

ADOPTING MOLECULAR TOOLS FOR DIAGNOSIS AND MONITORING MALIGNANT HEMATOLOGICAL DISEASE: FROM MORPHOLOGY TO GENETIC-MOLECULAR PROFILE

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RESUMEN •

La diversidad clínica y biológica de los cánceres hematológicos depende principalmente del origen celular sanguíneo y la etapa de diferenciación ontogénica, pero estos cambios son sólo la expresión de profundas alteraciones genéticas y moleculares produciendo un panorama muy complejo de perfiles distintivos clínicos, bioquímicos, morfológicos, cromosómicos y moleculares. Ahora, podemos redefinir algunos cánceres hematológicos dependiendo de marcas específicas moleculares y genéticas, pero la mayor parte de las enfermedades malignas hematológicas aun requieren los métodos mas tradicionales como la biometría hemática, la revisión morfológica y la citometría, en conjunto con el uso juicioso de técnicas mas sofisticadas. Este trabajo revisará los métodos mas usados en el estudio diagnóstico y seguimiento de los principales problemas hemato-oncológicos.

ABSTRACT •

THE CLINICAL and biological diversity among hematological cancers depends mainly on the cellular origin and stage of their ontogenic differentiation; those changes represent the expression of profound genetic and molecular disturbances becoming a very complex landscape of clinical, biochemical, morphologic, chromosomal and molecular distinctive profiles. Now we can redefine some hematological cancers depending on specific molecular or genetic imprints, but most malignant hematological diseases still deserve the traditional and useful complete blood count, morphological review and cytometry, together with the judiciously use of more sophisticated techniques. This work will review the methods used in the work-up for the diagnosis and monitoring of hematological malignant diseases.

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INTRODUCTION •

The Malignant Hematological Disease

The diagnosis and monitoring of hematologic cancers is a hallmark of biomedical sciences mainly by the complete expression of the disease at all levels such as molecular, genetic, epigenetic, proteomic, cell and tissue compartments, as well the disease implication on tissular, organic and systemic levels. This implies the use of laboratory tools to characterize the disease, diagnosing and monitoring it. Moreover, the many stages of normal hematopoietic differentiation and the disruption of normal growth, differentiation and apoptosis, give rise to a number of biologically and clinically distinct cancers, currently revisited by World Health Organization with proposals and recategorizing systems (1,2). Most new classifications systems now include very refined and sophisticated methods, becoming a necessity for the clinician to understand the most important laboratory techniques as well as their prognostic and treatment implications.

During the last 20 years, the diagnosis of hematologic cancers emerged from cytomorphology (and histopathology) alone to a comprehensive bundle of different methods that are necessary not only for the diagnosis and classification but also for individual treatment decisions. For example, for acute leukemia diagnosis, there are some algorithms that combine cytomorphology and cytochemistry with immunophenotyping accompanied by cytogenetics and molecular genetic methods that deserve to be established in the laboratory setting (3,4). Since methods, not only add important information for diagnosis, but also define markers for minimal residual disease (MRD) studies, this has to be considered at the first point of any analysis in every single patient. Furthermore, diagnostic results are equivalent to the most important prognostic parameters helping the clinician to take decisions (5).

So, the biological and clinical diversity of hematologic cancers implies the use of a diversity of techniques. As new techniques have been estab-

lished within the last decade, and were brought to routine use, it seems necessary to define a comprehensive global approach in the diagnosis of hematologic cancers. This could include a step-wise procedure in the lab flow in order to save time and money without losing important and detailed information, this procedure must be validated by each laboratory and institution.

After introducing the different techniques in the diagnosis and their respective results, this paper will review the most relevant techniques in the hemato-oncology diagnosis and monitoring, a laboratory work-up will also be suggested, it includes a comprehensive diagnosis that can be the basis for classification, therapeutic decisions, MRD studies, leading to prognostic markers and being cost effective if applied in a step-wise workflow.

BLOOD SMEAR •

An examination of the blood smear (or film) may be requested by physicians or initiated by laboratory staff. With the development of sophisticated automated blood-cell analyzers, the proportion of blood-count samples that require a blood smear has steadily diminished and in many clinical settings is now 10 to 15 percent or less, this is true mainly in general medicine practice, but the proportion could rise in some special situations such as in the oncology setting. Nevertheless, the blood smear remains a crucial diagnostic aid (6).

For maximal information to be derived from a blood smear, the examination should be performed by a skilled person, either a laboratory scientist or a medically qualified hematologist or pathologist. In Europe, only laboratory-trained staff members generally “read” a blood smear, whereas in the United States, physicians have often done this (7). In Mexico, hematologists as well clinical pathologists are commonly well trained and are currently reviewing blood smears and also bone marrow smears.

In comparison with the procedure for an automated blood count, the examination of a blood smear is labor-intensive and therefore relatively expensive and must be used judiciously.

A physician-initiated request for a blood smear is usually a response to perceived clinical features or to an abnormality shown in a previous complete blood count. A laboratory initiated request for a blood smear is usually the result of an abnormality in the complete blood count or a response to “flags” produced by an automated instrument.

Less often, it is a response to clinical details given with the request for a complete blood count when the physician has not specifically requested examination of a smear. For example, a laboratory might have a policy of always examining a blood smear if the clinical details indicate lymphadenopathy or splenomegaly. The International Society for Laboratory Hematology has published consensus criteria (available at www.islh.org) for the laboratory-initiated review of blood smears on the basis of the results of the automated blood count. The indications for smear review differ according to the age and sex of the patient, whether the request is an initial or a subsequent one, and whether there has been a clinically significant change from a previous validated result (referred to as a failed delta check). All laboratories should have a protocol for the examination of a laboratory-initiated blood smear, which can reasonably be based on the criteria of the International Society for Laboratory Hematology. Regulatory groups should permit the examination of a blood smear when such protocols indicate that it is necessary (6,7). The National Cancer Institute of Mexico has the policy to review all blood smears as requested by the physician and also the smears with profound cytopenias or leukocytosis and thrombocytosis.

