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Gene expression signatures in follicular lymphoma: are they ready for the clinic?

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The lymphoma field has fully embraced the molecular era for several years, the result of a number of studies utilizing genome-wide analysis platforms including gene expression profiling, array comparative genomic hybridization (aCGH), single nucleotide polymorphisms (SNP) arrays and several more novel technologies that provide information about methylation status and the epigenome.[1-4] As these new technologies became available, lymphoid cancers were a relatively early priority, in part because of the ease of sample acquisition and the availability of retrospective fresh-frozen biopsy material.

Heterogeneity of follicular lymphoma

Follicular lymphoma (FL) is the most common indolent non-Hodgkin's lymphoma (NHL). It typically presents as advanced-stage disease and in this setting is considered imminently treatable, but not curable with standard chemotherapy or immuno-chemotherapy regimens used today. There is growing evidence that the current era of therapy has produced improvements in overall survival.[5] Survival following diagnosis is markedly heterogeneous, with median survivals in the order of 8-10 years. Currently we have no reliable means to distinguish those patients who will die within three years of diagnosis from those who will be alive with little morbidity and requiring little if any therapy 25 years after diagnosis. These strikingly disparate outcomes in FL underlie the fury of activity surrounding the discovery of prognostic and predictive markers. The identification of molecular biomarkers that would help us better predict clinical outcome and risk of transformation would be a welcome addition, if indeed they could improve the prediction of prognosis beyond our current multi-parameter clinical indices.[6] An ideal biomarker would not only identify patients with inferior

survival or increased risk for transformation to a more aggressive lymphoma, but would also be relevant to the biology of the disease and provide a potential therapeutic target. For example, the discovery of Her2-Neu in breast cancer fulfills all of these criteria.

Predicting prognosis in follicular lymphoma

Clinical prognostic models are of value for estimating risk and comparing patient characteristics between clinical trials, but are notoriously unreliable for individual patients. For example, the Follicular Lymphoma International Prognostic Index (FLIPI), comprising five clinical variables including age, stage, hemoglobin, serum LDH and number of nodal sites is a useful clinical adjunct, but is of minimal value for individualizing therapy for patients with FL.[6] Histological transformation represents a real clinical concern for physicians treating FL patients, as it is a dominant clinical event in the course of FL. Survival following transformation is significantly inferior in comparison to those patients who do not transform.[7] Recent estimates suggest that transformation occurs at a steady rate of 3% per year following diagnosis, but this may be affected by the treatment regimen used. Clinical parameters such as the FLIPI indices may also be predictive of transformation, but few if any biological factors have been shown to reproducibly predict risk of transformation. The identification of robust and reproducible biomarkers capable of predicting overall survival and risk of transformation in FL would be of great value for individualizing therapy and by definition would provide an initial list of potential candidate genes for the development of targeted therapies.

The need for robust biomarkers in follicular lymphoma

Currently there is a paucity of clinically useful biomarkers in FL. This has created a wide disparity between the numbers of publications that claim to identify prognostic biomarkers and their relative impact on clinical practice. Like so many other areas of medicine, even the most promising of candidates fail to be validated when analyzed in a completely unrelated patient cohort. Most simply do not stand up under statistical scrutiny. This is in part due to various obstacles; most notably the lack of rational study design and hypothesis-driven research used to survey candidate biomarkers in the first place (see Table 1). The net result of all of this scholarly activity is a state of perpetual confusion for our clinical colleagues. This wide gap between credible biomarkers and the large number of published candidates could be significantly lessened by the development of a largely unmet research need. Large-scale tissue microarrays (TMAs) constructed using well defined clinical cohorts of FL patients treated with uniform therapy from multiple institutions would provide an invaluable platform on which potentially useful biomarkers could be surveyed. This approach, together with a logical list of candidate biomarkers, would likely be sufficiently powered to draw meaningful conclusions and allow the development of a combined clinical-biological prognostic model that could then be used to assign risk in individual patients with FL.

Table 1. Obstacles to finding novel prognostic biomarkers in follicular lymphoma.

