

REVIEW ARTICLE

GENOMIC MEDICINE

Alan E. Guttmacher, M.D., and Francis S. Collins, M.D., Ph.D., *Editors*Molecular Diagnosis
of the Hematologic Cancers

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THE DIAGNOSIS OF THE HEMATOLOGIC CANCERS PRESENTS A DAUNTING challenge. The many stages of normal hematopoietic differentiation give rise to a number of biologically and clinically distinct cancers. 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, arousing the suspicion 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. The approach relies on the fact that only a fraction of the genes encoded in the genome of each cell are expressed — that is, actively transcribed into messenger RNA (mRNA) (Fig. 1A). The abundance of mRNA for each gene depends on a cell's lineage and stage of differentiation, on the activity of intracellular 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.

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 sample¹ (Fig. 1B). A variety of analytic techniques are used to classify cancers on the basis of their gene-expression profiles.^{2,3} 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 (Fig. 2A). In a supervised approach, statistical methods are used to relate gene-expression data and clinical data (Fig. 2B). 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. This is not a comprehensive review of hematologic cancers; rather, it will provide examples of how gene-expression profiling has been used to provide a framework for the molecular diagnosis of these cancers.

MOLECULAR DIAGNOSIS OF NON-HODGKIN'S LYMPHOMA

DIFFUSE LARGE-B-CELL LYMPHOMA

Some cases of diffuse large-B-cell lymphoma respond well to multiagent chemotherapy,⁵ but this lymphoma nonetheless remains a perplexing clinical puzzle, since roughly

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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.^{4,6,7} To make sense of this variability, genes were classified into expression signatures⁸ — 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.

One gene-expression signature that varies markedly among diffuse large-B-cell lymphomas is the germinal-center B-cell signature.^{4,6} 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^{4,6} (Fig. 3A). The germinal-center B-cell–like subgroup (approximately 50 percent 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 percent of cases) instead 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.^{4,10} 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 subgroups. Activation of the nuclear factor- κ B signaling pathway is a feature of the activated B-cell–like subgroup but not the other subgroups, and in-

terference with this pathway selectively kills this type of diffuse large-B-cell lymphoma.¹¹

