An introduction to molecular pathology

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Molecular pathology: time for a transformation?

Discoveries in both basic and applied science over the past five years are revolutionizing our understanding of cancer. Pivotal discovery projects, including the ICGC/TCGA consortia, are rapidly providing information on the broad mutational landscape of cancer, revealing ever-increasing levels of complexity [1–8]. Multiple mutational events (between 50 and 200) are identified in early cancers [1–8], highlighting a far greater degree of molecular diversity within cancers than previously thought. Recent reports, based on copy-number variation (CNV) data alone, suggest the existence of at least 10 molecular subtypes of early breast cancer [9, 10]. Each of these subtypes may respond differently to different chemotherapy approaches and this could explain to a large degree the relatively slow progress over recent years in improving outcomes for patients with early breast cancer. Linked research has identified a greater degree of polyclonality within cancers than previously appreciated [3, 4, 6]. There are increasing numbers of reports which document the existence of multiple subclones within patients which may be identified contemporaneously (for example, by single cell analysis [11–13]) or by sampling patients at different times or tumor sites [4, 6, 11]. We now recognize, perhaps as never before, the true molecular complexity, diversity and heterogeneity of even “common” cancers such as breast, colorectal, lung and prostate.

In response to such discoveries, clinical researchers and pharmaceutical companies have been adapting, albeit slowly, their strategies for the development of novel therapeutic agents. The past 10 to 15 years have seen the implementation of targeted therapeutics, specifically directed against molecular events which are pivotal drivers in subsets of cancers. This has led to implementation of an increasing number of molecular diagnostic approaches to predict therapeutic response and target molecular therapies [14–21]. However, the success of molecularly targeted therapies such as Herceptin, Gleevec, Iressa, etc. [22, 23] has, paradoxically, highlighted the growing gap between clinical validation of therapeutic agents and validation of diagnostic tests. Over 30 years after the introduction of tamoxifen as an ER-targeted therapy and over 10 years after the introduction of Herceptin as a HER2-targeted therapy, debate as to the optimal methods for diagnostic testing, the accuracy and interpretation of “personalized diagnostics” remains an area of continuing controversy [24–28]. For neither therapeutic approach was the predictive diagnostic test, currently used in diagnostic pathology laboratories, prospectively validated. Many, indeed the majority of, current molecular diagnostic tests were retrospectively, and some argue poorly, validated prior to implementation. Nonetheless, “targeted” or personalized therapy is rightly viewed as an essential component of future improvements in treatments for individual patients. The rapidly expanding portfolio of targeted therapies, either in pre-clinical development or undergoing testing through clinical trials, represents the potential for an extremely rapid extension of the spectrum and number of diagnostic molecular pathology tests which may be required to implement targeted therapies in the future. For these advances in knowledge to impact on patient management in the clinic, they must be translated into novel diagnostic approaches which match clinical needs, improve patient outcomes and impact on healthcare in a cost-effective manner.
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Personalized medicine requires personalized diagnostics

*Personalized medicine* is the buzz word of patients, clinical managers, diagnosticians and healthcare professionals at all levels. As pathologists and diagnosticians, we recognize that future progress in personalized or targeted medicine depends entirely upon and requires significant and rapid progress in “personalized diagnostics.” Major advances in diagnostic anatomical pathology are essential if we are to deliver new diagnostic approaches to support targeted treatment for cancer patients. Such advances will, nonetheless, require to be fully validated before being implemented into modern healthcare practices. Fundamentally, what was true at the turn of the twentieth century remains true today – *accurate and appropriate diagnosis is fundamental to the successful treatment of disease* – “As is your pathology, so is your medicine” (William Osler, 1849–1919). What has changed dramatically over the past 100 years is our understanding of the scope of the challenge and of the diversity of cancer as a group of diseases, and our ability to address these challenges through a rapid acceleration in technology which puts us in a unique position to provide the molecular diagnostic tools for the twenty-first century which will accelerate improvements in healthcare for the coming generations of cancer sufferers.

It is our conviction that to achieve these goals diagnostic pathologists will need to focus on the delivery of novel “fit for purpose” multiparametric, functional molecular diagnostic assays and, in parallel, deliver established tests to a consistently high standard and continue the process of developing future diagnostic assays. This requires a revolution in anatomical molecular pathology analogous to the rapid development of clinical biochemistry in the 1980s and 1990s. Anatomic pathologists are uniquely placed to facilitate and drive this transformation in diagnostic molecular pathology through a multidisciplinary approach to the development, validation and implementation of complex molecular diagnostic methods. However, the challenge ahead will require both a multidisciplinary collaboration between pathologists, scientist and ancillary laboratory staff and a readiness to innovate and improve existing diagnostic pathology approaches. Failure to rapidly grasp these challenges may lead to further erosion of the pivotal role of anatomical pathology in the management of disease, both within and beyond the scope of cancer diagnostics.

