

DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIA

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Many adverse prognostic factors have been identified in acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML), including an increased leukocyte count and certain genetic features of the leukemic blast cells.⁽¹⁾ Yet none of the features can reliably identify patients who require additional and more intensive therapy to avert relapse. While contemporary treatment induces clinical remission in up to 98% of children with ALL and 85% of those with AML,⁽¹⁾ mathematical models predict that some of the patients may still have as many as 10¹⁰ leukemic cells.⁽²⁾ Currently, such variations in leukemia burden cannot be accommodated in clinical management strategies. That is, patients with high numbers of residual leukemic cells may receive essentially the same type and intensity of therapy as patients with much smaller leukemia infiltrates or no leukemia at all.

Recent advances in molecular techniques and immunologic assays have fueled the study of minimal residual disease (MRD, defined leukemic cells undetectable by morphologic examination).⁽³⁾ In theory, early detection of residual leukemia before the emergence of drug-resistant blasts would allow more timely, hence more effective, therapeutic intervention. This review summarizes recent advances in the development of MRD assays and their clinical application.

Methodological Considerations

Cell Karyotyping

Improved cytogenetic techniques can identify clonal chromosomal abnormalities in 90% or more of ALL or AML cases at diagnosis. The use of karyotyping methods to monitor MRD, however, is laborious and depends on the number of analyzable metaphases. Although the sensitivity of this approach is of the same order as that of morphologic examination ($\approx 5\%$), its accuracy is superior. With use of chromosome-specific and gene-specific probes to identify numeric and structural chromosomal abnormalities, fluorescence in situ hybridization (FISH) techniques provide interpretable results on interphase (nondividing) cells, increasing the chance of identifying residual leukemic cells with a low proliferative rate. Nonetheless, because of certain inherent technical difficulties, the sensitivity of MRD analysis by FISH approaches only 1%.⁽⁴⁾ Flow cytometric analysis of cells labeled with dyes specific for adenine-thymidine and guanine-cytosine-rich DNA can also be used in karyotyping, but this approach is not more sensitive than the above methods.⁽⁴⁾ The nuclear DNA content of cells, determined by flow cytometric analysis of cells labeled with DNA-binding fluorochromes (e.g., propidium iodide), affords another measure of MRD in most hypodiploid and hyperdiploid cases, but its sensitivity varies according to the extent of aneuploidy.

Cell Culture Techniques

Although culture of remission bone marrow has been used to detect MRD in both AML and ALL, this approach has numerous shortcomings.⁽³⁾ First, the culture conditions often reflect poorly defined components, and the lack of standardized methods of culture preparation makes it difficult to establish a clinically useful threshold level of clonogenic leukemic cells that can be applied in prospective studies. Even when clonogenic cells are detected, additional characterization of each individual colony, by karyotyping, immunophenotyping, antigen-receptor gene analysis or the polymerase chain reaction (PCR), is necessary to establish the leukemic origin of the colonies. In this regard, modified colony assays, based on cell sorting or PCR examination of the plucked colonies, were recently developed to study MRD in ALL.⁽⁵⁾ Finally, leukemic blasts that have the potential to cause relapse may fail to grow in culture systems; indeed, only a limited number of leukemic colony-forming cells can be expected to grow in available in vitro systems.

Detection of Antigen-Receptor Gene Rearrangements

In cases of ALL, clonal rearrangements of the variable, diversity, and junctional gene segments of the immunoglobulin (Ig) heavy-chain gene or T-cell receptor (TCR) genes can be used as markers of MRD if one applies PCR amplification and nucleotide sequencing. The PCR-amplified signal can be detected in ethidium bromide-stained gels when leukemia-specific primers are used, or by radioisotope-labeled clonospecific probes when consensus primers are employed. Enzymatic gene amplification by PCR can detect rearranged genes with greater sensitivity (one leukemic cell per 10³ to 10⁵ normal cells) than Southern blot analysis of nonamplified DNA (one per 20 to 100 cells). However, the utility of this method is confounded by the presence of multiple patterns of gene rearrangement at diagnosis (oligoclonality) or changes in gene rearrangements during the course of the disease (clonal evolution). Recent studies suggest that, with monitoring of two or more functional regions of multiple genes (e.g., immunoglobulin, T-cell receptor-g, and/or T-cell receptor-d), PCR detection of MRD is possible in approximately 90% of cases and reduces the false-negative rate due to clonal evolution to 10% or less.^(6,7)