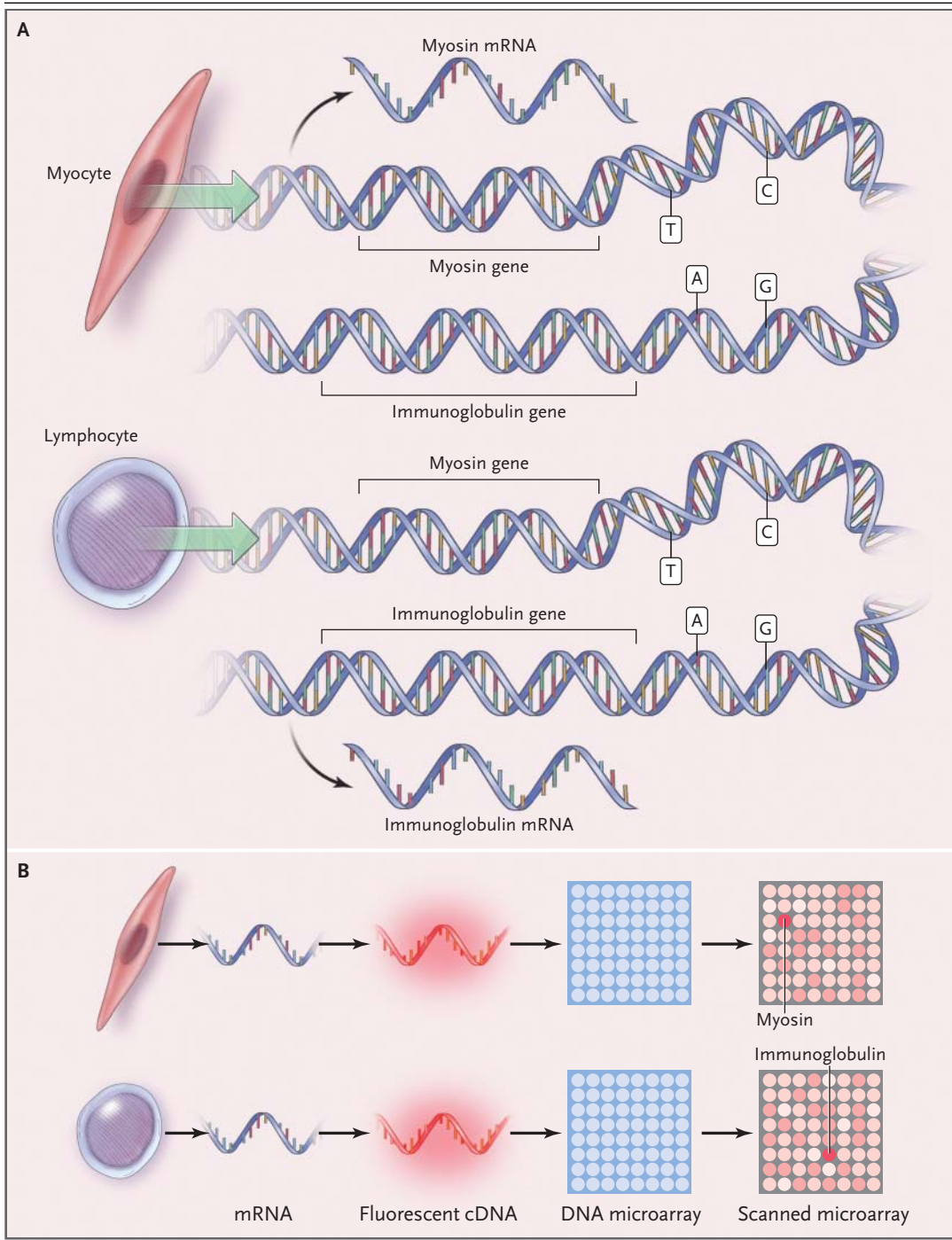
The subgroups defined with the use of gene-expression signatures are clinically distinct as well: patients with the germinal-center B-cell–like form have a higher rate of overall survival five years after chemotherapy than do patients in the other subgroups^{4,6} (Fig. 3A). This clinical distinction based on gene-expression profiles was evident even after the patients were classified according to the International Prognostic Index,^{4,6} a well-established predictor of outcome in diffuse large-B-cell lymphoma.¹²

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

Figure 1 (facing page). Differential Expression of Messenger RNA (mRNA) by Different Types of Cells (Panel A) and Gene-Expression Profiling Using DNA Microarrays (Panel B).

