Predicting Outcome in Follicular Lymphoma by Using Interactive Gene Pairs

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Abstract Purpose: Follicular lymphoma is a common lymphoma of adults. Although its course is often indolent, a substantial proportion of patients have a poor prognosis, often due to rapid progression or transformation to a more aggressive lymphoma. Currently available clinical prognostic scores, such as the follicular lymphoma international prognostic index, are not able to optimally predict transformation or poor outcome. Experimental Design: Gene expression profiling was done on primary lymphoma biopsy samples. Results: Using a statistically conservative approach, predictive interaction analysis, we have identified pairs of interacting genes that predict poor outcome, measured as death within 5 years of diagnosis. The best gene pair performs 31,000-fold better than any single gene or the follicular lymphoma international prognostic index in our data set. Many gene pairs achieve outcome prediction accuracies exceeding 85% in extensive cross-validation and noise sensitivity computational analyses. Many genes repeatedly appear in top-ranking pairs, suggesting that they reproducibly provide predictive capability. Conclusions: The evidence reported here may provide the basis for an expression-based, multi-gene test for predicting poor follicular lymphoma outcomes.

Follicular lymphoma (FL) is the second most prevalent form of non-Hodgkin’s lymphoma in North America, responsible for 24% to 40% of lymphoma cases (1, 2). FL is considered an indolent lymphoma with most patients enjoying prolonged survival (3, 4). However, some cases pursue a more aggressive clinical course. Therefore, FL serves as a model for the identification of biomarkers that predict clinical course. It is reasonable to expect that such markers may serve to identify subgroups of FL patients who will benefit from therapeutic regimens more closely tailored to the biology and severity of their disease. Current strategies to stratify FL into clinically relevant subtypes, including histologic grading and application of clinical variables such as those used to calculate the follicular lymphoma international prognostic index (FLIPI), offer only modest prognostic capability and clinical utility (5). New prognostic tests based more directly on disease biology are needed.

Earlier studies have identified candidate biomarkers of outcome, including bcl2 (6–13), p53 (6, 14–16), and myc status (17). Considered independently, none of these candidates are markedly superior to the clinical scores already available. More recently, results from global gene expression profiling studies using primary tumor samples have uncovered alterations in specific signal transduction pathways and contributions from nonneoplastic cells in the tumor microenvironment that correlate with clinical variables (18–25). Although these studies have used different measures of clinical outcome or tumor aggressiveness and generated largely nonoverlapping lists of contributing genes, the recurrent involvement of genes from common canonical pathways tends to validate this general approach.

We did gene expression profiling of FL biopsy samples and were able to identify pairs of genes whose expression pattern was predictive of poor prognosis. Using predictive interaction analysis (PIA; ref. 26), we identified several gene pairs whose expression patterns are predictive of death within 5 years of diagnosis with Student’s t test at \( P < 10^{-7} \), and outcome prediction accuracies exceeding 85%, a performance far superior to that achieved by the FLIPI score on our data set.

Materials and Methods

Samples and pathology review. Cases of FL were identified retrospectively by searching the surgical pathology archive of Kingston General Hospital, Ontario, Canada. The primary criteria for inclusion in
the study were (a) the availability of frozen biopsy tissue amenable to the purification of high-quality RNA, and (b) the availability of adequate clinical information, including clinical baseline and outcome data based on follow-up for at least 5 years. Forty-one cases were identified in this manner. A portion of biopsy tissue was snap-frozen in cryovials containing Tissue Tek optimal cutting temperature compound in an isopentane bath shortly after excision and maintained thereafter at -80°C. The routine and immunostained histology slides were retrieved and reviewed by two pathologists (P. Farmer and D. LeBrun) in order to confirm the diagnosis of FL and ensure consistent grading according to WHO criteria. Ethical approval for the use of the archival samples and the associated clinical data was obtained from the local ethics review board.

Clinical details. Clinical charts were available for review from all of the patients. Baseline data collected included age at diagnosis, sex, Eastern Cooperative Oncology Group performance status, stage and grade, presence of bulky disease, presence of more than five involved lymph node areas >3 cm in size, number of extranodal sites involved, bone marrow involvement, lactate dehydrogenase levels, hemoglobin, WBC count, differential and platelet count. The date of diagnosis, treatment details, time to transformation, time to death, and time to last follow-up visit were also noted (Table 1). Prognostic index scores were calculated using FLIPI.