Table 1. Specific Genetic Abnormalities in Acute Leukaemia

Translocation	Genes Involved	Freq. (%) [*] Adult	Freq. (%) [*] Childhood
B-lineage ALL			
t(9;22)(q34;q11)	BCR-ABL	15-25	3-5
t(1;19)(q23;p13.3)	E2A-PBX1	3-5	5-6
t(17;19)(q22;p13)	E2A-HLF	< 1	< 1
t(5;14)(q31;q32)	IL3 (IGH)	< 1	< 1
t(4;11)(q21;q23)	MLL-AF4	5	2

t(12;21)(p12-13;q22)	TEL-AML1	Unknown	25**
11q23 translocations	MLL-AF<	Unknown	2
B-cell ALL			
t(8;14)(q24;q32)	MYC (IGH)	5-8	1-2
t(2;8)(q12;q24)	MYC (IGk)	1	< 1
t(8;22)(q24;q11)	MYC (IGl)	1	< 1
T-cell ALL			
TAL	TAL-SIL	5-7	4
t(8;14)(q24;q11)	MYC (TCRD) < 1		< 1
t(10;14)(q24;q11)	HOX11 (TCRD)	3	< 1
t(7;10)(q35;q24)	HOX11 (TCRB)	< 1	< 1
t(11;14)(p15;q11)	TTG1 (TCRD)	< 1	< 1
t(7;11)(q35;p13)	TTG2 (TCRB)	< 1	< 1
t(11;14)(p13;q11)	TTG2 (TCRD)	1	1
t(1;7)(p32;q35)	TAL1 (TCRB)	< 1	< 1
t(1;14)(p32;q11)	TAL1 (TCRD)	1	< 1
t(7;9)(q34;q32)	TAL2 (TCRB)	< 1	< 1
t(7;19)(q34;p13)	LYL1 (TCRB)	< 1	< 1
t(1;7)(p34;q34)	LCK (TCRB)	< 1	< 1
t(7;9)(q34;q34)	TAN1 (TCRB)	< 1	< 1
AML			
t(3;21)(q26;q22)	AML1-EAP	< 1	< 1

t(8;21)(q22;q22)	AML1-ETO	6-12	8-15
t(6;9)(p23;q34)	DEK-CAN	1	< 1
t(9;22)(q34;q11)	BCR-ABL	3	< 1
t(15;17)(q21;q21)	PML-RARA	5-15	6-10
t(11;17)(q23;q21)	PLZF-RARA	< 1	< 1
inv(16)(p13q22)	CBFb-MYH11	3	8-15
inv(3)(q21q26)	EVI1	1	< 1
t(9;11)(p21-22;q23)	MLL-AF9	1-2	7-9
t(1;22)(p13;q13)	Unknown	< 1	2-3

* Based on literature review

** Based on molecular analysis

More than 20 partner genes are involved in fusion events with MLL

Detection of Chromosomal Breakpoints by PCR

eukemia-specific chromosomal translocations provide disease markers that can be detected by PCR analysis at a high level of sensitivity (10⁻⁴ to 10⁻⁶). If the designed PCR primers matching conserved regions that flank the translocation are separated by a relatively short stretch of DNA (no more than a few hundred base pairs), then genomic DNA can be used. Otherwise, a cDNA fusion transcript, from which intervening sequences have been spliced, is a preferable target for reverse transcription-PCR (RT-PCR) analysis. To date, up to 50% of ALL and 45% of AML cases in children have identifiable chromosomal translocations that are candidates for PCR analysis (Table 1). It should be noted that the most common chromosomal alterations both in B-lineage ALL (TEL-AML1) and T-cell ALL (TAL-SIL) represent cryptic translocations that are not evident at the karyotypic level and can only be identified by molecular analysis. Because of their potential clinical implications, we and others prospectively screen for certain translocations in all newly diagnosed patients: i.e., BCR-ABL, E2A-PBX1, MLL-AF4 and TEL-AML1 in ALL, and AML1-ETO, PML-RARA, CBFb-MYH11 and MLL-AF9 in AML.

