CHAPTER 5 IMMUNOPHENOTYPING AND PROGNOSTIC MARKERS

5.1 Immunohistochemistry

5.1.1 Introduction

Immunophenotyping of frozen section material, once the mainstay of lymphoma immunohistochemistry, has been superseded by improvements in antigen retrieval techniques\(^1,2\) and the development of antibodies that recognise fixation resistant epitopes. The detection of antigens in paraffin sections obviates the requirement for fresh material for immunohistochemistry, provides a much better morphological context for the interpretation of immunostains, and has enabled immunophenotyping of paraffin-embedded archival material\(^3,4\).

5.1.2 Choice of antibody panels

Diagnostic antibodies that are immunoreactive in paraffin sections are as follows (many of these are enhanced by some form of heat-induced antigen retrieval procedure)\(^5,5\):

- **Markers to exclude simulators of lymphoma**: CD45, cytokeratin, S100, HMB45, Melan-A (CD30, CD20 and CD43 as a second line)
- **Markers of B-lymphocytes**: CD20 (L26), Cd75 (LN1), CD79a
- **Markers of T-lymphocytes**: UCHL1 (CD45RO), MT1 (CD43), polyclonal CD3, OPD4, βF1 (TCR\(\beta\) chain), CD4, CD8, CD5
- **Markers of Reed-Sternberg cells**: CD15, CD30, CD75 (LN2), peanut agglutinin, Fascin, LMP1, (negative for CD45, Oct2, Bob1)
- **Markers of immature lymphocytes**: TdT (paraffin sections and imprints), CD79a, CD43, CD10, cytoplasmic CD3, CD34
- **Immunoglobulin restriction**: Kappa and Lambda light chains (Microwave retrieval with 4 M urea solution combined with protease digestion (imprints and paraffin sections)
- **Markers of myeloid cells and monocytes**: anti-myeloperoxidase, anti-neutrophil elastase, antilysozyme, CD34, CD68, Mac387, Ham56, CD43 (expressed but not specific)
- **Markers of plasma cells**: CD38, CD79a, CD138, monoclonal clg, EMA, CD45 (weak), CD30.
- **Markers of dendritic reticulum cells, Langerhans cells and interdigitating reticulum cells**: CD21, CD23, CD35, S100, CD1a
- **Others**: bcl-6, ALK protein, CD30, EBV-LMP1, EBER (in situ hybridisation), cyclin D1 (antigen retrieval in EDTA pH8.0, preferably at 120°C), CD10 (CALLA), CD23, MIB1, CD56, CD10, PAX5 (BSAP), Oct2, Bob1, MUM1.

**Panels for specific lymphoid neoplasms**

- **Follicular lymphoma versus follicular hyperplasia**: bcl-2, CD45RA (MT2), immunoglobulin light chain restriction, CD21 or CD35 (DRC pattern)
- **For small cell lymphoma**: CD3, CD5, CD10, CD43, CD23, bcl-2, cyclin D1, CD10 (CALLA), CD21 or CD35 (DRC pattern).
• **For blastic lymphomas**: TdT, CD1, CD3, CD4/CD8, CD5, CD10, CD20, CD34, CD43, CD79a, cyclinD1. MIB1 may be useful to identify high proliferation index, Myeloperoxidase.

• **For large-cell lymphoma**: CD45, CD20, CD3, CD43, (MIB1, bcl-2, MUM1, CD10, bcl-6 as prognostic indicators).

• **For classical or LP Hodgkin lymphoma**: CD15, CD20, CD30, CD43, CD45, ALK, CD57, EMA, (Negative CD45, Oct2, Bob1).

• **For anaplastic large-cell lymphoma**: CD45, EMA, ALK1, CD3, CD45RO, CD4, CD8, CD20, CD30, CD15, cytotoxic antigens (TIA1, perforin, or lysozyme).

5.1.3 **Interpretation**

Interpretation of immunostained sections must be done in conjunction with adequate positive and negative control sections. It requires an understanding of the specific staining patterns unique to each antibody. Although immunophenotyping may be relatively simple in the case of diffuse, monomorphous lymphomas, the interpretation of polymorphous infiltrates with complex immunoarchitecture may be problematic. More detailed discussion of immunophenotypic interpretation is beyond the scope of these guidelines.