Sample acquisition	- Ascertainment bias - Small sample sizes - Single institution studies - Short follow-up times - Heterogeneity in treatments - Inclusion of post treatment samples - Lack of validation cohorts - Lack of germline DNA to differentiate germline *vs.* somatic mutations/copy number changes
Technical	- Differences in sample type: e.g. fresh frozen *vs.* formalin fixed paraffin embedded tissue - Variation in tumor content: purified FL cells *vs.* whole biopsy samples - Differences in techniques (array CGH *vs.* GEP *vs.* TMA) - Differences in array platforms (custom *vs.* commercial) - Differences in sensitivity, thresholds, normalization methods, bioinformatic analysis tools - High false-positive rate due to multiple testing

Pathological features of follicular lymphoma

Nodal FL is currently separated into grades based on the number of large transformed B cells (centroblasts) localized within the neoplastic follicles. Currently we recognize three grades of FL; grade 1, grade 2 and grade 3, with grade 3 divided into 3a and 3b.[8] The latter has a truly follicular architecture, but the follicles contain almost exclusively large centroblasts. Grade 3a FL shows an admixture of centroblasts and small centrocytes, with ≥15 centroblasts per high-power field within the neoplastic follicles.

Studies that have examined the entire morphological spectrum of FL have recognized a number of general findings. Firstly, the proliferation rate typically increases as you increase in grade. Although the relationship between grade and proliferative activity is imperfect, the two features are clearly correlated. Exceptions do occur and these might have clinical consequences.[9] The total number of secondary cytogenetics alterations increases with grade, as does the frequency of polyploidy.[10,11] The presence of diffuse areas tend to increase with grade, and are particularly common in grade 3b FL. A careful examination of most of these features suggests that FL represents a continuum, making reproducible distinctions very difficult.

Any diffuse component of large B cells merits a separate diagnosis of diffuse large B cell lymphoma (DLBCL) and should drive the choice of initial therapy. Follicular dendritic cells (FDCs) comprise the scaffolding of both benign and malignant follicles and additionally function to present antigen to B and T cells within lymphoid follicles. These cells express a number of unique antigens and can easily be recognized in paraffin sections with immunohistochemical (IHC) studies that target CD21, CD23 or CD35. Use of these reagents is of paramount importance to distinguish true follicle formation from *pseudofollicles* e.g. nodular areas that may mimic follicles.

Follicular lymphoma: grade 3a versus 3b

Despite the evidence above that supports a continuum, a number of publications suggest that biological features distinguish grade 3a from 3b FL.[12] An overview of these features is shown in Table 2. The implication from published data is that grade 3a FL is more aligned with grades 1 and 2, while 3b is the *follicular variant* of DLBCL. One conclusion from these studies would be that FL should be divided into two major categories; grades 1 through 3a FL (a biological continuum) versus grade 3b FL. However, clinical studies have not substantiated this claim.[13, 14] Areas with a diffuse growth pattern have inferior survival, but in purely follicular cases, 3a versus 3b FL distinctions have not proven to be of clinical value.[14] Several studies that have assessed the clinical impact of growth pattern in grade 3 FL have not used IHC for FDCs, questioning the accuracy of these distinctions.[12, 14] Moreover, reproducible grading of FL is notoriously unreliable. Grade 3a FL typically shares the characteristic immunophenotype of low-grade FL with expression of CD10, while many grade 3b FL cases lack expression of CD10 and may indeed express MUM1/IRF-4, characteristic of cells undergoing plasmacytic differentiation similar to the Activated B Cell (ABC) type of DLBCL. Several recent publications suggest that grade 3 FL lacking a t(14;18) often harbor 3q27/*BCL6* translocations and share the same immunophenotype (e.g. $CD10^-/MUM1^+$).[15] At the cytogenetic level, grade 3a FL usually shows a t(14;18) and additional secondary changes indicative of clonal evolution, while grade 3b has a significant association with *BCL6* translocations involving chromosome 3q27. Moreover, both *BCL2* and *BCL6* translocations can occur in the same cells of grade 1 and 2 FL, but are mutually exclusive in grade 3b FL, a finding largely similar to *de novo* DLBCL (unpublished observations, 2008).[16] Thus, from a number of perspectives, grade 3b FL mimics the molecular and phenotypic characteristics of *de novo* DLBCL. The clinical expectations from these data might be that grade 3b FL would behave more aggressively, respond well to adriamycin-containing regimens and their use would produce a typical plateau on the disease-specific or failure-free survival curve for at least some of the patients, similar to DLBCL. However, published data to support this hypothesis are simply lacking. It is a generally held belief that all grade 3 FL cases should be distinguished from the largely overlapping grade 1 and 2 FL subset. Indeed, this is the obvious implication from the World Health Organization publication based on the survival curves in the chapter on FL (ref. #8, page 166). Most studies of purely follicular grade 3 FL indicate that 80% or more of these cases are grade 3a. Indeed, a purely follicular variant of grade 3b FL is quite a rare lymphoid tumor. The overall and failure-free survival curves from the WHO based on the use of adriamycin-containing regimens imply that grade 3 FL patients benefit from more intensive chemotherapy. Of note, no obvious plateau is apparent from these curves. These data are derived from the large international lymphoma classification project designed to validate the REAL classification of NHLs and were never published as a peer-reviewed manuscript. Based on the likely frequency of grade 3a FL amongst all follicular large cell lymphomas, it would seem logical to split FL into two major categories, grade 1 and 2 representing one group and grade 3a/3b FL the other. Based on gene expression profiling of a small number of purely follicular grade 3b FL cases in this issue of the journal, Piccaluga and colleagues reached similar conclusions.[17] However, these data should be viewed with caution, as the number of cases analyzed was very small. Nonetheless, despite mounting biological data to the contrary, it would seem prudent to distinguish two major categories of FL for clinical purposes (grade 1 and 2 FL *vs.* grade 3).

