

Molecular Diagnostics of Malignant Disorders

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Abstract

Expansion of our understanding of the molecular basis of cancer has enabled us to apply molecular techniques to categorize malignancies into more uniform, informative groups. In this review we describe the basic molecular techniques used for this purpose, including Southern blotting, polymerase chain reaction (PCR) and quantitative PCR, fluorescence in situ hybridization, DNA microarrays, and proteomics. The main applications of these techniques in the modern management of acute leukemia, chronic myeloid leukemia, lymphomas, and breast cancer are summarized.

Malignant diseases are traditionally diagnosed by light microscopic examination of tumor tissue obtained by biopsy or at surgical resection. In parallel to the increase in our understanding of the molecular basis of cancer, our ability to categorize malignant conditions on a molecular level into uniform, informative groups has greatly expanded. Increasingly, these advances are enhancing precision in diagnosis and determination of prognosis and are having an impact on therapeutic decisions. The purpose of this review is to describe the molecular techniques utilized in the diagnosis of malignancies and to discuss their applications to specific disorders.

MOLECULAR TECHNIQUES

Southern Blotting

The genetic information of the cell is encoded within chromosomes in the form of DNA. DNA is a complex molecule that is composed of nucleotides arranged in a linear sequence. Each nucleotide contains a nucleic acid subunit bound to a deoxyribose sugar. Within chromosomes, 2 complementary strands of nucleotides are bound by hydrogen bonds, with strict pairing of adenine with thymine nucleotides and guanosine with cytosine nucleotides. The complementary strands of nucleotides bind one another in antiparallel orientation with the 5' end of one strand binding to the 3' end of the complementary (sometimes termed "negative") strand.

Several molecular techniques take advantage of the tendency of complementary strands of nucleic acid sequences to associate (anneal) in a highly specific manner. Southern blotting is a technique in which DNA is isolated from cells and digested into smaller fragments with 1 or more restriction enzymes (enzymes derived from bacteria that generate double-stranded breaks in the DNA at specific sites). The DNA fragments are then separated according to size using gel electrophoresis, and denatured using alkaline buffer to generate single stranded DNA fragments. The single-stranded DNA fragments are then transferred from the gel and immobilized to a nylon or nitrocellulose membrane. A specific sequence of interest may then be detected on the membrane after hybridization to a radioactively labeled single-stranded DNA fragment complementary to the sequence of interest, termed a "probe." The probe anneals to the complementary fragment immobilized on the membrane and can be detected by exposing the membrane to radiographic film.¹ Southern blotting was one of the first molecular techniques to be developed, and is still the

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avored technique for the detection of clonal rearrangements of immunoglobulin and T-cell receptor genes, where it aids in the diagnosis of some lymphoproliferative disorders (see below). However, for other purposes, Southern blot analysis has largely been superseded by newer techniques.

Cytogenetics Techniques

Conventional Cytogenetics

Recurring chromosomal abnormalities are the hallmark of many malignant disorders, especially those of hematopoietic origin. In some cases, the resultant molecular changes have been shown to be central to the pathogenesis of the malignant transformation. Detection of these abnormalities aids in the diagnosis of many cancers, provides valuable prognostic information, and increasingly guides therapy.

Conventional cytogenetic techniques can detect additions or deletions of whole chromosomes or parts of chromosomes, and translocations of fragments among chromosomes. Standard chromosomal analysis is conducted on dividing cells in metaphase after Geimsa staining. Individual chromosomes are identified by their specific banding patterns, and abnormalities of number and structure can be detected. Under most circumstances, 20 metaphase spreads are examined by the cytopathologist.

Unique and novel chromosomal derangements can be detected using standard cytogenetics. However, the technique has limited sensitivity, since it can detect only large abnormalities that affect at least 5% of the cells examined. Furthermore, the technique requires active cell division to allow the preparation of metaphase spreads. Consequently, more advanced techniques have been developed to address these limitations. These include fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization as outlined below.

FISH

FISH uses the technique of DNA hybridization to improve the sensitivity of cytogenetics. Specific single-stranded DNA probes are fluorescently labeled and hybridized to the tissue of interest, such as peripheral blood, bone marrow, or tumor tissue. The sample is then examined using a fluorescent microscope. FISH can be used to detect numeric abnormalities of chromosomes (trisomies and monosomies) by use of probes that bind specifically to the centromere of each chromosome. FISH is also used to detect specific chromosomal translocations that may be suspected based on the clinical syndrome and/or tumor morphology. Examples include the t(15;17) in acute promyelocytic leukemia, the t(9;22) in chronic myeloid leukemia, and the t(8;14) in Burkitt lymphoma. The pattern of hybridization of the probes indicates the presence of a specific translocation, and can be used to estimate the proportion of malignant relative to nonmalignant cells. FISH is significantly more sensitive than conventional cytogenetics for the detection of known chromosomal abnormalities.² A further advantage of this technique is that it does not require cell division and it can be performed on fixed tissue. However, it will only provide information

on the presence of specific abnormalities related to the probes chosen. In contrast, conventional cytogenetic studies detect chromosomal abnormalities that may not have been expected based on the morphology and/or for which specific probes are not available.

