Genomic Applications in Pathology

George Jabboure Netto
Karen L. Kaul
Editors

Second Edition

Springer

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Second Edition 2019

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ISBN 978-3-319-96829-2 ISBN 978-3-319-96830-8 (eBook)
https://doi.org/10.1007/978-3-319-96830-8

Library of Congress Control Number: 2018962532

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

In the memory of my father whose wisdom will always guide me.

With love and thanks to Tony and our children Nick, Emilia and Alexis.

Preface

The pathologist has an increasingly central role in the management of cancer patients in the era of personalized oncology. Molecular diagnostic and genomic applications are rapidly penetrating the daily practice of the pathologist as the list of actionable genetic alterations in solid and hematologic malignancies continues to expand. At the same time, a paradigm shift in the diagnostic approach for inherited genetic diseases, infectious diseases, and pharmacogenetics is unfolding. As a result, a plethora of clinical genomic applications is being rapidly implemented in diagnostic molecular pathology laboratories as we move closer to the anticipated reality of "precision medicine."

This textbook provides a comprehensive resource of genomic applications to practicing molecular pathologists and hematopathologists, general and subspecialized practicing pathologists, as well as pathology trainees. The target audience also includes oncologists, geneticists, and other medical and surgical clinicians. The 33 chapters encompass a state-of-the-art review of the scientific principles underlying current and emerging genomic technologies and the bioinformatics approaches required to effectively analyze the daunting amount of data generated by next-generation sequencing. Implementation roadmaps for various clinical assays including single gene, gene panel, whole exome, and whole genome assays are addressed. Topics related to reporting and the pathologist's and laboratorian's role in the interpretation and clinical integration of genomic test results are discussed. Practice-related considerations including the regulatory framework, reimbursement, and legal and ethical issues as related to genomic testing are also included. Importantly, chapters on genomic applications for site-specific solid tumors and hematologic and lymphoid neoplasms provide a review with practical and actionable information regarding the latest advances. Finally, genomic applications in pharmacogenomics, inherited genetic diseases, and infectious diseases are also highlighted.

As this most exciting field continues to evolve rapidly, the information in this textbook provides an up-to-date framework for the transition of next-generation sequencing applications from bench to bedside, for genomic assay development, and for responsible implementation of genome-scale testing. We hope that you will enjoy the keen insights from our 62 expert authors and that this text will prove to be a valuable tool in your practice, as it is to ours.

Birmingham, AL, USA — George Jabboure Netto
Palo Alto, CA, USA — Iris Schrijver

Preface

In the few short years since the first edition of our volume on *Genomic Applications in Pathology*, the list of actionable genetic alterations in solid and hematologic malignancies has continued to expand in an unparalleled pace. Equally, momentous advances of the molecular approaches to the study of inherited genetic diseases and pharmacogenetics are unfolding. "Precision pathology" is now an integral part of the practice of "precision medicine."

The current expanded 39 chapters' volume provides the most up-to-date comprehensive discussion of established and emerging genomic technologies and their clinical implementation in molecular diagnostics. As in its first edition, the book places significant emphasis on implementation roadmaps for various clinical assays including single gene, gene panel, transcriptome sequencing, circulating tumor cells and cell-free DNA sequencing, whole exome, and whole genome assays. Detailed guidance on the central role of the pathologist in the interpretation, reporting, and clinical integration of genomic tests is provided. Expert opinions to help navigate growing compliance, reimbursement, and legal and ethical issues are shared in dedicated chapters.

The latest advances in genomic applications in oncologic diseases are addressed in an organ-based format covering the entire spectrum of solid and hematologic neoplasms according to the most current practice guidelines. Dedicated chapters to genomic applications in inherited diseases, sequencing cell-free DNA in maternal circulations, infectious diseases, pharmacogenomics, and the microbiome are also provided.

In a collective effort of 98 expert authors, our textbook will serve as a comprehensive resource for practicing molecular pathologists, general and subspecialized practicing anatomic and clinical pathologists, as well as pathology trainees. The wider target audience continues to include oncologists, geneticists, and other medical and surgical clinicians.

Birmingham, AL, USA — George Jabboure Netto
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Part I

Genomic Technologies

1 Practicing Pathology in the Post-genomic Era: Challenges and Opportunities

Karen L. Kaul

Introduction

Medicine and the field of pathology are both rapidly changing. In this era following sequencing of the human genome, rapid advances in knowledge applicable to patient care occur constantly, as the molecular basis for constitutional and somatic disease is elucidated, along with targets for more effective treatment. This is the era of precision medicine. Considerable technologic advances coupled with our rapidly expanding knowledge allow clinical laboratories to provide more information than ever before, within a time frame that allows efficient patient care and improved outcomes. The combination of new knowledge regarding the molecular basis of disease, the novel technologies, and the growing ability to use archival formalin-fixed paraffin-embedded (FFPE) samples and those that are obtainable by noninvasive methods will facilitate further growth in this area. This genomic information, along with treatments targeting the specific molecular defect(s) causing the disease, makes up the new discipline known as precision medicine.

