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LABORATORY DIAGNOSIS AND MONITORING OF LEUKAEMIAS AND LYMPHOMAS AT MOLECULAR LEVEL

Renata Zadro

Corresponding author's address:
Assist. Prof. Renata Zadro, PhD
Clinical Institute of Laboratory Diagnosis
Zagreb University School of Medicine and Clinical Hospital Center
Kispaticeva 12
10000 Zagreb, Croatia

Leukaemias and lymphomas are clonal disorders of the haemat-opoietic cell characterized by somatically acquired genetic alterations. The discovery that molecular abnormalities are involved in their pathogenesis has greatly improved our understanding of these diseases. In leukaemias, more than 300 chromosomal rearrangements have been detected and more than 100 of these have been cloned and characterized until now.
Molecular abnormalities in Haematologic malignancies are diverse but can be grouped in two categories:
- abnormal rearrangemments caused by chromosome translocations, inversions and duplications which usually result in oncogene activation; the result of these rearrangements can be either fusion proteins or deregulated expression of genes;
- mutations and deletions of tumour suppressor genes (e.g. p53, atm).

Additionally, normal rearrangements in lymphocyte antigen receptor genes can serve in detection of clonality that highly correlates with (but is not equal to) malignancy.
Perhaps the best studied is t(9;22) translocation, giving rise to the bcr/abl fusion protein. Fusion of BCR to ABL results in constitutive activation of ABL that is necessary and sufficient for induction of chronic myelogenous leukaemia (CML). At least 95% of CML cases carry t(9;22) which is not pathognomonic for CML, as it is also present in 15%-30% of adult and 5% of pediatric ALL and in 2% of AML. The t(9;22) is a reciprocal translocation in which a large segment of the Abelson proto-oncogene (abl) at 9q34 is juxtaposed within the breakpoint cluster region gene (bcr) at 22q11, resulting in bcr-abl fusion gene (Figure 1).

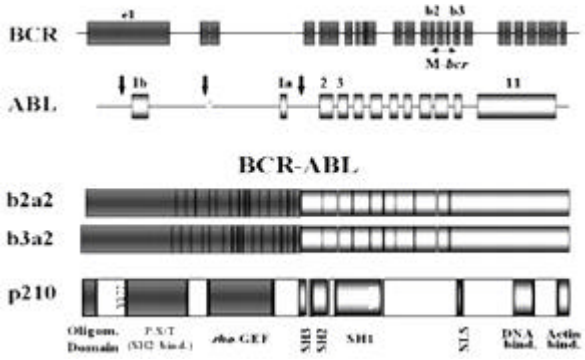


Figure 1. Structure of the genomic BCR and ABL loci with their respective breakpoint cluster regions (M-bcr in BCR and those indicated by vertical arrows in ABL), of the chimeric BCR-ABL mRNA transcripts (with b3a2 or b2a2 junctions), and of the p210 fusion protein. Reprinted from Kantarjian et al. with permission.

Transcription from this gene yields chimeric mRNA molecules. The final product is a protein with elevated tyrosine kinase activity that seems to exert its effects by interfering with cellular signal transduction pathways normally involved in the control of cell death and proliferation and cell-cell adhesion. The BCR-ABL fusion protein can vary from 190 kDa to 230 kDa, depending on the site of the breakpoint in the BCR gene. In almost all CML the breakpoints in the BCR gene are found within the M-bcr region with hybrid BCR-ABL transcript of 8.5 kb containing either BCR exon b2 or b3 and ABL exon 2. This mRNA encodes the 210 kDa BCR-ABL protein (p210). The majority of CML patients have transcripts with b3-a2 or b3-a2 junctions. In 5% of cases, both b3-a2 and b2-a2 transcripts can be formed as a result of alternative splicing. In very rare cases of Ph+ CML, the breakpoint in the BCR gene involves the ALL-associated m-bcr region, which results in production of smaller p190 fusion protein. A very small proportion of Ph+ CML patients display a larger BCR-ABL fusion transcript that is caused by breakpoints in the micro breakpoint cluster region (?-bcr) and results in a larger fusion protein p230.