SKY

SKY uses 24 differentially labeled fluorescent probes to “paint” each individual chromosome in metaphase spreads. The data are analyzed by spectroscopy, allowing the distinction of overlapping spectra to provide a complete map of the genome of the cell. This advanced genome-screening technique allows the most accurate assessment of chromosome number and complexity, as well as permitting the detection of cryptic translocations that are too small to identify by conventional cytogenetics. SKY has been a valuable research tool for analyzing the complexity of genetic events in neoplasia, and is beginning to be used for the analysis of clinical specimens.^{3,4}

Comparative Genomic Hybridization

Conventional cytogenetics, FISH-based screening methods, and SKY all depend on the availability of metaphases in the malignant cells, and may not be informative in samples with a low mitotic rate. In comparative genomic hybridization (CGH), differentially labeled genomic tumor DNA and control genomic DNA are hybridized to normal DNA. The ratio between the binding of normal DNA and tumor DNA can be measured and this enables the detection of gains and losses in DNA sequences in the malignant tissue. Array-CGH (also known as matrix-CGH) uses mapped genomic clones, cDNA, polymerase chain reaction (PCR) products, or synthetic oligonucleotides as the target DNA. It is the most powerful tool for the detection and localization of losses and gains in genetic material. Its main application currently is as a research tool for cancer cytogenetics, especially in solid tumors, but it holds significant potential in clinical genetics as an aid to conventional cytogenetic banding and FISH analysis.⁵⁻⁷

Polymerase Chain Reaction

PCR is a powerful technique in which a small piece of DNA of interest can be greatly amplified. The DNA sequence is identified by 2 flanking primers, which are short pieces of single-stranded DNA, one complementary to a sequence on the 5' strand of the DNA to be amplified, and one complementary to a sequence on the 3' strand. The DNA sequence that lies between the 2 primers is then amplified by the following method: The DNA template is heated and its 2 strands separated. The temperature is then lowered, enabling the primers to anneal to their complementary sequences of DNA. The DNA sequence between the 2 primers is then synthesized using a temperature-stable DNA polymerase (Taq polymerase). This process occurs on each strand of DNA, resulting in 2 identical copies of the original DNA, thus doubling the amount of the DNA sequence of interest. The cycle is then repeated by heating the DNA to separate the strands, cooling it to enable the primers to anneal and then synthesizing the DNA sequence between the primers using Taq polymerase. This process is

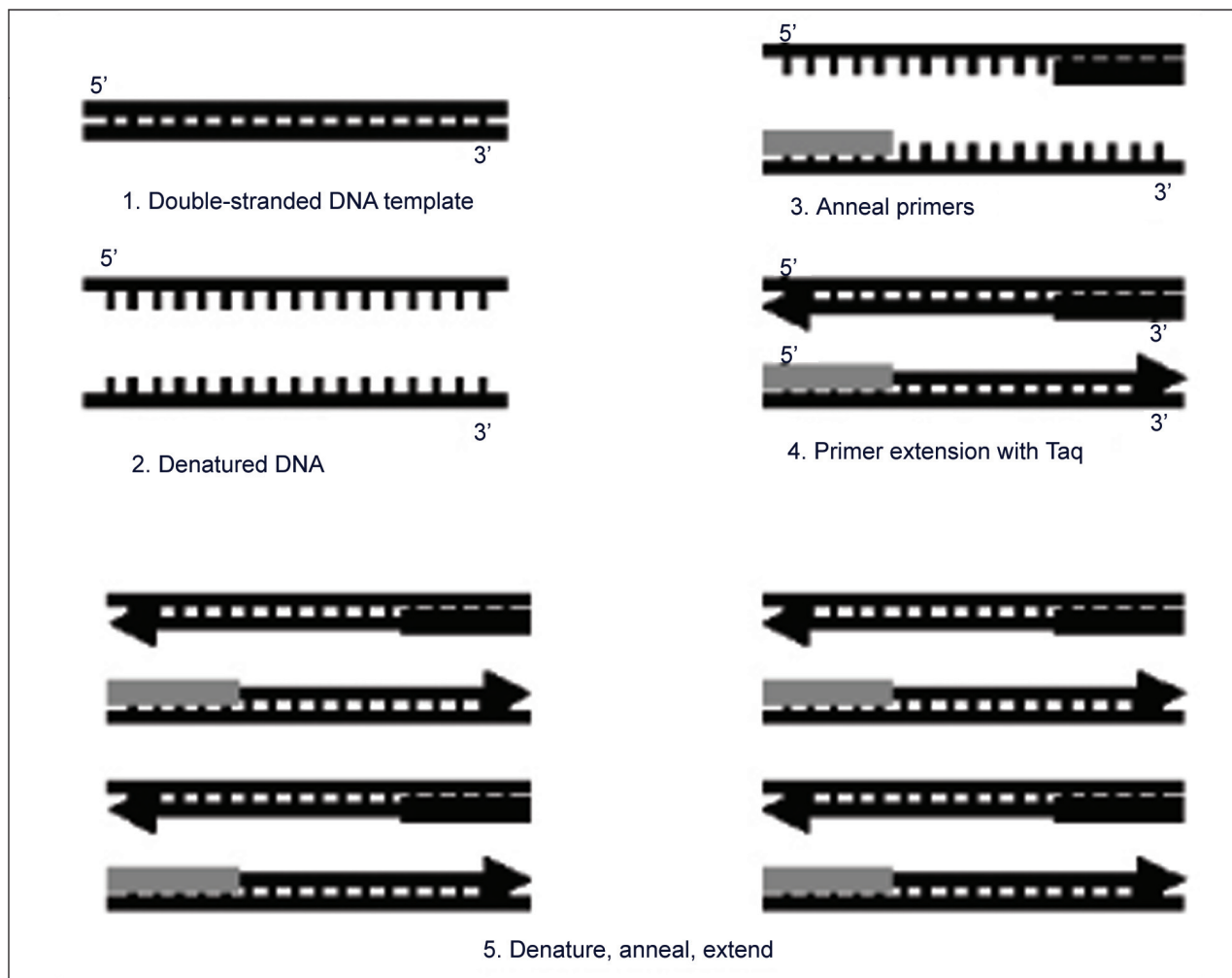


Figure 1. The polymerase chain reaction.

repeated for multiple cycles, each time doubling the amount of the DNA of interest, and as a result, a minute quantity of DNA can be greatly amplified (Figure 1). The PCR product is then analyzed by gel electrophoresis and directly visualized or detected by Southern blot analysis. It can also be isolated from the gel and further analyzed or cloned.

