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22.2.2 Diagnostic techniques in the assessment of haematological malignancies 5181 and retention of maturing haematopoietic cells within the marrow as shown by targeting of the gene in mice. Mobilization of stem cells from the marrow to the peripheral blood by G-CSF is thought to be secondary to reduction of SDF-1 transcripts and proteolytic cleavage of the protein. Finally, high cell surface expression of CD47 is also important in allowing circulating HSPCs to evade phagocytosis. Instead of needing to collect BM as a source of HSPC, 'mo- bilized' peripheral blood HSPCs can be collected by apheresis. Different preparative regimens and growth factors (e.g. G-CSF) can be used to increase the number of HSPCs collected, with enu- meration of CD34+ cells used as a surrogate marker of HSPCs. Within the CD34+ population, most cells are progenitors and the frequency of true HSCs is exceedingly rare. Plerixafor, a drug that blocks the chemokine receptor CXCR4 from binding to SDF-1, is now used clinically when needed to increase the numbers of cir- culating HSPCs (given with G-CSF). Ways to improve homing to the marrow and/or engraftment are also being studied, particu- larly with the use of umbilical cord blood transplants. These in- clude ex vivo fucosylation of cells to enhance selectin interactions, inhibition of the extracellular peptidase (CD26), which cleaves CXCL12/SDF-1, or pretreatment of donor cells with modified prostaglandin E2 to expand HSCs and to improve homing to the marrow. Thus studies of stem cell–niche interactions may ultim- ately impact clinical medicine, reducing the numbers of stem cells needed for transplantation through more efficient mobilization, homing, and engraftment. FURTHER READING Cellular basis of haematopoiesis Bonig H, Papyannopoulou T (2012). Mobilization of hematopoietic stem/progenitor cells: general principles and molecular mechan- isms. Methods Mol Biol, 904, 1–14. Orkin SH, Zon LI (2008). Hematopoiesis: an evolving

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22.2.2 Diagnostic techniques in the assessment of haematological malignancies

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ESSENTIALS

The diagnosis of haematological malignancies requires an under- standing of the diseases and the uses and limitations of the range of available investigations. The relative importance of different investi- gations varies by disease entity. The blood count is one of the most widely used tests in all of medi- cine and often the first indication of an underlying haematological malignancy. Some blood count features are ‘diagnostic’ and others may give an indication of a bone marrow defect. Morphological assessment of a stained blood film adds value to an abnormal blood count. It may identify abnormal morphology of red cells, leucocytes, or platelets which may be specific and diag- nostic (e.g. lymphoma cells), or give clues suggesting a diagnosis (e.g. red cell rouleaux formation in plasma cell dyscrasias). The bone marrow aspirate (liquid sample) gives cytological detail, whereas the trephine biopsy provides information about marrow cellularity, architecture, cellular distribution, and extent of fibrosis. Immunophenotyping detects cellular antigens in clinical samples and is essential in the diagnosis and classification of haematological malignancies. It is also used for disease staging and monitoring, to detect surrogate markers of genetic aberrations, identify potential immunotherapeutic targets, and to aid prognostic prediction. Cytogenetics assesses the number and structure of whole chromosomes (e.g. the presence of chromosomal translocations) and chromosomal regions in neoplastic cells and is performed to diagnose and classify some haematological malignancies.

 Molecular genetic methods facilitate the detection of mutations, rearrangements, or translocations in genes. Applications in malig- nant haematology include confirming clonality, detecting disease- associated genotypes, determining prognosis, disease monitoring following therapy, predicting imminent clinical relapse, and identifying patients

