bone marrow aspiration. Stromal cells are not involved in the hematopoietic reconstitution after BMT and are therefore of donor origin (Athanasou et al. 1990). Since only the sex chromosomes are monitored more parameters should be taken into account like immunophenotyping, cell sorting, and subsequent in situ hybridization or DNA/RNA analysis, to elucidate the nature of these cells .

From the present study it can be concluded that FISH is a valuable tool for the sequential analysis of bone marrow samples from leukemia patients whose leukemia cells carry numerical chromosomal aberrations. The speed and ease with which FISH can be performed gives the procedure an advantage over conventional cytogenetic analysis and makes it an attractive technique for the assessment of treatment efficacy. The FISH method is in particular valuable on top of morphological criteria in those leukemias where no other specific characteristics are available to distinguish leukemic cells from the normal marrow cells.

Several points of consideration in detecting "minimal residual disease" have to be made with respect to the interpretation of the results. FISH data may not correlate with the actual disease progression as observed by morphology because of the possible presence of cytogenetically different sub-clones that might behave differently under chemotherapy. Furthermore, with the choice of the DNA probes, conventional cytogenetic data must be interpreted with care. Complex karyotypes might indicate the absence of chromosomes although the region for which the probe is specific is still present (e.g. in a translocation product or a marker chromosome).

FISH as it is used in this study will depend largely on the conventional cytogenetic analysis for the proper choice of probes. Conventional cytogenetic analysis gives highly detailed information from a small number of cells. FISH on the other hand provides less complex information from large numbers of cells. Both methods should therefore be used to supplement each other.

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