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Detection and monitoring of trisomy 8 by fluorescence *in situ* hybridization in acute meyloid leukemia: a multicentric study

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Abstract

Background and Objective. **The role of fluorescence *in situ* hybridization (FISH) in the detection and monitoring of trisomy 8 (+8) in acute myelogenous leukemia (AML) has not been defined exactly. This multicentric study was performed in order to: i) analyze the sensitivity of interphase FISH with respect to conventional chromosome analysis (CCA) in detecting +8; ii) compare the results of FISH and CCA in the quantitation of the frequency of +8-positive cells; iii) analyze the possible role of FISH in the cytogenetic follow-up of patients with +8.**

Design and Methods. **One hundred and ninety-eight nonconsecutive patients with a diagnosis of AML seen at five centers over a 3-year period were studied by CCA and FISH with a chromosome 8-specific centromeric probe. Two hundred interphase cells were scored in each test and the cut-off for the recognition of +8 was set at 3%. An irrelevant pericentromeric probe was used as negative control in those cases with an apparently normal karyotype and trisomy 8 in interphase cells. FISH studies were conducted at diagnosis and, in 14 cases with +8, on 1-5 occasions during follow-up.**

Results. **Karyotype aberrations were seen in 121 cases (61.1%), with +8 being present in 38 of them (16 as the sole aberration). Interphase FISH detected +8 in 37/38 cases; in a patient with 1/10 metaphases with +8, 2.3% interphase cells with 3 signals were seen. Fourteen additional cases with occult +8 were detected by FISH, which showed 4-22% interphase cells with three signals; 6 patients had an abnormal karyotype without +8, 3 had a normal karyotype, 5 had no analyzable mitoses. In 24 cases with >15 analyzable metaphases, percent variations between CCA and FISH in the estimation of the size of the trisomic clone ranged between 0.4% and 51%, median value 22%. Underestimation of the percent of trisomy 8 by FISH occurred in all 10 cases with >90% +8 metaphases. In 7/14 cases investigated sequentially, FISH detected 5-35% trisomic cells in the BM after induction therapy (4 CR, 3 PR); 4 cases relapsed with +8 at 8-15 months. The absence of +8 in remission marrows was documented in the remaining 7 cases, 4 of which relapsed at 20-32 months.**

Interpretation and Conclusions. **It is concluded that FISH was a valuable method in this multicentric study since it showed greater sensitivity than CCA in detecting minor clones with +8, in patients with both normal and abnormal karyotypes. The role of FISH in the cytogenetic follow-up of trisomies in AML patients may be promising.**

Key words: acute myeloid leukemia, FISH, trisomy 8

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The detection of clonal chromosome abnormalities plays an important role in the diagnostic work-up of hematologic malignancies.[1-4]

Since it is independent of *in vitro* cell division, interphase fluorescence *in situ* hybridization (FISH) is more sensitive than conventional chromosome analysis (CCA) in detecting some primary chromosome changes in lymphoproliferative disorders with a low mitotic index.[5-7] Interphase FISH has also been consistently shown to be a specific and sensitive method for the study of numerical changes in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).[8] However, that this molecular cytogenetic method possesses greater sensitivity than CCA has not been clearly documented in myeloid neoplasias,[9,10] nor has its role in a cytogenetic follow-up been defined exactly.

Trisomy 8 is the most frequent numerical abnormality in AML, occurring in 5-6% of cases as an isolated anomaly[11] and in up to 20% of all cases in association with other chromosome changes.[12,13]

To better define the role of FISH in the detection of trisomy 8 in AML, this multicentric study was performed to address the following issues: a) the assessment of the sensitivity and specificity of this

molecular cytogenetic technique as compared to classical banding analysis; b) a comparison of results obtained by FISH and CCA in estimating the size of a trisomic clone; c) the significance of sequential FISH analysis in AML patients attaining hematologic remission after induction therapy.

