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Editorial note:

In this issue of Topical Update, Dr. Cheuk takes us through the evolution of applications of molecular methods in pathology using examples in haematolymphoid pathology. He also explains the new technique of microarray with its various abilities in understanding diseases and application on individual patients. This article illustrates how advances in basic sciences and informatics technology can be harnessed and applied in the diagnostic laboratories. We welcome any feedback or suggestions. Please direct them to Dr. Polly Lam (e-mail: lamwy@ha.org.hk) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Impact of Molecular Methods in the Diagnosis of Lymphomas

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Overview of conventional molecular techniques in lymphomas

The use of molecular techniques in hematolymphoid pathology started with cloning of the immunoglobulin and T cell receptor genes. [1] This is followed by the cloning of a number of translocation breakpoints in some common lymphoma types.[2-4] Assay of chromosomal breakpoints not only helps in confirming a clonal proliferation but also provides an indication of the type of lymphoma. The main application is to establish clonality or lineage of a lymphoid proliferation.

Southern blot analysis was the standard technique in molecular studies. The advent of the polymerase chain reaction (PCR) provides an alternative technical approach to Southern blot analysis, allowing molecular studies to be performed in many diagnostic laboratories. PCR technique is technically simpler, has a much faster turnaround time, requires a much smaller quantity of clinical materials, and can be performed on archival, formalin-fixed, paraffin-embedded samples (Figure 1).[5] Advances in PCR techniques allow accurate quantitation of the template (real time PCR) and make it possible to use RNA as the starting material (reverse

transcriptase PCR).[6] Fluorescence in situ hybridization (FISH) utilizes oligonucleotide probes to localize specific chromosomal segment so that translocation can be visualized under the fluorescence microscope.[7] This "interphase cytogenetics" technique obviates the need of fresh specimen and cell culture and revolutionizes the traditional cytogenetics.[8] Although FISH may not be as sensitive as PCR-based methods, it is superior in detecting complex karyotypic abnormalities involving multiple fusion partners and has lower false negative rates in detection of chromosomal translocations in some lymphoma types.

At this juncture, molecular technologies not only provide diagnostic aid, but also data useful in prognosis and clinical management. For example, for gastric mucosa-associated lymphoid tissue (MALT) lymphoma, the presence of API2/MALT1 gene translocation indicates that the tumor is unlikely to respond to Helicobacter-eradication therapy, [9, 10]yet progression to a large cell lymphoma is very rare. [11] Quantitation of t(14;18) translocation products and Epstein-Barr virus DNA in patient's plasma can be used to detect minimal residual disease and monitor the clinical course of patients with follicular lymphoma and NK/T cell lymphoma respectively. [12, 13]

Microarray technique

Yet the world is about to witness another major breakthrough in molecular biology. Like many important technological advances in the past, this major breakthrough is made possible with three contemporary developments, namely, the completion of the Human Genome Project,[14, 15] the availability of high-throughput array-based technique, [16] and the advancement of sophisticated bioinformatics strategies (Table 1).[17] The microarray technique uses gene-specific probes that represent thousands of individual genes. The probes are arrayed on an inert substrate and quantities of individual genes in a target sample are assayed. RNA is extracted from the tumor, labeled with fluorescent dye and allowed to hybridize to the arrays. Images are registered by confocal laser scanning. The relative fluorescence intensity of each gene-specific probe is a measure of the level of expression of the particular gene. A greater degree of hybridization manifests as more intense signal, implying a higher level of expression. The data are typically presented in a matrix in which each row represents a particular gene and each column represents a tumor sample. In the most common convention, the color codes used are based on the log ratio for each sample measured compared with a control sample; log-value close to zero are rendered in black, greater than zero in red (indicating upregulation) and negative values in green (downregulation) (Figure 2). DNA samples can also be analyzed to look for amplifications or deletions of genes, or to detect known DNA sequence mutations.