Blood smears must always be examined in case of unexplained leukocytosis, lymphocytosis, or monocytosis or if the flagging system of an automated instrument suggests the presence of blast cells. Depending on the instrument and the

practice of the local laboratory, a flag for atypical or variant lymphocytes may also be an indication for examination of a blood smear, since this flag is sometimes indicative of the presence of blast cells. Low rather than high counts likewise are an indication for a smear, since they may be indicative of aplastic anemia, acute leukemia, hairy-cell leukemia, or infiltration of non-hematopoietic malignant cells into the bone marrow. The role of blood smear in the diagnosis of leukemia and lymphoma is to suggest a likely diagnosis or range of diagnoses, to indicate which additional tests should be performed, and to provide a morphologic context without which immunophenotyping and other sophisticated investigations cannot be interpreted.

For two conditions, Burkitt’s lymphoma and acute promyelocytic leukemia, a blood smear is of particular importance because it facilitates rapid diagnosis and specific treatment (8) (Fig. 1).

Sometimes the blood smear provides the primary or the only evidence of a specific diagnosis, such as myelodysplastic syndrome, leukemia, lymphoma, or hemolytic anemia. It is important, if possible, to store blood smears for long time

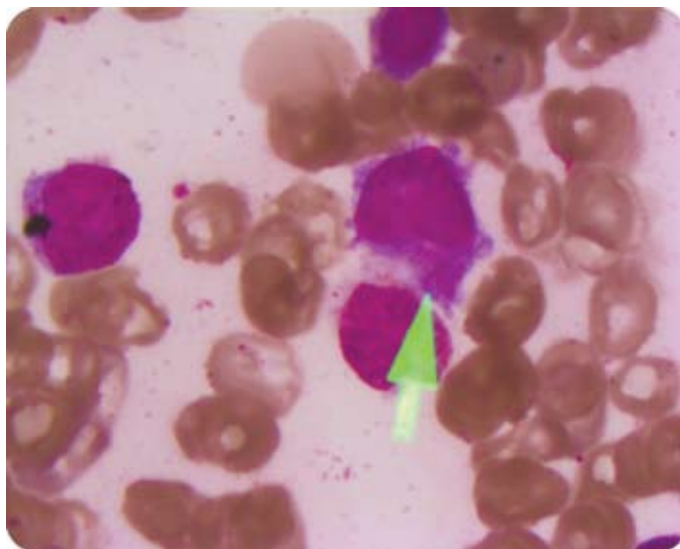


FIGURE 1 •
Promyelocyte with a characteristic Auer rod in blood smear from a patient with Acute promyelocytic leukemia. The arrow shows the Auer rod (100X)

period, just as a tissue that provides a histological diagnosis. In the practice, such storage is easily achieved if a patient has also had a bone marrow aspirate (since a blood smear should always be kept in this case), but it is harder to achieve if the blood smear alone has provided the diagnosis. Individual laboratories should have a mechanism to make possible the retention of blood smear as part of the medical record, smears themselves or an image derived from them. Some laboratories retain all smears that have been reviewed by hematologists or pathologists; this can create a storage problem, and it is likely that digital images of important abnormal smears will be stored.

The convenient and inexpensive capture and storage of relevant macroscopic and microscopic digital images for patient care is increasingly popular, especially with the present emphasis on quality assurance and rapid, accurate medical diagnosis. In this regard, high-resolution digital photography is essential for remote consultation and diagnosis by telepathology, and may provide new avenues for computer-assisted diagnosis (7)

BONE MARROW EXAMINATION •

Peripheral blood examination and other routine laboratory assays do not always provide enough information for the diagnosis of hematologic disease. In some patients direct microscopic examination of the bone marrow is required for confirmation of a suspected clinical diagnosis or monitoring the course of medical therapy. Occasional patients also require bone marrow collection for special studies, such as cytogenetic analysis, flow cytometry, molecular studies, or microbiologic cultures (9).

A bone marrow examination is a critical part of the evaluation of patients with a variety of hematopoietic and non-hematopoietic diseases. It is performed for diagnostic purposes in patients with splenomegaly, dysproteinemia, suspected lysosomal storage disease, an unexplained deficiency or excess of peripheral blood leukocytes or platelets, or the presence of immature or mor-

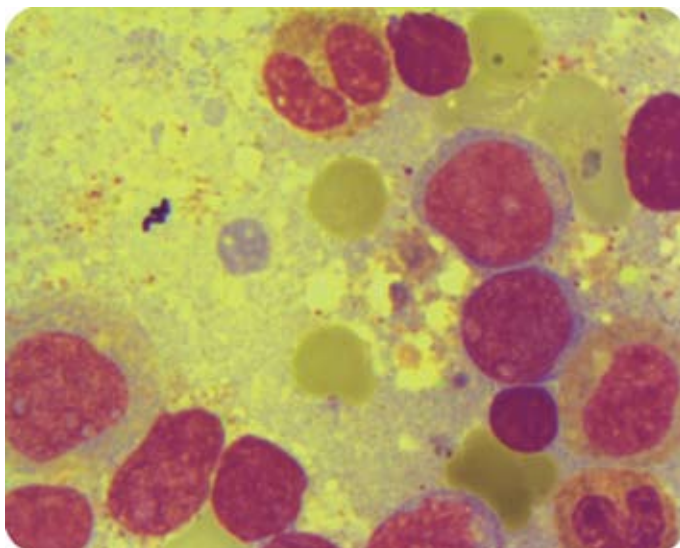


FIGURE 2 •

Myeloblasts. This image shows several myeloblasts, typical from M2 with clear nucleolus (100X)

phologically atypical cells in the peripheral blood (Fig. 2). Anemic patients are seldom subject to bone marrow examinations unless the cause is not apparent after a variety of other laboratory assays have been performed, or the disease does not respond to appropriate therapy.

The bone marrow examination may also be requested to obtain material for microbiologic culture in patients with unexplained fever (“fever of unknown origin”), HIV infection and AIDS, or other diseases, and to search for infectious organisms, neoplasms, granulomatous disease, or other lesions in these patients. Bone marrow examination is also part of the staging process for newly diagnosed patients with lymphoproliferative diseases and certain non-hematopoietic malignancies such as neuroblastoma and other childhood tumors (8). Examination of the bone marrow is performed to determine the extent of marrow damage in patients exposed to radiation, drugs, chemicals, and other myelotoxic agents. Moreover, marrow evaluation is essential to determine the efficacy of treatment and to monitor the recovery process in patients undergoing bone marrow transplantation or marrow-ablative chemotherapy.

