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Prediction of Survival in Follicular Lymphoma Based on Molecular Features of Tumor-Infiltrating Immune Cells

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ABSTRACT

BACKGROUND

Patients with follicular lymphoma may survive for periods of less than 1 year to more than 20 years after diagnosis. We used gene-expression profiles of tumor-biopsy specimens obtained at diagnosis to develop a molecular predictor of the length of survival.

METHODS

Gene-expression profiling was performed on 191 biopsy specimens obtained from patients with untreated follicular lymphoma. Supervised methods were used to discover expression patterns associated with the length of survival in a training set of 95 specimens. A molecular predictor of survival was constructed from these genes and validated in an independent test set of 96 specimens.

RESULTS

Individual genes that predicted the length of survival were grouped into gene-expression signatures on the basis of their expression in the training set, and two such signatures were used to construct a survival predictor. The two signatures allowed patients with specimens in the test set to be divided into four quartiles with widely disparate median lengths of survival (13.6, 11.1, 10.8, and 3.9 years), independently of clinical prognostic variables. Flow cytometry showed that these signatures reflected gene expression by nonmalignant tumor-infiltrating immune cells.

CONCLUSIONS

The length of survival among patients with follicular lymphoma correlates with the molecular features of nonmalignant immune cells present in the tumor at diagnosis.

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Follicular lymphoma is the second most common form of non-Hodgkin's lymphoma, accounting for about 22 percent of all cases.[1] The clinical course of follicular lymphoma is variable: in some patients the disease is indolent and slowly progressive over a period of many years, with waxing and waning lymphadenopathy, whereas in others the disease progresses rapidly, often with transformation to aggressive lymphoma and early death.[2,3] Management includes observation, chemotherapy, hematopoietic stem-cell transplantation, and immunologic therapies based on antibodies to B cells[4-7] or idiotype vaccines.[8-10] There is no conclusive evidence that any of these approaches offers a clinically significant survival advantage, and hence there is no agreement concerning the best treatment.[2]

The molecular and cellular mechanisms responsible for the clinical heterogeneity of follicular lymphoma are unknown. The tumor arises from a germinal-center B cell that, in the majority of cases, has acquired a t(14;18) translocation that deregulates *BCL2*, a key gene in the regulation of cell death. Some tumors subsequently accumulate further oncogenic aberrations that have been associated with transformation to diffuse large-B-cell lymphoma.[11] However, it is unclear whether these random genetic events account for the clinical heterogeneity of the disease. Several clinical factors are associated with prognosis in follicular lymphoma, and some of them constitute the International Prognostic Index (IPI).[12-16] However, prognostic models based on clinical variables have not been successful in determining the best initial treatment.

An understanding of the molecular biology that underlies the survival differences among patients with follicular lymphoma might provide a more accurate and rational method of risk stratification to guide treatment and might suggest new therapeutic approaches as well. We conducted a study to determine whether the length of survival among patients with follicular lymphoma can be predicted by the gene-expression profiles of the tumors at diagnosis. By whole-genome microarray analysis of gene expression, we constructed a multivariate model of survival that revealed aspects of the biology of follicular lymphoma that influenced the length of survival.

METHODS

PATIENTS

Fresh-frozen tumor-biopsy specimens and clinical data from 191 untreated patients who had received a diagnosis of follicular lymphoma between 1974 and 2001 were obtained from seven institutions in North America and Europe and studied according to a protocol approved by the National Cancer Institute's institutional review board. The patients had undergone a variety of standard treatments after biopsy, including various chemotherapy regimens (such as those containing anthracyclines and purine analogues) and autologous stem-cell transplantation, or had been followed with observation. The median age at diagnosis was 51 years (range, 23 to 81), and the median follow-up time was 6.6 years (range, less than 1.0 to 28.2); the median follow-up time among patients alive at last follow-up was 8.1 years. Additional clinical characteristics of the patients are listed in Table 1.

GENE-EXPRESSION PROFILING

RNA was extracted from the biopsy specimens as previously described[17] and was examined for gene expression with the use of Affymetrix U133A and U133B microarrays. Lymphoid subpopulations were purified and stimulated.[17] Monocytes were purified from lymphopheresis specimens by magnetic sorting for CD14+ cells (Miltenyi Biotec). Cell suspensions obtained from fresh biopsy specimens were separated by fluorescence-activated cell sorting into a CD19+, malignant subpopulation and a CD19−, nonmalignant subpopulation. Two rounds of linear amplification from total RNA were performed (Ambion).