Materials and Methods

Patients

One hundred and ninety-eight nonconsecutive patients presenting at the University Institutes of Hematology in Ferrara (77 cases), Rome (41 cases), Perugia (40 cases), Bologna (18 cases), and Pavia (22 cases) between 1993 and 1996 with a diagnosis of *de novo* AML or AML transformation of previous MDS (AML-MDS) were included in the present study. The FAB criteria for the recognition of different MDS and AML subtypes were used.[14-16] Patients were treated with different anthracycline-containing regimens. Elderly patients were treated with low-dose cytarabine.

Cytogenetic analysis

Routine cytogenetic investigations were performed according to standard methods[17] as part of the diagnostic work-up on bone marrow (BM) samples, which were usually separated by centrifugation over a Ficoll gradient and cultured for 4-48 hours in RPMI1640 additioned with 20-30% fetal calf serum. At least 10 metaphases were karyotyped in those cases having clonal chromosome aberrations. A minimum of 15 metaphases without chromosome changes, and usually twenty or more, were required for a case to be classified as *normal*. Karyotypes were described according to the ISCN nomenclature.[18]

FISH studies

FISH was carried out according to previously described protocols,[6] using a chromosome 8-specific pericentromeric probe (D2Z8; Institute for Human Genetics, Rotterdam, The Netherlands). This procedure was performed at diagnosis in all cases on the same samples that were submitted to cytogenetic analysis, as well as on normocellular BM after chemotherapy in 14 patients with +8 who attained complete hematologic remission (CR) (i.e < 5% BM blast cells) or partial response (PR) (i.e. 5-20% BM blast cells). The FISH experiments were conducted in the cytogenetic laboratories of each center, where the reactivity of the chromosome 8-probe with normal control samples was assessed. To reduce the heterogeneity of experimental conditions and interobserver variability in signal screening, a preliminary two-day meeting was organized at the cytogenetic laboratory in Perugia, with members of each participating center performing FISH experiments and interpreting hybridization results on normal control samples and on trisomic samples. Based on the results of these tests and on previous experience with centromeric probes used at the participating laboratories, the cut-off for recognizing trisomy 8 was set at 3% interphase cells showing 3 well-delineated signals. Two hundred interphase cells were scored in each experiment and FISH was repeated in those slides with no signal in more than 20% of the cells.

In those patients without +8 in their karyotypes who showed trisomy 8 in interphase cells, an irrelevant centromeric probe (chromosome 6) was used to rule out polyploidy.

Table 1. Recurrent chromosomal abnormalities at diagnosis in 121/198 cases of AML with clonal abnormalities.*

Chromosome abnormality	*Total no. of cases*	*Single abnormality*	*Subtype FAB (no. of cases)*
+8	38	16	M0(3); M1(7), M2(7), M3(4) M4(11), M5(7)
–7	9	0	M1(1), M2(3), M4(2), M5(3)
5q–	7	0	M0(1), M1(2), M2(1), M4(3)
11q23 aberrations	8	1	M2(3), M3(1), M4(4)
t(15;17)(q21;q21)	7	5	M3(7)
+11	5	0	M0(1), M1(2), M2(1), M5(1)
t(8;21)(q21;q21)	6	4	M2(4), M4(2)
inv(16)(p13q22)	4	2	M2(1), M4eos(3)
–18	4	0	M2(1), M4(3)
+18	3	0	M1(1), M4(1), M5(1)
+21	2	0	M1(1), M4(1)
t(9;22)(q34;q11)	2	2	M1(1), M5(1)

**Only those aberrations seen in at least 2 patients are included. For this reason the sum in column II does not add up to 121.*

Results

a) CCA and FISH at diagnosis

One hundred and sixty-nine cases presented with *de novo* AML, whereas AML-MDS was diagnosed in 29 patients with a previous history of RA (2), RARS (2), RAEB and RAEB-t (25).

Clonal chromosome anomalies were detected in 121 patients (61.1%) and normal karyotypes in 59 cases (29.8%). Inadequate mitotic yield was observed in 18 cases (9.1%). Recurrent primary chromosome anomalies are shown in Table 1, in correlation with FAB subtypes.