5.2 **Flow cytometry**

5.2.1 **Introduction**

Flow cytometry is the technique whereby suspensions of intact cells are stained with a range of fluorescent antibodies and exposed in single file to a laser light source at a specific wavelength. By measuring individual cell fluorescence and light scatter, the expression of surface antigens can be correlated with cell size and structure. Computer analysis and gating of the individual cell data enables the detection and characterisation of abnormal immunophenotypes.

The technique may be performed on a surgical biopsy of lymph node or extranodal tumour tissue, blood, bone marrow aspirate, fine-needle aspirate or other fluid sample.

Flow cytometry is particularly useful in the assessment of fine-needle aspiration (FNA) samples to establish cell type, lineage and B-cell clonality. It is also useful in following a specific cell phenotype in monitoring residual disease.

5.2.2 **Technical aspects**

Intact tissue slices undergo dissociation into cell suspensions in the flow laboratory. This step is omitted if a cell suspension has been prepared at the time of triage (refer to Chapter 4).

Flow cytometry can be applied to FNA biopsies and blood and bone marrow samples without the need to isolate mononuclear cells in suspension, thus simplifying laboratory procedures and making immunophenotyping of high-risk samples, such as HIV samples, safer. For these and many other specimens (e.g. fine-needle aspirate specimens) red cell lysis is required to remove contaminating red cells. An appropriate lysis reagent (e.g. ammonium chloride) is used to lyse the red cells without denaturing or destroying cellular antigens. Density gradient centrifugation (e.g. Ficoll hypaque) can also be used to remove red cells and dead cells from specimens. This method also concentrates the cells of interest.

For peripheral blood and bone marrow aspirate specimens, a stained smear should be available for morphologic assessment. For tissue, fine-needle aspirates and fluid samples, a cytocentrifuge preparation of the cell suspension should be made. These can be assessed morphologically before analysing by flow cytometry, which ensures adequacy of the specimen and guides antibody selection.
for cell analysis. Cellular viability should also be checked on the cell suspension. This can be done using fluorescent dyes such as propidium iodide, 7-AAD, or Trypan blue exclusion.

Isotype controls should be included for all analyses. These are negative controls that ensure that there is no non-specific binding of the primary antibody to the cell population of interest. Most samples analysed will contain some negative cells (i.e. normal cells that do not express the antigen of interest) that also act as internal negative controls.

Gating on the lymphoid cells of interest can be done using one of two methods:

1. Cell size (forward scatter) and cell complexity (side scatter), or
2. CD45 expression and cell complexity (side scatter).

Increased forward scatter and side scatter are seen in large-cell lymphomas. Most lymphoma samples have the same CD45 expression as normal lymphoid cells.

### 5.2.3 Choice of antibody panels

Antibodies used in the flow cytometric assessment of lymphomas recognise T-cell antigens (e.g. CD3, CD4, CD5, CD7, CD8, CD1a, T-cell receptor), B cells (e.g. CD10, CD19, CD20, CD23, CD79b, FMC7, IgM, kappa and lambda light chains) and differentiation related antigens (e.g. TdT). B-cell lymphomas are identified by the expression of B-cell associated antigens and light chain (kappa or lambda) restriction, indicating clonality. Many lymphomas exhibit characteristic phenotypes that assist in disease classification.

Assessment of flow cytometry requires interpretation of the cell phenotype, together with the gating and cell morphology. To interpret flow cytometry, it is important to understand cellular antigen expression in normal differentiation. Neoplastic cells may display the same phenotype as their normal counterpart. However, some malignancies acquire an antigen not normally expressed (e.g. CD2 expression in acute myeloid leukaemia), or have aberrant loss of an expected antigen (e.g. loss of CD7 in T-cell malignancies).

The number and type of antibodies included in the panel would depend upon:

- The clinical question, for example, initial diagnosis of lymphoma, follow-up studies in someone with established diagnosis, subtyping, detection of minimal residual disease or others, such as the number of T helper cells post-chemotherapy in a patient with known lymphoma.
- The type of flow instrument, for example, three or more colours
- Cost considerations

Table 5.1 shows the panel recommended by the British Committee for Standards in Haematology for the diagnosis of chronic/mature lymphoproliferative disorders.
Table 5.1  Diagnosis of chronic/mature lymphoproliferative disorders