There are relatively few contraindications for the bone marrow procedure. Acquired or congeni-

tal coagulation factor deficiencies and other coagulation abnormalities are considered contraindications by some physicians. Factor replacement therapy prior to bone marrow examination and hospital observation for 24 hours after the procedure may be indicated in these patients. Patients receiving anticoagulants should have prothrombin time (PT) or activated partial thromboplastin time (aPTT) values within the therapeutic range for warfarin or heparin. Isolated thrombocytopenia is not a contraindication to the bone marrow examination if the procedure is properly performed and technical difficulties are not encountered. Other contraindications include infection or previous radiation therapy at the sample site and poor patient cooperation. Sternal bone marrow aspiration is completely contraindicated in patients with diseases associated with bone resorption, including multiple myeloma. Also, trephine biopsy will never be performed in the sternum (9).

The bone marrow aspirate and biopsy are critical specimens for the diagnosis and monitoring of hematologic diseases. The posterior iliac crests are the preferred sites to obtain bone marrow for diagnostic purposes, since the pelvic bone has a large volume of bone marrow, there are no vital organs in close proximity, and most patients are more comfortable in the lateral decubitus position. During bone marrow examination, liquid bone marrow is aspirated from the bone marrow cavity with a syringe, and a solid core of bone marrow is obtained with a special needle.

Smears are prepared from the aspirated material and stained by the Wright-Giemsa technique for the evaluation of bone marrow cell morphology, while the bone marrow core is fixed, sectioned on microscope slides, and stained with the hematoxylin-and-eosin ("H&E") technique for assessment of cellularity and the presence of focal lesions such as malignant lymphoma, metastatic carcinoma, and granulomata. In some patients, aspirated bone marrow is also used for special procedures, including flow cytometric immunophenotypic analysis and cytogenetic analysis.

The bone marrow aspirate smears can also be stained by cytochemical or immunohistochemical techniques, while immunohistochemical or other special stains are often performed on the bone marrow biopsy.

Most patients tolerate the procedure well if adequate local anesthesia is performed by injecting lidocaine solution into the skin and over the periosteum of the procurement site. However, some patients require pre-medication to reduce anxiety over the procedure. Complications of the procedure are extremely rare if this is performed properly and the patient possesses an adequate hemostatic system. Bleeding, with the development of a subcutaneous hematoma, is the most common complication, usually in a patient with an unrecognized coagulopathy. Osteomyelitis, retroperitoneal hemorrhage, and other complications have been rarely reported. The recent development of bone marrow biopsy needles with internal snares to capture the bone marrow core is expected to reduce the discomfort of the procedure and increase specimen adequacy (9).

Again, as well as blood smear was outlined, the interpretation of bone marrow morphology requires a very trained staff, hematologist and pathologist reviewing the slides but is important to ensure quality control from the sample procedure, the staining quality and assure the diagnosis with different observers and case-discussion rounds in order to correlate not only the bone marrow morphology results, but also with the clinical picture and other laboratory results.

IMMUNOPHENOTYPING •

Starting from peripheral blood smears, bone marrow cytomorphology, histopathology review of biopsies (lymph nodes and other tissues), cytochemical tests and immunohistochemical panel, for some malignancies it is mandatory to further perform multiparameter flow cytometry (MFC), and metaphase cytogenetics in every case, in which some markers are well described. The lat-

ter has to be accompanied by FISH and also by PCR analysis or even screening for specific molecular markers. For some diseases, MFC is a very useful and informative tool, like in AML where it leads to very important information about subtypes, as well as in ALL for classification and stratification to adapted therapy (3,4,8,10).

Flow cytometry is a technique of quantitative single cell analysis. The flow cytometer was developed in the 1970's and rapidly became an essential instrument for the biologic sciences. Spurred by the HIV pandemic and a plethora of discoveries in hematology, specialized flow cytometers for use in the clinical laboratory were developed by several manufacturers. The major clinical application of flow cytometry is diagnosis of hematologic malignancy, but a wide variety of other applications exist, such as reticulocyte enumeration and cell function analysis.

The technique of analyzing individual cells in a fluidic channel was first described by Wallace Coulter in the 1950's, and applied to automated blood cell counting. Subsequent developments in the fields of computer science, laser technology, monoclonal antibody production, cytochemistry, and fluorochrome chemistry led to the development of the flow cytometer two decades later. Because the first commercial flow cytometers were large, complex, expensive, and difficult to operate and expensive to maintain, they were primarily used in the research laboratory.

However, the enormous value of the flow cytometer in the medical and biologic sciences was quickly appreciated, and its cost and complexity gradually decreased as its analytic capability increased. The present "state-of-the-art" flow cytometers are capable of analyzing up to 13 parameters (forward scatter, side scatter, 11 colors of immunofluorescence) per cell at rates up to 100,000 cells per second. Automation and robotics is increasingly being applied to flow cytometry to reduce analytic cost and improve efficiency (8,10).

Prepared single cell or particle suspensions are necessary for flow cytometric analysis. Various immunofluorescent dyes or antibodies can be attached to the antigen or protein of interest. The suspension of cells or particles is aspirated into a flow cell where, surrounded by a narrow fluid stream, they pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence if the cell contains a naturally fluorescent substance or one or more fluorochrome-labeled antibodies are attached to surface or internal cell structures. Light scatter is dependent on the internal structure of the cell and its size and shape. Fluorescent substances absorb light of an appropriate wavelength and re-emit light of a different wavelength. Fluorescein isothiocyanate (FITC), Texas red, and phycoerythrin (PE) are the most common fluorescent dyes used in the biomedical sciences. Light and/or fluorescence scatter signals are detected by a series of photodiodes and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector. The resulting electrical pulses are digitized, and the data is stored, analyzed, and displayed through a computer system. The end result is quantitative information about every cell analyzed. Since large numbers of cells are analyzed in a short period of time (>1,000/sec), statistically valid information about cell populations is quickly obtained.

The identification and quantitation of cellular antigens with fluorochrome-labeled monoclonal antibodies ("immunophenotyping") is one of the most important applications of the flow cytometer. Immunophenotypic analysis is critical to the initial diagnosis and classification of the acute leukemias, chronic lymphoproliferative diseases, and malignant lymphomas since treatment strategy often depends upon antigenic parameters. In addition, immunophenotypic analysis provides prognostic information not available by other techniques, provides a sensitive means to monitor the progress of patients after chemotherapy or bone marrow transplantation, and often permits the detection of minimal residual disease. Flow cytometric

analysis of apoptosis, multidrug resistance, leukemia-specific chimeric proteins, cytokine receptors and other parameters may provide additional diagnostic or prognostic information in the near future (10).