who are likely (or not) to respond to new targeted inhibitor therapies. Introduction The diagnosis of haematological malignancies is complex and evolving rapidly with many test types available. Optimal test util- ization requires an understanding of the diseases and the uses (and limitations) of the range of available investigations. Morphology, cell phenotyping, cytogenetics, and molecular genetics all have roles with their relative importance varying by disease entity. To use the tests appropriately requires an understanding of the principles of each test type and how they supplement traditional analyses such as blood count and morphological assessment of blood and bone marrow. This chapter provides a guide to the most frequently per- formed tests in the diagnostic assessment of haematological ma- lignancies as well as those that are currently under development. It covers the blood count and blood film, bone marrow examin- ation, immunophenotyping, cytogenetics, and molecular genetics (including 'next-generation' sequencing (NGS)). The reader is re- ferred to other chapters in this textbook that will discuss their appli- cation to the assessment haematological malignancies. The blood count Until the early 1960s the blood count was a manual, laborious test that required centrifugation, spectrophotometry, and cell counting using etched grids. Through modern technology and computing we now have high-throughput automated analysers which use flow technology, electrical impedance, optical light scatter, cytochemistry, and/or fluorescence to measure and count blood cells. These sophis- ticated blood count machines generate large amounts of quantitative numerical data including red cell indices, haemoglobin concentra- tions, reticulocyte counts, leucocyte counts with differentials, and platelet counts and indices. They also produce graphical data and 'flag' cells with abnormal features. Due to the low cost, simplicity, and easy access, the blood count is one of the most widely used tests in all of medicine. As it is commonly used as a 'screening test', this is often the first indication of an underlying haematological malig- nancy. Some blood count features are 'diagnostic'. Other results may give an indication of a bone marrow defect. These 'indicative' blood count features could be in one or more of red cells (anaemia or poly- cythaemia), leucocytes (-cytopenias or -cytoses; abnormal 'flags'), or platelets. Anaemia is a common presenting abnormality for many haem- atological malignancies as a result of reduced erythropoiesis. The red cells are generally normochromic and normocytic. Elevated mean cell volume (MCV) is seen with myelodysplasia and com- monly with plasma cell myeloma. Dimorphic red cells (elevated red cell distribution width) are seen in some cases of myelodysplasia such as those with ring sideroblasts. Polycythaemia vera, charac- terized by increased red cell production, presents with an elevated haemoglobin concentration and haematocrit; the red cells may be normocytic or hypochromic and microcytic (low MCV) if there is iron depletion. Abnormal leucocyte number and morphology are also common at diagnosis of a haematological malignancy. Neutrophilia, although most commonly secondary to infection, inflammation, haemor- rhage, or drugs, can also be seen in neoplastic disorders, particu- larly those of myeloid origin. A mild reactive neutrophilia can be seen as part of the acute phase response (e.g. in Hodgkin lymphoma) whereas a neutrophilia of 50–100 × 109/litre ('leukaemoid' re- action) may be in response to a nonhaematopoietic malignancy (e.g. carcinoma of lung, mesothelioma). Neutropenia may be iso- lated, occur in conjunction with anaemia or thrombocytopenia, or be part of a pancytopenia. Possible causes include failure or sup- pression of granulopoiesis due to bone marrow failure, fibrosis or infiltration, drug therapy, and toxins. The blood count may highlight 'left shift' due to the presence of immature or abnormal granulocytic progenitors in the circulation; this can occur in dysgranulopoiesis (e.g. myelodysplastic syndrome) or in response to peripheral consumption or destruction of mature neutrophils or their precursors (e.g. immune mechanisms) such as in lymphoid neoplasms or hypersplenism. Monocytosis (>1 × 109/litre) is a defining feature of chronic myelomonocytic leukaemia and juvenile myelomonocytic

leukaemia and can be seen in chronic myeloid leu- kaemia. Monocytopenia is rare but is seen in hairy cell leukaemia. Eosinophilia is most commonly secondary to reactions to aller- gens, parasites, or drugs. Primary eosinophil disorders (i.e. chronic eosinophilic leukaemia and myeloid or lymphoid disorders with abnormalities of PDGFRA, PDGFRB, or FGFR1) are rare but eo- sinophilia may be a ‘bystander’ feature in other haematological ma- lignancies (e.g. Hodgkin lymphoma). Peripheral blood basophilia is exceedingly rare, and, when present, should raise suspicion of a myeloproliferative neoplasm, in particular chronic myeloid leu- kaemia. Thrombocytopenia, common at presentation of a haemato- logical malignancy, is usually due to reduced megakaryopoiesis and platelet morphology is normal. Thrombocytosis can be seen with myeloproliferative neoplasms, although is more commonly seen in response to infection or inflammation. Pancytopenia, a reduction in all blood cells, suggests failure of normal haematopoiesis (inherited or acquired), bone marrow in- filtration, or reduced survival of all blood cells as a consequence of drugs or infections. The diagnostic possibilities that arise from an abnormal blood count must be interpreted in the context of the patient’s age, clinical scenario, and associated findings (e.g. physical examination, biochemistry). Blood film Morphological assessment of a stained blood film adds value to an abnormal blood count as it may provide an explanation for the quantitative and qualitative (i.e. ‘flags’) abnormalities. The film may identify abnormal morphology of red cells, leucocytes, or plate- lets which may be specific and diagnostic (e.g. lymphoma cells). Alternatively, they may be features that are associated with, but not diagnostic, of a clinical entity, that is, ‘the company the cells keep’.

