Sinchita Roy-Chowdhuri
Paul A. VanderLaan · John M. Stewart
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Molecular Diagnostics in Cytopathology

A Practical Handbook for the Practicing Pathologist

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Preface

This book is a compilation of high-yield molecular pathology topics relevant to the field of cytopathology in a user-friendly format that can be used as a reference handbook by training and practicing pathologists and laboratory personnel dealing with, and interested in, this evolving field.

Molecular diagnostics are increasingly used to help guide targeted therapy in solid organ tumors and hematologic malignancies. A large proportion of molecular testing is performed on limited-volume samples obtained via minimally invasive techniques, such as fine needle aspiration. Increasingly, cytopathologists play an essential role in this process, both in the triage of specimens during rapid on-site evaluation and in the evaluation of archival samples to determine suitability for ancillary testing. Therefore, it is imperative that practicing cytopathologists stay abreast of up-to-date diagnostic, prognostic, and predictive ancillary tests that can be used on limited cytologic material. This is a challenge since the landscape of known genomic alterations is constantly evolving and the subsequent set of testing options is ever expanding. In addition, many practicing cytopathologists have not had substantial molecular pathology training during residency or fellowship; therefore, the basic core principles of molecular testing may remain elusive.

The main focus of this book is to provide an overview of the principles of molecular diagnostics in context of cytopathology specimens together with a basic understanding and working knowledge of the available technology, platforms, and clinical applications. The initial sections of the book summarize the pre-analytic aspects of molecular testing, including

cytology specimen preparation and handling, specimen selection and evaluation, and workflow algorithms as well as the analytic considerations for a variety of nucleic acid and protein-based testing. The remaining section focuses on disease-specific molecular testing for various organ-based applications.

Molecular cytopathology is an expanding and evolving field. With the increasing demand for molecular testing on small specimens, cytopathologists need to understand how to efficiently select and triage the best tissue for testing as well as interpret the molecular test results in context of the morphology to cement the role of cytopathology as an independent and essential component of diagnostic and precision medicine.

We hope this book will serve as a practical handbook of clinical molecular diagnostics in context of the cytopathology specimen.

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Part I
Overview of Molecular Cytopathology

Chapter 1
Introduction: Overview of Current Molecular Diagnostic Testing on Cytology Samples

Michael H. Roh and Rashmi Kanagal-Shamanna

Abbreviations

ALK	Anaplastic lymphoma kinase or ALK receptor tyrosine kinase
BRAF	v-raf murine sarcoma viral oncogene homolog B
CEP	Centromeric probe
DDIT3	DNA damage-inducible transcript 3
EGFR	Epidermal growth factor receptor
EWSR1	Ewing sarcoma breakpoint region 1
FISH	Fluorescence *in situ* hybridization
FNA	Fine-needle aspiration
FOXO1	Forkhead box O1
FUS	Fused in sarcoma

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GNA11	Guanine nucleotide-binding protein subunit alpha-11
GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha
HER2	Human epidermal growth factor receptor 2 (*ERBB2*)
HPV	Human papillomavirus
KIT	KIT proto-oncogene receptor tyrosine kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
MDM2	Mouse double minute 2 homolog or E3 ubiquitin protein ligase homolog (mouse)
MEK1	Mitogen-activated protein kinase kinase 1
MYCN	v-myc myelocytomatosis viral oncogene homolog, neuroblastoma derived
NRAS	Neuroblastoma RAS viral oncogene
PDGFB	Platelet-derived growth factor β
PDGFRA	Platelet-derived growth factor receptor A
Pter	Terminal of chromosome short arm
Qter	Terminal of chromosome long arm
RNA	Ribonucleic acid
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase
SS18	Synovial sarcoma translocation chromosome 18
TFE3	Transcription factor binding to immunoglobulin heavy constant

Key Points

- Molecular testing plays a critical role in the work-up of both solid tumors and hematological malignancies for the purposes of diagnosis and biomarker identification for assessment of prognosis and therapeutic targets
- Pathologists have a central role in integrating appropriate molecular ancillary testing into routine cytopathology workflow to enable personalized medicine

