

# Molecular Diagnostics of Hematological Malignancies

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**ABBREVIATIONS:** DNA = deoxyribonucleic acid; mRNA = messenger ribonucleic acid; PCR = polymerase chain reaction; RT-PCR = reverse transcription polymerase chain reaction.

**INDEX TERMS:** diagnosis mutation; DNA; leukemia; lymphoma; molecular tests; RNA; translocation.

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Molecular diagnostics is a young discipline, and many of its earliest questions revolved around the diagnosis of hematological malignancies. Could identification of leukemias and lymphomas be aided by analysis of their DNA or RNA? The answer is clearly a resounding “Yes”, and we have moved on from simple diagnostic issues to ones of disease classification, prognosis, selection of therapy, and therapeutic efficacy. Along the way, a variety of methods has been employed, from the practically pre-historic (in molecular biology terms) Southern hybridization (sometimes still done with radioactivity!) to cutting edge gene expression arrays and mass spectroscopy. The availability of these tests, however, has not relieved the laboratory scientist from having to correlate the molecular diagnostic laboratory results with the patient’s condition and other laboratory findings. Indeed, the hematology arena—rich in data such as morphology, flow cytometry, and several kinds of cytogenetic tests—is perhaps the best example of the need for a comprehensive, integrated approach to diagnosis and

treatment. Of necessity, this review downplays many of the clinical and non-genetic aspects of hematological malignancies available elsewhere.<sup>1,2</sup> It focuses on a few of the most common entities (leukemias and lymphomas) illustrative of the variety of testing currently performed and on issues facing the molecular diagnostic laboratory in the near future. The future is likely to be one of increasing laboratory automation and sophistication—commercial kit-based rather than “home brew”, with more extensive gene sequencing and mutation identification, probably incorporating chip technology—until the molecular diagnostic laboratory begins to resemble more closely a contemporary clinical chemistry laboratory.

## CHRONIC MYELOGENOUS LEUKEMIA

Chronic myelogenous leukemia (CML) is an initially indolent hematopoietic stem cell disorder. It results in a proliferation of the full range of bone marrow hematopoietic cells that elevates the white blood cell count and fills the spleen. CML is paradigmatic of malignant disease in many ways, particularly in the molecular arena. It was the first human tumor in which a consistent cytogenetic alteration was recognized [the Philadelphia chromosome, t(9;22)(q34;q11)]. The genetic basis of the translocation was then shown to be a juxtaposition of two signal transduction genes, *BCR* (for breakpoint cluster region) and *ABL*, the Abelson murine leukemia virus proto-oncogene.<sup>3-5</sup> The *BCR/ABL* translocation is so specific for CML that its presence (or that of the Philadelphia chromosome) is now required for the diagnosis. Translocation of proto-oncogenes, particularly signal transduction genes, has since been identified as a common event in a variety of hematological neoplasms. These events often produce a chimeric protein using the malignant cell’s transcription machinery to splice the mRNAs, head to tail, from each of the translocation partners. More recently, the enzymatically active portion of the *BCR/ABL* chimeric protein, a serine/threonine kinase, has been targeted with specific inhibitory small molecules. The first of these, imatinib, has shown remarkable success in controlling the disease. Not to be outdone by therapeutic advances, CML cells—treated or not—frequently acquire additional genetic alterations and behave more aggressively, eventually ending in transformation to an acute leukemia with many blasts.

The molecular diagnostic laboratory plays a crucial role in CML diagnosis and management. As noted, the presence of the *BCR/ABL* translocation is diagnostic. This can be assayed by Southern hybridization with a probe to one of the translocation partners, but most laboratories now perform a PCR assay on RNA harvested from a patient's cells. Most breakpoints in the DNA fall, in *BCR*, between exons b2 and b3 or between b3 and b4, and in *ABL* upstream of the a2 exon. mRNA splicing brings the relatively small exons together, making an excellent target for reverse transcription-PCR. The amplification primers are made homologous to b2 and a2 and yield b2a2 or b3a2 amplicons (describing where the translocation occurred). Variant translocations, most notably in some cases of acute lymphoblastic leukemia, give different forms of the *BCR/ABL* transgene and chimeric protein.