The microarray allows, in one assay, the entire genome to be analyzed globally, so-called "gene profiling", instead of aiming at one or a few specific targets as in the traditional molecular techniques. This approach provides an overall view of the genomic make-up, and provides functional aspects of the genome in action. It not only identifies the aberrantly expressed genes, but also highlights functional groups of genes that are regulated in a similar fashion or involved in a common pathway that underlies many fundamental biologic processes such as lineage differentiation, proliferation, and survival, which may provide insight into the mechanistic aspects of the diseases being studied. The focus in genetic pathways rather than on single genes significantly enhances the power to understand molecular mechanisms of tumorigenesis, as the magnitude of changes in individual genes is very often too small to appear significant. [18]

Gene expression profiling in lymphomas

Diffuse large B-cell lymphoma (DLBCL) is the commonest, high-grade lymphoma with considerable clinical and biological heterogeneity within this diagnostic entity. [19, 20] Gene expressing profiling has identified at least three distinct molecular subgroups that are morphologically indistinguishable: germinal

center B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL and primary mediastinal B-cell lymphoma (PMBL), by their differences in expression of differentiation-related genes and oncogenetic pathways.[21-24] The five-year survival rates of patients with GCB DLBCL, ABC DLBCL and PMBL are 59%, 31% and 64% respectively. [21-24] PMBL, currently diagnosed predominantly based on clinical findings, demonstrates distinctive clinical features such as young age at presentation and involvement of the mediastinum or intra-thoracic structures.[25] By gene expression profiling, PMBL possess a distinct molecular signature in comparison to either GCB and ABC DLBCL.[22, 26] In addition, an unanticipated finding is that PMBL shares a strikingly similar expression profile with nodular sclerosis Hodgkin lymphoma, a tumor that is well known to demonstrate many clinicopathologic similarities to PMBL yet belonging to a different class of lymphoma.[22, 26] Gene expression profiling seems to offer a molecular explanation to this paradox. It has been proposed that PMBL and nodular sclerosis Hodgkin lymphoma may arise from a common precursor B cell in the thymus, or that these two entities may represent opposite ends of a biologic continuum, with the intermediate form manifested as mediastinal gray zone lymphoma.[27] Despite these molecular similarities, the gene expression profiles of the PMBL and nodular sclerosis Hodgkin lymphoma are still clearly distinguishable. [22, 26]

Apart from subclassifying DLBCL, gene expression profile also identifies genes whose expression levels correlate with survival regardless of DLBCL subgroups. [23, 28] Overexpression of genes like PRKCB1, PDE4B, bcl2 is associated with a poor outcome, whereas overexpression of bcl6 and LMO2 is associated with a good outcome, [23, 28, 29]although it is not surprising that some of these genes represent the same genes that distinguish DLBCL subgroups. Those genes that most correlate with survival have been selected out to create a panel of “survival predictor genes”. [23]A survival predictor score can be calculated based on the gene expression with significantly different 5-year survival. Meanwhile, monoclonal antibodies against protein products of genes that are useful in distinguishing GCB and ABC DLBCL have been developed, and subclassification of DLBCL based on immunohistochemical staining with a panel of these markers has been found to correlate with prognosis.[30] It shows that findings obtained from microarray studies can be applied using more accessible procedures carried out in the diagnostic laboratory.

Follicular lymphoma is the second most common and indolent lymphoma with a highly variable clinical course. Some patients may survive more than 15 years following diagnosis or even undergo spontaneous regression, whereas others may succumb in less than 5 years.[31-33] It has been shown that the length of survival of patients with follicular lymphoma can be predicted by gene expression profiling at the time of diagnosis. Two signatures, “immune response-1”, which is associated with a longer survival and “immune response-2”, which is associated with a shorter survival, have been identified.[34] These two signatures, interestingly, are not attributable to gene expression patterns of the neoplastic lymphoid cells, but reflect the character of the tumor-infiltrating immune cells. Immune response-1 indicates the presence of mainly T cells in the immune infiltrate, whereas immune-response-2 indicates an immune infiltrate that is relatively low in T-cell content and relatively enriched in macrophages and dendritic cells.[34]

Gene expression profiling can potentially subclassify the existing heterogeneous diagnostic categories into more homogeneous subgroups, and provide prognostically relevant parameters. At times, it may even outperform the diagnosis rendered by expert hematopathologists. A gene expression signature of Burkitt lymphoma has recently been established, which is characterized by high expression of c-myc target genes, expression of a subgroup of GCB genes, and low expression of MHC class I genes and NFκB target genes.[35, 36] Patients with Burkitt lymphoma diagnosed by gene profiling have a significantly better survival than those having high-grade B-cell lymphoma that lacks the Burkitt lymphoma signature (5-year survival 75% vs 39%), which is an expected clinical outcome for a correct distinction between Burkitt lymphoma and