RNA extraction and quality assessment. Total RNA was extracted from each frozen sample using Trizol (Qiagen) according to the manufacturer’s recommendation. Each sample was further purified using a RNEasy column (Qiagen). RNA concentration and A260/A280 ratios were determined using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies), and RNA integrity was measured using a 2100 Bioanalyzer (Agilent). Based on empirical data from our microarray center, only samples with RNA integrity numbers of at least 7 were used for microarray experimentation.

Microarrays. For each sample, 200 ng of total RNA was mixed with 2 μL of a 5,000-fold dilution of Agilent’s One-Color Spike-in RNA control. The mixture was amplified using the low-input RNA amplification kit (Agilent). Following amplification and labeling with Cy3, each sample was assessed on the Nanodrop ND-1000 to measure yield and specific activity. Only samples with yields of >1.5 μg cRNA and specific activities of >9.0 pmol Cy3/μg cRNA were processed further.

Successfully amplified and labeled samples were hybridized in a rotating oven to Agilent 44K Human Whole Genome microarrays according to the manufacturer’s instructions. Slides were scanned with an Agilent scanner and quantitated using Agilent Feature Extraction software, version 8.5.

Data analysis. Features with >10% missing values across all microarray slides were removed from the analysis. All preprocessing and analysis was carried out on the log10-transformed gene expression measurements. Interslide standardization was accomplished using trimmed-mean subtraction across all genes on each slide.

Single genes were analyzed for their ability to predict death within 5 years of diagnosis. Using the best 300 single-gene predictors, we carried out PIA (26) to examine whether any gene pair combinations showed statistically enhanced outcome prediction ability. Briefly, gene pairs were represented by the constructed single variables $v = x - y$ for competitive PIA models, and $u = x + y$ for synergistic PIA models, $x$ and $y$ being the log10 expression levels of each gene of any given pair. The single gene $x$ and $y$, and gene pair $v$ and $u$ variables were analyzed for their abilities to predict death within 5 years from diagnosis using the two-tailed, heteroscedastic Student’s t test. We defined two quantitative criteria for measuring outcome-prediction interaction effects. (a) “Stringent P value” gain was measured for comparison of gene pair performance to the best constituent single gene performance, and (b) “principal P value” gain was measured for comparison of gene pair performance to the null model which assumed that gene pair expression was not correlated within each class (27). Only gene pairs with both stringent and principal P value gains $\geq$10 times that of the best of their respective independent gene models were considered for further prioritization and analysis.

We computationally cross-validated by building a model on a training data subset and establishing the outcome classification accuracies on an independent test set. Cross-validation was carried out using conventional procedures (28) by randomly selecting 75% of the samples for training (12 positive and 19 negative), and 25% of the samples for testing (4 positive and 6 negative). For each gene pair, we made 5,000 distinct selections of training/test data set splits, and determined all four accuracy performance measures.

### Table 1. Baseline patient characteristics

<table>
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<th>Patient characteristics</th>
<th>41-Patient set, n (%)</th>
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<tr>
<td>Male</td>
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<tr>
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<td>Patients still alive</td>
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**NOTE:** Key baseline data collected on all patients and samples in the study are summarized.

*All other variables are baseline clinical characteristics of the patient at diagnosis.
† Two of these patients also received autologous stem cell transplant.
Conventional Kaplan-Meier survival analysis curves were generated post hoc using SPSS for Windows version 14.0 to compare overall survival among the 41 patients. Patient outcome class segregation was based on the best-performing gene pairs. For each selected gene pair, patients were classified by the PIA outcome prediction. The conventional log-rank test was used to assess whether the two groups had significantly different survival curves. Comparisons of survival based on FLIPI prognostic scores were also done. Due to the low number of patients in the FLIPI intermediate risk category (scores 2-3), these patients were pooled with low-risk patients (scores 0-1) for analysis purposes. Following stratification of the patient set by FLIPI scores, overall survival using the best-performing prognostic gene pairs was assessed.

Results

Clinical details. The average age of patients was 53 years (range, 35-87). Patient numbers were divided equally into low and high FLIPI scores. Treatment of patients varied, but in general, most patients (98%) received chemotherapy (median number of treatments was 2). Twenty-seven percent of patients received Rituximab (monoclonal antibody to CD20) either alone or in combination with chemotherapy. Two patients received an autologous stem cell transplant. Approximately half (44%) of the patients also received radiation therapy (2,000 cGy in 5 fractions or 3,600 cGy in 20 fractions), according to local standards of practice. Median follow-up was 6.1 years.