Once diagnosed, CML patients can in theory be followed by qualitative PCR assays for the presence of the transgene, both for prognostic purposes (minimal residual disease detection) and to assess therapeutic efficacy.<sup>6,7</sup> The utility of this approach has fallen into question because of the inconsistent clinical significance of detection of a transgene in different studies. Lately, a quantitative approach to *BCR/ABL* detection has been gaining favor leading to the concept of a “molecular remission” below a certain level of residual CML cells.<sup>8</sup> Simultaneous peripheral blood and bone marrow samples generally give comparable results.

When CML cells escape control by imatinib, they do so by a variety of mechanisms including expressing more of the transgene or developing mutations in the drug binding site.<sup>9</sup> It is likely that new sequence-based clinical tests will need to be developed to identify mutated and drug resistant clones in the near future. CML thus serves as an excellent example of the importance of the molecular diagnostic laboratory in management of hematological malignancies: for diagnosis, classification, prognosis, disease monitoring, and therapeutic effectiveness.

#### ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia (APL) is one subtype of the plethora of acute myeloid leukemias, proliferations of the most immature forms of non-lymphoid bone marrow cells. It is an infrequent entity, but important because it is another disease defined by a chromosomal alteration and because the therapy for it was the first example of a real “magic bullet”—therapy aimed squarely at the genetic cause of the malignancy. The

chromosomal alteration is the t(15;17)(q22;q12) uniting the promyelocytic leukemia gene (*PML*) to the retinoic acid receptor alpha gene (*RARα*), and the “magic bullet” is a form of vitamin A called all-trans retinoic acid (ATRA).<sup>10,11</sup> The *RARα* forms a heterodimer with a similar protein called RXR and incorporates itself into a complex that represses transcription and cell differentiation. Physiologic levels of retinoids permit relaxation of the complex, but this is not true when the chimeric *PML/RARα* is present. Supraphysiologic doses of ATRA can drive maturation of the leukemic cells yielding a temporary remission. The matured leukemic cells do not trigger the coagulopathy that is so characteristic of the immature promyelocytes, and therefore reduce the major cause of early mortality in APL patients.

As in CML, the defining translocation appears to be highly sensitive and specific for the diagnosis of APL. Reverse transcription-PCR can identify the several variants of *PML/RARα* by amplifying the spliced exons straddling the breakpoint. In combination with cytogenetics or fluorescence in situ hybridization (FISH), RT-PCR can screen out the rare variants that do not respond well to ATRA (most have translocations of other genes into *RARα*) and triage cases for appropriate chemotherapy. Monitoring of patients by qualitative analysis for relapse of disease (a relatively infrequent event in APL) has not proven successful due to both false positives and false negatives. Quantitative PCR assays may prove more useful, particularly when performed with internal calibrators, since there is some evidence that patients who convert more rapidly to *PML/RARα* negativity or who are negative prior to bone marrow transplantation have better outcomes. The best use of the molecular diagnostic laboratory may be in separating out the few APL patients who will need more aggressive therapy.<sup>12</sup>

With APL and CML as the paradigms, a large number of genes have now been identified as translocation partners in leukemias and lymphomas (Tables 1 and 2). Typically these fuse one or more transcription factor or tyrosine kinase genes and result in a putative disruption of a signaling or apoptotic pathway. Identification of these translocations is possible and, in some cases, important because of prognostic implications. The method used is typically an RT-PCR test or Southern hybridization. However, these tests are largely “boutique” assays performed by few investigators because of the overall infrequency and expense in setting-up and maintaining them. Widespread adoption of these tests probably awaits commercial kit production, preferably in a multiplex fashion.