PCR requires only very small amounts of template DNA. This renders it an extremely sensitive technique, applicable even when only small amounts of tissue are available or when disease burden is low. PCR is particularly suitable for the detection of minimal residual disease. However, the great sensitivity of the technique is also a potential limitation in that minuscule amounts of contaminating DNA can generate false-positive results. False-negative results can also occur, especially when primer design and/or temperature conditions are suboptimal. Strict controls are necessary to guard against incorrect interpretation.⁸

RT-PCR

The flow of genetic information proceeds from DNA to RNA and then to protein synthesis. Before a protein is synthesized, the DNA of the corresponding gene is transcribed into messenger RNA, which is modified and subsequently

translated into protein. This process is referred to as gene expression. Reverse transcriptase (RT)-PCR is a modification of the PCR technique for amplifying RNA sequences for analysis of gene expression. First, RNA is isolated from the tissue sample and transcribed into complementary DNA (cDNA) by a retroviral enzyme called reverse transcriptase. The sequence of interest in the cDNA can then be amplified by PCR.

Quantitative PCR

Quantitative PCR, in which the relative number of copies of a DNA sequence of interest is determined, is increasingly used to detect minimal residual disease in patients with hematologic malignancies in clinical remission. Initial quantitative PCR assays compared the final quantity of the PCR product of interest to the amount of product produced after simultaneous PCR amplification of a ubiquitously expressed gene such as actin. One important consideration in the interpretation of these assays is the fact that PCR amplification is exponential only when the reaction components are in excess. Accumulation of PCR product plateaus as the amounts of Taq enzyme and primers in the reaction become rate limiting. A more recently developed

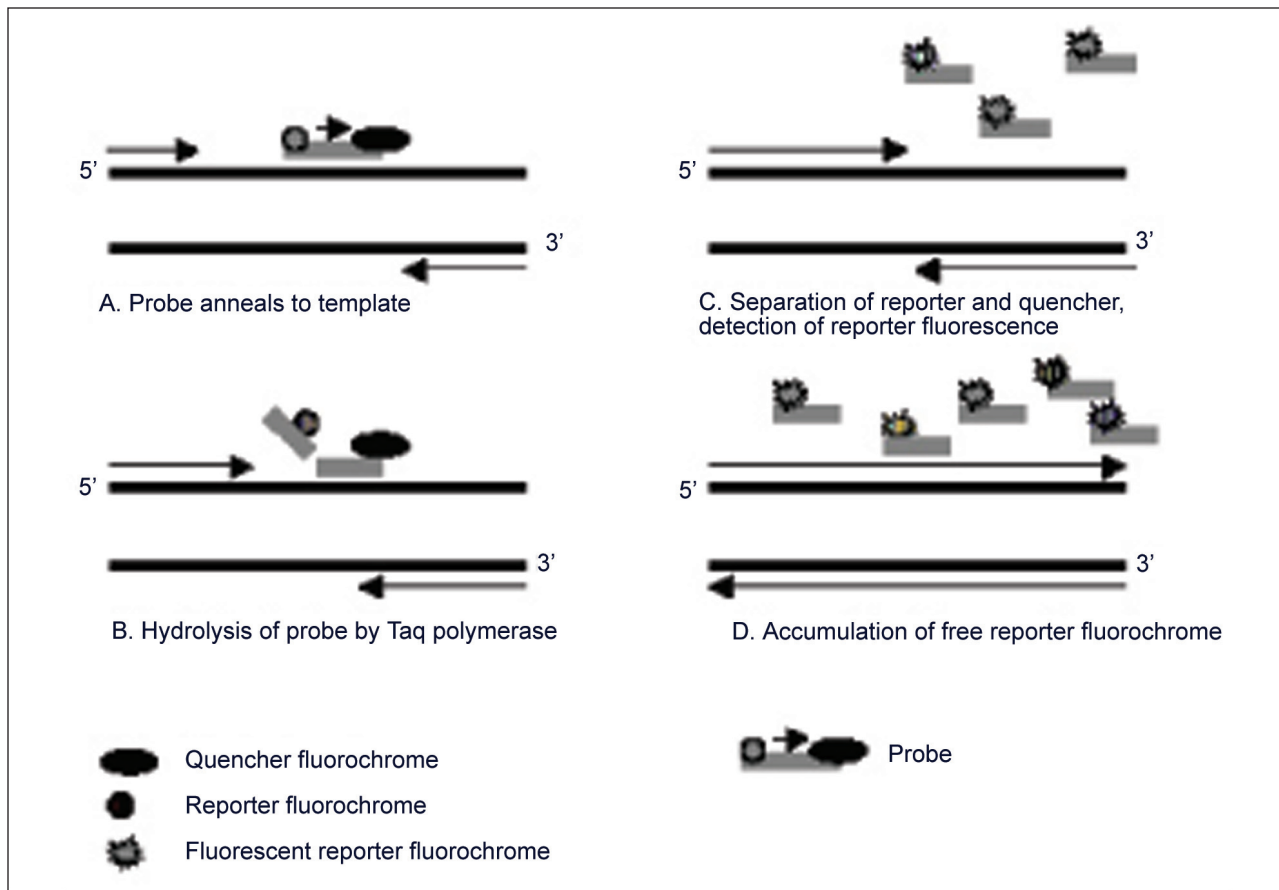


Figure 2. Quantitative polymerase chain reaction (PCR; hydrolysis probe method). The probe is conjugated with both a quencher and a reporter fluorochrome. The quencher absorbs the fluorescence of the reporter as long as the probe is intact. Upon amplification the probe is displaced and hydrolyzed by the Taq polymerase. Once the reporter and quencher are separated, the fluorescence becomes detectable. The amount of fluorescence increases with every PCR cycle.