STATISTICAL ANALYSIS

The statistical methods are described in detail in the Supplementary Appendix (available with the full text of this article at www.nejm.org) and in the Results section. The creation of the gene-expression–based multivariate model is outlined in Figure 1A. In brief, the biopsy specimens were divided into a training set (95 specimens) and a test set (96 specimens), which were balanced with respect to institution and the length of survival. All aspects of model development and all tests of association be-

Table 1. Clinical Characteristics of the Patients and Relative Risk of Death.*

Clinical Variable	Training Set of Specimens	Test Set of Specimens	Relative Risk of Death (95% CI)			
			Univariate Analysis	P Value	Multivariate Analysis	P Value
	% of patients					
Age (yr)						
≤60	64.5	70.2	1.00		1.00	
>60	35.5	29.8	1.90 (1.02–3.56)	0.04	2.21 (1.48–3.29)	<0.001
Stage						
I or II	33.3	25.5	1.00		1.00	
III or IV	66.7	74.5	1.31 (0.65–2.64)	0.45	2.31 (1.51–3.52)	<0.001
No. of extranodal sites						
<2	94.6	79.8	1.00		1.00	
≥2	5.4	20.2	1.58 (0.83–2.99)	0.16	2.21 (1.48–3.30)	<0.001
Lactate dehydrogenase†						
Normal	77.1	66.2	1.00		1.00	
Greater than normal	22.9	33.8	1.77 (0.97–3.24)	0.06	2.40 (1.57–3.67)	<0.001
ECOG performance status						
<2	90.6	87.5	1.00		1.00	
≥2	9.4	12.5	2.05 (0.89–4.71)	0.09	2.17 (1.40–3.35)	<0.001
Sex						
Male	41.9	64.9	1.00		1.00	
Female	58.1	35.1	1.62 (0.90–2.90)	0.10	2.17 (1.45–3.25)	<0.001
B symptoms‡						
Absent	82.8	78.7	1.00		1.00	
Present	17.2	21.3	2.05 (1.08–3.89)	0.03	2.10 (1.37–3.23)	<0.001
Tumor grade§				0.12	2.55 (1.63–3.99)	<0.001
1	45.0	43.4	1.00			
2	34.8	33.3	2.03 (1.04–3.96)			
3	20.2	23.3	1.39 (0.65–2.98)			
IPI score¶				0.03	2.28 (1.46–3.57)	<0.001
0 or 1	63.1	47.5	1.00			
2 or 3	33.3	45.0	2.07 (1.07–4.00)			
4 or 5	3.6	7.5	3.73 (1.18–11.18)			

* The univariate analyses included only the specified clinical variable; the multivariate analyses included the specified clinical variable and the survival-predictor score. Relative risks are for the patients with specimens in the test set and are based on a doubling in gene expression. CI denotes confidence interval, ECOG Eastern Cooperative Oncology Group, and IPI International Prognostic Index.

† The lactate dehydrogenase value was deemed greater than normal if it was greater than the upper limit of the normal reference range at each institution.

‡ B symptoms are weight loss, night sweats, and fever.

§ The relative risk of death for grades 2 and 3 was calculated with respect to the risk of death for grade 1. The P value is for all grades with grade used as a categorical variable.

¶ The relative risk of death for IPI score 2 or 3 and IPI score 4 or 5 was calculated with respect to the risk of death for IPI score 0 or 1. The P value is for all IPI risk groups with IPI risk group used as a categorical variable.

tween gene expression and survival were based solely on the data from the training set. No prior survival analysis or subgroup analysis was attempted with the test set.

The Cox model was used to identify genes associated with survival in the training set. The genes associated with a good prognosis and those associated with a poor prognosis were organized separately by hierarchical clustering,[18] and genes that had correlated expression patterns (r>0.5) were

grouped into gene-expression signatures. The expression levels of genes within a signature were averaged to create a "signature average" for each biopsy specimen. Two signatures, termed "immune-response 1" and "immune-response 2," were used to create a model in the training set in which a survival-predictor score was assigned to each patient. The score was calculated as follows: (2.71×immune-response 2 signature average)−(2.36×immune-response 1 signature average). A high survival-predictor score was associated with a poor outcome. This model was then evaluated for its association with survival in the test set.