Identification of gene pairs that predict death at 5 years. The identification of new treatments or biomarkers in FL has been complicated by the typically long survival associated with the disease, together with the waxing and remitting nature of the clinical course. These features have made it difficult for the field to agree on clinical end points that reflect underlying disease biology. We chose survival for 5 years after diagnosis as a clear, readily ascertainable end point. Of our 41 samples, 16 fell into the poor outcome group, and the remaining 25 were classified as good outcome.

After filtering missing and flagged values from the expression data sets, 23,216 features remained for analysis across all 41 samples. The top 300 performing single genes were selected by applying a two-tailed heteroscedastic Student’s t test for discriminating death within 5 years of diagnosis. The P values were obtained for each gene, and a false discovery rate (FDR) correction was applied to control for multiple comparisons. The top 20 ranking up- and down-regulated genes according to t test P values are shown in Figure 1. For comparison, we also report outcome prediction accuracies, as represented by sensitivity, positive predictive value, specificity, and negative predictive value. Average and SDs of the performance values of these 40 selected genes (bottom). Red, positive deviation from the mean (≥1 SD, light red; ≥2 SD, dark red); blue, negative deviations from the mean (<−1 SD, light blue; <−2 SD, dark blue).
associated with the predictive capabilities of these genes ranged from 0.013 to 9.3 \times 10^{-6} (Appendix 1; see Supplemental Data for the full list). These 300 genes served as the input for PIA. The top up-regulated and down-regulated gene expression profiles are shown in Fig. 1.

Notably, seven genes in this list were represented by more than one feature on the array, and were selected independently. The genes ST3GAL6 and PL2AGA are represented by a single probe sequence each, and are each spotted in 10 different locations over the chip. Eight of the 10 probe sequences were identified as having similar predictive value in our data set. Several other genes represented by either two (BMM, CASP4, DNGA) or three (TFF3, SRRM1) different probe sequences were identified as having similar predictive value.

We carried out PIA to examine whether any of the 44,850 gene pairs generated from the 300 single genes were able to discriminate the 5-year outcomes more reliably than either single gene of the pair (Appendix 2; see Supplemental Data for complete results). Only gene pairs with both stringent and principal P value gains of $\geq 10$ times that of their respective single genes models were considered for further analysis. Of the 303 gene pairs that passed both criteria, we observed 15 repeated gene pairs due to redundant features or genes represented by multiple probes on the array, as described above (Fig. 2A). P values and predictive accuracies were, respectively, geometrically and arithmetically averaged for these redundant gene pairs.

Overall, 271 nonredundant gene pairs (comprising 178 constituent single genes) remained, with P value gains ranging from $10^4$ to $10^8$ for discriminating 5-year outcomes. The top-performing gene pairs according to class discrimination t test performance are shown in Fig. 2B. The best-performing gene pair models show 1,000-fold smaller P values than the best single genes shown in Fig. 1. For both single and paired gene models, sensitivity and negative predictive value tended to be better than specificity and positive predictive value.

Plots for the best-performing competitive gene pair in terms of 5-year outcome prediction performance are presented in one- and two-dimensional visualizations in Fig. 3. The one-dimensional graphs in Fig. 3A display the mean and SDs for each class. The full two-dimensional display of the measurement data in Fig. 3B illustrates how much better the PIA separatrix (solid diagonal line) performs in separating the classes, compared with the single gene separatrices (horizontal and vertical broken lines). Similar interaction strengths were observed with the best synergistic gene pairs (data not shown).

**Prioritization of genes according to accuracy and cross-validation performance.** The PIA-derived gene pairs capture reproducible and statistically supported competitive and synergistic interactions between genes, providing t test P values for each predictive pair. More detailed results for the selected 303 gene pairs are reported in Appendix 3, including the 5,000 data splits cross-validation averages, SDs, and coefficients of variation for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the top 40 gene pairs.

**Kaplan-Meier survival analysis.** Following identification of the best-performing gene pairs (LOXL3 + NTS, RIPK5 + NOTCH2), we generated conventional Kaplan-Meier survival analysis curves to compare overall survival with segregation based on the combinatorial predictor. As shown in Fig. 4, representative gene pairs were able to divide patients into prognostic groups. As expected, the FLIPI high-risk group had significantly different overall survival than the combined intermediate/low-risk group (Fig. 5A). As shown in Fig. 5B and C, when patients were stratified on FLIPI risk category, followed by classification based on LOXL3 + NTS gene pair expression, patients in each FLIPI group were further divided into significantly different survival curve outcome groups. Ten patients were reclassified when evaluating with gene pairs compared with FLIPI. Six patients who were low-risk FLIPI were reclassified as high-risk with gene pairs and four patients who had a high-risk FLIPI were reclassified as low-risk according to gene pairs.

