IDENTIFYING A PROGNOSTIC TEST IN FOLLICULAR LYMPHOMA
USING A TISSUE MICROARRAY AND IMMUNOHISTOCHEMISTRY

By

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Abstract

Follicular lymphoma (FL) is an attractive model for discovering biomarkers and elucidating mechanisms of tumour progression. We hypothesized that alterations in the expression of proteins with known roles in cancer biology and hematological cells might correlate with clinical outcome and thereby shed light on biological mechanisms.

Sections from a tissue microarray (TMA) containing FL samples from 67 patients were immunostained for candidate biomarkers, including p53, p16\textsuperscript{INK4a}, Bcl-2, Bcl-6, MUM1, PML, phospho-ERK, and p27\textsuperscript{Kip1}. The Kaplan-Meier method and log-rank test were used to identify markers that correlate significantly (p<0.05) with overall survival (OS). The $\chi^2$ or Fisher exact test were used to examine associations between histological markers and baseline clinical features, including the Follicular Lymphoma International Prognostic Index (FLIPI) score. Expression of p16\textsuperscript{INK4a} or p53, or absent CD10 expression correlated with poor survival. Patients with p16\textsuperscript{INK4a}-negative tumours had a median OS of 13.4 years compared to 8.3 years for those with p16\textsuperscript{INK4a}-positive tumours (p=0.006). Expression of p16\textsuperscript{INK4a} was significantly associated with low hemoglobin, elevated serum lactate dehydrogenase (LDH), high histological grade, high cell proliferation index, presence of associated diffuse large B-cell lymphoma (DLBCL) and high-risk FLIPI classification. Our observation of a positive association between p16\textsuperscript{INK4a} expression and indicators of tumour aggressiveness is novel and perhaps
surprising since loss of the \textit{INK4a} tumour suppressor gene is one of the most frequently observed lesions in human cancers, including lymphoma. Expression of p16\textsuperscript{INK4a} may be part of a cellular response to unidentified pro-mitotic mutations, such as deleterious mutations of the \textit{RB} tumour suppressor gene, associated with more aggressive instances of FL. Immunostaining FL diagnostic biopsies for expression of p16\textsuperscript{INK4a} may serve as an informative prognostic biomarker to aid clinicians managing FL patients.
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List of Abbreviations

°C degrees Celsius
µm micrometre
ABC activated B-cell-like
array-CGH array comparative genomic hybridization
BSA bovine serum albumin
CDK-4/-6/-2 cyclin-dependent kinase-4/-6/-2
cGy centigray
dH$_2$O distilled water
DLBCL diffuse large B-cell lymphoma
ECOG European cooperative oncology group
EDTA ethylene diamine tetraacetic acid
FDC follicular dendritic cell
FL follicular lymphoma
FL1 follicular lymphoma, grade 1
FL2 follicular lymphoma, grade 2
FL3 follicular lymphoma, grade 3
FLIPI follicular lymphoma international prognostic index
GC germinal centre
GCB germinal centre B-cell-like
HPS hematoxylin, phyloxin and saffron
HRP horseradish peroxidase
IHC immunohistochemistry
IPI international prognostic index
IR-1 immune response-1
IR-2 immune response-2
LAM lymphoma associated macrophage
LDH lactate dehydrogenase
min minute
mm millimetre
NHL non-Hodgkin’s lymphoma
NR not reported
NS not significant
PI3K phosphatidylinositol 3-kinase
PIP$_2$ phosphatidylinositol (4,5) bisphosphate
PIP$_3$ phosphatidylinositol (3,4,5) triphosphate
RT room temperature
TBS tris-buffered saline
TMA tissue microarray
WHO world health organization
Chapter 1: Introduction

Follicular lymphoma (FL) is one of the most common lymphomas in the Western world [1]. Primarily a disease of older people, FL typically pursues a waxing and waning clinical course [2]. Although remissions are readily achieved with a variety of treatments, the lymphoma invariably recurs and patients eventually die of their disease [3, 4]. A subset of FL patients experiences a particularly aggressive disease which is often associated with transformation to diffuse large B-cell lymphoma (DLBCL). As a result there is a perceived need to be able to identify at diagnosis those patients who would most likely benefit from aggressive therapies and those who might be spared the noxious side-effects of such treatments and could be followed with close observation.

An immunohistochemistry-based test applied to diagnostic biopsy samples which could differentiate these patient groups would be a fast, easy and inexpensive method which could greatly benefit management of FL patients. Identification of such markers of outcome could also lead to further study of their biological role in lymphomagenesis and tumour progression. This, in turn, could lead to the development of novel therapeutics for FL patients.
Follicular Lymphoma

Non-Hodgkin’s lymphoma (NHL), encompassing numerous subtypes of lymphoid neoplasms, is the fifth most common cancer type for both Canadian males and females, with an estimated 7,000 new cases to be diagnosed in 2008 [5]. Between 1978 and the late 1990s, the age-standardized incidence rates of non-Hodgkin’s lymphomas increased by about 50% indicating an important need for effective studies which examine the risk factors for this disease and developing new treatment and management strategies for these patients. Follicular lymphoma, accounting for 22% of NHLs is second in incidence only to the more aggressive, but related tumour, diffuse large B-cell lymphoma [6]. Together these two diseases account for 53% of total NHL cases, further emphasizing the fact that improvements in prognosis and treatment for patients with FL could have a profound effect on a large number of individuals.

Follicular lymphoma typically presents in middle-aged to elderly individuals as lymph node enlargement, occurring as a result of an accumulation of neoplastic B-cells in a follicular growth pattern [2]. The diagnosis is made by a pathologist based on histological examination of a biopsy, often supplemented by immunological studies. The neoplastic cells in FL resemble the mature B-lymphocytes that populate the follicle centres of lymphoid follicles, according to morphological, immunophenotypic and molecular criteria. According to the World Health Organization (WHO) classification, a
higher grade is assigned to tumours with a greater number of centroblasts (the large
tumour cells) present per high-powered microscope field of view [7]. Patient
management is usually determined based on various clinical and histological factors
such as stage and grade of the cancer, prior therapies and age and overall health of the
patient. Treatment for this disease can range from a conservative ‘watch and wait’
approach to aggressive multi-agent chemotherapy and biological treatment regimens. FL
typically pursues an indolent clinical course characterized by cycles of remission and
recurrence [2]. Although most FL patients live for a relatively long time (median
survival is 8-10 years), survival varies widely and some cases of FL are aggressive [4].
Such a range is indicative of the heterogeneity of this disease, as is the fact that in a
number of cases, FL eventually undergoes transformation to a more aggressive
lymphoma. Transformation can manifest clinically as an increase in tumour burden
unresponsive to therapy or histologically as evolution into DLBCL. Histological
features that indicate transformation of FL to DLBCL include loss of the follicular
architecture and an accumulation of large centroblastic cells. DLBCL is characterized
histologically by diffuse (i.e. not follicular) infiltration by sheets of large neoplastic B-
cells and clinically by more aggressive behaviour than FL [2].
Germinal centres (GCs) of peripheral lymphoid organs are important sites of plasma cell and memory B-cell differentiation, which are crucial for the production of high-affinity antibodies for protection of the body against invading pathogens (reviewed in [8]). GCs are formed when naïve B-cells encounter antigen and become activated upon interaction with CD4-positive T-cells and antigen presenting cells and then migrate into primary follicles. Rapid proliferation of the activated B-cell occurs, creating a peripheral mantle zone and yielding the secondary follicle. The dark zone of the GC consists of large, highly proliferative B-cells called centroblasts and the light zone is comprised predominantly of centrocytes, smaller B-lymphocytes containing dark-staining, irregularly contoured nuclei. In the dark zone, centroblasts undergo clonal expansion and diversify their immunoglobulin gene variable regions by somatic hypermutation. This process involves error-prone DNA replication and introduces primarily single nucleotide exchanges. Multiple iterations of mutation are followed by a selection for B-cell clones. Survival signals are induced in those cells whose surface immunoglobulin protein can bind with high affinity to antigen presented on the surface of follicular dendritic cells. In the light zone, upon differentiation to centrocytes, those with low binding affinity subsequently undergo apoptosis. Somatic hypermutation has been shown to occur in non-immunoglobulin genes and as a result could potentially be involved in mutational events leading to an increased and uncontrolled proliferative
potential of germinal centre B-cells [9-11]. The normal germinal centre is a site of vigorous cellular proliferation, numerous genetic mutational events and abundant apoptosis. Therefore, should alterations in these processes occur, the germinal centre could be a likely site of neoplastic transformation.