Diagnostic molecular pathology: the challenge ahead

As will be clear from the review of molecular cell regulation (Chapter 2), the scope for both targeted therapeutics and targeted diagnostics is extensive. Even with existing targeted and conventional therapies, the range of candidate predictive and prognostic markers expands on a month-by-month basis. Validation of even a fraction of such markers will require further development of complex molecular diagnostic assays. Given the molecular complexity and heterogeneity of tumors, it is not unrealistic to propose that, within the next 5 to 10 years, diagnostic reporting of 10s to 100s of different molecular variants will be essential to provide relevant information to facilitate appropriate treatment decisions to be made.

Already significant numbers of “molecular diagnostic pathology” assays of varying utility and cost are being offered in different jurisdictions worldwide. These range from *in situ* based analysis of protein expression, gene copy-number/amplification, to mutational analysis, expression arrays of 10s to 100s of genes, etc. As outlined in the following chapters, the clinical challenge addressed ranges from prediction of response to specific therapies (mostly single gene/mutation assays at present), through prediction of residual risk or prognosis following treatment (increasingly multigene assays) to improved molecular classification (diagnosis) of tumors. As assays increase in complexity, we are experiencing a rapid switch from *in situ* manually or visually assessed approaches to multiplex analyses of mRNA, CNA and mutations. As the number of targeted therapies increase, so will the complexity and diversity of molecular diagnostic assays required to provide appropriate diagnostic information for personalized or targeted medicine approaches. In many cancers there is already a requirement for anatomical, expression, mutational and CNA data to be reported on the same tumor sample.

Through a glass darkly – microscopy is not the future of molecular pathology?

As a result, from a personal overview of molecular diagnostic pathology, I have reached the conclusion
that we are experiencing a critical transition point in our discipline – away from "slide-based" visual assessment of molecular markers towards high throughput and quantitative assessment of tumor markers in "liquid phase" assays such as multiplex polymerase chain reaction (PCR), targeted or whole genome sequencing and microarray platforms. The majority of chapters in this volume relating to molecular techniques represent this progressive switch. As molecular diagnostic assays are being developed, we do not conceptualize in situ approaches to mutation detection or methylation and increasingly expression and gene deletion or amplification (copy-number changes) are being addressed using moderate to high throughput liquid phase technologies (see Chapters 6 to 11). Currently, only protein (immunohistochemistry) and DNA/RNA hybridization technologies (see Chapter 5) continue to rely on in situ methodologies. However, these in situ methods represent some of the most challenging aspects of quality control among current molecular diagnostic assessments in clinical laboratories (see Chapter 4).

Since the advent of the light microscope almost 400 years ago, anatomical pathology has been built around the expertise of its practitioners in recognizing morphological patterns to discriminate tumor anatomical type, grade, local invasion, etc. With the advent of immunohistochemistry in the 1980s and in situ hybridization (ISH) in the 1990s, this expertise extended to assessment of protein expression (increasingly on a quantitative level) and gene alterations (deletions, fusions, amplifications). For many tumor types, pathological grade, type, protein expression (either qualitative or quantitative) or gene alterations by ISH are a pivotal part of the decision-making process in determining treatment. The challenge faced in performing subjective, visual assessments of the intensity of immunohistochemical staining, the degree of nuclear pleomorphisms or even the percentage of positive cells (either mitoses or MIB1/Ki67 stained) leads to significant inter-observer variation in each of these areas which can undermine, at least in part, the clinical utility of key pathological variables. Tumor grading, in almost all areas of pathology, is recognized as one of the most significant challenges facing diagnostic pathologists, resulting in considerable inter-observer variability (for example, [29, 30]). Indeed, a comprehensive review of breast cancer grading recognized that "despite the objective improvements that have been made to breast cancer grading methods, any assessment of morphological characteristics inevitably retains a subjective element" [29].

Equally challenging has been the standardization of molecular in situ assessments of protein expression, again particularly when such measures are used to direct treatment (for example, estrogen receptor in breast cancer or Ki67 as a marker of proliferation). Recent ASCO-CAP and expert panel guidelines [24, 27, 31] recognize the significant challenges in reducing to practice quantitative or even qualitative assessment of molecular markers where inter-observer variation and subjective interpretation of results is a critical component of the assay. This is further reflected and emphasized by the review of quality assurance practices for molecular in situ assays (Chapter 4). These challenges by no means undermine the pivotal role of the expert pathologist in providing critical diagnostic and predictive information through visual evaluation of tumor biopsies; they do, however, emphasize the critical need to adapt processes, even where improvements may simply increase the accuracy and reproducibility of current procedures and to re-evaluate procedures based on robust scientific evidence-based approaches to the diagnostic challenges ahead.