Table 1. Selected gene alterations in lymphoma

<i>Genes</i>	<b>Chromosomal translocation</b>	<b>Biologic function</b>	<b>Mechanism of activation or inactivation</b>	<b>Lymphoma type</b>
<i>API2/MALT1</i>	t(11;18)(q21;q21)	Regulator of apoptosis	Protein fusion	MALT
<i>BCL1</i>	t(11;14)(q13;q32)	Cyclin D1/ cell cycle regulator	Transcription deregulation	MCL
<i>BCL2</i>	t(14;18)(q32;q21)	Inner mitochondrial membrane protein, regulator of apoptosis	Transcription deregulation	FL; subset of DLCL
<i>BCL6</i>	t(3;v)(q27;v)*	Transcription factor	Transcription deregulation	DLCL
<i>BCL10</i>	t(1;14)(p22;q32)	Regulator of apoptosis	Protein truncation, transcription deregulation	MALT
<i>LYT10</i>	t(10;14)(q24;q32)	Transcription factor/ NF-kappa B family	Protein truncation	CTCL
<i>MYC</i>	t(8;14)(q24;q32); t(2;8)(p11;q24); t(8;22)(q24;q11)	Transcription factor	Transcription deregulation	BL
<i>NPM/ALK</i>	t(2;5)(p23;q35)	Nucleolar phosphoprotein, receptor tyrosine kinase	Protein fusion	ALCL
<i>PAX5</i>	t(9;14)(p13;q32)	Transcription factor	Transcription deregulation	LPL

**Legend for Tables 1 and 2:**

ALCL = anaplastic large cell lymphoma; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; ATLL = adult T-cell leukemia/lymphoma; BL = Burkitt lymphoma; CBF = core binding factor; CLL = B-cell chronic lymphocytic leukemia; CTCL = cutaneous T-cell lymphoma; DLCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; LPL = lymphoplasmacytoid lymphoma; MALT = extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; MCL = mantle cell lymphoma; NHL = non-Hodgkin lymphoma; TdT = terminal deoxynucleotidyl transferase.

\* More than 10 different partner chromosomal sites, including IgH, are identified.

† Other partner genes of *RARA* are *PLZF*, *NPM*, and *NUMA*.

‡ More than 30 chromosomal sites are involved.

§ 90-kb submicroscopic deletion that juxtaposes *TAL* to *SIL* creating a *SIL/TAL* fusion transcript

Modified from: Tam W, Dalla-Favera R. Protooncogenes and tumor suppressor genes in hematopoietic malignancies. In: Knowles DM. Neoplastic hematopathology. Philadelphia: Lippincott Williams & Wilkins; 2001:329-363.

**FOCUS: GENE-BASED DIAGNOSTICS**

**Table 2.** Selected gene alterations in leukemia

<i>Genes</i>	<b>Chromosomal translocation</b>	<b>Biologic function</b>	<b>Mechanism of activation or inactivation</b>	<b>Leukemia type</b>
<i>ETO/AML1</i>	t(8;21)(q22;q22)	CBF transcription factor alpha subunit, transcriptional co-repressor	Protein fusion	AML-M2
<i>BCR/ABL</i>	t(9;22)(q34;q11)	Serine/threonine protein kinase, GTPase activation, non-receptor tyrosine kinase (nuclear and cytoplasmic)	Protein fusion	Precursor B-ALL
<i>CBFB/MYH11</i>	inv(16)(p13q22)	CBF transcription factor beta subunit, smooth muscle myosin heavy chain	Protein fusion	AML-M4EO
<i>DEK/CAN</i>	t(6;9)(p23;q34)	Transcription regulator? (autoantigen), nucleoporin/nucleocytoplasmic transport	Protein fusion	TdT+ AML
<i>E2A/HLF</i>	t(17;19)(q22;p13)	Transcription factor (helix-loop-helix family), transcription factor (basic zipper family)	Protein fusion	Precursor B-ALL
<i>E2A/PBX1</i>	t(1;19)(q23;p13)	Transcription factor (helix-loop-helix family), transcription factor (homeobox family)	Protein fusion	Precursor B-ALL
<i>HOX11</i>	t(10;14)(q24;q11)	Transcription factor (homeobox family)	Transcription deregulation	Precursor T-ALL
<i>PML<sup>+</sup>/RARA</i>	t(15;17)(q22;q21)	Component of PML nuclear bodies, transcription factor (nuclear hormone receptor family)	Protein fusion	AML-M3 (APL)
<i>MLL/V<sup>+</sup></i>	t(11;v)(q23;v)	Transcription factor, variable	Protein fusion	AML-M5, M4, M1, Precursor B-ALL
<i>TAL1</i>	t(1;14)(p32;q11); 5' small deletion§	Transcription factor (helix-loop-helix-family)	Transcription deregulation	Precursor T-ALL
<i>TAN1</i>	t(7;9)(q34;q34)	Transmembrane receptor (Notch family)	Transcription deregulation, protein truncation	Precursor T-ALL
<i>TEL/AML1</i>	t(12;21)(p12;q22)	Transcription factor (ETS family), CBF transcription factor alpha subunit	Protein fusion	Precursor B-ALL
<i>TTG1</i>	t(11;14)(p15;q11)	Transcription factor (rhombotin gene family)	Transcription deregulation	Precursor T-ALL
<i>TTG2</i>	t(11;14)(p13;q11)	Transcription regulator (rhombotin gene family)	Transcription deregulation	Precursor T-ALL