Gene expression profiling in follicular lymphoma

Genome-wide transcriptome profiling of FL represents a powerful discovery tool for biomarker development. A number of studies have been published including both confirmatory and discordant data, not a surprise given the discussion above regarding reliable biomarkers and the vagaries of nucleic acid hybridization that is microarray technology.[1, 2] Moreover, a number of variables conspire to make interpretation of these studies difficult. The vast majority of published studies have used diagnostic biopsy samples from patients for which widely disparate treatment strategies were used, and thus fail to control for an important variable. In any given FL biopsy sample, the tumor cell content can vary

Table 2. Features distinguishing grade 3a versus 3b follicular lymphoma.

Feature	*Grade 3a*	*Grade 3b*
Morphology		
Centroblasts	Present, more than 15 per HPF, but admixed centrocytes	Almost exclusively large centroblasts
Diffuse areas	Uncommon	Frequent
Demarcation of follicles	Usually sharp	Poorly defined, may merge with areas of DLBCL
Bone marrow	Commonly involved, typically paratrabecular small centrocytes	Infrequent involvement. If positive, typically concordant large centroblasts
Immunophenotype	Typically $CD10^+$, $Bcl\text{-}6^+$, $Bcl\text{-}2^+$, $MUM1^-$, p53 usually negative and lack cytoplasmic immunoglobulin	Often $CD10^-$, $Bcl\text{-}6^+$, $Bcl\text{-}2^{+/-}$, $MUM1^{+/-}$, p53 expressed in 1/3 cases and cytoplasmic immunoglobulin often present
Cytogenetics	t(14;18) common, clonal evolution with numerous secondary changes	t(14;18) uncommon, *BCL6* translocations present, mutually exclusive of t(14;18)
Mean number of karyotypic alterations	6.5	8.9

enormously, as these biopsies contain large numbers of T cells, macrophages, FDCs and stromal cells. Very few of the studies have utilized the same commercial array platform; many being performed on custom microarrays. The comparability between probe sets and the unique genes they define are very problematic issues, making comparisons between studies difficult, if not impossible. Moreover, a plethora of bioinformatics strategies are available as data analysis tools that may indeed render completely different results. Rather dogmatic conclusions are often reached based on a small number of biological replicates and proper statistical design that includes both training and validation sets is not done. These experiments typically yield very large data output files and thus issues related to false discovery begin to emerge that can blur meaningful interpretation of these data.

