Variation in gene expression patterns in follicular lymphoma and the response to rituximab

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Analysis of the patterns of gene expression in follicular lymphomas from 24 patients suggested that two groups of tumors might be distinguished. All patients, whose biopsies were obtained before any treatment, were treated with rituximab, a monoclonal antibody directed against the B cell antigen, CD20. Gene expression patterns in the tumors that subsequently failed to respond to rituximab appeared more similar to those of normal lymphoid tissues than to gene expression patterns of tumors from rituximab responders. These findings suggest the possibility that the response of follicular lymphoma to rituximab treatment may be predicted from the gene expression pattern of tumors.

Follicular non-Hodgkin's lymphoma (NHL) is an indolent B cell malignancy with an annual incidence exceeding 10,000 cases in the United States. Although follicular lymphoma (FL) is frequently responsive to treatment, therapy is very rarely, if ever, curative. Rituximab, a chimeric IgG1 monoclonal antibody directed at the B cell antigen CD20, has become a mainstay of treatment for low-grade NHL; >400,000 patients worldwide have been treated with rituximab. Phase II trials of rituximab in patients with refractory or relapsed low grade or follicular NHL demonstrated a 50% response rate (1).

Despite this extensive clinical experience, the mechanism of action of rituximab remains unclear, as does the nature of resistance (2). Among the proposed mechanisms are antibody-dependent cell-mediated cytotoxicity (3), complement-mediated cytotoxicity (4), and direct cytotoxicity through modulating CD20 function (5–7). The association with resistance to rituximab treatment of a low-affinity variant of the Fc receptor (8) is suggestive of an immune mechanism, and remains the only plausible hint about the nature of resistance.

In this study, we examined whether gene expression profiling using cDNA microarrays could reveal biological diversity among follicular lymphomas and, more specifically, whether gene expression patterns in tumors might predict sensitivity to rituximab treatment.

Materials and Methods

Patient Characteristics. Patients were included in this study based on the availability of freshly frozen lymph node biopsy material containing enough mRNA to allow cDNA microarray analysis. Only patients with samples that had been obtained before any systemic therapy were included. In all cases the pathological diagnosis was follicular non-Hodgkin's lymphoma [follicular small cleaved (grade 1), follicular mixed (grade 2), or follicular large cell (grade 3) histology]. Each patient received rituximab treatment with documentation of clinical outcome. In all cases, biopsy and pathology review were performed at Stanford University Medical Center. Rituximab treatment was administered either at Stanford University Medical Center or by an outside oncologist.

Microarray Procedures. Freshly frozen lymph node samples were obtained from patients who underwent excisional biopsy at Stanford University Medical Center between 1984 and 1997, who subsequently received rituximab between 1994 and 2000 and whose clinical response to rituximab treatment had been recorded. Tonsil and spleen samples were similarly obtained from patients treated at Stanford University Medical Center in 2000 or 2001. Biopsy samples were stored frozen in optimal cutting temperature compound. Poly(A)$^+$ mRNA was obtained from biopsy samples after homogenization of tissue with the FASTTRACK 2.0 kit (Invitrogen). An experimental cDNA probe incorporating Cy5 dye was generated from mRNA from malignant and normal lymphoid tissues; a common reference cDNA probe incorporating Cy3 dye was from mRNA derived from a panel of cell lines and probes were hybridized to cDNA microarrays as described (9, 10). Two types of microarrays were used. Some experiments in this study used Stanford Human arrays comprised of 38,431 DNA spots of 38,276 unique cDNA clones, representing ≈31,139 unique Unigene clusters of which 16,152 correspond to unique named genes. Some experiments were conducted with lymphochip (LC) microarrays comprised of 37,632 DNA spots with 32,876 unique cDNA clones, representing ≈17,622 Unigene clusters of which at least 10,250 are unique named genes. More detailed information regarding microarray methods, and data selection, and analysis, as well as searchable figures and microarray data files, can be found at http://genome-www.stanford.edu/rituximab.