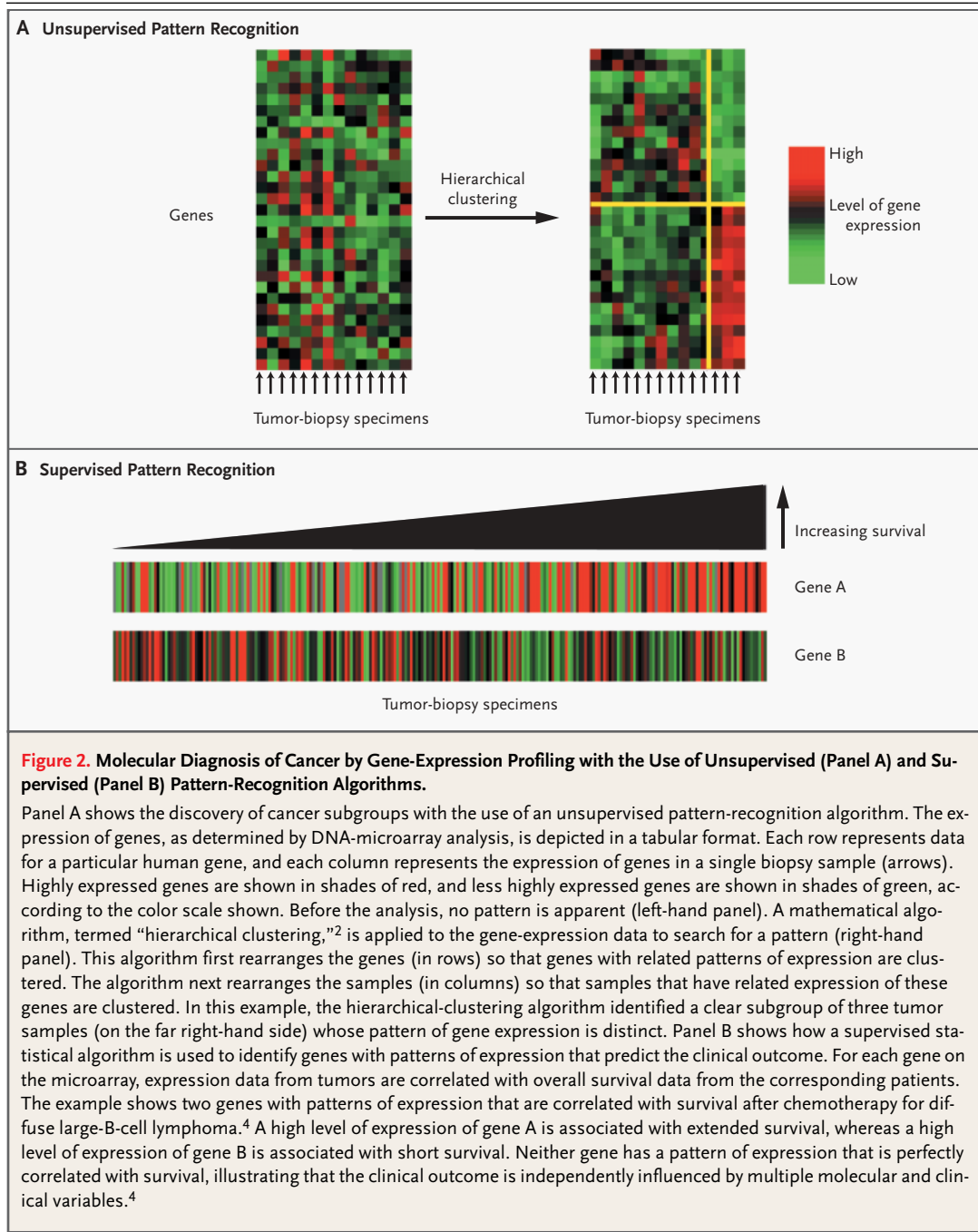
In Panel A, different types of cells, exemplified by a myocyte and a lymphocyte, express a distinct set of mRNAs from their genomes. Although the myocyte and lymphocyte possess the same inherited genomic DNA, distinct regulatory networks inside each cell cause different genes to be expressed as mRNA. The genes that encode myosin and immunoglobulin are among the most differentially expressed genes between these two types of cells. The mRNAs for other genes may be present in both types of cells, but at different levels, which may also affect the biology of the cells. Panel B shows the technique of gene-expression profiling, which uses DNA microarrays. First, mRNA is extracted from a cell and copied enzymatically to create a fluorescent complementary DNA (cDNA) probe representing the expressed genes in the cell. This probe is then incubated on the surface of a DNA microarray, which contains spots of DNA derived from thousands of distinct human genes. During the incubation, each cDNA molecule in the probe hybridizes to the microarray spot that represents its respective gene. The extent of hybridization of fluorescent cDNAs to each microarray spot is quantitated with use of a scanning fluorescence microscope. The levels of expression of more than 20,000 genes — in this example, the genes for myosin and immunoglobulin — can be measured in a single DNA-microarray experiment.