quantitative PCR technique, “real-time” PCR, measures the accumulation of PCR product during the exponential phase of amplification as it occurs, that is, in “real” time. This can be achieved by several methods, all of which are based on the principal that the emission of fluorescence increases in proportion to the amount of product accumulating in the PCR reaction. For example, quantitative PCR with hydrolysis probes uses an oligonucleotide probe that contains both fluorescent “reporter” and “quencher” dyes (Figure 2). The quencher fluorochrome absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact and the 2 dyes are in close proximity. The probe anneals to the target sequence, and as DNA polymerization proceeds by primer extension, it is cleaved from the template by the Taq polymerase and is also hydrolyzed by this enzyme. This results in the separation of the reporter and the quencher dyes and increases the intensity of the fluorescent signal. The number of cycles at which a threshold level of fluorescence is reached can be measured and is proportional to the initial number of copies of the target DNA in the sample.⁹ Several other methods of quantitative PCR, using other types of probes, are currently in use (reviewed in van der Velden.⁹)

DNA Microarray Technology

The cloning of the human genome and technological advances in molecular techniques have led to the development of DNA microarrays, which are powerful tools that can screen expression of thousands of genes in a single procedure. This technique has multiple potential applications in hematology/oncology, including the improved classification of tumor subsets, the identification of genes associated with chemotherapy resistance, and the design of new anticancer therapies based on novel therapeutic targets.^{10,11} There are 2 commonly used arrays, oligonucleotide and cDNA arrays. Both methods utilize small glass slides that are comprised of thousands of tiny squares. Multiple copies of a single oligonucleotide or cloned cDNA fragment are anchored to each square so that each slide contains thousands of genes. The Affymetrix oligonucleotide arrays contain synthetic short DNA fragments that have been synthesized in situ on the surface of the glass.¹² Synthetic oligonucleotides can also be robotically spotted onto the glass slides. cDNA arrays contain cloned cDNA fragments fixed to the slides.

Messenger RNA (mRNA) is extracted from the clinical sample and transcribed into cDNA that is then fluorescently labeled and hybridized to the glass slide array. There

are 2 main methods of analysis of the bound probe. In the Affymetrix system and in some spotted systems, the slides are scanned and the amount of specific mRNA in the experimental sample is calculated based on the intensity of the signal emitted by the dye from the sites on the slide that contain the gene of interest. Most spotted arrays use 2 samples, the experimental sample and a control sample. Each sample is labeled by a separate dye, and both are simultaneously hybridized to the slide. The ratio between the signals emitted by the 2 dyes is used to calculate the quantity of the cDNA of interest in the experimental sample.¹¹

Typically these experiments include a number of samples and thousands of genes. As a result they produce very large data sets, and sophisticated mathematical methods are needed for their analysis. Two main approaches have been developed to analyze microarray expression data: unsupervised and supervised learning. Unsupervised learning methods group samples based on similar patterns of expression without a priori knowledge of sample classification. In supervised learning the samples are analyzed in groups of interest, and expression patterns that distinguish between the groups are identified. These patterns are then used on an independent data set, and their ability to distinguish between the groups of interest is tested. For example, when comparing diffuse large B cell lymphoma samples for expression patterns that predict survival, unsupervised learning approaches use algorithms that look for differences between the samples without including patient outcome data. Supervised learning of the same data set includes methods of finding genes that are differentially expressed between samples from patients that have been already grouped by survival data.^{10,11}

Proteomics

Proteomic analysis couples modern mass-spectroscopy techniques and bioinformatics for the simultaneous analysis of the thousands of proteins present in a particular tissue or sample. Although more difficult and painstaking than gene expression profiling by cDNA or oligonucleotide microarray analysis, proteomics provides the most immediate picture of the array of proteins that govern cellular function. Several studies have demonstrated that protein expression does not always mirror mRNA expression, presumably due to pathways that control rates of translation and protein stability.^{13,14} Furthermore, although the amino acid sequence of proteins is encoded in the DNA, after translation many proteins undergo modifications such as glycosylation, phosphorylation, cleavage, and binding to other proteins. These post-translational modifications may be critical in determining cellular function. Posttranslational events are not captured by DNA microarray technology and can only be observed by direct examination of the proteins themselves.

In proteomic pattern profiling, proteins undergo ionization and are then separated on gels by both charge and mass. A mass-to-charge ratio can then be calculated for each protein. Supervised or unsupervised learning approaches, as described above, can then be used to identify patterns within the data.

Proteomics can be performed on small amounts of serum or body fluid and therefore, a major focus of ongoing research is the use of this technique as a screening tool for the early detection of cancer. Preliminary studies have demonstrated the potential of proteomics in the early detection of ovarian, breast, and prostate cancers, although the clinical applications of these findings are not yet established.¹⁵⁻¹⁷

APPLICATION OF MOLECULAR TECHNIQUES TO THE DIAGNOSIS AND CLASSIFICATION OF MALIGNANCY

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous disease, with subtypes that differ in their clinical course, prognosis, and response to therapy. The strongest prognostic indicator in AML is the presence or absence of specific cytogenetic abnormalities in the leukemic cells. Several pathognomonic chromosomal translocations have been identified that subclassify AML into prognostically distinct subgroups. As a result, the management of an individual patient is increasingly determined by the genetic subtype of his or her disease. Five-year survival ranges from 56% to 65% in patients with good-prognosis cytogenetics to only 12–26% in those with poor-prognosis karyotypes.^{18,19} Multiple studies have shown that molecular techniques can detect genetic abnormalities that are not apparent by conventional cytogenetic analysis, and these techniques can therefore provide essential information both on prognosis and on optimal clinical management.²⁰

Acute Promyelocytic Leukemia: The t(15;17)(q12;q11) translocation is pathognomonic for acute promyelocytic leukemia (APL). In this translocation, the promyelocytic leukemia (*PML*) gene on chromosome 15 fuses with the retinoic acid receptor- α (*RAR α*) gene on chromosome 17. The resultant chimeric fusion protein *PML/RAR α* inhibits the native *PML* and *RAR α* , which causes an arrest of retinoic-acid dependent transcription, inhibition of apoptotic pathways, and an arrest of myeloid maturation at the promyelocyte stage.²¹