Thirty-eight patients had karyotypes with +8 (16 as a single aberration, 22 with additional changes). In one of these cases, however, only 1 in 10 cells

Table 2. Detection of +8 by FISH in different cytogenetic groups identified by CCA.

	Median no. of metaphases (range)	*No. of case with +8 by FISH (% interphase cells with +8)*
38 cases with +8 by CCA	14 (10-25)	37 (10%-88%)
83 cases with abnormal karyotype without +8	18 (10-20)	6 (4%-21%)
59 cases with normal karyotype	20	3 (7%-16%)
18 patients with no mitoses	0	5 (6%-22%)

was found with +8. FISH detected >3% interphase cells with three signals (range 10-88%, see Table 2) in all 37 cases with clonal trisomy 8; 2.3% cells with three signals were found in the patient with a non-clonal +8 in the karyotype. As shown in Table 2, interphase FISH was able to detect a minor clone with +8 that had been missed at CCA in 14 cases: in 6 of these cases karyotypic abnormalities other than +8 were present (10-20 cells analyzed); in 3 cases karyotypes were normal (18, 21, 22 cells analyzed) and in 5 cases no analyzable mitoses were obtained. A normal karyotype was found in phytohemagglutinin-stimulated peripheral blood lymphocytes in these patients, ruling out constitutional mosaicism of +8 and normal cells.[19] Thus, a total of 52 cases (26.2%) presented trisomy 8 in this series.

b) percentage of +8 by CCA and by FISH

A comparative analysis of the percentage of trisomic cells as assessed by CCA and by FISH was performed in 24 cases with more than 15 metaphases (Figure 1). Observed variations ranged between 0.4% and 55%, median 22%. Interphase FISH underestimated the number of trisomic cells in all 10 cases with >90% +8 metaphase cells, with variations ranging between 14% and 55%, median 31%.

In the remaining 14 cases, underestimation by FISH occurred in 7 patients (13%-32% variation range, median 17%) and overestimation in 7 (0.4%-48% variation range, median value 8%).

c) +8 in remission samples

FISH studies were performed in 14 patients with +8 who attained PR or CR after chemotherapy. These cases were assessed by FISH on 1-5 occasions (median 2) during PR or CR.

In 7 cases FISH detected 5-35% trisomic cells in remission marrows (4 CR, 3 PR): 4 cases relapsed with trisomy 8 at 8-15 months, whereas 3 were in cytologic CR at 6-18 months. Fewer than 3% interphase cells with evidence of trisomy 8 were documented in the remaining 7 patients, 4 of whom relapsed at 20-32 months.

Discussion

Over the last 5 years, the sensitivity and specificity of interphase FISH for the detection of trisomy 8 in myeloid neoplasias has been investigated in studies which have shown that: a) the false-positive rate of FISH analysis is very low;[20,21] b) FISH may detect virtually all cases with clonal trisomy 8, revealing erroneous interpretations at banding analysis in some cases; c) FISH may reveal +8 in cases with uncertain results at karyotyping. In addition, some myeloid neoplasias with apparently normal karyotypes were shown by FISH to carry trisomy 8 in interphase cells in a study by Jenkins *et al.*,[20] whereas other studies failed to demonstrate the superiority of FISH in detecting trisomic clones in the presence of an optimal cytogenetic analysis.[21,22] The results obtained in our multicentric study partially confirm and extend