**Discussion**

Previous studies have identified common themes with respect to the genes whose expression levels differentiate outcomes in FL, including apoptosis-related genes, cell cycle genes, T-cell markers, and genes from signaling pathways that involve c-myc. Although it would seem that direct comparisons between expression profiling studies should begin to identify common expression patterns, such comparisons across different studies are impeded by platform-specific effects, differences in sample collection strategies and clinical end point selection, and limitations of statistical power in identifying individual genes. For example, we are not aware of other profiling studies on FL that used death within 5 years of diagnosis as the clinical end point. Therefore, the modest overlap between the genes that we identify here and those identified in other expression profiling studies of FL is perhaps not surprising. Although it will be important to validate our best-performing genes in an independent set of samples, the interarray consistency of gene expression measurements, as evidenced by the behavior of several multiply spotted genes, and the fact that the best-performing genes from our data set identified pathways that have been implicated in other FL studies is reassuring.

Our development of predictive biomarkers for poor outcome in FL differs from other approaches in several important respects. First, we made use of a one-color, long oligonucleotide microarray platform. The advent of long oligonucleotide arrays with sufficient internal controls to support one-color assays removes the confounding issue of a second dye. Unlike many microarray-based experimental designs, our analysis did not depend on clustering algorithms, and therefore, did not generate large agglomerated sets of genes as classification signatures. Rather, we took a direct approach of searching for specific gene pair combinations with predictive capabilities that exceeded what the best constituent single genes achieved. This method of analysis does not preclude the examination of our data using more conventional approaches to identify larger patterns of differentially expressed genes that correlate with outcome. The analysis of these larger profiles, and their relation to the smaller gene pair predictors identified here, are currently under way.

Our analysis approach was based on the expectation that whereas FL outcomes are likely too complex to be predicted by just one gene, a good predictive model could be built with a minimum number of genes using statistically conservative methods. The implementation of PIA limits gene interactions to...
pairs that are phenomenologically competitive or synergistic. These can be encoded simply as ratios or products, respectively, of the constituent single gene expression variables, generating a single variable representing each gene pair. The variables could be subjected to statistical tests to identify those that best distinguish outcome. The best gene pairs outperformed the best single genes in class-discriminating \( P \) values by \( \frac{1}{3} \) orders of magnitude (Figs. 1 and 2). PIA has been applied previously on human blood gene expression profiling data in developing predictive models of biological outcomes for the identification of marrow transplant donors whose recipients will be likely to experience graft versus host disease (26). In that study, the superior performance of gene pairs versus single genes was found with respect to strong clinical discrimination \( P \) values and robust accuracy, and was also shown through extensive computational cross-validation.

In this study, we determined the robust average accuracy of >5,000 independently generated training/test data set splits for the top gene pairs (28, 29) to be within \( \pm 2\% \) of the full data set accuracy. As cross-validation does not directly address the real clinical practice issue of biological noise, we artificially modified measured data sets with increasing levels of randomly added noise, and then determined the average cross-validation classification accuracies. Accuracies were largely robust if the added noise level was within 1 to 2 SDs of the measurement mean (data not shown).

Fig. 2. Color matrix plots illustrating gene pair performances. A, intensity profiles of replicated gene pairs found independently as combinations of microarray features with strong outcome discriminating \( P \) values.
Our top gene pair predictors were able to provide significant prognostic information, not only on a 5-year death binary outcome variable, but also in the classical continuous overall survival outcome variable as seen in the Kaplan-Meier plots. This type of analysis confirms that although a few cases are misclassified into 5-year death outcome classes based on their combinatorial gene expression values, this misclassification does not disrupt the prognostic power of the gene pair in a more familiar post hoc analysis of survival outcome. The possibility of using gene pair predictors in a clinical setting is strengthened by the fact that the prognostic information provided by these predictors is supplementary to the information gained by FLIPI.

Even with the novel experimental and analysis approaches, we found that genes selected by our methods were in keeping with the findings from previous studies. Many of the genes could be grouped into single pathways, including those related to apoptosis (NOTCH2, TFF3, CSF1, BIRC4, SRF), chemokine signaling (LIMK1, ROCK2, CCL13), cell growth and proliferation (BIRC4, BMX, CSF1, DGKA, TNFRSF6B, LMK1, SRF), and hematologic function (CCL13, CD47, CSF1, TNFRSF6B, GREM1, PLA2G4A, NOTCH2, CATA3, MMRN1, TSPAN8).