Statistical Analysis. Before statistical analysis, individual data points were median centered for each cDNA clone and filtered for data quality and signal at least 2-fold above the median in two or three of the samples in each data set, as described in the web supplement. Agglomerative hierarchical cluster analysis was applied to the gene axis and to the sample axis as described (11). Hierarchical cluster analysis of LC data revealed a technical artifact that resulted in samples segregating by the date of the experiment. Further investigation revealed that this artifact was likely caused by differences in the calibration of the two scanners used to read the arrays. Singular value decomposition was used to remove the pattern corresponding to this artifact before analysis (12) after missing data were estimated by using a K-nearest neighbors (KNN) impute algorithm with 12 nearest values (13). Supervised analysis taking into account known outcome to rituximab treatment was performed by using Wilcoxon rank sum test to generate a rank list of genes whose corresponding mRNA levels differ significantly in rituximab responders versus nonresponders (14).

Results

Patient Characteristics. Tumor samples from 24 patients were analyzed in this study. No significant differences in age or treatment history were observed between responders and nonresponders (Table 1). All patients except one received at least one course of chemotherapy before receiving rituximab (range, 0–6 prior courses). One patient had received a shared anti-

Abbreviations: FL, follicular lymphoma; LC, lymphochip.

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Table 1. Patient characteristics by response to rituximab treatment

Patient characteristics	PR/CR ($n = 12$)	MR/NR ($n = 12$)
Sex (M:F)	7:5	10:2
Age at diagnosis ± SD, years	45.3 ± 10.1	46.3 ± 13.2
Age at rituximab ± SD, years	52.2 ± 13.7	51.6 ± 9.2
Pathology		
FSC	7	9
FM	5	2
FLC	0	1
Mean courses of prior chemotherapy	2.4	2.4
Prior high dose chemotherapy	2	3

NR, no response; MR, minimal response; PR, partial response; CR, complete response; M, male; F, female; FSC, follicular small cleaved; FM, follicular mixed; FLC, follicular large cell. Age data are presented as mean age in years for each group ± standard deviation. None of the differences between the groups are statistically significant ($P > 0.35$ in all cases).

idiotype antibody as the sole prior treatment. For the treatment of FL, a course of rituximab typically consists of four weekly infusions of 375 mg/m^2; 21 of 24 patients received this dosing regimen, including all of the patients in the nonresponder group. In the partial response/complete response (PR/CR) group, two patients received a single dose of 375 mg/m^2 and one patient received eight weekly doses of 375 mg/m^2 with a documented near CR after 6 weeks of treatment. The overall response rate (CR+PR) for the patients in this study was 50%, which is similar to the overall response rate of 60% demonstrated for FL in the pivotal Phase II trial (1). Five patients achieved CR in response to rituximab, seven patients experienced PR, and 12 patients had no or minimal response.

Gene Expression Patterns Identify Two Subtypes of Follicular Lymphoma. An overview of the gene expression patterns from FL patients was generated by hierarchical cluster analysis of data from the first 16 FL patients analyzed together with samples from nonmalignant tonsil and spleen (Fig. 1*A*). The hierarchical cluster algorithm arranges tissue samples based on the degree of similarity in their gene expression patterns (11). In addition to tumor material obtained before treatment, we included tissue samples from later biopsies for four patients for whom such material was available. These repeat biopsies were obtained 2.5–6.5 years after the initial biopsy but before rituximab treatment. In all of the repeat biopsy cases, patients received one or more courses of chemotherapy between the initial and later biopsies and the histopathological diagnosis was the same for both biopsy samples.

To focus on genes differentially expressed in the samples, expression data from 2,037 unique genes whose expression differed 2-fold in at least three arrays were used for analysis. The dendrogram shown in Fig. 1*A* shows that the FL samples could be divided into two groups, with a subset of the FL samples exhibiting a gene expression pattern more similar to nonmalignant lymphoid tissues than to the other FL subtype. Hierarchical cluster analysis of the genes and tissue samples that contribute to the subclassification of FL samples are shown in Fig. 1*B*. The two subtypes of FL display gene expression patterns similar to or opposite those of nonmalignant spleen. It should be emphasized that the observed patterns of gene expression reflect all of the cells in the tumor, not only the tumor cells themselves. Interpretation of expression differences may include the differential presence or absence of other cell types, as has been found for breast cancer (9).

Rituximab Nonresponders Display Gene Expression Patterns Characteristic of Normal Lymphoid Tissue. Having divided the FL samples into two groups based on gene expression patterns, we sought to determine whether these subtypes correspond to clinical differences in individual patients, in particular, in response to rituximab treatment. We found that the rituximab nonresponders (orange in Fig. 1) were disproportionately distributed between the two FL clusters. Most of the rituximab nonresponders clustered with normal tonsil and spleen tissue ($P < 0.005$, Fisher's exact test). These findings indicate that there is biological diversity in FL lymph nodes from different patients before rituximab treatment and that the specific gene expression patterns defining the two groups described may be useful in predicting outcome to rituximab treatment.

