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*Recent Advances in the Cytobiology of Leukemias**

DETECTION OF NUMERICAL ABERRATIONS IN HEMATOLOGIC NEOPLASIAS BY FLUORESCENCE IN SITU HYBRIDIZATION

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ABSTRACT

Background and Objective. Over the last 5 years, fluorescence *in situ* hybridization (FISH) techniques have had an important impact on molecular cytogenetic diagnosis, providing a better understanding of the role of numerical aberrations in hemopoietic neoplasms. The objective of this article is to analyze the clinical applications of FISH in the management of hemopoietic malignancies.

Evidence and Information Sources. The material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index® and Medline®, and personal published and unpublished data.

State of art. FISH technology has the advantage of being relatively simple, fast and flexible. Published data and ongoing prospective studies show that, under well-controlled experimental conditions, interphase FISH is more sensitive than conventional metaphase analysis in the detection of numerical abnormalities. Due to the relatively high rate of false positive results, FISH cannot be used for the study of minimal residual disease. However, since molecular strategies for the detection of small-sized aneuploid clones have not been developed yet, FISH represents a useful adjunct to conventional cytogenetics, especially for the quantitation of the size of abnormal clones during the course of the disease and to monitor XX/XY chimerism following sex mis-matched bone marrow transplantation. Different approaches to the study of multiple cell-lineage involvement by chromosome changes have been developed that take advantage of FISH techniques by: a) simultaneous FISH and membrane immunophenotyping of cytologic and histologic preparations; b) two-step analysis based on assessment of the morphology of cells on panoptical stains, with subsequent hybridization and relocation of previously identified cells; c) FISH analysis of enriched cell fractions obtained by cell sorting or by separation of bone marrow cells on a density gradient, and d) study of single hemopoietic colonies grown in semisolid media.

Perspectives. New molecular cytogenetic techniques, such as dual color FISH *comparative genomic hybridization*, are at hand that will greatly improve the diagnostic power of cytogenetics and make FISH increasingly useful in research laboratories as well as in clinical practice.

Key words: chromosomes, cytogenetics, FISH, leukemia, lymphoma, hematologic malignancies

Major advances in the detection of clonal chromosome changes in hemopoietic neoplasms have occurred throughout the last decade. Variation of standard culture methods according to the type of malignancy under investigation, amelioration of banding techniques and the employment of growth factors that promote *in vitro* cell divisions have resulted in a significant improvement in the interpretation of abnormal karyotypes.

Recently, fluorescence *in situ* hybridization (FISH) techniques have been developed that have had an important impact in most cytogenetic laboratories. FISH can be applied to both fresh and frozen cells, to preparations for conventional chromosome analysis and to archival material, including blood smears and histologic sections. The advantages of this molecular cytogenetic technique may be summarized as follows: a) it permits the study of chromosome changes by scoring large numbers of non-dividing cells in a relatively short time; b) it enables the cytogeneticist to better define the nature of complex structural changes in metaphase cells; c) it allows estimation of the size of abnormal clones in interphase cells, and d) it may assist in determining

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the lineage of cells carrying a chromosome abnormality. All these issues have been the subject of recent reviews.[1-3]

Perhaps the major limitation to FISH is that only one abnormality can be tested in each hybridization experiment, making karyotype definition impractical with this technique. This limit has been partially overcome by modifications in the methodology that significantly reduce the costs and may permit the introduction of this method in routine screening for the detection of numerical abnormalities.[4] As summarized in Table 1, molecular cytogenetic diagnosis of human tumors is largely dependent on the availability of different types of probes that recognize different structures on human chromosomes.[5]

With regard to the detection of numerical clonal changes in hemopoietic neoplasms, three major areas of investigation that deserve attention have been pursued over the last 5 years: 1) the sensitivity of FISH in the detection of the most frequent numerical abnomalities in lymphoid and myeloid neoplasias; 2) the understanding of lineage involvement by chromosome changes; 3) the possible role of FISH in the study of residual disease in leukemia and lymphoma.

