Cytological and molecular diagnosis of lymphoma

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With the advances in molecular pathology, the cell as a morphological and functional unit has become essential in the diagnosis of lymphoma. Conventional staining, preparation, and interpretation of cells, as seen in fine needle aspiration cytology (FNAC), often used as a first line investigation of lymphadenopathy, is being supplemented with an array of immunocytochemical and molecular analyses, aimed not only at a more precise disease definition, but also at recognising factors that can predict prognosis and response to treatment. Accepting the pitfalls of conventional cytomorphology, this review looks at molecular changes characteristic to particular lymphomas and explores the currently available technology for their detection, with particular reference to cytological material. Future protocols for the diagnosis and management of patients with lymphadenopathy should include FNAC as an initial investigation, followed by immunocytochemistry and molecular investigations. Tissue biopsy, the conventional method of diagnosis, may be avoided in selected cases.

with often limited material for phenotyping, it is worth noting that 90% of NHLs are B cell, of which more than half are either diffuse large B cell lymphomas (DLBLs) or follicular lymphomas (FLs) (31% and 22%, respectively).³ According to the World Health Organisation classification, the morphological diagnosis of NHL relies largely on cytological detail, although the development of new technologies has helped to define several clinical entities. Morphology alone is often not sufficient for a definitive diagnosis. Recently published recommendations for the reporting of lymphoid neoplasms suggest that, in addition to clinical features and morphology, the final report may include: immunophenotype, karyotype, and molecular characteristics.⁴ The following is a short resume of the major molecular findings in some of the more common NHLs.

“Morphology alone is often not sufficient for a definitive diagnosis”

The molecular pathogenesis of small lymphocytic lymphoma/B cell chronic lymphocytic leukaemia is largely unknown. Deletions of chromosome 13q14 occur in approximately 60% of cases.⁵ Mutations of the p53 gene occur in 10% of cases, the frequency of p53 inactivation increasing in the late stages of disease, suggesting that it may be involved in tumour progression.⁶ Approximately 50% of lymphoplasmacytic lymphomas are associated with the t(9;14)(p13;q32) translocation, often in cases associated with Waldenström's macroglobulinaemia.⁷ The translocation involves genomic regions containing the immunoglobulin heavy chain (IgH) gene on chromosome 14q32 and PAX-5 (paired homeobox-5) gene on chromosome 9p13,⁸ which encodes a protein involved in the control of B cell proliferation and differentiation.⁹ Mantle zone lymphoma (MZL) is frequently associated with the t(11;14)(q13;q32) translocation. This translocation involves Bcl-1 at 11q13 and the IgH locus at 14q32, leading to deregulation of Bcl-1 (also known as PRAD1), which encodes cyclin D1. In B cell NHL, cyclin D1

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MOLECULAR CHARACTERISTICS OF NON-HODGKIN LYMPHOMA

Currently, non-Hodgkin lymphomas (NHLs) are diagnosed according to the World Health Organisation classification.² This classification still broadly divides lymphomas according to phenotype into B and T cell types. For cytopathologists

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Abbreviations: ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; BL, Burkitt lymphoma; DLBL, diffuse large B cell lymphoma; FISH, fluorescence in situ hybridisation; FL, follicular lymphoma; FNAC, fine needle aspiration cytology; IgH, immunoglobulin heavy chain; MZL, mantle zone lymphoma; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction

overexpression is restricted to mantle cell lymphomas and provides a useful marker for this tumour.^{10–11} FL is characterised by chromosomal translocations of the Bcl-2 gene—t(14;18)(q32;q21) on 18q21 is detected in 80–90% of cases.¹² Because Bcl-2 controls cellular apoptosis, overexpression may lead to the abnormal survival of B cells. Other genetic abnormalities found in FL are deletions of chromosome 6 at 6q27, which occurs in approximately 20% of cases.¹³ Histological transformation of FL into an aggressive lymphoma is often associated with p53 mutations/deletions, sometimes p16 inactivation and, rarely, c-MYC rearrangements or chromosome 6q deletions.^{13–14} The most common genetic alteration of mucosa associated lymphoid tissue lymphoma is the t(11;18)(q21;q21) translocation (seen in ~50% of cases).¹⁵ More rarely, t(1;14)(p22;q32) translocation leads to deregulation of the Bcl-10 gene, a negative regulator of apoptosis.^{16–17} DLBL is characterised by a variety of morphological and clinical characteristics, suggesting an underlying pathogenetic heterogeneity. Some of the more frequent molecular characteristics are chromosomal mutations and translocations of the Bcl-6 gene, which is seen in most cases, with 3q27 breaks irrespective of the chromosome involved. Bcl-6 is required for germinal centre formation and the B cell immune response. Approximately 25% of DLBLs have chromosomal rearrangements of Bcl-2. These are similar to those associated with FL and are mutually exclusive of Bcl-6. Deletions of chromosome 6 are also frequently seen in DLBL, in addition to somatic hypermutation of protooncogenes PIM-1, cMYC, PAX-5, and RhoH/TTF.¹⁸ Translocations involving c-MYC and one of the IgH loci are found in all cases of sporadic, endemic, and human immunodeficiency virus associated Burkitt lymphoma (BL).¹⁹ Other genetic lesions in BL include infection of the tumour by Epstein-Barr virus (100% endemic, 30% sporadic), inactivation of p53 (30–40%) and p16 (30–40%) tumour suppressor genes, mutations of Bcl-6, and deletions of 6q.²⁰ Anaplastic large cell lymphoma (ALCL) is typically associated with the t(2;5)(p23;q35) translocation, which includes fusion of the NPM (nucleophosmin) gene on 5q35 to a novel anaplastic lymphoma kinase (ALK) gene on 2p23, which results in phosphorylation of intracellular targets and triggers malignant transformation.²¹ The ALK gene is not expressed on normal T cells and has both diagnostic and prognostic importance.²²