has been used to develop robust predictors of prognosis in mantle-cell lymphoma¹³ and diffuse large-B-cell lymphoma.^{4,7}

Mantle-cell lymphoma constitutes approximately 8 percent of cases of non-Hodgkin's lymphomas but a much larger fraction of deaths from lympho-

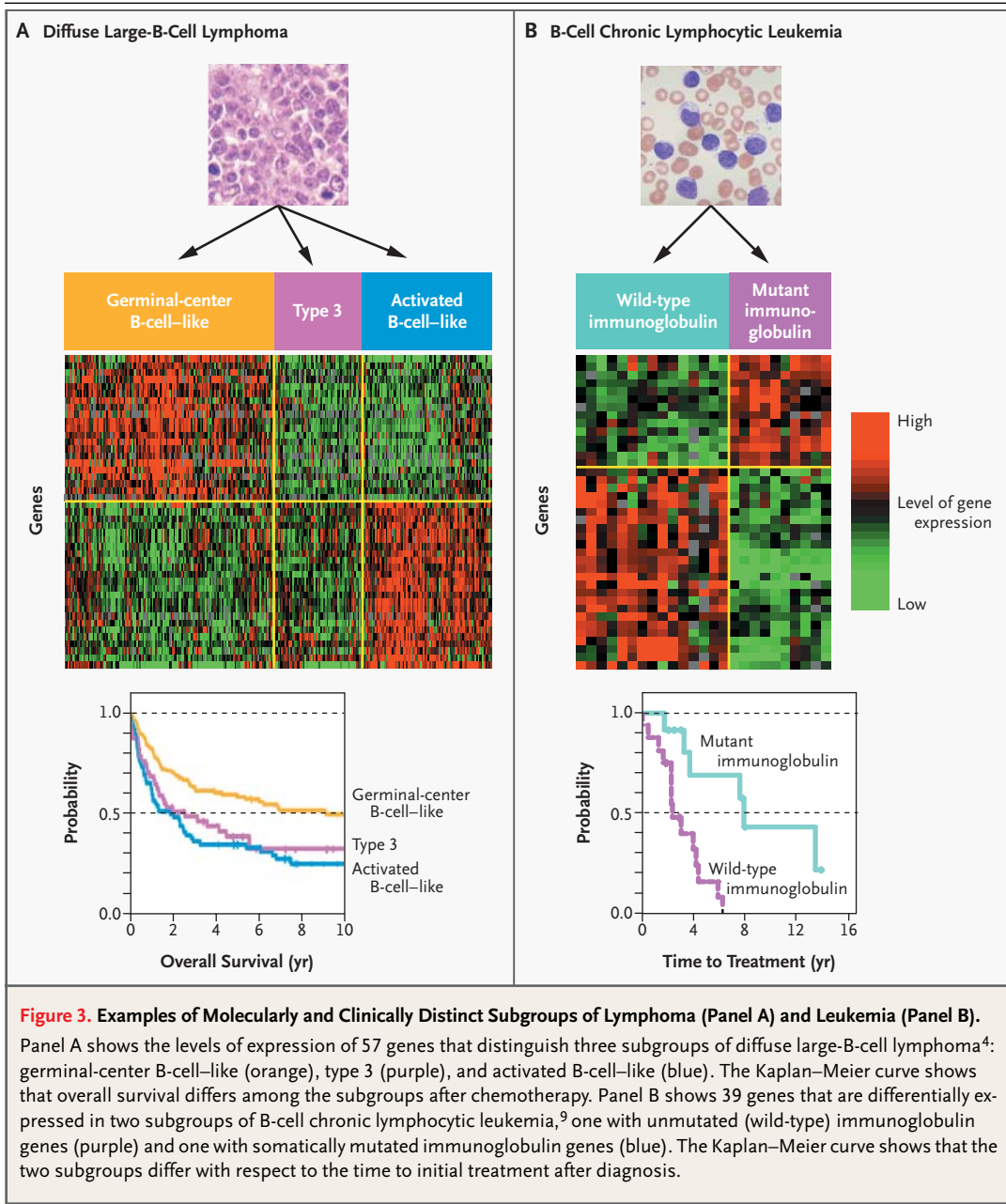
ma, since current therapy is not curative. The length of survival among patients with mantle-cell lymphoma is quite variable, ranging from less than 1 year to more than 10 years.¹³ Gene-expression profiling revealed a strong association between the expression of genes in the "proliferation" signature and surviv-