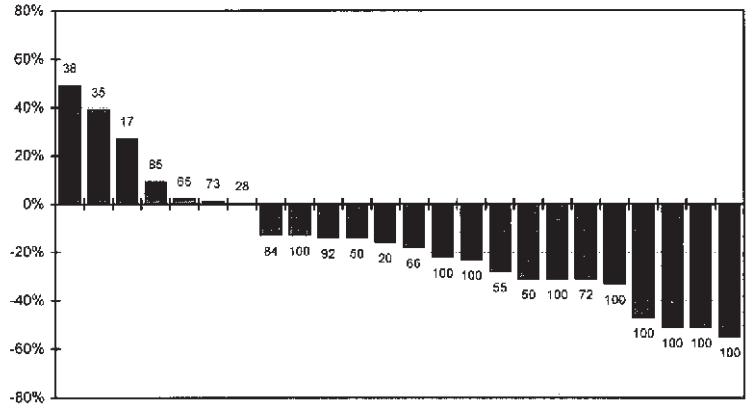

Figure 1. Percentage differences (y-axis) in the frequency of +8-positive cells as assessed by FISH and by CCA in 24 patients. The numbers at the top/bottom of each bar represent the percentage of +8-positive cells by CCA. Note that FISH overestimated the percentage of +8-positivity in 7 cases (% variation range: 0.4-48%), whereas underestimation was observed in the remaining 17 cases (variations ranging between 14% and 55%).

Table 3: Monitoring of trisomy 8 by FISH during the course of disease.

case	FAB	Age	Outcome of induction	Karyotype (diagnosis)	% +8 at diagnosis (FISH)	FISH % +8 remission (months) 6	12	18	24	30	% +8 relapse; duration of remission (months)	
1	M 4	60	CR	normal	11%	5%					17%	(9)
2	M 4	60	PR	normal	14%	7%	10%				23%	(15)
3	M5	81	PR	+8	74%	25%					88%	(8)
4	M 2	60	CR	+8	60%	5.6%					68%	(8)
5	M 4	45	PR	+8	48%	16%	15%	18%			/	(18+)
6	M 5	67	CR	+8	97%	35%					/	(6+)
7	M2	70	CR	normal	16%	10%					/	(10+)
8	M 2	73	CR	normal	7%	1%	3	6%			17%	(20)
9	M 5	27	CR	+8	59%	1%					19%	(20)
10	M 4	33	CR	+8	77%	0%	1%				52%	(24)
11	M 2	65	CR	+8	64%	5%	2%	2%	3%	7%	70%	(32)
12	M 3	21	CR	+8	53%	1%					72%	(NA)
13	M 5	50	CR	+8	5%	1%	6%				/	(12+)
14	M 3	22	CR	+8	58%	2%	1%	ND			/	(18+)

Abbreviations: NA = not available, ND: not done; + indicates that the patient is in continuous complete (or partial) remission.

these observations.

Despite the fact that previous studies have found that the false-positive rate for trisomy in interphase cells may be as low as 1% or less,[20] we agreed on a 3% cut-off value because of the multicentric nature of the study and because procedures for limiting the false-positivity rate, such as co-hybridization of the chromosome 8-probe and of a control probe, were not adopted.

At diagnosis, FISH was able to detect interphase cells with three signals targeting the chromosome 8 centromere in 37/38 cases with +8 in their karyotypes. The only patient with +8 in the karyotype (1 out of 10 cells) who was not recognized by FISH had a minor trisomic clone that accounted for 2.3% of interphase nuclei. This very high sensitivity rate, which was obtained both in *de novo* AML and in AML-MDS , is in line with data reported in single-institution studies[10, 21, 23] and shows that FISH can be safely employed for the detection of numerical changes in multicentric trials.[24]

The comparison between CCA and FISH in estimating the size of the trisomic clone deserves attention. As shown in Figure 1, underestimation by FISH with respect to CCA occurred frequently in those cases with a high percentage of trisomic metaphase cells. At least 4 factors may account for these observed discrepancies: a) sub-optimal hybridization, precluding unequivocal identification of trisomy 8 in some cells; b) the presence of lymphocytes, especially in those BM samples prepared for FISH by centrifugation over a Ficoll gradient that retained blast and mononuclear cells; c) differences in the *in vitro* mitotic index of distinct clones, influencing the abnormal-to-normal metaphase ratio; d) the relatively low number of karyotypes analyzed.