RESULTS

CONSTRUCTION OF A PREDICTOR OF SURVIVAL BASED ON GENE EXPRESSION

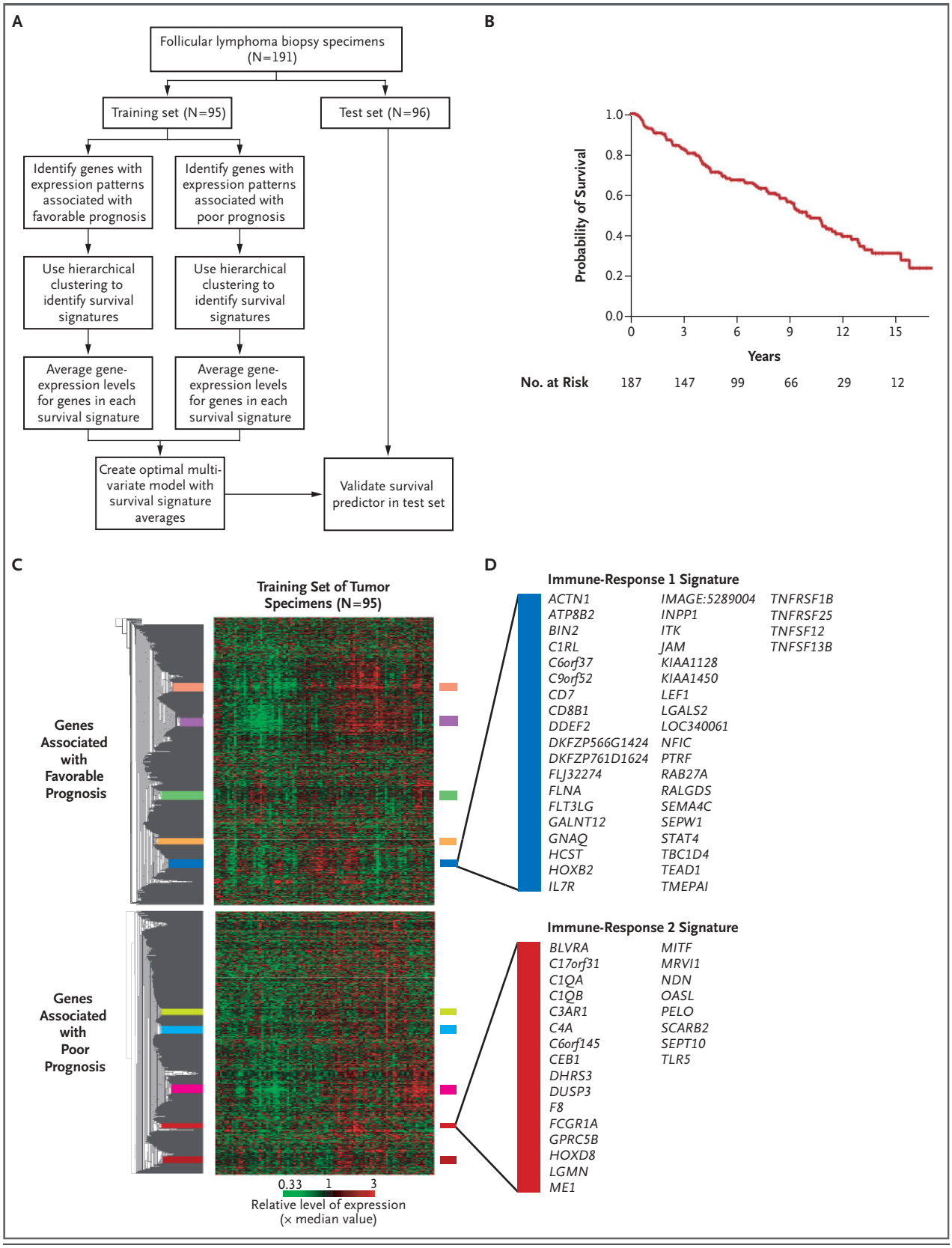
To devise a gene-expression–based model of survival in follicular lymphoma, we developed an analytical method, called survival signature analysis, which is a modification of a method previously used to create a molecular predictor of survival in patients with diffuse large-B-cell lymphoma.[19] The method is summarized in Figure 1A. Its key feature is the identification of gene-expression signatures, which are sets of coordinately expressed genes that can reflect the cell of origin of the cancer, the nature of the nonmalignant cells in the biopsy specimen, and the oncogenic mechanisms responsible for the cancer.[20] Survival signature analysis begins with the identification of genes having expression patterns that are statistically associated with survival. A hierarchical-clustering algorithm is then used to identify subsets of these genes with expression patterns that are correlated among the cancer specimens: these subsets are operationally defined as survival-associated signatures. By evaluating a limited number of survival-associated signatures, we aimed to mitigate the multiple-comparisons problem that is inherent in the use of large gene-expression data sets to create statistical models of survival.[21]