A wide variety of monoclonal antibodies against cellular antigens are available for the immunophenotypic analysis of hematological malignancies. In order to establish a B- or T-cell clonality, a panel of antibodies is used. A pan-B-cell panel would include CD19, CD20, and CD22 and a pan-T-cell panel would include CD2, CD3, CD4 and/or CD7, while additional antibody panels might be necessary to establish the presence of a specific lymphoproliferative disorder. Monoclonal antibodies against leukocyte common antigen (CD45) are often included in the panel to differentiate hematological malignancies from other neoplasms and to detect populations of blast cells, since almost all leukemic cell populations exhibit decreased (dim) CD45 expression compared to normal leukocytes. The CD34 and HLA-DR antigens are markers for hematopoietic stem cells used for the diagnosis of acute leukemia and quality assurance in bone marrow transplantation. Most of the remaining leukocyte surface antigens are lineage associated, but not specific to a single lineage or stage of cellular maturation (3,4).

Flow cytometry is used as a simple, rapid method for detection of minimal residual disease (MRD), the persistence of malignant cells in the bone marrow or other tissues of patients with hematologic malignancies after remission at levels below the limit of detection by conventional morphologic assessment. It is believed that these residual malignant cells are the source of disease relapse in many patients, although additional therapy to eradicate very small numbers of residual cells does not improve survival in all patients. Researchers are actively evaluating the significance of MDR.

Laboratory techniques for the detection of MDR must meet four criteria, which include sensitivity (detection limit of at least 10^{-3} cells), specificity (ability to differentiate normal and malignant cells), reproducibility, and applicability (easy

standardization and rapid collection of results). Morphologic evaluation, with an overall detection limit of approximately 5%, is clearly not suitable for the detection of MDR (8,10,11). However, immunophenotypic analysis, cytogenetics, fluorescence in situ hybridization (FISH), Southern blotting, polymerase chain reaction (PCR), and other techniques with detection limits of 10^{-2} to 10^{-4} cells have been applied, as well as the clonogenic assay, which has a detection limit of $\leq 10^{-4}$. Flow cytometric analysis is less sensitive than the polymerase chain technique for MRD, but it is simple and rapid to perform, provides quantitative data, and has adequate sensitivity in many leukemia cases. Flow cytometric analysis detects the presence of aberrant immunophenotypic features that are not characteristic of normal cell populations in the specimen under study. For example, the discovery of CD10+, TdT+, or CD34+ cells in the cerebrospinal fluid is diagnostic of MRD, since immature leukocytes with these markers are not normally present in the CSF.

The expression of TdT, cytoplasmic CD3, CD1a, or the dual phenotype CD4+/CD8+ by bone marrow cells is diagnostic of residual MRD in T-ALL, since cells with these phenotypes are normally confined to the thymus. The detection of B-ALL MRD is more difficult, since small numbers of immature B-cells are normally present in the bone marrow. But, the majority of B-ALL cases have aberrant antigenic features, including cross-lineage antigen expression (i.e., TdT, T-cell, or myeloid antigens), asynchronous antigen expression, or changes in the level of antigen expression (i.e. "dropped" or overexpressed antigens). The search for new markers and techniques of immunophenotypic analysis for MRD is also underway by several investigators (3).

CONVENTIONAL KARYOTYPING AND MOLECULAR CYTOGENETICS •

In 50–70% of patients with lymphomas and acute leukemias and in the vast majority of patients with chronic leukemias, acquired clonal chromosome aberrations can be observed after meta-

phase analysis. The cytogenetic results at diagnosis provide the most important single parameter for prognosis so far (3,4). Numerous recurrent karyotype abnormalities have been described.

These findings in the chromosomal level are followed and complemented in some parts by molecular studies that have identified genes involved in leukemogenesis. Even more, molecular markers, such as MLL partial tandem duplications (MLL-PTD) or FLT3 length mutations (FLT3-LM) in AML, or BCR-ABL in ALL (and CML) were found to characterize specific subtypes and complete the panel of genetic markers. The identification of specific chromosomal abnormalities or molecular markers and their correlation to cytomorphologic features as well as to clinical outcome led to a new understanding of hematologic cancers as a heterogeneous group of different biological entities (11,2).

The importance of cytogenetic and molecular genetic findings for the classification and for the understanding of pathogenetic mechanisms is increasingly appreciated in the clinical context and was translated also into the new WHO classification that uses cytogenetic abnormalities as a major criterion in most of the not well describe entities (3,11-13) (Fig. 3).

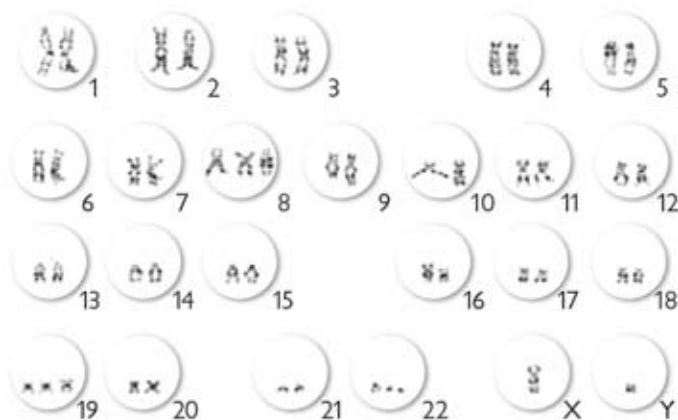


FIGURE 3 •
Abnormal Karyogram: Karyotype analysis with G banding in a patient with progression of chronic myeloid leukemia. It is easy to distinguish trisomy of chromosomes 8 and 19, double Philadelphia chromosome and apparently loss of Y chromosome.

Several genetic testing methods are used in hematologic cancers and include Conventional Cytogenetics (CC), fluorescent *in situ* hybridization (FISH), reverse transcriptase-polymerase chain reaction (RT-PCR), Southern blots that detect the BCR/ABL gene fusion at the genomic DNA level, Western blots that detect the BCR/ABL fusion protein, and genomic DNA PCR, which amplifies the fusion gene directly from the genome. CC usually requires bone marrow aspiration that is an invasive procedure. Other limitations of CC include sample inadequacy, the presence of associated myelofibrosis, dependence on actively dividing cells (metaphasic cells), which is particularly relevant in the presence of a myelosuppressive drug therapy such as IFN- α or alkylants like hydroxyurea, and suboptimal sensitivity for the purposes of minimal residual disease (MRD) assessment (1–5%).