Methodological aspects

Technical steps that permit reproducible detection of targeted DNA sequences by FISH have been widely reported in the literature.[6] The selection of adequate controls in each laboratory is critical for correct interpretation of results, especially when detecting minor clones or when the size of an abnormal clone is under scrutiny.

The reactivity of each test probe to normal BM and/or PB samples must be assessed in order to set the cut-off point for positivity for monosomy or trisomy. Experiments carried out so far in normal samples have found a false positivity rate of approximately 1% for trisomy and of 2-3% for monosomy. There is agreement that the cut-off point for recognizing a case affected by trisomy or monosomy should be set at the upper 95% confidential limit[1] (i.e. approximately 3% for trisomy and 7-10% for monosomy).

Another issue key to avoiding false positive results is the need to assess the target DNA characteristics by hybridizing the test DNA with a control probe. This appears to be of major importance when estimating the size of an abnormal clone, since ineffective hybridization due to sample degradation may occur. Dual color FISH with simultaneous hybridization of the test probe and of an adequate control probe may be a reasonably good approach for safe interpretation of data deriving from signal screening.

Sensitivity of FISH in the detection of numerical changes

Lymphoid neoplasias

A growing body of evidence has accumulated in the early 90's that FISH is more sensitive than conventional chromosome analysis in the cytogenetic assessment of low-grade B-cell lymphoid neoplasms. Following the first reports documenting that some patients with B-cell chronic lymphocytic leukemia (CLL) with normal karyotypes may carry trisomy 12 in interphase cells,[7,8] a number of studies have demonstrated the following:

a) 5-25% of B-CLL without analyzable metaphases or with normal karyotypes have in fact trisomy 12.[9] This anomaly has been found in frequent association with atypical morphology.[10] In most cases only a fraction of the neoplastic B-lymphocytes carries +12. This abnormality is usually detectable at presentation and does not appear to be acquired at disease evolution. Although the relative size of the trisomic clone did not significantly change over time in some untreated patients,[11,12] an increased percentage of trisomic cells was observed in one patient after reduction of the lymphocyte count had been obtained by alkylating agents, possibly suggesting drug resistance by the trisomic clone;[8]

b) numerical chromosome changes can be found in the large majority of multiple myelomas (MM),[13,14] the most frequent being trisomy 3, 7 and 11 and monosomies involving chromosomes 17 and X. It is noteworthy that results of cytogenetic analysis in MM have been disappointing due to the low mitotic rate of malignant plasma cells, although significant progress can be provided by growth factors such as interleukin-6 and granuloctyte-monocyte colony-stimulating factor (GM-CSF);[15,16]

c) in a similar fashion, many monoclonal gammopathies of undetermined significance (MGUS) can be shown to be aneuploid.[17,18] Interestingly, involvement of the same chromosomes that are usually missing or present in extra copies in MM has been shown to occur in this clonal proliferation. These preliminary data suggest that similar cytogenetic events may underlie the develoment of MGUS and MM, raising the possibility that as the chromosome changes relevant to myelomagenesis are identified, chromosome studies may predict the evolution of the former condition into the latter;

d) chromosome changes that specifically involve $CD30^+$ Reed-Sternberg cells and spare reactive lymphocytes are consistently detectable in Hodgkin's disease.[19] In those cases with concurrent FISH and karyotyping, a 100% concordance rate was obtained with these two complementary techniques, revealing a variety of clonal chromosome anomalies whose significance is gradually being elucidated;

Table 1. Most frequently used DNA probes for fluorescent in situ hybridization.