Two features of the dendrogram in Fig. 1 indicate that the clustering of tissue samples based on gene expression patterns is reflective of biologically relevant similarities between samples. First, pairs of normal spleen and tonsil tissue cluster very closely together. Second, FL lymph node biopsies from the same individual cluster together in three of the four cases (see black bars in Fig. 1*A*) despite the passage of time and treatment with systemic chemotherapy between biopsies. An exception was the two samples obtained from patient 13 which did not cluster together (Fig. 1*A*, black arrows) with the pretreatment biopsy clustering with the responders' portion of the dendrogram and the later sample displaying a gene expression pattern more similar to that of rituximab nonresponders and normal lymphoid tissue. The latter sample was predictive of the patient's actual outcome after rituximab treatment; the patient did not respond. This observation suggests that the gene expression phenotype of FL can change over time.

Genes with Significantly Different Expression In Rituximab Responders Versus Nonresponders. To better understand differences in gene expression in involved lymph nodes from rituximab responders versus nonresponders, we used supervised statistical analysis to determine which genes had the most significant differences in expression between the two groups. Having observed that differences in gene expression in FL subtypes correlate with expression patterns in normal lymphoid tissues, samples from the original 16 patients and an additional 8 patient samples were analyzed on LC microarrays enriched for genes expressed in lymphoid cells and genes known or suspected to be important in the immune response or cancer (10). A list of genes whose expression differed between responders and nonresponders with a P value <0.005, as determined by Wilcoxon rank-sum test (14), was chosen. In cases where expression of a given gene was measured on both Stanford Human and LC microarrays, genes were included if the P value was less than 0.005 in either the Stanford Human or LC data set and a cDNA from the same Unigene cluster displayed a P value of <0.05 in the other dataset. Genes that were measured in only one data set were included if the P value is <0.005. The results of this analysis are presented in Tables 2 and 3. By these criteria, 71 genes had significantly higher expression in rituximab nonresponders versus responders; 53 named genes are on this list and 35 of the Unigene clusters represented were measured on both arrays. A total of 27 genes were more highly expressed by these criteria in rituximab responders; of these, 11 are named genes and 5 were measured on both arrays. Of note, CD20 expression was well measured and did not correlate with outcome to rituximab treatment.

Many of the genes with higher expression in tissue from rituximab nonresponders appear to be involved in cellular immune response and inflammation, specifically those encoding cytokine, tumor necrosis factor, and T cell receptor signaling, and complement proteins (Table 4). Given the limitations of available experimental evidence, this simplistic classification cannot account for the complexity of function or regulation of these genes; perhaps, mediators of cellular immune response, such T cells, macrophages, monocytes, and natural killer cells, may be relatively more abundant and/or more active in lymph node tissue of rituximab nonresponders. Several mRNAs for proteins involved in the complement cascade are more abundant