Genomic-scale gene-expression profiling of tumor-biopsy specimens obtained from 191 patients with untreated follicular lymphoma was performed. The overall survival of this cohort is depicted in Figure 1B. To create a model of survival based on gene expression, we divided the specimens into a training set, which was used to develop the model, and a test set, which was used to evaluate its reproducibility. Within the training set, the Cox proportional-hazards model was used to identify survival-predictor genes with expression levels associated with long survival (good-prognosis genes) or short survival (poor-prognosis genes). A hierarchical-clustering algorithm was used to identify gene-expression signatures within the good-prognosis and poor-prognosis gene sets, according to the genes' patterns of expression among all the specimens in the training set. Ten clusters of coordinately regulated genes were observed in the good-prognosis gene set or in the poor-prognosis gene set (Fig. 1C). We averaged the expression levels of the component genes within each signature, thereby creating a signature average for each patient.

To create a multivariate model of survival, we generated different combinations of the 10 gene-expression signature averages and evaluated them for their ability to predict survival within the training set. Among models consisting of two signatures, we noted an exceptionally strong statistical synergy between one signature from the good-prognosis group and one from the poor-prognosis group. These signatures were termed immune-response 1 and immune-response 2 on the basis of the biologic function of certain genes within each signature (as discussed below). Although these signatures were not the best predictors of survival individually, the binary model formed with them was more predictive of survival than any other binary model. Together, these two signatures were highly predictive of survival in the training set ($P<0.001$).

Figure 1 (facing page). Survival and Genes Associated with Prognosis in Follicular Lymphoma.

Panel A shows an overview of survival signature analysis, the approach used for the development and validation of a survival predictor based on gene expression. Panel B shows a Kaplan–Meier survival curve for all the patients for whom these data were available. Panel C shows the hierarchical clustering of survival-associated genes according to their expression in the training set of 95 follicular lymphoma biopsy specimens. The data are represented in a grid format in which each column represents a single case of follicular lymphoma, and each row a single gene. The relative level of gene expression is depicted according to the color scale shown. The dendrogram shows the degree to which the expression pattern of each gene is correlated with that of the other genes; the colored bars represent sets of coordinately regulated genes, defined as gene-expression signatures. To the right of the dendrogram, the genes making up the immune-response 1 and immune-response 2 signatures that formed the survivor-predictor model are listed.



Therefore, we decided to base our model on these two signatures and to test whether any other signatures added to the statistical significance of the model, using a step-up procedure.[22] Of the remaining eight signatures, only one contributed significantly to the model in the training set ($P<0.01$), resulting in a three-variable model of survival.

This model was associated with survival in a highly statistically significant fashion in both the training set ($P<0.001$) and the test set ($P=0.003$). However, only the immune-response 1 and immune-response 2 gene-expression signatures contributed to the predictive power of the model in both sets (Table 2), and the remaining signature was therefore dropped from the model. The two-signature model was significantly associated with survival among patients whose specimens were included in the training set ($P<0.001$) and those whose specimens were included in the test set ($P<0.001$), thus confirming the model's reproducibility. For each patient, the model generated a survival-predictor score, which ranged from −0.20 to 4.56 (SD, 0.94) in the test set. Each unit increase in the survival-predictor score was associated with an increase in the relative risk of death by a factor of 2.27 (95 percent confidence interval, 1.51 to 3.39) in the test set.

To visualize the predictive power of the model, we ranked the patients according to their survival-predictor scores and divided them into four equal quartiles accordingly. Kaplan–Meier plots of overall survival showed clear differences in survival according to quartile among patients whose specimens were included in the test set (Fig. 2A). The survival medians for the quartiles were as follows: quartile 1, 13.6 years; quartile 2, 11.1 years; quartile 3, 10.8 years; and quartile 4, 3.9 years.