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Many challenges come along with the tremendous potential offered by precision medicine. The rapid evolution of knowledge means that clinical laboratories must often update assays to be consistent with standard of care as defined by consensus guidelines and routine practice. The pathology community, along with clinical practitioners, must become aware of these new advances and fluent in their applications to patient care. Regulatory oversight and limitations to reimbursement continue to present challenges to clinical laboratories performing genomic testing. Outcome studies illustrating the advantages of incorporating genomic data into the care of patients are sorely needed. This chapter will outline the current state and major issues facing genomic pathology, including these opportunities and challenges, as a preface to the more detailed discussion in the subsequent chapters.

Molecular Targets

Precision medicine entails measurement of personalized information for each patient using a host of new diagnostic tools made possible by recent advances in analytic methods. DNA, RNA, and protein targets have been employed thus far. The speed and cost of analysis, coupled with the ability to accurately analyze formalin-fixed paraffin-embedded tissue samples, have led to the predominant use of DNA analysis, fluorescent

G. J. Netto, K. L. Kaul (eds.), *Genomic Applications in Pathology*,
https://doi.org/10.1007/978-3-319-96830-8_1

in situ hybridization (FISH), and immunohistochemical (IHC) stains, though mRNA and miRNA targets are also utilized.

Genomic or DNA Targets

DNA remains the primary analytic target used in precision diagnostics. DNA offers the advantages of stability, relative ease of recovery, and extensive and growing knowledge regarding mutations and alterations that are clinically relevant. These alterations include single nucleotide substitutions or mutations, copy number variants, deletions, translocations, and other chromosomal rearrangements [1]. General analytic approaches and specific platforms differ in their ability to detect these abnormalities, so assay selection will require understanding of the data needed as well as the clinical application.

Specific genomic alterations are known to have a causal role in the development of many tumors [1]. Data from The Cancer Genome Atlas (TCGA) project and other efforts has been instrumental in reshaping the classification of many cancers [2]. In 2016, for example, new World Health Organization (WHO) classifications for brain tumors and lymphoma were published, demonstrating that knowledge of genomic changes is fundamental to the diagnosis and prognosis of a growing number of tumor types [3, 4].

Beyond diagnosis, specific knowledge of genomic alterations is often necessary for appropriate treatment planning. For an increasing number of malignancies, a standard and required part of the pathology report is the notation of important gene alterations that make the tumor susceptible to targeted therapy. The prototypic example in solid tumors is KRAS, a GTPase critical in signal transduction, which is mutated in a wide range of tumor types [5]. A landmark study presented at the American Society of Clinical Oncology (ASCO) meeting over a decade ago reported that patients with metastatic colorectal cancer harboring a mutated KRAS failed to respond to targeted therapy with cetuximab [6, 7]. Molecular pathology labs worked quickly to develop and validate reliable clinical KRAS assays [8, 9]. In 2009, the National Comprehensive Cancer Network (NCCN) and ASCO together recommended mutational profiling of KRAS exons 12 and13 before institution of anti-EGFR therapy for patients with metastatic colorectal cancer. It is since the standard of care to assess formalin-fixed paraffin-embedded tumor tissues from patients with metastatic colon cancer for *KRAS* mutation status [10, 11]. A few years later, new data demonstrated that mutation analysis of other RAS genes was also needed [12, 13]. With the constant evolution of the molecular knowledge base, molecular pathology diagnostic laboratories must be prepared to adjust laboratory assays and clinical practice accordingly and ensure quality assay performance as clinical needs evolve [14].

RNA

While DNA is perhaps most frequently analyzed molecular target, mRNA can also be extremely useful. mRNA offers the potential advantage of smaller analytic size since it is generated post-splicing of exons. It thus can allow a more simplified approach to analysis of large genes with many exons or detection of translocations or deletions. However, mRNA is labile and will degrade at variable rates following sample collection or tissue devascularization. Pre-analytic issues such as ischemic and transport time may thus have a significant impact on the quality of the results [15]. Recently, smaller noncoding RNA molecules known as miRNA have been studied for potential clinical use; miRNA offers the advantage of high stability in tissue and other biologic samples, along with effective recovery from FFPE samples. miRNA is also variably expressed in different tissues and tumors, allowing expression profiling as a research and clinical tool; this approach has been used to generate risk scores predictive of tumor progression [16, 17]. Transcriptome analysis by next-generation sequencing (NGS), such as RNA-Seq, is an important approach to the study of complex gene expression and is increasingly playing important role in biomarker analysis and discovery.

