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REVIEW

Developments in the immunophenotypic analysis of haematological malignancies

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ABSTRACT

Immunophenotyping is the method by which antibodies are used to detect cellular antigens in clinical samples. Although the major role is in the diagnosis and classification of haematological malignancies, applications have expanded over the past decade. Immunophenotyping is now used extensively for disease staging and monitoring, to detect surrogate markers of genetic aberrations, to identify potential immuno-therapeutic targets and to aid prognostic prediction. This expansion in applications has resulted from developments in antibodies, methodology, automation and data handling. In this review we describe recent advances in both the technology and applications for the analysis of haematological malignancies. We highlight the importance of the expanding repertoire of testing capability for diagnostic, prognostic and therapeutic applications. The impact and significance of immunophenotyping in the assessment of haematological neoplasms are evident.

1. Introduction

Immunophenotyping dates back to 1941 when Coons et al. first demonstrated the use of fluorescently labelled polyclonal antibodies to localise cellular antigens in tissue sections [1]. This was widely applied to fresh frozen tissue sections in the 1950s and, in the 1970s, began to be used for leukaemia diagnosis utilising both slide-based methods and fluorescent microscopy. Immunofluorescent labelling enabled assessment of multiple antigens simultaneously as well as their subcellular localisation. This led to the birth of modern flow cytometry for the analysis of haematological malignancies. Flow cytometry requires only a small sample volume, and allows many cellular parameters to be assessed simultaneously. Both surface membrane and intracellular antigens can be assessed, and the assessment is both quantitative and sensitive. Due to developments with the use of multiple lasers, new fluorochromes and high sample speeds, flow cytometry is highly sensitive for the identification of discrete populations even when present in small numbers [2,3]. Flow cytometry offers high diagnostic precision and sensitivity and has applications for determination of cell lineage and differentiation stage, identification of a disease-associated phenotype, and disease monitoring to sensitivities of 10^{-3}–10^{-4}. Since the 1990s multiparameter flow cytometric immunophenotyping has become the technique of choice, as it is the sole technique that fulfils the requirements for high speed, broad applicability at diagnosis and during follow-up, and accurate focusing on the malignant cell population using membrane-bound and intracellular proteins as targets.

For some haematological applications a major limitation of flow cytometry is the inability to directly correlate cell morphology, bone marrow architecture and phenotype. For diagnostic tissue pathology, including haematopathology, major breakthroughs occurred in the 1970s and 1980s with the development of staining techniques utilising enzyme labels and permanent chromogenic substrates. These immunoenzyme methods, based on horseradish peroxidase and alkaline phosphatase, enable both the cell membrane and intracellular antigens to be visualized simultaneously with tissue architecture and cell morphology using standard light microscopy [4–7]. Over the ensuing 40 years, there have been significant developments in these methods, resulting in their routine and extensive use in diagnostic pathology. Immunocytochemical methods are widely available, affordable, are performed *in situ* in human tissue and have numerous applications. This methodology, which also enables the detection and sub-cellular localisation of cellular antigens ("biomarkers"), has had a significant impact on our ability to diagnose haemopoietic neoplasms and provide clinicians with enhanced ability to manage patients.

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Immunophenotyping, whether performed by flow cytometry or immunocytochemistry, is a form of "*in situ* proteomics". This is now a proven crucial tool for the detailed characterisation of haematological malignancies at diagnosis and throughout the course of the disease. Testing is now performed in developed and developing countries for a range of applications, on a variety of automated platforms and for a broad repertoire of antigens. In this review we describe recent technological developments in immunophenotyping. We also present a review of the major applications as companion diagnostics in the diagnostic assessment, prognostic stratification and providing information regarding therapeutic approach and monitoring of haemopoietic neoplasms.

2. Technical aspects

Many impressive and important technological developments have taken place over the past decade, including the number and range of antibodies, new hardware, automation and analytical tools. These have all resulted in higher throughput, increased sensitivity of testing, more detailed cell characterisation and greater standardisation of testing.

2.1. Antibodies

Monoclonal antibodies were first generated over three decades ago but there are still no antibodies that are specific for haemopoietic neoplasms. As such, panels of antibodies to a broad range of cellular molecules are generally used to diagnose and classify disease. This gives high specificity and sensitivity for the classification of specific haematological malignancies and identification of molecules of clinical significance. Antibody panels are utilised based on the antigens that give the most useful information for the specific application. The majority are those that generate diagnostically useful information, such as cell lineage and stage of differentiation. The selection of the most appropriate panel of antibodies is largely determined by the specimen type (i.e. fresh cells or fixed paraffin-embedded tissue) and the detection method (i.e. flow cytometry, immunoenzyme or immunofluorescence).