\textit{t(14;18) Translocation}

It has long been known that the somatic chromosomal translocation \textit{t(14;18)(q32;q21)}, present in 70-90\% of FL cases, and juxtaposing the immunoglobulin heavy chain gene with the \textit{BCL2} gene, is a key molecular event related to the development of FL [12]. The outcome of this translocation is the constitutive expression of the \textit{BCL2} gene, resulting in overexpression of its protein product which functions to inhibit apoptotic cell death. While it is widely believed that in the majority of cases the translocation contributes significantly to the pathogenesis of FL, it is also evident that there are additional mutations contributing to the onset of cancer. This is supported by evidence showing that many normal, healthy individuals contain circulating B-cells possessing the translocation [13]. The authors of this study propose that these cells have sustained an initial oncogenic “hit”, but have yet to receive the additional, superimposed mutations required for full oncogenic transformation. Overexpression of the Bcl-2 protein leads to an accumulation of follicle centre cells with increased survival, and it is likely that following further oncogenic lesions, full transformation to FL occurs.
Follicular Lymphoma Tumourigenesis

The t(14;18) translocation, likely occurring as a result of an error in V(D)J recombination, and resulting in increased expression of the anti-apoptotic protein Bcl-2, has long been thought to be an initiating event in the pathogenesis of follicular lymphoma [14]. Since its discovery, some further evidence has emerged about the process of follicular lymphoma tumourigenesis, however much is yet to be elucidated. The current literature suggests a variable, multi-hit process including Bcl-2 expression, along with the involvement of the tumour microenvironment (Figure 1) [15]. With evidence that numerous healthy individuals carry circulating cells containing the translocation, it was clear that further events were needed for tumour development [13]. Until recently it was thought that these circulating cells were naïve B-cells that had not yet been exposed to antigenic stimulation in a germinal centre reaction. However, Roulland and colleagues have shown that these circulating t(14;18)-positive cells have undergone class-switch recombination, a process undergone during the germinal centre reaction [16]. A clonal population of these t(14;18)-positive FL-like cells can persist in a healthy individual for at least three years and perhaps signifies an early stage of lymphomagenesis. These findings suggest that the t(14;18)-positive FL-like cells can respond to stimulation by an antigen which may be involved, along with Bcl-2 expression, in their increased survival and therefore risk of undergoing further oncogenic mutations. Follicular dendritic cells and T-cells in the tumour
Figure 1. Follicular lymphoma tumourigenesis.

The t(14;18) translocation leading to Bcl-2 expression likely occurs in a naïve B-cell. This B-cell can encounter antigen and within a germinal centre reaction, differentiate into a memory B-cell through interaction with a T-cell and a follicular dendritic (FDC) cell. It is these memory B-cells which constitute the majority of the t(14;18)-positive cells found in the circulation of healthy individuals. Stimulation of these cells by antigen may contribute to malignant transformation and upon further oncogenic lesions, a tumour may develop. Adapted from [15].
microenvironment might contribute to encouraging or restraining malignant
transformation. This idea is supported by findings based on gene expression profiling
indicating a prognostic difference related to the presence or absence of benign
macrophages in the tumour microenvironment [17, 18].

Observations of early involvement of FL in lymph node biopsies have been described as
a type of *in situ* follicular lymphoma [19]. These biopsies contain some Bcl-2-positive
erginal centres, among mostly reactive, Bcl-2-negative follicles. In some cases these
individuals were found to have concomitant FL in other nodes, however in others, no
obvious disease was detected. On repeat biopsy, four patients were later found to have
developed follicular lymphoma, and in one case this occurred as many as 6 years later
[19]. This *in situ* follicular lymphoma likely represents an intermediate step in
tumourigenesis where t(14;18)-positive cells have sustained a secondary oncogenic hit
and have begun the process of malignant transformation. Through the balance of
proliferation and the action of tumour suppressor mechanisms, such as oncogene-
induced cellular senescence, the tumour growth is sustained for a variable amount of
time and upon further aberrations, the tumour is able to bypass growth inhibition. The
variable occurrence, nature and timing of these processes probably contribute to the
heterogeneity among patients which is observed clinically. Further study to delineate
these differences will potentially aid in directing a more tailored approach to management of FL patients.

**Transformation of Follicular Lymphoma to Diffuse Large B-cell Lymphoma**

Explorations into the cellular changes associated with transformation of FL to DLBCL have revealed that mutations of the *TP53* gene are associated with transformation [20, 21]. Defective p53 affects the cell’s ability to induce cell-cycle arrest in order to repair DNA damage or to induce apoptosis. Cells with defective p53 will have a selective growth advantage and are therefore more likely to acquire other mutations which may lead to transformation into an aggressive lymphoma [21]. The *BCL6* gene encodes a nuclear transcriptional repressor of various target genes that are involved in germinal centre formation and regulation of lymphocyte function and differentiation [22]. Mutations in the 5′ non-coding regulatory region of the *BCL6* oncogene and chromosomal translocations affecting the same gene, resulting in the deregulation of Bcl-6 expression have been associated with transformation of FL to DLBCL [23, 24]. These mutations may be associated with a selective growth advantage that gives rise to a more aggressive population of cells. The *CDKN2A* and *CDKN2B* genes encode the cyclin-dependent kinase inhibitors p16\(^{\text{INK4a}}\) and p15\(^{\text{INK4b}}\), respectively, and these proteins act to inhibit the cyclin-dependent kinases 4 or 6 resulting in blockage of cell
cycle progression prior to the G1/S transition. Loss of the function of p16\textsuperscript{INK4a} or p15\textsuperscript{INK4b} could lead to uncontrolled cell growth and alterations at these genetic loci have been associated with transformation of FL to DLBCL [25, 26].

**Prognostic Tools for Follicular Lymphoma**

A vast range of options is available for the management of FL patients. Therefore, a need exists for tools with which clinicians might gain important information about each patient’s case and make informed decisions about the most appropriate care for that individual. In the case of FL, these tools are especially important due to the apparent clinical heterogeneity among patients and the range of ways that these patients can be managed. Patients with an indolent tumour might not receive any therapy and could simply be followed for many years. Patients with an aggressive tumour likely to transform to DLBCL or otherwise result in shorter survival might be better served by aggressive treatment based on the expectation that the benefits will outweigh the adverse effects. Prognostic and predictive tools are important instruments that allow clinicians to more accurately tailor care and treatment to each individual patient.
Grade

According to the WHO criteria, conventional histological grading involves the counting of the large centroblast cells. Grade 1 tumours have 0-5 centroblasts per high-powered field; grade 2 tumours have 6-15 and grade 3 FL has greater than 15 centroblasts [7]. Some have suggested that tumour grade can stratify patients into more prognostically homogeneous groups, as patients with grade 3 FL tend to have shorter time until relapse than those with grade 1 or 2 diseases [7]. As a result, patients with grade 3 disease are often perceived as having an aggressive lymphoma and are treated with multi-agent chemotherapy and the anti-CD20 monoclonal antibody Rituximab while patients with grade 1 or 2 disease may simply be managed in a ‘watch and wait’ type manner. Clearly then, tumour grading has a significant impact on directing patient management.

However, there is some uncertainty as to the prognostic benefit of tumour grading, as investigators have been unable to show a difference in progression-free or overall survival across tumour grades [7, 27, 28]. In addition, tumour grading suffers from relatively poor reproducibility, indicating a need for alternative assays that might supplement current prognostic tools [6].

Clinical Parameters

The Follicular Lymphoma International Prognostic Index (FLIPI) was developed when it became apparent that the International Prognostic Index (IPI) did not adequately
stratify FL patients with different outcomes, as the majority of individuals were considered low-risk on this scale [29]. This was not surprising considering the IPI was initially developed to aid clinicians in assessing the prognosis of patients with aggressive lymphoma subtypes, namely DLBCL [30]. Thus, the FLIPI was subsequently developed and was geared specifically to patients with indolent diseases, particularly FL. While some of the adverse factors in this algorithm overlap with those included in the IPI, such as age at diagnosis, advanced stage and serum lactate dehydrogenase level, the FLIPI also includes hemoglobin level and whether there are greater than four nodal areas of disease [29, 30]. Using a numerical score calculated from the five adverse factors, the FLIPI then divides patients into three risk categories and is informative for clinicians in directing management of their patients. Little is known about how variation in clinical characteristics observed across FL patients which translates into differing FLIPI risk groups is related to underlying biological differences in the tumours of these patients. Further study is needed to identify the biological factors that underlie the observed clinical heterogeneity among individuals. The identification of such factors could lead to improved prognostic indices and/or identify novel therapeutic targets.
Biomarkers in Follicular Lymphoma

A recent study which seems to have had a profound impact on the field of prognostic biomarkers in FL found that the tumour microenvironment seemed to play a large role in the outcome difference among indolent and aggressive cases of FL [17]. Gene expression profiling of FL tumour samples revealed that expression signatures that were able to distinguish patients with good and poor outcome were resulting from non-neoplastic cells in the tumour environment. Immune response signature-1 (IR-1) appeared to reflect the contribution of reactive T-cells and conferred a favourable outcome, while immune response signature-2 (IR-2) conferred a poor outcome and was associated with benign macrophages in the lymph node biopsies [17]. As a follow-up to this study, Farinha and colleagues enumerated CD68-positive macrophages in the tumour environment using a tissue microray of a cohort of FL biopsy samples. Consistent with the results of the original gene expression study, they found that tumours containing a higher number of lymphoma-associated macrophages (LAM) had a poorer overall survival than patients whose tumours had fewer macrophages [18]. Further studies have examined the prognostic information that could be gained from assessing other cells in the tumour microenvironment. Low-grade lymphomas have been shown to have more clearly defined CD21-positive follicular dendritic cell (FDC) networks than high-grade lymphomas and a number of tumours lost the FDC network
prior to transformation [31]. A high FOXP3-positive regulatory T-cell count in the
tumour environment has been associated with longer survival of FL patients [32].