Known genetic aberrations have been included in World Health Organization (WHO) classification of acute leukaemias and Revised European-American Lymphoma (REAL) classification of lymphomas. Genetic characterization became an essential part of the diagnostic work-up in order to define the risk of relapse and assign patients to distinct treatment options.

The WHO classification recognizes in acute myeloid leukaemia (AML) four subgroups with distinct prognostic outcomes (Table 1):

- AML with recurrent genetic abnormalities including t(8;21), t(15;17), inv (16) or t(16;16) and 11q23 abnormalities; - AML with multilineage dysplasia;

- therapy related AML and MDS and AML classified as FAB subtype M0-M7 -

Table 1. The most common chromosomal translocations in acute myeloid leukaemia

FAB	Abnormality	Fusion gene	Frequency (%)	Detection system	Prognostic outcome
M2	t(8;21)(q22;q22)	AML1-ETO	12	FISH, RT-PCR	Favorable
M3	t(15;17)(q22;q21) t(11;17)(q23;q21) t(5;17)(q35;q21)	PML-RARa PLZF-RARa NPM-RARa	8 <1 <1	FISH, RT-PCR RT-PCR RT-PCR	Favorable Unfavorable ?
M4Eo	inv(16)(p13q22)	CBFb-MYH11	10	FISH, RT-PCR	Favorable
M4 or M5	t(9;11)(q22;q23) t(v;11)(v;q23)	MLL-AF9 MLL-var.	2 <1	FISH, RT-PCR	Unfavorable/ intermediate
var. FAB	t(9;22)(q34;q11) Duplication "	BCR-ABL MLL FLT3	1-2 5 25	FISH, RT-PCR RT-PCR RT-PCR	Unfavorable

The revised WHO classification of ALL relies upon initial immunophenotypic characterization that defines pre B, preT and Burkitt cell leukaemia. Each entity is then characterized with distinct cytogenetic subgroups (Table 2). The result of translocations in pre B-cell forms is the generation of fusion genes (proteins). In pre T-cell ALL most translocations result in deregulated expression of genes juxtaposed to heterologous promoters, usually of a T-cell receptor gene.

Table 2. The most common chromosomal translocations in acute lymphoblastic leukaemia

Disease	Abnormality	Fusion gene	Frequency (pediatric) (%)	Detection system	Prognostic outcome
pre B	t(9,22)(q34,q11) t(12,21)(p13;q22) t(4,11)(q21,q23)	BCR-ABL TEL-AML1 MLL-AF4	25(5) <1(20) <1(2)	FISH, RT-PCR	Unfavourable
pre T	Microdeletion 1p32	SIL-TAL1	16(26)	FISH, RT-PCR	Unfavourable
Burkitt	t(8,14)(p24,q32)	MYC-IgH	<1	FISH	Intermediate

The majority of B cell lymphomas and a minority of T cell lymphomas are characterized by recurring chromosome translocations (Table 3). Many involve immunoglobulin or T cell receptor loci with various partner chromosomes and lead to abnormal proto-oncogene expression. A few result in the production of a novel fusion protein. Aneuploidy and deletion of specific chromosome regions are common secondary chromosomal events which are rarely specific to a particular type of lymphoma but provide valuable prognostic information.

Table 3. Chromosomal rearrangemets in non-Hodgkin lymphomas

Hystological type	Translocation	Genes involved	Molecular analysis
MCL	t(11;14)(q13;q32)	BCL1 IgH	PCR
FL>DLCL	t(14;18)(q32;q21)	BCL2 IgH	PCR, FISH
CLL/SLL	t(14;19)(q32;q13)	BCL3 IgH	
DLCL>FL	t(3;14)(q27;q32)	BCL6 IgH	Southern, FISH
ALCL	t(2;5)(p23;q35)	NPM ALK	RT-PCR
LPL	t(9;14)(p13;q32)	PAX5 IgH	FISH, Southern

MCL- mantle cell lymphoma; FL- folicular lymphoma; DLCL-difuse large cell lymphoma; CLL/SLL- chronic lymphocytic leukaemia/ small cell lymphoma; ALCL-anaplastic large cell lymphoma; LPL-lymphoplasmocyoid lymphoma.

