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The Use of FISH with Chromosome-specific Repetitive DNA Probes for the Follow-up of Leukemia Patients

Correlations and Discrepancies with Bone Marrow Cytology

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ABSTRACT: *The use of fluorescence in situ hybridization (FISH) for the purpose of repeated follow-up examination of bone marrow samples from 38 leukemia patients was investigated. On the basis of conventional cytogenetic analysis, patients with acute leukemia whose leukemic cells carried numerical chromosomal aberrations were selected and followed with repetitive DNA probes that specifically hybridize to one chromosome type. Repeated cytogenetic metaphase analyses would have been laborious and not sensitive or quantitative enough to follow declining numbers of aberrant cells. FISH, as an interphase cytogenetic technique, provides a rapid and simple alternative with high sensitivity. Although FISH data before and after chemotherapy were in agreement with bone marrow cytology in 30 of 38 patients, discrepancies were noticed in specific cases. These could be explained by the presence of cytogenetically distinct subclones that behave differently during treatment, the presence of differentiated leukemic cells, changes in the chromosomal constitution caused by clonal relapse, or the fact that a numerical aberration is found by conventional chromosome banding analysis while the target region to which the probe is directed is still present in the nucleus as a diploid set.*

INTRODUCTION

The ability to monitor leukemia patients 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. One way of discriminating normal from aberrant cells is by fluorescence in situ hybridization (FISH) on interphase cells. FISH allows the recognition of the centromeres on the chromosomes as clearly localized and brightly fluorescent spots in metaphase spreads or in nuclei [1–4]. The number of centromeres present in the cell will be reflected by the number of fluorescent spots per nucleus. This provides a means to enumerate the copy number of chromosomes [1, 4–8]. For this reason FISH has developed as a rapid and relatively accurate alternative to conventional cytogenetic analysis [9–11]. In combination with immunophenotyping, it can give information about cell lineage, the lymphocyte subpopulation, and the genotype from serially followed sex-mismatched bone marrow transplants or demonstrate the presence of leukemia cells with the host phenotype [12].

Cytogenetically, 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 crises numerical chromosomal changes are observed in 70% of the cases [13, 14].

The fact that a considerable number of leukemias show numerical chromosomal aberrations, the speed and ease of performance, and the relative accuracy has made FISH an attractive method for patient monitoring. In this study we have examined the feasibility of the FISH procedure for the follow-up of leukemia patients. For our study we selected patients with numerical chromosomal aberrations in their leukemic cells as judged by conventional cytogenetic analysis. They were followed with FISH and cytology from the time of diagnosis through the phase of

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complete remission (detection of minimal residual disease) until relapse, if this occurred. Although in general a clear correlation was found between bone marrow cytology, conventional cytogenetic analysis, and FISH, in several cases unexpected results were obtained. This report emphasizes these specific discrepancies and discusses implications for future routine bone marrow analysis of leukemia patients with FISH.

MATERIALS AND METHODS

Samples

Bone marrow samples were obtained by an aspiration from the iliac crest. Cytology was performed after May-Grünwald Giemsa staining of the bone marrow smears. Bone marrow for the FISH study was collected in sterile heparinized flasks. During the process of cell separation the samples were kept at 4°C. The red cells were lysed by addition of excess buffer, consisting of 155 mM NH_4Cl, 0.1 mM EDTA, and 11.9 mM $NaHCO_3$. After centrifugation, the nucleated cells were washed twice in Hanks' HEPES-buffered balanced salt solution.

Fluorescence in Situ Hybridization

Cell suspensions were treated with hypotonic buffer (0.075 M KCl) for 10 minutes at room temperature. Suspensions were centrifuged and the cells were fixed with methanol/acetic acid under continuous vortexing. Fixation was repeated three times. After fixation the cell suspensions were dropped onto cleaned microscope slides and the area was marked with a diamond-tipped pen.

Complete plasmid with insert was labeled with biotin-16-dUTP using the nick-translation procedure [15]. Probes used in this study were chosen on the basis of the aberrations found in the patients and are listed in Table 1 [16–23]. All probes recognized chromosome-specific repetitive DNA sequences. The average probe fragment sizes after nick-translation ranged from 200–400 base pairs.