While many studies have focused on cells within the tumour environment, others have
been more interested in identifying prognostic differences within the neoplastic cellular
compartment of the tumour. One such study involving 73 cases of FL found that patients
whose tumours had high expression of BCL6 and CD10 had a higher overall survival
[33]. There has also been interest in the fact that while many FL tumours contain the
t(14;18) translocation resulting in expression of the anti-apoptotic Bcl-2 protein, not all
tumours do and perhaps this contributes to the clinical heterogeneity among FL patients.
It has been shown that patients with tumours lacking the translocation had significantly
better overall and disease-free survivals [34]. However, others have been unable to find
a significant survival difference associated with either the translocation [35] or
expression of the Bcl-2 protein in tumour cells [18, 36].

While the prognostic test of enumerating benign macrophages seemingly emerged as the
most exciting and perhaps likely candidate for clinical use, follow-up studies have
revealed some interesting results. Recent validation of its use as a prognostic test in
patients uniformly treated with Rituximab, the anti-CD20 antibody therapy which has
had a significant impact on survival of FL patients, in combination with
chemotherapeutic regimens has indicated that in these circumstances there is no longer a prognostic association with macrophage content in the tumour environment [37, 38]. It is clear that further studies are needed to assess the benefits of prognostic biomarkers for use in directing clinical management of FL patients.

**Immunohistochemistry and Tissue Microarrays**

*Immunohistochemistry*

IHC was first developed in the early 1940s by Coons and Jones who used a fluorescent dye-labeled antibody to identify an antigen in tissue [39]. Upon the discovery of the tight binding interaction between avidin (or streptavidin) and biotin, the technique was improved in allowing amplification of the signal to occur. It wasn’t until the 1970s that the use of IHC became commonplace in anatomic pathology and since then it has been routinely and widely used in hospital histology laboratories, as well as by researchers [40]. It is the norm for histology labs to have the ability to regularly stain for over a hundred different markers and this list continues to increase as novel diagnostic markers are identified and high-quality antibodies for use in formalin-fixed paraffin-embedded tissues are generated. The use of IHC is especially important in lymphoma diagnosis to aid pathologists in the difficult task of identifying the tumour subtype. There exist
numerous clinically heterogeneous lymphoma subtypes and accurate diagnosis is especially important for prognosis and directing appropriate management for each patient. A relatively simple and inexpensive IHC test can have profound impact on the ease with which pathologists can make an accurate diagnosis, especially with the increasing use of small needle biopsy specimens and the difficulty with which an accurate diagnosis and tumour grade may be determined from such a small sample [41]. Additionally, the increasing development and use of targeted therapies for cancer treatment has further augmented the clinical uses of IHC in allowing identification of patients likely to benefit from such therapies. Studies examining the use of markers of prognosis are also prevalent, indicating a likely expansion of the panel of markers available for use by clinicians.

Tissue Microarrays

The technique of creating tissue microarrays (TMAs) was first described in 1998 as a high-throughput means of arranging numerous tissue samples on a single slide [42]. The development of this technique has greatly facilitated morphological examination, immunohistochemical analysis and fluorescence in situ hybridization of multiple tissue samples. It allows for the simultaneous examination of numerous tissue samples, meaning that all samples are exposed to the same experimental conditions. It is much more cost effective with respect to immunohistochemical reagents, as numerous cases
are present on one slide. It minimizes the amount of valuable archived tissue used, while arraying in duplicate ensures that an adequate amount of tumour tissue is included. Therefore, the economy of scale offered by the use of TMAs makes it feasible to survey large numbers of tissue samples for expression of large numbers of proteins in a timely, cost-effective manner [43].

**Rationale**

One of the most common lymphomas in the Western world [1], FL typically pursues an indolent clinical course characterized by cycles of remission and recurrence, however conventional therapies do not have a great impact on survival and the vast majority of patients eventually die of their disease [4]. Although most FL patients live for a relatively long time, survival varies widely and some cases of FL are aggressive. Furthermore, FL commonly transforms to more aggressive disease after a variable period of time. Since treatment options for lymphoma are diverse, ranging from a conservative "watch and wait" approach to relatively aggressive multi-agent chemotherapy, an opportunity exists to identify subgroups of FL patients at diagnosis who stand to benefit from particular treatment strategies. For example, patients expected to survive with their disease for many years might be spared the morbidity associated with aggressive therapy, which might be more appropriately reserved for those with
aggressive disease. Therefore, FL patients are in a particular position to benefit from the
discovery of new biomarkers that can inform clinical management. Furthermore, as a
neoplasm with a variable, often prolonged, but generally unyielding clinical course, FL
serves as a potentially informative clinical model with which to investigate the biology
of tumour progression.

The dramatic advances in cancer biology and the emergence of new technology
platforms in recent years have stimulated attempts to identify informative FL
biomarkers based on the intrinsic biology of the disease. While these investigations have
shed light on biological factors that may underlie different clinical outcomes in FL, the
study-to-study reproducibility of the genes, or even signaling pathways, implicated by
the results has been modest, making it difficult to determine which genes or pathways
might be candidates as the basis of clinical tests. Gene expression microarray studies
may be weakened by errors of multiple testing (related to the difficulty of discerning
patterns based on evaluating the expression levels of thousands of genes across a
relatively small set of cases), sample-to-sample variability with respect to the
proportions of neoplastic versus non-neoplastic cells (this problem is especially relevant
to FL, where infiltrating non-neoplastic cells frequently represent a relatively major
population), and the vagaries of different microarray platforms. Relative to gene
expression profiling, the combination of TMAs and immunohistochemistry offers
several advantages for the identification of biomarkers in FL. The risk of errors related
to multiple hypotheses testing is reduced by evaluating a relatively small number of
proteins with established relevance to FL or cancer. Biomarker expression may be
evaluated within morphologically-defined populations of cells, thereby reducing the
potentially confusing effects related to expression in the non-neoplastic cells. This
approach can be applied to available archival, paraffin-embedded tissue samples and
protein expression is determined directly, rather than inferred from transcript
abundance. Finally, as a simple, robust test that is already used extensively in the
pathological diagnosis of cancers, IHC-based tests are simple and economically feasible
to incorporate into clinical practice.

**Hypothesis**

It is hypothesized that alterations in the expression of proteins with known roles in
hematological cells or cancer biology might correlate with clinical outcome and thereby
shed light on biological mechanisms of FL tumour progression. These biomarkers could
potentially be used to supplement prognostic information gained by currently used
clinical indices.
Objectives

(1) Use tissue microarrays and immunohistochemistry to identify proteins whose expression in FL cells correlates with patient survival.

(2) Elucidate the biological or clinical significance of these proteins by determining correlations with baseline clinical variables and known measures of tumour aggressiveness.

(3) Explore changes in biomarker expression in FL over time or upon transformation to a higher grade FL or DLBCL.
Chapter 2: Materials & Methods

Patient Selection

Sixty-seven cases of FL were identified retrospectively by searching the pathology archive of Kingston General Hospital, Ontario, Canada. Cases were selected for study based on the availability of frozen and paraffin-embedded tissue samples and associated clinical data. Frozen samples were used in a related study using Agilent gene expression microarrays. Subsequent searching of the same archive identified patients for whom paraffin-embedded tissue blocks of multiple biopsies (pre- and post-transformation) were available. Approval for the use of these resources was obtained from the Queen’s University Research Ethics Board.

Clinical Information

Chart reviews were done to collect patient outcome information and baseline clinical parameters for the initial cohort of follicular lymphoma patients. Information collected included: age at diagnosis, sex, Ann Arbor stage, Eastern Cooperative Oncology Group (ECOG) performance status, nodal involvement of disease, serum lactate dehydrogenase (LDH) levels, presence of bulky disease, extranodal involvement of disease and presence of B symptoms (fever, night sweats and weight loss). Date of death or last
clinical follow-up visit were noted, as was the date of transformation to aggressive
disease if it had occurred. The Follicular Lymphoma International Prognostic Index
(FLIPI) adverse factors were determined and a FLIPI score was calculated and a risk
category assigned where the required clinical data were available.

**Tissue Microarray Construction**

Routine diagnostic hematoxylin, phyloxin and saffron (HPS) and immunostained slides
were retrieved and reviewed independently by two experienced lymph node
pathologists; the pathological diagnosis and histological grade were established by
consensus according to WHO criteria [7]. Tumours were assessed for the presence or
absence of adjacent areas of DLBCL and also evaluated for the presence of zones of
monocytoid B-cells in the tumour sample. The regions of representative tumour tissue
were marked on the HPS stained slides and then the paraffin-embedded blocks to guide
TMA construction. Duplicate TMAs of the initial cohort of FL patients were constructed
using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). For each
TMA, duplicate 0.6 mm cores of tissue were removed from each tumour block from
within the marked area and arrayed systematically in the recipient paraffin block. The
TMA of matched pre-transformation FL and post-transformation DLBCL or post-
progression FL tumours was constructed using 1 mm cores. This increased core size was
used to optimize the amount of tumour tissue available for assessment in the TMA and since there were relatively few cases on this array, space was not an issue.