It has also been proposed that the culture conditions used to prepare cells for CC may influence the cell cycle of Ph-positive cells differently than Ph-negative cells, resulting in a measured leukemia burden, which is not truly representative. For these and other reasons, FISH and RT-PCR are becoming more popular tests for both the diagnosis and monitoring. Nevertheless, CC is the mainstay of genetic diagnostic and is always necessary for panel and workup for initial diagnosis and follow-up (12-14).

Bone marrow is the tissue of choice when using conventional G-banded analysis, which enables a complete picture of the human genome at a glance (Mark, 2005). At diagnosis, 90–95% of cases of CML have the characteristic translocation, t(9;22)(q34;q11.2). Variant Philadelphia translocations and cryptic rearrangements are also possible and account for the remaining cases. Secondary chromosomal abnormalities in CML include trisomy 8, i(17q), +Ph, and trisomy 19. Less common chromosomal abnormalities found in CML include -7, -17, +17, +21, -Y and t(3;21)(q26.2;q22). These numerical and structural chromosomal rearrangements can usually be detected readily by conventional cytogenetics via G-banding and/or molecular cytogenetics via FISH.

CC is a very important tool for hematologic malignancies diagnosis; lymphoma and lymphoproliferative disorders, multiple myeloma and acute leukemia are now well defined and in some cases also well characterized by some particular cytogenetical alterations. WHO classification incorporate some of these alterations and in very large proportion of cases prognostic implications are described. Some cytogenetical markers could serve as MRD tracers (in particular in some forms of acute and chronic leukemias) (3,11,12).

FISH can be performed on a variety of specimen types such as peripheral blood, bone marrow, and pathological sections. FISH utilizes fluorescent DNA probes to detect the location of genes directly in the genome in either metaphase and/or interphase cells (Fig. 4). Although these cells are often collected from bone marrow aspirations, as with CC, a growing body of literature is exploring the fidelity of FISH from peripheral blood. Additionally, FISH allows for a larger sample of cells to be harvested than CC, thus reducing much of the sampling inadequacies associated with the latter technique.

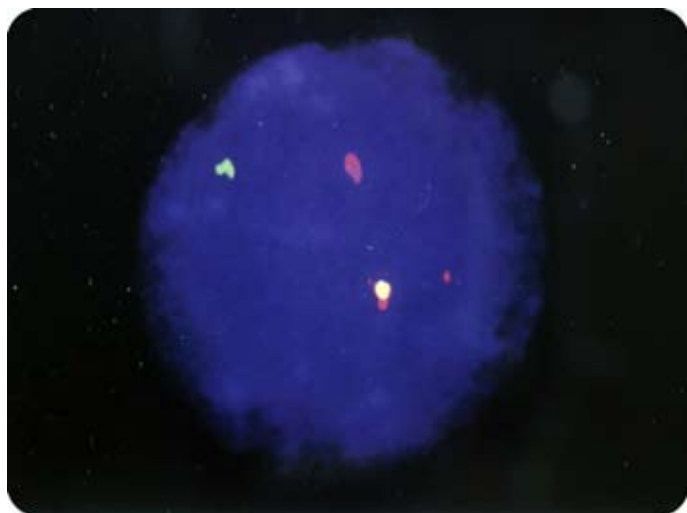


FIGURE 4•

Flourescent In-situ Hybridization. Interphasic cell with red signal from ABL gen (chromosome 9) and the sequence of BCR gene with green-acqua. The yellow signal is the product of the green and red fusion (Bcr-Abl), Philadelphia translocation.

Traditional FISH (also known as S-FISH or dual-FISH) is a two-color technique in which a fluorescent probe as well as a second or more fluorescent probes are utilized with contrasting color to detect the position of the respective genes.

Although the fluorophore signals appear to localize in a random fashion in normal cells, co-localization of probes results in a single fusion signal, indicating the genetic juxtapositioning or abnormal presence, absence or location of some genes associated with the malignancy. S-FISH is applicable to both metaphase and interphase cells and demonstrates a false positive threshold of 1–6.5%. In addition, because the scoring of potential fusion signals is subjective, S-FISH is also limited by a significant number of false negatives. These shortcomings have needed several adaptations to increase its sensitivity and its value as a molecular detection and monitoring tool. Triple probe FISH (or three-color FISH) increases the sensitivity of the two-probe S-FISH technique by introducing a third probe that spans the breakpoints. Each probe is labeled with a separate, distinct fluorochrome, and positive cells are distinguished by the loss of co-localization of the third probe in addition to the fusion signal that is the hallmark of S-FISH.

This two-step verification process allows for an increased sensitivity in the detection of positive cells with a false positive rate of 0.065 – 0.27% (SD 0.08–0.15%). Double FISH (or D-FISH) utilizes two additional probes, in addition to the two used in S-FISH. D-FISH yields a double fusion signal because the four probes bind to their respective counterparts loci. This further reduces the risk of both false positives and false negatives compared to S-FISH. Finally, hypermetaphase FISH (HMF) involves placing 500p cells in colcemid, a mitotic arresting agent, which allows additional chromosomal analyses to be conducted during a single collection (12, 14, 15) (Fig. 5).

RT-PCR•

Clinical assessment, blood counts, blood smears morphology, bone marrow examination, immunophenotyping, CC and FISH analysis have been

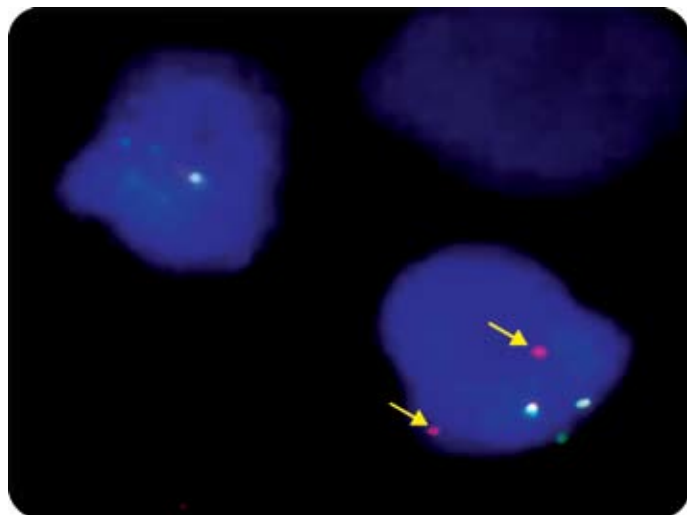


FIGURE 5 •
FISH analysis. Interphasic cells showing the Bcr-Abl fusion. The yellow arrow shows a cell containing double fusion and extra-copies from Abl signal (red dots).