Legend included in Table 1.

### LYMPHOMAS AND LYMPHOBLASTIC LEUKEMIAS: ANTIGEN RECEPTOR GENE REARRANGEMENT

The oldest molecular tests in the diagnostic laboratory's armamentarium involve the antigen receptor genes. These genes—immunoglobulin heavy and light chains in B cells and the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  T cell receptors—play a crucial role in the immune system. They normally undergo a process of rearrangement (splicing of DNA) using specialized cellular machinery active only in lymphocytes. While not causative of malignancy like the translocations discussed above, the gene rearrangement process does provide useful diagnostic information. Antigen receptor rearrangement 1) identifies cells as lymphoid in origin; 2) identifies the lineage, B or T cell; and 3) demonstrates clonality of a population of cells.<sup>13</sup> Clonality, or descent of a population of daughter cells from a single mother cell, is an important clinical criterion since it correlates with malignancy.

Gene rearrangements were originally detected by Southern hybridization. In this process, microgram quantities of DNA from a test tissue are purified and digested with restriction enzymes, then electrophoresed to separate fragments on the basis of size. The DNA is transferred (or "blotted") to a membrane support, where it is hybridized with a fluorescent or radioactive probe. The probes are cloned fragments of the gene of interest, either immunoglobulin or T cell receptor. When no gene rearrangement has occurred, all human cells will have the same "germline" pattern of bands on the membrane because of the constancy of the restriction digestion sites. Benign lymphocytes will rearrange their antigen receptor genes randomly, creating a random assortment of digestion fragment sizes and a smear on the membrane. It is only when a large proportion of the lymphocytes (greater than three percent to five percent in most laboratories) are clonal and carry identical rearrangements that the restriction fragments "pile up" at a single location on the membrane, yielding a discrete rearranged band.

Gene rearrangement analysis can also be performed by PCR using primers made to more or less conserved regions of the immunoglobulin or T cell receptor genes that span the spliced portion of DNA. Clonal cells are again recognized as a dominant population of amplicons above a background of benign lymphocyte rearrangements. The availability of clonality detection by PCR has great advantages; e.g., small biopsies and archival specimens in pathology files can both be successfully analyzed. As expected, though, there are trade-offs: cross-contamination of samples is much more

problematic with amplification methods, and the sensitivity of the PCR assays is less (50% to 80% compared to Southern hybridization) because the primers do not bind to all possible rearranged DNAs. However, when a rearranged band is amplifiable it can be used as a tumor specific marker and patients can be assayed for minimal residual disease. Both sensitivity and specificity issues have been addressed by improved detection methods, including the use of capillary gel electrophoresis, heteroduplex analysis, and sequencing.

Since gene rearrangements are indicators of a tumor's lineage, the assay can be used as a tool to explore tumors of uncertain origin. The most compelling example of this involves Hodgkin disease, a historically recognized malignancy that characteristically has more benign inflammatory cells than cytologically malignant ones. Nearly a century of argument over the cell of origin was recently resolved in favor of the B lymphocyte. Single tumor cells were plucked from tissue samples by microscopic dissection, then amplified by PCR to show typical immunoglobulin heavy chain gene rearrangements.<sup>14</sup> The same rearrangements were found in multiple cells from a single case; furthermore, sequencing demonstrated that different subtypes of Hodgkin disease had mutations indicative of different stages of normal B lymphocyte development. Thus, a kind of molecular classification of tumor subtypes is possible.