During this continuously evolving era of precision medicine, there have been tremendous advances in our understanding of the molecular genetic aberrations (e.g., mutations, gene amplifications, and gene rearrangements) that underlie and drive the growth and spread of various cancers. This has been complemented by advances in molecular diagnostic technologies available to pathologists and laboratory personnel to interrogate and detect these genetic abnormalities. Concurrent advances in minimally invasive interventional procedures have resulted in an increasing reliance on small biopsies along with exfoliative and fine-needle aspiration (FNA) cytology specimens not only for diagnostic purposes but also for ancillary molecular testing to guide the increasingly "personalized" management of patients with cancer [1–4]. In addition, the rapid development of new high-throughput and multiplex molecular testing modalities holds the promise to address the issue of simultaneously testing for a multitude of different genomic abnormalities utilizing cytology samples and small biopsies [5–8]. Furthermore, the recognition of tumor heterogeneity at the molecular level places an increasing demand for these procedures, as will the need to analyze multiple samples from the same patient over time in order to monitor the pattern of evolution of molecular abnormalities [9]. This would serve to reevaluate patients with cancers which have stopped responding to targeted therapy in order to identify new alterations which may be amenable to a switch in a targeted therapeutic regimen. Consequently, the importance of effectively integrating molecular ancillary testing with anatomic pathology workflow, particularly with regard to cytologic and small biopsy specimens, has never been greater.

Section II of this book will broadly address the practical considerations of cytology specimen processing in the molecular laboratory. Namely, chapters will be dedicated to the types of preparatory protocols and platforms utilized for molecular ancillary testing (Chap. 2), sample acquisition and test requisition (Chap. 3), specimen assessment/selection

along with triaging workflows for this purpose (Chap. 4), technical aspects of the various molecular diagnostic techniques and the practical implications for each (Chaps. 5, 6, and 7), and the interpretation of molecular diagnostic results along with how they correlate with cytopathologic diagnoses (Chap. 8).

Section III of this book will discuss the clinical settings in which molecular testing dovetails with the evaluation of cytology and small biopsy specimens, with respect to specific organ systems. In one aspect, molecular ancillary tests can be seen as adjuncts to aid in the diagnosis of small biopsies and cytology specimens [10–19]. Some key examples, not intended to be exhaustive, are listed in Table 1.1. The classic example is human papillomavirus (HPV) testing applied to gynecologic cytology specimens, which can be particularly useful to help guide the management of patients with indeterminate Pap test results [20]. HPV testing is also being increasingly applied in the diagnostic work-up of head and neck squamous cell carcinomas [21–24]. Detailed discussion of these applications will be addressed in Chaps. 9 and 10. Next, a variety of molecular diagnostic approaches have emerged to aid in the diagnosis of indeterminate thyroid FNAs [10, 15–17, 25–29]. These include high-throughput analysis of gene mutations and gene rearrangements, gene expression classifier analysis, and microRNA expression profiling (Chap. 12). Fluorescence *in situ* hybridization (FISH) analysis has also emerged as a useful diagnostic adjunct in the evaluation of pancreatobiliary brushings (Chap. 15) and urinary tract cytology specimens (Chap. 16) [11, 12, 18]. FISH testing also serves an instrumental role in the diagnosis and subclassification of salivary gland neoplasms (Chap. 14) and sarcomas (Chap. 18) in FNA and small biopsy samples [14, 30, 31].

Alternatively, molecular diagnostic adjuncts can be utilized to interrogate biomarkers that provide information pertinent to prognosis and/or targeted therapeutic strategies [1, 32–43]. Salient examples are listed in Table 1.2 and include molecular testing of non-small cell lung carcinomas for *EGFR* and *BRAF* gene mutations along with *ALK* and *ROS1* gene rearrangements (Chap. 11); testing of metastatic

TABLE 1.1 Examples of molecular testing as diagnostic adjuncts applied to cytology and small biopsy specimens

Context	Molecular diagnostic adjuncts
Liquid-based cervical cytology	HPV testing
Head and neck squamous cell carcinoma	HPV testing
Thyroid FNA cytology	Gene mutation and rearrangement analysis Gene expression classifier testing microRNA analysis
Urine cytology	FISH probe set (CEP3, CEP7, CEP17, 9q21)
Pancreatobiliary brushing cytology	FISH probe set (CEP3, CEP7, CEP17, 9q21) FISH probe set (1q21, 7p12, 8q24, 9p21)
Renal cell carcinoma (examples below)	FISH probes
• Clear cell renal cell carcinoma	3pter/3qter
• Papillary renal cell carcinoma	CEP1, CEP7, CEP17
• Chromophobe renal cell carcinoma	CEP1, CEP7, CEP17
• Translocation-associated renal cell carcinoma	*TFE3*
Soft tissue tumors (examples below)	FISH probes and RT-PCR assays
• Alveolar rhabdomyosarcoma	*FOXO1*
• Alveolar soft part sarcoma	*TFE3*