Probes	*Hybridization pattern and comments*
Alphoid or satellite probes	Strong signal (target sequences present in hundreds of copies) located in the centromeric region of each individual chromosome; with few exceptions probes are available that allow recognition of each autosome or sex- chromosome in interphase and metaphase cells
Telomere probes	Medium intensity signal deriving from hybridization to the chromosome-specific repeating DNA units that form the telomeres
Cosmid probes	Hybridize to homologous sequences, giving a weak signal (they usually hybridize to single copy genes). They can be used for the demonstration of small-sized deletions or trisomies involving the complementary DNA sequence, or to detect by dual color FISH the translocation of the target sequence in proximity of another genomic region
Yeast artificial chromosome (YAC) clones	Large human insert targeting up to Mbs homologous DNA. They give a medium intensity signal and when a translocation breaks the complementary DNA locus, 3 signals in interphase cells are generated, one deriving from the normal allele and two deriving from the allele involved in the translocation.
Chromosome-specific libraries	They recognize sequences distributed throughout a given chromosome, "painting" the entire chromosome in metaphase cells. They are useful for detecting translocation of portions of the target chromosome
Chromosome band-specific microdissection libraries	They recognize DNA sequences of a chromosomal band, detecting subtle translocations involving the target region

e) numerical chromosome changes occur infrequently in hairy cell leukemia (HCL),[20] although it is worth noting that only some of the centromere-specific probes currently available have been tested systematically. In a recent study, total or partial trisomy 5 was reported in 8/36 cases, a finding that warrants future investigation by FISH.[21] Trisomy 12 was found in one case of hairy cell-variant displaying cytologic features that are intermediate between classical HCL and prolymphocytic leukemia.[12]

Myeloid neoplasias

The addition of FISH to existing karyotyping procedures has led to a more definite assessment of the cytogenetic profile of myelodysplastic syndromes (MDS) and acute myelogenous leukemias (AML).[22]

The sensitivity of FISH in the detection of numerical abnormalities in myeloid neoplasias has been the subject of several investigations. In a study of 216 patients by Jenkins and coworkers,[23] including 61 cases with trisomy 8 identified by karyotypes, 55 showed evidence of trisomy 8 by interphase FISH, a sensitivity of 90.2%. Interestingly, in all 6 cases with negative FISH, only a minority of metaphase cells carried trisomy 8, suggesting that a minor subclone had escaped detection by interphase cytogenetic analysis. Moreover, the specificity of FISH proved to be satisfactory with respect to conventional chromosome analysis, since 2 normal signals targeting the centromere of chromosome 8 were seen in the majority of interphase cells in 140/142 cases with normal karyotype. In 2 cases in this study, FISH proved to be superior to chromosome analysis by demonstrating some interphase cells with three chromosome-8-centromere signals, a finding confirmed at subsequent karyotyping of a large number of cells.

In other studies, FISH provided valuable information concerning the presence of trisomy 8 in some cases with non-diagnostic karyotypes, but it did not detect more patients with +8 than conventional cytogenetic techniques in those cases with an adequate mitotic yield.[24] Similar findings were described by two French groups, both indicating that FISH was able to detect minor clones with monosomy 7 in MDS that had escaped detection at metaphase analysis.[25,26]

In an attempt to better define the role of FISH in the detection of numerical abnormalities in AML, a prospective study is being carried out by the cytogenetic committee of the GIMEMA Italian cooperative study group to compare the efficacy of conventional cytogenetics and FISH in the detection of +8, the most common numerical change in AML. From January 1995 to June 1996, five centers (Ferrara, Pavia, Perugia, Bologna, Rome) studied 167 newly diagnosed AML patients enrolled in the AML10 GIMEMA-EORTC protocol and found metaphase and interphase cells with clonal trisomy 8 in 32 cases (18.4%). In 13 additional cases, the presence of a subclone with +8 was demonstrated by FISH analysis, showing that FISH is very efficient in detecting small-sized clones with trisomy 8.

Chromosome anomalies and lineage involvement

Basically, four approaches has been adopted that assist in assigning chromosome anomalies to a specific cell lineage: 1) simultaneous FISH and mem-

brane immunophenotyping on cytologic and histologic preparations, also referred to as the MAC technique[27] or the FICTION method;[28-30] 2) two-step analysis based on assessment of the morphology of cells on panoptical stains, with subsequent hybridization using an appropriate probe and relocation of previously identified cells;[31,32] 3) FISH analysis of enriched cell fractions obtained by cell sorting[33] or by separation of bone marrow cells on a density gradient;[34] 4) FISH study of single hemopoietic colonies grown in semisolid media.[35,36] (see Table 2 for some relevant achievements).