The role of apoptosis in the development of FL is well documented, with multiple studies suggesting that down-regulation of proapoptotic and up-regulation of antiapoptotic genes are associated with poor outcome in FL (2, 17, 18, 20, 25). In our data, several proapoptotic genes are among the most consistently down-regulated genes, including BCLAF1 (30), BIRC4 (also XIAP; ref. 31), and RIPK5 (32), whereas the up-regulated genes include antiapoptotic genes NOTCH2 (33, 34), TFF3 (35, 36), CSF1 (37, 38), and BMX (39, 40).

Some of our candidate genes have been implicated specifically in the development of human cancers, including lymphoma. For instance, overexpression of NOTCH2 has been noted in a number of human lymphoma cell lines (41, 42), and seems to be involved in increased cell survival and proliferation (34). In mouse, NOTCH2 activity has been shown to be required for proper B cell development, suggesting a role in cellular proliferation and differentiation (43, 44), TFF3 overexpression.
has been implicated in increasing invasiveness and decreasing apoptosis in a number of human cell lines (45, 46). In addition, the expression of PLA2G3, part of the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway, has been shown to reliably distinguish diffuse large B cell lymphoma (DLBCL) from FL cases (20), and seems to have a role in stimulating both tumor cell growth (47) and angiogenesis (48), and protecting against apoptosis (49).

Fig. 3. Detailed PIA model performance characterization for the top-performing competitive gene pair of NOTCH2 + RIPK5. A, measurement values, means, and SD bars for each of the four PIA model variables, i.e., single genes x, y and gene pairs u, v. Gene x corresponds to NOTCH2 (single gene, \( P = 10^{-2.7} \)) and gene y corresponds to RIPK5 (single gene, \( P = 10^{-3.6} \)). B, detailed two-dimensional scatterplots of PIA models for the same top-performing competitive gene pair. The two-dimensional visualization illustrates the diagonal position of the PIA separatrix with slope +1 for the competitive model, in comparison with the one-dimensional model separatrices (vertical and horizontal broken lines).
patterns linked to outcome. In that study, an immune response 1 signature was associated with good outcome, whereas an immune response 2 signature was associated with poor outcome. It was hypothesized that the expression patterns in

Fig. 4. Overall survival of patients grouped by combinatorial gene pair classification. Kaplan-Meier analysis of overall survival for 41 patients grouped according to outcome classification based on LOXL3 and NTS gene pair (A) and NOTCH2 and RIPK5 (B). Log-rank statistical tests were used to assess the difference between the two survival curves.

Fig. 5. Overall survival of patients grouped according to FLIPI risk category. Kaplan-Meier analysis of overall survival, comparing pooled low and intermediate FLIPI risk versus high FLIPI risk (n = 41, A), and comparing LOXL3 and NTS PIA-based gene pair outcome classification in combined FLIPI low-risk and intermediate-risk patients (n = 18, B), and FLIPI high-risk patients (C). Log-rank statistical tests were used to assess the differences between the two survival curves.
the good outcome signature were derived from T cells and monocytes, whereas that in the poor outcome signature was derived from monocytes and dendritic cells (21). Because we also did not microdissect our samples, it could be expected that the same phenomenon would apply to our samples. It is, therefore, of interest to note that TNFRSF6B, one of the family of TNFRSF genes that were overexpressed in the good outcome group in the previous study, is overexpressed in our good outcome group as well. However, it was not possible to determine with certainty the cell type that was driving the expression of key genes identified in this study as we did not fractionate the samples according to cell type.

Overall, our findings complement the existing literature on the classification of FLs into outcome groups. Our study, however, has provided some evidence for the possibility of using a small number of genes in statistically conservative gene pair models to achieve a high predictive accuracy when using death within a short period of time as an end point. Further refinement of this approach, using additional samples and a larger clinical data set, will permit the generation of a usable prognostic tool that can be applied in clinical settings to assist clinicians in the development of individualized patient care.

Appendix 1

Performance summary of top 300 single genes discriminating death after 5-year outcome.

Appendix 2

Performance summary of 303 of the best selected PIA gene pairs discriminating death after 5-year outcome.

Appendix 3

Performance summary of 5,000-fold cross-validation results from 303 of the best PIA gene pairs for accuracies of 5-year outcome discrimination.

References

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