In the majority of patients with APL, all-trans retinoic acid (ATRA), administered in pharmacologic doses, can override the inhibition of *PML/RAR α* , and induce maturation of blasts. The early use of ATRA in combination with chemotherapy has led to a significant improvement in the cure rate of APL. Thus the prompt diagnosis of this subtype is critical in the management of these patients.^{22,23}

In most APL patients the diagnosis is suspected from the characteristic morphology of the blasts and the clinical picture, even before the results of the cytogenetic analysis are obtained. The microgranular variant, which occurs in 15–20% of cases, may be more difficult to recognize morphologically because the blasts do not have the characteristic large azurophilic granules. The diagnosis is usually suggested by either flow cytometry or by immunohistochemistry using antibodies against the *PML* protein.²⁴

Molecular techniques can be useful in patients in whom morphology combined with flow cytometry and/or immu-

nohistochemistry suggests APL, but cytogenetic analysis is either not successful, or does not demonstrate t(15;17).^{24,25} In some patients with APL, the *RAR α* gene is fused to other genes, including promyelocytic leukemia zinc finger protein (*PLZF*) on chromosome 11, nucleophosmin (*NPM*) on chromosome 5, nuclear mitotic apparatus (*NuMa*) on chromosome 11, or signal transducer and activators of transcription (*STAT*) 5b on chromosome 17. The sensitivity of these variants to ATRA is less predictable, and the overall prognosis is less favorable. In a study of 611 patients with newly diagnosed AML in which morphology suggested APL, 9% had a successful cytogenetic analysis that did not demonstrate the t(15;17). Sixty of these patients were studied in a central workshop by FISH, RT-PCR, and/or Southern blotting and were found to have either molecular evidence of *PML/RAR α* fusion (42/60), *PLZF/RAR α* rearrangements (11/60), or *NPM/RAR α* translocations (2/60).²⁶

Another potential use for molecular techniques in APL is to identify the breakpoint site in the *PML* gene. Most studies have shown that survival is shorter in patients with an intron 3 breakpoint and a shorter isoform compared with the long isoform which correlates to an intron 6 breakpoint.^{27,28}

One of the most important applications of molecular techniques in APL is the detection of minimal residual disease. In an Italian multicenter study,²³ RT-PCR for *PML/RAR α* was performed in 163 patients at multiple time points after induction and consolidation therapy. In all patients, the transcript was undetectable after consolidation; however, during follow-up it became detectable in 21, and 20 of these patients relapsed. Relapse occurred in only 8 patients of the 142 who did not have detectable *PML/RAR α* during surveillance. Treating patients with chemotherapy during molecular relapse resulted in excellent 2-year survival, compared with historical controls.

In summary, molecular techniques play several roles in the modern management of APL, including the detection of *PML/RAR α* in patients without informative cytogenetics, the identification of alternative *RAR α* translocations, and the postremission monitoring for minimal residual disease.

AML With Core Binding Factor Translocations t(8;21), inv(16), and t(16;16): Approximately 10–15% of de novo AML patients have translocations that result in fusion proteins involving subunits of the core binding factor (CBF) transcription factor complex. These translocations fuse either *CBF α* , located on chromosome 21q22, with *ETO*, located on chromosome 8, or *CBF β* , located on chromosome 16q22, with smooth muscle myosin heavy chain (*SMMHC*) on chromosome 16. The resultant chimeric proteins produced by the translocations inhibit the normal function of CBF, which is a transcription factor involved in the regulation of multiple genes important in normal hematopoiesis. Patients with these translocations have a better prognosis, especially when treated with high-dose cytarabine as consolidation.²⁹

Screening patients with newly diagnosed AML for these

translocations by RT-PCR will detect CBF abnormalities in cases in which conventional cytogenetics are not informative.³⁰⁻³² However, the percent of patients detected by RT-PCR who would not have been identified by conventional cytogenetics varies between studies, with a range between 37% to less than 10%. The reasons for the discordant results between the studies are probably related to the quality of the cytogenetic studies and the extracted RNA (reviewed by Rose and Berliner²⁰).

In contrast to APL, RT-PCR for the *CBF α -ETO* translocation to detect minimal residual disease is not useful in predicting disease outcome.³³⁻³⁵ Patients in remission following either chemotherapy or stem cell transplantation can have detectable *CBF α -ETO* fusion transcripts that persist long term. This may reflect the biology of the role of the translocation product, which is thought to be a necessary but insufficient pathogenetic event in the evolution of AML.³⁶ A stable level of RT-PCR detectable *CBF α -ETO* transcripts in the setting of treated t(8;21)-associated AML is not predictive of imminent relapse. However, conversion from a negative to a positive study, or increasing levels of fusion transcripts do predict relapse.³³

Chronic Myelogenous Leukemia

Diagnosis: The hallmark of chronic myelogenous leukemia (CML) is the Philadelphia chromosome (Ph), which is a translocation between chromosomes 9 and 22 t(9;22)(q34;q11). This results in the fusion of the 3' end of the *c-abl* gene to the 5' end of the *bcr* gene.³⁷ The *c-abl* gene encodes a tyrosine kinase which, when fused to *bcr*, becomes constitutively active and relocated from the nucleus to the cytoplasm. Approximately 5–10% of patients with the typical clinical presentation of CML do not have cytogenetic evidence of Ph. About one third of these patients will have a cytogenetically occult *bcr-abl* gene, which can be detected by RT-PCR or FISH. These patients have a clinical course and response to therapy which is indistinguishable from Ph-positive CML.³⁷⁻³⁹ Therefore, RT-PCR for the *bcr/abl* translocation should be performed in all patients in whom the diagnosis of CML is suspected, but in whom Ph is not observed.