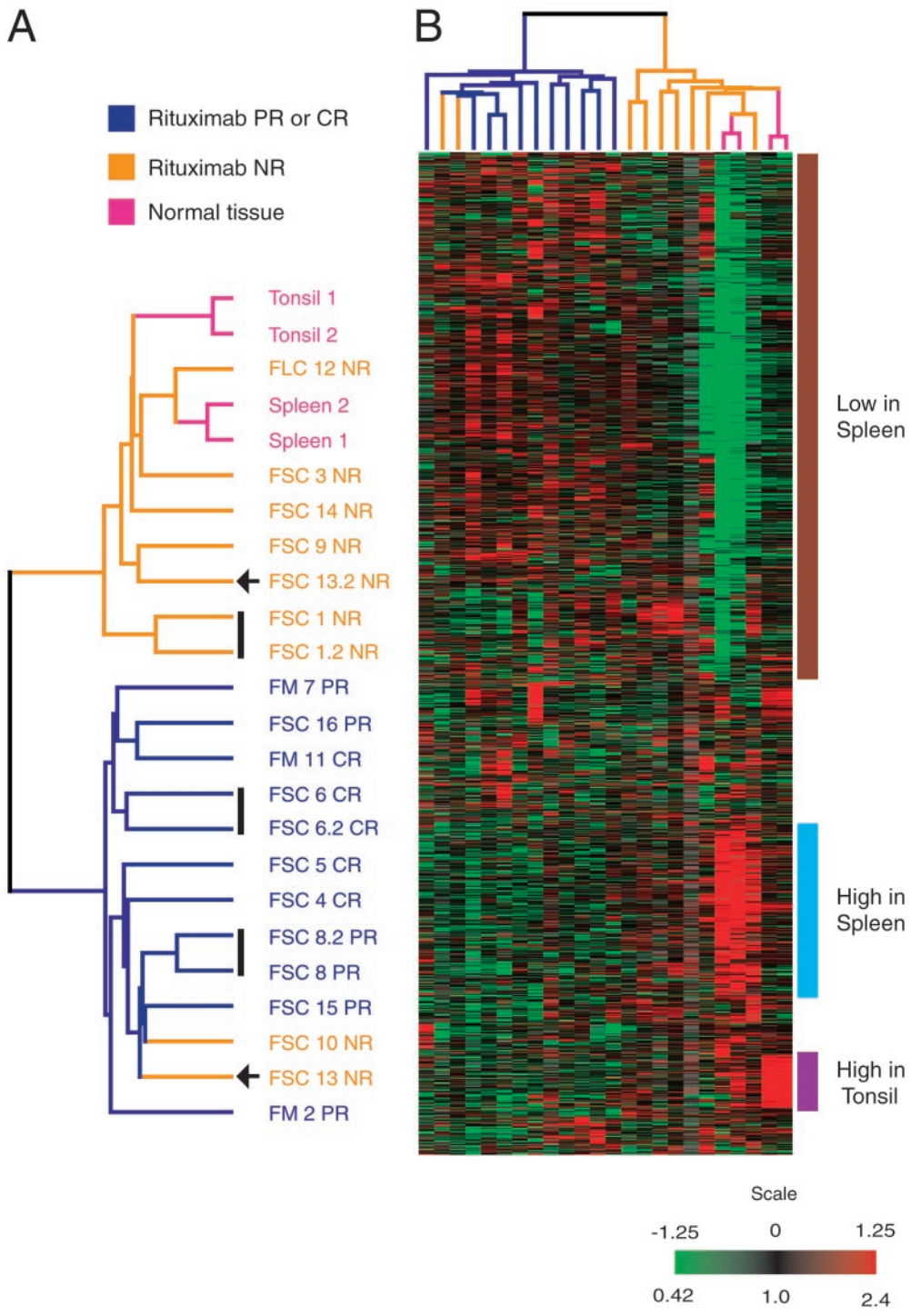

Fig. 1. Hierarchical cluster analysis of gene expression patterns in follicular lymphoma. (*A*) Patterns of gene expression in FL lymph nodes from rituximab responders cluster with normal lymphoid tissue. A total of 20 FL lymph node samples from 16 patients and four normal lymphoid tissues (two tonsil and two spleen) from four different patients were sorted by hierarchical clustering based on similarity of gene expression. The resulting dendrogram is shown. Patient pathological diagnosis or normal tissue type, and response to rituximab treatment are shown. Samples are color coded by response to rituximab (for FL samples) or normal tissue for simplicity. For four patients, paired samples are presented; biopsy samples obtained later are identified by patient number plus ''.2''. In three cases (patients 6, 8, and 18), paired samples clustered together (black bars). The two samples from patient 13 clustered on separate branches of the dendrogram (arrows). (*B*) Patterns of gene expression in FL samples and normal lymphoid tissue. Hierarchical clustering of genes and samples was performed. Variation of gene expression measured with 2,037 genes in 20 FL and four nonmalignant lymphoid tissue samples. Data are presented in a matrix format; each row represents a particular cDNA and each column is an individual FL or normal lymphoid tissue sample. For each sample, the ratio of the abundance of the mRNA measured by each cDNA clone to the median abundance of the mRNA across all tissue samples is represented by color in the corresponding cell in the matrix. Green represents transcript levels less than the median, black represents transcript levels equal to the median, and red represents transcript levels greater than the median. Color saturation represents the magnitude of the ratio relative to the median for each cell (see scale). Colored bars the to right of the matrix define groups of genes with similar expression patterns in normal lymphoid tissues, with blue denoting genes displaying relatively higher expression in spleen, purple denoting genes with higher expression in tonsil, and brown denoting genes with lower expression in spleen. Data can be viewed on our web site at http://genome-www.stanford.edu/rituximab/.