the mainstay of disease diagnosis and monitoring in hematologic malignancies. Response criteria based on those results are critical for clinical assessment and follow-up, but recently more sensitive molecular methods have been incorporated, this is especially true for RT-PCR. They have proved to be very sensitive for diagnosis and good predictors of long term response. Over the past 12 years, several groups have developed quantitative RT-PCR assays to measure transcript levels, in the blood, marrow and some other tissues that enabled the dynamics of residual disease to be monitored over time and have provided a viable alternative for disease monitoring. The transcript level correlates with the number of malignant cells present in the blood, marrow and tissues that can be used as an accurate measure of the response to therapy. The leukemia, myeloma or lymphoma specific transcripts are excellent targets for molecular monitoring by quantitative PCR. In some cases there is no need for patient-specific primers because nearly all patients of specific diseases have one of two transcripts types (some patients have more than one) which differ by just one or two exons. But, some other cases will require several primers and need and special work-up (13,16).

The early quantitative PCR studies used titration assays, which incorporated competitive targets. Internal controls were introduced for some methods to control the variation in the quality of samples. The copy number was normalized to the number of control gene transcripts and the value expressed as a percentage ratio. But for the most hematologic cancers, there is no quantitative reliable method developed, so usually the reports are commonly in terms of presence or absence or with a semi-quantitative scale (16,17).

The technique is also used to monitor patients after bone marrow transplantation where rising levels preceded cytogenetic and hematological relapse and low or decreasing levels predicted remission. It was also used as a sensitive indicator of response to donor leukocyte infusion following relapse after bone marrow transplantation (18).

The terms PCR relapse or PCR progression were proposed to identify patients with pending relapse. This term refers to the rise in transcripts levels in consecutive analyses, which reflects the inaccuracy inherent in the competitive PCR technique. Despite the apparent value of the competitive PCR assays for monitoring treatment response, its use was limited to specialized laboratories.

Thoughtful experimental design and careful validation of competitive PCR techniques were absolutely critical for reliable results (17). The technique was time consuming because multiple PCR reactions were required to titrate the sample with the competitor and extensive post PCR manipulation was necessary. The introduction of real-time PCR techniques in the late 1990's considerably simplified quantitative PCR analysis and have largely replaced the cumbersome competitive quantitative procedures. The kinetics of PCR are followed during amplification rather than at end-point, which eliminates the need for co-amplification of a competitor. The fluorescent based technology enhances the reproducibility since quantitation is determined during the exponential phase of the PCR. The dynamic range is extended to over five orders of magnitude

and real-time detection of the accumulating product eliminates the need for post PCR manipulation.

Thus the possibility of PCR contamination is limited. The real-time technique is performed on an analyzer that incorporates a thermal cycler, fluorescence detection and result calculation, which has greatly simplified quantitative PCR.

Nevertheless, the inherent technical complexities of quantitative PCR should not be overlooked and the successful application of real-time technology for reliable quantitation requires careful assay design and validation of all aspects of the procedure. For sensitive and reproducible results high quality RNA is essential. The appropriate selection of standards and control genes is of particular importance. It must be recognized that differences in the amplification efficiencies between DNA plasmid standards and the cDNA target can invalidate results. Normalization to the control gene compensates for variations in the quality of the RNA and for differences in the efficiency between reverse transcription reactions.

The control gene must therefore degrade at the same rate as the target for accurate normalization. Appropriate design of primer and probe sequences are required to exclude the amplification of contaminating DNA and to avoid hybridization at the polymorphic site in (16-19). Rigorous precautions to exclude PCR contamination are still necessary when monitoring minimal residual disease.

The clinical usefulness of transcripts quantitation by RQ-PCR has been demonstrated by several studies. RQ-PCR analysis of patients treated with imatinib has shown a strong correlation between the percentage of Ph-positive metaphases in the bone marrow and simultaneous study of peripheral blood BCR-ABL levels measured by RQ-PCR. Early reduction of BCR-ABL transcript levels predicts cytogenetic response in chronic phase CML patients treated with imatinib and the reduction of BCR-ABL correlates with prognosis (16).

The RQ-PCR methods vary in respect to the type of instrument used, the primer and probe location, the real-time chemistry and the control gene. These differences can lead to a variation in the sensitivity and measurement reliability between methods. It is essential that each laboratory establish these limits for their method to allow accurate interpretation of serial monitoring (17). The estimation of measurement reliability and appropriate quality assurance according to international standards are important aspects of the development of any method used to monitor patients. However, for the measurement of vast majority of transcripts used in hematology setting by quantitative PCR, certified international reference and control materials are currently not available. Several groups have been working towards methods' standardization, and are developing guidelines for data analysis and for the reporting of minimal residual disease (17).

GENOMICS •

Inherited DNA-sequence variants do not appear to have a prominent causative role; rather, these diverse cancers are typically initiated by acquired alterations to the genome of the cancer cell, such as chromosomal translocations, mutations, and deletions. The diagnosis of the hematologic cancers is commonly based on morphologic evaluation supplemented by analysis of a few molecular markers. However, in some diagnostic categories defined in this fashion, the response of patients to treatment is markedly heterogeneous, suggesting that there can be several molecularly distinct diseases within the same morphologic category.

Gene-expression profiling is a genomics technique that has proved effective in deciphering this biologic and clinical diversity of several tumors. The approach relies on the fact that only a fraction of genes encoded in the genome of each cell are expressed — that is, actively transcribed into messenger RNA (mRNA). The abundance of mRNA for each gene depends on a cell's lineage and stage of differentiation, on the activity of in-

tracellular regulatory pathways, and on the influence of extracellular stimuli. To a large extent, the complement of mRNAs in a cell dictates its complement of proteins, and consequently, gene expression is a major determinant of the biology of normal and malignant cells (13).