Minimal Residual Disease: FISH, PCR, and especially real-time quantitative PCR are increasingly used in the monitoring of minimal residual disease in CML, both in patients treated with imatinib mesylate and after allogeneic stem cell transplant. Imatinib results in a cytogenetic remission in over 70% of patients in chronic phase CML. However, in the vast majority of these patients, the *bcr/abl* translocation can still be detected using molecular techniques such as FISH or RT-PCR. Using a quantitative real-time RT-PCR test, Hughes et al⁴⁰ demonstrated that patients who had at least a 3-log reduction in the number of *bcr/abl* transcripts in peripheral blood after 1 year of therapy with imatinib had a lower chance of relapse compared with patients with a higher number of *bcr/abl* transcripts. Quantitative PCR for *bcr/abl* in peripheral blood is becoming increasingly available in commercial and hospital-based laboratories, and will most

likely become the standard method of monitoring patients with CML receiving treatment with imatinib. Patients who have a rise in the number of *bcr/abl* transcripts on imatinib therapy should be considered for alternative therapy such as allogeneic stem cell transplant.

In patients who have undergone allogeneic transplant for CML, quantitative PCR is used to monitor the change in the level of *bcr/abl* transcript over time.^{41,42} Low levels of *bcr/abl* transcripts can be detected by PCR in the majority of patients following stem cell transplant for CML, especially in the first 6 months. Some *bcr/abl* transcripts may persist at a stable level long term.^{43,44} However, several studies have indicated that an increase in the *bcr/abl* transcript number by quantitative PCR is predictive of relapse in patients post transplantation, and may be an indication for interventions such as donor lymphocyte infusion.^{42,45}

Acute Lymphoblastic Leukemia

As in myeloid leukemias, genetic alterations present in lymphoblastic leukemia cells profoundly influence their clinical behavior. Molecular studies have provided important insights into the pathogenesis of the disease and are increasingly used in the diagnosis and the management of acute lymphoblastic leukemia (ALL). In childhood ALL, where the majority of patients can be cured of their leukemia, molecular studies permit the identification of patients at high risk for disease recurrence, for whom intensified therapy is appropriate, from patients with favorable features who may be spared unnecessarily toxic therapy.

Molecular Risk Stratification in ALL: ALL is a molecularly heterogeneous disorder. The frequency of specific cytogenetic derangements in ALL varies with the age of the patient.⁴⁶ For example, t(9;22) is the most common molecular derangement in adults with ALL, where it is detected in approximately 25% of cases. This translocation is present in only about 3% of children with B cell precursor ALL. The presence of this translocation is an adverse prognostic feature with 5-year event-free survival of only approximately 25% in children and of less than 10% in adults treated with conventional multi-agent chemotherapy. Similarly, it has been found that translocations involving the *MLL* gene on chromosome 11q23 have negative prognostic significance. *MLL* combines with various fusion partners, the most common of which is the *AF4* gene on chromosome 4, generating t(4;11). *MLL* gene rearrangements are characteristic of ALL in infants (approximately 50% of ALL arising in the first year of life harbors an *MLL* rearrangement), but they also occur in about 10% of ALL cases in older children and adults. This cytogenetic feature portends a poor prognosis with conventional chemotherapy, but the addition of high-dose cytarabine may improve outcome for these patients.⁴⁷ The higher incidence of adverse cytogenetic features in adults and young infants compared with childhood ALL explains, in part, the large gap in outcome among these groups of patients. When adverse molecular features are identified in patients with ALL, therapy may be intensified and transplantation is often

considered.

As described above, conventional cytogenetic studies are insensitive to subtle chromosomal rearrangements. Newer and more sensitive molecular studies are used to complement cytogenetic studies in the diagnostic workup of ALL. These studies have the power to detect “cryptic” abnormalities, when conventional cytogenetics fail or are reported as normal. An important example is the t(12;21) translocation, which is rarely detected on routine cytogenetics of ALL cells. However, when specific FISH or PCR probes are applied, it has been shown that this translocation is present in about 22% of children with ALL and about 2% of adult cases.⁴⁸ This translocation creates a fusion between 2 transcription factors, the *TEL* gene on chromosome 12 and the *AML1* gene on chromosome 21. Patients with this abnormality appear to have a relatively favorable outcome with modern chemotherapy protocols. Abnormalities of chromosome number also commonly occur in ALL and have prognostic significance. Hyperdiploidy (>50 chromosomes) occurs in approximately 25% of children with ALL and these children are at very low risk of treatment failure. Ploidy can be determined by conventional cytogenetics, by flow cytometry or by use of centromere-specific FISH probes. Molecular studies including FISH and/or PCR to detect t(9;22), t(12;21), and 11q23 rearrangements are now a standard component in the diagnostic evaluation of childhood ALL. Because of the high incidence and prognostic significance of t(9;22) in adults, specific molecular tests for this translocation are obtained in all adult patients with ALL.

Ph-Positive ALL Versus CML With Lymphoid Blast Crisis: Patients with CML may experience lymphoid blast crisis (ie, ALL) arising at variable latency from the diagnosis of their CML. Lymphoid blast crisis may in fact present in the absence of a preceding clinically appreciated stable phase. ALL in this setting is morphologically indistinguishable from ALL that arises de novo. Molecular techniques can aid in discriminating between CML in blast crisis and Ph-positive ALL. As described above, the cytogenetic hallmark of CML is Ph, which fuses the *bcr* gene to the *c-abl* gene in the translocation t(9;22). In most cases of CML, the *bcr/abl* fusion transcript encodes a chimeric oncoprotein of 210kD. This transcript may be identified using RT-PCR. The transcript encoding the p210 oncoprotein is also detectable in approximately half of the adults with Ph-positive ALL. However, in the remaining 50% of adults and the majority of children with Ph-positive ALL, the *bcr-abl* translocation encodes a 190 kD protein. Using RT-PCR, this translocation may be specifically identified. Distinguishing between these 2 diseases is important in the monitoring of residual disease before and after hematopoietic cell transplantation and in the use of donor lymphocyte infusion after transplantation.^{46,49}