Directly before the hybridization procedure, the 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 histone 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 × 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, 0.1 mg/mL sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate, and 2 × SSC in 50% formamide at pH 7. Probes were denatured in the hybridization mixture for 10 minutes at 90°C and placed 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 for 4 hours. After hybridization, slides were washed 3 times for 2 minutes in 2 × SSC followed by a stringent washing in 50% formamide in 2 × SSC. Five minutes at 45°C was used routinely but with probes known for high nonspecific binding, stringency was increased by either increase of the temperature, formamide concentration, or time of washing. Slides were washed in 2 × SSC and finally placed in 4 × 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 (Vector, Burlingame, CA), 5 μg/mL in 5% non-fat dry milk in SSC-T with 0.002% sodium-azide, for 20 minutes at 37°C. Slides were washed three 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 (Vector), 5 μg/mL in 5% non-fat dry milk, for 20 minutes at 37°C. After washing, the Av-FITC incubation step was repeated. Nuclear DNA was counterstained with propidium-iodide (1 μg/mL) in Slowfade (Molecular Probes, Eugene, OR).

Scoring of Fluorescent Spots

A Zeiss Axioskop-20 microscope was used. Screening of the slides was performed with a 63 × objective lens. Every

Table 1 Mean number of fluorescent spots per nucleus as observed by fluorescence in situ hybridization on peripheral blood cells from healthy male and female individuals (350 nuclei were scored per probe)

Probe for chromosome[c]	Frequency of fluorescent spots per nucleus (%)[a]				
	0	1	2	3	Other[b]
(n = 5 males)					
7	0.6	2.4	93.6	0.7	2.7
	0.5	*1.1*	*2.0*	*0.3*	*1.0*
8	0.8	2.7	93.3	1.0	2.2
	0.8	*1.1*	*1.9*	*0.7*	*0.5*
9	0.6	1.1	96.6	0.4	1.3
	0.4	*0.2*	*1.0*	*0.5*	*1.9*
10	0.3	1.7	95.6	0.4	2.0
	0.4	*0.9*	*1.9*	*0.1*	*0.8*
11	0.3	2.0	94.8	0.6	2.3
	0.5	*0.7*	*2.1*	*0.5*	*1.5*
17	0.7	2.5	93.2	0.6	3.0
	0.6	*0.9*	*2.6*	*0.4*	*2.0*
18	0.3	1.4	94.9	0.6	2.8
	0.2	*0.2*	*2.1*	*0.5*	*2.2*
X	0.9	97.7	0.2	0.0	1.2
	0.7	*1.0*	*0.3*	*0.0*	*0.9*
Y	0.6	98.7	0.2	0.0	0.5
	0.3	*0.4*	*0.3*	*0.0*	*0.4*
(n = 4 females)					
X	0.6	1.5	95.2	0.6	2.1
	0.2	*0.2*	*0.8*	*0.3*	*1.0*
Y	99.6	0.2	0.0	0.0	0.2
	0.5	*0.2*	*0.0*	*0.0*	*0.3*

[a] Average values are given. Standard deviation in italics.

[b] This column shows the percentage of nuclei that did not display a discrete number of spots. In this group unclassifiable nuclei and split spots are taken together.

[c] References in Materials and Methods.

nucleus in the field of view was taken into account. Per slide, 300–500 nuclei were scored. When nuclei could not be classified to a group with a discrete number of spots they 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. Each subsequent sample was scored per patient by the same observer.

Controls

For each probe in this study control values were derived from the peripheral blood from five healthy male individuals. In addition, four healthy female samples were tested with X and Y probes only.

RESULTS

Table 1 shows the performance of the probes used in this study. When tested on peripheral blood from five healthy men and four healthy women none of the probes reached 100% diploidy when the number of FISH signals per nucleus was counted. For the autosomes, the two spots that can be expected per nucleus are observed in 93.2–96.6% of the cells. For the sex chromosomes (one spot per nucleus for both X and Y chromosome) in male cells the values are 98.7%. In female cells (two or zero spots for X and Y probes, respectively), 95.2% and 99.6% are the values of the expected number of spots in healthy cells. This phenomenon is considered to determine the lower detection level of the method.

The FISH results performed on the bone marrow cells from the leukemia patients could be classified into three groups: one in which bone marrow cytology and FISH showed good correlation, a second group in which the leukemic cell number as determined by FISH was lower when compared to the number of blast cells found by cytology, and a third group in which the number of leukemic cells as determined by FISH was higher when compared to the number of blasts. Furthermore, with multiple hybridizations performed in some cases with different repetitive DNA probes, it was shown that noncorrelating fluctuations exist when different probes were tested in one patient.