**Sectioning & Immunohistochemistry**

Prior to sectioning, the TMA block was cooled on ice for a few minutes and using a Leica microtome, 4µm thick histological sections were cut. The sections were then mounted onto glass slides which were baked overnight in a 52°C oven and stored at 4°C until staining was done. Staining occurred within a maximum of one week following sectioning to minimize the risk of loss of antigenicity. The slides were immunohistochemically stained with a range of antibodies (Table 1).

Immunohistochemical staining was done either using the biotin-streptavidin immunoperoxidase technique manually with the Universal LSAB+, HRP kit (Dako, Glostrup, Denmark) or using an automated immunostainer, (Ventana, Tucson, AZ) according the manufacturer’s instructions. Double colour-immunostaining was achieved using Ventana’s ultraView alkaline phosphatase and horseradish peroxidase linked secondary reagents. For manual staining, sections were deparaffinized and rehydrated in three, successive five minute baths of toluene, followed by ten minutes in two 100% ethanol baths, an 85% ethanol bath and a final 70% ethanol bath, followed by a five
Table 1. Antibodies and staining conditions used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antigen Retrieval</th>
<th>Clone</th>
<th>1° Ab Supplier</th>
<th>1° Ab Dilution</th>
<th>1° Ab Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>EDTA</td>
<td>Bp53-11</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>16 min at 37°C</td>
</tr>
<tr>
<td>p16^Nfkα</td>
<td>EDTA</td>
<td>16p04</td>
<td>Cell Marque</td>
<td>1:10</td>
<td>28 min at 37°C</td>
</tr>
<tr>
<td>PML</td>
<td>EDTA</td>
<td>rabbit pAb</td>
<td>Dr. Peter Greer</td>
<td>1:100</td>
<td>overnight at 4°C</td>
</tr>
<tr>
<td>p27^Kip1</td>
<td>EDTA</td>
<td>F-8</td>
<td>Santa Cruz</td>
<td>1:10</td>
<td>overnight at 4°C</td>
</tr>
<tr>
<td>p63</td>
<td>EDTA</td>
<td>4A4</td>
<td>LabVision</td>
<td>1:200</td>
<td>32 min at 37°C</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>EDTA</td>
<td>100/D5</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>30 min at 37°C</td>
</tr>
<tr>
<td>Bcl-6</td>
<td>EDTA</td>
<td>P1F6</td>
<td>Vector</td>
<td>1:20</td>
<td>32 min at 37°C</td>
</tr>
<tr>
<td>pERK</td>
<td>Citrate</td>
<td>20G11</td>
<td>Cell Signaling</td>
<td>1:100</td>
<td>overnight at 4°C</td>
</tr>
<tr>
<td>MUM1</td>
<td>EDTA</td>
<td>MUM1p</td>
<td>Abcam</td>
<td>1:20</td>
<td>32 min at RT</td>
</tr>
<tr>
<td>CD43</td>
<td>EDTA</td>
<td>L60</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>28 min at 37°C</td>
</tr>
<tr>
<td>CD79a</td>
<td>EDTA</td>
<td>JCB117</td>
<td>Cell Marque</td>
<td>Prediluted</td>
<td>28 min at 37°C</td>
</tr>
<tr>
<td>CD10</td>
<td>EDTA</td>
<td>56C6</td>
<td>Ventana</td>
<td>Prediluted</td>
<td>24 min at 37°C</td>
</tr>
<tr>
<td>Ki67</td>
<td>Citrate</td>
<td>SP6</td>
<td>Cell Marque</td>
<td>Prediluted</td>
<td>32 min at 37°C</td>
</tr>
<tr>
<td>CD68</td>
<td>EDTA</td>
<td>KP1</td>
<td>Dako</td>
<td>1:60</td>
<td>16 min at 37°C</td>
</tr>
<tr>
<td>CD21</td>
<td>EDTA</td>
<td>2G9</td>
<td>Vector</td>
<td>1:20</td>
<td>30 min at 37°C</td>
</tr>
<tr>
<td>CD3</td>
<td>EDTA</td>
<td>PS1</td>
<td>Ventana</td>
<td>1:100</td>
<td>28 min at 37°C</td>
</tr>
</tbody>
</table>

EDTA, ethylene diamine tetraacetic acid; min, minutes; RT, room temperature
minute water wash. Antigen retrieval was done by placing the sections in 90-95°C Dako Target Retrieval solutions, as indicated in Table 1, for 30 minutes using a kitchen steamer (T-Fal, Scarborough, Canada). Following antigen retrieval, the sections were allowed to cool for 20 minutes at room temperature and subsequently washed with tris-buffered saline (TBS) three times and placed in a TBS buffer bath. The slides were gently wiped around the specimen and placed in a humidified chamber. Following each of the subsequent wash steps, the slide was similarly washed and dried, and great care was taken to not allow the tissue to dry out during this process between each incubation step. Three percent hydrogen peroxide in water, to block endogenous peroxidase activity, was applied directly onto the tissue and allowed to incubate for five minutes at room temperature. This was followed by three gentle washes with TBS and a dip in 0.025% Triton X-100 in TBS. The sections were then blocked in 3% bovine serum albumin (BSA) in TBS for one hour at room temperature. This was followed by incubation of the sections with the primary antibody which had been diluted in 1% BSA in TBS. Concentrations and conditions of this step varied for each antibody and are detailed in Table 1. Negative control slides were incubated similarly with only 1% BSA in TBS. A cocktail of biotinylated secondary antibodies (anti-mouse, anti-rabbit and anti-goat) (Dako Universal LSAB+, HRP Kit) was applied to each section and allowed to incubate in the humidified chamber at room temperature for thirty minutes. This was followed by incubation with streptavidin conjugated to horseradish peroxidase (HRP)
for thirty minutes at room temperature. The hydrogen peroxide-activated
diaminobenzidine solution intensified with copper, which generated the signal, was
applied and allowed to incubate for ten minutes. The sections were then washed with
distilled water (dH2O) and quickly dipped in hematoxylin for counterstaining. Following
a five minute water wash, the slides were dipped in ammonia water ten times with a
subsequent water wash for two minutes. The sections were then dehydrated by being
dipped ten times in a 70% ethanol bath, 80% ethanol bath and two 100% ethanol baths,
followed by ten dips in three toluene baths. The sections were coverslipped using
Permount mounting medium and the other slides remained in toluene while this was
being done for each slide. They were then allowed to dry at room temperature, typically
overnight.

**Scoring**

The immunostained slides were scored visually for staining in the neoplastic B-cells
only, except for enumeration of benign (CD3-positive) T-cells or (CD68-positive)
macrophages, and evaluation of the integrity of (CD21-positive) follicular dendritic cell
networks. This scoring was done in a manner blinded to patient outcome. While two
cores were arrayed for each case, some were lost due to technical reasons or due to the
lack of apparent tumour tissue in the sample. One score was assigned per case based on
any tumour tissue present, whether it was from one or two cores. A standard four-point scoring system was used to evaluate the intensity or prevalence of staining for most markers. Generally, a score of ‘zero’ was assigned for negative staining; ‘one’ for those cases with no data due to loss of the tissue core or no apparent tumour in the sample; ‘two’ for weakly positive staining and ‘three’ for strongly, positive staining. The score definitions varied somewhat for some markers, as detailed in Table 2.