22.2.2 Diagnostic techniques in the assessment of haematological malignancies 5183 These accompanying abnormalities (e.g. red cell rouleaux formation in plasma cell dyscrasias) may help generate a provisional diagnosis. Some of the blood film abnormalities that may indicate a malignant bone marrow disorders are described in the following paragraphs. Abnormal leucocytes in the blood may be diagnostic of a haem- atological malignancy. Abnormal lymphoid cells on a blood film are most commonly reactive (e.g. secondary to viral infection) but on rare occasions may be neoplastic. Some malignant cells have characteristic morphology (e.g. hairy cell leukaemia, follicular lymphoma—see also Chapter 22.4.3). However, this is not always the case and it can be challenging on morphology alone to distin- guish between reactive and neoplastic cells. Correlation with clin- ical history and serology is commonly required and, in unresolved situations, flow cytometric immunophenotyping may be indi- cated (see ‘Flow cytometric immunophenotyping’). Most reactive lymphocytoses are of T-cells whereas neoplastic proliferations are more commonly of a B-cell lineage and have restricted kappa/ lambda light-chain expression. The presence of circulating abnormal (‘dysplastic’) neutrophils (e.g. abnormal size, granularity, nuclear segmentation, or chro- matin condensation) indicates dysgranulopoiesis within the bone marrow. The presence of isolated dysplastic promyelocytes in the absence of other neutrophil precursors is highly suggestive of acute promyelocytic leukaemia, which also commonly presents with pan- cytopenia. Blast cells are not normally present in the blood. Their presence may indicate recovery from bone marrow failure, severe sepsis, cytokine administration, or underlying bone marrow path- ology. They may also indicate acute leukaemia, myelodysplastic syndromes, myelodysplastic/myeloproliferative neoplasms, chronic myeloid leukaemia, primary myelofibrosis, or bone marrow in- filtration by a nonhaematopoietic malignancy. More than 20% blast cells in the blood (or bone marrow) defines acute leukaemia. Plasma cells do not commonly appear in the circulation and their presence indicates a florid B-cell response to infection or a B-cell neoplasm with plasmacytoid differentiation (i.e. plasma cell mye- loma, plasma cell leukaemia, or

lymphoplasmacytic lymphoma/ Waldenström macroglobulinaemia). Plasma cells may be accompanied by cytopenias, high red cell MCV, leucoerythroblastic film, rouleaux formation, and background protein staining of the film. Additional biochemical investigations (e.g. serum protein analysis) and bone marrow examination may be required. Although red cells and platelets are rarely directly involved in the malignancy per se, there are commonly 'accompanying' abnormalities in these lineages. Red blood cell morphological abnormal- ities include rouleaux (plasma cell neoplasms), spherocytes and red cell agglutination (autoimmune conditions with a mature B-cell neoplasm), teardrop poikilocytes (myelofibrosis or metastatic in- filtration of the marrow), and hyposplenic features (splenic infiltra- tion). Circulating erythroblasts (nucleated red cells) with abnormal morphology imply bone marrow dyserythropoiesis suggestive of myelodysplastic syndrome or acute myeloid leukaemia. Abnormal platelet morphology occurs with bone marrow dysmegakaryopoiesis such as in the myeloproliferative neoplasms and myelodysplastic syndromes. A leucoerythroblastic blood film (i.e. presence of erythroid and leucocyte precursors in the blood) is seen with bone marrow infiltration (i.e. haematological malignancy, metastatic in- filtrate, or marrow fibrosis), severe sepsis, cytokine administration, and prolonged hypoxia. There may be accompanying other features (as previously mentioned) that may shed light on the diagnosis. From this it can be seen that blood film review is crucial: the findings may be diagnostic, and, if not, they guide the next step in the inves- tigation process. Is a bone marrow examination required to reach a final diagnosis or can flow cytometric immunophenotyping be used to determine the diagnosis? Bone marrow examination Examination of the bone marrow may be required to determine the cause of unexplained blood count or film abnormalities or to confirm a diagnosis suspected from the blood count and film. Indications for a bone marrow examination have been developed by the International Council for Standardization in Haematology and are summarized in Box 22.2.2.1. Bone marrow aspirate and trephine biopsy specimens are generally both taken and these pro- vide complementary information. The aspirate (liquid sample) gives cytological detail, whereas the trephine biopsy provides information about the marrow cellularity, architecture, cellular distribution, and extent of fibrosis. For some disorders, the aspirate may provide suffi- cient information without the need for a trephine biopsy (e.g. acute leukaemia). For others, the trephine biopsy is the prime diagnostic material (e.g. lymphoma staging and myelofibrosis). In addition to morphology, the marrow sample can be used for ancillary biological tests required to reach a diagnosis and World Health Organization (WHO) classification (i.e. immunophenotyping and molecular gen- etics). It is beyond the scope of this chapter to describe the bone marrow morphological findings in haematological malignancies as this will be addressed in accompanying chapters. Immunophenotyping Immunophenotyping is the method by which antibodies are used to detect cellular antigens in clinical samples and is essential in the diagnosis and classification of haematological malignancies (as per WHO criteria). It is also used for disease staging and monitoring, to detect surrogate markers of genetic aberrations, identify poten- tial immunotherapeutic targets, and to aid prognostic prediction Box 22.2.2.1 Indications for bone marrow examination in the diagnosis and assessment of haematological malignancies • Investigation of unexplained cytopenia/s or pancytopenia • Investigation of unexplained blood film morphological abnormalities • Investigation of a paraproteinaemia • To confirm a diagnosis of a haematological malignancy made on peripheral blood • To classify a haematological malignancy • To determine the extent of bone marrow involvement by a haemato- logical malignancy • Bone marrow staging of lymphoma • To obtain prognostic information based on the pattern of marrow infiltration • To obtain specimens for ancillary studies in the investigation of haem- atological malignancies • Post-therapy and post-transplant assessment of haematological malignancies