Various clinical variables were significantly associated with the probability of survival, including the IPI and some of its components and the presence or absence of B symptoms (i.e., weight loss, night sweats, or fever) (Table 1). The gene-expression–based model predicted the probability of survival independently of each of the clinical variables. The Kaplan–Meier plot shown in Figure 2B illustrates the association of the IPI with the probability of survival. Among patients with specimens in the test set who were at low risk (IPI score, 0 or 1) and those who were at intermediate risk (IPI score, 2 or 3), the gene-expression–based survival model stratified patients into groups differing by more than five years in median survival (Fig. 2C). The high-risk group (IPI score, 4 or 5) comprised less than 5 percent of the patients and was omitted from this analysis. These results demonstrate that the gene-expression–based model does not act as a surrogate for clinical variables that are known to predict survival in follicular lymphoma; rather, the gene-expression–based model identifies distinct biologic attributes of the tumors that are associated with survival.

Table 2. Predictive Power of Gene-Expression Signatures in Follicular Lymphoma.*

Gene-Expression Signature	P Value for Contribution to Model in Test Set	Relative Risk of Death (95% CI)*	Effect of Increased Gene Expression on Survival
Immune-response 1	<0.001	0.15 (0.05–0.46)	Favorable
Immune-response 2	<0.001	9.35 (3.02–28.90)	Unfavorable

* Relative risks are for patients with specimens in the test set and are based on a doubling of the signature expression as a component in the two-variable survival-predictor model. CI denotes confidence interval.

CELLULAR ORIGIN OF SURVIVAL-ASSOCIATED GENE-EXPRESSION SIGNATURES

The signatures in the survival model were named on the basis of the biologic function of certain genes within each signature. The immune-response 1 signature includes genes encoding T-cell markers (e.g., *CD7*, *CD8B1*, *ITK*, *LEF1*, and *STAT4*) and genes that are highly expressed in macrophages (e.g., *ACTN1* and *TNFSF13B*). Notably, the immune-response 1 signature is not merely a surrogate for the number of T cells in the tumor-biopsy specimen, since many other standard T-cell genes (e.g., *CD2*, *CD4*, *LAT*, *TRIM*, and *SH2D1A*) were not associated with survival. The immune-response 2 signature includes genes known to be preferentially expressed in macrophages, dendritic cells, or both (e.g., *TLR5*, *FCGR1A*, *SEPT10*, *LGMN*, and *C3AR1*).

To identify directly the cells that expressed these signatures within the tumor-biopsy specimens, the CD19+, malignant cells were separated from the CD19−, nonmalignant cells by flow sorting, and each subpopulation was profiled for gene expression. Figure 3A shows the difference in the gene-expression signature averages between the CD19+ and CD19− subpopulations from four patients. A germinal-center B-cell signature was constructed from genes known to be overexpressed at this stage of B-cell differentiation[20] (specifically, *MME*,