From 38 patients with acute leukemia, bone marrow samples were investigated at diagnosis or thereafter. In 30 cases the FISH findings were in accordance with what

Table 2 Discrepancies found between FISH and bone marrow cytology in eight patients

Patient	Diagnosis									
1	AML M1	% of cells with +10	**43**	**0**	**0**	0	0	0	**0**	**0**
		% of blasts	**90**	**16**	**14**	4	3	3	**19**	**8**
		Time (months)	0	1	2.5	4	5	5.5	9	9.5
2	ALL	% of cells with +17	**33**	**38**						
		% of cells with +X+X	**72**	**54**						
		% of cells with +Y	**74**	**55**						
		% of blasts	**95**	**95**						
		Time (months)	0	1						
3	RAEB	% of cells with −7	**68**	**4**	**17**	**17**				
		% of cells with −17	**5**	**5**	**2**	**2**				
		% of blasts	**18**	**0**	ND	Biopsy				
		Time (months)	17	18.5	19	20				
4	AML M2	% of cells with +8+8	**0**	0	0	0	0			
		% of blasts	**6**	5	4.6	0.8	3.3			
		Time (months)	5	6	10	16	20			
5	AML M2	% of cells with −Y	**98**	**11**	1	**5**	3			
		% of blasts	**44**	**4**	5	**0**	3			
		Time (months)	0	1.5	3	4	4.5			
6	AML M1	% of cells with −7	**1**	1	**5**					
		% of cells with +8	37	1	0					
		% of blasts	**35**	5	**3**					
		Time (months)	0	1.5	3					
7	ALL	% of cells with +7	**1**							
		% of cells with +8	13							
		% of cells with +11	25							
		% of cells with +18	**2**							
		% of cells with +Y	15							
		% of blasts	ND							
		Time (months)	14							
8	MM[a]	% of cells with +7	**78**							
		% of cells with −8	**2.4**							
		% of cells with +9	**81**							
		% of blasts	ND							
		Time (months)	5							

[a] MM, Multiple myeloma. Unexpected values are in bold.

could be expected on the basis of the phase of the disease and bone marrow cytology. In general, both FISH data and cytology data showed a sharp decrease in the percentage of aberrant cells or blasts after the first course of chemotherapy (data not shown).

In Table 2 the results are given from eight patients in which a clear discrepancy exists between conventional cytogenetic analysis, bone marrow cytology, and FISH results. In this table time indicates the timepoints after diagnosis at which samples were collected for FISH analysis. Guided by conventional cytogenetic analysis, probes were chosen that allowed recognition of numerical aberrations. These aberrations are indicated in column 3. In the corresponding rows the percentages of cells that showed the expected aberrations as judged by FISH are indicated at the various timepoints. The data points for which discrepancies exist between bone marrow cytology (percentage of blast) and FISH or among multiple FISH results are in bold. At those timepoints where bone marrow cytology data were not available this is indicated by ND in Table 2. Detailed cytogenetic analyses of these eight patients at the time of diagnosis is presented in Table 3.

In patient 1, a 90% blasts 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 (43%). After the first course of chemotherapy, the cells with a trisomy 10 as observed by FISH disappeared. Yet, only a partial remission was achieved as judged by cytologic analysis (16% blasts). From this timepoint with FISH no aberrant cells could be detected. The second treatment course did not further reduce the number of blasts in the bone marrow (14%). A complete remission was achieved only after the third course of chemotherapy (4 months after diagnosis). None months after diagnosis an increase in blasts was observed (19%), while the analysis for trisomy 10 with FISH remained negative.

In the leukemic cells of patient 2 multiple numerical aberrations were found. This patient was tested with probes for chromosome 17, X, and Y. Two timepoints were available. For each timepoint and for each probe the percentage of cells with aberrant FISH signals appeared to be lower than the percentage of blast found by cytology.

In patient 3, with a monosomy for chromosome 17 found by conventional cytogenetic analysis in 19 of 25 metaphases with aberrant karyotype, a similar observation was made. At 17 months after diagnosis the bone marrow contained 5% of cells with a monosomy 17 when analyzed by FISH but 18% blasts by cytology.