 (Table 22.2.2.1). Immunophenotyping can be performed on single cells in solution by flow cytometry or on sections of bone marrow trephine biopsies by immunohistochemistry. Immunophenotyping can be used to establish the lineage and stage of differentiation of cells and provide a surrogate of clonality. It can make or confirm a diagnosis based on ‘classical’ disease-associated phenotypic pro- files and classify according to WHO definitions. The technology and antibody panels used to achieve this vary by sample type (fresh or fixed), the suspected neoplasm, and the information required to best characterize the cells of interest. Flow cytometric immunophenotyping Flow cytometric immunophenotyping is the technique of choice for the assessment of cells in blood or aspirated bone marrow. It requires only a small sample, performs high-speed analysis of large numbers of cells, and allows many cellular parameters to be as- sessed simultaneously. It assesses individual cells in suspension for the presence (or absence) of specific antigens. The sample is incu- bated with preselected antibodies, each of which has an attached fluorophore. Following exposure to a laser beam, the cells with bound antibody (and fluorophore) emit light at a specific wave- length which is captured by detectors. This signal is captured and indicates the presence of the relevant antigen. Since morphology cannot be assessed, cells of interest are ‘gated’, i.e., electronically selected based on predefined criteria. This can be on light scatter properties (related to cell size and internal complexity) and/or fluorescence (i.e. antigen expression, such as CD45). Both surface membrane and intracellular antigens (cytoplasmic and nuclear) can be assessed (Fig. 22.2.2.1). Flow cytometers are commonly fitted with three or more lasers and there are many fluorophores available for use. Hence, with this combination, it is possible to assess eight or more antigens simultaneously in one cell. Flow cytometry can therefore provide high diagnostic precision and sensitivity. It can identify disease-associated phenotypes and be used for low-level disease monitoring (i.e. ability to detect one cell with a specific phenotype in 10 000 cells). Imaging flow cytometry is a new technological development which further refines flow cytometry but is yet to find its place in diagnostic practice. In addition to generating standard flow cytometric data, these instruments capture high-resolution im- ages of each cell using digital cameras. This enables the cells being studied to be directly visualized, thereby overcoming the major limitation of ‘standard’ flow cytometry. The cell imagery compo- nent opens possibilities for further study of neoplastic cells, such as detecting colocalized cellular molecules, ‘spot’ counting (e.g. intracellular molecules), integrating phenotype and fluorescent in situ hybridization (FISH), and studying biological process (e.g. cell cycle, mitosis, or apoptosis). Immunohistochemistry Immunohistochemistry (also known as immunocytochemistry) is another immunophenotyping method but where the testing is performed on sections of tissue, in this case bone marrow tre- phines or other haematological biopsies. It is performed using antibodies (usually monoclonal) and antigen–antibody binding is detected with an enzyme (i.e. horseradish peroxidase or alka- line phosphatase) and a chromogenic substrate. Cells of interest are identified by their morphology and location by standard light microscopy. The presence (or absence) of a chromogenic colour reaction shows whether the antigen in question is expressed. Both cell membrane and intracellular antigens can be detected. Fig. 22.2.2.1 Example of flow cytometry of a case of acute myeloid leukaemia. The gated leukaemic (blast) cells on CD45 and side scatter (purple) are then shown to express CD33 and myeloperoxidase. The cells are HLA-DR negative and few express CD34 antigen. Table 22.2.2.1 Clinical applications of immunophenotyping Diagnosis and classification Determine cell lineage Determine stage of cell differentiation Classical disease-associated phenotypes Aberrant antigen expression Clonality assessment Undifferentiated neoplasms Prognostic prediction Antigen expression and prognostic stratification Staging Extent of disease Rare event analysis Surrogate

phenotype–genotype correlation Integrated phenotype and genotype Therapeutic applications Detection of potential immunotherapeutic targets Minimal/measurable residual disease assessment Early detection of disease relapse Bone marrow regeneration following therapy