**Data Analysis**

Overall survival for each patient was calculated using the Kaplan-Meier method using the time from the date of diagnosis to the date of death from any cause. Some subjects were censored at the date of last follow-up. Each marker was assessed for its ability to divide patients into groups with significantly different overall survival. Survival curves were generated which allowed comparison of the groups and the log-rank test was used to assess their similarity. All missing values (i.e., score of 1) were dropped from this analysis. Cases assigned a score of 2 (i.e., an intermediate score) were combined with cases that scored 0 or 3 based on their tendency to co-segregate on survival analysis thus creating a binary (i.e., positive versus negative) score for each marker. Markers for which the intermediate scores of 2 were deemed ‘positive’ include: CD10, CD43, Bcl-2, Bcl-6, p27\(^{KIP1}\), p16\(^{INK4a}\), Ki67, p63, CD68-positive macrophages and CD21-positive
Table 2. Definitions of scores for immunohistochemical staining.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>&lt; 5% cells staining</td>
<td>uninterpretable</td>
<td>5-50% of cells showing weak to moderately intense staining</td>
<td>&gt; 5% of cells with strong staining or &gt; 50% with weak to moderately intense staining</td>
</tr>
<tr>
<td>CD79a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27 (^{kip})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16 (^{INK4a})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>&lt; 1% of cells staining</td>
<td>uninterpretable</td>
<td>1-10% of cells</td>
<td>&gt; 10% of cells</td>
</tr>
<tr>
<td>MUM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign T-cells(^{*})</td>
<td>≤ 175 cells</td>
<td>uninterpretable</td>
<td>175-300 cells</td>
<td>≥ 301 cells</td>
</tr>
<tr>
<td>Macrophages(^{**})</td>
<td>≤ 25 cells</td>
<td>uninterpretable</td>
<td>26-125 cells</td>
<td>≥ 126 cells</td>
</tr>
<tr>
<td>FDC Network(^{***})</td>
<td>no visible networks</td>
<td>uninterpretable</td>
<td>somewhat disrupted</td>
<td>dense, intricate network</td>
</tr>
<tr>
<td>Ki67</td>
<td>&lt; 30% of cells</td>
<td>uninterpretable</td>
<td>31% - 50%</td>
<td>&gt; 50% of cells</td>
</tr>
<tr>
<td>p63</td>
<td>no cells</td>
<td>uninterpretable</td>
<td>few scattered cells</td>
<td>many and/or intensely positive cells</td>
</tr>
<tr>
<td>PML bodies</td>
<td>no PML bodies</td>
<td>uninterpretable</td>
<td>------</td>
<td>PML bodies in many cells</td>
</tr>
<tr>
<td>phospho-ERK</td>
<td>no cells positive</td>
<td>uninterpretable</td>
<td>------</td>
<td>some cells staining</td>
</tr>
</tbody>
</table>

FDC, follicular dendritic cell
\(^{*}\)CD3-positive T-cells
\(^{**}\)CD68-positive macrophages
\(^{***}\)CD21-positive follicular dendritic cells
FDC network. Those which were classified as negative include: CD79a, PML, p53, MUM1 and benign CD3-positive T-cells. Differences in overall survival based on tumour grade (Grade 1 or 2 versus grade 3) and FLIPI (low and intermediate risk versus high risk) were also examined. Associations among significant prognostic markers and clinical variables were undertaken using the $\chi^2$-test or Fisher’s exact test. For this analysis, the clinical variables and tumour grade were grouped based on their previously published prognostic categories. The statistical software package SPSS (Version 14.0, Chicago, IL) was used for all statistical analysis and $p \leq 0.05$ was considered statistically significant.
Chapter 3: Results

Clinical characteristics

Sixty-seven cases of FL were identified for study. The median age at diagnosis was 58 years (range 34-87 years) and patients were evenly distributed with respect to sex (33 males and 34 females, Table 3). The patients had a median overall survival of 13.4 years (95% CI: 7-19.7). When the analysis was undertaken, 25 patients had died and the remaining 42 patients had a median follow-up time of 5.7 years (range 2-21 years).

Patients’ general health and quality of life at diagnosis were relatively good, as 84% had an ECOG performance status of one or less. It was possible to determine a FLIPI score for 56 patients: 46%, 14% and 39% fell into the low-, intermediate- and high-risk categories, respectively. For analysis purposes, the low and intermediate risk categories were grouped together and these patients had a median overall survival of 28 years, whereas FLIPI high-risk patients had a relatively short median overall survival of 2.4 years (p<0.001) (Figure 2). Treatment of patients varied, but in general, most patients (91%) received chemotherapy. Forty percent of patients received Rituximab (monoclonal antibody to CD20) either alone or in combination with chemotherapy.

Seven patients received an autologous stem cell transplant. Approximately a third (34%) 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.
Table 3. Baseline patient characteristics and treatment modalities.

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>Patient Set % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>49% (33)</td>
</tr>
<tr>
<td>Female</td>
<td>51% (34)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 60 years</td>
<td>54% (36)</td>
</tr>
<tr>
<td>&gt; 60 years</td>
<td>46% (31)</td>
</tr>
<tr>
<td><strong>Ann Arbor Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I or II</td>
<td>39% (26)</td>
</tr>
<tr>
<td>III or IV</td>
<td>58% (39)</td>
</tr>
<tr>
<td>NR</td>
<td>3% (2)</td>
</tr>
<tr>
<td><strong>Hemoglobin Level</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; 120 g/L</td>
<td>24% (16)</td>
</tr>
<tr>
<td>≥ 120 g/L</td>
<td>69% (46)</td>
</tr>
<tr>
<td>NR</td>
<td>7% (5)</td>
</tr>
<tr>
<td><strong>Nodal Areas</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 4</td>
<td>54% (36)</td>
</tr>
<tr>
<td>&gt; 4</td>
<td>42% (28)</td>
</tr>
<tr>
<td>NR</td>
<td>4% (3)</td>
</tr>
<tr>
<td><strong>Serum LDH</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>67% (45)</td>
</tr>
<tr>
<td>Elevated</td>
<td>10% (7)</td>
</tr>
<tr>
<td>NR</td>
<td>22% (15)</td>
</tr>
<tr>
<td><strong>Performance Status</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 1</td>
<td>84% (56)</td>
</tr>
<tr>
<td>&gt; 1</td>
<td>12% (8)</td>
</tr>
<tr>
<td>NR</td>
<td>4% (3)</td>
</tr>
<tr>
<td><strong>Extranodal Sites</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 1</td>
<td>88% (59)</td>
</tr>
<tr>
<td>&gt; 1</td>
<td>6% (4)</td>
</tr>
<tr>
<td>NR</td>
<td>6% (4)</td>
</tr>
<tr>
<td><strong>Tumour Grade</strong></td>
<td></td>
</tr>
<tr>
<td>1 or 2</td>
<td>58% (39)</td>
</tr>
<tr>
<td>3</td>
<td>39% (26)</td>
</tr>
<tr>
<td>NR</td>
<td>3% (2)</td>
</tr>
<tr>
<td><strong>Chemotherapy</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>91% (61)</td>
</tr>
<tr>
<td>No</td>
<td>9% (6)</td>
</tr>
<tr>
<td><strong>Radiotherapy</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34% (23)</td>
</tr>
<tr>
<td>No</td>
<td>66% (44)</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; NR, not reported.
Figure 2. Overall survival according to tumour grade and FLIPI risk groups.
Kaplan-Meier analysis and the log-rank test were used to assess the difference in overall survival of FL patients on the basis of expression of (A) tumour grade, and (B) the Follicular Lymphoma International Prognostic Index (FLIPI). Patients were censored at the time of their last follow-up.
Histopathological correlates of survival

Histological grading according to WHO criteria was evaluated as a means of predicting survival [7]. Twenty-four cases were scored as grade 1, 15 as grade 2, and 26 as grade 3 (23 as 3a and 3 as 3b). Histological grade could not be assessed reliably in two cases due to poor morphological preservation. Patients with grades 1 or 2 FL enjoyed a median overall survival of 13.4 years, whereas patients with grade 3 disease had a median survival of 11 years (p=0.04) (Figure 2). Therefore, the correlation between histological grading and survival was statistically significant, but modest in this series.

The presence of prominent zones of monocytoid B-cells in FL has been shown to correlate with shorter survival [44]. Seven of our 64 evaluable samples contained zones of monocytoid cells constituting greater than 5% of the lymphoid sample examined. These were often disposed peripherally in relation to neoplastic lymphoid follicles. The median overall survival was 4.4 years and 13.4 years (p=0.37), respectively, for patients whose lymphomas did and did not contain a significant element of monocytoid change. Therefore, although these cases manifested a trend toward shorter survival, the difference did not reach statistical significance in this relatively small series.
**Immunohistochemical correlates of survival**

Paraffin-embedded biopsy tissue from all 67 cases was represented in a TMA, sections from which were immunostained with a large panel of primary antibodies. The primary antibodies were chosen based on the known relevance of their protein targets to cancer or lymphoid biology and their utility when used in conjunction with formalin-fixed, paraffin-embedded tissue sections. For most markers, results were scored based on apparent expression in lymphoma cells according to the criteria listed in Table 2. Some markers, including the status of CD21-positive follicular dendritic cell networks and enumeration of infiltrating CD3-positive T-cells or CD68-positive macrophages, were scored based on the staining in apparently non-neoplastic, host-derived cells. At least 80% of the cases in the TMA were evaluable for each marker. The correlation between marker status and patient survival is summarized in Table 4.