8.1 Methodological approaches for detecting leukaemia/lymphoma-associated abnormal rearrangements

Haematologic malignancies are analyzed and classified on the basis of properties including morphology, cell surface markers, immunohistochemistry, and cytogenetic abnormalities. Additionally, the knowledge about haematopoietic differentiation and genetic abnormalities in Haematologic malignancies have aided in establishment and interpretation of gene expression data.

Structural changes affecting chromosomes can be analyzed by using a variety of techniques including comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). Finer resolution of these alterations can be obtained by using the polymerase chain reaction (PCR), DNA sequencing, and genomic and cDNA array analysis.
Table 4 provides a listing of some currently used techniques in molecular diagnostics today.

Table 4. Summary of techniques for molecular analysis of hematopoietic disorders

Tests for Genome-Wide Screening of Chromosomal Abnormalities
Spectral karyotyping (multicolor fluorescence *in situ* hybridization)
Comparative genomic hybridization
Tests Targeting Specific Chromosomal Abnormalities
PCR (polymerase chain reaction analysis of DNA)
RT-PCR (reverse transcriptase PCR analysis of RNA)
Real-time PCR
Genotyping for single nucleotide polymorphisms (PCR-SSP)
Fluorescence in situ hybridization (FISH)
Tests for Gene Expression Profiling
Global microarrays
Focused microarrays
Microarray of amplified RNA from microdissection
Molecular Tests for Minimal Residual Disease Detection
Nested PCR
Quantitative real-time PCR

Conventional karyotyping still has a paramount role in diagnosis and classification of human leukaemias and lymphomas. Besides chromosome translocations, other clinically important aberrations are detectable using this technique, allowing implementation of patient stratification in prognostic groups – particularly in AML (Table 1). The study of the full karyotype on metaphases provides important information on distinct lesions that characterize particular leukaemia/lymphoma while additional numerical and structural aberrations carry biological and clinical relevance.

FISH is a very useful technique for detection of targeted chromosomal abnormalities around the time of initial diagnosis or at relapse, when there is a relatively high level of abnormal cells. It can be performed on blood, bone marrow, tissue touch preparations, body fluids, and paraffin-embedded fixed tissue. FISH can be done with metaphase or interphase preparations so it overcomes the need for metaphases that is the biggest problem with lymphoma and chronic leukaemia samples. FISH assays are particularly useful in detection of chromosomal translocations that are not amenable to PCR due to widely distributed breakpoints. FISH is not useful for detection of low-level minimal residual disease (MRD) as the sensitivity is only 10-2.

Reverse transcription (RT)-PCR is a well-established technique for identifying very small amounts of specific mRNA transcripts. The

application of this technique has permitted researchers to detect cancerous cells at levels well below the threshold of the light microscope.

Table 5. Sensitivity of various detection methods

Method	Sensitivity (1 cell in n cells)
Cytogenetics	1/25
Interphase FISH	1/500
Immunophenotyping	$1/10^2 - 10^4$
Nested PCR	$1/10^3 - 10^6$
Real time PCR	$1/10^3 - 10^5$
Microsatellite PCR	$1/10^2 - 10^4$

Additionally, the accurate quantification of target sequences is possible using automated systems. RT-PCR is widely used in routine genetic diagnosis and in assessment of the response to treatment. Quantitative real-time PCR measures the number of target DNA copies in automated manner using a fluorescence analyzer and is particularly useful for MRD studies.

8.2 Minimal residual disease in Haematological malignancies

Although many patients with Haematologic malignancies achieve a complete clinical remission and remission by morphologic and immunologic criteria, a relatively high proportion of them will ultimately relapse. A persistent malignant cellular population present at low level, below the limit of detection of standard techniques, is the cause of this relapse and is called minimal residual disease (MRD). Several studies have shown that detection and quantification of residual tumour cells significantly correlate with clinical outcome. The quantitative measurement of the decrease in the leukemic cell load during the initial phases of treatment has a high prognostic value.