Role of FISH in the study of residual disease

Due to the relatively high rate of false positive results with current techniques, there is a general consensus in the literature that FISH can detect trisomic or monosomic clones accounting for approximately >3% and >10% of total cellularity, respectively. Although estimation of the size of abnormal clones must be undertaken with caution, it is worth pointing out that automated systems for signal screening are being developed which will probably improve the sensitivity of FISH, allowing enumeration of large numbers of cells.[37] In addition, increased sensitivity has recently been reported in a study of acute lymphoblastic leukemia in remission that employed dual color FISH and two centromere-specific probes in each experiment, along with an adequate selection of positive and negative controls.[38]

FISH has the obvious advantage over chromosome analysis of being independent of the cell-cycle status, offering the possibility of assessing the entire cell population of mitotic and interphase cells. Since reproducible molecular strategies that permit detection of small-sized aneuploid clones have not been developed yet, FISH represents a useful adjunct to conventional cytogenetics, especially for quantitation of the size of abnormal clones during the course of the disease.[39]

An interesting approach to the study of residual disease was undertaken by Anastasi *et al*.[40] who studied suspicious blast cells that persisted in the marrow of two ALL cases in bordeline cytologic remission that presented a hyperdiploid karyotype with trisomy 17 at diagnosis. The immature cells were identified morphologically and relocated under fluorescence microscopy following FISH with a chromosome-17-specific probe. These authors were thus able to document the persistence of immature cells with trisomy 17 in one patient who relapsed shortly thereafter, and demonstrate a normal chromosomal complement in the other patient who maintained a prolonged remission.

FISH is also increasingly being used to monitor XX/XY chimerism or to identify the origin of cells in relapse following sex mis-matched bone marrow transplantation.[41,42] Interestingly, a 0.1% sensitivity for the detection of Y-bearing cells in male recipients transplanted with a female marrow was reported by van Dekken *et al*.[43]

FISH may also play a relevant role in the cytogenetic study of mobilized progenitor blood cells, and studies comparing this molecular cytogenetic approach with conventional cytogenetics have recently been described.[44]

Table 2. Outcome of investigations of lineage involvement by numerical chromosome aberrations.

Method	*Results*	*References*
Simultaneous FISH and membrane immunophenotyping	Heterogeneity of myeloid lineage involvement by -7 and +8 in MDS. The B- and T-cell lineages are not affected	29,33,54,55
	Involvement of the myeloid lineage by +8 in polycythemia vera	56
	Trisomy 7 affects tumor-infiltrating CD4+ lymphocytes in tumors and Ki1+ lymphomas	30
	Numerical abnormalities specifically involve CD30+ Sternberg-like cells in Hodgkin's disease	19
	+12 involve CD11c+cells in hairy cell variant	12
Morphologic study and FISH analysis of previously identified cells	Assignment of numerical aberrations to residual suspicious blast cells after remission induction in ALL; correlation with early relapse	40
	Numerical changes involve the myeloid and not the lymphoid lineage in MDS and AML	32
	Both trisomic cells and disomic cells are sensitive to in vivo GM-CSF stimulation in MDS with +8	31
	Demonstration of different patterns of lineage involvement by numerical aberrations in de novo AML with respect to AML following MDS	57
	Numerical abnormalities in CML lymphoid blast crisis may involve the differentiating myeloid cells and spare the lymphoblasts in some patients. The blast cells are always involved in myeloid blast crisis	58
	+8 in blast crisis of CML affects multiple cell lineages. Disease progession is associated with expansion of the +8 component	59
	In myelodysplastic syndromes, monosomy 7 can occur in cells capable of differentiation along granulocytic and monocytic lineages, but not along the lymphocytic lineage.	61
Analysis of enriched cell fractions	The CD34+ stem cell compartment in AML may carry the same numerical abnormalities as the deriving neoplastic clone in both CD34+ and CD34- leukemias	60
	Trisomy 11 may involve both the granulomonocytic and the erythroid lineage in AML-M4 with trilineage myelodysplasia	34