Some of the IHC antibodies were for leukocyte markers (CD79a, CD10, CD43 and MUM1) used in the pathological diagnosis and characterization of lymphomas. CD10 is expressed in normal follicle centre B-cells and the majority of FL cells. Loss of CD10 expression in neoplastic follicle centre cells was observed in tumours from 10 patients (17%). Patients with CD10-negative FL had shorter median overall survival relative to CD10-positive cases (2.6 years versus 11 years, p=0.04, Table 4 and Figure 3).
Table 4. Correlation of immunohistochemical results and survival.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Score</th>
<th>Median Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive % (n)</td>
<td>Negative % (n)</td>
</tr>
<tr>
<td>p53</td>
<td>11% (7)</td>
<td>89% (58)</td>
</tr>
<tr>
<td>p16^{INK4A}</td>
<td>32% (21)</td>
<td>68% (45)</td>
</tr>
<tr>
<td>PML</td>
<td>45% (26)</td>
<td>55% (32)</td>
</tr>
<tr>
<td>PML bodies</td>
<td>52% (33)</td>
<td>48% (30)</td>
</tr>
<tr>
<td>p27^{kip1}</td>
<td>91% (49)</td>
<td>9% (5)</td>
</tr>
<tr>
<td>p63</td>
<td>49% (33)</td>
<td>51% (34)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>62% (39)</td>
<td>38% (24)</td>
</tr>
<tr>
<td>Bcl-6</td>
<td>78% (49)</td>
<td>22% (14)</td>
</tr>
<tr>
<td>pERK</td>
<td>25% (16)</td>
<td>75% (48)</td>
</tr>
<tr>
<td>MUM1</td>
<td>5% (3)</td>
<td>95% (58)</td>
</tr>
<tr>
<td>CD43</td>
<td>16% (9)</td>
<td>84% (47)</td>
</tr>
<tr>
<td>CD79a</td>
<td>76% (45)</td>
<td>24% (14)</td>
</tr>
<tr>
<td>CD10</td>
<td>83% (50)</td>
<td>17% (10)</td>
</tr>
<tr>
<td>Ki67</td>
<td>72% (47)</td>
<td>28% (18)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>50% (33)</td>
<td>50% (33)</td>
</tr>
<tr>
<td>FDC Network</td>
<td>79% (50)</td>
<td>21% (13)</td>
</tr>
<tr>
<td>T-cells</td>
<td>17% (9)</td>
<td>83% (45)</td>
</tr>
</tbody>
</table>

NS, not significant (p>0.05)
FDC, follicular dendritic cell
Figure 3. Overall survival according to the expression of p16\textsuperscript{INK4a}, p53 or CD10 in lymphoma cells. Kaplan-Meier analysis and the log-rank test were used to assess the difference in overall survival of FL patients on the basis of expression of (A) p16\textsuperscript{INK4a}, (B) p53 and (C) CD10. Patients were censored at the time of their last follow-up.
Assessment of the host-derived cells in the tumour microenvironment revealed variable numbers of infiltrating, CD68-expressing macrophages and CD3-expressing T-cells and variable prominence and integrity of networks of CD21-expressing follicular dendritic cells. Most cases retained the integrity of their CD21-expressing follicular dendritic cell networks, while 21% of cases lacked apparent organization of these cell connections. None of the parameters relating to host-derived, non-neoplastic cells correlated significantly with overall survival.

Most antibodies used for IHC were directed against proteins with demonstrated or potential tumour suppressor (p53, p63, p16^{INK4a}, p27^{KIP1} and PML) or oncogenic (Bcl-2, Bcl-6 and phospho-ERK) activity. Significant associations with survival were observed for expression of p53 and p16^{INK4a} (Table 4 and Figure 3). Staining for the p53 tumour suppressor was observed in 7 (11%) cases. Signal was exclusively nuclear and in a variable portion of lymphoma cells (Figure 4). Patients with p53-positive tumours experienced shorter median overall survival relative to those with p53-negative lymphomas (2.6 versus 13.4 years, p=0.01). Correlating p53 expression status with other parameters, including the clinical parameters used in ascertaining FLIPI-defined risk groups, indicated a significant association with low baseline hemoglobin levels (Table 5). A combined survival analysis of tumours positive for p53 and/or p16^{INK4a} protein compared to tumours negative for both proteins showed that expression of one or both
Figure 4. Immunohistology findings from two cases stained for p53 protein.
(A) Low-magnification view of a FL sample with no apparent p53-expressing cells and a higher magnification view of the same case in (C). (B) A low magnification view of a different case showing numerous p53-positive cells within the tumour sample. A higher magnification view of the same case can be seen in (D).
Table 5. Correlation of p16INK4a, p53 or CD10 expression with clinical parameters.

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LDH, lactate dehydrogenase; DLBCL, diffuse large B-cell lymphoma; NS, not significant (p>0.05)
proteins conferred a significantly worse outcome than tumours negative for both (Figure 5).

Expression of p16^{INK4a} correlates with tumour progression and shorter survival

Twenty-one (32%) of the assessable samples expressed detectable p16^{INK4a} protein (Table 4). These patients had a significantly shorter median overall survival relative to p16^{INK4a}-negative cases (8.3 versus 13.4 years, p=0.006, Figure 3). Previous studies have found that deletion or transcriptional silencing through promoter hypermethylation of the INK4a locus correlates with histological transformation in FL [25, 45]. Therefore our observation of a positive correlation between expression of p16^{INK4a} protein and shorter survival is novel and merits further investigation.

The prevalence of immunostained cells in p16^{INK4a}-positive cases varied from nearly 100% in a minority of cases to, more commonly, a "mosaic" pattern in which fewer, but still a substantial population of cells within neoplastic follicles was stained (Figure 6 A to C). Cells that expressed p16^{INK4a} generally presented an appearance consistent with small or large follicle centre lymphocytes. However, the relatively low prevalence of stained cells in some cases raised the question as to which of the several other cell types known to reside within lymphoid follicles (including T-cells, macrophages and follicular
Figure 5. Overall survival of patients grouped by a combined p16\textsuperscript{INK4a} & p53 IHC score. Kaplan-Meier analysis suggests that a combined p16\textsuperscript{INK4a} and p53 immunostaining score reveals a difference in overall survival. Patients with absent staining for both proteins had a significantly (p=0.01) better outcome when compared to tumours that expressed detectable p16\textsuperscript{INK4a} or p53.
Figure 6. Representative immunohistology findings from p16^{INK4a}-positive cases.

(A) Low-magnification view of a FL sample with prevalent p16^{INK4a}-expressing cells. Note the preferential segregation of immunostained cells to the neoplastic lymphoid follicles. (B) Higher magnification view showing pan-cellular localization of signal within apparently lymphoid cells. (C) Another p16^{INK4a}-positive case illustrating a relatively low prevalence of p16^{INK4a}-expressing cells. Once again, note the apparent segregation of immunoreactive cells within the follicle. (D), (E) and (F) Immunostaining results for, respectively, CD20 (brown signal), p16^{INK4a} (red signal) and co-detection of both markers in a single case of FL. In image (F), note that p16^{INK4a} is expressed in B-(CD20-positive) cells in most instances.
dendritic cells) were expressing $p16^{\text{INK4a}}$. Two-colour immunostaining of two representative cases for $p16^{\text{INK4a}}$ and CD20 confirmed that the majority of $p16^{\text{INK4a}}$-expressing cells were of B-lineage and belonged, therefore, to the neoplastic cell population (Figure 6 D to F).

In correlating $p16^{\text{INK4a}}$ expression with clinical and other findings, expression was significantly associated with lower hemoglobin and elevated lactate dehydrogenase levels at baseline, increased cell proliferation index as determined by a higher prevalence of Ki67 expression, grade 3/3 histology, high risk FLIPI classification, and the presence of an associated component of DLBCL within the biopsy sample (Table 4). Assessment in these patients, excluding those with a component of DLBCL at diagnosis, of the difference in time to histologically or clinically documented transformation to aggressive disease revealed a trend towards shorter time to transformation for patients with $p16^{\text{INK4a}}$-positive tumours. While this result is not statistically significant, it is based on a very small sample of patients and further study of this correlation in a larger series is warranted. These correlations suggest a relationship between $p16^{\text{INK4a}}$ expression and transformation of FL to more aggressive disease.

In order to explore a possible relationship between $p16^{\text{INK4a}}$ expression and tumour progression, we identified seven subjects from whom several lymphoma samples were
available. These represented: FL before and after transformation to DLBCL, five cases; progression from grade 1/3 to grade 3 FL, one case; and, grade 2/3 FL sampled 10 years following an initial diagnosis of grade 1 disease, one case. Samples from representative paraffin blocks were incorporated into a TMA, sections from which were immunostained for $p16^{INK4a}$. The staining results were then scored in a blinded manner, using the criteria employed in the original survey. In four subjects (patients 2, 5, 6 and 7), both pre- and post-transformation samples expressed $p16^{INK4a}$ (Figure 7). In two patients (patients 3 and 4), lymphoma cells converted from $p16^{INK4a}$-negative to -positive; in one of these instances (patient 4) this correlated with transformation to DLBCL. In the seventh patient (patient 1), the three samples were obtained over a brief period of only 2 years and were consistently $p16^{INK4a}$-negative. Therefore, the findings in this small dataset indicate that induction of $p16^{INK4a}$ expression can occur when FL cases are followed over time and it sometimes correlates with histologically documented transformation to more aggressive disease. Our failure to document loss of $p16^{INK4a}$ expression over time or on histological transformation seems especially noteworthy, considering published evidence predicting loss of $p16^{INK4a}$ expression on transformation [25, 45].
Figure 7. Tumour progression and transformation timeline for seven patients.