Protein

Pathologists have been using proteins and protein expression as an adjunct to histology for decades. The use of single target immunostains for cellular proteins became routine in the 1980s, with multiplexed detection developed thereafter [18]. More advanced proteomic approaches using mass spectrometry on tissue sections are under development and may impact the practice of anatomic pathology in the future [19]. Protein-directed approaches may be quite complementary to genomic studies as genomic and transcriptomic alterations ultimately impact protein structure and expression.

Analytic Methods

Laboratories use a variety of methods to detect mutations and other genomic abnormalities. Analytic approaches have advanced significantly in recent years. Sanger sequencing, pyrosequencing, allele-specific amplification, PCR-melt curve analysis, and multiplexed methods such as SNaPshot, dHPLC, and SNP arrays have all been utilized to characterize tumors for mutations [20]. The development of NGS platforms that address the speed, cost, and throughput needs of the clinical laboratory settings has paved the way for NGS to become a routine approach used for analysis of tumors and germline samples. Simultaneous interrogation of multiple genes, whether by targeted gene panels or broader analysis, is a more efficient and cost-effective way to profile tumors and other samples and make optimal use of the small-sized samples often available.

At present, two commercial platforms have captured the majority of the clinical market: most labs are utilizing either Illumina or ThermoFisher Ion Torrent technology for NGS. These platforms differ with respect to chemistry, DNA input requirements, time for analysis, and sample throughout. They have differing strengths and weaknesses for clinical analysis [21–23]. Each manufacturer offers a range of platforms suitable for small, targeted analyses to larger, high-throughput, and whole genome analysis. Novel, more rapid, and single molecule approaches may reach mainstream clinical utilization in the future. A summary of the features of various NGS platforms is provided in Table 1.1 [24].

Table 1.1 Summary of currently available instruments for clinical next generation sequencing

Manufacturer	Model	Sequencing chemistry	Analytic capacity	Analytic time	Instrument cost
Illumina					
	MiniSeq	Synthesis	7.5 Gb	4–24 h	49K
	MiSeq	Synthesis	15 Gb	1–2 days	100K
	NextSeq	Synthesis	120 Gb	1–3 days	
	HiSeq	Synthesis	1500 Gb	3 days	125K
	Firefly	Single channel Semiconductor	1 Gb	3–13 days	30K
ThermoFisher Ion Torrent					
	PGM	Semiconductor	30 MB–2 Gb	2–7 h	80K
	S5	Semiconductor	0.5–15 Gb	2–4 h	65K
	Proton	Semiconductor	10–15 GB	2 h	
Pacific Biosciences		Long-read sequencing			
	Sequel		0.5–1 Gb		350K
	RSII		5–10 Gb	20 min	700K
Oxford Nanopore		Single molecule Nanopore sensing			
	PromethION		128 Gb	1–48 h	Minimal
	Minion		21 Gb	1–48 h	Minimal

Adapted by permission from Springer Nature, Kaul [24], and from Perkel and Fung [39], and corporate websites

Interpretation of Data/ Informatics Pipeline

The immense amount of complex data generated by next-generation sequencing instrumentation is a challenge to laboratories and healthcare institutions, requiring concomitant investments and advances in informatics and laboratory systems able to handle this data. Interpretation of the data is also a challenge, requiring multiple steps and informatics tools used in tandem to generate a final result from the raw data. Collectively, these software tools are called the "bioinformatics pipeline" and are a critical part of sample analysis and generation of results [25]. The goal for clinical laboratories performing NGS is that the correct result be generated in each analysis and in each laboratory. The complexity of the informatics pipeline for NGS, and the fact that labs may use different components and settings in the pipeline, makes this a potential source of variation. Thus, clinical validation of an NGS assay requires validation of the bioinformatics tools in addition to the "wet lab" portions of the assay that generate the raw sequence data.

Regardless of the sequencing platform used, primary data interpretation begins with raw data requiring signal/noise determination and production of sequence reads, which leads to generation of a FASTQ file. Quality scores can also be generated at this step and can be used for filtering of poor-quality results. Sequence reads are next assembled and aligned, and variant calls are performed, resulting in the VCF file, with generation of additional quality indicators such a depth of coverage. Lastly, variants are annotated, and associated gene variants and other alterations are done via database searches [20]. For clinical reporting, links to information on clinical utility and clinical trials are often useful and may be included in reports.