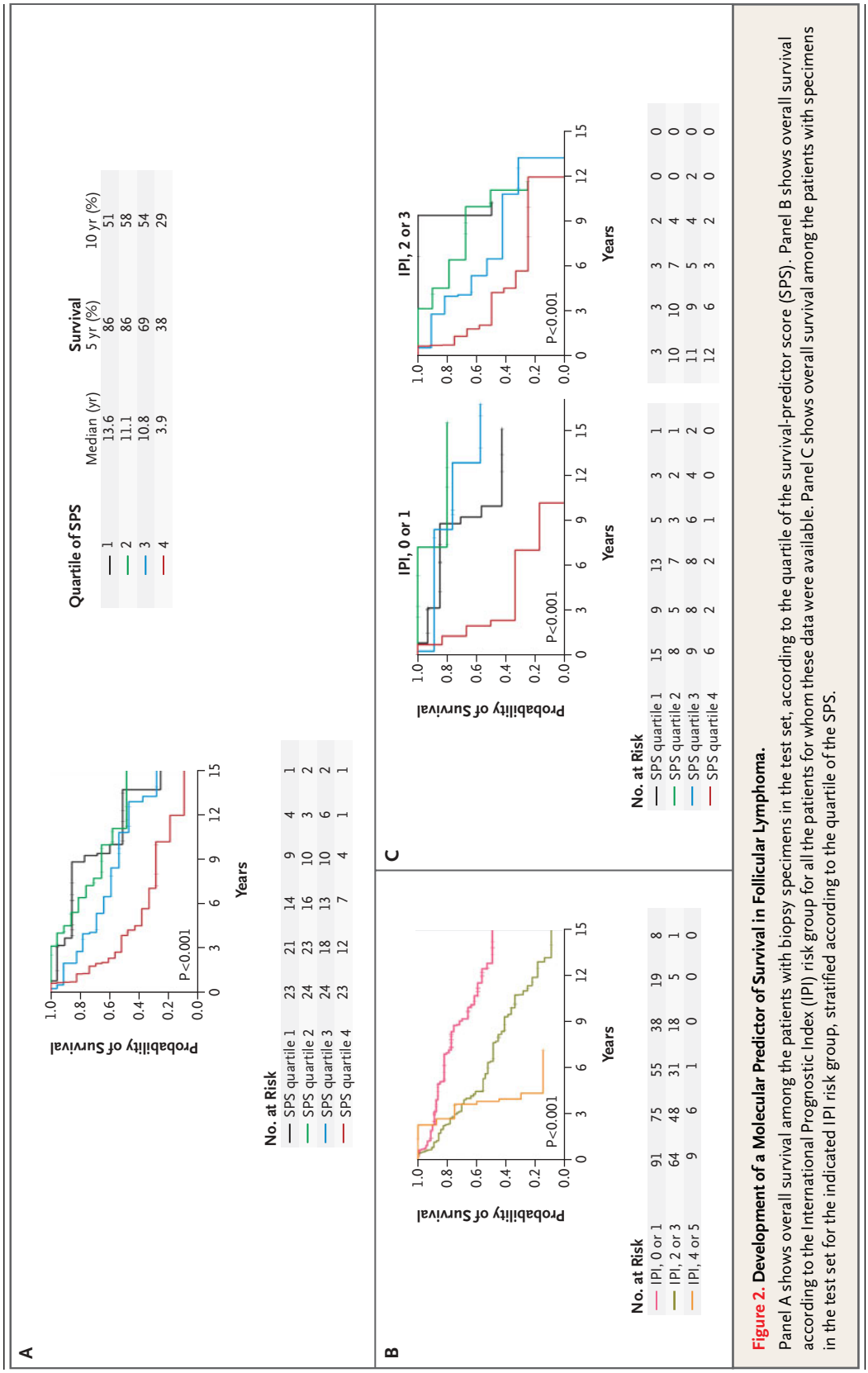


Figure 2. Development of a Molecular Predictor of Survival in Follicular Lymphoma.

Panel A shows overall survival among the patients with biopsy specimens in the test set, according to the quartile of the survival-predictor score (SPS). Panel B shows overall survival according to the International Prognostic Index (IPI) risk group for all the patients for whom these data were available. Panel C shows overall survival among the patients with specimens in the test set for the indicated IPI risk group, stratified according to the quartile of the SPS.

MEF2C, *BCL6*, *LMO2*, *PRSPAP2*, *MBD4*, *EBF*, and *MYBL1*). The malignant cells in follicular lymphoma are of germinal-center origin, and the CD19+, malignant fraction would therefore be expected to express this signature highly, as was found to be the case in the sorted samples (Fig. 3A). In contrast, the immune-response 1 and immune-response 2 signature averages were higher in the CD19–, nonmalignant cells from the tumors. Moreover, most of the component genes of the immune-response 1 and immune-response 2 signatures were expressed more highly in the CD19–, nonmalignant cells than in the CD19+, malignant cells (Fig. 3B).

To characterize the expression of the two survival-associated gene-expression signatures within the hematopoietic lineage, we profiled gene expression in various purified subpopulations derived from peripheral blood or tonsils (Fig. 3B). None of the genes in the immune-response 1 or immune-response 2 signatures were preferentially expressed in germinal-center B cells, the cell of origin of follicular lymphoma. Instead, many of the genes in the immune-response 1 signature were more highly expressed in T cells than in any of the B-cell or monocyte subpopulations, and others were more highly expressed in both T cells and monocytes than in B cells. Many of the genes within the immune-response 2 signature were more highly expressed in monocytes than in any of the lymphoid subpopulations. These findings support the notion that the immune-response 1 and immune-response 2 signatures reflect the biologic characteristics of the nonmalignant immune cells within the biopsy specimens.