PITFALLS IN THE CYTOMORPHOLOGICAL DIAGNOSIS OF LYMPHOMAS

Cytological assessment of the sample taken with FNAC is often the first line morphological investigation of lymphoma.^{23–25} Some lymphomas have characteristic cytological appearances, and it is generally possible to diagnose most lymphomas, at least in general terms, on the basis of the initial morphology.²⁶ With an overall accuracy rate of 96–99% and a typing accuracy rate of 96.5%, FNAC yields a high rate of conclusive cytological diagnoses in the assessment of metastatic malignancies, high grade NHL (79–90%), and Hodgkin's disease (with the exception of the lymphocytic predominance variant of Hodgkin disease), but has significant limitations in the assessment of low grade NHL and precise REAL classification typing.^{27–31} Some centres successfully use a combination of cytology and flow cytometry as an initial approach.^{32–36} The combination of FNAC and core biopsy in the diagnosis of lymphoma can further reduce the rate of inadequate samples and improve typing of lymphoma.³⁷ The techniques are best used to complement rather than compete with one another.

Examples of morphological difficulties include distinguishing the following: (1) low grade lymphoma from reactive lymph node hyperplasia; (2) variants of small lymphocytic

lymphomas and lymphoma from metastases, particularly of small round cell tumours, such as peripheral neuroectodermal tumour, rhabdomyosarcoma, and small cell carcinoma; (3) Hodgkin's disease from ALCL³⁸; and (4) ALCL from non-lymphoid tumours. Although most of these can be solved with the help of immunocytochemistry, there are sometimes problems with immunophenotyping on cytological preparations, partly as a result of the small amount of material available.^{39–40} Grading FLs on fine needle aspiration specimens cannot clearly distinguish between grades 2 and 3.^{41–42} Despite the best efforts of morphology and immunophenotyping, some lymphomas are impossible to diagnose on FNAC.⁴³

OBJECTIVES OF MOLECULAR DIAGNOSIS OF LYMPHOMA

The main objectives in the molecular diagnosis of lymphoma are the detection of clonality, translocations, or genetic abnormalities.⁴⁴ Assessment of clonality is often used for establishing the diagnosis of lymphoma and its differentiation from hyperplasia. This can be achieved by means of Southern blotting, the polymerase chain reaction (PCR), flow cytometry, and immunocytochemistry (light chain restriction).⁴⁵ The detection of chromosomal translocations can help support the diagnosis—for example, t(14;18)(q32;q21) in FL, t(11;14)(q13;q32) in MZL, and t(2;8), t(8;14) and t(8;22) in BL.⁴⁶ Gene rearrangement is sometimes used in the diagnosis of minimal residual disease.

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Molecular techniques that are presently used in selected cases, either singly or in combination, are PCR, fluorescent in situ hybridisation (FISH), comparative genomic hybridisation, and nucleic acid microarrays/gene profiling.^{46–47}

Polymerase chain reaction

The PCR technique and its modifications have become the mainstay of diagnostic and research medicine. The technique allows amplification of nucleic acid sequences both for purposes of disease detection (analytical PCR) and for the preparation of hybridisation probes and sequencing templates (preparative PCR). The application of PCR to cytological material provides a promising, relatively non-invasive approach to clinical molecular genetic analysis.^{48–49} It can be used to identify monoclonal rearrangements of the IgH or T cell receptor genes and translocations involving the Bcl-1 and Bcl-2 genes (figs 1–3).⁵⁰ The detection rate of t(14;18), the most common translocation in B cell lymphomas, which results in rearrangement of the IgH and Bcl-2 genes, has been examined in fresh and archival cytological smears, the results being highly comparable to those in excision biopsies.⁵¹ The use of PCR in the detection of clonal immunoglobulin and T cell receptor gene recombination was standardised in the BIOMED-2 report.⁵²