### FOLLICULAR LYMPHOMA

Follicular lymphoma is one of the most common hematopoietic malignancies in the Western world. A generally indolent form of lymphoma, it nevertheless spreads frequently to the bone marrow. As a B cell tumor, follicular lymphoma usually carries a normal immunoglobulin gene rearrangement as a marker of its origin and clonality. In addition, follicular lymphomas contain another rearrangement that is causative of the disease. This rearrangement,  $t(14;18)(q32;q21)$ , juxtaposes the immunoglobulin heavy chain gene on chromosome 14 to the *BCL2* gene on chromosome 18. Unlike the CML or APL translocations that form chimeric proteins, the  $t(14;18)$  results from a mistake of the B cell's gene rearrangement machinery and results in the joining of part of the immunoglobulin locus to an intact *BCL2* gene. The immunoglobulin gene promoter is active because it is in a maturing B cell, and this drives the overproduction of *BCL2* protein. The abundant *BCL2* forms part of a complex of proteins that inhibits apoptosis and yield an ever-expanding population of long-lived tumor cells. PCR assays have been developed that span the two main breakpoints in follicular



lymphoma and are in common use to assist with diagnosis. They have also been employed in minimal residual disease detection, particularly to assess the efficacy of new therapies. Because of their extreme sensitivity, they have been plagued by false positives due to a very low background of transgenes in healthy individuals. This specificity issue may be alleviated by quantitative assays.<sup>15</sup>

#### OTHER NON-HODGKIN LYMPHOMAS

Oncogene upregulation by translocation is a common theme in lymphomas (Table 1). This is undoubtedly due to the “unstable genome” created by the normal B and T cell antigen receptor gene recombination machinery. Although the enzymatic machinery cuts and rejoins DNA with high fidelity, the rare mistake that rejoins a promoter region to a proto-oncogene gives a growth advantage that can lead to malignancy. In some instances there are lymphoma-generating events in addition to the translocation, such as point mutations in the *MYC* gene in Burkitt lymphoma. This phenomenon highlights the multi-step path to full malignant transformation. Currently, gene sequencing is not a routine part of molecular diagnosis but this may change in the near future with the advent of more rapid and less expensive chip-based methods.

An area of current interest in hematology is the molecular classification of tumors. Physicians have long recognized the inadequacies of traditional classification schemes: entities are defined by microscopic appearance but do not always behave similarly, while targeted therapies (such as anti-CD20 antibodies directed against cell surface antigens) depend more on the molecular constitution of a cell than on its position in a list of tumor types. Toward this end, gene expression arrays are being used to group tumors on the basis of their mRNA populations, most notably in large cell lymphoma. In a landmark study, Alizadeh and colleagues used a gene expression array that they termed a “lymphochip” to distinguish two prognostically different groups, one with a germinal center-like expression pattern and the other that more closely resembled activated B cells.<sup>16</sup> The study identified both previously known and newly recognized expressed genes that, in aggregate, provided more information than standard risk factors alone. While this technology now resides more in the developmental side of the laboratory, it is likely that the information and perhaps methodologies from this approach will translate to the diagnostic laboratory in short order.

#### ACUTE MYELOID LEUKEMIA: *FLT3*

There are many other forms of acute myeloid leukemia in addition to APL described above. Although heterogeneous in their morphology, genetics and behavior, they share some features. A clinically relevant one is mutation in *FLT3*, a receptor-type tyrosine kinase gene expressed in stem cells. Activating mutations in *FLT3* are the most common in acute myeloid leukemia, turning up in roughly 30% of cases, frequently those that lack specific chromosomal translocations.<sup>17</sup> There are two varieties of mutation: a point mutation at amino acid D835, and an internal duplication of varied length. These mutations may develop throughout the course of disease and are associated with a poor prognosis. Rapid diagnostic methods are likely to be useful in prognosticating for patients with *FLT3* mutated acute leukemia.<sup>18</sup> Of particular importance is that small molecule inhibitors of *FLT3* are already in clinical trials, only a few years after the first descriptions of mutation.

#### CONCLUSION

The molecular diagnostic laboratory now plays an important role in the diagnosis of patients with hematological malignancies. Its importance will only grow as our understanding of the complexity and variety of these diseases grows, because our patients need this information for accurate prognosis and treatment.

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**FOCUS: GENE-BASED DIAGNOSTICS**

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