In the process of expression profiling, robotically printed DNA microarrays are used to measure the expression of tens of thousands of genes at a time; this creates a molecular profile of the RNA in a tumor sample. A variety of analytic techniques are used to classify cancers on the basis of their gene-expression profiles (21).

There are two general approaches. In an unsupervised approach, pattern-recognition algorithms are used to identify subgroups of tumors that have related gene-expression profiles. In a supervised approach, statistical methods are used to relate gene-expression data and clinical data. These methods have revealed unexpected subgroups within the diagnostic categories of the hematologic cancers that are based on morphology and have demonstrated that the response to therapy is dictated by multiple independent biologic features of a tumor (20).

DIFFUSE LARGE-B-CELL LYMPHOMA •

Some cases of diffuse large-B-cell lymphoma respond well to multidrug chemotherapy, but this lymphoma nonetheless remains a perplexing clinical puzzle, since roughly molecular diagnosis of non-Hodgkin's lymphoma 60 percent of cases are incurable. This observation raises the possibility that this single diagnostic category may harbor more than one molecular disease.

The gene-expression profiles of lymph-node-biopsy specimens from patients with morphologically identical diffuse large-B-cell lymphoma show pronounced variability, with no common set of genes expressed in all cases. To make sense of this variability, genes were classified into expression signatures that is, groups of genes with similar patterns of expression in a set of samples. Some signatures

include genes expressed in a particular type of cell or stage of differentiation, whereas other signatures include genes expressed during a particular biologic response, such as cellular proliferation or the activation of a cellular signaling pathway (13).

One gene-expression signature that varies markedly among diffuse large-B-cell lymphomas is the germinal-center B-cell signature. This signature characterizes B cells that are responding to a foreign antigen within the germinal-center microenvironment of secondary lymphoid organs. Among biopsy samples from patients with diffuse large-B-cell lymphoma, three biologically and clinically distinct subgroups have been identified. The germinal center B-cell-like subgroup (approximately 50% of cases) has high levels of expression of germinal-center B-cell signature genes, whereas the other two subgroups of diffuse large-B-cell lymphoma—termed activated B-cell-like and type 3—do not. The activated B-cell-like subgroup (approximately 30% of cases) expresses genes that are induced by mitogenic stimulation of blood B cells. The type 3 subgroup does not express genes characteristic of the other two subgroups and may yet be found to be heterogeneous. These findings suggest that the subgroups of diffuse large-B-cell lymphoma arise from different stages of normal B-cell development.

The notion that the gene-expression subgroups represent pathogenetically distinct types of diffuse large-B-cell lymphoma has been strongly supported by analysis of recurring chromosomal abnormalities in this cancer (13).

The t(14;18) translocation involving the BCL2 gene and the amplification of the c-rel gene on chromosome 2p are recurrent oncogenic events in germinal-center B-cell-like diffuse large-B-cell lymphoma, but they never occur in the other groups. Activation of the nuclear factor κB signaling pathway is a feature of the activated B-cell-like subgroup but not the other subgroups, and interference with this pathway selectively kills this type of diffuse large-B-cell lymphoma (20).

The subgroups defined with the use of gene expression signatures are clinically distinct as well: patients with the germinal-center B-cell–like form have a higher rate of overall survival five years after chemotherapy than do patients in the other subgroups. This clinical distinction based on gene-expression profiles was evident even after the patients were classified according to the International Prognostic Index, a well-established predictor of outcome in diffuse large-B-cell lymphoma.

PREDICTING THE CLINICAL OUTCOME •

The example of diffuse large-B-cell lymphoma demonstrates how an unsupervised analysis of gene expression data can reveal clinically distinct subgroups of tumors. In the complementary, supervised approach, clinical data are used to identify genes whose patterns of expression are correlated with the length of survival after diagnosis or with the likelihood that therapy will be curative. This approach has been used to develop robust predictors of prognosis in mantle-cell lymphoma and diffuse large-B-cell lymphoma (13).

Mantle-cell lymphoma constitutes approximately 8% of cases of non-Hodgkin’s lymphomas but a much larger fraction of deaths from lymphoma, since current therapy is not curative. The length of survival among patients with mantle-cell lymphoma is quite variable, ranging from less than 1 year to more than 10 years (22).

Gene-expression profiling revealed a strong association between the expression of genes in the “proliferation” signature and survival in mantle-cell lymphoma. The proliferation signature includes genes that are highly expressed in dividing cells but not in quiescent cells. The quartile of patients with the highest level of proliferation-signature expression had a median survival of 6.7 years, whereas the quartile with the lowest level of expression had a median survival of 0.8 year. The variable survival of patients with mantle-cell lymphoma is therefore largely dictated by a single aspect of tumor biology, the rate of cell division, which can be quantitated by gene expression profiling.

Although the subgroups of diffuse large-B-cell lymphoma have distinct survival rates, the statistical approach of supervised analysis identified additional molecular differences among the tumors that can account for much of the remaining heterogeneity in survival. This approach demonstrated that at least five distinct features of diffuse large-B-cell lymphomas influence the response to chemotherapy. Specifically, the levels of expression of the germinal-center B-cell signature, the proliferation signature, the major-histocompatibility-complex (MHC) class II signature, and the lymph-node signature were predictive of the clinical outcome, as was the level of expression of BMP6, a gene that does not belong to a defined expression signature. As in mantle-cell lymphoma, expression of the proliferation signature predicted a poor outcome (13,22). Predictive genes in two other signatures suggest that the host immune response has an important role in curative responses to chemotherapy.

Expression of the lymph-node–signature genes reflects the non-tumor cells in the diffuse large-B-cell lymphoma–biopsy specimen, including activated macrophages, natural killer cells, and stromal cells. A high level of expression of these genes predicts a favorable clinical outcome, suggesting that this reactive immune response is beneficial. The MHC class II signature includes genes encoding components of this critical antigen-presentation–protein complex, and decreased expression of these genes predicts a poor outcome. These findings suggest that some tumors may evade the immune response by down-regulating their antigen-presentation capacity.

These expression signatures can be combined to form a multivariate predictor of survival after chemotherapy for diffuse large-B-cell lymphoma. With the use of this approach, half the patients can be placed into a favorable-risk group, with a five-year survival rate of more than 70%; one quarter can be assigned to a poor-risk group, with a five-year survival rate of 15 percent; and the remaining patients are in an intermediate-risk group, with a five year survival rate of 34% (13,20,22).