DLBCL. However, in the same study, some cases of Burkitt lymphoma or atypical Burkitt lymphoma diagnosed by expert hematologists do not show the molecular signature of Burkitt lymphoma, whereas some cases considered to be definitely not Burkitt or atypical Burkitt lymphoma turn out to show the molecular signature of Burkitt lymphoma. These findings suggest that molecular diagnosis of Burkitt lymphoma may be more accurate and more consistent with the expected clinical outcome than conventional diagnosis based on morphology and immunohistochemistry, noting that the diagnostic criteria of this entity were originally derived from the latter.[37] Currently, the major types of lymphoma recognized by the WHO classification can be distinguished from one another by their gene expression profiles. A custom DNA microarray, LymphDx, constructed using approximately 2653 genes claims to be able to distinguish various lymphoma types and subgroups with a concordance of 95% to 100% to the diagnosis based on current methodology.[38]

Molecular technology and the practice of pathology

The world of pathology has witnessed several waves of technological advancement, e.g., the electron microscope and immunohistochemistry, that had profound impact in its practice.[39] At the early period, molecular technique represented no more than a supplement to the existing armamentarium of diagnostic aids in confirming the clonal nature of the tumor and putting the tumor into the existing categories of classification system. As the technology advances, the target of interest expands from an individual gene, chromosomal translocation, to the entire genome. The microarray findings not only purify various categories in the existing classification systems, but also refine and redefine new entities. The findings also evolve from diagnosis-oriented to individual patient-oriented, providing biological parameters that are relevant to prognosis, predicting response to certain therapy, and even identifying potential therapeutic targets in the future. The new information will undoubtedly be incorporated into the definition and diagnostic criteria in the future tumor classifications, similar to what immunohistochemistry has done to expedite adoption of REAL classification to replace the Working Formulation.[5, 40]

Conclusions

As the impact of diagnosis based on genomic features is beginning to be recognized, a post-genomic era has been proclaimed by some investigators.[41] This post-genomic era focuses on the epigenetic aspects of genome, that is, modulation of gene expression without changes in DNA sequence, such as DNA methylation, non-coding RNA, histone modification and chromatin remodeling. Their influence in our understanding of diseases is still limited, but the potential cannot be underestimated as epigenetic control sits in between the genotype governed by DNA sequence and phenotypes dictated by the pattern of gene expression. It appears that epigenetic alterations occur more readily in response to environmental factors than its genetic counterparts, with profound biological consequences.[42] The practice of pathology has been changing since the very beginning of this specialty, and is bound to evolve along the advancement in medicine. The role of the pathologist, however, remains unchanged if not becoming more and more important in the future, as there are no medical professionals who are more capable and appropriate than a pathologist to preside at this strategic position from the bench to the bedside in patient care.

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New lexicology
The first, and usually the more widespread, impact a technology brings are the new terms that come with it. Because of the Human Genome Project, the meaning of the word "genome", which refers to the complete collection of genes in an organism, is well known to most people nowadays. [14, 15] Genomics, therefore, is the study of genomes. Not long afterwards, as molecular biology spreads its influence, the vogue of the suffix "-omics", that is, the study of "-omes", becomes almost unstoppable. Oncogenomics is the study of cancer-related genome; proteomics, the totality of proteins; transcriptomics, the mRNA complement of an entire organism, tissue type, or cell; spliceomics, the alternative splicing protein isoforms; ORFeomics, the DNA sequences that begin with the initiation codon ATG, end with a nonsense codon, and contain no stop codon; kinomics, the protein kinase in a cell; metabolomics or metabonomics, the metabolites; lipidomics, the lipids; glycomics, the glycans, carbohydrate structures… The use of "-ome" and "-omics" is limited only by the imagination, and omeome refers to a complete set of "omes" and omician and omists are people who study omes and omics.[43]