Detection of MRD with Immunologic Methods

since virtually all antigens expressed on malignant cells are also present on corresponding normal cells, multiparameter flow cytometric analysis measuring two or more cell-surface antigens simultaneously is being used to detect the presence of leukemic cells.⁽³⁾ The rationale is that “aberrant” expression of certain combinations of antigens is a characteristic feature of some leukemias. Recently, several apparently

leukemia-specific monoclonal antibodies were used in single-color flow cytometric studies of MRD (e.g., the 7.1 antibody in cases defined by 11q23 abnormalities⁽⁸⁾ and the KOR-SA3544 reagent for Philadelphia chromosome-positive leukemia⁽⁹⁾). Because the latter antibody also reacts with myeloid cells, it must be used in combination with reagents that identify CD19+ TdT+ lymphoid progenitors. Since some antigens tend to be expressed more strongly on leukemic cells than on normal cells,⁽¹⁰⁾ their measurement by flow cytometry can be of value in assessing MRD. In general, flow cytometry allows the detection of at least one target cell in 10⁴ normal cells. Finally, certain immunophenotypes are leukemia-specific when the cells are found in “sanctuary” sites (e.g., TdT+ cells in cerebrospinal fluid). Table 2 summarizes the phenotypic combinations that can be used for productive study of residual leukemia.

Table 2. Immunophenotypic Combinations Used to Study MRD in Patients with Acute Leukemia

Disease	Phenotype*	Frequency in normal marrow Frequency (%)	(% positive cells ± SD)à
B-lineage ALL	TdT/CD10/CD13	7	0.02 ± 0.01
	TdT/CD10/CD33	8	0.03 ± 0.02
	TdT/CD10/CDw65	7	0.02 ± 0.01
	TdT/CD10/CD21	10	0.02 ± 0.01
	TdT/CD10/CD56	9	< 0.01
	TdT/cytoplasmic m/CD34	14	0.03 ± 0.01
	KOR-SA3544	10	< 0.01
	7.1	3	< 0.01
T-lineage ALL	TdT/cytoplasmic CD3	90	< 0.01
AML	CD34/CD56	20	< 0.01
	CDw65/CD34/TdT	15	< 0.01

* TdT and CD10 can be replaced by CD19 and CD34. Greater than 10% positive leukemic lymphoblasts.

à These positive cells had light-scattering properties typical of immature lymphoid or myeloid cells. The reduced expression of the indicated phenotypes by normal cells allows 1 leukemic cell to be identified among 10,000 normal marrow cells.

Sources of Error in MRD Detection

Even highly sensitive methods of MRD detection are limited in their application (Table 3). False-positive results due to contaminating traces of DNA are a potential source of error with PCR. Similar errors can arise from nonspecific hybridization of “clonospecific” probes to amplified DNA from normal lymphocytes in assays directed to Ig or TCR gene rearrangements. The same assays will yield false-negative findings if the Ig or TCR molecular target is deleted, or if additional rearrangements have emerged during the course of the disease (“clonal evolution”).

Table 3. Potential Sources of Error in Detection of MRD

Method	Type of error False-negative result	Type of error False-positive result
<i>In situ</i> hybridization	Poor hybridization	Aneuploidy in normal cells; artifactual co-localization of probes
Colony assays	Low proliferative activity; of leukemic cells apoptosis due to lack of stromal support	Colony formation from non-leukemic cells
PCR performed on antigen-receptor gene rearrangements	Degraded DNA; clonal evolution	Cross-hybridization with sequences from normal cells
PCR amplification of translocation breakpoints	Degraded RNA or DNA; low efficiency of reverse transcriptase	Contamination
Immunologic methods	Phenotypic switch	Antibody cross-reactivity

False-positive results with immunologic methods can derive from expression of “leukemia-associated” phenotypes by subsets of normal cells and from the use of antibodies with nonspecific reactivities, such as cells binding through their Fc portion. Phenotypic switches occurring at the time of relapse may result in false-negative results during MRD monitoring if the changes affect markers used in the original panel.

The heterogeneous distribution of leukemia in the body is another potential source of error, irrespective of the specific technique used. While there are obvious advantages

to the use of peripheral blood rather than bone marrow for the monitoring of MRD, the available evidence suggests that the latter is a more reliable tissue for analysis.⁽¹¹⁾

One of the aims of MRD investigation is to estimate the amount of residual tumor rather than to simply establish its presence. Immunologic methods, in situ hybridization with interphase cells and ploidy studies by flow cytometry allow precise quantitation of the proportion of abnormal cells within a given population, while PCR analysis appears less accurate.