(continued)

Table 1.1 (continued)

Context	Molecular diagnostic adjuncts
• Clear cell sarcoma	*EWSR1*
• Dermatofibrosarcoma protuberans	*PDGFB*
• Desmoplastic small round cell tumor	*EWSR1*
• Ewing sarcoma	*EWSR1*
• Myxoid/round cell liposarcoma	*DDIT3/FUS*, *DDIT3/EWSR1*
• Synovial sarcoma	*SS18*
• Well-differentiated liposarcoma	*MDM2*
Small B-cell lymphomas	
• Lymphoplasmacytic lymphoma	*MYD88*, *CXCR4* mutation analysis
• Follicular lymphoma	t(14;18)(q32;q21) *IGH/BCL2*
• Mantle cell lymphoma	t(11;14)(q13;q32) *CCND1/IGH*
• Hairy cell leukemia	*BRAF* mutation analysis
• Extranodal marginal zone lymphoma	t(11;18)(q21;q21) *BIRC3/MALT1*; t(14;18)(q32;q21) *IGH/MALT1*; t(3;14)(p14.1;q32) *FOXP1/IGH*; t(1;14)(p22;q32) *BCL10/IGH*
Burkitt lymphoma	t(8;14)(q24;q32) *MYC/IGH*
Extranodal NK/T-cell lymphoma, nasal type	EBV testing
ALK-positive T-cell lymphoma	*ALK* rearrangement

Table 1.2 Examples of molecular testing as prognostic/theranostic adjuncts applied to cytology and small biopsy specimens

Context	Molecular diagnostic adjuncts
Non-small cell lung carcinoma	*EGFR* and *BRAF* mutation testing *ALK* and *ROS1* rearrangement testing PD-L1 testing
Melanoma	*BRAF*, *KIT*, *GNAQ*, *GNA11*, *NRAS*, *MEK1* mutation testing
Colon adenocarcinoma	*KRAS*, *BRAF* mutation testing
Breast carcinoma	*ERBB2* amplification
Gastroesophageal adenocarcinoma	*ERBB2* amplification
Gastrointestinal stromal tumor	*KIT* and *PDGFRA* mutation testing
Neuroblastoma	*MYCN* amplification
Inflammatory myofibroblastic tumor	*ALK* rearrangement
Papillary thyroid carcinoma	*BRAF* mutation testing
Lymphoplasmacytic lymphoma	*MYD88*, *CXCR4* mutation testing
Diffuse large B-cell lymphoma	*MYC* rearrangement FISH testing Gene expression classifier testing Gene mutation analysis for *CARD11*, *CD79A*, *CD79B*, *EZH2*, *MYC*, *MYD88*, *TP53*, etc.
High-grade B-cell lymphoma	*MYC*, *BCL2*, *BCL6* rearrangement

(continued)

Table 1.2 (continued)

Context	Molecular diagnostic adjuncts
Chronic lymphocytic leukemia/small lymphocytic lymphoma	Deletions of *ATM*, D13S319, and *TP53*; trisomy 12 Gene mutation analysis for *ATM*, *BIRC3*, *TP53*, *NOTCH1*, *SF3B1*, etc. Drug resistance mutation testing for *BTK*, *PLCG2* genes
Myeloid sarcomas	FISH testing for multiple rearrangements Gene mutation analysis including *ASXL1*, *CALR*, *DNMT3A*, *FLT3*, *IDH1/2*, *JAK2*, *KIT*, *MPL*, *NPM1*, *RUNX1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, etc.

melanomas for various mutations including *BRAF*, *NRAS*, and *KIT* gene mutations; *ERBB2* gene amplification testing for breast (Chap. 13) and gastroesophageal carcinomas; and a multitude of molecular testing for pediatric tumors (Chap. 19) for which *MYCN* gene amplification testing in neuroblastomas represents a classic example.