22.2.2 Diagnostic techniques in the assessment of haematological malignancies 5185 Immunohistochemistry is widely used to refine and classify the diagnosis of haematological malignancies. Other applications include lymphoma staging, detecting antigens associated with disease prognosis and potential immunotherapeutic targets, and disease monitoring. Cytogenetics Cytogenetics is performed to diagnose and classify a number of haematological malignancies according to WHO criteria. It as- sesses the number and structure of whole chromosomes (e.g. the presence of chromosomal translocations) and chromosomal re- gions in neoplastic cells. The ‘gold standard’ tool for basic genetic diagnosis of haematological malignancies is karyotyping. This de- pends on the presence of dividing cells in the sample and is there- fore generally performed on aspirated bone marrow. Although the resolution of karyotyping is limited, and generally only 20 cells are studied, it provides a global analysis of the entire genome. Some karyotypic abnormalities provide a definitive diagnosis (e.g. the Philadelphia chromosome arising from t(9;22)(q34;q11) in chronic myeloid leukaemia). Other karyotypic changes have prog- nostic significance. In childhood lymphoblastic leukaemia, for ex- ample, near-haploidy (<30 chromosomes) is associated with a poor prognosis whereas hyperdiploidy (51–65 chromosomes) carries a good prognosis. Karyotyping is used at diagnosis but since only few cells are analysed it lacks sensitivity when only small numbers of abnormal cells are present (e.g. monitoring for residual disease following therapy). In recent years, a range of FISH and high-resolution array- based techniques have become integrated into the broader field of cytogenetics. These identify chromosomal regions and can be performed on nondividing cells (including smears and tissue sections). This is particularly useful for haematological malignan- cies with a low proliferative index, when chromosome morphology is poor or when full chromosomal analysis is unsuccessful. FISH is based on a single-stranded DNA probe annealing to its comple- mentary sequence in a target genome; it thereby detects and local- izes specific DNA sequences in cell metaphases or interphase cells. Fluorescent probes (i.e. whole chromosome paints or locus-specific probes) are used and these bind to those parts of the chromo- some with which they show a high degree of sequence homology. Binding of the probe to chromosomes is visualized by fluorescence microscopy. In some clinical scenarios it may be necessary to carry out FISH on specific cell types. To achieve this, phenotyping and genotyping can be integrated in a single analysis. This method is called ‘FICTION’ (Fluorescence Immunophenotyping and inter- phase Cytogenetics as a Tool for the Investigation of Neoplasms) and allows specific genetic abnormalities (e.g. gene transloca- tions, numerical abnormalities) to be assessed in cells highlighted (or identified) by their phenotype. Although complex, FICTION increases the accuracy over standard FISH since the genetic ab- erration is assessed only in the cell population of interest. Array- based comparative genomic hybridization and single nucleotide polymorphism arrays are two other genetic methods that can be applied to haematological malignancies. These have facilitated the identification of novel chromosomal abnormalities but have not yet been integrated into clinical practice. Molecular genetics A major change that has occurred over the past 10 years has been the integration of molecular genetic methods into the diagnostic workup of haematological malignancies. We now have a vast array of techniques in our diagnostic armamentarium to facilitate the de- tection of mutations, rearrangements, or translocations in genes. Specific chromosomal changes and perturbed genes can be detected down to single base changes in individual genes, moving diagnostics from morphology to mutation.