ACUTE LEUKEMIAS •

The molecular diagnosis of leukemias began with the recognition and analysis of recurrent chromosomal translocations. The genes discovered at the translocation break points have drawn attention to critical regulatory pathways in hematopoietic cells that can cause cancer when they are deregulated. In many acute leukemias, translocations fuse genes that reside on the two partner chromosomes, creating a chimeric gene with novel oncogenic properties (3,4).

Chromosomal translocations have been used to identify patients with acute leukemia with distinct clinical outcomes. In acute myeloid leukemia (AML), for instance, the presence of a t(8;21) translocation or a chromosome 16 inversion identifies patients with a comparatively good prognosis, whereas the t(9;22) translocation is associated with a poor outcome. It is important to note that chromosomal translocations have been used to identify patients who will benefit from intensifying the dose of chemotherapy (11).

Despite these prognostic and therapeutic successes, chromosomal translocations account for only part of the varied clinical behavior of acute leukemia, for several reasons. First, other genetic aberrations can be functionally equivalent to a translocation, thus diminishing the prognostic power of a translocation as a single variable. Second, additional oncogenic abnormalities may accumulate in a leukemia that alter its responsiveness to therapy. For example, mutations in the gene encoding the *flt3* receptor tyrosine kinase have been associated with response to treatment in patients with AML. Furthermore, *flt3* mutations that activate the kinase are present in some cases of acute lymphoblastic leukemia (ALL) with a t(4;14) translocation, rendering them susceptible to killing by *flt3* inhibitors (23,24).

Finally, a sizable fraction of the acute leukemias have none of the defined recurrent translocations. Gene-expression profiling has been used as an alternative

approach to mapping chromosomal translocations. In pediatric B-cell ALL, gene-expression signatures have been identified that correlate with six different chromosomal abnormalities (13, 23).

These gene-expression signatures can be combined with the use of statistical algorithms to predict chromosomal abnormalities with 96 to 100 percent accuracy.

Likewise, in adult AML, a gene-expression-based predictor has been created that can identify three different chromosomal translocations with a high rate of accuracy (13,23,24).

Gene-expression predictors can also identify patients with AML who have isolated trisomy 8. These encouraging results demonstrate that DNA microarrays can be used to diagnose most chromosomal abnormalities in acute leukemias and could potentially substitute for the multiple diagnostic tests for these abnormalities that are currently required (139).

An oncogene likely to be causally related to T-cell ALL can be deregulated by chromosomal translocations in some cases but by alternative mechanisms in others. For example, the *HOX11* oncogene is involved in recurrent but infrequent translocations in T-cell ALL, but gene-expression profiling revealed that some cases of T-cell ALL overexpress *HOX11* without any detectable chromosomal abnormalities in this gene. All leukemias that overexpress *HOX11* have a common gene-expression signature, suggesting that they are biologically similar. Most important, patients with leukemias that overexpress *HOX11* have a favorable outcome, as compared with patients with other types of T-cell ALL, whether or not the overexpression is due to translocation, indicating the clinical superiority of expression profiling over identification of the translocation (23).

Two adverse events after the treatment of acute leukemias are relapse and the development of secondary leukemias. In B-cell ALL, gene-expression profiling at the time of diagnosis provided

information that could predict which patients would relapse and which would remain in continuous complete remission. Interestingly, no patterns of gene expression have been found to predict relapse in all subtypes of ALL. Rather, relapse was predicted by the expression of different genes in each leukemic subtype, emphasizing once again their divergent biologic characteristics. Secondary AML arises as a consequence of treatment in some patients with ALL, and this complication could also be predicted on the basis of gene-expression profiling in the subgroup of B-cell ALL with the t(12;21) translocation.

Although these predictors of clinical outcome will need to be validated in independent data sets, these findings suggest that treatment stratification based on gene-expression profiling can be initiated at the time of the initial diagnosis of ALL (13).

CHRONIC LYMPHOCYTIC LEUKEMIA •

The most common leukemia in humans — chronic lymphocytic leukemia (CLL) — is an indolent but inexorable disease with no cure. Studies of immunoglobulin gene mutations in CLL cells raised the intriguing hypothesis that CLL might be two distinct diseases (25).

The presence of somatic mutations in the immunoglobulin genes of CLL cells defined a group of patients who had stable or slowly progressing disease requiring late or no treatment. By contrast, the absence of immunoglobulin gene mutations in CLL cells defined a group of patients who had a progressive clinical course requiring early treatment. These two subtypes of CLL may also differ with respect to oncogenic mechanisms, since deletion of the ATM locus on chromosome 11q is associated with the absence of immunoglobulin gene mutations in CLL and with shortened survival in some patients (26).

Despite these clinical and molecular differences between the subtypes of CLL, gene-expression profiling revealed that CLL cells express a common gene-expression signature that differentiates this form of

leukemia from other lymphoid cancers and from normal lymphoid subpopulations (13,27).

This signature is shared by all cases of CLL, irrespective of the immunoglobulin gene mutation status, suggesting that CLL should be considered a single disease entity. Nonetheless, given the clear clinical differences between the two subtypes of CLL, a hunt was made for genes that correlated with this distinction (13).

Roughly 160 genes were found whose levels of expression differed significantly between the two subtypes. Expression of the single most discriminating gene, ZAP-70, distinguished these two subtypes with 93 percent accuracy. Whereas analysis of the immunoglobulin gene sequence would be a challenging and expensive test to introduce into routine clinical practice, a quantitative RT-PCR assay or protein-based assay for the expression of ZAP-70 is feasible (25).

What kind of technology will be used for the molecular diagnosis of cancer in the future? Our experience with gene-expression profiling has taught us two clear lessons: multiple genes need to be studied to distinguish most types of cancer, and quantitative measurement of molecular differences among tumors results in clinically important diagnostic and prognostic distinctions. An important goal will therefore be to develop a platform for routine clinical diagnosis that can quantitatively measure the expression of a few hundred genes. Such a diagnostic platform would allow us quickly to translate what we have learned about important molecular subgroups within each hematologic cancer. As we design new clinical trials, however, we must include genomic-scale gene-expression profiling in order to identify the genes that influence the response to the agents under investigation. In this fashion, we can iteratively refine the molecular diagnosis of the hematologic cancers on the basis of new advances in treatment and thus eventually reach the goal of tailored therapies for molecularly defined diseases (13,25,27).

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