Methods to detect MRD include technologies designed to detect residual malignant cells beyond the sensitivity of conventional approaches (Table 5). Ideally, techniques used for MRD detection should have a sensitivity level in the $10^5 - 10^6$ range. Only a few commonly used techniques are sensitive enough for detection of MRD in leukaemias and lymphomas. Currently, PCR based methods represent the most widely accepted technologies for MRD detection. Over the past 15 years, quantitative PCR assays were developed. Competitive RT-PCR employed to monitor patients after transplantation or treatment with specific agents are time-consuming and cumbersome. Quantification of residual disease has been simplified with the introduction of real-time PCR methodologies and machines. Nested PCR and quantitative real-time PCR can be used for disease-associated translocations. If there is not a good translocation target for PCR analysis, patient-specific gene rearrangements may be targeted.

8.3 DNA microarrays

In the past several years, experiments using DNA microarrays have contributed to an increasingly refined molecular classification of Haematologic malignancies. Quantitative information about the expression of thousand genes can be generated with rapidity and reproducibility.

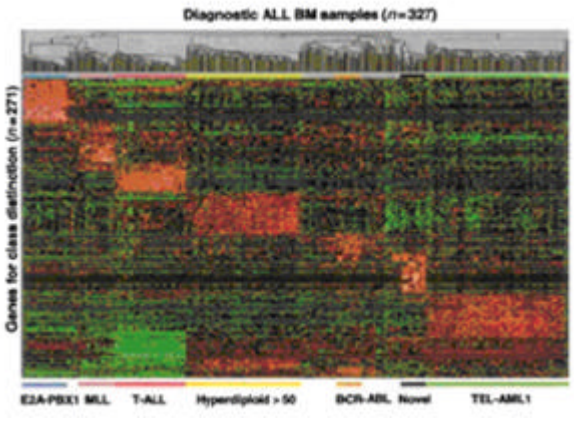
In DNA microarray experiments, DNA probes (cDNA fragments, generated by PCR of cDNA clone inserts) are arrayed on a platform (glass slide, nylon membrane, silicone wafer). After target cDNA or cRNA generated from sample RNA and labeled with fluorescent dye or biotin is hybridized to the microarray, a scanner measures fluorescence at the site of each unique probe.

Gene microarrays have been used to profile acute leukaemias and have identified expression signatures characteristic of AML and ALL. The same approach has been used to profile subsets of both these leukaemias. It was demonstrated that expression patterns are strongly linked to karyotypic status for t(8;21), t(15;17), inv(16), 11q23 and normal controls. Profiles for ALL with rearrangement of the MLL gene clearly distinguished this category from ALLs and AMLs without MLL. In ALL, expression profiles subdivided ALL into T-ALL, hyperploid, BCR-ABL, E2A-PBX1, TEL-AML, MLL and one previously unrecognized subset of ALL (Figure 2).

Diffuse large B-cell lymphoma (DLBCL) is one disease in which attempts to define subgroups on the basis of morphology have largely failed. Using DNA microarrays two molecularly distinct forms of DLBCL were identified which had gene expression patterns indicative of differnt stages of B-cell differentiation: one type expressed genes characteristic of germinal centre B cells (GC-like DLBCL) while the second type expressed genes normally induced during in vitro activation of peripheral blood B cells (PB-like DLBCL). It was found that GC-like DLBCLs have a more favorable outcome compared with the PB-like DLBCLs, suggesting that putative cell of origin might be predictive of response to treatment in this disease.

Gene microarray helps to progress the study of Haematological malignancy in the area of classification and outcome prediction, hich then enables tailoring and earlier application of treatment for the benefit of patient.

Figure 2. Expression profiling-based classification of AML and ALL subtypes. Reprinted from Yeoh et al. with permission.



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