Fluorescence in situ hybridisation

FISH is based on the principle that many lymphomas are characterised by complex karyotypic changes. Specific FISH probes are useful to determine characteristic or identifying marker chromosomes.⁵³ Metaphase FISH uses intact chromosomes on metaphase cells so that chromosomal localisation can be checked. Interphase FISH exploits intact interphase nuclei.⁵⁴ This method is particularly suitable for cytological imprints and can detect numerical and structural chromosomal abnormalities.^{55–57} Fibre FISH is used on isolated DNA molecules, maps deletions, insertions, or translocations, but

Figure 1 Cutaneous T cell lymphoma. A patient with a history of T cell cutaneous non-Hodgkin lymphoma presented with a nodule in the buttock and forearm. (A) Low power view of a fine needle aspiration sample showing a mixture of lymphoid cells (May-Gruenwald and Giemsa (MGG) stain; original magnification, $\times 400$). (B) High power view shows medium sized lymphocytes admixed with follicle centre cells (MGG stain; original magnification, $\times 600$). (C) Polymerase chain reaction amplification of the T cell receptor γ chain gene shows a biallelic rearrangement (lanes 3 and 4), confirming the presence of neoplastic clone(s).

can be used only on fresh or frozen tissue and therefore is also applicable to cytology.^{58 59}

Comparative genomic hybridisation

This method detects regions in the genome undergoing quantitative changes; that is, copy number gains and losses. Different tumour types show distinct comparative genomic hybridisation patterns of gains and losses. Comparative genomic hybridisation is not used extensively in lymphomas but is used to analyse leukaemias.⁶⁰

Microarrays/gene profiling

This exploits the fact that the characteristics of a tumour and its clinical behaviour are determined by the unique set of genetic lesions contained within a cell. A microarray is an orderly arrangement of samples or probes immobilised on to a matrix. Genetic lesions are reflected in the pattern of mRNA

expression and are its molecular “signatures”. Uncovering the molecular characteristics of a tumour cell may help not only in diagnosis and classification but also in prognosis and treatment.^{61–63} Microarray technology can be broadly divided into two distinct areas: arrays of nucleic acid spots (DNA microarray) and tissue microarray. DNA and tissue microarrays are complementary techniques. Tissue microarray allows validation of the DNA microarray results and the possible identification of immunocytochemical surrogates of gene expression profiling. For example, by using a DNA microarray, DLBL can be divided into three prognostically important subgroups: germinal centre B cell type, activated B cell type, and a group with type 3 gene expression profiles. Staining with a panel of antibodies, Hans *et al* showed that the expression of Bcl-6 or CD10 was associated with better overall survival, whereas the expression of MUM1 and cyclin D2 was associated with worse overall survival. Using

Figure 2 Follicular lymphoma (FL). Two patients with a history of FL had pleural effusions, which were suspected of being recurrences. (A) Polymerase chain reaction shows a background smear in case 1 (lanes 2 and 3) and amplification of IgH in case 2 (lanes 4 and 5). (B) Case 1: small and medium size lymphocytes. (C) Case 2: a mixture of small and medium sized lymphocytes (May-Gruenwald and Giemsa stain; original magnification, $\times 600$).

Figure 3 Mucosa associated T cell lymphoma (MALT). (A) Patient with a history of MALT lymphoma excision 10 years previously. (B) The patient presented with a swelling in the other parotid gland, which was suspected of being a MALT lesion. (C) Polymerase chain reaction of IgH shows a monoclonal band, thereby confirming the diagnosis. Lane 1, negative control; lane 2, monoclonal control; lanes 3 and 4, fine needle aspiration samples from MALT; lane 5, polyclonal control. (D) Fine needle aspiration smear showing a dense population of lymphoid cells with prominence of residual follicle centre fragments. (E) High power view showing a monotonous population of centrocyte-like cells. The patient gave her full permission for this photograph to be published.

immunocytochemistry, cases were subclassified as germinal centre B cell type and non-germinal centre B cell type, with the germinal centre B cell type having a better overall survival.⁶⁴ DNA microarrays can rapidly provide information on many genes at the mRNA level. It has been possible to use small volume samples from lung and breast FNAC in microarrays.^{65–67} The amount of RNA that can be recovered from FNAC is comparable to that from a core biopsy.⁶⁸ Genetic abnormalities may provide information beyond the expression of mRNA and protein—for example, the overexpression of MYC in BL, which can also be found in many normal cells and lymphomas lacking translocation.⁶⁹