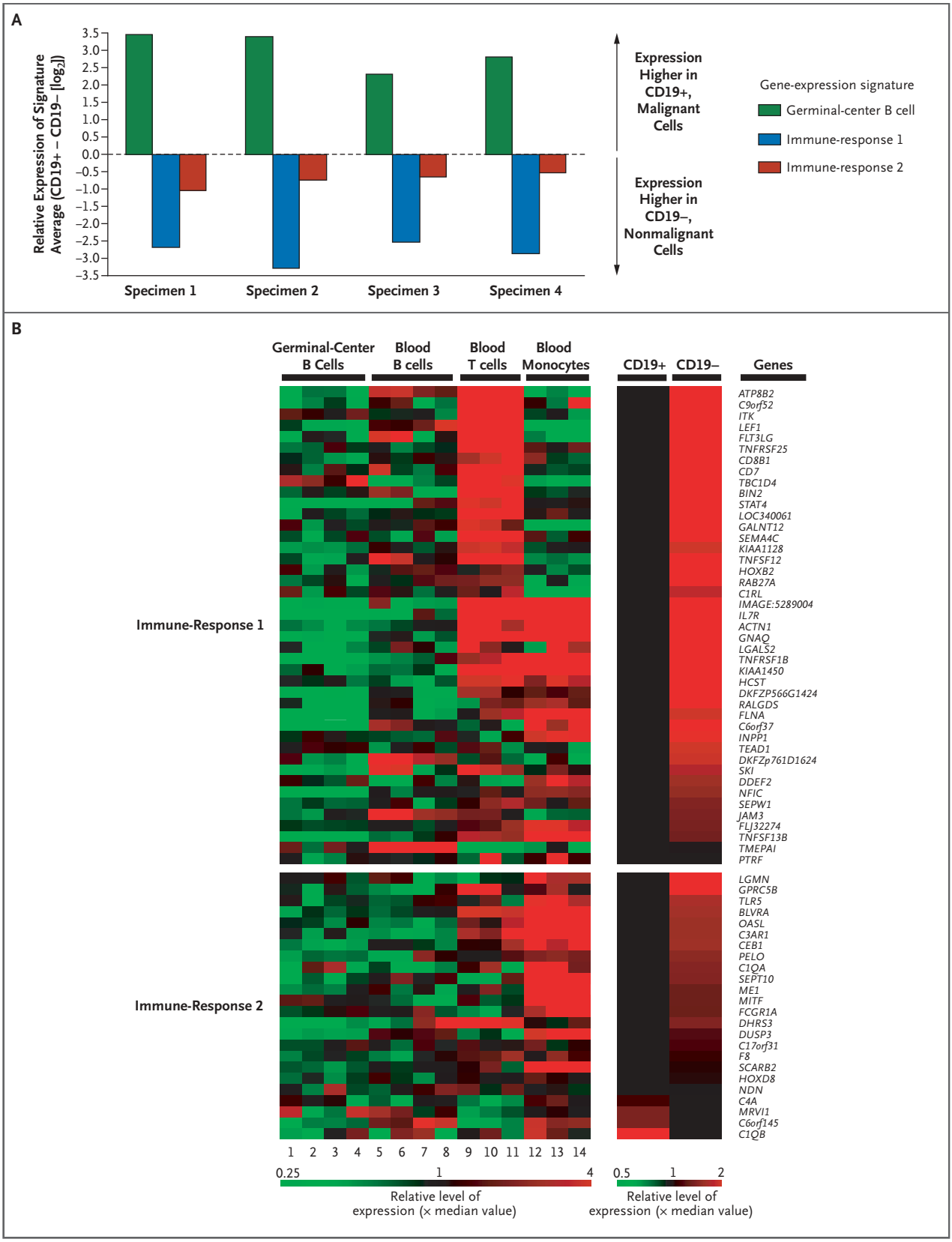
Figure 3 (facing page). Cellular Origin of the Survival-Associated Gene-Expression Signatures.

Panel A shows the relative expression of the survival-associated signature averages in the CD19+ and CD19– subpopulation of cells isolated from four biopsy specimens from patients with follicular lymphoma. Panel B shows the expression of individual genes in the survival-associated gene-expression signatures. The left-hand portion of Panel B depicts gene expression in purified normal immune-cell populations. Lanes 1, 2, and 3 contain total tonsillar germinal-center B cells; lane 4 contains tonsillar germinal-center CD77+ centroblasts; lanes 5 and 6 contain peripheral-blood B cells obtained before stimulation; lane 7 contains peripheral-blood B cells obtained 24 hours after anti-IgM stimulation; lane 8 contains peripheral-blood B cells obtained 48 hours after anti-IgM stimulation; lanes 9 and 10 contain peripheral-blood T cells obtained before stimulation; lane 11 contains peripheral-blood T cells obtained seven days after anti-CD3 stimulation; and lanes 12, 13, and 14 contain peripheral-blood monocytes. The right-hand portion of Panel B depicts the average expression of each gene in the CD19+ and CD19– subpopulations isolated from the tumor-biopsy specimens. The relative level of gene expression is depicted according to the color scales shown.