The first column on the left shows the original pathological diagnosis and the numerical headings for the other columns indicate the number of years elapsed subsequent to the original diagnosis. Grey and black boxes represent p16\textsuperscript{INK4a}-negative and -positive status, respectively. FL1, follicular lymphoma grade 1/3; FL2, follicular lymphoma grade 2/3; FL3, follicular lymphoma grade 3/3; DLBCL, diffuse large B-cell lymphoma.
Chapter 4: Discussion

Follicular lymphoma is a heterogeneous disease typically characterized by cycles of relapse and remission, while in some cases exhibiting aggressive behaviour and early death. By identifying proteins whose expression correlates with clinical outcome, we hope to contribute to the development of diagnostic algorithms capable of identifying groups of patients likely to benefit from clinical management strategies that are more specifically tailored to their particular disease. In recent years, the FLIPI has been useful in aiding physicians in making treatment decisions for FL patients. However, little is known about how the FLIPI factors are related to the underlying biological differences among indolent and aggressive tumours. Differences in expression of tumour protein markers are likely to be more closely related to the observed clinical heterogeneity among FL patients. In the clinical setting, immunohistochemistry on formalin-fixed paraffin-embedded tumour samples is already performed routinely, making the implementation of IHC tests for new proteins relatively straightforward and inexpensive. New algorithms incorporating protein marker testing and clinical variables such as those used in the FLIPI could potentially predict outcome information more accurately. This could spare some patients toxic therapies while ensuring that aggressive cases are treated appropriately. Prognostic protein biomarkers may also help to elucidate the biological mechanisms that underlie the observed heterogeneous clinical outcomes.
in follicular lymphoma. This would inform subsequent efforts to identify novel therapeutic targets. It was with these initials aims that we set out to examine possible prognostic biomarkers in FL.

It was observed that absence of CD10 expression in FL cells correlated with shorter overall survival. This is consistent with published results showing that weak or absent CD10 staining in FL cells is more common in grade 3/3 tumours [46] and associated with shorter overall survival, disease-specific survival and time to treatment failure [33]. Consistent with these earlier results, patients in our study whose tumours lacked expression of CD10 had a significantly shorter median overall survival of 2.6 years compared to 11 years for those with CD10-positive tumours (p=0.04). CD10 is a membrane-bound glycoprotein, an established marker of germinal centre B-cells, and one of several markers whose expression defines the germinal centre B-cell-like (GCB) subset of DLBCLs, associated in some studies with a relatively favorable prognosis [47]. Post-transformation FL tumours retain a germinal centre phenotype. Thus, instances of DLBCL that arise by transformation of FL invariably belong to the GCB DLBCL subtype [26]. Our finding that CD10 expression in FL confers a longer survival is interesting, considering CD10 expression is a defining marker of the favourable, GCB subtype of DLBCL [47]. CD10-negative FL and activated B-cell (ABC) DLBCL may
share underlying biological features that contribute to their relatively aggressive clinical
behaviour.

Cellular senescence is a concept that was first introduced after the observation that
primary cells propagated in tissue culture stop proliferating after a finite, apparently pre-
determined number of cell divisions [48]. It is a stable, perhaps irreversible state of cell
cycle arrest induced by diverse stimuli [49-51]. Senescent cells become enlarged and
flattened, and cannot divide, but remain metabolically active. Besides propagation in
tissue culture, this process can be activated in response to various types of cellular stress
such as telomere shortening, DNA damage, oxidative stress and oncogenic mutations
(reviewed in [52]). Senescence has been proposed as a tumour suppressor mechanism
that aids in the inhibition of tumourigenesis [53]. It is believed that cells that lose the
ability to senesce are unable to activate this failsafe mechanism leading to proliferation
and accumulation of malignant cells.

Cells harboring growth stimulating oncogenic mutations induce senescence through the
p16\(^{\text{INK4a}}\)/Rb pathway, the p14\(^{\text{ARF}}\)/p53/p21\(^{\text{Cip1}}\) pathway and the PTEN/p27\(^{\text{KIP1}}\) pathway
(reviewed in [54]). The p16\(^{\text{INK4a}}\) protein competes with cyclin D to bind to cyclin-
dependent kinase-4 (CDK-4) or -6, thus inhibiting the kinase from phosphorylating Rb.
Rb is then maintained in its active form, which represses expression of genes needed for
cell-cycle progression thereby bringing about cell cycle arrest (Figure 8) [54]. The p53
tumour suppressor is activated in response to DNA damage or other conditions that
induce senescence and is involved in transcriptional induction of p21^{Cip1}, an inhibitor of
various cyclin/CDK complexes. Hdm2, (also known as Mdm2, the murine form) a
protein that facilitates p53 degradation is sequestered by the p14^{ARF} tumour suppressor
protein that is induced by oncogenic signals and DNA damage (reviewed in [54]).
Oncogenic growth stimulation can also upregulate expression of the PML tumour
suppressor, which stimulates p53 activity [55]. PTEN is a lipid phosphatase that opposes
the actions of phosphatidylinositol 3-kinase (PI3K) in catalyzing the conversion of
phosphatidylinositol (4,5) bisphosphate (PIP2) to phosphatidylinositol (3,4,5)
triphosphate (PIP3) by dephosphorylating PIP3. Through its involvement in this
conversion, the PTEN tumour suppressor is involved in regulation of the CDK-2
inhibitor p27^{Kip1} [56]. p27^{Kip1} induces G1 cell cycle arrest and its accumulation is
associated with a senescence phenotype [57]. Alterations in these pathways could affect
a cell’s ability to restrict proliferation as a result of inappropriate growth signals.

Oncogene-induced senescence has previously been examined as a tumour suppressor
mechanism. Examination of the presence of oncogene-induced senescence markers in
lung adenomas and lung adenocarcinomas revealed that numerous cells in the
Figure 8. The p16\textsuperscript{INK4a}-Rb pathway.

In order to induce cell-cycle arrest, p16\textsuperscript{INK4a} inhibits pRb phosphorylation by the CDK-4/-6 and Cyclin D complex. This in turn keeps pRb in its active form and E2F is kept from activating transcription, which thereby inhibits progression of the cell-cycle. In situations where pRb has been inactivated, expression of p16\textsuperscript{INK4a} is induced, perhaps via loss of pRb-mediated repression. This results in high levels of p16\textsuperscript{INK4a} not able to induce cell-cycle arrest due to the requirement of downstream pRb function.
premalignant lesions stained for p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b}, while the malignant tumours were essentially negative [58]. Michaloglou and colleagues looked for markers of senescence in human naevi, benign tumours of melanocytes that frequently have oncogenic mutations. The naevus cells expressed senescence associated β-galactosidase and p16\textsuperscript{INK4a}, while normal melanocytes did not, suggesting that oncogene-induced senescence is involved in growth suppression of premalignant moles. Upon transformation to fully malignant melanoma cells, p16\textsuperscript{INK4a} expression is no longer apparent. It is hypothesized that loss of p16\textsuperscript{INK4a} expression likely reflects the tumour acquiring the ability to avoid the protective function of the senescence machinery [59].

It is well known that alterations to the function of the p53 protein occur in numerous tumour types and are involved in cancer development. Defective p53 affects the cell’s ability to induce cell cycle arrest in order to repair DNA damage, induce senescence or to induce apoptosis. Cells with defective p53 will have a selective growth advantage and in the context of a FL tumour cell, are then more likely to acquire other mutations that, in turn, may lead to progression into an aggressive lymphoma and consequent shorter survival. Previous studies have associated \textit{TP53} mutations with FL progression and transformation to DLBCL [20, 21, 60]. Consistent with these findings, numerous studies have associated p53 expression detectable by immunohistochemistry with higher grade FL tumours, [31, 61, 62] transformation to DLBCL [63] and accelerated tumour
progression [36]. The presence of immunohistologically detectable p53 generally correlates with the presence of missense mutations exerting deleterious effects on p53 function. These studies detecting p53 protein by IHC used relatively small sample sizes (from 7 to 43 cases) and either did not comment on, or failed to detect a relationship between p53 expression and overall survival. Considering these earlier results, it is somewhat surprising that we did not observe a correlation between IHC-detectable p53 expression and histological grade. This may simply be attributable to the relatively small number of p53-positive cases in our series. To the best of our knowledge, ours is the first demonstration of a direct, statistically significant correlation between p53 protein expression and shorter overall survival in FL patients.

This is the first report of an adverse prognosis in association with expression of p16\(^{INK4a}\) in FL cells. The p16\(^{INK4a}\) protein mediates pausing in the G1 phase of the cell cycle through binding to cyclin-dependent kinases 4 or 6 (CDK4/6), thereby preventing their association with D-cyclins and consequent phosphorylation of Rb. Consistent with its primary function in controlling cell proliferation, \(INK4a\) is a bone fide tumour suppressor gene deleted or otherwise inactivated in a considerable number of neoplasms (reviewed in [64]). Increased expression of p16\(^{INK4a}\) has been associated with poor outcome in patients with ductal carcinoma in situ [65], thyroid cancer [66], and ovarian carcinoma [67]. However, to our knowledge, there are no previous studies that assess
p16\textsuperscript{INK4a} protein status in a large set of FL patients to examine its association with clinical aggressiveness and survival.