While the sequences generated will remain static, the interpretation may change over time as new information is gathered regarding variants, adding complexity to the management of genomic data. Similarly, the clinical significance of recognized mutations and variants may evolve as new treatments and trials develop. Thus, unlike other laboratory reports, the interpretive component of NGS studies may be subject to reinterpretation, in which case careful tracking of versions and dates will be important for clinical and potentially liability purposes. While germline sequencing results will remain constant (though the clinical importance of variants will change), the genomes of tumors are known to evolve, particularly under the selective pressure of treatment. No current guidelines exist that recommend schedules for re-biopsy and NGS analysis.

Scope of Analysis

Studies suggest that the human genome includes 30,000 genes, of which 20,000 might be involved in carcinogenesis [3]. These genes sort into 1 of 12 classes such as signal transduction, cell cycle control, and other functions. One of the evolving questions is how broad an analysis should be performed for somatic or constitutional disorders. Genomic analysis can target a single locus, a gene panel, an exome, or a whole genome. The current clinical needs for analysis of multiple genes have driven the move to NGS in place of individual gene assays, but there remains discussion as to what is "actionable" and how many genes to include. Consensus treatment guidelines generally include multiple gene targets for which strong evidence of clinical utility has accumulated [26, 27]. Beyond that, there are many genes that might be included but which lack definitive evidence such as that obtained from a prospective clinical trial. Analysis beyond a targeted gene panel might include these genes implicated in cancer or perhaps the entire coding or transcribed portion of the genome. This whole exome sequencing (WES) would encompass all coding regions of the genome, similar to transcriptome analysis (which starts with reverse transcription and amplification of mRNA). Both would yield information on mutations, while transcriptome analysis also yields information on gene expression [28]. These analyses would not include regulatory regions, sequences that might alter splicing, or other areas within the so called dark matter, which constitutes the majority of the

Table 1.2 Genomic analysis in tumors

	Targeted analysis	Whole exome	Whole genome	Transcriptome
Includes	Known hotspots 50–200 genes	Coding regions of 20 K genes	Coding and noncoding	Transcribed genes (mRNA)
Detects	Miss new mutations	Translocations Splice variants	Regulatory regions Structural variants	Expression patterns Pathway analysis Splice variants
Utility	Established clinical utility	Clinical utility evolving Discovery?	Clinical utility? Discovery Variants of unknown significance	Clinical Utility evolving Discovery
Analysis	Complex Known targets	More complex Map to genes/pathways	Very complex Map to genes/pathways Determine relevance of variants	Complex Map to pathways
Cost	$	$$	$$$	$$
Clinical QC	Clinical QC, PT available	?	??	??

Adapted from [24]
Adapted by permission from Springer Nature, Kaul [24]

genome and which to date we know relatively little about. The most expansive analysis would of course be sequencing the whole genome (WGS), which at this time is generally viewed as a research tool. These approaches are summarized in Table 1.2 [24].

Most clinical sequence analysis currently covers a panel of 50–500 genes inclusive of known hotspots and genes implicated in cancer. The more broad panels include genes for which less is known and may thus generate variants of undetermined significance (VUS), which present interpretive challenges for clinical labs. Beyond clinical utility, cost and reimbursement issues may influence the chosen size of the NGS panel. Conversely, broader analysis such as WGS offers the opportunity to gain discovery data on tumor samples that may add to our knowledge and future clinical use. As our knowledge expands and the cost of analysis declines, it is anticipated that broad genomic analysis will become more routine.

Other approaches may be needed to fully characterize genomic abnormalities in tumors, providing complementary information to NGS. Complete analysis may require additional approaches such as fluorescent in situ hybridization, arrays, or other approaches for assessment of structural abnormalities and copy number variations. Novel sequence variations may require extensive in silico and even functional studies to determine significance.

An additional consideration is the sequencing of paired tumor and normal DNA. While the allele frequency in NGS can provide evidence regarding whether a nucleotide variant is somatic or germline, certainty may require the paired analysis of both tumor and non-tumor samples.

Genomics Education

Training Students

As we enter an era in which incorporation of genomic information becomes a routine part of medical care, all physicians will need a basic education in the molecular basis of disease, diagnosis, treatment, and disease monitoring. Medical schools are beginning to incorporate this foundation [29].

Training Clinicians

These advances are broadly revolutionizing the practice of medicine, especially oncology. The pace of change, the rapid accumulation of new