Similar to solid tumors, molecular testing is a critical adjunct for diagnosis and personalized therapy in hematological malignancies [44]. This is reflected in the recently revised 2017 WHO classification system for lymphoid and myeloid disorders where genetic results form a basis for diagnosis and classification in multiple disease groups [45, 46]. Cytology FNA is routinely used for work-up of tissue-based hematological malignancies including lymphomas and leukemias and provides ample opportunity for molecular testing [5, 47]. Typical scenarios are summarized below, while Chap. 17 will address these in greater detail.

Differentiation of low-grade lymphomas and reactive lymphoid proliferations is, by far, the most challenging task for pathologists. Molecular tools can be extremely helpful to identify clonal markers that can facilitate this distinction. These include detection of monoclonal VDJ rearrangements in immunoglobulin heavy chain (*IgH*), T-cell receptor (*TCR*)

beta or gamma receptor genes, as well as specific gene rearrangements and gene mutations in B-cell and T-cell lymphomas [47–56]. In the right clinical and morphologic context, FISH or PCR studies for characteristic gene rearrangements are helpful in sub-typing of small B-cell lymphomas [such as t(11;14)(q13;q32) *IGH/CCND1* in mantle cell lymphoma, t(14;18)(q32;q21) *IGH@-BCL2* in follicular lymphoma, etc.], certain large B-cell lymphomas such as Burkitt lymphoma [t(8;14)(q24;q32) *MYC-IGH@*], and *ALK*-positive T-cell lymphomas [t(2;5)(p23;q35)] [47, 51, 57, 58]. Assessment of translocations by PCR enables evaluation of measurable (minimal) residual disease [59]. Molecular testing is essential to identify biomarkers to assess prognosis and determine the choice of therapy. Some examples, which are in no way comprehensive, include molecular testing of lymphoplasmacytic lymphoma for mutations in *MYD88* and *CXCR4* [60]; testing of large B-cell lymphomas for *MYC* gene rearrangement as well as gene expression profile for distinguishing between germinal center and activated B-cell phenotype [61, 62]; testing of high-grade B-cell lymphomas for rearrangements and copy number changes in *MYC*, *BCL2*, and *BCL6* genes [63, 64]; testing of T-cell lymphomas for *ALK* gene rearrangement (58); and testing of myeloid sarcomas for recurrent genetic translocations such as t(15;17) and t(9;22) *BCR/ABL* and various gene mutations such as *FLT3* and *IDH1/2* [46, 65, 66]. Further, in the context of newly developed targeted therapies, testing for gene mutations that confer resistance to drugs such as *BTK* mutations in chronic lymphocytic leukemia/small lymphocytic lymphomas is emerging as a standard modality [67]. In most cases, mutation testing for multiple genes is best done using a multi-gene sequencing assay such as next-generation sequencing. Molecular testing for malignancy associated viruses, specifically Epstein-Barr virus, plays an important role in diagnosis and monitoring of a variety of hematological conditions including Hodgkin and non-Hodgkin lymphoma, immunodeficiency-associated lymphoproliferative disorders including post-stem cell transplant [68, 69]. These applications will be further elaborated in Chap. 17.

Although some molecular tests serve more as diagnostic aids (Table 1.1) and others serve more to provide prognostic/theranostic information (Table 1.2), as mentioned above, these two roles of molecular diagnostic tests applied to cytology specimens and small biopsies should not be seen as necessarily mutually exclusive. As the repertoire of targeted therapeutic regimens expands, based on our evolving scientific knowledge surrounding molecular biomarkers and targets, these two roles will become increasingly intertwined.

The objective of this book is several-fold. First, the advantages and limitations of cytology specimens will be discussed to assist in the understanding, on the part of pathologists and laboratory personnel, of how best to leverage these patient samples for diagnosis and molecular testing. Second, practical knowledge surrounding the acquisition and laboratory processing of these specimens, along with triage and workflow algorithms, will be shared. This information will be cemented by providing details surrounding the actual testing methodologies utilized for molecular diagnostic evaluation of these samples (Section II). Finally, clinically relevant contexts in which molecular diagnostics and cytopathology are becoming increasingly intertwined will be discussed (Section III).

It is the hope of the editors of this book and the individual chapter authors that the reader can utilize this book as a practical guide to develop best practice algorithms by which molecular testing is applied to the diagnosis and work-up of cytology and small biopsy specimens. Understanding the pre-analytic and analytic aspects of molecular testing for cytology samples will enable pathologists and laboratory personnel to best determine how samples are managed and triaged to successfully obtain the optimum amount of diagnostic, prognostic, and theranostic information for each patient sample.

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