Clinical Application of MRD Monitoring

Acute Lymphoid Leukemia

Most studies evaluating the clinical utility of PCR analysis for MRD monitoring have been retrospective and based on TCR gene rearrangements. A single positive PCR result early in remission is not necessarily correlated with long-term response to treatment.^(12,13) Likewise, the absence of MRD during treatment or even at the end of therapy by PCR analysis does not ensure a durable remission.⁽¹⁴⁾ However, the persistence or an increase in positivity on repeated analysis is closely associated with a subsequent and sometimes very late relapse.⁽¹⁵⁾ In one study, clinical relapses occurred in two patients more than eight years after positive post-treatment PCR results,⁽¹⁶⁾ suggesting that remnants of the leukemic clone can survive in a quiescent state for years before giving rise to overt relapse. Using semiquantitative PCR analysis, some investigators have correlated higher levels of residual disease in the immediate postinduction period with an increased likelihood of hematologic and sometimes combined relapses.^(17,18) Similarly, using cell sorting with colony assays for leukemic progenitor cells, others showed that high concentrations of leukemic cells in bone marrow were predictive of relapse in patients who were undergoing autologous transplantation.⁽⁵⁾

In ALL, a number of leukemia-specific fusion transcripts can be amplified by PCR, but only BCR-ABL has been systematically applied in the study of MRD. Most of the cases analyzed had received either allogeneic or autologous bone marrow rescue. Among these patients, BCR-ABL transcripts disappeared after transplantation and remained undetectable in long-term disease-free survivors, but re-emerged in those who subsequently developed a clinical relapse.^(19,20)

In contrast to PCR assays, a single positive result by immunologic methods appeared to be highly predictive of subsequent relapse.⁽²¹⁾ In our ongoing prospective study (median follow-up, 2.5 years), four of 20 patients with positive immunologic assay results upon attaining a morphologic remission after induction therapy have relapsed, compared with only one of 71 patients with a negative result; a positive result at 32 weeks of morphologic remission appears to be even more predictive of subsequent outcome (D. Campana and C-H. Pui, unpublished data). Finally, although routine examination of cerebrospinal fluid by immunologic methods would probably not be cost-effective, this approach can lend a diagnostic edge when morphologic findings are equivocal.⁽³⁾

Acute Myeloid Leukemia

A number of studies have established the predictive value of PCR reactivity in acute promyelocytic leukemia with the PML-RARA fusion gene.⁽²²⁾ Indeed, PCR

negativity should be regarded as a goal in the clinical management of this disease. The clinical value of PCR monitoring in acute myeloblastic leukemia with the t(8;21) and AML-ETO fusion gene is less certain, as the fusion transcripts may be detected in bone marrow or even peripheral blood from patients who have completed all treatment and remained in remission for as long as eight years.⁽²³⁾ This result contrasts with that of a cytogenetic study in which all patients with detectable t(8;21) metaphases eventually relapsed.⁽²⁴⁾ Whether expression of the chimeric protein does not itself constitute a transformed phenotype or whether the “quiescent cells” in patients with only PCR reactivity (hence MRD at a very low level) was suppressed by host immunity is unclear. This finding also raises questions about the validity of the detection of fusion-gene products as a predictor of clinical outcome in some leukemias. Studies of PCR analysis of MRD in patients with the CBFb-MYH11 or the MLL-AF9, two other common subtypes of AML, have been limited to only a few patients. Finally, several studies have shown that persistence or increase of phenotypically abnormal cells generally was followed by overt relapse.⁽²⁵⁾

Summary and Perspective

Minimal residual disease can be monitored by several different techniques, but none is satisfactory in all patients. Rather, to accommodate more than 90% of the cases, one must apply multiple techniques. A recent study revealed increased WT1 expression in the leukemic cells of all 45 AML and 22 ALL cases examined by semiquantitative RT-PCR, with serial measurements of gene expression correlating with treatment outcome in nine AML cases tested.⁽²⁶⁾ Additional studies are needed to establish the clinical utility of this potential new marker of MRD detection. Although immunologic methods appear to be quite specific, their wider application will require identification of additional leukemia-associated phenotypes and preferably leukemia-specific antigens. It has become clear that the mere detection of MRD by PCR, especially during early remission, does not predict relapse. In this regard, recent PCR studies disclosed the presence of a BCL2-IGH or BCR-ABL fusion gene in apparently healthy individuals, with the frequency of PCR positivity increasing with age.^(27,28) Thus, expression of a fusion transcript does not necessarily connote malignant proliferation. There is clearly a need for carefully controlled prospective studies to evaluate the predictive value of serial and, perhaps, quantitative MRD determinations. The expectation is that accurate determination of MRD will have a profound impact on the clinical management of patients with leukemia.

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