Table 2. Genes expressed at significantly higher levels in rituximab nonresponders versus responders

Gene or clone name	*P* value on LC	*P* value on SH
IMAGE:2021765	0.0002	N.M.
NPDC1	0.0002	0.0018
TNFRSF1B	0.0003	0.0150
CCND1	0.0248	0.0004
DUSP6	0.0047	0.0004
C4B	0.0006	0.0025
JUNB	0.0006	0.0041
PTPRM	0.0006	0.0035
FLJ20967	N.M.	0.0006
IMAGE:4100953	N.M.	0.0006
NGFRAP1	0.0007	0.0009
DAAM2	N.M.	0.0009
DKFZp564C2063	0.0248	0.0009
SSI-3	0.0010	0.0018
RET	0.0011	0.0320
MLPH	N.M.	0.0013
C1S	0.0013	0.0018
TRB@	0.0016	0.0404
CAV1	0.0065	0.0018
IFITM1	0.0047	0.0018
IMAGE:3899550	N.M.	0.0018
KIAA1223	N.M.	0.0018
RAB38	N.M.	0.0018
UACA	0.0040	0.0018
C1QR1	0.0019	0.0086
SNX9	0.0019	0.0114
IMAGE:1371537	0.0023	N.M.
MOX2	0.0023	N.M.
IMAGE:27277	N.M.	0.0025
FLJ23221	N.M.	0.0025
FLJ23705	N.M.	0.0025
LOC115908	N.M.	0.0025
LOC51087	N.M.	0.0025
NCF4	N.M.	0.0025
TYROBP	0.0077	0.0025
BHMT	0.0028	N.M.
PTD004	0.0028	0.0064
Similar to MM20	0.0028	N.M.
SLC21A9	0.0028	N.M.
SPP1	0.0028	N.M.
TNFSF10	0.0028	0.0195
CRY1	0.0033	N.M.
NK4	0.0033	0.0064

P values are determined by Wilcoxon rank sum test. SH and LC refer to different microarray configurations. ''N.M.'' indicates that expression of this cDNA clone or Unigene cluster was not measured on the corresponding array type.

Table 3. Genes expressed at significantly higher levels in rituximab responders versus nonresponders

Gene or clone name	*P* value on LC	*P* value on SH
IMAGE:1582330	N.M.	0.0006
IMAGE:1475660	N.M.	0.0006
IMAGE:1504098	N.M.	0.0009
PRO0650	N.M.	0.0009
IMAGE:626773	N.M.	0.0009
IMAGE:460189	N.M.	0.0009
IMAGE:22374	N.M.	0.0013
Similar to MGR7	N.M.	0.0013
KIAA0317	N.M.	0.0018
H2BFG	N.M.	0.0018
RRM2B	N.M.	0.0018
DKFZp434K1210	N.M.	0.0018
IMAGE:1891596	N.M.	0.0018
BRI3BP	0.0470	0.0025
FREB	0.0470	0.0025
DKFZp564D113	N.M.	0.0025
UBQLN1	N.M.	0.0025
IMAGE:826372	N.M.	0.0025
DKFZp434B1620	N.M.	0.0025
GLE1L	N.M.	0.0030
H2BFB	0.0065	0.0035
ST14	0.0416	0.0035
IMAGE:752612	N.M.	0.0035
BLNK	0.0416	0.0048
IMAGE:1560875	N.M.	0.0048
BLCAP	N.M.	0.0048
IMAGE:814273	N.M.	0.0048

P values are determined by Wilcoxon rank sum test. SH and LC refer to different microarray configurations. ''N.M.'' indicates that expression of this cDNA clone or Unigene cluster was not measured on the corresponding array type.

in nonresponders; the complement cascade can mediate direct cytotoxicity and/or act to stimulate cell-mediated cytotoxicity by opsonization of target cells. We were unable to identify a compelling physiological link among the 11 named genes with significantly increased expression in rituximab responders.