al in mantle-cell lymphoma.¹³ The proliferation signature includes genes that are more highly expressed in dividing cells than in quiescent cells (Fig. 4A). 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

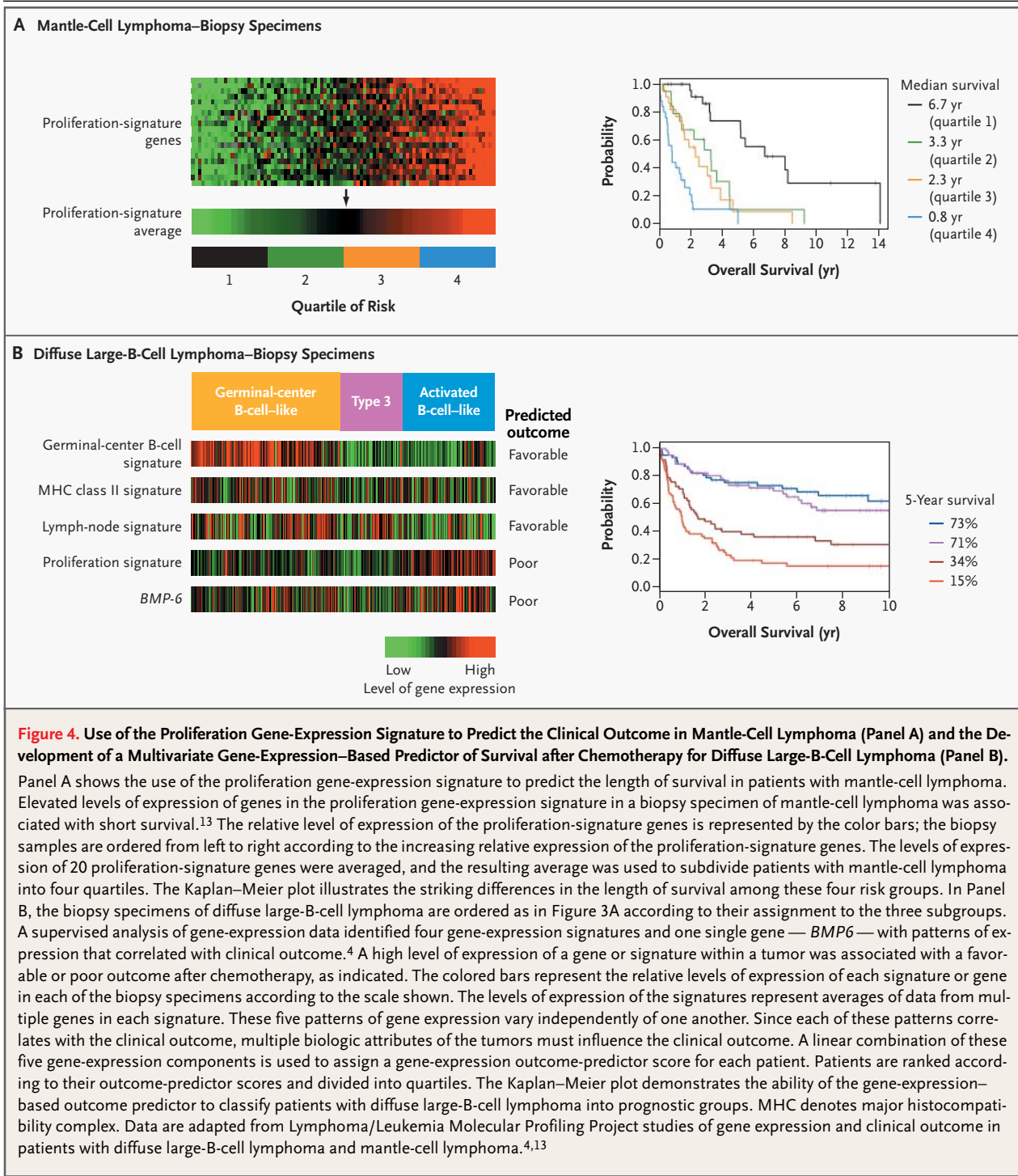
of 0.8 year (Fig. 4A). The variable survival of patients with mantle-cell lymphoma is therefore largely dictated by a single aspect of tumor biology, the rate of cell division, which can be quantitated by gene-expression profiling.