A gene signature is best defined as a group of coordinately expressed genes that define either a cell type or a cellular function. For example, a T cell signature is a group of genes that define cells as T cells. The list could include a number of genes that code for pan-T cell surface antigens (*CD2*, *CD3*, *CD4*, *CD7*, etc), genes that code for components of the T cell antigen receptor complex (TCR α, TCR β, etc) and a number of signaling molecules that function downstream of that complex (e.g. *LAT*, *ZAP-70*, etc). Similarly, cellular proliferation can be defined by a group of genes that are coordinately expressed when benign or malignant cells are actively cycling.[18] The list could include genes such as *RAN*, *CDC2*, *DNA Pol E2*, *Topo II*, *PCNA* and *Ki-67* to name a few. Additionally, there are groups of genes that are expressed differently based on their cell of origin and reflect the activity of the B cell in that stage of development.[19] For instance, naïve B cells, B cells undergoing somatic hypermutation (SHM), memory B cells and plasma cells have very different functions and characteristic expression profiles. Thus, the lymphomas emanating from these cells would reflect in part these gene expression profiles such that CLL, FL, MCL and myeloma would cluster as distinct disease entities. Similarly, the proliferation signature in MCL may be different than a proliferation signature in DLBCL. Finally, a gene signature can define a specific biological entity within a lymphoma subtype. For example, cyclin D1-negative MCL was defined on the basis of identifying cases with characteristic gene expression for MCL that lacked expression of cyclin D1 and did not have a t(11;14)(q13;q32).[20]

Lessons learned from gene expression profiling?

So what have we learned from gene expression profiling of FL and can we short-list a number of candidate biomarkers that could be used now to improve the prediction of survival and risk of histological transformation? A number of recent studies have been published that do shed some light on the biology of FL. In a large profiling study by Dave et al., expression profiles that implicated a dominant role for non-neoplastic cells as major contributors to FL survival were discovered.[1] These were referred to as immune response-1 and immune response-2, implicating T cells and a macrophage/monocyte signature, respectively. A prognostic role for cells in the microenvironment was not totally unexpected in FL, but somewhat surprising was the finding that gene expression at the time of diagnosis could so dramatically predict overall survival. Traditional thinking suggested that survival in FL was much more influenced by stochastic events occurring in these tumor genomes over time, many of which would not have been evident at the time of diagnosis. Glas and colleagues performed a slightly different study taking advantage of FL samples with sequential biopsies and built an 81-gene molecular predictor for determin-

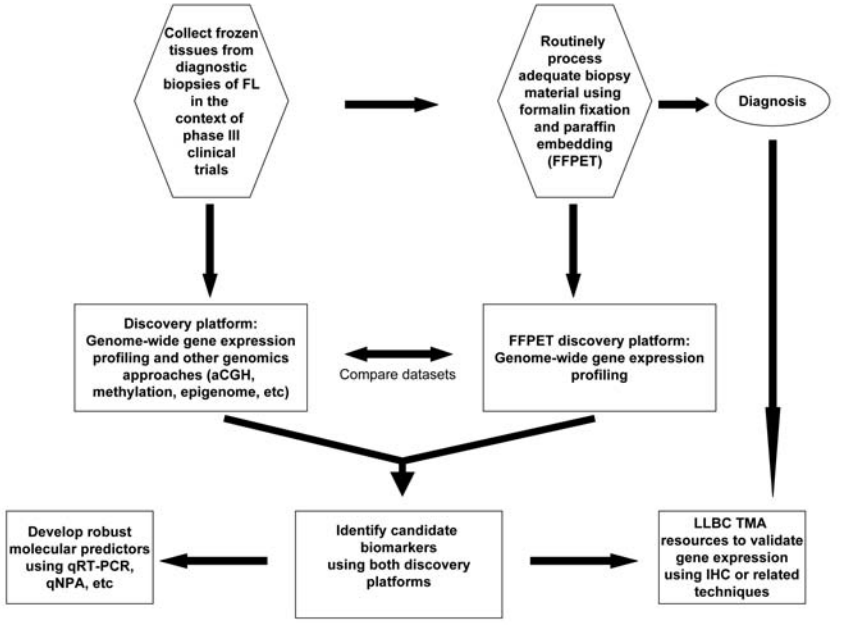

Figure 1. Proposed algorithm for incorporating gene expression profiling into phase III clinical trial design. Mandatory collection of both fresh and fixed tissues would be part of the clinical trial design. Genome-wide expression profiling arrays would be used for novel gene discovery and would be performed in parallel using the fresh-frozen biopsy sample and formalin-fixed paraffin embedded tissues (FFPET). The latter would determine the feasibility of these assays using routinely available archived material. If successful, large numbers of completed clinical trials could be retrospectively analyzed using this routine source of diagnostic biopsy material. The candidate biomarkers could then be incorporated into molecular predictors, with models built and tested using strategies such as quantitative reverse transcriptase PCR (qRT-PCR), quantitative nuclease protection assays (qNPA) or others. Lastly, protein expression could be determined for candidate biomarkers with subsequent validation of their clinical impact using large scale tissue microarray (TMA) resources such as those available from the Lunenburg Lymphoma Biomarker Consortium (LLBC). This strategy would also allow a determination of the *in situ* pattern of gene expression.