<table>
<thead>
<tr>
<th>First line:</th>
<th>B-cell</th>
<th>T-cell</th>
<th>B-cell and T-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>CD2</td>
<td>CD5</td>
<td></td>
</tr>
<tr>
<td>CD23, FMC7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SmIg* (kappa/lambda)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD22*, CD79b*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Second line:</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c, CD25</td>
<td></td>
<td>Cyt Ig (kappa/lambda)</td>
<td>CD3, CD7</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CD103, HC2</td>
<td></td>
<td>CD79a, CD138</td>
<td>CD4, CD8</td>
<td>CD25</td>
</tr>
</tbody>
</table>

Source: modified from British Committee for Standards in Haematology (BCSH)\textsuperscript{10}  
*Intensity of membrane expression. I = disorders with villous cells; II = disorders with suspected lymphoplasmacytic or plasma cell differentiation; III = T-cell disorders; IV = suspected mantle cell and unclassifiable B-cell lymphomas. Optional markers: natural killer associated (CD16, CD56, CD11b, CD57); thymic markers (TdT); markers associated with activated T-cells (CD25); cytotoxic T-cell or NK marker (TIA-1).

The panel of antibodies recommended for a routine clinical laboratory includes most of the following: CD5, CD19, CD20, CD10, CD23, CD22, CD16, CD56, CD3, CD4, CD8, FMC7, CD103, CD25, CD11c, CD7 and CD79b antibodies.

### 5.2.4 Findings in specific diseases

#### B-cell neoplasms

One of the important applications of flow cytometry in haematology is to establish whether the B cells in a sample are monoclonal. This is performed by demonstrating light chain restriction (i.e. B-cell population expressing only kappa or lambda light chain) of the B cells present in the sample. A kappa: lambda ratio of 3:1 or <1:2 is strongly suggestive of the presence of a monoclonal B-cell population. This can be applied to peripheral blood, bone marrow, fine-needle aspirate, tissue or fluid samples. B-cell clonality can be backed up by demonstration of a characteristic phenotype of specific B-cell lymphomas/leukaemias.

Table 5.2  Low-grade B-cell lymphomas: immunophenotypic features\textsuperscript{11}

<table>
<thead>
<tr>
<th>Lymphoma type</th>
<th>SIG</th>
<th>CIG</th>
<th>CD5</th>
<th>CD10</th>
<th>CD23</th>
<th>CD43+</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL/SLL</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lymphoplasmacytoid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Follicle center</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-/+</td>
<td>-</td>
</tr>
<tr>
<td>Marginal zone</td>
<td>+</td>
<td>40%+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

\textsuperscript{11}  + = 90% positive; +/- = >50-% positive; +/- = <50% positive; - = <10% positive

It should be noted that clonal B-cell populations phenotypically resembling CLL/SLL may rarely be detected in clinically healthy individuals with normal blood parameters.\textsuperscript{12}

#### T-cell neoplasms

The flow cytometric assessment of T-cell lymphoproliferative disorders is more difficult than for the B-cell malignancies as few have a characteristic phenotype. However, many T-cell malignancies show atypical T-cell phenotypes with aberrant antigen acquisition or loss. Examples include aberrant loss of an expected T-cell antigen (typically CD5 or CD7), and loss of or co-expression of CD4 and CD8. Where the malignancy makes up the majority of cells present in the sample, flow cytometry can
usually establish the phenotype of the abnormal cell. If, however, the malignant T-cell population makes up only a small proportion of cells in the sample, it is usually not feasible to detect these against the background of normal T cells.

For T-cell processes there is no comparable phenotypic marker for monoclonality. Monoclonal antibodies to V\(\beta\) repertoire antigens can be used by flow cytometry. Restricted V\(\beta\) repertoire expression can be used as a screening test for T-cell monoclonality. The definitive demonstration of T-cell clonality is dependent on molecular biological techniques (Southern blotting or PCR).\(^{13}\)

**Hodgkin lymphoma**

In Hodgkin lymphoma (HL), the neoplastic cell population often represents as little as 1% of the total number of cells in suspension. For this reason, and the fact that neoplastic Hodgkin cells are CD45 negative and usually express a ‘null’ cell phenotype, flow cytometry findings are non-contributory in this disease. Flow is therefore not generally helpful in Hodgkin lymphoma except by excluding a monoclonal or any other immunophenotypically aberrant lymphoid cell population.