Table 1. New lexicology

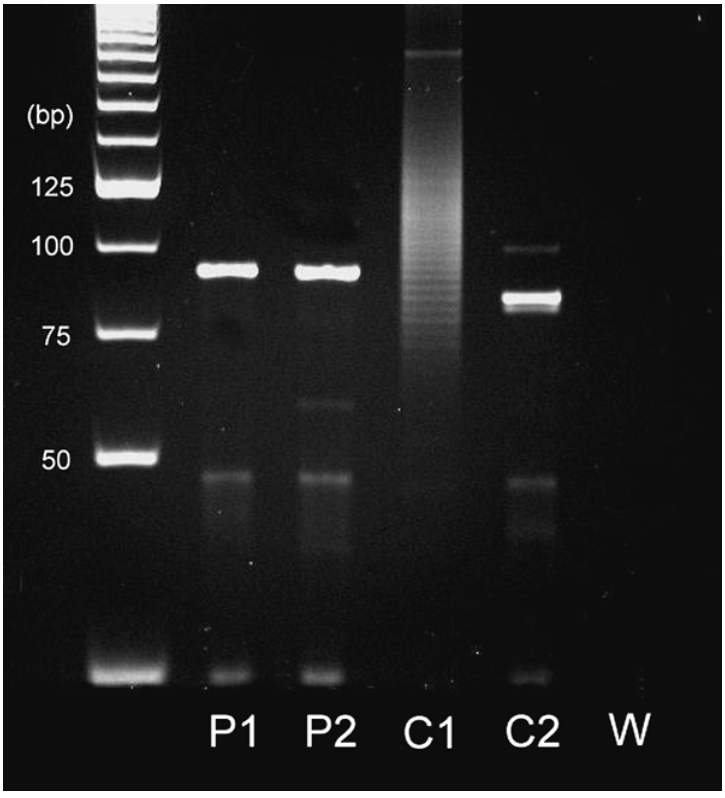

Figure 1

PCR for immunoglobulin gene rearrangement. Left lane, molecular size ladder (bp, base pair); lanes P1 and P2, tumor sample in duplicate showing a single band of identical size; lance C1, polyclonal positive control; lane C2, B-cell lymphoma positive control; lane W, water negative control.

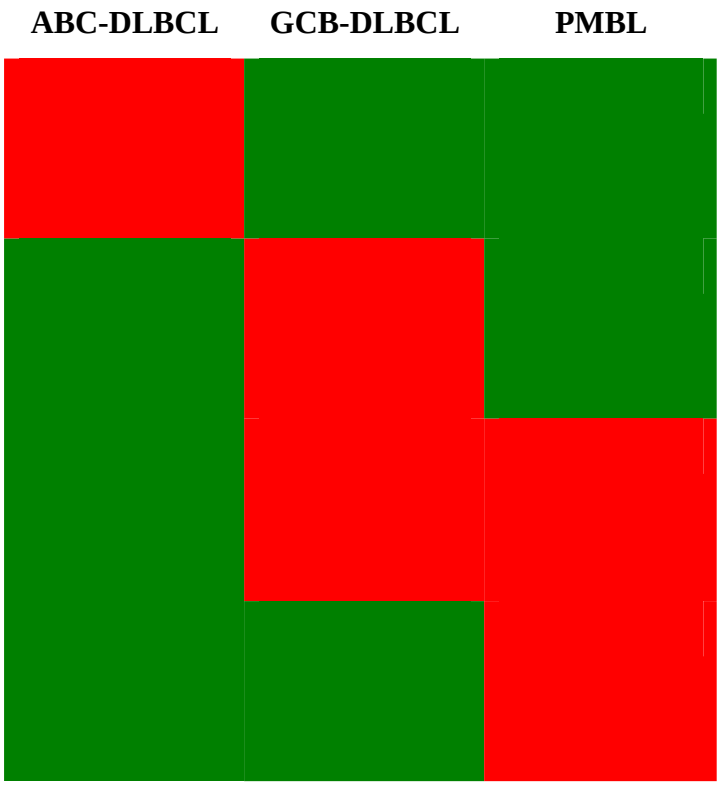

Figure 2.

Three distinct subgroups of DLBCL represented in separate columns. Each row represents a group of differentially expressed genes among these subgroups. Red indicates overexpression and green indicates underexpression.