Discussion

Variation in gene expression patterns of FL lymph nodes suggests that it may be useful to divide these tumors into two groups. We suppose that these subtypes are reflective of basic biological differences between FL samples from different individuals. As mentioned before, the differences could be the result, at least in part, of the differential admixture of cells other than the malignant B cells that comprise most of the tumor. Thus, although it is quite possible that the subtypes identified simply represent molecular heterogeneity in the malignant cells, the finding that one subtype displays an expression pattern more similar to nonmalignant lymphoid tissue raises the possibility that the subtypes may, in fact, result from interactions between the malignant B cells and other cell types in the host. On the basis of the genes differing most in their expression between the two subtypes of tumors, it is possible that the differences may involve either the participation or the activities of cells involved in host immune response to the tumors. In any case, on the basis of this preliminary study, the subtype (and the response to rituximab) appears to be determined by the time of initial diagnosis and distinctive features of the gene expression pattern of a given individual's lymphoma appear to be recognizably retained over an interval of years.

Gene expression profiling studies of various malignancies have identified previously unappreciated subtypes within accepted pathological diagnoses and, in some cases, the prognoses of these subtypes varied significantly (10, 15–17). Several models of rituximab resistance are consistent with our findings. If rituximab nonresponders are capable of mounting a cellular immune response to their tumor before therapy, the FL cells may have been selected to evade this immune response and this may translate into an ability to evade any antibody-dependent cell-mediated cytotoxicity (ADCC) response subsequently triggered by rituximab. Alternatively, it has been proposed that FL cells may be able to recruit a cellular microenvironment similar to that in the germinal center, which facilitates the growth of the lymphoma cells (18). Following this line of reasoning, the list of genes with higher expression in rituximab nonresponders may indicate that these tumors may more effectively induce a growth stimulating microenvironment and this more favorable microenvironment may make these cells less sensitive to killing by

Table 4. A subset of genes with significantly higher expression in rituximab nonresponders versus responders are listed by biological function

Cytokine signaling
STAT4
STAT-induced STAT inhibitor 3
Secreted phosphoprotein 1 (osteopontin)
MRC OX-2 antigen
Small inducible cytokine, subfamily B, member 1 (GRO1)
Small inducible cytokine, subfamily A, member 2
Natural killer cell transcript 4
IFN-induced transmembrane protein 1 (LEU13)
IFN-induced transmembrane protein 2
IFN-induced transmembrane protein 3
IL-2 inducible T cell kinase
Protein tyrosine kinase binding protein
Neutrophil cytosolic factor 4
Complement
Complement component 4B
Complement component 1, s subcomponent
Complement component 1, q subcomponent, receptor 1
T cell receptor signaling
T cell receptor β
ζ-chain (TCR) associated protein kinase (70 kDa)
Tumor necrosis factor signaling
Tumor necrosis factor receptor superfamily, member 1B
Tumor necrosis factor, α-induced protein 2
Jun B protooncogene
Tumor necrosis factor (ligand) superfamily, member 10
Growth arrest and DNA damage-inducible gene, β
FOS protooncogene
p75NTR-associated cell death executor

A review of the literature for all 53 named genes in Table 2 identified 25 genes whose products have been demonstrated to function in cytokine, tumor necrosis factor, T cell receptor signaling, or complement function. STAT, signal transducer and activator of transcription.

rituximab through ADCC, complement-mediated cytotoxicity, or induction of apoptosis.

Prognostic tests allowing the prediction of response to specific therapeutic agents would be of great utility, as they would allow physicians to choose therapy to maximize patient benefit while avoiding unnecessary delay, toxicity, and expense. Our findings suggest that it may be possible, at the time of the initial diagnosis of follicular lymphoma, to predict whether patients will later respond to rituximab treatment for relapsed or refractory disease. The data described here suggest that analysis of gene expression patterns in tumors may allow prediction of the sensitivity of tumors to particular antitumor agents and elucidate the biology underlying resistance to a given therapy. In fact, the possibility of dividing FL into molecularly distinct subtypes may confer additional useful information on differences among these tumors, such as response to therapies other than rituximab. Clearly, our findings require validation on a larger, independent patient cohort. A better understanding of the nature of resistance to rituximab treatment may allow more effective use of this powerful agent. For instance, it is possible that patients who are resistant to standard rituximab dosing may benefit from rituximab administered at a different dose or schedule, or may respond to rituximab in combination with other agents. The ability to prospectively identify patients who are relatively resistant to rituximab would facilitate clinical trials to determine the optimal treatment for such patients.

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