Applications in malignant haema- tology include confirming clonality, detecting disease-associated genotypes, determining prognosis, disease monitoring following therapy, and predicting imminent clinical relapse. Molecular gen- etic tests are also increasingly being used to identify patients who are likely to respond to new targeted inhibitor therapies. Some of the techniques that are used in or are applicable to haematology practice are described. Polymerase chain reaction Polymerase chain reaction (PCR), by which DNA is amplified to generate thousands of copies of the same sequence, is one of the most widely used techniques. A series of 25 to 40 amplification cycles are repeated at different temperatures. Each cycle commences with ini- tial denaturation of the complementary DNA strands at high tem- perature. The temperature is then lowered to allow annealing of the added test primers of known sequence to the single-stranded sample DNA template. Addition of DNA polymerase to the template–primer hybrid results in DNA synthesis. Through repeated cycling, multiple copies of the same DNA sequence are generated. The amplified DNA products can be visualized by one of a number of methods (e.g. gel electrophoresis or fragment analysis) or further analysed by a var- iety of other downstream techniques. PCR is rapid, inexpensive, and a simple means of producing relatively large numbers of copies of DNA molecules derived from all haematological sample types (i.e. blood and bone marrow) even when the DNA is of relatively poor quality (e.g. extracted from paraffin-embedded material or air-dried smears scraped from glass slides). JAK2 V617F mutation detection for the investigation of possible myeloproliferative neoplasms is an example of a commonly performed diagnostic PCR test. Multiplex PCR Multiplex PCR uses multiple different primer sets such that a number of target regions can be assessed in a single PCR reac- tion. The amplified DNA regions (amplicons) that are generated in the cycling reaction are specific for each of the different DNA sequences. One common application is the assessment of clonality in lymphoid cell proliferations. B-cell clonality can be identified by immunoglobulin (IG) heavy- and light-chain gene rearrangement and T-cell clonality by rearrangements of the T-cell receptor (TCR) gene. During differentiation of B- and T-progenitor cells, DNA re- arrangements of the IG and TCR genes result in massive diversity of genotypically different cells. This process of gene rearrangement of the V, D, and J domains can be utilized for the detection of clonal lymphoid cells since all the neoplastic cells will have undergone the same IG or TCR gene rearrangement. In a polyclonal population, all the lymphoid cells will have undergone different rearrange- ments. Multiplex PCR assays that use multiple primers have been

 developed for the most widely used V, D, and J domains (such as the BIOMED-2 PCR protocols). These protocols are used to assess IG and TCR gene rearrangements in the investigation of lymphoid proliferations in fresh (blood and bone marrow) and formalin-fixed paraffin-embedded tissue. Nested PCR Nested PCR is performed by two consecutive PCR reactions using two different primer pairs, both covering the region of interest. As a result, nested PCR yields high analytical specificity and sensitivity (as low as one cell in a million). Due to this exquisite sensitivity, nested PCR is used for residual disease monitoring applications fol- lowing therapy. Quantitative real-time PCR Quantitative real-time PCR (RQ-PCR) is used to quantify gene ex- pression. RQ-PCR follows the general principles of PCR, but the amplified DNA is detected in ‘real time’ as the reaction progresses cycle by cycle. It gives a precise measurement of the amount of a spe- cific DNA or RNA in a sample. Quantitation is usually performed by comparing the expression with that of a control (‘normalized’) gene. The method is highly sensitive (one cell in 1000–1 000 000) and is used for residual disease monitoring. It is particularly useful to monitor changes in level of gene expression during and following therapy (e.g. BCR-ABL1 in chronic myeloid leukaemia) and as an ‘early warning system’ to predict disease relapse (e.g. PML-RARA in acute