A second observation, outlined in brief above, is that the breadth of molecular assessments required will rapidly expand to exceed the capacity and capability of existing pathology laboratories if we remain focused on in situ approaches. Already, it can be challenging to examine multiple molecular markers on limited tissue samples available through diagnostic core biopsies. With a static or even shrinking budget, pathologists are called upon to deliver even more diagnostic results. Even with improvement in image-guided biopsy, microtomy and rapid processing of in situ assays, it is impossible to envisage a workflow which would allow high throughput analysis of 10s to 100s of markers per case which is reliant upon in situ approaches and manual or visual assessment by pathologists. This in parallel with the rapid expansion of mutational assays which cannot currently be assessed by in situ methods argues strongly against the continued development of slide-based assays for future molecular pathology.

Does this mean that the age of the microscope is past? Nearly 400 years after van Leeuwenhoek, is it time to abandon morphological assessment of tumor pathology? Will novel molecular approaches completely replace or supplant anatomical pathology?
I would strongly argue that this is not the case, and that while the future of pathology is increasingly bound up with molecular diagnostic approaches which require quantitative, accurate and reproducible assessment of multiple molecular components of tumors, these approaches will both develop alongside and require a firm foundation in anatomical pathology. As will be apparent in multiple examples from Chapters 12 to 22, molecular diagnostic approaches augment and supplement anatomical pathology assessments and are frequently, if not uniformly, dependent upon them. Detection of HER2 amplification in early breast cancer is essential to direct use of anti-HER2 therapies, but HER2 amplification in pre-invasive ductal carcinomas (DCIS) is more frequent than in invasive cancers [32, 33]. Similarly, assessment of HER2 expression in gastric cancers requires close linkage to the anatomical pathology context of disease. Discrimination between tumor subtypes in ovarian, breast, lung and other organs may ultimately drive the selection of appropriate treatments and molecular diagnostic assays. Furthermore, expression and mutation of key genes can be rapidly and quantitatively assessed by PCR and other methodologies, but normal tissues also express many of the key driver genes and indiscriminate homogenization of cancer biopsies can compromise molecular diagnostic approaches by dilution of the invasive tumor component with normal tissue (stroma, lymphocytes, etc.). Finally, tumor heterogeneity where subclones of invasive tumors may be molecularly distinct presents a clear challenge to novel diagnostic approaches.

The challenge ahead, therefore, is not to forget the past, but to build upon it. It is to develop a cooperative multidisciplinary approach to anatomical and molecular diagnostic pathology which incorporates the highest standards of care for the patient. Novel diagnostic assays must be accurate, reproducible, portable and rapidly scalable if we are to address coming challenges in molecularly targeted therapeutics [34–37]. Existing anatomical pathology approaches will survive only if they match these criteria as well as or to a greater extent than candidate molecular assays. Novel molecular approaches will only be viable to the degree that they extend our ability to manage patients beyond the significant information derived by “conventional” morphological and pathological assessments. To address the increasing complexity of cancer, to improve risk stratification and to accelerate delivery of personalized medicine will require a refocusing and repurposing of diagnostic anatomic and molecular pathology as a discipline at the center, rather than the periphery of modern healthcare. We expect, over the next three to five years, to see an acceleration of delivery of both single gene and pathway or panel-based multiparametric tests which are focused on delivery of personalized medicine. Within or beyond five years, it seems possible, even likely, that multiparametric molecular profiling of cancers will be the norm rather than the exception. The future of anatomical pathology is increasingly linked to development of molecular diagnostic assays which will deliver accurate information to direct clinical decisions by both the patients and their physicians.

Personalized pathology for personalized medicine

As we witness an ever-expanding repertoire of molecular diagnostic tests, it appears that the days of “simple” diagnostic pathology are far behind us. Simply expanding the taxonomy of cancer to include molecular features has significant potential, but unless this expanded classification is linked to treatment decisions it will, ultimately, fail to impact on targeted treatment. In addition to addressing the conventional question, “what kind of cancer is it?” – diagnostic medicine must address two further questions: 1. How should I treat it? and 2. which drugs are likely to be the most effective?