### 5.3 Prognostic markers

#### 5.3.1 Introduction

Although the WHO classification divides lymphomas into apparently distinct entities, many are heterogenous. For example, the t(14;18), which is characteristic of follicular lymphoma, is found in a substantial minority of diffuse large B-cell lymphomas (DLBCLs) suggesting that this group of lymphomas encompasses more than one entity. This heterogeneity is also reflected in the clinical behaviour of DLBCL, as 50–60% are cured with anthracycline-containing regimens, while the remaining 40–50% are not cured. The search for markers of prognostic significance has concentrated mainly on DLBCL, but more recently, the molecular events in small lymphocytic lymphoma/CLL have been shown to have highly significant prognostic importance.

#### 5.3.2 Diffuse large B-cell lymphoma

A number of markers have been examined as possible prognostic indicators in DLBCLs, but no single candidate has achieved universal acceptance. For each of the following markers, the cut-off percentages used for defining positivity are critical, and the use of different thresholds may account for some of the apparently contradictory findings in some series.\(^{14}\)

**Proliferative index as measured by Ki-67 fraction**

The Ki-67 antigen that identifies cells in the G\(_1\), S, G\(_2\) and M phases of the cell cycle has been used to identify the proliferative index in a large variety of lymphomas, in particular DLBCLs. Studies performed on frozen sections have produced inconsistent results, but most suggest that a proliferation index of >80% is a poor prognostic indicator, independent of the international prognostic index (IPI) group.\(^{15}\) More recent series using paraffin sections have produced similar findings.\(^{16}\)

In contrast, a study of relapsed lymphomas not restricted to DLBCL\(^{17}\) found that patients with tumour proliferation rates of <80% were significantly more likely to have no response to therapy, fail to achieve a complete response, and tend to have shorter progression-free survival and overall survival, than patients with a higher proliferation index.

**p53 alterations**

p53 alterations in DLBCL are more difficult to assess because of the different methods of analysis, including immunostaining, loss of heterozygosity analysis, single strand conformational polymorphism analysis, and direct sequencing. Immunostaining represents the simplest means of studying p53. Multivariate analysis in one study revealed shorter overall survival for those patients with p53 mutations in the low and low–intermediate IPI group.\(^{18}\) Others suggest that p53 protein
expression is not an independent risk factor for CR and survival\textsuperscript{19,20}. Studies correlating drug resistance with p53 expression have yielded conflicting results\textsuperscript{17,18}. The development of p53 expression in some lymphomas, however, may be associated with tumour progression.\textsuperscript{21}

\section*{bcl-2}

There is no evidence that \textit{bcl-2} translocation per se has prognostic significance in DLBCL. Expression of \textit{bcl-2} protein, however, has been shown to be a significant adverse prognostic indicator in DLBCL\textsuperscript{22,23} and has been used to further stratify cases defined as intermediate risk by IPI.\textsuperscript{24} Speculation that the anti-apoptotic effect of \textit{bcl-2} expression may mediate drug resistance\textsuperscript{25} is supported by animal models.\textsuperscript{26} A more recent study of elderly patients has indicated that the adverse prognostic effect of \textit{bcl-2} protein expression in DLBCL is annulled by the addition of rituximab to standard combination chemotherapy treatment protocols.\textsuperscript{27}

In a study of relapsed lymphomas not restricted to DLBCL, however, \textit{bcl-2} protein expression was found to be a surrogate marker for low proliferation index and to have no independent effect upon drug resistance, progression-free survival or overall survival.\textsuperscript{17}

\section*{bcl-6}

Studies attempting to correlate \textit{bcl-6} translocation with prognosis in DLBCL have produced conflicting results.\textsuperscript{28,29} \textit{Bcl-6} gene expression, however, has been associated with significantly improved survival in DLBCL in two recent series\textsuperscript{30,31}. In the former study, the patients with high \textit{bcl-6} gene expression showed longer overall survival in multivariate analysis with and without elements of the IPI compared to the group with low \textit{bcl-6} expression, both by real-time reverse transcriptase PCR and immunostaining.