Antibodies routinely applied in diagnostics recognise molecules that are cell lineage-specific (e.g. CD3 T-cell; CD20 B-cell) or associated (e.g. CD2, CD7, CD11c, CD56), or those associated with the stage of haemopoietic differentiation (e.g. CD34, terminal deoxynucleotidyl transferase [TdT]). Antibodies directed to non-lineage associated cellular components, proliferation or cell cycle associated (e.g. Ki67) and apoptosis-associated molecules (e.g. Bcl-2; Bcl-XL) are also widely utilised.

Gene expression cDNA array technology led to the discovery of differentially regulated genes between different neoplastic entities that could assist in disease classification and stratification. Monoclonal antibodies were then generated to the products of significantly dysregulated genes that had been shown to have diagnostic, prognostic or therapeutic relevance. New disease-associated biomarkers were generated to products of genes involved with cell cycle regulation, proliferation, apoptosis, transcription factors and signalling. Some of these "novel" monoclonal antibodies are now being applied by immunophenotyping for clinical use. One example is *SOX11*, a gene encoding a transcription factor which is highly expressed in aggressive (conventional) forms of mantle cell lymphoma but under-expressed in indolent cases. SOX11 protein expression correlates with this gene expression profile. Immunophenotyping for SOX11 using monoclonal antibodies is now applied at diagnosis for prognostic stratification of mantle cell lymphoma [8].

This approach to the generation of new antibodies has expanded the repertoire of diagnostically useful biomarkers for the assessment of haematological malignancies which now enables detection of:

1. Cell enzymes (e.g. myeloperoxidase; tartrate-resistant acid phosphatase)
2. Adhesion molecules (e.g. CD54 [ICAM-1])
3. Protein signalling (e.g. phosphorylated STAT or Akt signalling molecules)
4. Oncogene products (e.g. Cyclin D1; PML protein)
5. Transcription factors (e.g. SOX11)
6. Fusion gene products (e.g. *BCR*/*ABL*; *PML*/*RARA*)
7. Chemokines (e.g. CXCL13)
8. Mutation-specific (e.g. *BRAF* V600E)

With such a vast number of antibodies to an extensive repertoire of antigens, how are antibody panels devised? Overall panel design is not straightforward and there is poor standardisation between institutions. The panel of antibodies will be determined by:

1. Patient clinical history
2. The provisional diagnosis based on cell morphology and/or pattern of bone marrow infiltration
3. Antibodies which will discriminate between disorders
4. The clinical question being asked, i.e. diagnosis, staging, monitoring, prognosis or detecting a potential therapeutic target
5. Whether the phenotype of the abnormal population has been established from previous investigations
6. The sample type being analysed (i.e. smears or fixed bone marrow trephine biopsy)
7. The immunophenotyping method to be used

Standard panels can be structured to address cell lineage, stage of differentiation, to identify disease-associated antigenic profiles and cover common diagnostic problems (e.g. small lymphocytic or blast cell populations) [9–14]. Very large and extremely small focussed panels should be avoided as these can be misleading. Most authors now recommend that rather than applying all antibodies at presentation, sequential use of a comprehensive primary panel followed by a more focused secondary panel provides an alternative and practical approach [10,11,14]. This averts the need for a large number of possibly inappropriate antibodies being used up-front thereby being more cost effective. Applying small primary antibody panels followed by focused secondary panels is equally applicable to flow cytometry and the other immunophenotyping methods. Many attempts have been made to introduce guidelines for antibody panels by consensus groups. The 2006 Bethesda International Consensus Conference on Flow Cytometric Immunophenotyping of Hematolymphoid Neoplasia guidelines recommended using an initial short primary panel followed by a more focused secondary panel to further characterise abnormal populations identified [9]. The European Leukaemia Net (ELN) has also developed consensus guidelines for immunophenotyping of acute leukaemia, lymphoproliferative disorders and myelodysplasia using antibody panels [12,13]. The comprehensive nature of the panels resulted in these being divided into those which were regarded as "mandatory" and others as "complementary". More recently the EuroFlow Consortium published its guidance for antibody panels for 3-laser, 8-colour flow cytometry for acute and chronic leukaemia panels [15,16]. These panels consist of extremely large lists of antibodies that address a large range of clinical questions, but not all of the antibodies included are essential. Because of the size, as well as the antibodies being restrictive in terms of fluorochromes and manufacturers, the EuroFlow Consortium panels have also not gained universal acceptance [16,17].

2.2. Immunophenotyping platforms

2.2.1. Flow cytometry

There have been significant and major developments in clinical flow cytometry since the introduction of the first fluorescence-based flow cytometers 40 years ago. Increased numbers of cellular parameters can be assessed simultaneously and with greater speed of acquisition generating huge amounts of information. These advances can be attributed to parallel achievements in fluorochromes, hardware and data analysis. Fluorochromes, or the fluorescent probe conjugated