Minimal Residual Disease Detection in ALL: With modern induction regimens, the majority of patients with newly diagnosed ALL achieve remission, defined as less than 5% bone marrow blasts. However, residual leukemia cells per-

sist in these patients below the level of detection by light microscopy and may lead to recurrence of the leukemia. Increasingly sensitive techniques have been applied to patients undergoing treatment for ALL to detect residual leukemia with the goal of identifying patients that are at high risk of treatment failure. Several different strategies have been successfully applied, including immunophenotyping (flow cytometry⁵⁰), FISH, and PCR techniques. Whereas conventional cytogenetic techniques have the power to detect 1 malignant cell in 20–40 metaphases analyzed, these more sensitive techniques may detect 1 malignant cell in up to 10⁴–10⁶ nucleated cells. FISH and PCR-based studies have been developed to detect leukemia-associated translocations (eg, t(9;22) and t(12;21)⁵¹) and clone-specific rearrangements of the immunoglobulin and T-cell receptor loci.⁵² In a study that followed 104 children with ALL who had clone-specific quantitative PCR performed on bone marrow on day 29 of induction therapy, each of the 16 children that relapsed had higher levels of minimal residual disease (MRD) than those that stayed in remission.⁵³ None of the children with MRD levels less than 0.01% relapsed within the 4-year median follow-up period. Therapeutic research protocols will soon begin to incorporate MRD into treatment algorithms for children with ALL.

Microarrays in ALL: Applications to Diagnosis and Treatment: DNA microarray studies have yielded important new insights into leukemia pathogenesis and will likely lead to the rational development of new therapies in the future. Early studies demonstrated that the distinction between ALL and AML could be accurately made on the basis of gene expression profiles.⁵⁴ Subsequent studies demonstrated that microarray technology has the power to discriminate among several subtypes of ALL including: *E2A-PBX*, *MLL*, T-ALL, hyperdiploid, *bcr/abl*, and *TEL-AML1*.⁵⁵ The application of DNA microarray technology to the evaluation of infant ALL with rearrangement of the *MLL* gene located at chromosome 11q23 demonstrated a molecular signature distinct from ALL and AML, with molecular features of both. In addition, this work identified the *FLT-3* gene as one of the genes distinguishing infants with *MLL*-associated ALL from those with non-*MLL*-rearranged, pre-B-cell ALL.⁵⁶ Subsequent work has confirmed abnormalities in *FLT-3* in approximately one third of these infants and led to the evaluation of *FLT-3* as a therapeutic target in these leukemias.⁵⁷ Gene expression profiling studies are now also being applied to predict response to chemotherapy in patients with ALL. One recent study used supervised clustering analysis based on in vitro sensitivity data to identify sets of genes whose expression profiles were correlated with treatment outcome in children with ALL.⁵⁸ The pattern of expression of these genes was translated into a combined drug resistance gene expression score for each patient. Those patients with “high” scores had a significantly inferior outcome compared with those with “low” scores. It is expected that these types of studies may ultimately be used to tailor therapy for patients with ALL.

Lymphomas

Gene Rearrangement Studies

B and T lymphocytes rearrange their immunoglobulin (Ig) and T-cell receptor (TCR) loci, respectively, by a complex process of rearrangement and deletion of DNA sequences. This process serves as a mechanism for generating antibody and cellular diversity, enabling the immune system to respond to the enormous array of antigenic challenges that it faces. This process gives each lymphocyte a unique molecular “signature” associated with the final gene product. The biologic effect is the production of a unique protein that can respond to a specific antigenic challenge to the cell.

When a lymphoid cell undergoes malignant transformation, all its daughter cells will share the same unique sequence of immunoglobulin or TCR genes. This accumulation of identically rearranged Ig or TCR gene loci gives the resulting tumor the same unique molecular “signature” that can be detected by molecular techniques. Clonal rearrangement of TCR or Ig genes can be detected by Southern blot analysis or by PCR. PCR is the most widely used in the clinical setting, however, its sensitivity is only 70–80%. False-negative results occur because of the difficulty in designing primers that will reliably anneal to different variable regions of the immunoglobulin and TCR genes. Although Southern blotting has less false-positive and false-negative results, it is now rarely used outside the research setting because it is labor intensive and requires a relatively large amount of good quality DNA.^{59,60}

Gene rearrangement studies provide a sensitive means of proving the lymphoid origin of cells in question, and demonstrating their clonality. This can be utilized in diagnosing a lymphoproliferative disorder when the cells are poorly differentiated and lack the necessary surface markers to identify their origin. Clonality in B-cell proliferations can usually be established by the light chain expression pattern of the cells. Because Ig genes are expressed on the cell surface and have a clonal expression of either κ or λ light chains, clonal populations of B-cells can be detected by flow cytometry for light chain expression, obviating the need for molecular studies in most patients.⁶¹ However, the TCR does not have a similar clonally determined marker. Consequently, gene rearrangement studies can be essential in distinguishing polyclonal from monoclonal T-cell proliferations in morphologically ambiguous cases.