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^{4,7} (Fig. 4B). This approach demonstrated that at least five distinct features of diffuse large-B-cell lymphomas influence the response to chemotherapy.⁴ 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 sig-

nature 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. 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 nontumor 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.⁴ 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 percent; 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 percent (Fig. 4B).

MOLECULAR DIAGNOSIS OF LEUKEMIAS

ACUTE LEUKEMIAS

The molecular diagnosis of leukemias began with the recognition and analysis of recurrent chromosomal translocations.^{14,15} 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 dysregulated. In many acute leukemias, translocations fuse genes that reside on the two partner chromosomes, creating a chimeric gene with novel oncogenic properties.

Chromosomal translocations have been used to identify patients with acute leukemia with distinct clinical outcomes.^{16,17} 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.¹⁷ It is important to note that chromosomal translocations have been used to identify patients who will benefit from intensifying the dose of chemotherapy.¹⁸⁻²⁰

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 transloca-

tion,^{21,22} 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.²³⁻²⁶ 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.²⁷ Finally, a sizable fraction of the acute leukemias have none of the defined recurrent translocations.^{16,17}

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.^{28,29} These gene-expression signatures can be combined with the use of statistical algorithms to predict chromosomal abnormalities with 96 to 100 percent accuracy.²⁹ 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.³⁰ Gene-expression predictors can also identify patients with AML who have isolated trisomy 8.³¹ 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.

An oncogene likely to be causally related to T-cell ALL can be dysregulated by chromosomal translocations in some cases but by alternative mechanisms in others.²² 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 profiling²² over identification of the translocation.

Two adverse events after the treatment of acute leukemias are relapse and the development of sec-

ondary 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.²⁹ 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.²⁹ 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.

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.^{32,33} 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 CLL³⁴⁻³⁶ and with shortened survival in some patients.³⁷

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.^{9,38} This signature is shared by all cases of CLL, irrespective of the immunoglobulin gene mutation status, sug-

gesting 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.^{9,38} Roughly 160 genes were found whose levels of expression differed significantly between the two subtypes⁹ (Fig. 3B). Expression of the single most discriminating gene, ZAP-70, distinguished these two subtypes with 93 percent accuracy.^{9,39} Whereas analysis of the immunoglobulin gene sequence would be a challenging and expensive test to introduce into routine clinical practice, a quantitative reverse-transcriptase–polymerase-chain-reaction assay or protein-based assay for the expression of ZAP-70 is feasible.^{39,40}

TRANSLATING MOLECULAR DIAGNOSIS INTO A CLINICAL REALITY

What form 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.

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CORRECTION

Molecular Diagnosis of the Hematologic Cancers

Molecular Diagnosis of the Hematologic Cancers . On page 1780, the sentence beginning on line 4 of the left-hand column should have read "The quartile of patients with the lowest level of proliferation-signature expression had a median survival of 6.7 years, whereas the quartile with the highest level of expression had a median survival of 0.8 year," rather than "highest" and "lowest," respectively, as printed.