A recent and larger study of 128 cases using a different threshold for positivity (10\% versus 25\%), however, failed to show prognostic significance for \textit{bcl-6} expression in DLBCL.\textsuperscript{23}

\section*{CD10}

CD10 expression in DLBCL has been examined in several recent studies using both immunohistochemistry and flow cytometry, with contradictory results.\textsuperscript{14} The largest study performed to date, however, has shown a significantly better survival for CD10\textsuperscript{+} versus CD10\textsuperscript{-} DLBCL when selected for low-risk IPI. CD10 expression did not predict for survival in the high-risk IPI patients, however.\textsuperscript{32} CD10 expression appears to correlate with presence of the t(14;18)(q32;q21) in DLBCL, and, in combination with \textit{bcl-6}, it has been shown to be a surrogate for the ‘germinal centre’ or ‘GC phenotype’ identified in cDNA microarray studies.\textsuperscript{33,34} Combining \textit{bcl-2} negativity with this GC phenotype appears to further enhance survival in the intermediate-risk IPI group\textsuperscript{24}. Contradictory findings in some studies\textsuperscript{27} may be partly explained by the recent observation that late relapse in DLBCL is more often observed in cases with a GC phenotype.\textsuperscript{35}

\section*{MUM1}

The MUM1/IRF protein is normally expressed in plasma cells and late GC B-cells. In microarray studies, MUM1 clusters within the ‘activated B-like DLBCL’ rather than GC-like group\textsuperscript{29}, and the combined staining for \textit{bcl-6}, CD10 and MUM1 in tissue sections has been shown to be predictive for these prognostic groups.\textsuperscript{30} The same study, using tissue microarrays, found that the expression of MUM1 correlates with a poor clinical outcome in DLBCL.\textsuperscript{30}

\section*{cDNA microarray studies}

Gene expression profiling has stratified cases of DLBCL into highly significant prognostic groups independent of IPI, principally those of ‘germinal centre’ versus ‘activated B-cell’ profiles (see Section 6.2.6).

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The same approach applied to mediastinal large B-cell lymphoma (MLBCL) has demonstrated a distinctive gene expression signature quite unlike other forms of DLBCL, and more in keeping with that of classical Hodgkin lymphoma.\textsuperscript{36,37} 

At present, cDNA microarray studies currently have practical application in only a few specialised centres. It is expected that more widely applicable surrogate markers using immunoperoxidase techniques will follow from this technology. 

\subsection*{5.3.3 Other lymphomas} 

**Small lymphocytic lymphoma (SLL)/chronic lymphocytic leukaemia (CLL)** 

CLL can be divided into two highly-significant prognostic groups according to the presence or absence of somatic mutations in the expressed immunoglobulin heavy chain variable (IgVH) regions.\textsuperscript{38} The expression of ZAP-70 (zeta-associated protein 70), a tyrosine kinase protein normally expressed in T and NK cells, has been shown to correlate inversely with IgVH mutation status.\textsuperscript{39,40} The expression of ZAP-70 measured by flow cytometry and immunohistochemistry has been shown to correlate closely with ZAP-70 mRNA expression and unmutated IgVH gene status.\textsuperscript{41} 

**Mantle cell lymphoma** 

The proliferation index in mantle cell lymphoma (MCL), determined by immunohistochemical staining for the Ki-67 antigen, has been shown in multivariate analysis to have prognostic significance.\textsuperscript{42} Over-expression of survivin, an inhibitor of apoptosis, can be detected by mRNA or immunohistochemistry. It has also been shown in multivariate analysis to have a significantly adverse effect on survival, but less than that of proliferative index.\textsuperscript{43} 

Studies of IgVH mutation status analogous to CLL have produced controversial results.\textsuperscript{44,45} Moreover, no surrogate marker of mutational status analogous to ZAP-70 in CLL has yet been found in MCL. 

Recently, cDNA microarray studies have characterised the gene signatures of MCL and created a survival predictor based on gene-expression for this disease.\textsuperscript{46,47} At this stage, however, there is no surrogate marker identified for widespread use in routine diagnostic laboratories. 

**CD30+ anaplastic large-cell lymphoma (ALCL)** 

Although not generally considered a prognostic marker, ALK1, is detectable immunohistochemically in paraffin sections. ALK1 expression in ALCL has been shown to be strongly associated with better prognosis.\textsuperscript{48,49} Expression of CD56 in a subset of CD30+ ALCL has been shown in multivariate analysis to correlate with poor survival independently of ALK expression or IPI.\textsuperscript{50} MUC-1 expression has also been shown to correlate with poor survival in ALK negative, but not ALK positive cases.\textsuperscript{51} 

\subsection*{5.4 References} 


12. Rawstron AC, Green MJ, Kuzmicki A et al. Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts. Blood 2002; 100: 635-9.


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