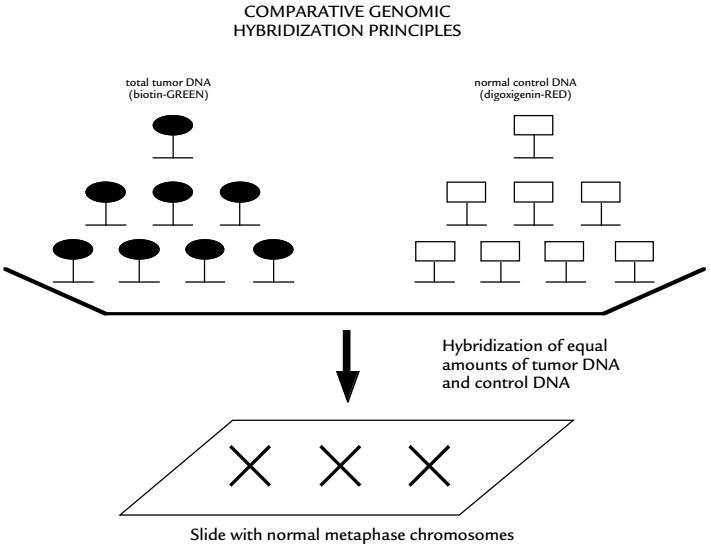

Figure 1. Principles of comparative genomic hybridization. Equal amounts of total tumor DNA and normal control DNA are co-hybridized in a single experiment to normal metaphase chromosomes. The sample DNA and the control DNA are differently labelled (i.e. biotion- and digoxigenin-labelled). If over- or under-representation of an entire chromosome or a part of one is present in the tumor sample, an imbalance in the green-to-red fluorescence intensity ratio will be obtained along the corresponding metaphase chromosome, indicating where the DNA gain or loss has occurred in the test tumor. For instance, trisomy or monosomy 8 in the tumor DNA in the example depicted in the figure would result in a fluorescence shift towards green or red, respectively, on chromosome 8 in the metaphase plate, which can be safely recognized by a subsequent banding technique. Quantitative fluorescence digital analysis is necessary for objective interpretation of results.

Perspectives and conclusions

Developments in molecular genetic techniques are at hand that will greatly improve the diagnostic power of FISH by making combinations of probes available for detection of the majority of primary chromosome changes in hemopoietic neoplasms.[45,46] Dual color FISH revealing simultaneous hybridization of differently labelled probes (i.e. test probe and control probe) to target DNA sequences is likely to improve the sensitivity and specificity of this technique by reducing the rate of false positive and false negative results.[47,48]

A novel approach to molecular cytogenetic diagnosis has been developed that may potentially modify the strategy for detecting numerical chromosome changes. Indeed the so-called *comparative genomic hybridization* (CGH)[49] allows rapid and comprehensive assessment, in a single experiment, of chromosomal DNA gain or loss occurring in a tumor sample. The rationale behind CGH is that by co-hybridizing equal amounts of biotin-labelled tumor DNA and digoxigenin-labelled normal DNA to normal metaphases, different ratios of green-to-red fluorescence intensity can be obtained along each metaphase chromosome, depending on the relative amounts of DNA present in the tumor and in the control sample (see Figure 1). The application of this technique, which requires cytogenetic, molecular genetic and cytofluorimetric expertise, is not yet widespread[50] and efforts aimed at standardizing automated systems for image detection and analysis are being carried out.[51] Over the last 4 years preliminary data have accumulated showing that CGH may be of value for the study of numerical aberrations in fresh samples and in formalin-fixed tissues, as well as for the assessment of clonal evolution and for the identification of DNA amplification sites in different types of human tumors.[52] Although balanced translocations escape detection at CGH, hematologic neoplasms with a low mitotic index such as B-CLL and low-grade non Hodgkin's lymphomas are excellent candidates for CGH study, and interesting preliminary data have been reported.[53]

Thus established methods and newly developed techniques are making FISH a major tool for the refinement of cytogenetic diagnosis, particularly useful in clinical practice for monitoring the size of numerically abnormal clones.

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