Numerous studies have examined changes to the status of the \textit{INK4a} gene in B-cell lymphomas. Some have shown an increased prevalence of gene alterations, primarily promoter hypermethylation and homozygous deletions, in primary or transformed aggressive lymphomas compared to indolent tumours [45, 68]. These alterations to the \textit{INK4a} gene have been associated with a loss of p16\textsuperscript{INK4a} protein, low-to-high grade tumour progression, [69] and poorer prognosis in B-cell lymphomas [70]. While these studies are informative, the cases that were examined represent a variety of different histological subtypes and the sample sizes were generally small. Recent changes to lymphoma classification might also impact the results of these studies with respect to differences among subtypes.

With respect to FL, the existing literature suggests a model in which expression of p16\textsuperscript{INK4a} helps enforce the cell cycle-arrested (G\textsubscript{0}) state in which most neoplastic cells exist during the indolent phase of the disease. Subsequent inactivation of the \textit{INK4a} gene, for example by deletion, mutation or promoter hypermethylation, contributes to transformation to histologically or clinically more aggressive disease associated with accelerated cell proliferation. Thus, Elenitoba-Johnson and colleagues reported that
histological transformation of FL was associated with loss of heterozygosity at the
\textit{INK4a} locus and decreased expression of \textit{p16\textsuperscript{INK4a}} [25]. Deletions at the \textit{INK4a} locus
were detected in eight of eleven post-transformation DLBCL samples, however none of
the corresponding pre-transformation samples exhibited loss of heterozygosity or
homozygous deletions at that locus. All nine of the interpretable low-grade FL cases
expressed \textit{p16\textsuperscript{INK4a}} as assessed by immunohistochemistry. Five of the corresponding
post-transformation tumours showed loss of or diminished \textit{p16\textsuperscript{INK4a}} expression and each
of these cases showed homozygous deletions at the \textit{INK4a} locus. Our observation that
\textit{p16\textsuperscript{INK4a}} is expressed in a clinically and histologically aggressive minority of FL cases
seems inconsistent with this model.

Experimental and correlative studies have documented the existence of a reciprocal
relationship in the expression of \textit{p16\textsuperscript{INK4a}} and pRb. Whereas \textit{p16\textsuperscript{INK4a}} clearly acts to
maintain pRb in a functional state by preventing its phosphorylation by CDK4/6,
inactivation of pRb stimulates transcription of \textit{INK4a}, possibly by relieving the gene
from direct, pRb-mediated transcriptional repression (Figure 8) [71-74]. Loss of pRb
function is prevalent in advanced human cancers and may occur directly, through
mutation or inactivation of the \textit{pRB} gene, or indirectly through alterations of upstream
mediators, including deletion or other deleterious alterations of \textit{INK4a} leading to
inactivation of the pRb protein by CDK-mediated phosphorylation [75, 76]. We
speculate that some cases of particularly aggressive FL arise through activation of mitogenic signaling pathways. These could result in compensatory transcriptional induction of INK4a and pRb-dependent cell cycle arrest. Subsequent steps in tumour progression result in inactivation of critical components of the pRb signaling axis. Thus, we suspect that up-regulation of p16INK4a expression in a minority of FL cases, as reported here, reflects underlying dysfunction in the Rb pathway likely due, in at least some cases, to deletion or inactivation of pRB. This does not preclude the possible existence of a separate set of relatively aggressive FL cases in which p16INK4a is not expressed consequent to INK4a inactivation by various mechanisms.

**Summary and Conclusions**

Irrespective of the underlying mechanisms, our findings suggest that immunohistological detection of p16INK4a identifies a relatively large group of FL patients with particularly aggressive disease. Tumours with positive p16INK4a protein expression in our series tended to be of higher grade, more proliferative as assessed by Ki67 staining, associated with adverse FLIPI scores, and associated with a poorer overall survival. Increased expression of the p16INK4a tumour suppressor protein in poor outcome FL cases may reflect induction of the INK4a gene downstream of pro-mitotic signals as a result of the mutational activation of unidentified oncogenes. The growth-
suppressive potential of p16\textsuperscript{INK4a} requires intact pRb function in order to become manifest, and a reciprocal regulatory relationship exists between hyperexpression and the failure of cells to respond appropriately by growth arrest. Testing diagnostic FL biopsies by IHC for p16\textsuperscript{INK4a} protein expression is inexpensive and simple to perform and interpret. Furthermore, the pathological diagnosis of lymphoma is made with increasing frequency based on small needle biopsies, as opposed to more conventional excised lymph node specimens [41]. In contrast to conventional histological grading or molecular tests involving RNA-based gene expression profiles, IHC for p16\textsuperscript{INK4a} can be carried out using minute, fixed, paraffin-embedded samples of the type obtained using cutting biopsy needles.

**Future Directions**

Further investigation is required to validate our findings on a separate set of data based on patients managed on a standardized protocol and to determine the potential of the assay to predict clinical responses to particular therapeutic regimens. Importantly, assessment of this assay should be done on a cohort of patients who have all been managed similarly with Rituximab, an anti-CD20 antibody therapy that has recently had great impact on survival of patients with FL and other B-cell malignancies. Validation on a larger sample of patients would also allow for further multivariate analysis of the
biological and clinical prognostic markers, which has been limited in our study due to its modest size for a study of this nature. This type of larger scale study would likely prove difficult in a single centre, such as our own, due to the number of patients required and length of follow-up needed to discern accurate results. Collaboration with a clinical trial could allow for an independent validation study on a large set of uniformly treated patients with complete clinical and follow-up data available. Comparisons between the various treatment arms could also indicate whether the benefit of the prognostic test if validated in the standard treatment arm is retained following use of the experimental treatment. With complete clinical outcome data easily available for patients enrolled in a clinical trial, surrogate endpoints of survival, such as progression-free survival, could also be examined and would aid in the difficulty of performing these types of studies on FL patients due to the relatively long survival of individuals with indolent disease.

Within this study, further validation of interesting markers of outcome identified by others, such as the involvement of cells in the tumour microenvironment, might also be performed. In conjunction, a validation study of the related gene expression microarray results previously performed by our group, might also be feasible.

Further study to assess status of the p16^{INK4a}-Rb pathway in these tumours and its correlation with p16^{INK4a} expression and outcome would be an informative next step to examine the biological basis of these clinically heterogeneous tumours. Analysis of the
gene expression changes also associated with p16\textsuperscript{INK4a} protein expression in FL tumours may reveal some interesting genes related to the biological difference between p16\textsuperscript{INK4a}-positive and p16\textsuperscript{INK4a}-negative tumours and as a result, perhaps the difference between indolent and aggressive FL tumours.

Analysis of matched samples from tumours that have transformed or progressed would benefit from increased sample numbers, as these samples are relatively rare. Further study of the underlying genetic differences between these matched tumours is being assessed by array comparative gene hybridization (array-CGH) in our lab. Correlations among these results and p16\textsuperscript{INK4a} protein expression status could prove interesting.

These results could lead to further study to identify targets of novel therapeutics for FL patients. The design of small molecules and monoclonal antibody therapies which target specific proteins in the tumour cell have emerged in relatively recent years as an exciting trend in drug development. These agents are being tested in clinical trials and in some cases used routinely in a variety of tumour types, alone or in combination with classical chemotherapeutic agents. Further studies examining the biological basis for the clinical heterogeneity among FL patients would not only allow further refinement of prognostic algorithms, but could allow development of such agents. Treatments which
could target such pathways and which could effectively “convert” an aggressive tumour into an indolent tumour would have enormous therapeutic benefits.

**Significance**

Follicular lymphoma is a disease that affects numerous individuals each year. While some patients will enjoy a relatively long survival, they experience recurring periods of remission and relapse and they will ultimately die of their disease. Others will undergo rapid transformation to aggressive disease, and likely experience a short survival. There is a need for the development of new algorithms which might help identify at diagnosis important prognostic information to predict if a patient’s tumour will remain indolent or become aggressive. The benefit of tumour grading is questionable, it’s poorly reproducible among pathologists, tedious and time consuming and increasingly hard to ascertain with the frequent use of small, needle biopsies. While the FLIPI is informative, even among patients treated with Rituximab, little is known about the relationship between the tumour and the adverse factors that make up the FLIPI. Assessment of expression of tumour protein biomarkers could be incorporated into its algorithm for a prognostic test more closely related to the tumour. We have identified p16\(^{\text{INK4a}}\) as a novel marker of prognosis in FL tumours. Detectable expression of p16\(^{\text{INK4a}}\) protein in FL biopsies correlates with inferior survival, high risk FLIPI score, adjacent diffuse
areas in the tumour sample, and high tumour grade. A simple immunohistochemical-based assay could be performed and along with other indices, inform clinicians for the management of these patients. Patients with indolent tumours could be spared the side effects of toxic treatment regimens and aggressive tumours would be treated appropriately. These findings may also lead to further investigations of the biological basis of clinical heterogeneity among FL patients that could lead to the development of novel therapeutics.
Literature Cited