THE IMPORTANCE OF MOLECULAR DIAGNOSIS OF LYMPHOMAS

New developments in the diagnosis of lymphoma help us to understand their pathogenesis, refine their classification, and obtain diagnoses.^{70–71} They also help in staging and prognosis and in the development of new treatments.⁷² The stage and place of translocation may determine the nature of the disease—for example, stem cells, pre-germinal, post-germinal.⁷³ Molecular investigations have helped to define entities—for example: (1) the t(14;18) translocation and Bcl-2 expression have been useful in distinguishing reactive from neoplastic proliferations; (2) t(11;14), which encodes cyclin D1, has helped to define MZL as an important and clinically distinct category of B cell lymphoma; (3) ALCL, previously “malignant histiocytosis”, has also been defined

by the specific t(2;5) translocation. Others are non-specific, such as the t(14;18) translocation seen in FL.

Molecular investigations also play a role in assessing prognosis—for example, the t(2;5) translocation and overexpression of ALK in ALCL conveys a good prognosis, whereas the t(1;14) and t(11;18) translocations and overexpression of Bcl-10 in gastric mucosa associated lymphoid tissue lymphoma conveys an adverse prognosis.⁷⁴ In B cell chronic lymphocytic leukaemia, the presence of an unmutated IgV(H) gene is strongly associated with the expression of ZAP-70 (70 kDa ζ associated protein). ZAP-70 is a strong predictor of the need for treatment in B cell chronic lymphocytic leukaemia and can be detected in the routine laboratory by means of flow cytometry.^{75–77} As discussed earlier, gene expression profiling has revealed prognostically important groups of DLBL, with Bcl-10 and CD10 expression conveying better prognosis.^{64 78 79}

“The t(2;5) translocation and overexpression of ALK in anaplastic large cell lymphoma conveys a good prognosis”

Genetic abnormalities can also be used in staging and the detection of minimal residual disease—for example, t(14;18) Bcl-2/IgH rearrangement for follow up of bone marrow transplants for FL and FISH for cyclin D1/Bcl-1 rearrangement for follow up of patients with MZL.⁸⁰ However, “molecular recurrence” of FL, as detected by means of direct

sequencing of Bcl-2/IgH translocation, may be unrelated to the original clone and may lead to false positive results.⁸¹

Genetic abnormalities may also be exploited for developing new treatments, such as STI571 in chronic myeloid leukaemia, and drugs that could target fusion proteins such as NPM/ALK or inhibit cell cycle proteins such as cyclin D1.⁸²

The molecular profile can also be used to predict the response to chemotherapy.⁸³ Shipp *et al* suggest a "supervised learning classification", which involves searching for specific reporter genes for the pre-set parameter of interest—for example, survival.^{84–85} The resulting "microarray classifier" is a better predictor of patient survival than the international prognostic index. If the combination of genes associated with a particular disease is known, molecular investigations may also be used for a disease screen, with the potential of discovering new genes associated with prognostically important categories.^{86–87}

Whatever the promise of new technology, molecular techniques need experts to standardise tests (sensitivity and specificity), learn the pitfalls in interpretation, accept limitations, and interpret the results in the context of other data. In the future, microarray technology will probably become the cornerstone of molecular research. The key question is whether we can harness to our needs all the information that will become available.⁸⁸

TO BIOPSY OR NOT TO BIOPSY THE LYMPH NODE

Future algorithms for the diagnosis of lymphoma should include FNAC as a preferred method of initial lymphoma diagnostic triage and RNA or protein extraction, followed by high throughput genomic or proteomic analysis and tissue biopsy, where necessary (fig 4). FNAC is an accurate method in the diagnosis of lymphomas, particularly when the cytological diagnosis is corroborated by immunocytochemistry and molecular investigations. However, increasing use of FNAC for primary diagnosis and classification of lymphomas may result in the loss of archival tissue for complementary analyses, reclassification, and research purposes. Therefore, FNAC should retain its role in triaging benign from malignant lymphadenopathies, most frequently metastatic carcinoma. In the minority of cases where lymphoma is suspected, FNAC should be followed by tissue biopsy. In

cases of recurrence, staging, and detection of minimal residual disease, cytological material complemented by ancillary studies may suffice for diagnosis.

"In the future, microarray technology will probably become the cornerstone of molecular research"

In conclusion, cytomorphology is an important manifestation of gene expression. Our vision can be refined by looking at slides that have been subjected to new techniques.⁸⁹ Functional genomics will probably have a lasting impact on the pathology laboratory and we should embrace it with eager anticipation.⁹⁰

The patient gave full permission for the reproduction of fig 3.

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Figure 4 Algorithm for the diagnosis of lymphoma. FISH, fluorescence in situ hybridisation; FNAC, fine needle aspiration cytology; PCR, polymerase chain reaction; PSA, prostate specific antigen; Tg, thyroglobulin.

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