promyelocytic leukaemia). Reverse transcription PCR Reverse transcription PCR (RT-PCR) uses mRNA instead of DNA as the starting point and can be used to identify the expression of a gene or the sequence of an RNA transcript. It is particularly ap- plicable to haematological malignancies for mutation detection and for the detection of chimeric mRNA resulting from chromosomal translocations. An example is quantitation of BCR-ABL1 transcripts in chronic myeloid leukaemia. Gene expression analysis Gene expression analysis, or microarray-based gene expression pro- filing, allows the simultaneous assessment of the expression of many thousands of genes, and, potentially, every gene within a cell. This has some benefits in assessing haematological malignancies but is not routinely used due to the high test cost. Gene expression analysis has largely been superseded by newer technologies which are more rapid, cost-effective, and simpler to perform. Sequencing Sequencing is the ultimate molecular genetic test. The Human Genome Project, completed over a decade ago, determined the com- plete sequence of base pairs making up human DNA. One of the out- comes has been the ability to identify genetic variants or mutations in malignancies. DNA sequencing methods have changed substantially over time. The ‘first-generation’ sequencing, or Sanger sequencing, is based on the incorporation of fluorescently labelled A, C, G, and T nucleotides during DNA synthesis using DNA polymerase. This remains the ‘gold standard’ method for diagnostic applications but has many limitations. These include the time to perform, low throughput, being labour intensive, and having low sensitivity (the limit of detection of a genetic variant is one cell in five). Massively parallel DNA sequencing Massively parallel DNA sequencing, commonly known as NGS, was introduced in the early 2000s and is a radical change from ‘standard’ Sanger sequencing. NGS records the sequence of DNA while the strand is being synthesized (so-called sequencing by syn- thesis). All DNA fragments in the starting material are sequenced simultaneously thereby generating vast amounts of output and rapidly. NGS can be used to sequence entire human genomes or large fractions, such as the exome. It is now finding its place in diagnostic practice. It has potential for large-scale introduction and to replace other molecular testing approaches. At present, most clinical NGS is ‘targeted’, that is, only a selected number of genes (tens to hundreds) are sequenced. Targeted sequencing is the most likely format that will be incorporated into practice. The principle of NGS is that large numbers (millions or billions) of genome fragments that have been sheared into 100 to 400 base- pair lengths are sequenced in parallel. The fragments are ampli- fied and fluorescence emission or hydrogen ion release from an incorporated nucleotide determines the genomic sequence. Each fragment of DNA is sequenced multiple times: the term ‘coverage’ or ‘read depth’ is a reflection of the number of times a specific re- gion of a gene has been sequenced (these and other NGS terms are shown in Table 22.2.2.2). To ensure accuracy, most technolo- gies aim for greater than 30-fold coverage at greater than 90% of bases for whole genome NGS, 100-fold for exome and 1000- fold for targeted NGS. The sequencing reads are ‘mapped’ to a reference genome and variants (i.e. putative mutations) called (Fig. 22.2.2.2). A ‘variant call’ is a conclusion that there is a nu- cleotide difference from the reference at a given position in the genome. DNA sequencing variants (i.e. mutations, insertions, de- letions, translocations, or copy number variations) may indicate the presence of a mutation or a genetic association. NGS methods are cheap, high throughput, and rapid (with a run time of hours to a few days depending on the technology used). There are a number of commercial platforms available, which differ in their methods for preparing the template for sequencing, the sequencing method, detection (i.e. imaging), and data analysis. All generate huge amounts of data and data interpretation is com- plex requiring bioinformatics expertise. Errors can occur in the technical performance, mapping, and variant calling. The whole human genome, or targeted regions, can be analysed and produce sequences at a subgenomic level in a

clinically useful time frame. Massively parallel sequencing is now beginning to be used clinically to characterize individual patient tumours and to select therapies based on the identified mutations (Box 22.2.2.2). NGS can be used to perform whole-genome, whole-exome, or targeted sequencing. Whole-genome sequencing Whole-genome sequencing determines the complete DNA sequence (coding and noncoding regions) of cells in a sample. Genetic vari- ants are detected by comparing somatic variants between the neo- plastic population and the germline (normal cells from the same individual; Fig. 22.2.2.2). As it is comprehensive, it is an attractive approach, but it generates huge amounts of data making analysis and

interpretation complex. The first report of NGS of a haematological malignancy determined the full sequence and mutation profile of a case of cytogenetically normal acute myeloid leukaemia. This was a major breakthrough and illustrative of the enormous potential for clinical application in the assessment of malignancies. Future devel- opments and cost reductions may lead to it moving from research applications to becoming a clinical tool in guiding management. Whole-exome sequencing Whole-exome sequencing is where only the protein-coding regions of a gene are sequenced. There are 180 000 exons, constituting 1% of the human genome. Mutations in these coding regions of the DNA are likely to of greater significance than in the noncoding regions. The sequencing technology used for exons is the same as for other NGS approaches (i.e. whole genome and targeted). Whole-exome sequencing is quicker and cheaper than whole-genome sequencing. It is generally used as a discovery tool and also has not yet found a place in routine assessment of haematological malignancies. Targeted sequencing Targeted sequencing determines the sequence of predefined selected genes and has an analytical sensitivity of one cell in 50 to 100. The genes assessed are determined by the disease type, the application (i.e. diagnostic, classification, and therapeutic options), and published data. Targeted analysis is usually per- formed to screen for somatic mutation ‘hotspots’ of genes or re- current gene fusions known to occur in the malignant process. Due to the targeted approach, it cannot detect mutations out- side the genes being analysed. Several technologies are available that selectively enrich for relevant genes/regions (target enrich- ment) before NGS is performed. Targeted sequencing is quicker, cheaper, more reliable, and simpler to interpret than whole- genome and whole-exome methods. Targeted sequencing paves the way for panels of genes to be assessed simultaneously; this may obviate the need for individual PCR assays which are cur- rently performed when assessing haematological malignancies. It also offers the advantage of being able to add additional ‘targets’ to the panel as new discoveries are made. Put simply, targeted NGS offers the capability for a holistic ‘multiplex’ genomic plat- form that could replace the current sequential testing methods which are laborious, expensive, and time-consuming. A targeted amplicon sequencing panel for myeloid neoplasms, for example, would include the most relevant ‘key’ gene targets known to be mutated in these neoplasms (e.g. NPM1, JAK2, RUNX1, IDH1/2, and SF3B1). One comprehensive test could be performed which would include all relevant genes required for the diagnosis, clas- sification, and ongoing management. Similar panels could be es- tablished for lymphoid neoplasms. Table 22.2.2.2 Glossary of terms used in genomics and sequencing Adapters Short DNA oligonucleotides that contain the primer sites used by the sequencer to generate the sequencing read Amplicons Amplified regions of DNA Amplification A selective increase in the number of copies of a gene Copy number variation (CNV) The number of copies of a gene varies from one individual to another which may result from insertions, deletions, duplications, and complex variants Deletion Mutation resulting in the loss of part of a chromosome or a gene