Expression Profiling in Lymphoproliferative Disorders

Microarray studies are advancing our ability to classify lymphomas into groups with more uniform prognosis. Diffuse large B-cell lymphoma (DLBCL), which is the most common aggressive lymphoma in adults, is a clinically heterogeneous disease for which more accurate prognostic indices are needed. Alizadeh et al⁶² created a specialized cDNA microarray, the “Lymphochip,” using genes from a germinal center B-cell library, from lymphoma libraries, and genes known to be involved in lymphocyte biology. Using unsupervised learning algorithms, 2 molecularly distinct forms

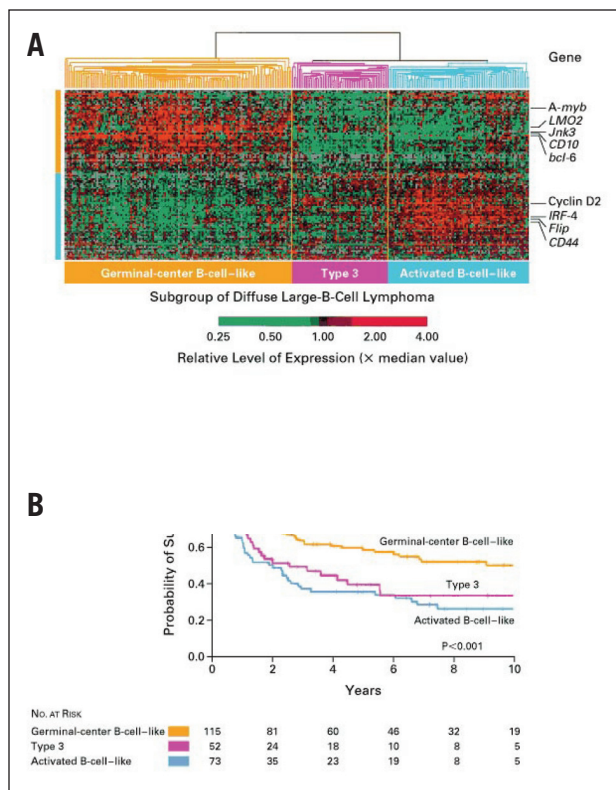


Figure 3. Diffuse large B-cell lymphoma (DLBCL) grouped by gene expression profiles. A. Hierarchical clustering of expression profiles of 100 genes in 240 cases of DLBCL. Each column represents a single patient and each row represents a single gene. Red areas indicate increased expression and green areas decreased expression. Specific genes that are expressed in germinal-center like DLBCL and in activated DLBCL are listed on the right. B. Kaplan–Meier estimates of overall survival in the 240 previously untreated patients, according to the expression profile subgroup.

Modified with permission from Rosenwald et al.⁶³

of DLBCL were identified. One type expressed germinal center B-cell genes, and the second type expressed genes known to be induced during B-cell activation. Patients with the germinal-center-like DLBCL had a better prognosis than the ones with the activated B-cell-like lymphoma. These results were confirmed by Rosenwald et al⁶³ using the same microarray in 240 DLBCL samples. These researchers then used supervised learning to identify 17 genes that could group the patients into 4 risk categories with a 5-year survival ranging from 15% to 73% (Figure 3). Shipp et al⁶⁴ used supervised learning methods and an oligonucleotide gene microarray to identify other genes that predicted outcome in a series of DLBCL.

It is still unclear which model and method will be the most useful in predicting prognosis in DLBCL. However, it is likely that microarray technology will add to, and perhaps replace, the International Prognostic Index and other clinical and pathologic tools that are currently in use for predicting prognosis in lymphoproliferative disorders.

Molecular Diagnostics in Solid Tumors

Breast Cancer

Our understanding of the molecular basis of solid tumors has lagged behind that of the malignancies of hematopoietic origin, reflecting the genetic complexity of solid tumorigenesis. Thus, the use of molecular techniques as guides to prognosis and choice of therapy is not yet as widespread. However, some genetic features of solid tumor cells have been elucidated and are candidates for analysis by molecular techniques. Perhaps the most rapid progress in this regard has been made in breast cancer, a highly heterogeneous disease in which our traditional methods of subtyping are relatively crude. The most prominent example of the progress made in the molecular profiling of breast cancer is the identification of the *HER2/neu* oncogene, a member of the epidermal growth factor receptor family. Multiple studies have shown overexpression of this oncogene to be a poor prognostic factor in breast cancer. Furthermore, therapy with trastuzumab (Herceptin, Genentech), a monoclonal humanized murine antibody targeted against the extracellular portion of *HER2/neu*, improves survival in patients whose tumors overexpress *HER2/neu*. Detection of amplification of the *HER2/neu* oncogene by FISH analysis has become a standard test for determining prognosis and for predicting response to trastuzumab therapy.⁶⁵

Expression Profiling in Breast Cancer

The majority of patients with early stage breast cancer are treated with adjuvant therapy, although only a minority benefit from this approach.⁶⁶ Preliminary data in breast cancer suggests that expression profiling holds promise both in identifying the patients with early stage breast cancer who are at risk for relapse, and therefore would benefit from adjuvant therapy, and in predicting response to chemotherapy. In a pivotal study, van't Veer et al⁶⁷ performed gene expression analysis in 78 women with node-negative breast cancer and, using supervised learning, identified 70 genes that could predict which women would develop metastatic disease at 5 years. The expression profile of these 70 genes was evaluated in 295 patients with breast cancer and found to be a strong independent predictor of disease outcome and survival. The 10-year survival rates for the patients with good-prognosis expression profile was $94.5 \pm 2.6\%$, compared with $54.6 \pm 4.4\%$ in the poor-prognosis group.⁶⁸ Prospective adjuvant breast cancer trials are being planned to assess the validity of the microarray techniques in predicting prognosis and assigning adjuvant therapy.⁶⁹

Preliminary data using gene expression microarray data to predict sensitivity to chemotherapeutic agents have also been published, and suggest that, in the future, we may be able to tailor chemotherapy and decrease the number of patients who receive unnecessary therapy. For example, using supervised learning on samples from 24 breast cancer patients, Chang et al⁷⁰ identified 92 genes that could predict docetaxel response in 6 independent samples.

Conclusion

This review focused on the malignancies for which molecular techniques have made the largest contribution to-date to the determination of diagnosis and prognosis. During the near future we will likely witness an explosion of new data regarding the application of these techniques to an increasing number of malignancies. With the development of more targeted agents, cancer therapy will become less empiric and more rational, as the molecular analysis of a specific tumor will help guide the choice of therapy. Thus it is anticipated that our reliance on molecular techniques in the diagnosis and management of malignant disorders will expand rapidly over the next few years. These developments will likely greatly improve the quality of care delivered to patients with cancer.

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