In patient 4, who was diagnosed with a tetrasomy for chromosome 8, FISH with the centromere probe for chromosome 8 did not reveal cells with three or four spots during the entire period of follow-up. One month after the fourth chemotherapy course, at t = 5 months (earlier sample points were not available for FISH), 6% blasts at this stage indicated a partial remission. Thereafter a continuous complete remission was observed (less than 5% blasts).

In patient 5 the percentage of aberrant cells that was observed at diagnosis with FISH was much higher than the percentage of blasts found with cytology, i.e., 98% vs. 44%, respectively. The percentage of cells characterized by −Y 4 and 4.5 months later, after chemotherapy, was 5 and 3, respectively, which is well above the lower detection level of this probe (see Table 1). Blast cell frequencies less than 5% indicate morphologic complete remission at these timepoints. Proof of imminent relapse was not

Table 3 Detailed cytogenetic analysis of eight patients in which no correlation was found between numerical chromosomal aberrations as measured by FISH and bone marrow cytology

Patient number (age at diagnosis)	Diagnosis	Karyotype at diagnosis
1 (25)	AML M1	47,XX,+10,t(11;17)(q23;q24)[37]
2 (18)	ALL	46,XY,t(2;10)(q32.2;q11)c[19] 56,XY,+X,+X,+Y,t(2;10)c+4,+5,+6,+14,+17,+21,+21[19]
3 (56)	RAEB	45,XY,der(5)t(5;17)(q21;q12),−17[2] 44,XY,idem,−7,[14] 43,XY,idem,−7,−14,der(19)t(14;19)(q13;q13)[3] 46,XY[6]
4 (70)	AML M2	48,XX,+8,+8,t(11;17)(p15;q23)[8] 46,XX[10]
5 (50)	AML M2	45,X,−Y,t(8;2;16;21)(q22;q32;q13;q22)[31]
6 (45)	AML M1	46,XY,t(1;15)(p21;23),t(3;5)(q23;q12),ins(4;12)(q28;p?),dic(5;17)(q11;p11),der(7)t(7;18;14)(7q11;18q12→q23;14q12→qter),+8,−14,del(18)(q12q23),add(19)(q23),+21[32] 46,XY[8]
7 (17)	ALL	60–68 <3n>,XY,add(2)(p16),−3,−5[6],add(9)(q34),+10,+12,−13,−16,−17,+del(18)(q21),−20,−21,+22,+2mar[cp10] 46,XY[5]
8 (45)	MM	50,XY,del(2q),+7,−8,+9,+11,der(12)t(8;12)(q?;p?),−13,+15,der(16)t(1;16)(q21;q?),+17+mar[4] 46,XY[11]

obtained since this patient died of a pneumonitis shortly after the last bone marrow sample was taken.

The same phenomenon was observed in patient 3. In this patient, 68% of the cells carried a monosomy for chromosome 7 at 17 months after initial diagnosis, while at this stage 18% blast cells were found in the bone marrow. Chemotherapy was given and after an initial decrease in numerically aberrant cell numbers, again an increase was observed after 19–20 months, which concurs with the rise of the percentage of blast cells in the bone marrow as judged by bone marrow biopsy. At 19 months an exact percentage of blasts could not be given; at 20 months, biopsy indicated an increase in the percentage of blasts ranging between 10% and 20%.

In a number of patients multiple numerical aberrations allowed the investigation with more than one chromosome-specific probe. In patients 2, 3, 7, and 8, the fluctuations that were observed in percentages of cells with aberrant numbers of spots for the various probes that were used did not correlate with each other. In patient 3, in contrast to the observations with the chromosome 7-specific probe, FISH with a probe specific for chromosome 17 (which was reported to be the second numerical aberration) showed low numbers of aberrant cells at all timepoints and no increase at the time of relapse.

DISCUSSION

Metaphase cytogenetic analysis provides detailed information about the karyotype of a limited number of cells. Interphase cytogenetic analysis such as FISH, on the other hand, provides limited information about the karyotype of a large number of cells. 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 are studied that are derived directly from the patient, selective outgrowth of subpopulations during short-term culture is prevented and a representative percentage of aberrant cells as present 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.