The finding of cytogenetically undetected +8 in 14 out of 160 patients (8.75%) revealed a high incidence of occult trisomy 8 in this series as compared to previous studies.[9, 21] Even though dual color FISH was not performed in this study, the hybridization of these cases with an irrelevant centromeric probe ruled out the presence of polyploidy or sub-optimal hybridization with erroneous counting of artifactual spots. Our 14 cases with occult +8 carried a minor trisomic clone that represented 4%-22% (median 11%) of interphase cells. Interestingly, while eight of these cases had a normal karyotype or no analyzable metaphases, suggesting that the abnormal clone had a low *in vitro* mitotic index, karyotype aberrations without +8 were detected by CCA in the remaining six cases that had 4-21% tri-signalled interphase cells. Analysis of > 30 G-banded mitotic figures was performed in 4 patients for whom additional fixed cells were available, and this revealed at least 1 metaphase with an extra copy of chromosome 8 as an additional aberration. The possibility should also be considered that, in some cases with complex karyotypes, hybridization of the chromosome 8-centromeric probe to interphase cells could yield 3 signals in the presence of supernumerary chromosome markers with the chromosome 8 centromere.

Thus, FISH may be a useful complement to CCA for the detection of occult aneuploid clones not only in cases with a normal karyotype or an inadequate mitotic yield, but in those with abnormal karyotypes as well. It is worth noting that follow-up data in 4 cases with occult +8 documented the reduction/disappearance of the trisomic clone after induction therapy, followed by its regrowth at relapse (cases #1, 2, 7, 8; see Table 3), suggesting that the acquisition of + 8 provided the leukemic clone with a relative growth advantage both at diagnosis and at relapse. Fluctuations of the size of the trisomy 8-positive clone is a well-documented phenomenon in MDS,[25,26] indicating that +8 in myeloid neoplasias may frequently be a secondary event, possibly associated with unidentified genetic defects that play a role in the evolution of the malignant clone.

The employment of FISH to detect occult trisomy 8 in some cases may in part account for the relatively high incidence of this anomaly observed in our series.[11,13] An additional explanation may derive from the consideration that the incidence of karyotype anomalies, including +8, was previously found to be associated with environmental exposure to myelotoxic agents.[27] We therefore undertook a retrospective analysis of the frequency of exposure to myelotoxic agents in the patients seen at the major contributing center in this study. On review of the clinical records reporting information about professional exposure, we found 29/77 patients who had been exposed to pesticides or solvents for more than 5 years. A statistically significant association (p=.007) was found between +8 and exposure to myelotoxic agents (13/21 patients with trisomy 8 could be classified as *exposed*, as compared with 16/56 patients without trisomy 8). Interestingly, trisomy 8 was present as an additional change in 8/11 exposed patients with analyzable karyotypes, suggesting that the development of multiple chromosome aberrations is associated with prolonged exposure to myelotoxic agents.

Due to the limited number of cases analyzed and to the relatively short period of observation, our data on the role of FISH follow-up in post-remission samples must be interpreted with caution. Reduction in the size of the trisomic clone was invariably observed after induction treatment, with a shorter CR duration (median 8 months) in 4 assessable cases with residual trisomy 8-positive cells than in 4 assessable patients without +8 in remission marrows who relapsed at 20 (2 cases), 24 and 32 months. It is worth noting that a gradual increase in the percentage of trisomic cells was observed in the remission phase in 3 cases (#2, 8, 11) who eventually relapsed, suggesting that the expansion of the cytogenetically abnornmal clone may herald hematologic relapse.

In conclusion, FISH proved to be very efficient in detecting trisomy 8 in this multicentric study, showing greater sensitivity than CCA in detecting minor trisomic clones both in those with normal karyotypes and in patients with chromosome anomalies other than +8. While several technical factors may preclude a precise quantitation of the frequency of +8 cells by FISH and by CCA, the role of FISH in the cytogenetic follow-up of trisomies in AML appears to be promising, especially in view of the lack of more sensitive molecular genetic methods for the detection of aneuploidy.

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GLC, AC, GA, MM, CMec, PB and AZ were responsible for the conception of the study, its design, funding, and data handling. RB, MGR, AB, GMR, NP, NT, CC, CMatt, RLS, PC, EG and MN performed cytogenetic and FISH analyses. All the authors contributed to the analysis and writing of the paper. With respect to the order in which the names appear, the authors are grouped according to centers and these latter appear according to the number of cases contributed. The last name indicates the principal clinician involved and the senior author.

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Disclosures

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