DISCUSSION

In this study, we identified a molecular predictor of the length of survival in patients with follicular lymphoma — a predictor that may prove useful clinically. The molecular features of follicular lymphoma at the time of diagnosis dictated, to a large degree, the aggressiveness of the disease and the duration of survival, suggesting that the random acquisition of oncogenic abnormalities after diagnosis does not have a major effect on survival. The gene-expression signatures that were associated with survival were not surrogates for clinical prognostic variables. Rather, these signatures identified biologic attributes of the tumors that influenced survival. Unexpectedly, the gene-expression signatures that predicted survival were derived from nonmalignant cells in the tumors. This observation points to an important interplay between the host immune system and the malignant cells in this form of cancer.

How might this molecular predictor of survival be used clinically? The survival predictor can identify a substantial subgroup of patients who have an indolent form of the disease (more than 75 percent of the overall population of patients with follicular lymphoma), among whom the median survival after diagnosis is more than 10 years. This is a subgroup of patients for whom our survival predictor would provide valuable prognostic information and for whom watchful waiting is appropriate. In the quartile with the least favorable prognosis, patients survived a median of only 3.9 years; for these patients, newer treatments in the context of clinical trials should be considered. Indeed, the molecular predictor could be used to design clinical trials that have achievable end points. Since, overall, patients with follicular lymphoma survive a median of more than 10 years, it has been difficult to complete clinical trials in which overall survival is the primary end point. Now, however, a clinical trial could be designed to enroll only those patients in the quartile with the least favorable prognosis, a strategy that would allow assessment of overall survival.



Our analytical approach, survival signature analysis, focused on sets of coordinately regulated genes known as gene-expression signatures.[20] Surprisingly, the two gene-expression signatures that predicted survival, immune-response 1 and immune-response 2, comprised genes expressed by nonmalignant tumor-infiltrating cells. The immune-response 1 signature included several T-cell–restricted genes but was not merely a measure of the number of tumor-infiltrating T cells, since a signature of pan–T-cell genes was not associated with survival. The immune-response 1 signature also included genes that were more highly expressed in monocytes than in T cells, suggesting that it reflected a mixture of immune cells. The immune-response 2 signature did not include T-cell–restricted genes but rather genes that are highly expressed in monocytes, dendritic cells, or both.[23-28] The statistical synergy of these two signatures in the survival model suggests that their relative contribution to the tumor's gene-expression profile — not their absolute expression levels — is of primary importance. In other words, the nature of the infiltrating immune cells was the predominant feature of the tumor that predicted the length of survival.

There is considerable clinical evidence that immune responses are important in follicular lymphoma. In some cases, the lymphoma regresses spontaneously,[29] an observation that has also been made in melanoma and renal-cell carcinoma and that may indicate an effective antitumor immune response. The response of follicular lymphomas to idiotype vaccines also highlights the potential of the immune system to recognize and counteract this type of lymphoma.[8-10] Although these findings suggest that the clinical course of follicular lymphoma can be modulated by immune responses, our study provides a molecular signature of the type of immune response that is associated with long-term survival.

It is also possible that the lymph-node cells responsible for the immune-response 1 signature provide trophic signals that promote the survival or proliferation of the malignant cells. This signature could represent a variant germinal-center reaction that includes T cells, follicular dendritic cells, and the malignant cells. The dependence of the malignant cells on these environmental signals may prevent them from leaving the lymph node, possibly accounting for the association between the immune-response 1 signature and prolonged survival. An understanding of the nature of these trophic signals provided by the microenvironment in follicular lymphoma could provide new targets for therapy.

In a pilot study involving 26 patients with follicular lymphoma who were treated with rituximab, the expression of certain genes was associated with responsiveness to this treatment,[30] but these genes do not overlap appreciably with our survival-predictor genes and do not predict overall survival in our series (data not shown). Clearly, future investigations should evaluate these molecular predictors of survival in a prospective fashion.

Our work provides a molecular tool to investigate aspects of the immune response to follicular lymphoma that may positively or negatively influence the pace of the disease. The genes in the immune-response signatures can be used as markers to identify subpopulations of immune cells that may promote or antagonize the proliferation or survival of the malignant clone.

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