ing the immediate post-biopsy clinical behavior.[2] Indeed, a finding featured in this work was the inability to predict either long-term survival or risk of transformation, in contrast to the earlier work by Dave and colleagues. These two somewhat discordant publications have never been adequately reconciled, adding further to the confusion concerning clinically relevant biomarkers in FL. A number of subsequent publications have used different technologies (gene expression profiling, IHC, flow cytometry) to determine prognostically important biomarkers in FL, but the results remain inconclusive, in part due to discrepant results.[21-23] These strategies do not measure the same features, make assumptions that gene and protein expression are perfectly matched, while IHC studies often fail to report on immunoarchitectural features (the distribution of cells in relation to the malignant follicles) that might be more important than the total numbers of cells. Flow cytometry techniques offer the possibility of more objectively enumerating many thousands of cells, but interpretation of these data is very much operator-dependent and similarly does not address aspects of immunoarchitecture.

Treatment is a prognostic variable

Treatment has largely not been considered an important prognostic variable in FL. The overwhelming percentage of biomarker studies in FL include patients treated with markedly different regimens. The recent addition of rituximab to chemotherapy suggests a survival advantage for the first time in several decades for patients with FL.[24] Thus, all biomarker studies will need to be re-visited in the current era of therapy. Evidence in support of this hypothesis is already emerging.[25,26]

Clinical translation of new knowledge in follicular lymphoma

So, are we ready to introduce a combined clinical-biological index for risk-stratification of FL patients and is there a robust and reproducible biomarker assay available at this time to facilitate clinical translation of all we have learned from microarray and related experiments in FL? The obvious answer is no. Is there a way forward that may allow us to reach this laudable goal in the near future? The obvious answer, at least to this reviewer, is yes. The algorithm detailed in Figure 1 suggests a possible scenario for success. Genome-wide microarray studies offer a powerful discovery tool and should,whenever possible, be built into the design of all or most phase III clinical trials in FL. The design of these studies must mandate that adequate biopsies are performed to allow such analyses to be performed. Similar approaches should be considered for those population-based registries where uniform therapy is used and the practice of sequential biopsy is considered optimal medical practice. These approaches offer the possibility of unraveling the molecular mystery of histological transformation, a frequently dominant clinical event in the life of FL patients. Making these correlative science studies mandatory for phase III trial design will significantly increase the likelihood of discovering new targets for future therapies. Parallel efforts to adapt microarray technology to allow the analysis of formalin-fixed paraffin embedded tissues (FFPET) will be equally important, as fresh/frozen tissue is simply not available for the majority of patients with FL. Importantly, although genome-wide microarray strategies may be critical as a discovery platform, the clinical translation of this new knowledge may take an entirely different form. For example, more robust and reproducible assay techniques such as reverse transcriptase PCR (RT-PCR), quantitative nuclease protection assays or possibly IHC may be more applicable to the routine laboratory setting.[27, 28] Such approaches have recently been shown to predict survival in FL. Lastly, most biomarkers are developed in the setting of single institution studies, often not properly powered to adequately address the question, frequently in a situation of variable treatment with patient selection and referral bias; the net result is that most biomarkers cannot be validated in an independent cohort. This all too common scenario creates an atmosphere of confusion and more importantly does not benefit patients. Consortia such as the Lunenburg Lymphoma Biomarker Consortium (LLBC) will be critical to overcoming this obstacle, by analyzing important candidate biomarkers using large-scale TMA resources including patients pooled from several phase III clinical trials from both North America and Europe.[29] The increase in sample size offered by this approach will allow statistically robust biomarkers to be determined and, together with technical advancements, will likely provide the necessary ingredients for success. With time, the long anticipated combined clinical-biological index will be achieved that may indeed allow for more targeted therapies and provide the patient with FL and the treating clinician with a much improved expectation of survival.

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