The presence of cells with an aberrant number of spots in healthy individuals sets the threshold for the lower detection level of aberrant cells in leukemia samples. Different statistical tests have been evaluated to determine the number of cells that has to be counted to observe significant differences between control samples and samples with aberrant cells [24]. Once approximately 400–500 cells per sample are analyzed, the lower detection level is on the order of 1–5%. In this study, bone marrow was analyzed that was derived from patients during and after remission-induction therapy. Whether FISH values obtained from bone marrow regenerated after chemotherapy can be compared to the peripheral blood values from healthy individuals remains questionable. The treatments that the patients undergo can very well be of influence on the level of so-called false positive or negative signals in such bone marrow cells. Comparing our control studies (Table 1) with reports by others [25, 26], it can at least be concluded that our FISH methodology yields comparable results.

To be able to reduce the detection level additional parameters are required. Such can be performed when FISH is combined with flow sorting. Until now this technique has worked only in sex-mismatched bone-marrow–transplanted patients where a discrimination can be made on the basis of the sex chromosomes [27].

In our study of 38 patients, we found that FISH data were generally in agreement with data obtained by cytology. Nevertheless, some patients had higher while others had lower numbers of leukemic cells, as determined by FISH, than the percentage of blasts counted in the bone marrow smears. The presence of a higher percentage of aberrant cells, as judged by FISH, in patients that morphologically are in complete remission (less than 5% blasts), can only be explained by the fact that cells that have lost their blast-like appearance carry the numerical aberration that is detected by FISH. A lower number of aberrant cells, as determined by FISH, 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. In addition, subclones with specific numerical aberrations can selectively be eliminated by chemotherapy in case they are more sensitive to the treatment. Furthermore, new clones may appear at relapse. Finally, blood taken with the bone marrow aspiration for FISH might cause a slight underrepresentation of the percentage of blasts. The cases from our studies illustrate these points and are discussed in further detail below.

In patient 1 at diagnosis, 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 marrow. This observation strongly suggests the presence of at least two subclones in the patient, of which one is carrying a trisomy for chromosome 10. Nevertheless, conventional cytogenetic analysis revealed an extra chromosome 10 in all the analyzed metaphases. Since conventional cytogenetics is based on a smaller number of cells and since only the dividing fraction can be studied, the nontrisomy-containing subpopulation of the leukemic cells might have remained unnoticed. In subsequent marrow studies the percentage of cells with a trisomy 10 as determined with FISH dropped and remained at an undetectable level while the cytology data revealed the presence of blast cells at several stages during the follow-up period. Apparently, the trisomy-10–containing leukemic subclone was more sensitive to the chemotherapy applied and was therefore eradicated.

In contrast, the diagnosis sample of patient 5, with a loss of the Y chromosome in all leukemic cells, 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 a complete remission (4% blasts in the bone marrow), 11% 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. This is often observed in these types of leukemia with a translocation involving chromosomes 8 and 21. The question whether these cells are fully differentiated or still retain leukemic potential remains unsolved.

Patient 3, characterized by a monosomy 7 and 17, showed a phenomenon similar to that observed in patient 5. 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. Striking is the fact that the fluctuation in the frequency of cells carrying a monosomy 17 correlated neither with the fluctuations in the percentage of blast cells nor with the percentage of cells with a −7 found by FISH at the same timepoints. Either the percentage of cells carrying the −17 is too small to observe significant fluctuations in cell numbers, or the centromere region of chromosome 17 is present and therefore in situ hybridization on interphase nuclei, applying the probe for the α-satellite DNA from chromosome 17, results in two fluorescent spots.

The karyotype analysis of patient 6 initially revealed the loss of one chromosome 7. This was not in concordance with the percentage of cells carrying the trisomy for chromosome 8. In a later stage it was found by chromosome painting on metaphases from this patient that a part of chromosome 7 including the centromere was present (indicated as der(7) in Table 3).

FISH is a valuable tool for the sequential analysis of bone marrow samples from leukemia patients whose leukemic cells carry numerical chromosomal aberrations. The speed and ease with which FISH can be performed make it an attractive technique for the assessment of treatment efficacy. The FISH method, in particular, is valuable on top of morphologic criteria in those leukemias where no other specific characteristics are available to distinguish leukemic cells from normal marrow cells. Discrepancies between FISH and cytology add valuable information and may be helpful in understanding the disease progression but are at the same time a potential source of erroneous conclusions. This paper demonstrates that care should be taken with respect to the choice of probes and that results should be interpreted with caution. FISH as it is used in this study depends on conventional cytogenetic analysis for the proper choice of probes. Both methods should therefore be used as supplements to each other.

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