Prognostic, residual risk and predictive diagnostic assays

These, apparently simple, questions encompass a wide range of diagnostic challenges, firstly to determine the elements of tumor pathology and biology which impact on the natural course of disease, to determine the extent of treatment including surgery, local treatment and systemic treatment with cytotoxic and increasingly targeted agents. Patients with low-grade, organ-confined cancer are frequently low risk and can avoid some of the toxic sequelae of adjuvant chemotherapy. Conversely, patients with high-grade disease frequently benefit from aggressive treatment options. The use of conventional histopathological approaches to assess disease prognosis, or risk of recurrence following surgery and local
therapy, has been a mainstay of treatment decision making for decades. Increasingly, molecular diagnostic assays, including multiplex assays, are impacting in this area. In breast cancer, where endocrine treatment is associated with relatively minor side effects, such assays increasingly inform patients of the "residual risk" of recurrence following local resection, radiotherapy and adjuvant endocrine therapy to aid in decisions relating to additional therapeutic options.

However, increasingly, with the advent of molecularly targeted therapies, predictive diagnostic assays, which discriminate between patients likely to benefit from treatments and those who require alternative treatment approaches, are required. These may be those developed in the context of specific molecularly targeted therapies, or possibly in the context of so-called "conventional" chemotherapies. The goal there is not risk assessment in the context of disease progression or prognosis, but specifically aiding treatment selection based on the molecular make up of particular tumors and the existence of molecularly targeted therapies.

Clearly, the individual diagnostic context will differ between tumor types; however, the key challenges of prognosis, residual risk and predicting response to therapeutic agents cross tumor boundaries. To design appropriate diagnostic assays, it will be critical to relate these to the appropriate disease context based on a clear understanding of the objective for which the test is being developed.

Prognosis or residual risk

Almost all patients who receive a diagnosis of cancer have one major question in common with their physician. How likely is it that this cancer will kill me? Some “cancers,” including superficial transitional cell carcinomas of the urinary bladder and non-melanoma skin cancers, rarely result in death, even if treated conservatively. Others, including pancreatic, ovarian and lung tumors are frequently diagnosed late in the disease course and are associated with high mortality. However, many patients, probably the majority, are faced with the dilemma of balancing their risk of succumbing to their disease against the potential costs and benefits of treatment. For most situations, surgical resection is a starting point, with progressively more aggressive local (radiotherapy) and systemic (chemotherapy) treatments given the greater the risk of disease recurrence following surgery.

Strictly speaking, prognosis relates to the study of the natural history of a disease in the absence of intervention. Even the most basic form of intervention, surgery, changes the disease pattern. The result is that in almost every context cancer diagnosis, both conventional and molecular, is used to assess the residual risk of relapse following treatment. If that residual risk is low following surgery or local treatment, then additional adjuvant chemotherapy may be more harmful than beneficial.

Treatment of cancer in the “adjuvant” setting aims to eradicate residual tumor cells at sites distant from the primary tumor, if present, which are not detected and are not removed by conventional surgical approaches. By definition, therefore, adjuvant chemotherapy treats risk of disease – not its actual presence. For every patient for whom treatment is necessary may experience harmful side effects for no personal benefit. A significant minority of patients exposed to adjuvant chemotherapy are not destined for the cancer to recur even if untreated and molecular profiles are increasingly used to propose stratification of cancers and treatment selection [15, 38–42]. Even for patients who benefit from polychemotherapy, therapeutic choices are largely based on risk and average benefit across populations. Lacking a clear understanding of the mechanisms by which drugs act, and therefore the molecular features of tumors which markers prospectively select for response, reduces the ability to rationally develop predictive diagnostic assays. Pathology remains, as ever, rooted in both the understanding of disease mechanisms and the subsequent development of rational diagnostic approaches to disease [21].

Multiple strategies have been applied, particularly in early breast cancer, to develop tools to assess “residual recurrence risk” after surgery, radiotherapy and endocrine therapy to inform the choice between endocrine therapy and/or chemotherapy. These range from algorithms based on historical clinicopathological features (stage, grade, etc.) and patient factors (age, co-morbidities), such as “Adjuvant Online!” [43], through studies exploring the impact of in situ biomarkers on risk of relapse [44, 45], to complex multiparameter diagnostic assays such as Oncotype DX [46]. As this area of research evolves, the proliferation of multiple competing, and modestly selective, risk stratifiers [47] presents a twofold
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The developments highlighted above have, over the past decade or more, begun to impact on the delivery of molecular pathology services. Increasingly, anatomical pathology laboratories are adopting molecular pathology tests and implementing them into their routine workflow. However, increasingly complex, multiparametric assays are being offered by central, commercial laboratories as a solution to the perceived and actual challenges of disseminated molecular testing. More critically, when the “conventional” challenges outlined above (risk stratification, more accurate prediction/selection of both conventional and targeted therapeutics) are aligned with recent advances delineating the molecular landscape and the extent of molecular heterogeneity within common cancers, the scope of the challenge facing molecular pathology both in the clinic and at the research/validation phase becomes daunting.