Duplication A type of mutation resulting in the production of one or more copies of a chromosomal region or gene Exon Coding region of a gene FICTION Fluorescence Immunophenotyping and interphase Cytogenetics as a Tool for the Investigation of Neoplasms FISH Fluorescent in situ hybridization Genome The complete set of genetic instructions within a cell Indel Insertion or deletion of bases of DNA that cause a shift in a reading frame. A combination of insertion and deletion Insertion A type of mutation resulting in the addition of genetic material Intron Noncoding portion of a gene Library A collection of DNA or complementary DNA (cDNA) fragments prepared for sequencing Library preparation Method to prepare DNA or RNA for next-generation sequencing Massively parallel sequencing Sequencing of many DNA templates simultaneously Mutation A change in the structure of a gene that is different from the reference leading to a variant Read depth Number of times a nucleotide is read Single nucleotide polymorphism A single base difference when the same DNA sequence is compared between individuals Targeted sequencing Sequencing of a selected subset of interest of a genome Translocations Rearrangement of parts between nonhomologous translocations Variants Putative mutations Variants of uncertain significance An alteration in the sequence of a gene the significance of which is unclear Whole-exome sequencing Sequencing of coding regions of the genome Whole-genome sequencing Sequencing of the entire genome, both coding and noncoding regions

SECTION 22 Haematological disorders 5188 These new sequencing technologies offer the prospect of cheaper, faster approaches to the genomic analysis of haematological malig- nancies. However, there are obstacles to be overcome before testing can become mainstream. Clinical laboratory standards, consensus guidelines, and integrated reporting methods need to be devel- oped. These are essential to ensure quality of testing and accurate relevant reporting especially as the aim is to detect clinically rele- vant genomic alterations of diagnostic, prognostic, or therapeutic significance. NGS panels and clear diagnostic algorithms could revolutionize diagnostic testing and lead to clinical applications and personalized pharmacogenomics. Future developments The field of diagnostic testing in haematological malignancies has come a long way since the first descriptions of leukaemia in the mid- nineteenth century. Diagnostic assessment now requires the inte- gration of a number of testing modalities ranging from ‘basic’ tests (the blood count) to manual skill (morphology) and advanced ‘high- technology’ approaches (immunophenotyping and genomics). All deliver information about the biology of the neoplastic cell population from ‘morphology to mutation’. It is the integration of these data that leads to an accurate diagnosis and optimized management of a haem- atological malignancy. Current costs may preclude the full profile of tests being performed on all cases in all settings. As we go forward, we will develop rational diagnostic algorithms and only apply those diagnostic techniques that are required for modern clinical practice. FURTHER READING Bacher U, Schnittger S, Haferlach T (2010). Molecular genetics in acute myeloid leukemia. Curr Opin Oncol, 22, 646–55. Bacher U, et al. (2018). Challenges in the introduction of next- generation sequencing (NGS) for diagnostics of myeloid malignan- cies into clinical routine use. Blood Cancer Journal, 8, 113. Calvo KR, McCoy CS, Stetler-Stevenson M (2011). Flow cytometry immunophenotyping of hematolymphoid neoplasia. Methods Mol Biol, 699, 295–316. Craig FE, Foon KA (2008). Flow cytometric immunophenotyping for hematologic malignancies. Blood, 111, 3941–67. Erber WN (ed) (2010). Diagnostic techniques in hematological malig- nancies. Cambridge University Press, Cambridge. Heel K, et al. (2013). Developments in the immunophenotypic analysis of haematological malignancies. Blood Rev, 27, 193–207. Huret JL, et al. (2013). Atlas of genetics and cytogenetics in oncology and haematology in 2013. Nucleic Acids Res, 41 Database issue, D920–4. Johnsen JM, Nickerson DA, Reiner AP (2013). Massively parallel sequencing: the new frontier of hematologic genomics. Blood, 122, 3268–75. Kuo FC

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