The goal for the future delivery of molecular pathology is to focus research, both technical and applied, towards the development of clinically applicable and accurate diagnostics for a highly complex disease. Future diagnostic approaches will have to recognize the broad spectrum of molecular events which drive cancers (mutations, CNVs, transcriptional and proteomic changes), to address the increasing fragmentation/subtyping of so-called “common cancers” and to match a rapidly expanding pharmacopeia of conventional and targeted drugs to support the expected advent of personalized medicine.

Molecular pathology – the coming transformation

Summary

Personalized medicine requires personalized diagnostics which in turn requires robust, validated diagnostic tests which can be applied in routine diagnostic pathology laboratories. This is critical to the delivery of personalized medicine in cancer where novel diagnostics will drive improvements in therapeutic targeting by: improved assessment of risk (to exclude patients from unnecessary or harmful treatments); validation of predictive diagnostic assays to better personalize current therapeutic options; and development of theranostics, pairing diagnostics with drugs to accelerate delivery of targeted therapies.

Anatomical pathology is central to the future delivery of accurate diagnostic approaches to disease, particularly for cancer. This will require a rapid adoption of novel approaches to diagnostic medicine throughout the profession, from training to quality assurance programs. Anatomical pathologists are, as always, central to the appropriate treatment of disease because they deliver critical diagnostic information to inform treatment choice. Development of future approaches to personalized medicine cannot be delivered without rapid development and investment in anatomical molecular pathology. While progress has been slow in the past, there is now a wealth of opportunity to address the key challenges facing molecular anatomical pathology.

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References


Overview
Within the human body exists a diversity of cells that perform specialized functions yet contain the same genetic material as all other cells. How, then, does a liver cell "know" how to function as a liver cell and a skin cell, with the same genetic blueprint, function in an entirely different manner? Cellular function is controlled by a highly complex and interconnected network of intracellular signaling pathways. Since all cells, apart from differentiated B-cells and enucleated red blood cells, contain the same DNA “codeset” to direct cellular function, it is clear that for different cellular functions to be manifest some signaling pathways must be activated and others deactivated. Activation of critical pathways, such as metabolic pathways, is common to all cells in the body; however, within cells, specific functional pathways may be “imprinted” during embryological development and differentiation, or modified by specific environmental or developmental cues. The process of silencing some pathways and activating others is fundamental to the development of specific tissue types. The resulting cell is functionally organized, which is a defining feature of a multicellular organism. Despite developmental commitment, however, it is becoming increasingly evident that even terminally differentiated cells retain a degree of developmental plasticity. In normal tissues, for example, monocytes may differentiate into either dendritic cells or macrophages depending on the cytokines produced by infected tissues. In response to extracellular stimuli, metastatic cancer cells may undergo epithelial-to-mesenchymal transition to detach from the tumor and migrate to distant tissues.

Cancer is a disease of deregulation of cellular control pathways and, therefore, to understand cancer it is essential that we understand the regulation of normal cellular function and its broad diversity. This chapter will provide an overview of molecular pathways, regulatory mechanisms and signaling networks that control normal cell function, and outline the challenges to development of therapeutic agents targeting deregulation of key pathways in pathological conditions such as cancer. We will begin by describing the concept of receptor signaling and a simple, linear signal transduction pathway. Additionally, we will discuss more complex pathways, how they engage in signaling networks during cross talk and describe regulatory inputs that control the effects of signaling events. To finish, we will discuss how complex molecular changes within an organism contribute to the continuing “evolution” of adaptive mechanisms in pathological conditions such as cancer.

Signaling pathways
Receptor signaling turns on a switch
Signaling pathways convey information from the extracellular environment to the interior of the cell that leads to a particular cellular response. Most signaling pathways involve a physical interaction between two components: a ligand and a receptor. Ligands, which may be secreted or membrane bound, bind to receptors on another or the same cell. A receptor molecule is usually a transmembrane protein that not only binds a ligand, but also acts to transmit the signal. In many cases, the intermediate molecules are protein or lipid kinases, which phosphorylate a target molecule to activate it. Signals are propagated like waves, which induce reversible changes to signaling intermediates: one activated kinase phosphorylates the next, which in turn phosphorylates another, triggering a chain reaction in a